

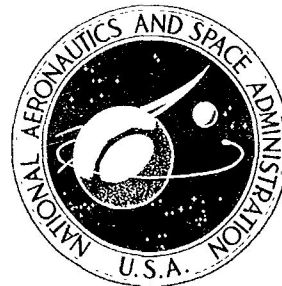
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# CO<sub>2</sub>: CHEMICAL, BIOCHEMICAL, AND PHYSIOLOGICAL ASPECTS

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# CO<sub>2</sub>: CHEMICAL, BIOCHEMICAL, AND PHYSIOLOGICAL ASPECTS

*Edited by*

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*A symposium held at*  
*Haverford College*  
*Haverford, Pennsylvania*  
*August 20-21, 1968*



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## FOREWORD

During the 1920's and 1930's there was a wave of investigation into the biochemistry of  $\text{CO}_2$ : into the acid-base reactions of plasma and whole blood, the interaction of  $\text{CO}_2$  with  $\text{O}_2$ -hemoglobin equilibria, the hydration-dehydration reactions of  $\text{CO}_2$  culminating in the discovery of carbonic anhydrase, the reaction of  $\text{CO}_2$  and hemoglobin to form carbamate, and the overall transport of  $\text{CO}_2$  by the blood. These researches provided fundamental data on the physiological chemistry of blood and the foundation of clinical chemistry, as well as techniques for a decade of clinical investigation.

Since that very active period, these topics have been relegated to the background in favor of more modern and more exciting subjects, largely because they were considered completely understood: the contempt for the familiar. However, during the past several years there has been a resurgence of interest in specific aspects of the chemistry and physiology of  $\text{CO}_2$ . New measurements have been made with modern instruments of the thermodynamic and kinetic constants of the reactions of  $\text{CO}_2$  with buffer systems. The equilibrium and velocity constants for the carbamates of simple peptides and of hemoglobin, including the influence of oxygenation on the latter, have been reinvestigated. Blocking the terminal alpha-amino groups in hemoglobin has been found to eliminate carbamate formation at physiological pH. Carbonic anhydrase has been intensively investigated, the amino acid sequences of different forms are in process of determination, the crystal structure of one form is being delineated with increasing refinement, and detailed chemical mechanisms of its catalysis are proposed for experimental test. Details of enzymic carboxylation have been investigated, including the determination of whether  $\text{CO}_2$  or  $\text{HCO}_3^-$  is the active species. Although the equilibration of  $P_{\text{CO}_2}$  between alveolar gas and end capillary blood in the alveolus is a pillar of physiological dogma, investigations in a number of laboratories have demonstrated that, under some conditions at least, the  $\text{CO}_2$  tensions in blood and gas may be significantly different.

In view of these developments and because no major meeting on these subjects had taken place for many years, we believed it would be worthwhile to bring together those active in these particular fields. Because many interested investigators were expected to be in the United States in the fall of 1968 to attend the 24th International Physiological Congress in Washington, D.C., a conference on  $\text{CO}_2$  was arranged for August 20 and 21 at Haverford College. Generous support was provided by the National Aeronautics and Space Administration and the National Heart Institute. The problem of biochemical production of  $\text{CO}_2$  was not included because of its magnitude. Even so, the interest in carbonic anhydrase alone was so great that it became necessary to extend the duration of the conference half a day.

This volume includes the formal presentations as well as the discussions that followed. Every effort has been made to present it as soon as possible, and we gratefully acknowledge the cooperation and assistance of the National Aeronautics and Space Administration in publishing these proceedings.

The task of organizing the conference and compiling the proceedings fell largely upon Miss Michele Ramirez, who was assisted by Mrs. Florence Fisher, Mrs. Ruth Manwaring, Mrs. Evelyn Connolly, Miss Carol Shaffer, and Mr. John H. Read. We gratefully acknowledge their successful efforts. Miss Pamela Brown was of invaluable assistance in editing the whole.

R. E. FORSTER  
J. T. EDSALL  
A. B. OTIS  
F. J. W. ROUGHTON

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*SESSION I*

**Theoretical Aspects of CO<sub>2</sub> Chemistry**

*Chairman: F. J. W. ROUGHTON*



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## Introductory Remarks

F. J. W. ROUGHTON

As one of the three assistant organizers of this conference, I should like to open on two personal notes. First, I would like to express our warmest thanks to the main organizer, Dr. Robert E. Forster, his secretaries, and other local colleagues for all the work they have done and the finances they have raised, to bring us together in this beautiful Haverford campus, for what is likely to be a rewarding meeting. Only those who have been local secretaries to an international gathering such as this can appreciate the almost interminable efforts imposed by such a job. Thank you, Dr. Forster, your wife and family, your secretaries, and all your other local talent—"troops" as you have been calling them—for all you have already done for us and all you are going to do for us in the next 2 days.

Second, I should like to say how delighted I am at last to be in the thick of a conference on carbon dioxide in chemistry and animal biology.

During the last 5 years I have taken part in two very successful conferences on oxygen in biology, and like some others at those meetings, I have hoped rather wistfully that some day would come the turn of the junior partner, carbon dioxide.

Here at last we are and I trust that many of you, if not all, will contribute happily to one or more of the five main sections into which we have divided the conference.

Since 1930 my colleagues and I, at one time or another, have published papers in every one of these five fields and I greatly appreciate the privilege, in my 70th year, of being in the chair for two of our sessions, together with the opportunity of being brought up to date in a very wide range of topics, about which I once knew or thought I knew something. Without further ado, I now open the first two sessions of the conference.

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## Distribution of Carbon Isotopes and Their Biochemical and Geochemical Significance<sup>1</sup>

SAMUEL EPSTEIN

California Institute of Technology<sup>2</sup>

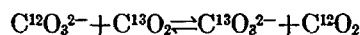
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Most elements are composed of two or more stable isotopes. For example, carbon has two stable isotopes of masses 12 and 13, and oxygen has three isotopes of masses 16, 17, and 18. Carbon dioxide is, thus, a mixture of different isotopic species such as:  $C^{12}O^{16}O^{16}$ ,  $C^{13}O^{16}O^{16}$ ,  $C^{12}O^{16}O^{18}$ , etc. The chemical and physical properties of the different isotopic species of a compound are sufficiently similar so that it is customary to ignore the isotopic constitution of a compound when its properties are described. On the other hand, it is well known that there exist differences in the thermodynamic and kinetic properties of the isotopic species of a chemical compound (Urey, 1947), the differences in the thermodynamic properties being primarily a result of the effect of mass on the vibrational frequencies of the compound or crystal. Because of this effect, there occur measurable isotopic fractionations associated with many processes in nature and in the laboratory for elements such as H, C, N, O, and S. The geochemist has made use of natural variations in the isotopic composition of the lighter elements to study natural processes that have taken place in the recent and ancient history of the Earth. To illustrate some of these uses will be the aim of this paper, with particular reference to carbon isotope fractionation.

Different isotopic species of a compound may also react at different rates resulting in isotopic

fractionation during nonequilibrium reactions (Bigeleisen, 1949). The effect of mass on rates of diffusion and on molecular velocities undoubtedly caused isotopic variations in natural substances as well.

The equilibrium distribution of the isotopes of carbon between two compounds can be defined by considering an isotope exchange reaction between the two compounds:



The equilibrium constant (or fractionation factor  $\alpha$ ) for this reaction is

$$\alpha = (C^{13}O_3^{2-}/C^{12}O_3^{2-}) / (C^{13}O_2/C^{12}O_2)$$

If the standard Gibbs free-energy change for the above reaction is not equal to 0, then  $\alpha$  is either smaller or greater than 1, which in turn means that at equilibrium, the  $C^{13}/C^{12}$  ratio of the carbon dioxide can be respectively lower or greater than that of the carbonate. In fact,  $\alpha$  is equal to about 1.008 for this exchange reaction. Namely, at equilibrium, the  $C^{13}/C^{12}$  ratio is greater in the carbonate by about 0.8 percent, or 8 per mil, relative to that of the carbon dioxide. Although this is a relatively small isotopic fractionation, a precise mass spectrometer is now available which permits one to measure with relatively high precision  $\alpha$  and the change of  $\alpha$  with temperature.

The isotopic effects in chemical kinetic reactions are also expressed in terms of a "fractionation factor" for an "exchange reaction" between the reactant and activated complex in accord with Eyring's transition-state hypothesis. The fractionation factor then represents the ratio of the

<sup>1</sup>Financial assistance for this research was provided by National Science Foundation grant GB 7517 and U.S. Atomic Energy Commission contract AT(04-3)-427.

<sup>2</sup>Division of Geologic Sciences, Contribution No. 1572.

rate constant of reaction for the different isotopic species considered.

Isotope fractionations associated with kinetic effects have been observed in many cases (Melander, 1960). From a geochemical point of view, an interesting case is that observed for the O<sup>18</sup>/O<sup>16</sup> ratio in oxygen dissolved in the ocean. Rakestraw, Rudd, and Dole (1951) observed that the O<sup>18</sup>/O<sup>16</sup> ratio increases with depth, whereas the concentration of oxygen decreases proportionately, indicating that during oxidation of organic debris in the ocean, the O<sup>16</sup> is preferentially used. The kinetic fractionation factor that they calculated suggests that the rates of reaction are dependent upon the square root of the mass of the oxygen molecular isotopic species.

#### Mass Spectrometry and Notation

Small differences in the C<sup>13</sup>/C<sup>12</sup> ratio are measured on an isotope ratio mass spectrometer of the Nier (1947) type, which has been specifically modified for this purpose by McKinney, McCrea, Epstein, Allen, and Urey (1950). The instrument compares the C<sup>13</sup>/C<sup>12</sup> ratio between two carbon dioxide gases. One gas serves as a standard, the other is the unknown. Actually, the ratio 45/44 (C<sup>13</sup>O<sup>16</sup>O<sup>16</sup>/C<sup>12</sup>O<sup>16</sup>O<sup>16</sup>) is compared between the gases. This ratio is the same as the ratio C<sup>13</sup>/C<sup>12</sup>, provided some known minor corrections are made (see Craig, 1957). The difference in the C<sup>13</sup>/C<sup>12</sup> ratio between the two gases is reported in terms of δ-values where:

$$\delta = \left\{ \frac{C^{13}/C^{12}(\text{sample})}{C^{13}/C^{12}(\text{standard})} - 1 \right\} \times 1000$$

A δ-value of +1.0 means that the C<sup>13</sup>/C<sup>12</sup> ratio in the sample is 1 per mil greater than that of the standard, and a δ-value of -1.0 means that the C<sup>13</sup>/C<sup>12</sup> ratio of the sample is 1 per mil lower than that of the standard. The precision of the measurements permits the detection of a difference of 0.1 per mil in δ-value. The standard gas used for the measurements discussed here is the carbon dioxide extracted from a fossil skeleton called *Belemnitella americana*, a standard originally used in the Chicago laboratories of Professor Harold C. Urey.

The fractionation factor α for an equilibrium system of two chemical compounds A and B is

related to δ by the expression:

$$\alpha = \frac{(1 + \delta_A/1000)}{(1 + \delta_B/1000)}$$

In many cases, the carbon dioxide preparation procedures determine the meaningful accuracy of δ-measurements. Essentially, the most desirable procedure for preparing carbon dioxide for the mass spectrometer is the conversion of the carbon compound to carbon dioxide quantitatively and without introducing or retaining gaseous impurities. Impurities of masses other than 45 and 46 can still affect the reliability of the δ-values.

#### The C<sup>13</sup>/C<sup>12</sup> Ratio of Some of the Natural Reservoirs

The first limited survey of the C<sup>13</sup>/C<sup>12</sup> ratio in natural materials was made by Nier and Gulbransen (1939). Craig (1953), using the more sensitive mass spectrometer, made an extensive survey of the C<sup>13</sup>/C<sup>12</sup> ratio using a large variety of sources of carbon compounds. Since then, many additional carbon isotope data have been added to the literature to give a better understanding of the natural variations of the C<sup>13</sup>/C<sup>12</sup> ratio. Figure 1 summarizes, essentially, Craig's δ-values for carbon in the most common types of natural carbon reservoirs. This illustration shows that the range of the δ-values of the C<sup>13</sup>/C<sup>12</sup> ratio is about 35 per mil which is about 350 times larger than the precision of measurement of the ratio.

The range of the δ-values for carbon is even larger if one takes into account the isotope data

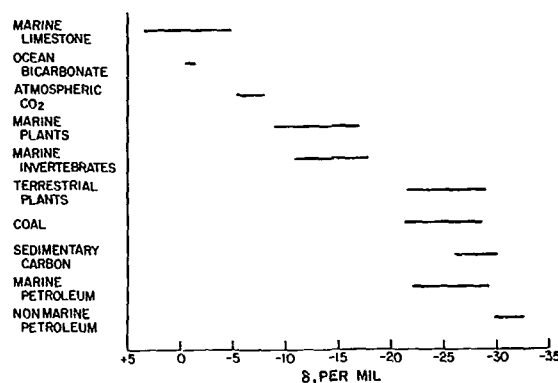


FIGURE 1.—The approximate ranges of the C<sup>13</sup>/C<sup>12</sup> ratios (δ-values) for carbon from various reservoirs. (Data mostly from Craig, 1953.)

for some of the more rare natural occurrences of carbon. For example, Clayton (1963) found a  $\delta$ -value of about +60 per mil for a carbonate mineral in a carbonaceous chondritic meteorite, while Silverman and Epstein (1958) measured a  $\delta$ -value of -49.3 for a natural gas from the Red Wash field in Utah. There is, therefore, at least a 115-per-mil variation in the  $C^{13}/C^{12}$  ratio in natural materials.

### Carbon Isotope Fractionation Processes

As figure 1 shows, the  $C^{13}/C^{12}$  ratios of the natural carbon reservoirs fall into several groups. The carbonate-bicarbonate compounds are the most enriched in  $C^{13}$ , next is atmospheric carbon dioxide, followed by marine animals, algae, terrestrial plants, marine petroleum, carbon found in shales (sedimentary carbon), and, finally, non-marine petroleum. The groupings are primarily a result of a few basic isotope fractionation processes. The smallest is the equilibrium fractionation factor associated with the carbon dioxide and dissolved ocean bicarbonate. This fractionation factor is about 1.007, resulting in an enrichment of 7 per mil in the bicarbonate. There is little fractionation between the bicarbonate and carbonate in the ocean, perhaps 1 or 2 per mil. The equilibration between the bicarbonate and the atmospheric carbon dioxide is rapid, so that the  $\delta$ -values of the atmospheric carbon dioxide and the dissolved carbonate in the ocean remain nearly constant and are controlled by the  $\alpha$  between the carbon dioxide and carbonate. The rapid rate of the equilibration between atmospheric carbon dioxide and the carbon in the ocean has been established by the  $C^{14}$  measurements of the two reservoirs (Suess, 1953). The relatively large range for the  $\delta$ -values of atmospheric carbon dioxide shown in figure 1 is primarily caused by large diurnal and possible seasonal variations in the  $C^{13}/C^{12}$  ratio in highly vegetated areas (Keeling, 1958) and to industrial contributions of isotopically light combusted petroleum in industrial areas (Friedman and Irsa, 1967). Normally the  $\delta$ -value of rural atmospheric  $CO_2$  is essentially constant over the world.

The other main fractionation processes for natural carbon are those associated with photosynthesis and the conversion of living organic

matter to sedimentary carbon. Fractionation caused by photosynthesis in the marine environment appears to be about 11 per mil, and in the terrestrial environment, about 20 per mil.

In addition, there appears to be an isotopic fractionation associated with the conversion of biogenic material to petroleum and to carbonaceous material found in sediments (primarily shales). The exact reason for this isotope effect has not as yet been worked out, but several possibilities exist. The decomposition of biogenic material may be accompanied by an isotope effect such that, for example,  $C^{13}$ -rich cellulose or lignin may decompose at a more rapid rate compared to the  $C^{12}$ -rich cellulose or lignin. The  $C^{12}$ -rich cellulose or lignin may then be retained preferentially in the sediments and be rearranged to form the carbonaceous material found in the sediments. The second possibility is based on the fact that, in a plant, the  $C^{13}/C^{12}$  ratio is 5 to 8 per mil lower for the lipid fraction relative to that of the rest of the plant (Park and Epstein, 1961), and that the lipids will be retained preferentially to form the carbonaceous material in sediments. It is possible, then, that the lipid fraction of living plants or animals provides the material for the petroleum reservoirs of the world.

### The $C^{13}/C^{12}$ Ratio of Ancient Carbon

With the knowledge of the  $C^{13}/C^{12}$  ratio fractionations associated with the few key processes discussed above, it is possible to apply carbon isotope data of ancient carbons to determine the magnitude of the various carbon reservoirs in the past history of the Earth, because the isotopic composition of any of the reservoirs on the Earth depends upon the fractionation factors between reservoirs and upon the relative amount of carbon in each reservoir. Also, it may be possible to ascertain at what time in the history of the Earth the contribution of biogenic carbon to the total carbon of the Earth became important. The average isotopic composition of the total carbon of the Earth is determined primarily by sedimentary carbonates and organic carbon in sediments (mostly shales) because, according to Rubey (1951), these two sources represent 99 percent of the total carbon in the Earth's carbon cycle. The carbon in the carbonates is about 73 percent of the

TABLE 1—Carbon Isotope Ratios ( $\delta$ -Values) in Sediments (Shales, Slates, and Chert) as Determined by Hoering (1967)

| Sediment                     | Location                              | Approximate age, million years | $\delta$ for C <sup>13</sup> |
|------------------------------|---------------------------------------|--------------------------------|------------------------------|
| Unconsolidated sediment..... | Aransas Bay, Tex.                     | Recent                         | <sup>a</sup> -19.4           |
| Oil shale.....               | Green River formation, de Beque, Col. | 40                             | <sup>a</sup> -29.8           |
| Shale.....                   | Woodford formation, Okla.             | 280                            | <sup>a</sup> -28.8           |
| Shale.....                   | Chattanooga formation, Tenn.          | 320                            | <sup>a</sup> -28.5           |
| Shale.....                   | Alum formation, Sweden                | 500                            | <sup>a</sup> -28.4           |
| Shale.....                   | Nonesuch formation, Mich.             | 1100                           | -29.3                        |
| Slate.....                   | Thompson formation, Minn.             | 1600                           | -30.4                        |
| Shale.....                   | McMinn formation, Australia           | 1600                           | -31.7                        |
| Chert.....                   | Gunflint formation, Ontario, Canada   | 1800                           | -30.2                        |
| Slate.....                   | Onwantin formation, Ontario, Canada   | 1700                           | -22.3                        |
| Shale.....                   | Ventersdrop System, South Africa      | 2100                           | -37.9                        |
| Slate.....                   | Knife Lake formation, Mich.           | 2600                           | -35.6                        |
| Shale.....                   | Soudan formation, Minn.               | 2000                           | -37.4                        |
| Slate.....                   | Soudan formation, Minn.               | -----                          | <sup>a</sup> -35.8           |
| Shale.....                   | Swazilan System Fig Tree formation    | 3000                           | -28.0                        |

<sup>a</sup> (Benzene-methanol treated).

total. Using a material balance calculation where the  $\delta$ -value of carbonate is taken to be 0 and that for shales about -27, the  $\delta$ -value of the Earth's carbon should be about -7 per mil. If, in the early history of the Earth, the carbon was present almost entirely as carbonates, the  $\delta$ -value for these carbonates would be -7 per mil. It follows that if the isotopic differences between the reservoirs remained constant, the  $\delta$ -value of the atmospheric carbon dioxide would be -14 per mil and that of the sedimentary carbon, -34 per mil. Conversely if, at some time, the amount of carbon in the shale would be the dominant reservoir, then the carbon in the shales would have a  $\delta$ -value of about -7 per mil. The  $\delta$ -value of the carbonates would be +20 per mil, and that of atmospheric carbon dioxide, +13 per mil. It follows that a lower proportion of biogenic carbon than that of the present day would result in a correspondingly more negative  $\delta$ -value for the biogenic carbon. The  $\delta$ -value of old carbon might then provide evidence as to the time when biological processes began to play an important role in the carbon cycle of Earth.

There are difficulties in obtaining the necessary data for the above considerations. It is not certain that the isotopic composition of very old carbon is preserved over many millions of years. It is possible that photosynthesis and the processes

associated with the formation of shale carbon were accompanied by unexpected isotopic fractionations. There is also the possibility that some of the carbon associated with very old shales was secondary carbon added at a much later time after the shales were formed. Nevertheless, it is still interesting to examine isotopic data available for some very ancient organic matter. Hoering (1967) has analyzed organic carbon from some of the oldest known shale and slate (metamorphosed shale) formations, in order to evaluate the possibility of the existence of biogenic carbon at that time. The age and  $\delta$ -values given by Hoering for these carbons are listed in table 1 and figure 2.<sup>3</sup> It would appear that the older carbon has a lower  $\delta$ -value, suggesting that around 2 billion years ago, the Earth contained a relatively small fraction of biogenic material. The ratio of sedimentary to carbonate carbon reached its present value about 1200 million years ago. The problems of interpretation of isotope data for carbon as old as 1 billion years are discussed in some detail by Hoering, and it would appear that more information is necessary regarding the biochemistry

<sup>3</sup> The organic material that can be washed out or extracted by benzene-methanol is ignored, because in most cases the material that can be extracted represents a minor contribution to the total carbon of the shale.

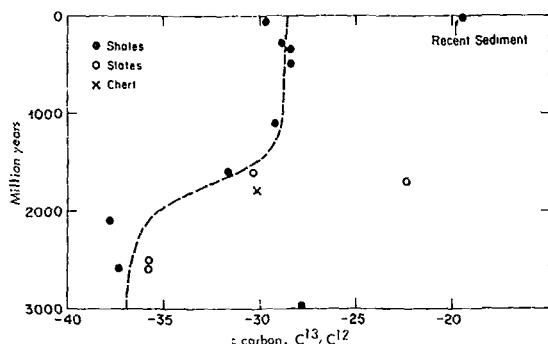


FIGURE 2.—A plot of Hoering's (1967)  $C^{13}/C^{12}$  data for shales and slates of various ages.

of shales and the isotopic fractionations associated with biochemical processes. Hoering's data, however, serve to illustrate an interesting application of carbon isotope measurements to an interesting geobiological problem.

It is relevant to point out that the  $\delta$ -value for carbon in carbonatites is about  $-5$  to  $-8$  per mil (Taylor, Frechen, and Degens, 1967). Carbonatites are carbonate rocks considered by many geologists to be deep seated and of magmatic origin and, thus, could possibly be the primary inorganic carbon of the Earth. Isotopically, carbonatites qualify to be the early source of carbon associated with the early formation of biogenic materials because their  $\delta$ -values are very similar to the  $-7$  calculated for the average  $\delta$ -value of the Earth's carbon.

#### CARBON ISOTOPIC FRACTIONATION IN THE PHOTOSYNTHETIC FIXATION OF $CO_2$

Considerations of carbon isotope variations in natural materials point out the importance of understanding the isotopic fractionations associated with a large number of biological processes. The lack of this knowledge makes interpretation of natural isotopic variations very difficult. One attempt to study a biological process to gain understanding of natural carbon isotopic variations is described below. At the same time, these experiments may illustrate how isotopic fractionations can be used in conjunction with biological research.

Park and Epstein (1960) made an attempt to determine the mechanism which causes the carbon

isotope fractionation associated with photosynthesis. Such a mechanism had to explain the existing isotope information: (1) the magnitude of the fractionation factor between carbon dioxide and plants; (2) the range in  $\delta$ -values of plants; (3) the differences in  $\delta$ -value for plants found in close proximity to one another, implying that the isotopic composition of carbon dioxide is not the only factor in the range of the  $\delta$ -value of the plant; and (4) the difference in  $\delta$ -value for marine and nonmarine plants. In addition, there were several results obtained by additional experiments which also had to be consistent with the proposed mechanism.

Baertchi (1952, 1953) determined that the photosynthetic isotope fractionation is not caused simply by the diffusion of carbon dioxide through the atmosphere to the surface of the plant. He showed that absorption of carbon dioxide from the atmosphere into a solution of barium hydroxide caused a maximum fractionation of only 14 per mil, as compared with a maximum fractionation of 26 per mil he observed for a bean plant grown under controlled laboratory conditions.

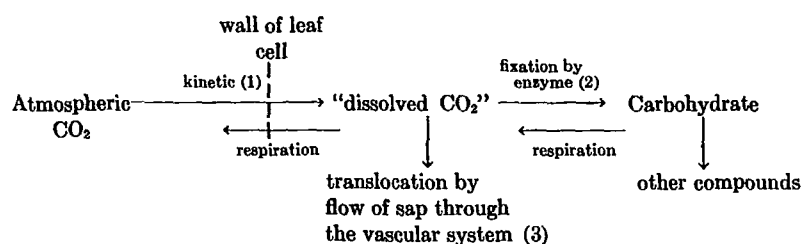
Experiments, where tomato plants were grown in a closed box from seed in vermiculite and watered with nutrient solution (Hoagland's) in an atmosphere containing carbon dioxide of known  $\delta$ -value and concentration, gave  $\alpha$ -values between carbon dioxide and plant of 1.0279 and 1.0285, representing about a 28-per-mil difference between the  $\delta$ -values of carbon dioxide and plant. This carbon dioxide air mixture was passed over the plant with sufficient speed so that neither the concentration nor the  $\delta$ -value of the carbon dioxide was changed appreciably during the growth of the plant. Plants, which were grown under concentrations of carbon dioxide ranging from 50 to one-half times the concentration present in the Earth's atmosphere, gave fractionation factors ranging from 1.0280 to 1.0247, respectively. The plant grown at half the atmospheric concentration of carbon dioxide gave an unusually low yield of plant growth (about one-fourth that of the other plants). This result will be discussed later in the light of the proposed model for carbon isotope fractionation during photosynthesis.

The  $\delta$ -values were then determined for carbon dioxide respired under dark conditions by tomato plants. Using a tomato plant whose total carbon

$\delta$ -value was  $-32.4$  per mil, the carbon dioxide respired during the first 10 minutes gave a  $\delta$ -value of  $-25.8$  per mil. As respiration continued, the  $\delta$ -value of the respired carbon dioxide continuously decreased. This result will also be discussed in the context of the proposed model.

Calvin and Bassham (1962) have shown that the primary fixation reaction in photosynthesis is the carboxylation of ribulose-1,5-diphosphate (RuDP) to yield two 3-phosphoglyceric acid (PGA) molecules, the reaction being enzyme catalyzed. Using a large excess of bicarbonate so-

lution as the source of the "dissolved CO<sub>2</sub>," RuDP was converted completely to PGA in the bicarbonate solution. With the knowledge of the  $\delta$ -values of the RuDP, the PGA, and the bicarbonate, it was possible to calculate the difference in the  $\delta$ -value of the bicarbonate and the  $\delta$ -value of carbon dioxide fixed by the RuDP to form PGA. The difference was 17.4 per mil, giving a fractionation factor of about 1.017. With this information, it was possible to suggest the following model to explain the isotopic fractionation associated with photosynthesis:



It was suggested by the authors that the step associated with absorption of carbon dioxide into plant cells can be accompanied by an isotope fractionation similar to that associated with the capture of carbon dioxide by a barium hydroxide solution. This fractionation factor would be about 1.014 (Baertchi, 1953, and Craig, 1953). Because atmospheric carbon dioxide has a  $\delta$ -value of  $-7$ , the dissolved CO<sub>2</sub> in the cytoplasm of the cell could have a  $\delta$ -value of  $-21$  per mil. Because the pH of cytoplasm is not high ( $\approx 7$ ), it is possible that exchange between atmospheric carbon dioxide and dissolved CO<sub>2</sub> in the cytoplasm may take place, resulting in a  $\delta$ -value for the dissolved CO<sub>2</sub> of approximately 0 per mil (the value for bicarbonate in equilibrium with atmospheric CO<sub>2</sub>). The  $\delta$ -value of the dissolved CO<sub>2</sub> in the cytoplasm, therefore, can range from  $-21$  to 0, depending on how rapidly the atmospheric carbon dioxide is fixed by the plant. Considering that plants can grow at different rates, it would not be surprising if the dissolved CO<sub>2</sub> could have a wide range in  $\delta$ -values. Thus, step 1 alone can account for the existing relatively large variation in the C<sup>13</sup>/C<sup>12</sup> ratio of plants.

It was possible to extract the dissolved CO<sub>2</sub> from photosynthesizing tomato plants to deter-

mine whether the  $\delta$ -value of this material is in the range of 0 to  $-21$ . The extraction of the dissolved CO<sub>2</sub> was accomplished by rapidly cutting tomato leaves into a liquid nitrogen bath. With appropriate precautions to eliminate the addition of contaminating carbon dioxide, the dissolved CO<sub>2</sub> was extracted by permitting 1 N sulfuric acid to react with the leaves at temperatures of about 0 C. The  $\delta$ -values for the extracted carbon dioxide ranged from  $-9.5$  to  $-17.5$ . To indicate that carbon dioxide extracted in this manner represents carbon dioxide introduced into the cytoplasm, the experiment was repeated with respiring tomato plants. In this case, the average  $\delta$ -value for the extracted carbon dioxide was  $-23.5$ , as compared with the respired  $\delta$ -values averaging  $-25.8$ . It seems reasonable to conclude that the extracted carbon dioxide from the cytoplasm represents the intermediate dissolved CO<sub>2</sub> in both respiration and photosynthesis. The second carbon isotope fractionating step in photosynthesis is the enzyme-catalyzed fixation process adding the dissolved CO<sub>2</sub> to RuDP to form PGA.

Whenever there are two successive kinetic steps in a reaction, the isotope fractionations associated with the two steps cannot be additive. This fact becomes obvious if step 1 is considered

as the slow rate-controlling step and step 2 as a rapid one. Namely, as soon as the carbon dioxide is absorbed by the cytoplasm, it is immediately converted to the primary photosynthetic product. In such a case, only the first step would cause isotopic fractionation, because total conversion of carbon from one form to another cannot be accompanied by fractionation. The plant would have a minimum  $\delta$ -value of  $-21$  per mil. On the other hand, if step 2 were the rate-controlling one, then the dissolved  $\text{CO}_2$  in the cytoplasm would have the opportunity to equilibrate continuously with atmospheric carbon dioxide and acquire and maintain a  $\delta$ -value of about 0 per mil, the approximate equilibrium  $\delta$ -value of bicarbonate when in equilibrium with atmospheric carbon dioxide. In this case, the isotope fractionating step would be due to the enzyme reaction (step 2) which would result in the  $\delta$ -value for the plant of about  $-17$  per mil. Any combination of intermediate rates for steps 1 and 2 would give  $\delta$ -values for the plant ranging between  $-17$  and  $-21$ . To permit the addition of isotopic fractionation by the two successive kinetic steps, it is necessary to introduce a third step which, during photosynthesis, could eliminate part of the dissolved  $\text{CO}_2$  from the plant. The suggested step 3 is the translocation of dissolved  $\text{CO}_2$  by the flow of sap through the vascular system of the plant.

If the pumping of carbon dioxide through the root system is relatively rapid, and if step 1 is the slow rate-controlling step, there will not be a complete incorporation of dissolved  $\text{CO}_2$  to form PGA by the rapid step 2. The isotopically light carbon dioxide will be fixed photosynthetically because of isotopic fractionation and the remaining  $\text{C}^{13}$ -enriched carbon dioxide in the cytoplasm will be pumped away through the roots. Ideally, then, step 1 will give an isotope fractionation of 1.014 and the enzyme-catalyzed step 2 will add a fractionation factor of 1.017 resulting in a maximum overall fractionation factor of the photosynthetic fixation of atmospheric carbon dioxide of 1.031. The plant will then acquire the minimum  $\delta$ -value of  $-38$  per mil.

Similarly, if step 2 is the slow rate-controlling step, the equilibration of the dissolved  $\text{CO}_2$  with atmospheric carbon dioxide will not take place in step 1 because the dissolved  $\text{CO}_2$  will be continuously pumped away by way of step 3. Provided

there is no isotopic fractionation associated with step 3, the minimum  $\delta$ -value of  $-38$  for plants can be attained under these conditions as well. Naturally, if the  $\delta$ -value of atmospheric carbon dioxide varies, the  $\delta$ -value of the plant can vary to maintain the  $\alpha$ -value. The rapid evolution of carbon dioxide through the root systems of the plants during the period of exposure to light has been observed by Harris and van Bavel (1957). In addition, Galimov (1966) has shown that carbon dioxide sampled from sandy and sandy loam soils in forests gives  $\delta$ -values more typical of  $\delta$ -values for dissolved  $\text{CO}_2$  rather than  $\delta$ -values expected for respired carbon dioxide. Galimov explains his results on the basis of the above model; namely, that well-developed roots in this soil pump  $\text{CO}_2$  into the soil.

It is also possible to assign a priori the maximum  $\delta$ -values for plants in the extreme case that plants can incorporate nearly all the carbon dioxide in the air that is available to them. If the translocation of carbon dioxide is small, the quantitative transfer of  $\text{CO}_2$  to plants would be void of an isotope fractionation and the  $\delta$ -value of the plants can be similar to that of atmospheric carbon dioxide, namely,  $-7$  per mil. In the oceans where the bicarbonate  $\delta$ -values are around 0 or  $-1$  per mil, the marine plants can also have  $\delta$ -values of 0. It is, therefore, possible that plants may have  $\delta$ -values ranging from about  $-38$  to 0 per mil. This phenomenon explains the existing range in the  $\delta$ -values for marine and nonmarine plants. The value of  $\alpha = 1.028$  observed for the tomato plants grown under laboratory conditions gives a  $\delta$ -value of  $-35$  for the plants. This value is also within the range indicated above.

Variability of the  $\delta$ -values of the plants growing within a narrow area can also be explained. Because the fractionation associated with photosynthesis is so sensitive to the relative rates of steps 1, 2, and 3, the  $\delta$ -values of atmospheric carbon dioxide alone will not determine the  $\delta$ -value of the plants.

The presence of a  $\text{C}^{13}$ -enriched dissolved  $\text{CO}_2$  in the cytoplasm (relative to total plant and respired  $\text{CO}_2$ ) explains the nature of change in the  $\delta$ -values of carbon dioxide as respiration of the tomato plants proceeds. During respiration, the isotopically heavier dissolved  $\text{CO}_2$  was being swept out of the cytoplasm by the respired carbon



dioxide so that the first carbon dioxide sample collected was the richest in C<sup>13</sup>, and this enrichment decreased as the proportion of the C<sup>13</sup>-poor respired carbon dioxide increased.

It is also possible to explain the difference between the  $\delta$ -values for marine and terrestrial plants. If we assume that there is adequate communication between the cytoplasm and ocean water in the marine environment, then the enzyme step 2 will have as its starting point a  $\delta$ -value for carbon dioxide of about 0. The most negative  $\delta$ -value for marine plants should be about  $-17$  or  $-18$  per mil. This result is in agreement with the data of Craig (1953). Recent work by Degens, Guillard, Sackett, and Reppman (1968) showed that some marine planktonic diatoms have  $\delta$ -values lower than  $-17$  per mil. They have proposed that these plants use carbon dioxide gas dissolved in ocean water. Because this carbon dioxide has a lower  $\delta$ -value than that of bicarbonate, the plankton will acquire more negative  $\delta$ -values. There are other variables, such as temperature and pH associated with the  $\delta$ -value acquired by the plankton. The reader is referred to the work of Degens et al. (1968) for further details. There is, of course, the difficulty of determining which form of carbon dioxide is incorporated into plants, but it is beyond the scope of this paper to try to discuss the possible ramifications of this problem on  $\delta$ -values of plants. The model proposed should be qualitatively independent of the nature of the carbon dioxide used by the plants, but the predicted isotopic compositions of the plants can vary, depending on whether plants use carbon dioxide or bicarbonate.

Previously, it has been pointed out that halving the natural concentration of carbon dioxide in the atmosphere decreased the isotopic fractionation factor during photosynthesis. There may be two reasons for this decrease. The rate of growth of the tomato plants appears to be so markedly decreased by the lowering of the concentration of carbon dioxide, that equilibration between the atmospheric carbon dioxide and the dissolved CO<sub>2</sub> in the cytoplasm is enhanced and, as was previously pointed out, such an effect would result in a lower fractionation factor. The other possibility is that the concentration of the carbon dioxide in the atmosphere was decreased during photosynthesis, and because removal of carbon

dioxide from the air would tend to increase the C<sup>13</sup> concentration of the air, the overall result would affect a C<sup>13</sup>-enrichment in the plant.

Several aspects of the variation of the C<sup>13</sup>/C<sup>12</sup> ratio of photosynthetic materials have been explained on the basis of the proposed model. It was also possible to use the model to predict the  $\delta$ -value of certain plants and to design an experiment for one of many possible tests of the model. As was pointed out previously, absence of the translocation step 3 would permit a fractionation factor between carbon dioxide and the plant of 1.017 to 1.021, provided the  $\delta$ -value of atmospheric carbon dioxide remains constant.

Certain terrestrial plants such as lichens lack a vascular system, and translocation of solutes is negligible. Therefore, one would predict that the  $\delta$ -values of lichens must range from about  $-17$  to  $-21$  per mil. At the time this work was done, lichens had not, as yet, been analyzed isotopically. Also, there were no  $\delta$ -values observed for any plants in this  $\delta$ -range. It, therefore, proved highly satisfactory to obtain  $\delta$ -values ranging from  $-18$  to  $-21.1$  per mil for seven lichens collected from three localities. In addition, a sample of grass and a leaf of *Quercus agrifolia* (both vascular plants) collected within a few feet of the lichen samples gave the normal  $\delta$ -values for plants of  $-26.0$  and  $-28.3$ .

The proposed model required that the carbon dioxide must be in the gaseous phase for step 1 to contribute to the carbon isotope fractionation during photosynthesis. It was, therefore, interesting to be able to grow two identical plants: one under water and one in the open air. In the plant grown under water, isotopic fractionation associated with step 1 of the model should be eliminated and the  $\delta$ -value of the plant should be about  $-17$  to  $-21$ , whereas the plant grown in air should have a  $\delta$ -value more normal to terrestrial plants. Two *Hydrophylla polysperma* plants, a common aquarium specimen, were grown side by side, one under water and the other in a moist atmosphere. Both systems were well ventilated with the same air supply. The  $\delta$ -value for new growth on the underwater sample was  $-21.7$ , whereas the  $\delta$ -values for the new air-grown leaves were  $-25.5$ ,  $-27.0$ , and  $-29.0$  per mil. Considering that laboratory air may have a somewhat lower  $\delta$ -value than  $-7$  because of industrial and respiratory con-

tamination, the  $-21.7$  value can be considered to be in the predicted range. The difference in  $\delta$ -value between the two plants was also as the model predicted.

The isotopic fractionations associated with the biochemical steps in converting PGA to cellulose, lignin, and lipids have not been investigated, but it seems reasonable that the formation of low  $C^{13}/C^{12}$  ratio lipids must be accompanied by an increase in the  $C^{13}/C^{12}$  ratio in some part of the total  $CO_2$  fixation and respiration processes. Lipid formation in plants is biochemically closely associated with plant respiration. It was, therefore, suggested by Park and Epstein (1961) that the  $C^{13}/C^{12}$  ratio decrease in the lipids is compensated for by a small increase in the  $C^{13}/C^{12}$  ratio of the respired carbon dioxide.

Since the above model was proposed, there do not appear to be any data that would contradict its validity. Considerably more carbon isotope data have been obtained for a large variety of plants. Smith and Epstein (unpublished results) have analyzed isotopically a large variety of species of terrestrial plants and found that there is a relatively consistent grouping of isotope data for the plant kingdom. With some exceptions, the grouping is related to the nature of the enzyme step 2. It is now known that photosynthesis is associated with intermediates other than RuDP and PGA and, as was pointed out previously, that the rates in step 2 differ markedly depending on the nature of the intermediates. If step 2 becomes so rapid and efficient that the translocation of carbon dioxide through the vascular system becomes relatively unimportant, the degree of carbon isotope fractionation would also decrease and the  $\delta$ -values for the plants would become less negative. This appears to be true in many cases; for example, corn fixes carbon dioxide rapidly and efficiently so that during the day, the concentration of carbon dioxide in air around cornfields can be reduced markedly. The  $\delta$ -value of corn is about  $-12$  per mil.

In summary, it appears that investigations of carbon isotope fractionations should be useful not only in studies of the fixation of carbon dioxide by plants, but could be extended to studies involving the interaction of carbon dioxide with many other biological systems.

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# Carbon Dioxide, Carbonic Acid, and Bicarbonate Ion: Physical Properties and Kinetics of Interconversion<sup>1</sup>

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The structure and certain properties of carbon dioxide, carbonic acid, and the bicarbonate ion are reviewed. The topics discussed include the solubility of CO<sub>2</sub> in water and in several other polar and nonpolar solvents and a comparison with other simple molecules, the thermodynamics of the hydration of CO<sub>2</sub> and the ionization of H<sub>2</sub>CO<sub>3</sub>, the velocity of hydration of CO<sub>2</sub> and the dehydration of bicarbonate ion or H<sub>2</sub>CO<sub>3</sub>, and the catalysis of the hydration and dehydration reactions by various inorganic and organic molecules and ions, including copper chelate complexes of peptides and related compounds. Certain unexplained discrepancies among the existing data are pointed out, with particular reference to the true ionization constant of H<sub>2</sub>CO<sub>3</sub> and the rate constants for the hydration and dehydration reactions at various temperatures.

As living organisms and producers of carbon dioxide, humans are a small part of the vast cycle that involves the production, consumption, and exchange of this substance among Earth, atmosphere, and ocean and plants and animals. As L. J. Henderson (1913) pointed out, two generations ago, in "The Fitness of the Environment" (ch. IV, p. 133):

Two chemical individuals stand alone in importance for the great biological cycle upon the earth. The one is water, the other carbon dioxide. . . . These two simple substances are the common source of every one of the complicated substances which are produced by living beings, and they are the common end products of the wearing away of all the constituents of protoplasm, and of the destruction of those materials which yield energy to the body.

As Henderson pointed out, carbon dioxide is unique among gases in distributing itself, in almost equal amounts per unit volume, between air and water. Thus it is constantly undergoing exchange between the atmosphere and the ocean;

and in the ocean it forms a component of the world's largest buffer solution, containing approximately 100 bicarbonate ions, and an appreciable quantity of carbonate ion, per molecule of CO<sub>2</sub>. The steady-state concentration of CO<sub>2</sub> in the air is stabilized by this vast ocean reservoir, and maintained by the opposing and nearly balanced processes of photosynthesis, respiration, combustion, and sedimentary deposition. (For an excellent discussion, see Brown, 1957.) If the steady state is not maintained—if production of CO<sub>2</sub> by respiration and combustion slightly exceeds its consumption by photosynthesis, as it appears may be the case at present—then thoughtful scientists ask questions and point to possible dangers for mankind. Congressmen have held hearings to ask the significance of the slowly rising concentration of carbon dioxide in the atmosphere.

At this meeting we shall not try to explore all these larger questions; our concerns are broad enough, however, because we shall consider the production and assimilation of carbon dioxide by living organisms, and the catalysts that accelerate this exchange. Before we turn to these more complex phenomena, let us look at the molecules involved, as the physical chemist sees them. Just

<sup>1</sup> The studies in our laboratory on the properties and reactions of carbon dioxide, particularly in their relations to carbonic anhydrase, were supported by grants GB 1255 and GB 5826 from the National Science Foundation, and grant HE 03169 from the U.S. Public Health Service.

over 10 years ago, Wyman and I (Edsall and Wyman, 1958) looked at the current status of the problem; and in 1960, David Kern wrote an admirable review on the hydration of carbon dioxide. Some of the puzzles that were then apparent are still unresolved; some of them will be pointed out and the knowledge achieved since 1960 will be reviewed.

### THE STRUCTURE OF CO<sub>2</sub>, THE CARBONATE ION, AND THE CARBOXYL GROUP

Figure 1 shows the structure of the molecules, ions, and groups that are the chief concern. We must note, however, that the CO<sub>2</sub> structure is based on spectroscopic data on the molecule in the gas phase, whereas the structure of the carbonate ion was determined by X-ray diffraction measurements on calcite crystals. We are concerned with molecules and ions in aqueous solutions, in which the dimensions may be slightly different from those given here. It is probably safe, however, to assume that such differences are minor.

Carbon dioxide is a symmetrical linear molecule

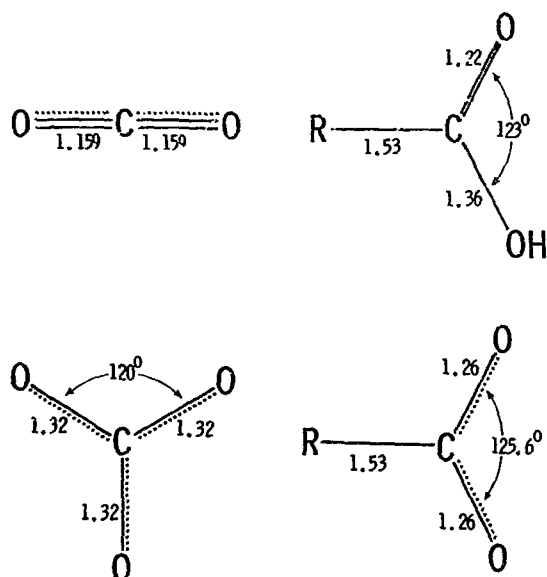


FIGURE 1.— Physical dimensions of carbon dioxide, the bicarbonate ion, and the carboxyl group in its ionized and un-ionized state. The R-C distance in the carboxyl group is for a C-C linkage. An N-C linkage, as in *N*-carboxybiotin, would be somewhat shorter.

with zero dipole moment. The C—O distances, 1.159 Å, are shorter than those found in a typical C=O double bond (1.22 Å) and indicate the contribution of resonance, as Pauling (1960) has pointed out. The carbonate ion is planar and symmetrical, with three equivalent C—O bonds, intermediate in character between single and double bonds. When it binds a proton to form the bicarbonate ion, the threefold symmetry is destroyed—the C—(OH) bond must become longer, while the other two C—O bonds acquire more double-bond character and shorten. The addition of a second proton to form H<sub>2</sub>CO<sub>3</sub> carries the process further. Even after both protons are bound, all the C—O bonds still retain enough double-bond character to maintain the planar triangular arrangement of the carbon and the three oxygens. Thus, when CO<sub>2</sub> is hydrated to form H<sub>2</sub>CO<sub>3</sub> or HCO<sub>3</sub><sup>-</sup>, the two C—O bonds must lengthen and must bend toward one another, the angle between them decreasing from 180° to about 120°. The relative slowness of the hydration and dehydration reactions must be associated with the necessity of making these electronic rearrangements. In contrast we may note that the hydration of SO<sub>2</sub> (Eigen, Kustin, and Maass, 1961) proceeds about 100 million times as rapidly as that of CO<sub>2</sub>. Sulfur dioxide is a bent triatomic molecule, with an O—S—O angle of 119.5°. The sulfite (SO<sub>3</sub><sup>2-</sup>) ion is a rather flat triangular pyramid with the sulfur atom at the apex. When it forms by hydration of SO<sub>2</sub>, the added oxygen forms a bond to the sulfur with very little angular distortion of the two S—O bonds already present (Edsall, 1968).

When carbon dioxide is released in metabolism, generally in the course of the Krebs tricarboxylic acid cycle, it arises from a carboxyl group; when it is incorporated, either in photosynthesis or in other incorporation reactions, it joins an organic molecule as a carboxyl group. In figure 1 the usual dimensions of the carboxyl group are shown in both ionized and un-ionized states. At physiological pH nearly all carboxyl groups are ionized, the two C—O bonds being equivalent in length because of resonance, slightly longer than a typical C=O double bond, with an angle of 125° to 126° between them. It is possible that some biochemical carboxylations and decarboxylations are associated with a transitory protonation of the carboxyl group. The dimensions of a typical un-

ionized carboxyl group with one double and one single C—O bond are also shown in figure 1. But, in any case, the biochemical processes of carboxylation and decarboxylation—if CO<sub>2</sub> rather than bicarbonate is the reactive species—require not only the formation or breakage of a covalent bond, usually a carbon-carbon bond, but also changes in the angle between the two C—O bonds, similar in magnitude to those occurring in the hydration and dehydration reactions.

#### SOLUBILITY OF CO<sub>2</sub> IN WATER AND IN MORE HYDROPHOBIC SOLVENTS; COMPARISON WITH OTHER GASES<sup>2</sup>

When carbon dioxide dissolves in water, it is present almost entirely as unhydrated CO<sub>2</sub>. As yet, however, we know little about the interactions between it and the surrounding water molecules. Does the CO<sub>2</sub> molecule induce orientation of water molecules around it, either by electrostatic interactions or by hydrophobic bonding or something analogous to it? Although the molecule as a whole is nonpolar, each of the two C—O bonds may act as a small dipole, with the oxygen at the negative end, and these dipoles could attract and orient surrounding water molecules. At present there is little basis for an attempt to answer these questions; but it is of interest to consider the relative affinity of CO<sub>2</sub> for water and for certain organic compounds, and to compare it in this respect with some simple molecules. Table I records the solubility of CO<sub>2</sub> in water, benzene, toluene, acetone, and ethanol at several temperatures. The data are expressed as cm<sup>3</sup> of CO<sub>2</sub> (reduced to 0 C<sup>3</sup> and 1 atmosphere) in solution per gram of solvent at 1-atmosphere pressure. In water this solubility coefficient  $\lambda$  is not far from unity; it is exactly unity a little below 15 C, and decreases with rising temperature. CO<sub>2</sub> is more than three times as soluble in the nonpolar solvents benzene, toluene, and heptane as it is in water. It is even more soluble in ethanol, and especially in acetone, mole-

cules which contain both hydrophobic and strongly polar groups.

Table II provides some comparable data for O<sub>2</sub>, where the two oxygen atoms are directly joined, rather than being separated by a carbon atom as in CO<sub>2</sub>. Here the actual solubility in water at a given gas pressure is, of course, far less than that for CO<sub>2</sub>. At a given temperature, CO<sub>2</sub> is some 30 times as soluble as O<sub>2</sub>. The affinity of O<sub>2</sub> for hydrophobic solvents, relative to water, is markedly greater than for CO<sub>2</sub>. This relative affinity is more directly shown in table III, which also provides comparable data for nitrogen and carbon monoxide. Whereas CO<sub>2</sub> is 3.5 times as soluble in heptane as in water, the other three gases are 17 to 22 times as soluble in heptane or hexane as in water. All these gases prefer acetone or ethanol to water as a solvent. However, O<sub>2</sub>, N<sub>2</sub>, and CO have less

TABLE I.—Solubility Coefficient  $\lambda^a$  of CO<sub>2</sub> in Several Solvents<sup>b</sup>

| <i>t</i> , C | Solvent |         |         |         |         |
|--------------|---------|---------|---------|---------|---------|
|              | Water   | Benzene | Toluene | Acetone | Ethanol |
| 0.....       | 1.676   |         | 3.55    |         |         |
| 15.....      | .989    | 2.81    | 2.70    | 9.3     | 4.0     |
| 25.....      | .746    | 2.57    | 2.42    | 7.4     | 3.35    |
| 50.....      | .429    |         |         |         |         |

<sup>a</sup>  $\lambda = \text{cm}^3 (\text{CO}_2) (0 \text{ C}, 1 \text{ atm}) / \text{g solvent} \times p (\text{atm})$ .

<sup>b</sup> All data in this table, and in tables II and III, are taken from Landolt-Börnstein "Zahlenwerte und Funktionen aus Physik, Chemie, Astronomie, Geophysik und Technik" Sechste Auflage. II. Bd. 2. Teil, Sec. 22261 (Springer-Verlag, Berlin: Göttingen-Heidelberg, 1962).

<sup>c</sup> At 25 C:  $\lambda$  in cyclohexane = 1.97, in heptane = 2.63.

TABLE II.—Solubility Coefficient  $\lambda$  of O<sub>2</sub> in Various Solvents<sup>a</sup>

| <i>t</i> , C | Solvent |         |             |         |         |
|--------------|---------|---------|-------------|---------|---------|
|              | Water   | Benzene | Cyclohexane | Acetone | Ethanol |
| 0.....       | 0.048   |         |             |         | 0.290   |
| 15.....      |         | 0.221   |             | 0.310   |         |
| 25.....      | .0273   | .226    | 0.323       | .316    | .273    |

<sup>a</sup> For the source of the data in this table, see table I.

<sup>2</sup> See note added in proof, p.26.

<sup>3</sup> In this volume we have denoted temperatures according to the convention used by the American Physiological Society which omits the degree sign in centigrade as redundant. We mention this point explicitly since this convention is different from that generally followed by chemical and biochemical journals.—The Editors.

TABLE III.—Solubility Ratios in Organic Solvents Relative to Water (25 C)<sup>a</sup>

| Solubility ratio              | Solvent           |                |                |                   |
|-------------------------------|-------------------|----------------|----------------|-------------------|
|                               | CO <sub>2</sub>   | O <sub>2</sub> | N <sub>2</sub> | CO                |
| Benzene/H <sub>2</sub> O..... | 3.45              | 8.3            | 9.6            | 8.7               |
| Hexane/H <sub>2</sub> O.....  | <sup>b</sup> 3.53 | 18.5           | 22.4           | <sup>b</sup> 17.4 |
| Acetone/H <sub>2</sub> O..... | 9.9               | 11.7           | 14.4           | 12.5              |
| Ethanol/H <sub>2</sub> O..... | 4.5               | 10.0           | 11.8           | 10.3              |
| 100% in H <sub>2</sub> O..... | 74.6              | 2.73           | 1.42           | 2.15              |

<sup>a</sup> For the source of the data in this table, see table I.

<sup>b</sup> In heptane.

affinity for these polar solvents than for the strictly nonpolar solvents, whereas CO<sub>2</sub> likes them better.

We should not try to draw too many conclusions from these data, but they do have some suggestive implications when we consider the binding of CO<sub>2</sub> to carbonic anhydrase. The CO<sub>2</sub> molecule dissolves much more readily in water than most gases do; it dissolves even better in hydrophobic solvents; and it dissolves most readily of all in solvents that contain polar groups, with C<sup>+</sup>-O<sup>-</sup> dipoles, adjoining nonpolar residues. This still leaves us a vast amount of latitude in specifying the active site of carbonic anhydrase, but it should provide us with a few clues.

#### THERMODYNAMICS OF HYDRATION AND IONIZATION

The ionization constant  $K$  of carbonic acid, expressed as

$$K_1 = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{CO}_2] + [\text{H}_2\text{CO}_3]} \quad (1)$$

has long been established with a high degree of accuracy (MacInnes and Belcher, 1933, 1935; Shedlovsky and MacInnes, 1935; Harned and Davis, 1943; Harned and Bonner, 1945). It will be discussed here only in its relation to other problems. We are concerned primarily with the true first ionization constant of carbonic acid,

$$K_{\text{H}_2\text{CO}_3} = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} \quad (2)$$

and with the hydration constant,

$$K_h = \frac{[\text{H}_2\text{CO}_3]}{[\text{CO}_2]} \quad (3)$$

These constants are of course interrelated by the equation:

$$\frac{K_1}{K_{\text{H}_2\text{CO}_3}} = \frac{K_h}{K_h + 1} \approx K_h \quad (4)$$

The approximation indicated on the right-hand side of equation (4) is a good one, because  $K_h$  is of the order of 0.003 or less. This equation defines  $K_h$  entirely in terms of the two ionization constants  $K_1$  and  $K_{\text{H}_2\text{CO}_3}$ . It is also possible to calculate  $K_h$  in an entirely independent way, from the ratio of the velocity constants for the hydration and the dehydration reactions. This question will be discussed later.

I know of no new measurements of these ionization constants since 1958. Therefore, I present in table IV the same data that Wyman and I used in 1958 (Edsall and Wyman, 1958, p. 561). The best direct measurements of  $K_{\text{H}_2\text{CO}_3}$  are probably those of Wissbrun, French, and Patterson (1954), which were obtained by conductance measurements at high field strengths (the Wien effect) extrapolated to zero field strength. They covered the temperature range from 5 to 45 C. For measurements of the heat of reaction involved in hydration (or dehydration) and in ionization, the measurements of Roughton (1941) are probably still unsurpassed.

The following point is noted: (1) the sum of the  $\Delta H$  values for reactions (2) and (3), from Roughton's thermal data, is in excellent agreement with the entirely independent data of Harned and Davis for reaction (1). This agreement is true also of the  $\Delta S_p$  values, and these findings strengthen confidence in Roughton's measurements. However, the temperature dependence of  $K_{\text{H}_2\text{CO}_3}$ , as reported by Wissbrun et al., leads to much lower values of  $\Delta H$  for this reaction than those reported

TABLE IV.—*Thermodynamics of Hydration and Ionization at 25 C<sup>a</sup>*

| Reaction   | <i>pK</i> | $\Delta G^b$ | $\Delta H^b$ | $\Delta S^b$ | $\Delta C_p^b$ |
|--|-----------|--------------|--------------|--------------|----------------|
| $K_A = [\text{H}_2\text{CO}_3]/[\text{CO}_2]$  | 2.59      | 3 530        | 1130         | - 8          | -63            |
| $K_{\text{H}_2\text{CO}_3} = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]}$ | 3.77      | 5 170        | 1010         | -14          | -28            |
| $K_1 = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{CO}_2]+[\text{H}_2\text{CO}_3]}$         | 6.352     | 8 666        | 2240         | -21.6        | -90            |
| $K_2 = \frac{[\text{H}^+][\text{CO}_3^{2-}]}{[\text{HCO}_3^-]}$                            | 10.329    | 14 092       | 3603         | -35.2        | -65            |

<sup>a</sup> The *pK* value of  $K_{\text{H}_2\text{CO}_3}$  is from Wissbrun et al. (1954); see also Berg and Patterson (1953). The values of  $\Delta H$  and  $\Delta S$  for this reaction are from Roughton (1941). Roughton (1941) also gave an estimate of  $K_{\text{H}_2\text{CO}_3}$  at 0 C as 3.60, which was based on kinetic data for hydration and dehydration, together with determinations of  $K_1$ .

The values of  $K_1$  and  $K_2$  are from Harned and Davis

(1943). (See also Harned and Bonner, 1945; MacInnes and Belcher, 1933, 1935; Shedlovsky and MacInnes, 1935.) The values of  $K_A$  are calculated from the other data by equation (4).

<sup>b</sup>  $\Delta G$  and  $\Delta H$  in cal mole<sup>-1</sup>,  $\Delta S$  and  $\Delta C_p$  in cal deg<sup>-1</sup> mole<sup>-1</sup>.

by Roughton and listed in table IV. They report  $\Delta H$  as 580 cal/mole at 0 C, 0 at 17, -412 at 27, and -880 at 37. These values are consistently more negative than those of Roughton by 1100 to 1500 cal/mole at each temperature. It will be noted that their values of  $\Delta C_p$  are large and negative, as are those of Roughton, but the consistent discrepancy in the  $\Delta H$  values is puzzling, and to my knowledge has not been explained. Tentatively I favor Roughton's  $\Delta H$  values, because they are based on direct thermal measurements, because he measures independently the heats of hydration and of ionization, and his data check so well with those determined by Harned and Davis.

It is to be expected that the hydration of  $\text{CO}_2$  should involve negative values of  $\Delta S$  and  $\Delta C_p$ . The joining of two molecules to form one reduces the translational and rotational degrees of freedom of the system. In the absence of compensatory interactions with the surrounding solvent molecules, this will inevitably decrease the entropy and heat capacity of the system. The ionization of  $\text{H}_2\text{CO}_3$  also leads to negative values of  $\Delta S$  and  $\Delta C_p$ ; here, of course, the effect is largely due to the immobilization of water molecules in the electric field of the ions produced by the reaction, and is characteristic of all such reactions in which two ions are formed from an uncharged molecule. The negative value of  $\Delta S$  is even larger for the second

ionization constant of carbonic acid, as shown in table IV.

Here the ionization constants  $K_1$  and  $K_{\text{H}_2\text{CO}_3}$  are considered at zero ionic strength. The influence of ionic strength on the apparent<sup>4</sup> ionization constants  $K_1'$  and  $K'_{\text{H}_2\text{CO}_3}$  has not been discussed. Harned and Davis (1943) and Harned and Bonner (1945) have reported, in detail, very precise measurements of  $K_1'$  over a wide range of ionic strength and temperature. Little is known directly concerning the ionic strength dependence of  $K'_{\text{H}_2\text{CO}_3}$ . The technique of Wissbrun et al. (1954) could be applied only in the absence of added electrolytes, except for the ions arising from the ionization of water and of  $\text{H}_2\text{CO}_3$  itself. Because the ionization of  $\text{H}_2\text{CO}_3$  involves the formation of an  $\text{H}_3\text{O}^+$  ion and a monovalent anion from a neutral molecule, we may expect that  $K'_{\text{H}_2\text{CO}_3}$  will vary with ionic strength much as the ionization of acetic acid does; at least the two acids should behave similarly at low ionic strengths, up

<sup>4</sup> For the acid-base reaction  $\text{HA} \rightleftharpoons \text{H}^+ + \text{A}$ , the relation between pH and  $pK'$  is given by the equation

$$\text{pH} = pK' + \log \frac{[\text{B}]}{[\text{A}]} = pK + \log \frac{[\text{B}]}{[\text{A}]} + \log \frac{\lambda_B}{\lambda_A}$$

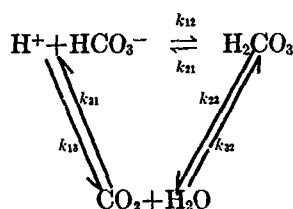
where  $A$  denotes an acid,  $B$  denotes its conjugate base, and  $\lambda_B$  and  $\lambda_A$  denote the activity coefficients of  $B$  and  $A$ , respectively (Edsall and Wyman, 1958, p. 442).



to about 0.15–0.2 *M*. The ionic strength dependence of  $K'_{\text{H}_2\text{CO}_3}$  is important for evaluating the rate constant for the dehydration of bicarbonate as well as in other biochemical problems. We may hope that future research will relieve our present ignorance of this important point.

### VELOCITY OF HYDRATION OF CO<sub>2</sub>

The hydration of CO<sub>2</sub> may lead to the formation of H<sub>2</sub>CO<sub>3</sub>, but it may also yield a hydrogen ion and a bicarbonate ion; a converse statement holds for the dehydration reaction. Therefore, like Eigen et al. (1961), we write the reaction scheme as:



This scheme has also been used by Ho and Sturtevant (1963) and by Gibbons and Edsall (1963).

We note immediately that the ratio of rate constants  $k_{21}/k_{12}$  equals the equilibrium constant  $K_{\text{H}_2\text{CO}_3}$ . Moreover, both  $k_{21}$  and  $k_{12}$  are far larger than any of the other four rate constants shown in the above scheme. Eigen and Hammes (1963) give the value of  $k_{12}$  at 25 C as  $4.7 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ ; from this value, and from the value of  $K_{\text{H}_2\text{CO}_3}$  in table IV, we obtain  $k_{21} \approx 8 \times 10^8 \text{ sec}^{-1}$ . Therefore, we may consider the reaction  $\text{H}^+ + \text{HCO}_3^- \rightleftharpoons \text{H}_2\text{CO}_3$  as being at equilibrium at all times while the other reactions are proceeding.

As yet we cannot determine individually the four rate constants  $k_{13}$ ,  $k_{31}$ ,  $k_{23}$ , and  $k_{32}$ . We can measure the overall rate of formation or disap-

pearance of CO<sub>2</sub>, as in manometric measurements, or we can measure the formation or disappearance of bicarbonate ion, usually by measuring the accompanying pH change. Thus we may write

$$\begin{aligned}
 \frac{-d(\text{CO}_2)}{dt} &= k'_{31}[\text{CO}_2] - k'_{13}[\text{H}^+][\text{HCO}_3^-] \\
 &= k_{\text{CO}_2}[\text{CO}_2] - k_{\text{H}_2\text{CO}_3}[\text{H}_2\text{CO}_3] \quad (5)
 \end{aligned}$$

In terms of the reaction scheme of Eigen et al. (1961), the rate constants in this equation are

$$k'_{31} \equiv k_{\text{CO}_2} = k_{31} + k_{32} \quad (6)$$

$$k'_{13} = k_{13} + (k_{23}/K'_{\text{H}_2\text{CO}_3}) \quad (7)$$

$$k_{\text{H}_2\text{CO}_3} = k'_{13}K'_{\text{H}_2\text{CO}_3} \quad (8)$$

In the following discussion the data will be formulated in terms of the rate constants  $k_{\text{CO}_2}$  and  $k_{\text{H}_2\text{CO}_3}$  (as was done by Edsall and Wyman, 1958). However, the relation of these constants to the more general reaction scheme of Eigen et al. (1961) should be borne in mind.

There have been several recent determinations of the hydration rate constant,  $k_{\text{CO}_2}$ , most of them at 25 C. In table V these are compared with some of the earlier values. The discrepancies are larger than might be expected. The highest value, that of Eigen et al. (see also Pinsent and Roughton, 1951), from temperature jump, is nearly 70 percent greater than that of Pinsent, Pearson, and Roughton (1956), which is based on the continuous flow thermal measurements of Roughton (1941), corrected for the contribution of the reaction  $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$ . Ho and Sturtevant (1963) and Gibbons and Edsall (1963) used stopped flow measurements, mixing CO<sub>2</sub> solutions with buffer solutions and following the resulting pH changes optically with an indicator. Their values

TABLE V.—Some Rate Constants for Hydration at 25 C

| $k_{\text{CO}_2} (\text{sec}^{-1}) \times 10^9$ | Authors   | Method           |
|---|---|------------------|
| 2.75  | Mills and Urey (1940)                               | Isotope exchange |
| 2.57  | Pinsent et al. (1956)                               | Thermal          |
| 3.58  | Ho and Sturtevant (1963)                            | pH; stopped flow |
| 3.75  | Gibbons and Edsall (1963)                           | pH; stopped flow |
| 4.3   | Eigen et al. (1961); and Eigen and de Maeyer (1963) | P- and T-jump    |

are closer to the higher ones of Eigen et al. (1961) than to the lower ones of Pinsent et al. (1956) and of Mills and Urey (1940).

There is good agreement among the various methods for the value of  $k_{\text{CO}_2}$  at 0 C, which is  $0.0021 \text{ sec}^{-1}$ . The mean energy of activation between 0 C and 25 C is 19 000 cal/mole if we take the value of Gibbons and Edsall for  $k_{\text{CO}_2}$  at 25 C; but Pinsent et al. (1956) conclude that it falls to an apparent value of 10 750 cal/mole at 38 C. Because their value of  $k_{\text{CO}_2}$  at 25 C is much lower than those of the later workers listed in table V, it would seem desirable to recheck the values of the rate constant at 38 C, which is of course of major physiological interest. The elegant isotope-exchange technique of Mills and Urey (1940) gave very reliable values at 0 C, but as the hydration rate increased with temperature, their data showed increasing scatter at higher temperatures and were subject to various uncertainties which they were careful to point out. Will subsequent technical advances make the isotope-exchange technique capable of yielding much more accurate results, not only at 0 C but at higher temperatures? Others at this conference could answer this question far better than I can; I would hope that this meeting might stimulate further work on the problem, especially at temperatures above 25 C.

#### THE REACTION: $\text{CO}_2 + \text{OH}^- \xrightarrow{k''} \text{HCO}_3^-$

This reaction is important, because it makes a significant contribution to the total rate of disappearance of  $\text{CO}_2$  in aqueous solution at pH values above about 7.5; above pH 10 it dominates the hydration reaction. It is also of interest in relation to the mechanism of the reaction catalyzed by carbonic anhydrase, which probably proceeds by the donation of  $\text{OH}^-$  to  $\text{CO}_2$  in the direction of hydration, and by the removal of  $\text{OH}^-$  from  $\text{HCO}_3^-$  in the dehydration reaction.

The most accurate values for the rate constant  $k''$  are undoubtedly those of Pinsent et al. (1956) by the rapid thermal method and those of Sirs (1958a, b) by a critical reexamination of the electrical conductivity and optical techniques. Sirs gives  $k'' = 8500 \text{ M}^{-1} \text{ sec}^{-1}$  at 25 C and Pinsent et al. (1956) give the energy of activation as 13 250 cal/mole.

The rate constant of the reverse reaction,

$\text{HCO}_3^- \rightarrow \text{CO}_2 + \text{OH}^-$ , is readily calculated, because it must be equal to  $k''K_w/K_1$ , where  $K_w = (\text{H}^+)(\text{OH}^-) = 1.008 \times 10^{-14}$  at 25 C and  $K_1$  is obtained from table IV. This value gives a rate constant of  $1.9 \times 10^{-4} \text{ sec}^{-1}$  at 25 C.

#### THE KINETICS OF THE DEHYDRATION REACTION

The kinetics of dehydration of  $\text{H}_2\text{CO}_3$ , like those of the hydration of  $\text{CO}_2$ , have been studied by continuous flow and stopped flow and by thermal, optical, and glass electrode measurements. The experimental procedures for producing the reaction fall into two main categories: a bicarbonate solution is rapidly mixed with hydrochloric acid in such proportions that the pH is low ( $< 5$ ) throughout the reaction; or the bicarbonate is mixed with a buffer, in the pH range 6 to 8. The principles of both methods have been set forth in the classic paper of Brinkman, Margaria, and Roughton (1933). The calculations for the procedure in the acid pH range are straightforward. The rate constant,  $k_{\text{H}_2\text{CO}_3}$ , is given by the equation

$$k_{\text{H}_2\text{CO}_3}(t_2 - t_1) = \left\{ 1 + \frac{K'_{\text{H}_2\text{CO}_3}}{b - a} \right\} \ln \frac{[\text{H}^+]_1}{[\text{H}^+]_2} + Q$$

where  $b$  is the concentration of  $\text{Na}^+\text{HCO}_3^-$  at time zero,  $a$  the concentration of  $\text{H}^+\text{Cl}^-$  at time zero,  $K'_{\text{H}_2\text{CO}_3}$  the apparent ionization constant of  $\text{H}_2\text{CO}_3$  at the ionic strength of the experiment,  $t_1$  and  $t_2$  the times after mixing, and  $Q$  a small term which is only a few percent of the first term on the right. In the first term,  $K'_{\text{H}_2\text{CO}_3}/(b - a)$  is only of the order of 0.05 under usual conditions; hence an uncertainty of several percent in the assumed value of  $K'_{\text{H}_2\text{CO}_3}$  makes little difference in the final value of  $k_{\text{H}_2\text{CO}_3}$ . On the other hand, near neutral pH, where  $\text{Na}^+\text{HCO}_3^-$  is mixed with buffer, the quantity directly obtained from the experiments is  $k'_{13}K'_{\text{H}_2\text{CO}_3}$  (see eq. (8)), and the uncertainties in the choice of the correct value of  $K'_{\text{H}_2\text{CO}_3}$ , at the ionic strength and temperature of the experiment, are much more serious than in the first procedure. Gibbons and Edsall (1963), for example, assumed the value of Wissbrun et al. (1954) for  $K_{\text{H}_2\text{CO}_3}$  at zero ionic strength, and then calculated  $K'_{\text{H}_2\text{CO}_3}$  at the ionic strength of the experiment, on the assumption that the ionization of

H<sub>2</sub>CO<sub>3</sub> varies with ionic strength in the same way as that of acetic acid. Questions may be raised about both these assumptions. I note, for instance, that Roughton (1941) has given an estimate of  $K_{H_2CO_3}$  that is significantly higher than that of Wissbrun et al. (see footnote to table IV).

Table VI shows several values obtained, at different temperatures, by the two procedures. If we compare the two sets of measurements, especially the data at or near 25 C, it is apparent that the measurements made at acid pH values tend to give higher values than those made at neutral pH for the dehydration rate constant. The value of 25.5 sec<sup>-1</sup> reported by Rossi-Bernardi and Berger (1968) is 1.85 times as large as the value of 13.7 sec<sup>-1</sup> reported by Gibbons and Edsall (1963). I have included a recent value of 20 sec<sup>-1</sup>

at 25 C, obtained by Dr. Raja Khalifah in our laboratory using the low pH method. Khalifah has made a series of determinations of  $k_{H_2CO_3}$  at different temperatures, from 8 to 45 C, and his data are plotted against 1/T in figure 2, together with the data of previous investigators (Khalifah, unpublished observations). It will be seen that the general agreement is good, but that the values measured in buffers in the pH 6 to 8 region do lie significantly below the others at 25 C. Khalifah's value of 20 sec<sup>-1</sup> at 25 C, however, is only slightly above that of Ho and Sturtevant (1963). We should note that the latter authors determined dehydration rates over the whole pH range from 4 to 7, and used all their data to determine a most probable value for the rate constant.

Dalziel (1953) and Sirs (1958a, b) calculated

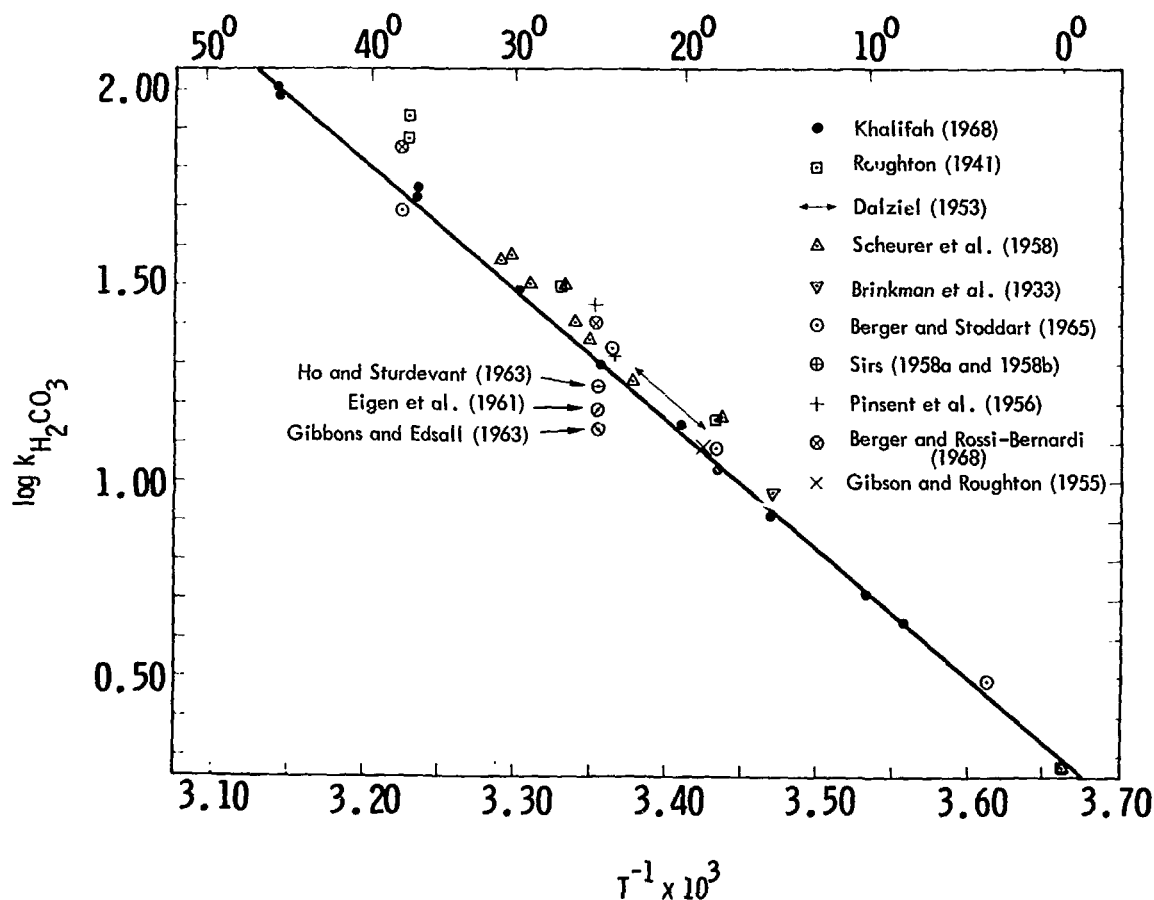


FIGURE 2.—The logarithm to the base 10 of the dehydration rate constant for H<sub>2</sub>CO<sub>3</sub> is plotted against the reciprocal of the absolute temperature.

from their data an energy of activation of 16 900 cal/mole for the dehydration process. Khalifah's data point to a slightly lower value, near 15 000, but they are subject to further refinement and checking.

#### THE RATIO $K_a = [\text{H}_2\text{CO}_3]/[\text{CO}_2]$ , FROM EQUILIBRIUM AND RATE MEASUREMENTS

This constant may be calculated from equation (4), as the ratio of  $K_1$  to  $K_{\text{H}_2\text{CO}_3}$ . It may also be calculated from the ratio of the two rate constants,  $k_{\text{CO}_2}/k_{\text{H}_2\text{CO}_3}$ . Table VII lists some of the results.

The values from kinetics, at 25 C, cover a range from  $1.01 \times 10^{-3}$  to  $2.85 \times 10^{-3}$ . The lowest value is deliberately chosen as a lower extreme, by combining the lowest recorded value of  $k_{\text{CO}_2}$  with the highest recorded value of  $k_{\text{H}_2\text{CO}_3}$  at this temperature. The value that agrees most closely with that obtained from the equilibrium data is that of Gibbons and Edsall, but I will not claim that it is necessarily the best, even though Dr. Gibbons did her work in our laboratory and I can testify to her experimental skill and judgment. If we used the recent measurements of Dr. Khalifah for  $k_{\text{H}_2\text{CO}_3}$  in our laboratory (table VI) to which I have already alluded, together with Dr. Gibbons'

TABLE VI.—Some Dehydration Rate Constants <sup>a</sup>

| $k_{\text{H}_2\text{CO}_3}$ (sec <sup>-1</sup> ) | $t$ , C | Reference                             | Method <sup>b</sup> |
|--|---------|---------------------------------------|---------------------|
| 14.6   | 17.8    | Roughton (1941).....                  | c.f.; thermal       |
| 31.0   | 27.0    | Roughton (1941).....                  | c.f.; thermal       |
| 80.0   | 36.6    | Roughton (1941).....                  | c.f.; thermal       |
| 12.3   | 18.0    | Dalziel (1953).....                   | c.f.; optical       |
| 12.3   | 18.0    | Sirs (1958).....                      | s.f.; electrode     |
| 25.9   | 24.1    | Berger and Stoddart (1965).....       | s.f.; thermal       |
| 20.6   | 24.1    | Berger and Stoddart (1965).....       | s.f.; optical       |
| 25.5   | 25.0    | Rossi-Bernardi and Berger (1968)..... | c.f.; electrode     |
| 72.0   | 37.0    | Rossi-Bernardi and Berger (1968)..... | c.f.; electrode     |
| 20.0   | 25.0    | Khalifah (1968).....                  | s.f.; optical       |
| <sup>c</sup> 15.1                                | 25.0    | Eigen et al. (1961).....              | T-jump              |
| <sup>c</sup> 17.5                                | 25.0    | Ho and Sturtevant (1963).....         | s.f.; optical       |
| <sup>c</sup> 13.7                                | 25.0    | Gibbons and Edsall (1963).....        | s.f.; optical       |

<sup>a</sup> This table is taken from Berger and Stoddart (1965) and Rossi-Bernardi and Berger (1968), with the addition of Khalifah's value at 25 C (unpublished observations).

<sup>b</sup> Abbreviations: c.f. = continuous flow; s.f. = stopped flow.

<sup>c</sup> Measurements made in buffer (pH 6 to 8). All others made at low pH; bicarbonate + HCl.

TABLE VII.—Values at 25 C of the Equilibrium Constant,  $K_a = [\text{H}_2\text{CO}_3]/[\text{CO}_2]$

| Ratio   | $K_a \times 10^3$  | Reference   |
|---|--------------------|---|
| Rate constants:                                     | 1.01 = 0.0257/25.5 | Pinsent et al. (1956); Rossi-Bernardi and Berger (1968) |
| $\frac{k_{\text{CO}_2}}{k_{\text{H}_2\text{CO}_3}}$ | 2.05 = 0.0358/17.5 | Ho and Sturtevant (1963)                                |
|   | 2.74 = 0.0375/13.7 | Gibbons and Edsall (1963)                               |
|   | 2.85 = 0.043/15.1  | Eigen et al. (1961)                                     |
| Equilibrium constants:                              |                    |   |
| $\frac{K_1}{K_{\text{H}_2\text{CO}_3}}$             | 2.57               | Wissbrun et al. (1954); Harned and Davis (1943)         |

value for  $k_{\text{CO}_2}$ , the value of  $K_A$  would be  $1.88 \times 10^{-3}$  instead of  $2.74 \times 10^{-3}$ , as listed in table VII. I can only conclude that there are significant discrepancies in the kinetic data, and careful work and thought will be required to clear them up.

### CATALYSIS OF THE HYDRATION AND DEHYDRATION REACTIONS

Since the fundamental work of Roughton and Booth (1938) and of Kiese and Hastings (1940) on the catalysis of the hydration and dehydration reactions by various bases, there have been several further studies that have provided some new points of view. Sharma and Danckwerts (1963) studied catalysis by a large number of buffer systems, in the hydration reaction, and described their data by the coefficients of the equation

$$\text{Rate (M sec}^{-1}\text{)} = (k_{\text{CO}_2} + k_B[B])[CO_2] \quad (9)$$

Here  $[B]$  is the molar concentration of the basic constituent of the buffer, and  $k_B$  is its catalytic coefficient<sup>5</sup> in  $\text{M}^{-1} \text{sec}^{-1}$ . They made measurements in phosphate, veronal, and carbonate buffers between pH 7.5 and 10.1, at 0°C, and concluded that, for a considerable group of bases whose conjugate acids release a proton from an -OH group, there is a nearly linear relation between the  $pK_A$  value of the conjugate acid and  $\log k_B$  of the base (the Brønsted equation).

$$\log k_B = 0.56 pK_A + \text{constant} \quad (10)$$

This group included the conjugate bases of telluric, germanic, arsenious, and silicic acids, with  $pK_A$  values ranging from 8.1 to 10.1, and also those of a group of aldehyde hydrates, including several (the hydrates of glyoxal, formaldehyde, and acetaldehyde) with  $pK_A$  values which they estimated as lying between 13 and 14. Because their highest experimental pH was 10.1, the actual concentration of the anions of these aldehyde hydrates was less than 0.1 percent of the concentration of the conjugate acid in all cases. They reported values of  $k_B$  as high as 8000 for formaldehyde hydrate, and 3000 for acetaldehyde hy-

drate. These figures are to be compared, for instance, with  $k_B = 1.3$  for sulfite, which was one of the most active of the catalysts studied by Roughton and Booth (1938). The estimates for both  $pK_A$  and  $k_B$ , for substances with such very high  $pK_A$  values, are obviously subject to considerable uncertainty. Indeed, Dennard and Williams (1966), in a critical reexamination of the problem, concluded that there was no convincing evidence of the validity of the Brønsted relation. Table VIII, which is taken from their paper, indicates a lack of any systematic correlation between  $pK_A$  and  $k_B$  values. We note that their catalytic coefficients are obtained from the equation.

$$\text{Rate (M sec}^{-1}\text{)} = k_{\text{CO}_2}(1 + k_{\text{OH}^-}[\text{OH}^-] + k'_{\text{HB}}[\text{HB}] + k_B[B])[CO_2] \quad (11)$$

where  $\text{HB}$  is the conjugate acid of the base  $B$ , and the units of  $k'_{\text{HB}}$  and  $k_B$  are  $\text{M}^{-1}$ . Thus  $k_B'$  is related to  $k_B$  of equation (9) by the equation

$$k_B' = k_B/k_{\text{CO}_2} \quad (12)$$

Dennard and Williams showed explicitly for a number of systems that  $k'_{\text{HB}}$  is zero within experimental error, which is indeed what most earlier workers concluded. They divided good catalysts into two groups:

(1) Group 1 consists of oxyanions of the highest oxidation state of a nonmetal in which there is no oxygen in the anion equivalent to that from which the proton has been removed. Examples are  $[\text{OTe}(\text{OH})_5]^-$ ,  $[\text{OGe}(\text{OH})_3]^-$ , and  $[\text{OSi}(\text{OH})_3]^-$ . These are all good catalysts; the negative charge remains localized on the oxygen atom from which a proton has been removed. On the other hand, in such anions as  $\text{HCO}_3^-$ , which is not a catalyst, and  $\text{HPO}_4^{2-}$ , which is a rather weak one, the negative charge is distributed by resonance over the oxygens, rather than being locally concentrated.

(2) Group 2 consists of oxyanions of lower oxidation states of nonmetals with at least one lone pair of electrons, such as  $\text{SO}_3^{2-}$ ,  $\text{SeO}_3^{2-}$ ,  $\text{ClO}^-$ , and  $\text{BrO}^-$ . These are the most powerful of all the nonenzymic catalysts that have been studied, except for some metal chelates which are certainly in the same class. These will now be considered.

The principal report on such chelates is that of Esther Breslow (1966). She studied the copper

<sup>5</sup> Presumably they corrected their data at higher pH values by taking account of the reaction of  $\text{CO}_2$  with  $\text{OH}^-$  ions. This would involve insertion in eq. (9) of a term  $k_{\text{OH}^-}[\text{OH}^-]$  on the right-hand side, but this is not explicitly stated in their paper.

TABLE VIII.—Some Catalytic Constants for Bases of Oxygen Acids in the Hydration<sup>a</sup> of CO<sub>2</sub><sup>b</sup>

[The data were obtained at 0 C and high ionic strengths (about 0.8); from Dennard and Williams (1966)]

| Base  | pK <sub>A</sub> | k <sub>B</sub> (M <sup>-1</sup> ) |
|---|-----------------|-----------------------------------|
| HPO <sub>4</sub> <sup>2-</sup> -----              | 7.2             | 10                                |
| H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ----- | 2.1             | 2                                 |
| HAsO <sub>4</sub> <sup>2-</sup> -----             | 7.0             | 5                                 |
| AsO <sub>4</sub> <sup>2-</sup> -----              | 9.2             | 2×10 <sup>3</sup>                 |
| SO <sub>3</sub> <sup>2-</sup> -----               | 7.2             | 1×10 <sup>3</sup>                 |
| Te(OH) <sub>4</sub> O <sup>-</sup> -----          | 8.0             | 700                               |
| ClO <sup>-</sup> -----                            | 7.5             | 5×10 <sup>4</sup>                 |
| BrO <sup>-</sup> -----                            | 8.5             | >10 <sup>4</sup>                  |
| Phenolate-----                                    | 10.0            | 10                                |

<sup>a</sup> Hydration rate (M sec<sup>-1</sup>) =  $k_{CO_2}[1 + k_{OH}[OH^-] + k_B(B)][CO_2]$ .

<sup>b</sup> No catalytic activity was shown by CO<sub>3</sub><sup>2-</sup>, HCO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, HS<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, H<sub>2</sub>PO<sub>3</sub><sup>-</sup>, pyrophosphate, benzenesulfonate, benzenesulfinate, and various organic anions.

(Cu<sup>2+</sup>) complexes of the di-, tri-, and tetra-peptides of glycine and also those of L-valylglycine and of glycylglycine ethyl ester. Cu-Gly-Gly, Cu-Val-Gly, and Cu-Gly-Gly ethyl ester were the most powerful catalysts. In terms of the equation

$$\text{Rate (M sec}^{-1}\text{)} = (k_{H_2O} + k_{OH}[OH^-] + k_{cat}(\text{catalyst}))[CO_2] \quad (13)$$

she found that  $k_{cat}$  for Cu-Gly-Gly increased with rising pH at 1.5 C from 0.11 M<sup>-1</sup> sec<sup>-1</sup> at pH 7 to 9.3 M<sup>-1</sup> sec<sup>-1</sup> at pH 9. For SO<sub>3</sub><sup>2-</sup> ion,  $k_{cat}$  was only 1.3 and did not change between pH 8 and 9. The copper of the complexes of Gly-Gly and Val-Gly at pH 9 is coordinated in solution with the α-amino group and with a peptide bond nitrogen from which the proton has been displaced. A water molecule bound to the copper releases a proton with a pK<sub>A</sub> of 9.7 at 0 C. Thus the data strongly suggest that the catalytic species in CO<sub>2</sub> hydration is Cu-Gly-Gly-OH<sup>-</sup>, and correspondingly for the other dipeptide derivatives. In the copper derivatives of triglycine and tetraglycine, Cu<sup>2+</sup> is coordinated to the α-amino group and to two peptide nitrogens, increasing to three for Cu<sup>2+</sup>-tetraglycine as the pH rises above 9.5. These compounds, unlike the Cu<sup>2+</sup>-dipeptide complexes, show no ionization of a coordinated water molecule in the pH range from 6 to 10.

Addition of imidazole (Im) to Cu<sup>2+</sup>-Gly-Gly leads to the formation of a mixed 1:1 complex with imidazole (Koltun, Fried, and Gurd, 1960), and Breslow found that this complex had lost nearly all catalytic activity. Its  $k_{cat}$  value was less than 0.3 at pH 9, as compared with 9.3 for Cu<sup>2+</sup>-Gly-Gly at this pH. The imidazole may displace bound water from the copper complex, and thereby prevent the formation of the Cu<sup>2+</sup>-Gly-Gly(OH<sup>-</sup>) derivative.

It is interesting to compare this inhibitory effect of imidazole on the catalysis of CO<sub>2</sub> hydration with the activating effect of imidazole on the Zn<sup>2+</sup>-catalyzed hydration of acetaldehyde (Pocker and Meany, 1967). Whereas  $k_{cat}$  for this hydration reaction is 0.108 M<sup>-1</sup> sec<sup>-1</sup> for aqueous Zn<sup>2+</sup>, it increases to 2.15 M<sup>-1</sup> sec<sup>-1</sup> in the presence of enough imidazole buffer to convert most of the zinc to Zn(Im)<sub>2</sub><sup>2+</sup>. Pocker and Meany suggest that this species is in mobile equilibrium with such species as Zn(Im)<sub>2</sub>(H<sub>2</sub>O)<sup>2+</sup> and Zn(Im)<sub>2</sub>(OH)<sup>+</sup>, and that it is the coordinated water or OH<sup>-</sup> in such complexes, which is catalytically effective.

Breslow's studies of catalysis are obviously suggestive of some proposed mechanisms for the action of carbonic anhydrase. The formation of HCO<sub>3</sub><sup>-</sup> from CO<sub>2</sub>, in either case, does not necessarily involve direct donation of the metal-bound OH<sup>-</sup> ion to carbon dioxide. Other, more indirect action is possible, such as general base catalysis by the metal-bound OH<sup>-</sup>, abstracting a proton from a neighboring water molecule while the oxygen of the latter makes a nucleophilic attack on the carbon of CO<sub>2</sub>. Breslow has discussed this and other possibilities. No peptide chelates of zinc have yet been shown to have catalytic activity with CO<sub>2</sub>. The X-ray determination of the structure of human carbonic anhydrase C should soon provide clear indications of the nature and arrangement of the ligands around the zinc that are required for catalytic efficiency. We may hope that soon the study of simple synthetic catalysts and the study of enzyme structure and mechanism, in the reactions of CO<sub>2</sub>, will converge.

#### ACKNOWLEDGMENTS

I am indebted to Dr. Raja G. Khalifah for his help in the preparation of this article, including

the assembly of the information in figure 2 which contains his own unpublished data on the dehydration reaction.

#### NOTE ADDED IN PROOF

Concerning the solubility of CO<sub>2</sub>, see J. H. Hildebrand and R. L. Scott, "The Solubility of Nonelectrolytes," third edition, Reinhold Publishing Corporation, New York, 1950; reprinted by Dover Publications, New York, 1964. On page 248 they tabulate the solubility of CO<sub>2</sub> in a number of solvents, and note (page 249) that "there is abundant evidence of solvation, particularly with solvents containing the carbonyl group."

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## DISCUSSION

**ROUGHTON:** In regard to the effect of temperature on  $K_{H_2CO_2}$  and the discrepancy between our work and that of Patterson, is there not also an absolute discrepancy in the numerical values of  $K_{H_2CO_2}$ ? The Patterson values, which you preferred both in your book and in your talk this afternoon, are markedly less than the values obtained by the rapid titration method. This discrepancy should be resolved.

**EDSALL:** That is certainly another discrepancy.

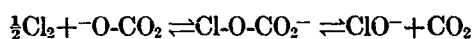
**HASTINGS:** I wish somebody here could explain how hypochlorous and hypobromous ions catalyze the hydration of  $CO_2$ . Last year I had occasion to try to bypass Warburg's method for accelerating  $CO_2$  uptake and buffering  $CO_2$  tension in the course of respiration. When I remembered this hypochlorous ion-hypobromous ion effect, it occurred to me that I might get around the addition of carbonic anhydrase, although it is not patented.

**ROUGHTON:** We did patent carbonic anhydrase, but unfortunately it turned out to be commercially valueless. On the other hand, the catalysis of  $CO_2$  uptake by arsenite solutions, which we failed to patent, has proved to be of considerable industrial importance.

**EDSALL:** The report by Dennard and Williams (1966), from which some of these data are taken, does have a short discussion of possible mechanisms. They are a bit inconclusive, but do discuss whether the catalysis involves a direct attack on the  $CO_2$ . They assume that the XO-anion must bind with the carbon temporarily and then become released again. They point out that the X in the case of hypochlorite and hypobromite halogen may be the atom that prefers to attach to the carbon directly, rather than the oxygen. This reaction can then induce the taking on of another oxygen from a water molecule. It still is not clear from their discussion why these anions should have such phenomenally high catalytic coefficients in comparison to some of the others here. I do not think that anyone would have predicted this in advance, even with all the modern knowledge of electronic structure.

**HASTINGS:** The key is thoroughness in doing everything, not brains.

**CAPLOW:** The special catalytic properties of hypochlorous acid may possibly be explained by considering a related system; namely, phosphoramidate acid. The unique thing about phosphoramidate hydrolysis is that it is especially susceptible to electrophilic catalysis. That is, Jencks has observed catalysis for hydrolysis, by hydronium ion, carbonyl compounds, and halonium ions. The special thing about the phosphoramidate structure is that there is an unshared electron pair on nitrogen which can coordinate with any of these electrophiles, thereby making the phosphoryl bond more susceptible to attack and the leaving group a stronger acid. The parallel in the carbonate structure is that it too contains an unshared electron pair for coordination with electrophiles. Reactions of carbonate might, therefore, be expected to be catalyzed by hydronium ion (observed), by aldehydes (this has not been observed although the reactive species in aldehyde hydrate catalysis for decarboxylation may be the free aldehyde), and halonium ions. A scheme for electrophilic catalysis by chlorine is outlined as follows:



In this scheme there is an initial reaction to form an acyl hypochlorite intermediate which breaks down to carbon dioxide and hypochlorite. The first reaction would probably be extremely rapid, and the second reaction involves a displacement of a relatively acidic group ( $pK$  7.5). This reaction would solve the principal problem in bicarbonate dehydration; that is, the difficulty in displacing the highly basic hydroxide ion ( $pK$  15.7). From microscopic reversibility, the reactive species for carbon dioxide hydration would be a hypochlorite ion, which could be expected to be considerably more reactive than water in addition to carbon dioxide. The second reaction would involve chloride removal of chloronium ion from the intermediate.

**POCKER:** There is no incorporation of oxygen from hypochlorous ion into  $HCO_2^-$  during the  $^{18}OCl^-$  catalyzed hydration of  $CO_2$ .

**CAPLOW:** I take it that you are considering the reaction in the direction of hydration. Obviously,

according to this mechanism, this incorporation should be observed. Is this research unpublished?

**POCKER:** Yes; these are our preliminary results.

**CAPLOW:** Well, then the theory would fail.

**POCKER:** It appeared to be a possible explanation, but it just did not work out.

**WANG:** I am not sure about this point, but according to Anbar and Taube's work, hypochlorite exchanges with water (*J. Am. Chem. Soc.*, **80**: 1073, 1958).

**POCKER:** In the absence of chloride ions, the hypochlorite exchange follows the expression,  $\text{rate} = k[\text{HOCl}]$ , where  $k$  is roughly  $1 \text{ min}^{-1}$  at 0 C. The basic form of HOCl, namely  $\text{OCl}^-$ , does not exchange.

**CAPLOW:** At what pH were reactions studied?

**POCKER:** The hydration of CO<sub>2</sub> by  $\text{OCl}^-$  was studied at pH values ranging from 8 to 11, but the test for <sup>18</sup>O incorporation was carried out only in the pH range of 10 to 11; here the rate of <sup>18</sup>O exchange with water is not very fast, but a correction had to be applied to the  $\text{HCO}_3^-$  derived from the term  $k_{\text{OH}^-}[\text{OH}^-]$  which is significant in this region.

**CAPLOW:** Well, at pH 8 a significant fraction of the hypochlorite would be in the form of the neutral acid; I would expect that this would exchange <sup>18</sup>O. It is obviously required that oxygen does not exchange out of hypochlorous acid during the experiment for your results.

**EDSALL:** To be conclusive, would you expect that, if it is the acid that is in the active form, the catalytic efficiency would be higher at lower pH?

**CAPLOW:** I have not as yet considered the effects of pH on the reaction. The hydrogen ion concentration will be reflected in the concentrations of Cl<sub>2</sub>, HOCl, HCO<sub>3</sub>, and CO<sub>3</sub><sup>2-</sup>. It is not unlikely that HOCl might serve as a chlorinating agent in the decarboxylation reaction.

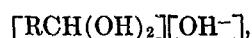
**HASTINGS:** We thought we connected it to the OCl.

**POCKER:** I think so, too. The kinetics are unclear in the acidic region. Above 8.5 or 9, decent kinetics can be obtained. But in the basic region, particularly at the higher pH values, the component due to hydroxide ions,  $k_{\text{OH}^-}[\text{OH}^-]$ , becomes very large. I do not believe in the high value quoted for  $k_{\text{OCl}^-}$  because this catalytic co-

efficient was found to vary with pH by a significant factor, so that it is not really a catalytic constant.

**EDSALL:** I think that Sharma and Danckwerts (1963) reported that the catalytic coefficient appeared to go down somewhat as the pH rose. I think they mentioned that Kiese and Hastings (1940) observed something like that too, but I do not remember.

**POCKER:** I would like to make some comments on the criticisms of the Brønsted equation as it applies to the general base-catalyzed hydration of CO<sub>2</sub>. It is difficult to obtain a satisfactory Brønsted plot for structurally dissimilar bases. Perhaps the best way out is to discuss first "respectable" buffers, namely, those found earlier by R. P. Bell (1966), to conform with the Brønsted relation in other reactions involving the hydration of carbonyl groups or the dehydration of the conjugate carbonyl hydrates. There has been some difference of opinion about the correct method of applying kinetic statistical corrections, but it has been shown by Bell and Evans (1966) that the simplest correct method is to use the equation,  $k_{B/q} = G_B(p/qK_A)^p$ , where  $K_A$  is the acid dissociation constant of the catalyst acid-base pair,  $p$ —the number of equivalent protons in the acid, and  $q$ —the number of equivalent basic sites in the base. Following our studies of the general acid-base and metal ion catalysis of aldehyde hydration (1967, 1968), we have undertaken a similar study of CO<sub>2</sub> hydration. Preliminary data indicate that with H<sub>2</sub>O, CH<sub>2</sub>Cl CO<sub>2</sub><sup>-</sup>, HCO<sub>2</sub><sup>-</sup>, CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, HPO<sub>4</sub><sup>2-</sup>, H<sub>2</sub>BO<sub>3</sub><sup>-</sup>, and OH<sup>-</sup> as "respectable" general bases, one can construct a reasonable plot which conforms with the Brønsted relation over an unusually wide range of  $pK$  values. There are, of course, a good number of catalysts which are off the line. Thus the catalytic coefficient of diethylmalonate dianion (C<sub>2</sub>H<sub>5</sub>)<sub>2</sub>C(CO<sub>2</sub>)<sub>2</sub><sup>2-</sup> is a full log unit below the line representing the Brønsted relationship and defined by the seven bases mentioned. Perhaps I should also add that there are a number of more complicated cases. Thus hydrates of aldehydes, and particularly of dialdehydes, appear to catalyze the hydration of carbonyl systems via two concurrent mechanisms; one involving the term



which can be ascribed to catalysis by the conjugate base  $\text{RCH}(\text{O}^-)\text{OH}$ , and the other involving the term  $[\text{RCH}(\text{OH})_2][\text{B}]$  where  $[\text{B}]$  stands for the concentration of a general base. Perhaps I should also mention that parallel studies in  $\text{D}_2\text{O}$  lead to the following provisional ratios:

$$k_0^{\text{H}_2\text{O}}/k_0^{\text{D}_2\text{O}} \approx 4; \quad k_{\text{OAc}^-}^{\text{H}_2\text{O}}/k_{\text{OAc}^-}^{\text{D}_2\text{O}} \approx 2.8;$$

$$k_{\text{HPO}_4^{2-}}^{\text{H}_2\text{O}}/k_{\text{HPO}_4^{2-}}^{\text{D}_2\text{O}} \approx 2.4$$

and

$$k_{\text{OH}^-}^{\text{H}_2\text{O}}/k_{\text{OH}^-}^{\text{D}_2\text{O}} \approx 0.8$$

**WANG:** Is this ratio for hydration of  $\text{CO}_2$  or aldehyde?

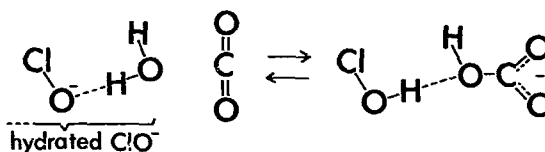
**POCKER:** For  $\text{CO}_2$ . In the hydration of acet-aldehyde,  $k_0^{\text{H}_2\text{O}}/k_0^{\text{D}_2\text{O}} \approx 3.9$  (Pocker and Meany, 1967). In the hydration of 4-pyridine aldehyde,  $k_0^{\text{H}_2\text{O}}/k_0^{\text{D}_2\text{O}} \approx 4.2$  (Pocker and Meany, in press). Kinetic deuterium isotope effects for the  $\text{CO}_2$  hydration are approximate, particularly the  $k_0^{\text{H}_2\text{O}}/k_0^{\text{D}_2\text{O}}$  ratio.

**WANG:** Was it your report about 3 years ago that gave a ratio of about 2?

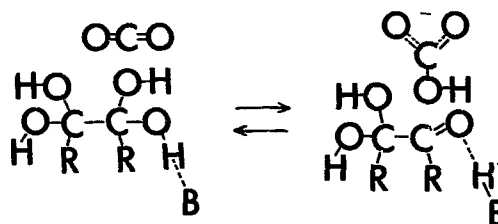
**POCKER:** You must be referring to the turnover number ratios in the enzymic process. I am discussing here the kinetic isotope effects obtained for general base catalysis.

**WANG:** Because we are discussing and speculating on various possibilities of why hypobromite, hypochlorite, arsenite, etc., catalyze the  $\text{CO}_2$  hydration so much faster than other anions, I would like to venture a speculation we had on this for some time, although I was never sure whether it was correct. Because Dr. Hastings is anxious to find out, I guess you would be willing to listen to something that may not turn out to be right. I just want to point out a new possibility. Let us consider the hydration of  $\text{CO}_2$  by the hydroxide ion. Now, this hydroxide ion is not unattached in solution; it is heavily hydrated. We could say, it is hydrogen bonded to water molecules. Consequently before this hydroxide ion can attack the carbon atom of the substrate in a nucleophilic reaction, it has to get rid of some of its hydration water. So in considering the activation step, the free energy of this partial dehydration must be included. One of the secrets of carbonic anhydrase is that the hydroxide ion bound to its zinc is already in juxtaposition to the bound  $\text{CO}_2$  so that the enzyme-substrate complex does not need as

much activation free energy. Now if you look at the other catalysts which are said to be so marvelous, they all interact strongly with solvent water as suggested by their high  $^{18}\text{O}$  exchange rate in neutral and acid solutions. Presumably they do not exist as bare  $\text{BrO}^-$ ,  $\text{ClO}^-$ ,  $\text{AsO}_2^-$  ions in solution but are hydrogen bonded to water molecules. (In the case of  $\text{AsO}_2^-$ , it could even be covalently bonded to an  $\text{OH}^-$  ion to form  $\text{HAsO}_3^{2-}$ .) Each of these hydrated species could, because of its low charge density, get very close to a  $\text{CO}_2$  molecule and rapidly produce an  $\text{OH}^-$  ion through facilitated proton transfer; this  $\text{OH}^-$  ion could begin its nucleophilic attack before it has time to become fully hydrated as illustrated below:



Likewise, the hydrated dialdehydes studied by Dr. Pocker could employ a similar trick when activated by a suitable base B as shown by the following scheme:

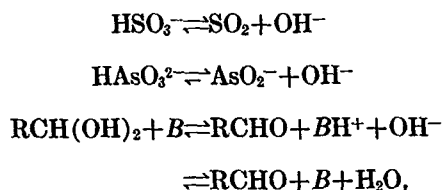


These are just qualitative ideas which may or may not be true. To find out, I have to do more work to translate them into quantitative relationships for comparison with experimental data.

**WANG:**<sup>3</sup> *An Approximate Theory of Base-Catalyzed Hydration of  $\text{CO}_2$ .* Because an  $\text{OH}^-$  in aqueous solution is normally H-bonded to its neighboring water molecules, its reaction with  $\text{CO}_2$  to form  $\text{HCO}_3^-$  must be preceded by the breaking of at least one of its H-bonds; i.e., the formation of an activated  $\text{OH}^-$  which is partially dehydrated. For this reason, molecules or ions which can rapidly release such an activation  $\text{OH}^-$

<sup>3</sup> Submitted later.

right next to a CO<sub>2</sub> molecule are expected to catalyze the hydration of the latter. For example, sulfite and arsenite, as remarked by Dr. Edsall, and hydrated aldehydes, as reported by Dr. Pocker, could catalyze the hydration of CO<sub>2</sub> through such a mechanism:



where  $B$  is a general base.

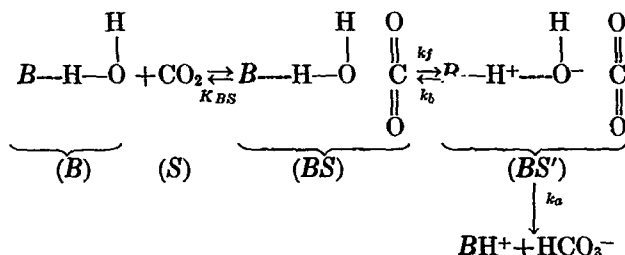
I wonder if the very high catalytic activity of hypochlorite and hypobromite ions, which Dr. Hering referred to, could also be due to some labile hydrated species of these ions.

Let us consider the hydration of CO<sub>2</sub> catalyzed by bases denoted by  $B_i$ , and write the general rate equation as

$$\text{Rate} = (k_{\text{CO}_2} + \sum_i k'_{B_i} [B_i]) [\text{CO}_2] \quad (1)$$

where  $k_{\text{CO}_2}$  is the pseudo-first-order rate constant of the uncatalyzed reaction,  $k'_{B_i}$  the second-order rate constant of the reaction catalyzed by the base  $B_i$ , and the summation is over all base species in solution, e.g., OH<sup>-</sup>, ClO<sup>-</sup>, HPO<sub>4</sub><sup>2-</sup>, CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, etc.

A possible mechanism for the base-catalyzed reaction which makes use of facilitated proton transfer (Wang, 1968) within the solvation shell of the catalyst molecule is illustrated by the following scheme, where for simplicity we consider only the reaction resulting from a single base  $B$ .



If  $B$  bears one unit of net negative charge,  $\text{BH}^+$  is actually neutral. For simplicity let us denote the conjugate acid by  $\text{HB}$  in the following discussion. The symbols  $S$ ,  $BS$ , and  $BS'$  denote substrate, catalyst-substrate complex, and active catalyst-substrate complex, respectively. The constants in the above scheme are defined as follows:

$$K_{BS} = \frac{[B][S]}{[BS]} \quad \text{at equilibrium}$$

$k_f$ ,  $k_b$ ,  $k_a$  = first-order rate constants for the reactions as shown;

$$K' \equiv \frac{k_f}{k_b} = \frac{K_w'}{K_{HB}'}$$

where  $K_w'$  and  $K_{HB}'$  are the theoretical ion product of H<sub>2</sub>O and theoretical dissociation constant of  $\text{HB}$ , respectively, in the catalyst-substrate complex  $BS$  shown above. If we confine our discussion to bases bearing a net negative charge, we may

use the approximation

$$\frac{K_w'}{K_{HB}'} \approx \frac{K_w}{K_{HB}}$$

where  $K_w$  and  $K_{HB}$  are the ion product of H<sub>2</sub>O and dissociation constant of  $\text{HB}$  in solution.

If we also assume  $k_b > k_a$ , so that

$$\frac{[BS']}{[BS]} \approx K'$$

we obtain for the reaction catalyzed by  $B$ ,

$$\begin{aligned} \text{Rate} &\equiv k_{B'} [B][S] = k_a [BS'] \approx K' k_a [BS] \\ &\approx \left( \frac{K'}{K_{BS}} \right) k_a [B][S] \end{aligned}$$

that is,

$$k_{B'} \approx \left( \frac{K'}{K_{BS}} \right) k_a \quad (2)$$

Equation (2) is for solutions in which all catalyst molecules exist in the basic form  $B$ . The following particular cases are of special interest.

(1) For different bases in  $H_2O$  solutions at pH values considerably above all values of  $pK_{HB}$ , we have in the following rewritten form of equation (2)

$$k_B' \approx \left( \frac{1}{K_{BS}} \right) \left( \frac{K_w}{K_{HB}} \right) k_a \quad (3)$$

$k_a$ ,  $K_w$   $\approx$  same constants for all bases, but  $K_{BS}$ ,  $K_{HB}$  may both vary with the nature of the base. Consequently, we do not expect a simple relationship between  $k_B'$  and  $pK_{HB}$  from equation (3).

Furthermore, if, in the pH range of the solutions studied, some of the catalyst molecules exist in protonated form, equation (2) is not applicable and the correlation between  $k_B'$  and  $pK_{HB}$  will be even more complex.

(2) For the same base in  $H_2O$  and  $D_2O$  solutions, respectively, at pH or  $pD \gg pK_{HB}$  or  $pK_{DB}$ , we have  $K_{BS}$ ,  $k_a \approx$  same constants in both solutions, and hence obtain from equation (2) the simple relationship

$$\gamma \equiv \frac{k_B'^H}{k_B'^D} \approx \frac{K'_H}{K'_D} \quad (4)$$

where the indices H and D refer to  $H_2O$  and  $D_2O$  solutions, respectively.

Assuming that the hydrogen isotope effect on the equilibrium between  $BS$  and  $BS'$  is mainly caused by the differences in zero-point vibrational energies, we can rewrite equation (4) in the form

$$\ln \gamma \approx - \frac{h}{2kT} \sum_i (\Delta\nu_i^H - \Delta\nu_i^D) \quad (5)$$

where  $\Delta\nu_i^H$  and  $\Delta\nu_i^D$  represent the changes in vibrational frequencies of the  $i$ th normal mode when  $BS$  becomes  $BS'$  in  $H_2O$  and in  $D_2O$  solutions, respectively; these are usually negative quantities. The summation  $\sum$  is over all normal vibrational modes of the catalyst-substrate complex. If the  $j$ th mode is not appreciably affected by the transformation  $BS \rightarrow BS'$ ,  $\Delta\nu_j^H$  and  $\Delta\nu_j^D$  are automatically zero. Similarly, if the  $k$ th mode is not appreciably affected by changing the solvent from  $H_2O$  to  $D_2O$ ,  $(\Delta\nu_k^H - \Delta\nu_k^D)$  is automatically zero. Consequently only a few terms on the right-hand side of equation (5) need be considered.

Denoting by  $\sum'$  the summation over these few appreciably affected terms and using the approximate relationship

$$\frac{\nu^H}{\nu^D} \approx 1.36 \quad \text{or} \quad \nu^H - \nu^D \approx 0.26\nu^H$$

for O—H frequencies, we can approximate equation (5) by

$$\ln \gamma \approx - \frac{0.13h}{kT} \sum'_i \Delta\nu_i^H \quad (6)$$

For bases of similar structure, the frequencies associated with the B—H bond in  $BS'$  tend to increase and approach the higher frequencies associated with the corresponding O—H bond in  $BS$  as the basic strength of  $B$  increases. Consequently, as the catalyst becomes a stronger base, the sum  $-\sum'_i \Delta\nu_i^H$  will be smaller, and hence  $\gamma$  will also be smaller according to equation (6).

Because of its simplicity, the quantitative correlation of measured  $\gamma$  values with the observed or estimated vibrational frequencies seems more promising than with the theoretical  $pK_{HB}$  values. Unfortunately the relevant data available in the literature seem insufficient for us to reach a definite conclusion on this point.

(3) In the extreme case where  $B$  is the  $OH^-$  ion itself,

$$-\sum'_i \Delta\nu_i^H \approx 0$$

and we obtain from equation (6) the interesting result

$$\gamma \approx 1$$

i.e., negligible isotope effect.

(4) For the opposite extreme case where  $B$  is an  $H_2O$  molecule (a very weak base), one might expect an unusually high value of  $\gamma$  from the above discussion. However, that would be an oversimplification because an order-of-magnitude calculation for this case shows that  $k_f$  of the above mechanism is even smaller than the observed pseudo-first-order rate constant  $k_{CO_2}$ . Consequently the uncatalyzed reaction must take place principally along a different reaction path (or paths), represented by the first term on the right-hand side of equation (1), e.g., nucleophilic attack at the carbon atom of  $CO_2$  by an  $H_2O$

molecule, concerted with, or followed by, proton transfer.

On the other hand, the present approximate theory could be the explanation of the observed catalysis of CO<sub>2</sub> hydration by an unusually wide range of anions.

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*SESSION II*

**Carbamate Reactions  
(With Special Reference to Hemoglobin)**

*Chairman:* F. J. W. ROUGHTON

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# The Carbamates of Glycylglycine and Glycylglycylglycine, Kinetics and Equilibria

N 70 - 23293

MOGENS BALLUND JENSEN

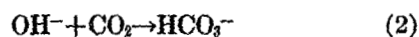
*The Royal Danish School of Pharmacy*

The velocity constants for the reaction between carbon dioxide and the amino groups in glycylglycine and glycylglycylglycine have been determined; the equilibrium constants for the equilibria between carbamates and carbonates have been calculated. The velocities of decomposition of the carbamates in a basic medium have been investigated, and the experimental values of the velocity constants have been compared with the values calculated from a theoretical expression.

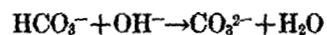
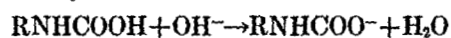
## REACTIONS OF CO<sub>2</sub> AND PEPTIDES

The carbamate reactions of glycine, glycylglycine, and glycylglycylglycine are of interest not only in themselves, but also as models for the carbamate reactions of the amino groups in hemoglobin and other proteins (Roughton and Rossi-Bernardi, 1966; Chipperfield, 1966).

The velocity constant for the reaction between carbon dioxide and the amino group in glycylglycine or glycylglycylglycine in aqueous solution can be determined experimentally by adding a small amount of carbon dioxide to a solution of one of these amines and sodium hydroxide. The carbon dioxide will react according to the equations



followed by



RNH<sub>2</sub> stands for the basic form of either glycylglycine or glycylglycylglycine and RNHCOOH for the corresponding carbamic acids. The solution is immediately analyzed for carbamate and carbonate and the velocity constant,  $k_{\text{CO}_2, \text{Am}}$  for reaction (1) is obtained from the expression

$$\frac{[\text{carbamate}]}{[\text{carbonate}]} = \frac{k_{\text{CO}_2, \text{Am}} \times [\text{RNH}_2]}{k_{\text{CO}_2, \text{OH}^-} \times [\text{OH}^-]}$$

where  $k_{\text{CO}_2, \text{OH}^-}$  is the velocity constant for reaction (2);  $[\text{RNH}_2]$  is the mean value of the initial and final concentrations of the amine, and  $[\text{OH}^-]$  is the mean value of the initial and final concentrations of hydroxyl ion.

In the experiments of Henriksen and Jensen (1967), the amine-sodium hydroxide solution was shaken with a mixture of air and carbon dioxide in a flask. This solution should be prepared immediately before use because hydrolysis of the peptide bonds takes place upon standing. The solution was analyzed for carbonate in the following way. About 10 ml of the solution were placed in a centrifuge tube and a barium chloride solution was added. The carbonate will precipitate as barium carbonate, whereas the carbamate remains in solution. The sample was centrifuged in a hand-driven centrifuge, and the barium carbonate was purified by repeated washing and centrifugation. A known volume of 0.1 N hydrochloric acid was added; then the centrifuge tube was placed in a boiling water bath with a fine stream of air bubbling through the sample in order to expel the carbon dioxide. Back titration was carried out with 0.1 N sodium hydroxide after cooling. The sum of carbamate and carbonate was determined in a similar way, the carbamate being converted to carbonate by heating the solution for some hours before the sample was analyzed; the amount of carbamate was obtained as a difference. Be-



cause carbon dioxide is taken up from the atmosphere during the experimental procedure, it was found necessary to carry out blank determinations. All the following data are calculated from the corrected carbonate determinations. Usually, the correction amounts to 1 to 4 percent of the carbonate content found directly.

The experimental data on the absorption of carbon dioxide by solutions containing sodium hydroxide and glycylglycine or glycylglycylglycine are given in table I. The table also contains the calculated values of  $k_{\text{CO}_2, \text{Am}}$  (liters  $\times$  moles<sup>-1</sup>  $\times$  min<sup>-1</sup>, Briggs' logarithms)<sup>1</sup> and, for the sake of comparison, the value of  $k_{\text{CO}_2, \text{Am}}$  for glycine determined by Jensen, Jensen, and Faurholt (1952).

<sup>1</sup> Chipperfield (1966) and other authors have used units of (liters  $\times$  moles<sup>-1</sup>  $\times$  sec<sup>-1</sup>, Napierian logarithms) for their velocity constant,  $k_2$ . To convert  $k_{\text{CO}_2, \text{Am}}$  to  $k_2$ , the numerical value of  $k_{\text{CO}_2, \text{Am}}$  should be multiplied by 2.303/60, i.e., by 0.0384.

## REACTIONS OF CARBAMATE AND CARBONATE

Carbamates are almost completely decomposed into carbon dioxide or carbonate in acid solution and in strongly basic solution, respectively; in weakly basic solution, equilibrium is established between the carbamate and carbonate. Table II presents the data from the equilibrium experiments carried out in solutions containing RNH<sub>3</sub><sup>+</sup> and RNH<sub>2</sub>. The carbamate solutions were prepared by shaking the amine-aminium ion solution with carbon dioxide. The solutions were analyzed for carbonate and carbamate in the aforementioned way. Table II also gives the equilibrium constant,  $K_{\text{Eq}}$ , for the reaction



and, in addition, the value of  $K_{\text{Eq}}$ <sup>2</sup> for glycine determined by Jensen et al. (1952).

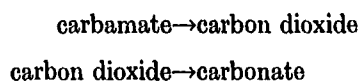
TABLE I.—Carbon Dioxide in Peptides and NaOH at 18 C

|                          | Initial solution   |                     | Ab-sorbed CO <sub>2</sub> , moles/liter | Percent carba-mate | Final solution     |                     | Mean               |                     | log $k_{\text{CO}_2, \text{Am}}$ |
|--------------------------|--------------------|---------------------|---|--------------------|--------------------|---------------------|--------------------|---------------------|----------------------------------|
|                          | [OH <sup>-</sup> ] | [RNH <sub>2</sub> ] |   |                    | [OH <sup>-</sup> ] | [RNH <sub>2</sub> ] | [OH <sup>-</sup> ] | [RNH <sub>2</sub> ] |                                  |
| Glycine.....             |                    |                     |   |                    |                    |                     |                    |                     | 5.05                             |
| Glycylglycine.....       | 0.097              | 0.099               | 0.0122                                  | 35.2               | 0.077              | 0.094               | 0.087              | 0.097               | 4.63                             |
|                          | .100               | .200                | .0195                                   | 47.4               | .070               | .191                | .085               | .195                | 4.61                             |
|                          | .110               | .290                | .0201                                   | 55.7               | .081               | .279                | .096               | .285                | 4.65                             |
| Glycylglycylglycine..... | .094               | .098                | .0192                                   | 27.5               | .061               | .093                | .078               | .096                | 4.51                             |
|                          | .099               | .150                | .0205                                   | 39.9               | .066               | .142                | .082               | .146                | 4.59                             |

TABLE II.—The Solution of Carbonate-Carbamate in Equilibrium at 18 C

|                     | Initial solution                 |              |                                  |                     | Percent carba-mate | Equilibrium                      |              |                                  |                     | log $K_{\text{Eq}}$ |
|---------------------|----------------------------------|--------------|----------------------------------|---------------------|--------------------|----------------------------------|--------------|----------------------------------|---------------------|---------------------|
|                     | [HCO <sub>3</sub> <sup>-</sup> ] | [Carba-mate] | [RNH <sub>3</sub> <sup>+</sup> ] | [RNH <sub>2</sub> ] |                    | [HCO <sub>3</sub> <sup>-</sup> ] | [Carba-mate] | [RNH <sub>3</sub> <sup>+</sup> ] | [RNH <sub>2</sub> ] |                     |
| Glycine.....        |                                  |              |                                  |                     |                    |                                  |              |                                  |                     | -1.48               |
| Glycylglycine       | 0.022                            |              | 0.040                            | 0.060               | 75.5               | 0.0052                           | 0.016        | 0.040                            | 0.044               | -1.85               |
|                     |                                  | 0.021        | .041                             | .038                | 77.7               | .0047                            | .016         | .041                             | .043                | -1.91               |
|                     |                                  | .015         | .055                             | .029                | 73.3               | .0048                            | .011         | .055                             | .034                | -1.81               |
| Glycylglycylglycine | .020                             |              | .10                              | .10                 | 88.4               | .0022                            | .017         | .10                              | .082                | -1.96               |
|                     |                                  | .020         | .040                             | .041                | 83.5               | .0033                            | .016         | .040                             | .044                | -2.06               |

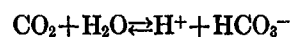
When  $k_{CO_2,Am}$  and  $K_{Eq}$  are known, it is possible to calculate the velocity of the decomposition of the carbamates to carbonate. The conversion is a two-step reaction:



and takes place according to a first-order scheme at fixed concentrations of amine and hydroxyl ion. The first-order velocity constant,  $k_{amate}$  can be calculated from the expression put forward by Lund and Faurholt (1948):

$$k_{amate} = \frac{k_{CO_2,Am} \times K_w \times 1/K_{CO_2} \times K_{Eq}}{[OH^-] + \frac{k_{CO_2,Am}}{k_{CO_2,OH^-}} \times [RNH_2]}$$

where,  $K_{CO_2}$  is the equilibrium constant for the reaction



and  $K_w$  is the dissociation constant of water.

Table III shows the experiments that were carried out at a pH of approximately 11. The solutions were analyzed for carbonate and carba-

TABLE III.—Velocity Constants for the Process: Carbamate → Carbonate at 18 C, pH = approximately 11

|                           | Initial solution |                     | Minutes | Percent carbamate left | $k_{amate}$ |
|---------------------------|------------------|---------------------|---------|------------------------|-------------|
|                           | [Carbamate]      | [RNH <sub>2</sub> ] |         |                        |             |
| Glycylglycine -----       | 0.019            | 0.079               | 0       | 100.0                  |             |
|                           |                  |                     | 1146    | 45.3                   | 0.000302    |
|                           |                  |                     | 1385    | 39.3                   | 0.000294    |
|                           |                  |                     | 1514    | 37.7                   | 0.000281    |
|                           |                  |                     | 2595    | 17.2                   | 0.000295    |
|                           |                  |                     | 2974    | 13.0                   | 0.000299    |
|                           |                  |                     | 3171    | 10.6                   | 0.000307    |
|                           |                  |                     | mean    |                        | 0.00030     |
|                           | calc.            |                     | 0.00032 |                        |             |
|                           | 0.021            | 0.18                | 0       | 100.0                  |             |
|                           |                  |                     | 1129    | 69.8                   | 0.000138    |
|                           |                  |                     | 1477    | 61.5                   | 0.000144    |
|                           |                  |                     | 2590    | 42.6                   | 0.000143    |
|                           |                  |                     | 2769    | 39.6                   | 0.000146    |
| 4440                      |                  |                     | 25.2    | 0.000134               |             |
| 5632                      |                  |                     | 14.2    | 0.000142               |             |
| mean                      |                  |                     |         | 0.00014                |             |
| calc.                     |                  | 0.00015             |         |                        |             |
| Glycylglycylglycine ----- | 0.019            | 0.081               | 0       | 100.0                  |             |
|                           |                  |                     | 225     | 89.4                   | 0.000220    |
|                           |                  |                     | 393     | 82.8                   | 0.000208    |
|                           |                  |                     | 1323    | 50.3                   | 0.000224    |
|                           |                  |                     | 1770    | 40.3                   | 0.000222    |
|                           |                  |                     | 3260    | 17.3                   | 0.000234    |
|                           |                  |                     | mean    |                        | 0.00022     |
|                           |                  |                     | calc.   |                        | 0.00023     |

mate in the aforementioned way. Table III also gives the experimental values of  $k_{\text{amate}}$  as well as the values calculated from the expression stated previously. It will be seen that there is good agreement between calculated and experimental values of  $k_{\text{amate}}$ .

#### COMPARISON WITH RESULTS BY OTHER METHODS

The velocity constants of the reactions between carbon dioxide and the amine groups of glycine and glycyglycine (but not glycyglycyglycine) have also been determined by Chipperfield (1966) by means of the rapid thermal method, which is, of course, quite independent of and different from the method used in the present paper. According to Chipperfield, the thermal results agree with the present ones within the limits of the combined accuracy of the two methods.

The equilibrium constant of the reaction be-

<sup>2</sup> Roughton and Rossi-Bernardi (1966) and others have used the equilibrium constant

$$K_e = \frac{[a_h][\text{RNHCOO}^-]}{[\text{CO}_2][\text{RNH}_2]}$$

where  $K_e = K_1/K_{Ea}$  and  $K_1$  is the apparent first ionization constant of carbonic acid; i.e.

$$K_1' \{ [\text{CO}_2] + [\text{H}_2\text{CO}_3] \} = a_h f [\text{HCO}_3^-]$$

tween carbon dioxide and the amine group of glycyglycine has also been measured by Roughton and Rossi-Bernardi (1966),<sup>2</sup> both by an independent CO<sub>2</sub> electrode method and by a "total equilibrium" method. A comparison of their results with those of the present method was considered by them to be reasonably good because of the inherent experimental errors in the several methods employed and of the theoretical uncertainty of the activity coefficient corrections.

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# Studies on the $pK$ and Rate of Dissociation of the Glycylglycine-Carbamic Acid Molecule<sup>1</sup>

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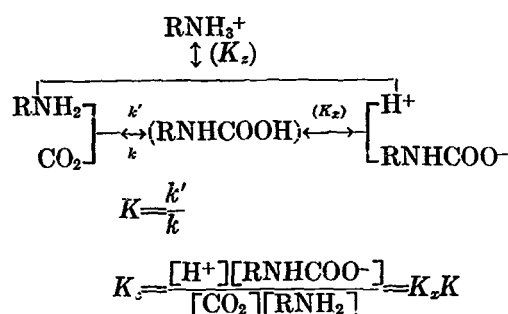
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The carbamate reactions of  $\text{CO}_2$  with amines are supposed to take place via the mechanisms shown here:



It will be noted that in this scheme,  $\text{CO}_2$  combines only with the unprotonated form of the amine,  $\text{RNH}_2$ , and not at all with the cationic form,  $\text{RNH}_3^+$ . The latter, however, participates indirectly in the overall process, by virtue of being always in "instantaneous" equilibrium with two of the primary reactants— $\text{RNH}_2$  and  $\text{H}^+$  ions.

Measurements of the following items have already been made for a large range of amines:

(a) The bimolecular velocity constant  $k'$  of the reaction  $\text{CO}_2 + \text{RNH}_2 \rightarrow \text{RNHCOOH}$

(b) The equilibrium constant  $K_c$  of the overall reaction  $\text{CO}_2 + \text{RNH}_2 \rightleftharpoons \text{RNHCOO}^- + \text{H}^+$

(c) The ionization constant  $K_x$  of the amines

Only preliminary estimates have been available as to:

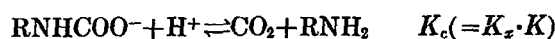
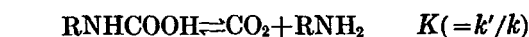
(d) The velocity constant  $k$  of the dissociation reaction  $\text{RNHCOOH} \rightarrow \text{CO}_2 + \text{RNH}_2$

(e) The ionization constant  $K_x$  of the unstable carbamic acids  $\text{RNHCOOH}$

The present paper reports kinetic measurements of  $k$ , and thence, by calculation, of  $K_x$ , which is equal to  $kK_c/k'$  (see below).

## Carbamate + Acid

### Equilibria:



$$\text{Note: } K_c = \frac{(\text{RNHCOO}^-)(\text{H}^+)}{(\text{CO}_2)(\text{RNH}_2)} \quad K_x = kK_c/k'$$

### Kinetics:

$$\ln (h_1/h_2) = 2k(t_2 - t_1) + \ln (h_1 + 2K_x)/(h_2 + 2K_x)$$

Before entering into the details of this new kinetic work, it may be useful to discuss the importance of knowing the values of  $K_x$  and  $k$ , and to review briefly the previous attempts to estimate  $K_x$ .

Faurholt (1925; see also his earlier references quoted in that paper), in the course of his early attempts to estimate  $K_x$  for the carbamic acid derivatives of ammonia and dimethylamine, pointed out that a knowledge of  $K_x$ , or at least of its order of magnitude, is needed for the proper physicochemical interpretation of experiments on aqueous solutions of carbamates. Values for  $pK_x$  would also be meaningful in discussions on the

<sup>1</sup>This research has been supported in part by a grant from the Consiglio Nazionale delle Ricerche, Rome.

chemical structure of the carbamic acids. From the more physiological angle, there is also the question as to how far the carbamic acids, especially of hemoglobin, can themselves act significantly as buffers under physiological conditions (e.g., pH 7.0–7.4), and this in turn requires a knowledge of  $pK_z$ .

Faurholt's early estimates were based on attempts to determine the degree of hydrolysis in carbamate solutions soon after (e.g., 1 minute) dissolving the solid carbamate in water, using for the purpose determinations of pH by colorimetry or electrical conductivity for the carbamic acid of ammonia (NH<sub>2</sub>COOH). At 0 C he obtained a preliminary value for  $pK_z$  of about 7.1, but admitted that this figure might be uncertain to the extent of 1 or 2 pH units. The next attempt by a rapid thermal method (Roughton, 1941), gave a value of  $pK_z$  for NH<sub>2</sub>COOH of around 5.8 at 0 C. More recently, Roughton and Rossi-Bernardi (1966) gave two independent indications that the  $pK_z$  for the carbamic acid of glycylglycine, which is a good model for hemoglobin, was not greater than 6.0.

#### DESCRIPTION OF METHOD AND TYPICAL EXPERIMENT AT 3–5 C

The present measurements have been made with a continuous flow Hartridge-Roughton rapid reaction method, the progress of the reaction being followed by glass electrode pH determinations over a period of 0 to 20 msec. This method, which is illustrated in figure 1, has been applied primarily to glycylglycine carbamic acid, though some experiments have also been done on the carbamic acids of ammonia and glycine. Glycylglycine was preferred because:

- (1) Its lower value of  $k$  (about 200 sec<sup>-1</sup> at 5 C, compared with 600 to 800 sec<sup>-1</sup> for ammonia and glycine);
- (2) In glycylglycine the —NH<sub>2</sub> group is adjacent to a CO—NH linkage and, consequently, has its  $pK_z$  lowered from about 10 (as in ammonia and glycine) to about 8.0, i.e., to a value of the same order as that of the terminal  $\alpha$ -NH<sub>2</sub> groups of the four chains in hemoglobin, which are also adjacent to CO—NH linkages. It is these terminal  $\alpha$ -NH<sub>2</sub> groups in hemoglobin that

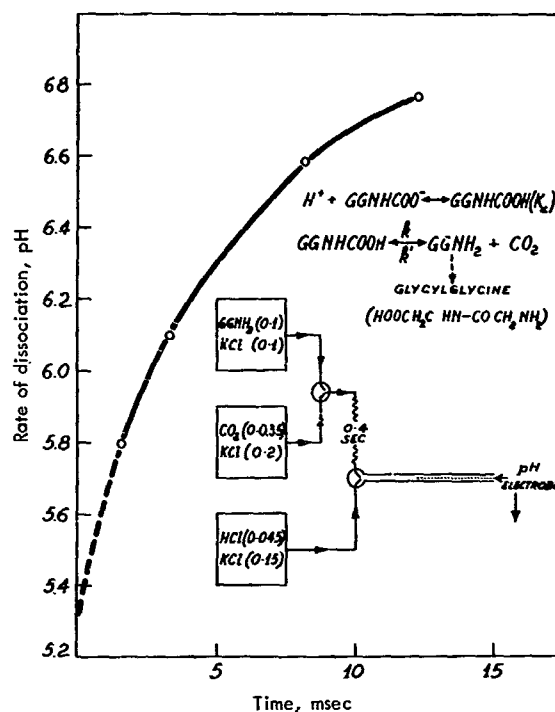


FIGURE 1.—Arrangement for measuring the rate of dissociation of carbamic acids, together with a graph of the results obtained in a typical experiment on the rate of dissociation of glycylglycine carbamic acid.

are believed to be solely responsible for carbamate formation under physiological conditions: for this reason glycylglycine is, as stated above, a good model for hemoglobin.

The details in a typical experiment are as follows.

A solution of sodium glycylglycinate (0.1 M) from one syringe was driven into a Hartridge-Roughton mixing chamber where it met with a CO<sub>2</sub> solution (e.g., 0.035 M) from a second syringe. The emerging mixed solution passed into a second mixing chamber where it met with a solution of 0.045 M HCl from a third syringe. The intermediary volume of tubing between the two mixing chambers was adjusted to give a lapsed time of about 0.4 second, when the three syringes were being driven in at their usual rates. This interval was sufficient, at 3–5 C, for practically all the CO<sub>2</sub> to be converted to glycylglycine carbamate during the passage of the fluid from the first to the second mixing chamber. In the

case of ammonia, for which the value of  $k'$  is much smaller than for glycine or glycyglycine, the lapsed time was increased to 3 or 4 seconds to insure complete carbamate equilibrium (but, of course, without significant bicarbonate or carbonate formation). The pH of the moving fluid emerging from the second mixer was measured at various distances along the observation tube by means of an exploratory glass electrode. The syringes and observation tubes were all jacketed by circulating water to maintain a temperature of 3–5 C. Appropriate amounts of KCl were added to each syringe to give an ionic strength of 0.15  $M$  in the fluid traversing the observation tube.

**THE KINETIC EQUATIONS FOR THE DISSOCIATION OF GLYCYLGLYCINE CARBAMIC ACID IN THE pH RANGE 5.0–7.0**

During the early stages, in which the velocity of the back reactions can be neglected, the rate of formation of  $CO_2$  from the dissociation of carbamic acid,  $RNHCOOH$ , is

$$\frac{d[CO_2]}{dt} = k[RNHCOOH] = \frac{k[RNHCOO^-]h}{K_x} \quad (1)$$

where

$$h = \text{hydrogen ion concentration}$$

$$R = -OOCH_2CHNCOCH_2.$$

The values of  $[H^+]$ ,  $[OH^-]$ , and  $[RNH_2]$  can be neglected in the pH range 5 to 7, and the condition for electroneutrality of the solution is

$$2[RNHCOO^-] + [RNHCOOH] + [Cl^-] = [K^+] \quad (2)$$

Similarly, the principle of conservation, as applied to the total carbon dioxide content of the solution, gives

$$[Total CO_2] = [RNHCOOH] + [RNHCOO^-] + [Free CO_2] \quad (3)$$

while as regards the total nitrogen content

$$[Total N] = [RNHCOOH] + [RNHCOO^-] + [RNH_3^+] \quad (4)$$

From equation (2), it follows that

$$[RNHCOOH] \left( 1 + \frac{2K_x}{h} \right) = [K^+] - [Cl^-] = \phi,$$

or

$$[RNHCOOH] = \frac{\phi h}{h + 2K_x} \quad (5)$$

From equations (1) and (3)

$$\begin{aligned} \frac{d[CO_2]}{dt} &= \frac{d}{dt} \{ [Total CO_2] \\ &\quad - [RNHCOOH] - [RNHCOO^-] \} \\ &= -\frac{d}{dt} \left\{ [RNHCOOH] \frac{h + K_x}{h} \right\} \quad (6) \end{aligned}$$

and therefore, from equations (1), (5), and (6)

$$\begin{aligned} \frac{d[CO_2]}{dt} &= k[RNHCOOH] = k \frac{\phi h}{h + 2K_x} \\ &= -\frac{d}{dt} \left\{ \phi \frac{h + K_x}{h + 2K_x} \right\} \quad (7) \end{aligned}$$

whence, after various simplifications

$$\frac{dh}{dt} = -\frac{h(h + 2K_x)}{K_x} \quad (8)$$

Integration of equation (8) over the range  $t_1 (h = h_1)$  to  $t_2 (h = h_2)$  then gives

$$\begin{aligned} \ln \frac{h_1}{h_2} &= 2k(t_2 - t_1) + \ln \left( \frac{h_1 + 2K_x}{h_2 + 2K_x} \right) \\ &= 2k(t_2 - t_1) + \ln \left( \frac{h_1 + 2kK_c/k'}{h_2 + 2kK_c/k'} \right) \quad (9) \end{aligned}$$

Since  $k'$  and  $K_c$  are known independently, the value of  $k$  can be computed from measurements of  $h$  at two times,  $t_1$  and  $t_2$ . In practice, a simple way of solving equation (9) for  $k$  is to start by neglecting the second term on the right-hand side, which is much smaller than the left-hand side term; the numerical value of  $k$  so obtained is inserted in the second term on the right-hand side and the equation solved again for  $k$ . Two or three such iterations give a satisfactorily convergent value for  $k$ .

**PRELIMINARY RESULTS FOR  $k$  AND  $K_z$   
(AT ABOUT 5 C)**

The time range used for glycylglycine was from 1.64 to 3.28 msec (corresponding pH range, 5.8 to 6.1). The results are believed to be more reliable than for ammonia and glycine in which, because of the far greater rate of dissociation of the corresponding carbamic acids, the time range had to be reduced to 1.34 to 1.68 msec (pH range, 6.4 to 6.8).

TABLE I.—Approximate Velocity and Ionization Constants of RNHCOOH

| Amine              | $k'$  | $pK_c$ | $k$   | $pK_z$           |
|--------------------|-------|--------|-------|------------------|
| Ammonia.....       | 100   | 6.0    | 560   | 5.2 <sub>s</sub> |
| Glycine.....       | 2500  | 4.7    | 830   | 5.1 <sub>s</sub> |
| Glycylglycine..... | 750   | 4.8    | 220   | 5.3 <sub>s</sub> |
| Acetic acid.....   | ----- | -----  | ----- | 4.77             |

Temperature about 5 C.

Table I shows the preliminary results thus obtained for  $k$  and  $pK_z$  in the case of ammonia, glycine, and glycylglycine at about 5 C and the independently measured values of  $k'$  and  $pK_c$  for these three amines. There is, as already mentioned, a considerable spread in the figures for  $k$  for the three amines, but the values of  $pK_z$ , on the other hand, are relatively constant, being 0.4 to 0.5 unit higher than the  $pK$  for acetic acid.

Since the above experiments were carried out, an improved reaction velocity apparatus with a shorter time resolution has been used. Results with the new apparatus have confirmed those previously obtained with the old: the next step will be to extend the experiments to a wider range of pH, temperature, etc., than has hitherto been feasible.

**EFFECT OF STAGNANT FILMS**

When a solid object is placed in a moving fluid, there is a tendency for a relatively stagnant layer of fluid to form at the interface. If a chemical reaction is occurring in the moving fluid, the time in which the reaction has taken place, as calculated from the average rate of fluid flow and the distance of the electrode from the mixer, may be

appreciably shorter, e.g., 3 to 5 msec, than the actual time of reaction of a fluid in immediate contact with the electrode. If, for any given experiment, such delay time  $\tau$  is constant for different positions of the electrode in the observation tube, then the application of equation (9) should not be affected, because  $t_1$  and  $t_2$  would have to be replaced by  $t_1 + \tau$  and  $t_2 + \tau$ , respectively, and time interval  $t_2 - t_1$  would thus remain unaltered.

**SIGNIFICANCE OF THE NUMERICAL  
VALUES FOR  $pK_z$**

Glycine, or amino-acetic acid, exists at pH around 5.0 primarily in the dipolar form ( $\text{NH}_3^+\text{CH}_2\text{COO}^-$ ), the classical un-ionized form ( $\text{NH}_2\text{CH}_2\text{COOH}$ ) only being present to less than 0.0004 percent (Cohn and Edsall, 1943). The alkaline  $pK$ , corresponding to the ionization  $\text{NH}_3^+\text{CH}_2\text{COO}^- \rightleftharpoons \text{NH}_2\text{CH}_2\text{COO}^- + \text{H}^+$ , is 9.78 at 25 C, whereas the acid  $pK$  (for the ionization  $\text{NH}_3^+\text{CH}_2\text{COO}^- + \text{H}^+ \rightleftharpoons \text{NH}_3^+\text{CH}_2\text{COOH}$ ) is 2.35 at 25 C. The ionization of carbamic acid, which might be regarded as amino formic acid, is very different. The present studies suggest that at pH about 5.0, it is largely in the classical form  $\text{NH}_2\text{COOH}$ , rather than the dipolar form  $\text{NH}_3^+\text{COO}^-$ , the existence of which is probably inhibited by resonance conditions, which are unable to operate in glycine (Dr. J. T. Edsall, private communication; see also Cohn and Edsall, 1943). Had the reaction  $\text{NH}_2\text{COO}^- + \text{H}^+ \rightarrow \text{NH}_3^+\text{COO}^-$  been significant, there might have been a large heat of ionization, as in the case of  $\text{NH}_2\text{CH}_2\text{COO}^- + \text{H}^+ \rightarrow \text{NH}_3^+\text{CH}_2\text{COO}^-$  (i.e., 10 500 calories per  $\text{H}^+$ ). Old, unpublished observations by Roughton, using the rapid thermal method, failed to reveal any such large heat of ionization, the situation being apparently analogous to the heat of acetic acid ionization,  $\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_3\text{COOH}$ , which is only of the order of 100 calories. These considerations, together with the fact that  $pK_z$  is so close to the  $pK$  of acetic acid, make it seem likely that the electrically neutral form is predominantly  $\text{NH}_2\text{COOH}$  rather than  $\text{NH}_3^+\text{COO}^-$ .

Theoretically the  $\text{NH}_2\text{COOH}$  form might be able, at more acidic pH, to add on a proton at the N atom to give  $\text{NH}_3^+\text{COOH}$ . No such reaction has, as yet, been demonstrated, but Dr. J. T.

Edsall (in a private discussion) has expressed the view that such "basic" power of carbamic acid should be similar to that of the urethanes ( $H_2NCOOR$ , where  $R=CH_3, C_2H_5$ , etc.), which are believed to be very weakly basic, rather like the acid amides,  $RCONH_2$ , the  $pK$ 's of which are in the range of 1.0.

The effect of temperature on the  $pK$  of acetic acid is slight, and if the same is true by analogy of the effect of temperature on  $pK_z$ , the value of the latter at mammalian body temperature, 37 C, would only be around 5.0 to 5.2 and, accordingly, the carbamic acids would have no significant buffer power under mammalian physiological conditions (pH 7.0-7.4, 37 C). If, therefore, with regard to  $pK_z$ , the carbamic acids of hemoglobin behave similarly to the carbonic acids of ammonia, glycine, and glycylglycine, there should be no need to take into account their buffer power under the conditions of  $CO_2$  transport in the

animal body; and the current practice of regarding the hemoglobin carbamic acids as "completely" ionized at physiological pH would certainly be valid. Such, however, would not have been the case had further work substantiated Faurholt's preliminary, though very guarded, estimate of  $pK_z=7.1$  for the ionization of carbamic acid.

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## Carbamate Formation and Breakdown—Kinetics and Mechanism<sup>1</sup>

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As part of a study of the chemical and kinetic properties of the N-carboxybiotin intermediate formed in enzyme-catalyzed carbon dioxide transfer reactions (Caplow, 1965; Caplow and Yager, 1967; Caplow, 1968), we have investigated the kinetic behavior of carbamates of widely varying structure and basicity. In these studies we used the previously developed barium-quenching technique (Faurholt, 1925) in which carbamates are separated from carbonate by the differential solubility of their barium salts. We have modified this procedure by use of radioactive carbon dioxide of high specific activity. This modification greatly facilitates the assay of carbamate and carbonate, which were separated using a Millipore filter installed on a hypodermic syringe. Because virtually no amine or hydroxide is consumed in the course of the reaction with radioactive gas, the reactions are pseudo-first order even at very low hydroxide and amine concentrations.

The products formed in the reaction of carbon dioxide with alkaline amine solutions are determined by the constants and concentrations in equation (1).

$$\frac{[\text{Carbamate}]}{[\text{Carbonate}]} = \frac{k_{\text{amine}}[\text{amine}]}{k_{\text{OH}^-}[\text{OH}^-]} \quad (1)$$

Using a previously determined value of  $k_{\text{OH}^-}$  equal to  $2400 \text{ M}^{-1} \text{ sec}^{-1}$  at  $10 \text{ C}$  (Sirs, 1958), we may readily determine the rate constant for reactions of amines with carbon dioxide. The results obtained in a typical experiment with hydrazine are plotted in figure 1 according to equation (1).

<sup>1</sup>This work was supported by grant GM-11820 from the National Institute of Health.

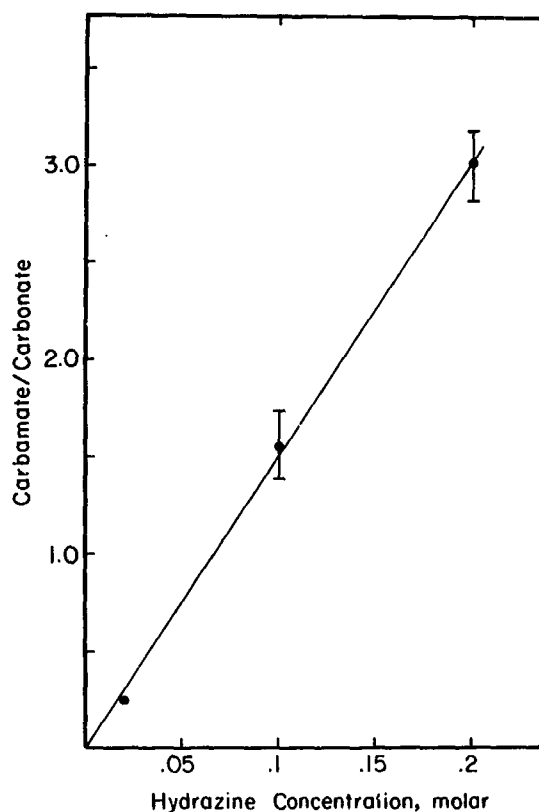


FIGURE 1.—Product ratio obtained in the reaction of hydrazine with carbon dioxide at pH 13.70. (Reprinted with the permission of the American Chemical Society.)

It was found, however, that in the reaction of several amines the second-order rate constant determined from the slope of a plot similar to the one given in figure 1 was not constant at varying hydroxide concentrations. The hydroxide dependence of the reaction of benzylamine is illus-

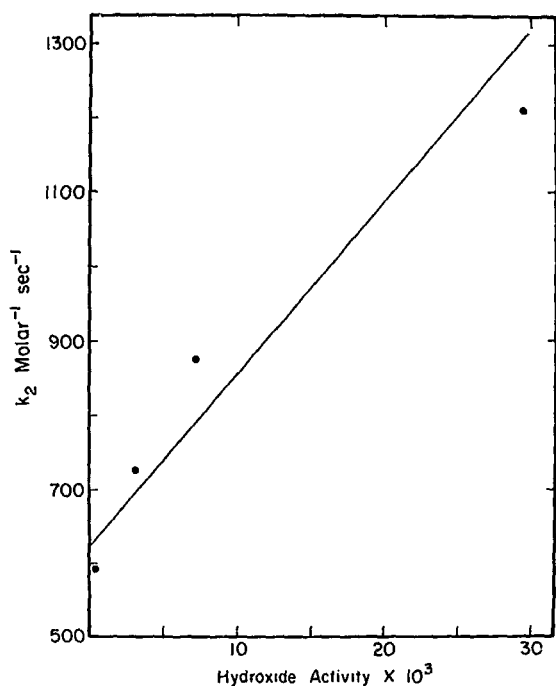


FIGURE 2.—Hydroxide ion dependence of the second-order rate constant for reaction of benzylamine with carbon dioxide. (Reprinted with the permission of the American Chemical Society.)

trated in figure 2. These results have been interpreted in terms of a hydroxide-catalyzed aminolysis reaction which has been included in the rate law of equation (2)

Carbamate

Carbonate

$$= \frac{k_{\text{amine}}[\text{amine}] + k'_{\text{amine}}[\text{amine}][\text{OH}^-]}{k_{\text{OH}^-}[\text{OH}^-]} \quad (2)$$

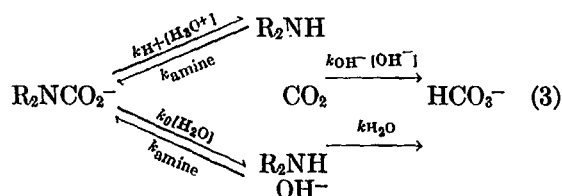
Third-order rate constants for aminolysis ( $k'_{\text{amine}}$ ) are summarized in table I. The hydroxide catalysis involves proton removal as part of the rate-limiting step because the amines are extremely nonacidic (Bell, 1959), and for the reaction of an amine anion to contribute significantly to the observed rate at the pH's studied, the second-order rate constants for reaction of the amine anion would have to greatly exceed that of a diffusion-controlled reaction. A Brønsted plot of the rate constants for uncatalyzed aminolysis is given in figure 3. Separate lines of slope 0.43

and 0.48 have been drawn for the reactions of primary and secondary amines and reactions of amines which, because of the  $\alpha$ -effect, have been found to react as a separate class of compounds in several acyl transfer reactions (Bruice, Donzel, Huffman, and Butler, 1967). Chipperfield (1966) has observed a Brønsted  $\beta$  of 0.262 for amino acid-carbon dioxide reactions.

TABLE I.—Third-Order Rate Constants for the Reaction of Amines With Carbon Dioxide at 10 C

| Amine                     | $k_{\text{amine}}, \text{M}^{-2} \text{sec}^{-1}$ |
|---------------------------|---|
| Acetohydrazide.....       | 15 000  |
| Aniline.....              | 388   |
| Benzylamine.....          | 20 800  |
| <i>n</i> -Butylamine..... | 5 300   |
| Hydroxylamine.....        | 13 000  |
| Morpholine.....           | 24 000  |
| Piperidine.....           | 57 000  |
| Trifluoroethylamine.....  | 22 000  |

A reaction scheme for the decomposition of carbamates is given in equation (3).



Decarboxylation proceeds via acid-catalyzed and acid-uncatalyzed pathways, which correspond, respectively, to the reverse of the uncatalyzed and hydroxide-catalyzed pathways observed in carbamate formation. Typical rate data obtained with morpholine carbamate are shown in figure 4. The specific acid catalyzed reaction predominates, even at very high pH, and the hydrogen ion dependence on the rate of decarboxylation of morpholine carbamate is given in figure 5. The equilibrium constant for carbamate formation (eq. (5))



reflects the tendency of an amine proton to be replaced by an alternate electrophile. This constant may be calculated from the ratio of rate

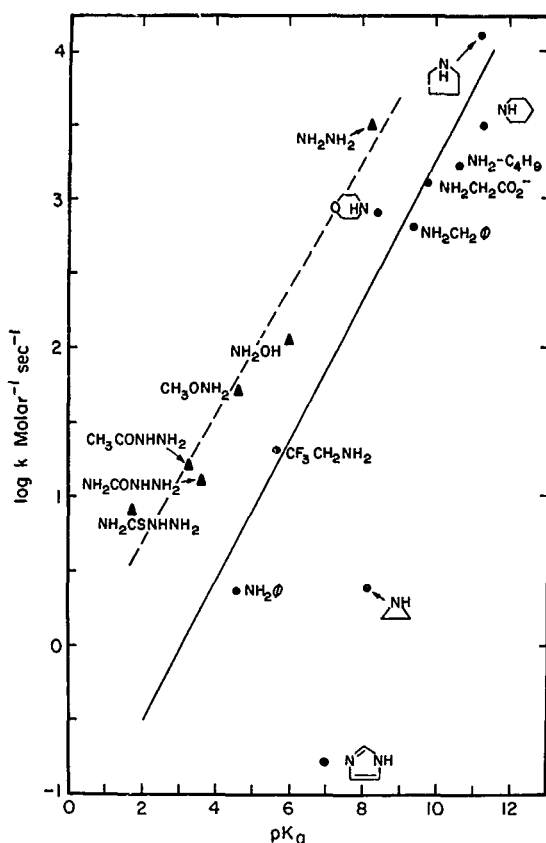


FIGURE 3.—Brønsted plot for the uncatalyzed reaction of amines with carbon dioxide at 10 C. (Reprinted with the permission of the American Chemical Society.)

constants. The results summarized in figure 6 indicate that, as in the case for replacement of an amine proton by a hydroxymethyl group (Kallen and Jencks, 1966), the equilibrium is insensitive to amine basicity and varies by a factor of only 2060 over a  $pK$  range of more than 10 units.

#### The Rate-Limiting Step

A mechanism for carbamate formation and breakdown is outlined in equation (6). In the direction of synthesis,

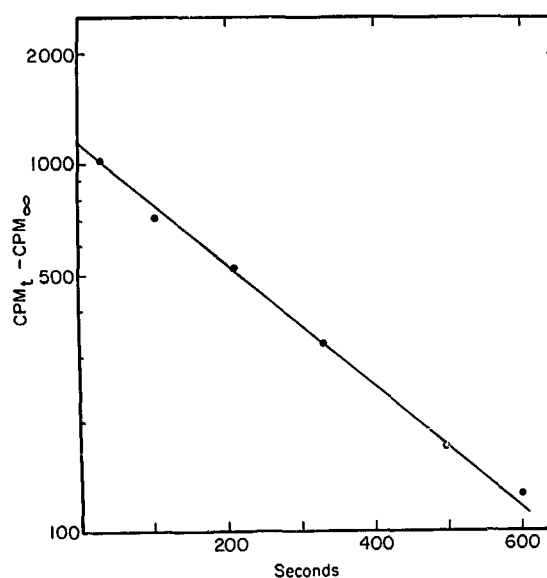
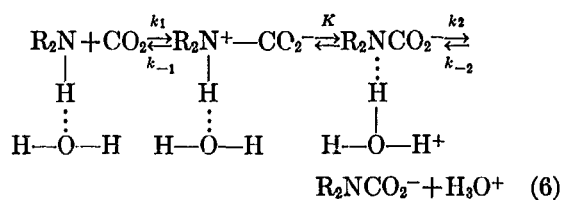


FIGURE 4.—Decarboxylation of morpholine carbamate at pH 10.72. (Reprinted with the permission of the American Chemical Society.)

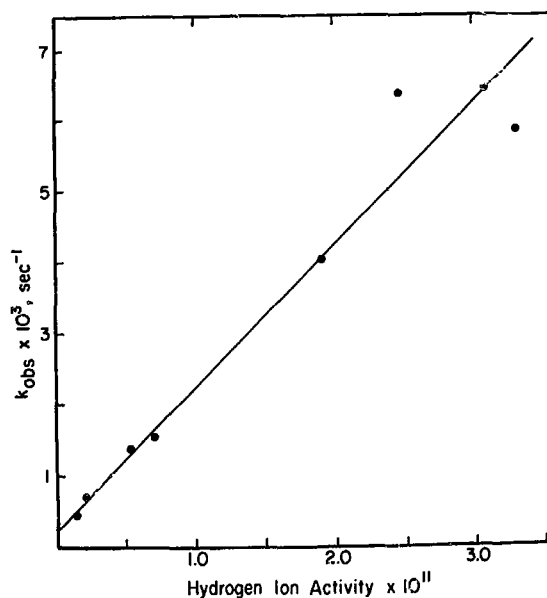


FIGURE 5.—Dependence of the first-order rate constant for decarboxylation of morpholine carbamate on the hydrogen-ion activity. (Reprinted with the permission of the American Chemical Society.)

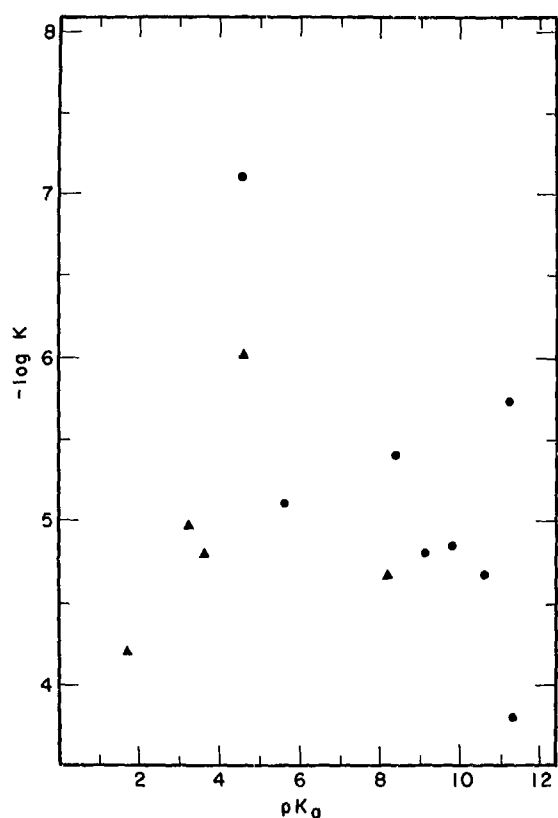
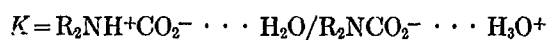


FIGURE 6.—Equilibrium constant for carbamate formation as a function of amine basicity. (Reprinted with the permission of the American Chemical Society.)

following carbon-nitrogen bond formation, the amine proton is transferred to a water molecule to give charged products that dissociate at a diffusion-limited rate. Proton transfer occurs within a hydrogen-bonded complex and is exceedingly rapid in the direction of favorable equilibrium. Rate laws for reactions under conditions where the reverse reaction is negligible are given in equations (7) and (8), where



Formation:

$$\begin{aligned} \text{Rate} &= k_{\text{amine}}[R_2NH][CO_2]; \\ k_{\text{amine}} &= k_1k_2 / (k_{-1}K + k_2) \end{aligned} \quad (7)$$

Breakdown:

$$\begin{aligned} \text{Rate} &= k_{H^+}[R_2NCO_2^-][H^+]; \\ k_{H^+} &= k_{-2}k_{-1}K / (k_{-1}K + k_2) \end{aligned} \quad (8)$$

According to this scheme, the nature of the rate-limiting step is determined by the relative size of  $k_2$  and  $k_{-1}K$ . That is, if dissociation of the hydrogen-bonded complex to carbamate and hydronium ion is faster than loss of carbon dioxide ( $k_2 > k_{-1}K$ ), the highest point in the energy profile, i.e., the rate-limiting step, will involve carbon-nitrogen bond formation or cleavage, depending on the direction in which the reaction is considered. If the reverse case holds and carbon dioxide expulsion is faster than proton loss ( $k_2 < k_{-1}K$ ), the rate-determining step will be loss and addition of a proton in the forward and reverse reaction, respectively. Evidence bearing on this question comes from the dependence of the rate of decarboxylation on amine basicity (fig. 7).

The Brønsted plot for hydronium ion-catalyzed decarboxylation reveals a complex pattern that may be fitted to two straight lines of slope 0.45 and 0.60, respectively, for reactions of carbamates formed from ordinary amines and amines of the hydroxylamine-hydrazine class. The fit to these lines is poor, especially for ordinary amines where approximately fivefold deviations are observed for four of the nine amines studied. These deviations are much greater than experimental error. Although the  $\alpha$ -effect appears to be of importance in the synthesis reaction, there is no requirement that this effect will be significant in the reaction in both directions. Looking at the overall results, it is apparent that the rates for weakly basic amines ( $pK$  -1 to 5.6) show a significantly greater dependence on basicity than do the more basic amines. The results suggest an alternate plot, shown in figure 7, in which the slope is 0.77 for amines of  $pK$  -1.05 to 5.6 and zero for more basic compounds. The results are better correlated with this more complicated assignment, the implications of which are discussed below.

Rates of reaction of weakly basic carbamates are clearly dependent on the amine  $pK$ . For these reactions, the  $k_2$  term predominates in the denominator of equations (7) and (8) so that  $k_{H^+} = k_{-2}k_{-1}K/k_2$ , and  $k_{\text{amine}} = k_1$ . Effects of basicity on the breakdown reaction are expected for  $k_{-1}$  and  $K$ , and these effects will be in the opposite direction; increases in basicity will increase  $K$  and decrease  $k_{-1}$ . The positive slope of the Brønsted plot for decarboxylation indicates

that the equilibrium ( $K$ ) is more sensitive to amine basicity than to the breakdown of the zwitterion. In the synthesis reaction the attack step is rate-limiting and, as expected, the reaction is increased by electron donation (fig. 3).

Results obtained with basic carbamates are difficult to interpret with certainty. A leveling out of the Brønsted plot is suggested from the result and is predicted when rates are equal to the rate of encounter of hydronium ion and carbamate. In terms of equation (8), when increases in basicity increase the equilibrium constant  $K$  so that the  $k_{-1}K$  term predominates in the denominator,  $k_{H^+} = k_{-2}$ . The constant  $k_{-2}$  represents the encounter of hydronium ion and carbamate, and this reaction is expected to be independent of basicity. The principal objections to the assignment  $k_{-1}K > k_2$  are that maximal rates are only

approximately  $10^8 \text{ M}^{-1} \text{ sec}^{-1}$  at 10 C and the  $\Delta H^*$  for hydronium ion-catalyzed decarboxylation of morpholine carbamate is 6.46 kcal/mole. This result is to be compared with the diffusion-limited reaction of hydronium ions with amines which is independent of basicity, with rates equal to approximately  $10^{10} \text{ M}^{-1}$  at 25 C (Eigen, 1964; Eigen, Kruse, Maass, and DeMaeyer, 1963). Values for  $\Delta H^*$  for diffusion-limited proton exchange are 2–4 kcal/mole (Lowenstein and Szoke, 1962; Luz and Meiboom, 1964a). Several factors may account for these differences. First, we have found previously that a hydronium ion is 20 times less reactive than predicted from the Brønsted relationship in the general acid-catalyzed decarboxylation of carboxyimidazolidone (Caplow and Yager, 1967). Hydronium ions may, for some reason, react relatively slowly with carbamates. Also, rate constants for proton exchange between ammonium ions and methyl-substituted ammonium ions and their conjugate bases, and the phosphate monoanion and dianion, which are presumably diffusion-limited processes, are in the range observed for carbamate breakdown (Grunwald and Ku, 1968; Luz and Meiboom, 1964b). Finally, because of electrostatic attraction and the fact that the carboxyl group is undoubtedly the basic locus of carbamates, the initial site of protonation by hydronium ions may be on the carboxyl function. If the generation of the catalytically active species having the proton on the nitrogen atom via intramolecular proton transfer is not faster than the dissociation of the hydrogen-bonded complex to carbamate and hydronium ions, a significant fraction of encounters between hydronium ions and carbamate may be unsuccessful. Proton transfer to the nitrogen atom in a neutral carbamic acid to give the zwitterion is thermodynamically unfavorable and may, therefore, be relatively slow. In a related reaction, in which the equilibrium constant is 1, the rate constant for proton transfer between oxygen atoms in acetic acid mediated by two solvent water molecules is only  $4.8 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$  at 25 C (Luz and Meiboom, 1963).

The assignment  $k_{-1}K > k_2$  requires that in the reaction of basic amines with carbon dioxide, the rate-limiting step is loss of a proton from the zwitterionic intermediate. This result is not altogether unexpected in light of results obtained in

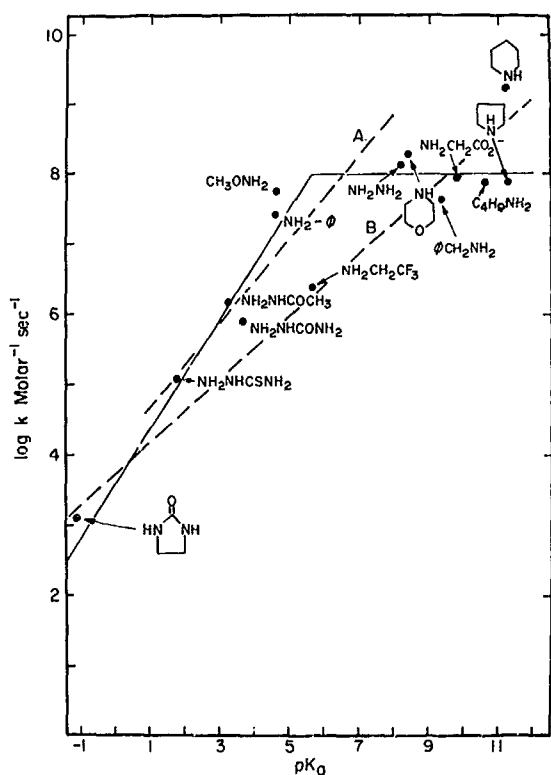


FIGURE 7. Dependence of the second-order rate constant for hydrogen-ion catalyzed decarboxylation of carbamates on the  $pK_a$  of the parent amine. The slope for curves A and B are 0.60 and 0.45, respectively; the slope of the solid line is 0.77 and 0. (Reprinted with the permission of the American Chemical Society.)

studies of the reaction of N-methylhydroxylamine with *p*-chlorobenzaldehyde (Reimann and Jencks, 1966). In this reaction it has been calculated that the loss of a proton from a zwitterionic carbinolamine intermediate would not occur fast enough to account for the observed rates, if it were not for the intervention of a special pathway in which there is an intramolecular proton transfer to the alkoxide function. A similar mechanism apparently holds in the hydration of acetaldehyde (Eigen, 1965). The reaction of amines with carbon dioxide differs from these reactions in that the formation of a basic center does not accompany amine attack; and as a result, loss of carbon dioxide from the zwitterionic carbamate may be faster than deprotonation. This reaction has precedent in the reaction of thiol anions with acetaldehyde, where breakdown of a hemithioacetal anion to starting materials is faster than protonation of the intermediate by solvent (Barnett and Jencks, 1967). Finally, a direct comparison may be made between the rates of proton and carbon dioxide loss from an identical base (hydroxide ion) by comparing the rates of ionization of water and decarboxylation of bicarbonate ions. The observed rate constant for the former reaction, which is a complex constant reflecting an unfavorable equilibrium prior to the rate-determining step, is  $2.5 \times 10^{-5} \text{ sec}^{-1}$  (Eigen et al., 1963). The rate constant for bicarbonate decomposition is  $1.9 \times 10^{-4} \text{ sec}^{-1}$  (Gibbons and Edsall, 1963).

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## DISCUSSION

**JENSEN:** We did not believe that the reaction of tris involved nitrogen attack since glycine derivatives give no carbamate at neutral pH.

**CAPLOW:** With respect to the tris reaction, the observation of hydroxide catalysis does not conclusively indicate oxygen reactions. Aminolysis may also be subject to hydroxide catalysis. There is an amine squared term in the rate law for many reactions involving acetyl transfer to amines which has been interpreted in terms of a mechanism in which one amine molecule catalyzes amine attack; I am not certain that your results are free of this extra reaction. We were able to work at very low amine concentrations,  $10^{-5}$  to  $10^{-6}$  M, because the  $\text{CO}_2$  concentration is essentially zero.

**POCKER:** In the carbamate reaction you had two kinetic terms—one first order in amine and the other first order in both amine and hydroxide ion. In the parallel reaction leading to bicarbonate, you had only a hydroxide ion term; yet the reaction in question is also general base catalyzed, and amines are very efficient in this respect. Were your experimental conditions such that the amine term could be neglected in the reaction leading to bicarbonate?

**CAPLOW:** That is related to what I have pointed out to Dr. Jensen, that an amine square term might be very important, but we work at very low amine concentrations, where it may be neglected. Also, I think we may rule out a contribution of this type because the plots of the product ratio versus the amine concentration are linear.

**POCKER:** In the general catalysis of the amine reaction, in addition to the hydroxide ion term  $k'_{\text{amine}}[\text{R}_2\text{NH}][\text{OH}^-]$ , are there additional terms, say second order in amine, such as  $k''_{\text{amine}}[\text{R}_2\text{NH}]^2$  or  $k'''_{\text{amine}}[\text{R}_2\text{NH}][\text{R}_3\text{N}]$ , or do you feel that these second-order terms can be neglected?

**CAPLOW:** We decided that there might be an amine anion component in reactions with carbon dioxide because, when we studied the decomposition of carboxyimidazolidone, we found that there was a hydrogen-ion-catalyzed as well as an uncatalyzed reaction. In the latter, the anion is the reaction species, and the reaction involves a direct displacement of the imidazolidone anion plus  $\text{CO}_2$ . This reaction must also occur in the

reverse direction. That is, we have to have an amine anion, or something equivalent, in the reaction of imidazolidone with carbon dioxide. According to the same reasoning, we have observed that the decarboxylation of carboxyimidazolidone is general acid catalyzed. In the reaction the products are imidazolidone,  $\text{CO}_2$ , and the conjugate base of the general acid. There must, therefore, be a general base-catalyzed aminolysis reaction. We have not been able to observe this process because we cannot measure any nucleophilicity in imidazolidone; it is less reactive than water.

**SIESJO:** I would like to make a comment on the quantitative significance of the presence of carbamate in tissues. Many people have tried to determine the pH of the tissue by acidifying the tissue and liberating the acid-labile  $\text{CO}_2$ . Then Conway and associates found in 1934 that at least half of the acid-labile  $\text{CO}_2$  of the tissue was soluble in the presence of barium salts at alkaline pH. Dr. Hastings did some experiments in muscle tissue to determine the  $pK$  of homogenates. When we repeated the experiments on brain tissue, we could not find any variation in  $pK$  with the amount of tissue. The conclusion then, as you have referred to these measurements, was that there were no carbamates present. Can anyone explain why Conway could get about 50 percent of the acid-labile  $\text{CO}_2$  barium soluble?

**HASTINGS:** Not I.

**ROUGHTON:** Dr. Edsall, have you any comment on our tentative values for the ionization constant for the carbamic acids. Do they look reasonable?

**EDSALL:** Yes; considering the sort of resonance you might expect to have with the amino group directly bound to the carboxyl. This resonance should shift the  $pK$  value to a value slightly above that of acetic acid.

**ROSSI-BERNARDI:** I would like to ask Dr. Siesjo if I understood his question correctly. You refer to the method of measuring intracellular pH by measuring the  $pK$  of carbonic acid?

**SIESJO:** Yes.

**ROSSI-BERNARDI:** I know that there is some old work in which it was said that a lot of  $\text{CO}_2$  would be bound to the muscle protein—since

then, nothing has been done. I would also be interested to know about your new work done on this subject.

**SIESJO:** We just repeated the measurements done by Davis and Hastings in 1939 by varying the amount of tissue homogenate, knowing the CO<sub>2</sub> tension, determining the amount of bicarbonate, and calculating the  $pK$ . If the tissues were forming carbamate compounds, then one would expect that the  $pK$  would decrease with increasing concentration of tissue in the homogenates, but we found no alteration at all in the  $pK$  value.

**ROSSI-BERNARDI:** This leads to the important question of whether myoglobin can react with CO<sub>2</sub>. Myoglobin certainly has one terminal amino group which would be expected to react with CO<sub>2</sub>. Unfortunately, as yet, no observations

have been made on this reaction; perhaps tomorrow we might return to this question.

**HASTINGS:** If there is a measurable amount of the CO<sub>2</sub> in muscle, tied down, this would grossly influence the intracellular pH. The evidence indicates that it must be about 6.8 or 6.9 rather than about 6.2.

**SIESJO:** Yes; because you get the same answer if you use, for example, DMO, which is not supposed to take part in such reactions.

**ROSSI-BERNARDI:** You have to be careful in measuring the  $pK$  of the carbonic acid, because a change in pH of 0.03 would make a significant amount of carbamate. To be sure that there is no CO<sub>2</sub> combination, your measurements must be accurate. In the case of hemoglobin, which is well known to combine carbamino-wise with CO<sub>2</sub>, it is difficult to measure the amount of carbamate by this method.



# Rate of Reaction of CO<sub>2</sub> With Human Hemoglobin

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N70-23296

## PRODUCTION OF CARBAMATE IN HEMOGLOBIN SOLUTION

We (Forster, Constantine, Craw, Rotman, and Klocke, 1968) sought to measure the rate of reaction of CO<sub>2</sub> with human hemoglobin to form carbamate under physiological conditions. To do this we rapidly mixed a solution of deoxygenated hemoglobin in water with a solution of CO<sub>2</sub> in water in a continuous-flow rapid-reaction velocity apparatus using a P<sub>CO<sub>2</sub></sub> electrode to follow the changes in dissolved [CO<sub>2</sub>] with time. A diagram of the apparatus is shown in figure 1. The hemoglobin solution was prepared by a method similar to that of Adair and Adair (1934) from 700 to 1000 ml of fresh human blood by centrifugation and dialysis. The final solution contained an average of 7.66 mM hemoglobin in water, with a residual [Na] of 10 to 20 meq. O<sub>2</sub> was pumped off, and an atmosphere of N<sub>2</sub> or He substituted. The resulting pH, about 7, was then adjusted to the desired values from 6.55 to 7.64 with HCl and KOH. The CO<sub>2</sub> solution was prepared by equilibrating water with 10 percent CO<sub>2</sub> gas at 37 C. Both the CO<sub>2</sub> and hemoglobin solutions included 1.1 mM acetazolamide (supplied through the courtesy of Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.) to inhibit the carbonic anhydrase.

The P<sub>CO<sub>2</sub></sub> was measured at reaction times from 0.007 to 0.260 second and the decrement from the initial value in the mixture, before any reaction had taken place, calculated. The reproducibility of this change in P<sub>CO<sub>2</sub></sub> was about 1 mm Hg.

The main findings are illustrated in figure 2. P<sub>CO<sub>2</sub></sub> fell rapidly to a plateau by 0.1 second. This must represent the formation of hemoglobin carbamate, because the uncatalyzed rate of CO<sub>2</sub>

hydration over the same period was negligible. The absolute decrement in P<sub>CO<sub>2</sub></sub> at 0.1 second has been used before to estimate the [hemoglobin carbamate] (Constantine, Rossi, and Roughton, 1963), because it was assumed that carbamate equilibrium existed by that time. This assumption is further supported by studies on glycyglycine carbamate in which the same K<sub>c</sub> (see eq. 5) was found by the electrode method as by the classical Ba precipitation method (Rossi-Bernardi and Roughton, 1966).

The next point is that the total amount of carbamate formed increased as the pH increased. This is predicted by theory, as discussed by Rossi-Bernardi and Roughton (1967). There are three pertinent equilibria:

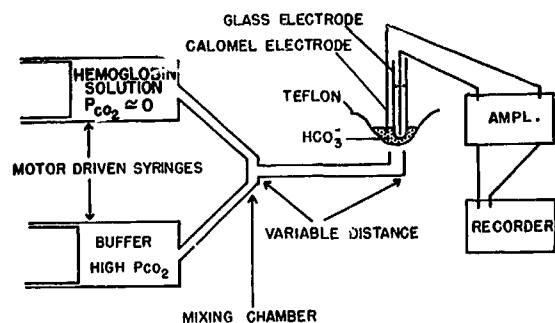
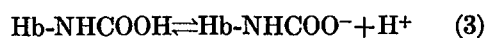
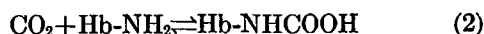


FIGURE 1.—The continuous-flow rapid-reaction apparatus uses the P<sub>CO<sub>2</sub></sub> electrode (Constantine, Craw, and Forster, 1965). The motor-driven syringes, mixing chamber, observation tube, and lower part of electrode are surrounded by a water bath maintained at a constant temperature.

Reaction (1) is important because CO<sub>2</sub> reacts only with the un-ionized amine. An acid ionization constant is defined as

$$K_2 = \frac{[\text{Hb-NH}_2][\text{H}^+]}{[\text{Hb-NH}_3^+]} \quad (4)$$

The  $pK$  of the acid ionization of hemoglobin carbamate, Hb-NHCOOH, is not known exactly, but is probably less than 6 (Roughton, 1964; Rossi-Bernardi and Roughton, 1967). It is therefore convenient to combine reactions (2) and (3) in one equilibrium constant,  $K_c$ , as follows:

$$K_c = \frac{[\text{H}^+][\text{Hb-NHCOO}^-]}{[\text{CO}_2][\text{Hb-NH}_2]} \quad (5)$$

It is assumed that there is one NH<sub>2</sub>-group

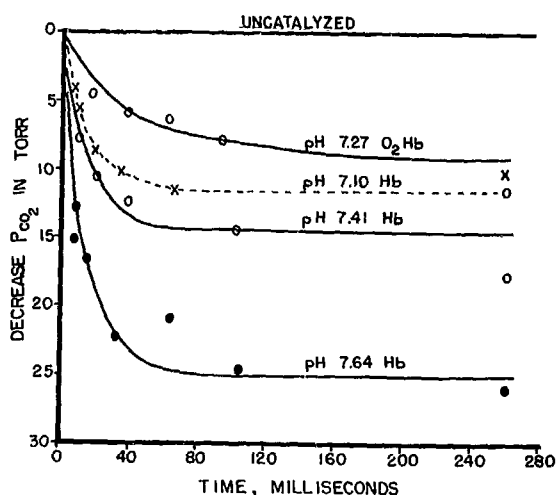


FIGURE 2.—The fall in  $P_{\text{CO}_2}$  with time following the rapid mixing of hemoglobin and CO<sub>2</sub> solutions. The ordinate is the decrease in  $P_{\text{CO}_2}$  at a given time compared to the calculated initial  $P_{\text{CO}_2}$  in the mixture before any reactions had taken place. The line at the top is a graph of the fall in  $P_{\text{CO}_2}$  because of uncatylyzed hydration, assuming a velocity constant of  $0.12 \text{ sec}^{-1}$  and an initial  $P_{\text{CO}_2}$  of 37 mm Hg. All the data points have been corrected for this process. The symbols indicate individual observations; the same symbol is used for all observations on a given hemoglobin solution. The pH indicated on the curves is that of the hemoglobin solution before mixing, which would approximate that during the entire reaction. The smooth curves are simple exponentials fitted by inspection to the data points. In one experiment, at pH 7 (indicated with 0), O<sub>2</sub> was added to raise the  $P_{\text{CO}_2}$  to 620 mm Hg and the pH fell from 7.41 to 7.27. (Reproduced with the permission of the *Journal of Biological Chemistry*.)

available to form carbamate for each molecule of iron in the hemoglobin molecule from pH 7 to 8 (Roughton, 1964). These relationships can be combined to provide an equation describing the fraction of the total amount of amine which has formed carbamate:

Fraction of total carbamate

$$= \frac{[\text{CO}_2]}{[\text{CO}_2] + \frac{[\text{H}^+]}{K_c} + \frac{[\text{H}^+]^2}{K_c K_2}} \quad (6)$$

Owing to the  $[\text{H}^+]^2$  term in the denominator, the fraction of carbamate can be very sensitive to changes in pH. The total amounts of carbamate we found were compatible with equation (6), with  $K_c = 2.4 \times 10^{-5}$  and  $K_2 = 7.2 \times 10^{-8}$  M. Figure 3 is a plot of the decrement in  $P_{\text{CO}_2}$  at 0.1 second, equivalent to the hemoglobin-carbamate formed, versus the pH at the start of the reaction. Because the total remaining  $P_{\text{CO}_2}$  at final equilibrium decreases as the amount of carbamate formed increases, all the decrements in  $P_{\text{CO}_2}$  are normalized to a standard  $P_{\text{CO}_2}$  of 35 mm Hg according to equation (6). The data have been normalized, as well, to a constant total  $[\text{Hb}]$  of 3.88 mM, the average value. Because there are two unknown constants,  $K_c$  and  $K_2$ , it is not remarkable that a fit can be secured. However, the values of  $K_c$  and  $K_2$  used to calculate the smooth curves in figure 3 are the best available estimates, including the independent data of other authors.

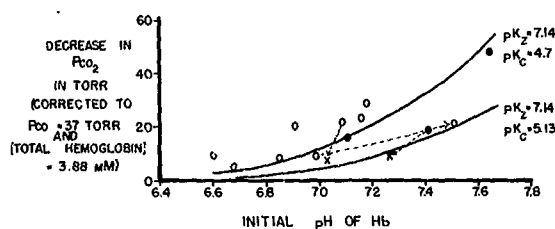


FIGURE 3.—The fall in  $P_{\text{CO}_2}$  at 0.1 second versus the pH of the hemoglobin solution at the start of the reaction. Each point has been corrected to a final equilibrated  $P_{\text{CO}_2}$  of 37 mm Hg and a hemoglobin concentration at 3.88 mM. The arrows connect data points from the same experiment. The solid circles were obtained with deoxygenated hemoglobin solution, the X's with oxygenated. The smooth curve is a plot of equation (6) using the indicated values for  $K_2$  and  $K_c$ . (Reproduced with the permission of the *Journal of Biological Chemistry*.)

Also shown in figures 2 and 3 is the decrease in carbamate formed when the hemoglobin is oxygenated.

The rate of carbamate formation should follow the relation

$$\frac{d[\text{Hb-NHCOO}^-]}{dt} = k_a[\text{CO}_2][\text{Hb-NH}_2] - k_d[\text{Hb-NHCOO}^-] \quad (7)$$

where  $k_a$  is the association velocity constant in M<sup>-1</sup> sec<sup>-1</sup>,  $k_d$  is the dissociation velocity constant in sec<sup>-1</sup>, and  $t$  is the time in seconds.

We calculated  $k_a$  from changes in  $P_{\text{CO}_2}$  with time by two methods. The simpler one is to measure  $d[\text{Hb-NHCOO}^-]/dt$ , which is the same as  $-d[\text{CO}_2]/dt$ , from a tangent to the curve of  $P_{\text{CO}_2}$  versus time at the start of the reaction, at which point the  $[\text{CO}_2]$  and pH are known from the composition of the reacting solutions, and  $[\text{Hb-NHCOO}^-]$  is negligible.  $[\text{Hb-NH}_2]$  can be calculated from  $[\text{H}^+]$  and equation (4), provided  $K_s$  is known. Because we have measurements at several different  $[\text{H}^+]$ , it is possible to calculate both  $K_s$  and  $k_a$  from our data. We can rearrange equation (4) to obtain

$$[\text{Hb-NH}_2] = \frac{[\text{Hb-NH}_2 + \text{Hb-NH}_3^+]}{K_s + [\text{H}^+]} K_s \quad (8)$$

$[\text{Hb-NH}_2 + \text{Hb-NH}_3^+]$  equals the total hemoglobin concentration,  $[\text{Hb}]$ , at the start of the process.

Substituting this in equation (7) and disregarding the dissociation term, we obtain

$$\frac{[\text{total Hb}]P_0}{dP/dt} = \frac{1}{k_a} + \frac{[\text{H}^+]}{K_s k_a} \quad (9)$$

where  $P_0$  is the initial CO<sub>2</sub> tension and  $dP/dt$ , its rate of change. Figure 4 is a plot of the left-hand term against  $[\text{H}^+]$ , the ordinate intercept equaling  $1/k_a$ , from which  $k_a = 11.7 \times 10^8$  M<sup>-1</sup> sec<sup>-1</sup>, and the abscissa intercept equaling  $-K_s$ , from which  $K_s = 4.5 \times 10^{-8}$  M. A similar equation was derived independently by Kernohan and Roughton (1968).

The second technique was to fit a simple exponential equation to the  $P_{\text{CO}_2}$  versus time curves and calculate  $k_a$  and  $K_s$  from the values of the exponent as a function of  $[\text{H}^+]$ . If we assume  $[\text{H}^+]$  is constant for the duration of the reaction (it actually increases less than 12 percent), equation

(7) has a simple exponential solution with the exponent  $k$ , equal to

$$k = k_a[\text{Hb-NH}_2] \frac{P_0}{P_0 - P_\infty} \quad (10)$$

where  $P_\infty$  is the  $P_{\text{CO}_2}$  at 0.1 second.

Equations (8) and (10) can be combined to give

$$\frac{[\text{total Hb}]P_0}{(P_0 - P_\infty)k} = \frac{1}{k_a} + \frac{[\text{H}^+]}{K_s k_a} \quad (11)$$

This equation can be solved graphically for  $K_s$  and  $k_a$  by plotting the left-hand term against  $[\text{H}^+]$ , analogously to figure 4, providing a value of  $9.8 \times 10^8$  M<sup>-1</sup> sec<sup>-1</sup> for  $k_a$  and  $1.02 \times 10^{-7}$  for  $K_s$ .

We believe the best estimates of  $k_a$  and  $K_s$  are combinations of the results from two methods;  $11\,000$  M<sup>-1</sup> sec<sup>-1</sup> for  $k_a$  and  $7.2 \times 10^{-8}$  for  $K_s$ , a  $pK_s$  of 7.14.

Because the total carbamate formed, that is, the decrease in  $[\text{CO}_2]$  at 0.1 second, is known as well as  $[\text{CO}_2]$ ,  $[\text{H}^+]$ , and  $K_s$ , equation (6) can theoretically be solved for  $K_c$ . Unfortunately our data are not sufficiently precise to provide an exact value. The estimates varied from  $2 \times 10^{-6}$  to  $8 \times 10^{-6}$  M. We believe it best to rely on the value of  $2.4 \times 10^{-5}$  M calculated by Rossi-Bernardi and Roughton (1967) from Ferguson's (1936) data.

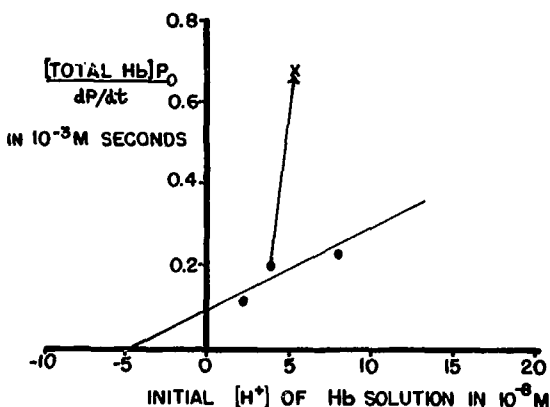


FIGURE 4.—Graphical solution of equation (9). The arrow connects a point obtained on deoxygenated (O) with one obtained on the same oxygenated (X) hemoglobin solution. The line is the least-mean-square regression for the data on deoxygenated hemoglobin solution. (Reproduced with the permission of the Journal of Biological Chemistry.)

The initial rate of formation of carbamate from O<sub>2</sub>Hb was less than that from deoxygenated hemoglobin, although pH, [CO<sub>2</sub>], and [total Hb] were kept the same. According to equation (7), this could be explained by a decrease in  $k_a$ , or in  $K_s$ , or in any combination thereof. We do not have sufficient data to make a useful plot of equation (9) (see fig. 4) and to determine  $k_a$  for O<sub>2</sub>Hb independently of  $K_s$ , so this question remains in abeyance.

#### PRODUCTION OF CARBAMATE IN RED CELLS

It should be possible to determine the rate of carbamate formation in intact red cells, and the necessary measurements have been reported by Constantine, Craw, and Forster (1965). They used a continuous-flow, rapid-reaction apparatus with a  $P_{CO_2}$  electrode to follow the uptake of CO<sub>2</sub> by a human red cell suspension under near-

physiological conditions in the presence and absence of acetazolamide and obtained results comparable to those we found with a hemoglobin solution (fig. 5). The upper curve was determined in the absence of carbonic anhydrase inhibitor and is dominated by the intracellular-catalyzed hydration of CO<sub>2</sub>. The lower curve, during which carbonic anhydrase was inhibited, represents the formation of carbamate inside the cells (complete by 0.2 sec) and the slow uncatalyzed hydration of CO<sub>2</sub>, primarily in the suspending fluid. If we assume that the intracellular [H<sup>+</sup>] is constant, which is reasonable in view of the great buffer capacity of the hemoglobin, and that the [CO<sub>2</sub>] is constant (actually it falls 11 percent), we can use the simple exponential solution for equation (7) given in equation (10). [Hemoglobin] was 0.45 mM in the reacting mixture. The pH was 7.5 and intracellularly approximates 7.3. The  $P_{CO_2}$  at the start was 35 mm Hg. The half time was 0.060 second. We obtained a value of

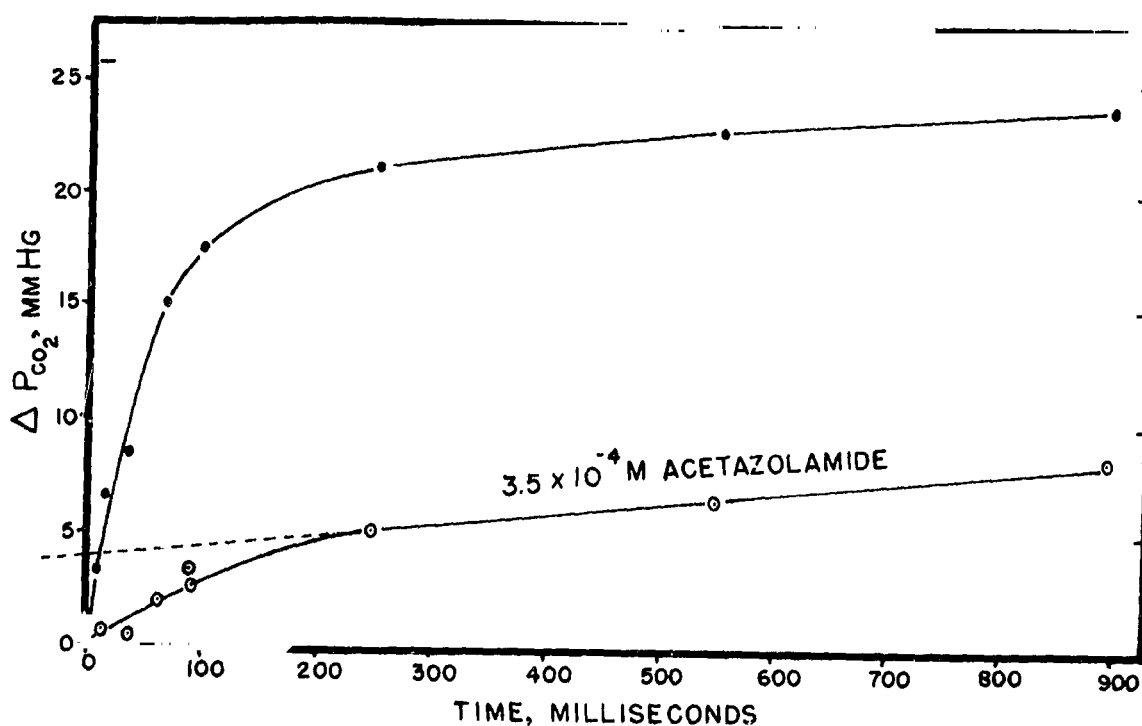


FIGURE 5.—The decrease in  $P_{CO_2}$  with time following the mixing of a 1:10 suspension of normal human blood in buffer at pH 8.66 and  $P_{CO_2}$  less than 2 mm Hg, with a 31.7 mM bicarbonate solution at pH 7.25 and  $P_{CO_2}$  71 mm Hg. Both solutions had zero  $P_{O_2}$ . Temperature was 37 C. The lower curve represents an experiment in which  $3.5 \times 10^{-4}$  M acetazolamide was added to both reactant mixtures. (Reproduced with the permission of the Journal of Biological Chemistry.)

4850 M<sup>-1</sup> sec<sup>-1</sup> for  $k_a$  with  $K_s = 7.2 \times 10^{-8}$ , which is in reasonable agreement with our value of 11 000 M<sup>-1</sup> sec<sup>-1</sup> in hemoglobin solution.

The velocity constant for the dissociation of hemoglobin carbamate,  $k_d$ , equals

$$\frac{k_a K_a}{K_c}$$

where  $K_a$  is the acid dissociation constant for the ionization of Hb-NHCOOH (eq. (3)). Accepting  $K_c$  as equal to  $2.4 \times 10^{-5}$  M,  $k_a$  as 11 000 M<sup>-1</sup> sec<sup>-1</sup>, and  $K_a$  as  $10^{-6}$  M or greater, a minimal value of  $k_d$  is 500 sec<sup>-1</sup>.

I conclude that the total amount of carbamate formed from human hemoglobin under near-physiological conditions follows the theoretical predictions. The best available estimate of the acid ionization constant for the pertinent amine groups,  $K_s$ , is  $7.2 \times 10^{-8}$ , that for the association constant for the reaction of CO<sub>2</sub> with hemoglobin-NH<sub>2</sub> is 11 000 M<sup>-1</sup> sec<sup>-1</sup>, and the corresponding dissociation constant is at least as great as 500 sec<sup>-1</sup>. For intact human red cells,  $k_a$  approximates 5000 M<sup>-1</sup> sec<sup>-1</sup>.

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## Kinetics of Carbamino Compound Formation in Red Cells and in Hemoglobin Solutions

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The continuous-flow rapid calorimeter has been used to measure the rates of reactions involving  $\text{CO}_2$  in bovine red cells and hemoglobin solutions. In the absence of a carbonic anhydrase inhibitor there is rapid heat evolution after  $\text{CO}_2$  solutions are mixed with a cell suspension or hemoglobin solution. In the presence of concentrations of acetazolamide sufficient to inhibit the carbonic anhydrase, less heat is evolved. In the latter case the heat evolution is solely due to the formation of hemoglobin carbamino compounds. The rate of formation of these compounds has been calculated; the rate constant for carbamino formation with deoxyhemoglobin is about twice that found for oxyhemoglobin. Some possible reasons for this difference are discussed. No significant difference is found between the rates for cell suspensions and for hemoglobin solutions measured under the same conditions.

The reactions of carbon dioxide with hemoglobin preparations and with intact red cells may be studied using the continuous-flow rapid calorimeter. When hemoglobin solutions or red-cell suspensions are mixed with carbon dioxide solutions in the apparatus shown schematically in figure 1, a rapid evolution of heat due mainly to the carbonic anhydrase-catalyzed hydration of carbon dioxide may be observed. In the presence of a carbonic anhydrase inhibitor such as acetazolamide, the amount of heat evolved is much smaller (fig. 2). The difference between the heat evolved in the absence and in the presence of the inhibitor may be attributed to the hydration reaction and may be used to estimate the rate of the reaction, and, hence, the activity of carbonic anhydrase.

The hydration reaction can be considered to occur in two stages:



The heat of reaction (1), which is endothermic, is 2200 calories per mole (Roughton, 1941). For the second stage of the reaction, hemoglobin is the predominant buffer under the conditions of the experiments. Chipperfield, Rossi-Bernardi, and Roughton (1967) have determined the heats of ionization for oxyhemoglobin and hemoglobin pre-

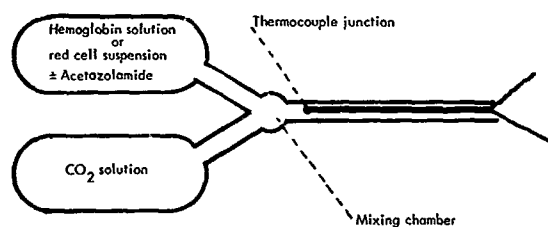


FIGURE 1.—Schematic diagram of method for measuring reaction rates. The temperature rise caused by reaction is the difference between the temperature observed when the solutions are mixed and the temperature the mixture would have if reaction did not occur. The latter may be calculated from the temperature of the two solutions and the composition of the mixture.

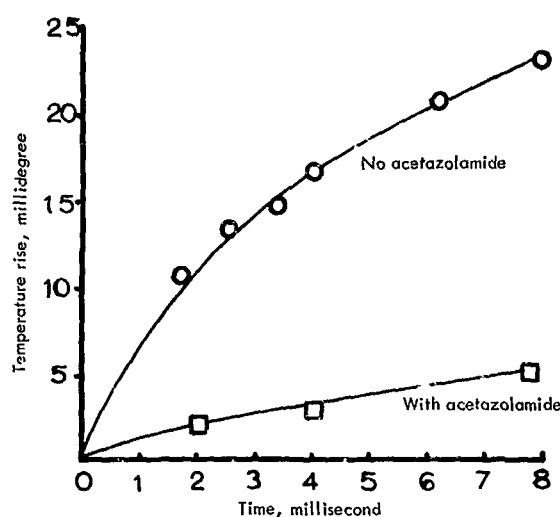


FIGURE 2.—Time course of heat evolution after mixing CO<sub>2</sub> solution with hemoglobin solutions, with and without carbonic anhydrase inhibitor.

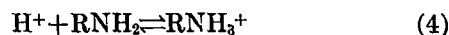
pared from bovine blood. Therefore the total heat of the hydration reaction is known, and the initial rate of CO<sub>2</sub> hydration may be calculated from the difference in the initial rates of heat evolution with acetazolamide absent and present.

As was found in previous studies, the carbonic anhydrase activity varies about threefold between individual blood samples. In concentrated hemoglobin solutions, the activity closely approximates that predicted from measurements using the optical stopped-flow technique on much more dilute solutions, if the activity is assumed to be proportional to the enzyme concentration. The activity was not influenced by the state of oxygenation of the hemoglobin. The activity in intact red cells appears to be about 30 percent higher than in hemoglobin solutions of the same concentration. In this comparison, corrections were made for the influence of different physicochemical conditions. Uncertainties in the corrections might account for most of the observed difference.

The smaller evolution of heat that occurs in the presence of acetazolamide is much more than can be accounted for by the uncatalyzed hydration reaction or by the residual activity of the enzyme and must be from the reaction of CO<sub>2</sub> with hemoglobin to form carbamino compounds.

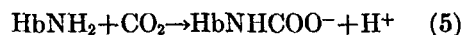
This evolution may be used to estimate the rate at which these compounds are formed in much the same way that Chipperfield (1966) did in his study of carbamate formation by a series of amino compounds.

This reaction may also be considered to occur in two stages:



The overall heat, which is the sum of the heats of reactions (3) and (4), may be determined by allowing the reaction of CO<sub>2</sub> with excess amine to go to completion. The heat of the second stage may be determined directly by measuring the heat of titration of the amine with hydrogen ions. Hence the heat of reaction (3) may be calculated. Chipperfield (1966) found that this varied from 3400 to 5100 calories per mole for a series of amino acids and peptides. The heat of reaction (4) was close to 10 700 calories per mole.

The formation of carbamino compounds by hemoglobin may also be broken down into two stages:



Reaction (6) is identical with reaction (2), the heat of which has been measured by Chipperfield et al. (1967). The heat change associated with reaction (5) cannot be measured directly, either by itself or together with the heat from reaction (6), as it is not possible to realize conditions under which reaction (5) will go to completion without the occurrence of other side reactions. It has, therefore, been necessary to assume that the heat of reaction (5) is in the same range as that found by Chipperfield (1966) for reaction (3). We feel justified in making this assumption, since it seems likely that this stage of carbamino formation by hemoglobin only accounts for about one-third of the total heat of the process and any uncertainties introduced by the assumption are correspondingly reduced. If, however, the assumption does prove to be in serious error, our experimental results will still be valid, although the interpretation of them will need revision. The possibility of the heat of reaction (5) being different for hemoglobin and oxyhemoglobin will be examined later.

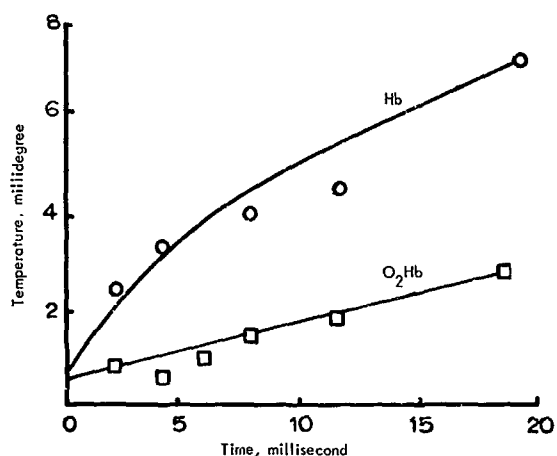


FIGURE 3.—Time course of heat evolution after mixing  $\text{CO}_2$  solution with suspensions of deoxygenated and oxygenated red cells containing carbonic anhydrase inhibitor.

Measurements on the rate of carbamino formation in red-cell suspensions and in hemoglobin solutions, both in the oxygenated and deoxygenated states, have been made. The reaction rates measured for hemoglobin solutions and for cell suspensions agree within experimental error. This agreement is direct evidence that the kinetics of the carbamino reactions of hemoglobin are the same for hemoglobin in the red cell as for hemoglobin in solution: the calculations of Roughton and Rupp (1958) had already shown that diffusion factors were very unlikely to affect the overall reaction rates in the red cell. As figure 3 shows, however, the rate of heat evolution with deoxyhemoglobin is about twice that observed with oxyhemoglobin under the same condition. When the reaction rates are calculated, the rate constant  $\phi$  also has about twice the value for deoxyhemoglobin that it has for oxyhemoglobin. Table I shows typical values of  $\phi$  for both forms. In calculating  $\phi$ , the total concentration of reacting amino groups is used, one per heme, regardless of whether they would be in the protonated or unprotonated state at the particular pH;  $\phi$  varies with pH, depending on the ionization constant of the amino group. We can calculate the rate constant  $k'$  for the reaction of  $\text{CO}_2$  with the unprotonated amino group only if we know the  $pK$  of the group. Because the ionization constants for these groups in bovine hemoglobin have not been definitely established, we have calculated  $k'$  for each of a

TABLE I.—Rate Constants for Carbamino Reactions in Bovine Hemoglobin Solutions at 25 C<sup>a</sup>

|                        | $\phi$ , the rate constant | $k'_{6.9}$ | $k'_{7.2}$ | $k'_{7.5}$ | $k'_{7.8}$ |
|------------------------|----------------------------|------------|------------|------------|------------|
| Hb. ....               | 670 (pH 7.43).....         | -----      | 1060       | 1460       | 2240       |
| O <sub>2</sub> Hb. . . | 270 (pH 7.37).....         | 362        | 458        | 635        | 996        |

<sup>a</sup> All rate constants are expressed in  $\text{M}^{-1} \text{sec}^{-1}$ . Subscripts are assumed  $pK$  values of the reacting amino groups.

series of possible  $pK$  values. These values are also presented in table I.

It is difficult to account for the difference between oxyhemoglobin and deoxyhemoglobin solely in terms of differences in the  $pK$  values of the reacting amino groups. If the heats of reaction are the same and the values of  $k'$  are the same, then it would be necessary for the  $pK$  of the groups on deoxyhemoglobin to be about 0.5 unit lower than the  $pK$  of the groups on oxyhemoglobin. This difference would mean a reversed Bohr effect for these groups.

If the groups showed a normal Bohr effect, then the difference in their rate of reaction with  $\text{CO}_2$  would need to be greater than twofold. R. J. Hill (personal communication) has suggested that the  $pK$  of the N-terminal amino groups in human oxyhemoglobin and deoxyhemoglobin is 6.72 and 7.71, respectively. If these values are applicable to bovine hemoglobin, then the unprotonated amino groups on deoxyhemoglobin must react with  $\text{CO}_2$  about five times faster than the same groups on oxyhemoglobin.

The difference in the observed heat evolution could be accounted for by a difference, not of reaction rate, but of heats of reaction. For this to be the case it would be necessary for the reaction of oxyhemoglobin in reaction (5) to have a heat that was some 5000 calories per mole less than the heat for the reaction involving deoxyhemoglobin. The reaction of oxyhemoglobin would then be slightly endothermic.

The difference in the observed course of heat evolution need not be accounted for by just one of these factors. It may be caused by a combination of some or all of them.

It has been assumed that all four of the terminal groups on the hemoglobin molecule react and that



they are equivalent in their reactivity with CO<sub>2</sub>. This need not be the case. Perutz (1965) has suggested that the change in conformation of the hemoglobin molecule that occurs when it combines with oxygen may make the terminal groups on the  $\beta$ -chains unavailable for reaction with CO<sub>2</sub>.

A fuller report on this work has appeared recently (Kernohan and Roughton, 1968).

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# The Estimation of Hemoglobin-CO<sub>2</sub> Compounds by Gel Filtration and Ion-Exchange Chromatography

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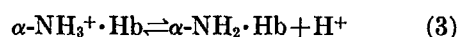
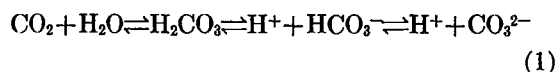
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Under physiological conditions of pH and  $P_{\text{CO}_2}$ , hemoglobin combines directly, to a limited extent, with dissolved CO<sub>2</sub> to form carbamino compounds (for discussion and references, see Roughton, 1964). The reaction is believed to be caused mainly by the four  $\alpha$ -amino groups of the hemoglobin molecule at the end of its four polypeptide chains.

In aqueous solution, CO<sub>2</sub> also reacts with water to form H<sub>2</sub>CO<sub>3</sub>, HCO<sub>3</sub><sup>-</sup>, and CO<sub>3</sub><sup>2-</sup>. Accordingly the scheme of the CO<sub>2</sub> reactions in hemoglobin solutions may be formulated as follows:



At physiological pH,  $P_{\text{CO}_2}$ , and temperature, the concentration of  $\alpha\text{-NHCOO}^- \cdot \text{Hb}$  (HbCO<sub>2</sub> for short) is only a small, though physicochemically and physiologically important, fraction of the total CO<sub>2</sub> in all forms (i.e., 5 to 10 percent). Thus a method for measuring HbCO<sub>2</sub> accurately is greatly needed, but finding a solution has proved difficult. This is particularly true of the only direct chemical method hitherto available for carbamate estimations in hemoglobin solu-

tion; namely, that described by Ferguson and Roughton (1934) and Ferguson (1936) over 30 years ago. Their method, which was adopted from Faurholt's (1925) classical barium precipitation technique for simpler amines, consists of rapidly adding to the chemical system represented by equations (1), (2), and (3) sufficient alkali to bring the solution to pH 12-13, thus converting all the H<sub>2</sub>CO<sub>3</sub> and HCO<sub>3</sub><sup>-</sup> to CO<sub>3</sub><sup>2-</sup>. The CO<sub>3</sub><sup>2-</sup> is then precipitated by adding BaCl<sub>2</sub>. The barium salts of carbamic acid are, however, soluble in water and, being sufficiently stable at this alkaline pH, can be separated from the BaCO<sub>3</sub> precipitate by centrifugation. The CO<sub>2</sub> of the supernatant fluid is then estimated gasometrically and the carbamino content of the original solution calculated.

In hemoglobin solutions there are, however, several complications not found in the simpler cases studied by Faurholt and his colleagues. Perhaps the most obvious of these is the action of hemoglobin as a "protective colloid" in hindering the formation of the BaCO<sub>3</sub> precipitate and hence its complete removal by centrifugation. Then again, because of the ever-present danger of protein denaturation, the pH of the hemoglobin solution, after mixture with the alkali, should not be allowed to exceed pH 12 at most, as compared with a pH of about 13, which is often used in

work with simpler amines. At the lower pH of 12, however, there is more danger of some of the dissolved CO<sub>2</sub> in the original hemoglobin solution turning into fresh protein carbamate on the addition of the alkali. This is because the competition of the OH<sup>-</sup> ions for CO<sub>2</sub> will be 10 times less effective, as compared with that of the uncharged amine groups of the hemoglobin, which, at pH 12, will include all the numerous ε-NH<sub>2</sub> groups of the lysine side chains as well as the four terminal α-NH<sub>2</sub> groups. Furthermore, at pH 12, the rate of dissociation of the carbamate to CO<sub>2</sub> and amine will be around 10 times faster than at pH 13. Elaborate precautions and blank corrections had to be applied to keep these disturbing factors to a minimum.

In the hope of improving the general accuracy of Ferguson and Roughton's method, and, in particular, of eliminating or greatly reducing the

magnitude of their blank corrections (which are especially serious in the case of oxygenated hemoglobin solutions with their normally low carbamate contents), we give in this communication a preliminary account of a new, and perhaps simpler, approach to the experimental problem of separating HbCO<sub>2</sub> from H<sub>2</sub>CO<sub>3</sub>, HCO<sub>3</sub><sup>-</sup>, and CO<sub>3</sub><sup>2-</sup>. The new method is based on a combined gel filtration and ion-exchange procedure, with the incorporation of some minor improvements in technique. With its aid we have been able to achieve a 99.5-percent separation of HbCO<sub>2</sub> from carbonate.

#### PRINCIPLE OF THE METHOD

A hemoglobin solution equilibrated with CO<sub>2</sub>, and thus containing HbCO<sub>2</sub>, H<sub>2</sub>CO<sub>3</sub>, CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, and CO<sub>3</sub><sup>2-</sup> in fixed proportions, is rapidly mixed with enough KOH to bring the pH of the

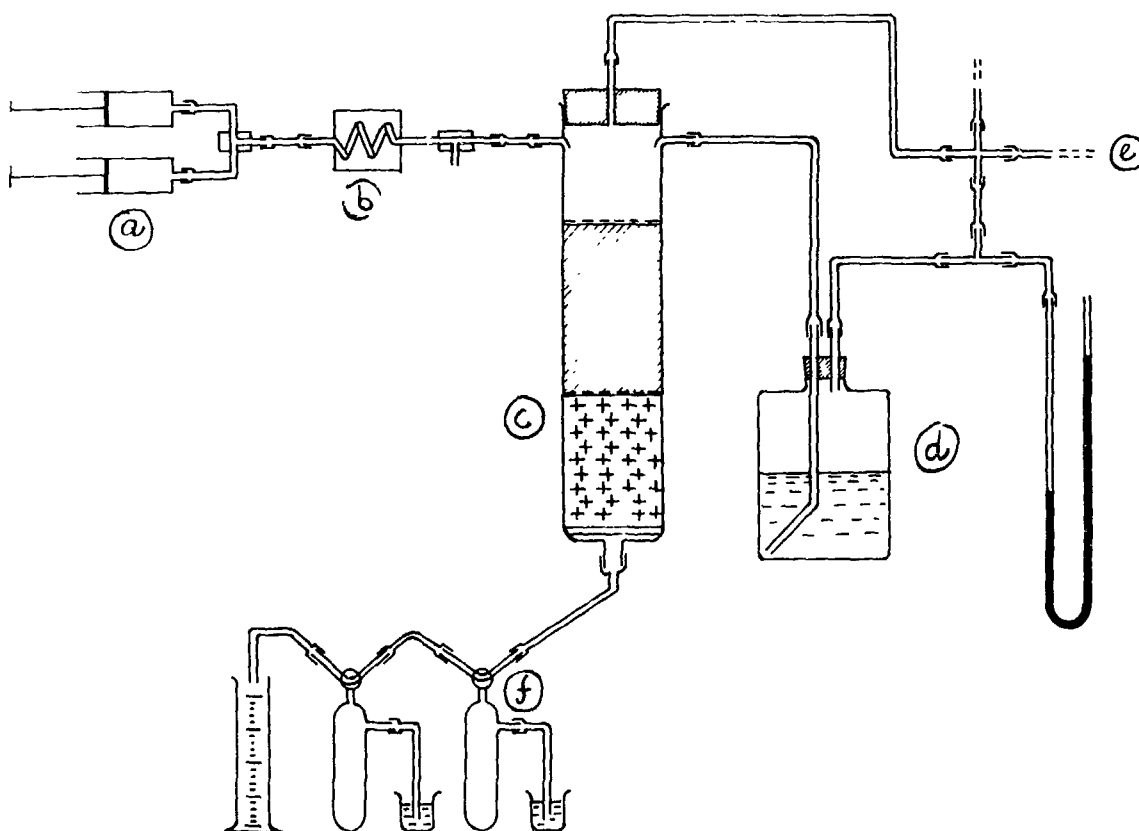


FIGURE 1.—Apparatus for gel filtration and ion exchange chromatography determination of hemoglobin-carbamate compounds: (a) syringes for Hb solution and KOH solution, (b) cooling coil, (c) column containing Sephadex and ion exchange phases, (d) eluting solution, (e) connection to compressed nitrogen, and (f) tubes for collection of samples from column.

mixed fluid to about 12, KOH being used in place of NaOH in view of the disturbing effect of Na<sup>+</sup> ions on the measurement of pH by the glass electrode at alkaline reactions. The rapid mixture is achieved by the use of two motor-driven syringes, the contents of which are driven into a Hartridge-Roughton mixing chamber, where the two solutions are mixed (at least 99 percent) in a millisecond or so (fig. 1(a)). The syringe containing the KOH is thermostatted at 0 C, whereas the hemoglobin solution, in the series of experiments described in this communication, was usually kept at 25 C. Thus immediately after mixing, the pH is brought to about 12 and the temperature to about 12.5 C. The effluent from the mixing chamber then passes through a stainless-steel spiral (fig. 1(b)) of 2-mm bore and 100-cm length, surrounded by a glass jacket containing an ice-salt solution at a temperature of about -12 C. This device allows the temperature of the effluent from the mixing chamber to be lowered from 12.5 to about 1.5 C in about 1 second. Within a few microseconds after mixing, the H<sub>2</sub>CO<sub>3</sub> and HCO<sub>3</sub><sup>-</sup> are completely transformed into CO<sub>3</sub><sup>2-</sup>. The HbCO<sub>2</sub> is, as already indicated, relatively stable under these conditions, for at pH 12 and 1.5 C, 20 percent dissociation requires an hour or so (Ferguson, 1936). Thus by applying this alkaline hemoglobin solution to a Sephadex G-25 column at 0 C, it should be possible to separate the HbCO<sub>2</sub> from CO<sub>3</sub><sup>2-</sup> before an appreciable amount of carbamate has been decomposed to CO<sub>2</sub> and thence to carbonate.

The column and the elution procedure are shown in figure 1. Figure 1(c) represents the separation column and figure 1(d), the reservoir containing the eluting solution. To increase the speed of separation, the column is operated under 20-30 cm Hg positive nitrogen pressure from a gas cylinder connected to the system via figure 1(e). The eluate is collected in glass reservoirs, filled previously with CO<sub>2</sub>-free nitrogen in high purity. Samples are collected at the appropriate time by rotating the stopcock at the top of each glass reservoir.

#### Experimental Details of a Typical Run

The column (fig. 1(c)), of dimensions 30 by 3 cm and surrounded by an ice jacket, contains

either Sephadex G-25 alone or Sephadex G-25 plus Dowex 1 x 8 cm in the OH<sup>-</sup> form and is washed with two volumes of the eluting solution (0.015 M KOH) that has been freed of CO<sub>2</sub> by the procedure described by Albert and Serjeant (1962). The liquid over the gel is then washed by first closing the reservoir (d) and then opening the nitrogen inlet at the top of the column. The upper part of the Sephadex column is protected by a disk made from stainless-steel mesh. The gas inlet is subsequently closed and the pressure in the column brought to that of the atmosphere.

The motor is then activated, and the content of the two syringes ejected directly into the column. The first portion of the mixed fluid is usually discarded through the three-way tap interposed between (b) and (c). The sample is then driven into the gel by positive nitrogen pressure (20-30 cm Hg), followed by the eluting solution. The collection of the samples is made anaerobically as above, and the samples are then transferred, also anaerobically, into a van Slyke manometric apparatus for estimation of total CO<sub>2</sub> content by the usual van Slyke-Neill procedure. The total time for the elution is about 3 minutes. The volume of the hemoglobin solution applied to the column was usually 10 to 15 ml.

## EXPERIMENTAL TESTS AND RESULTS

### Controls for the Separation of HbCO<sub>2</sub> From Carbonate

First the efficiency of the column was tested by mixing CO<sub>2</sub>-free hemoglobin solutions with 0.025, 0.05, 0.10, and 0.15 M carbonate solutions containing enough KOH to bring the pH of the mixed fluid to about 11.7-12.0. Bovine hemoglobin was used mainly in view of its relatively high resistance to alkaline denaturation. The hemoglobin concentration before mixing was 10-12 meq/O<sub>2</sub>/liter and was estimated as COHb at a 540-millimicron wavelength and also from the optical density at 280 millimicrons, as measured by a Beckman spectrophotometer (DU-2).

Results for a typical experiment are shown in figure 2. In this case only G-25 Sephadex (fine) was used in the separation column, the elution time being around 12 minutes. It is clear from this and several other similar experiments that

complete separation cannot be achieved with the Sephadex alone. In fact, the ratio of total CO<sub>2</sub> to hemoglobin in the samples was never less than 0.03 to 0.04. To achieve a better separation, a column containing G-25 (coarse) as a top layer and Dowex 1 x 8 ion-exchange resin in the OH<sup>-</sup> form in the bottom layer was used. The analytical grade Dowex 1 x 8 resin (obtained from Fluka A.G., Germany) was converted from the Cl<sup>-</sup> to the OH<sup>-</sup> form by repeated washing with CO<sub>2</sub>-free 1 M KOH. Thus, the Hb is first separated from the CO<sub>3</sub><sup>2-</sup> to the extent of 98 to 99 percent in the Sephadex phase, and the remaining traces of carbonate are then removed by ion exchange in the Dowex phase.

It should be noted that the Dowex resin alone (without the Sephadex) would not be practicable, because in this case all the anions of the hemoglobin solution (including Cl<sup>-</sup>) would be exchanged for OH<sup>-</sup>. The ionic strength of the hemoglobin solutions in our experiments was always

near physiological, i.e., 0.1 to 0.2 M, usually in KCl, and the ion exchange would thus produce a 0.1–0.2 M KOH-hemoglobin solution of a pH above the critical limit for denaturation of the protein. The inclusion of the Sephadex as the first phase in the separating column insures that all salts are retarded by the gel, leaving only traces of Cl<sup>-</sup> and CO<sub>3</sub><sup>2-</sup> to be exchanged for OH<sup>-</sup> in the Dowex phase.

The results of a typical experiment with the combined gel-ion exchange technique are presented in figure 3, wherein all the conditions were otherwise the same as in figure 2. It will be seen that now the carbonate is almost completely removed.

#### Application of the Method to the Actual Estimation of HbCO<sub>2</sub>

Similar experiments have been carried out with the hemoglobin solutions in "total" equilibrium with a CO<sub>2</sub> pressure of 45 mm Hg at 25 C. In this case the solution in one syringe contained

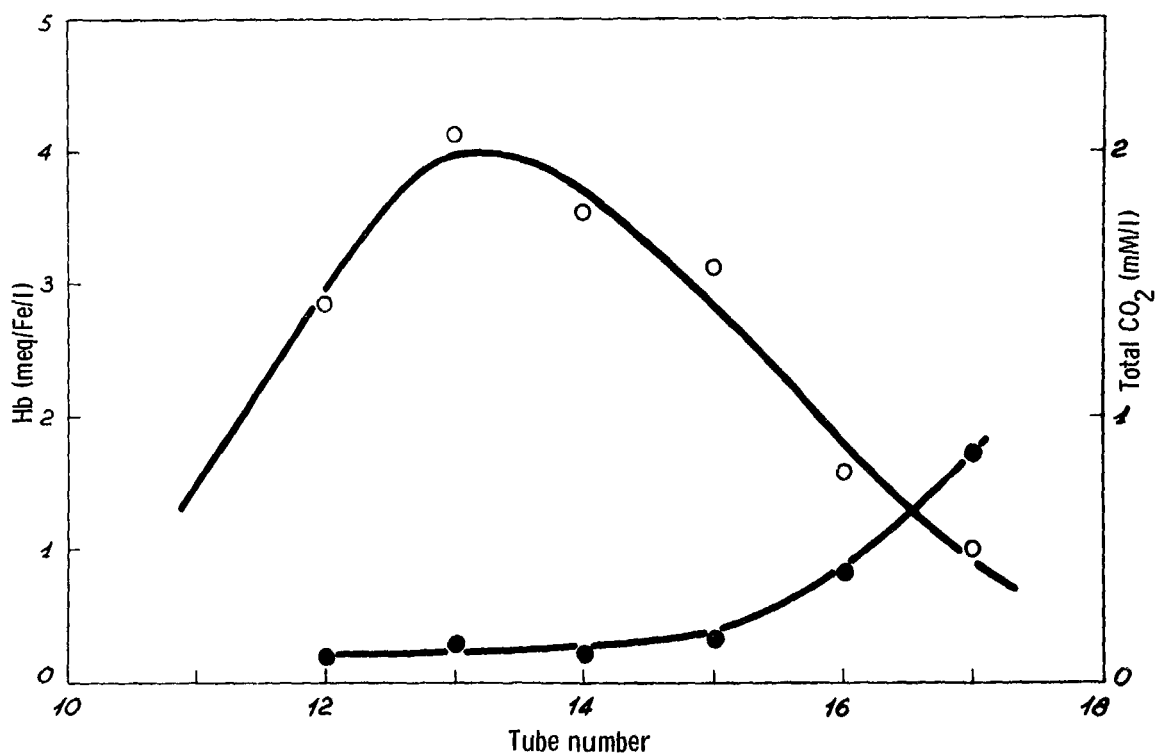


FIGURE 2.—Total Hb concentration and total CO<sub>2</sub> concentration in series of sampling tubes, with Sephadex only in the column (no ion-exchange phase). In the example shown, CO<sub>2</sub>-free Hb (11 meq/l) was mixed with 0.15 M carbonate in 0.15 M KOH.

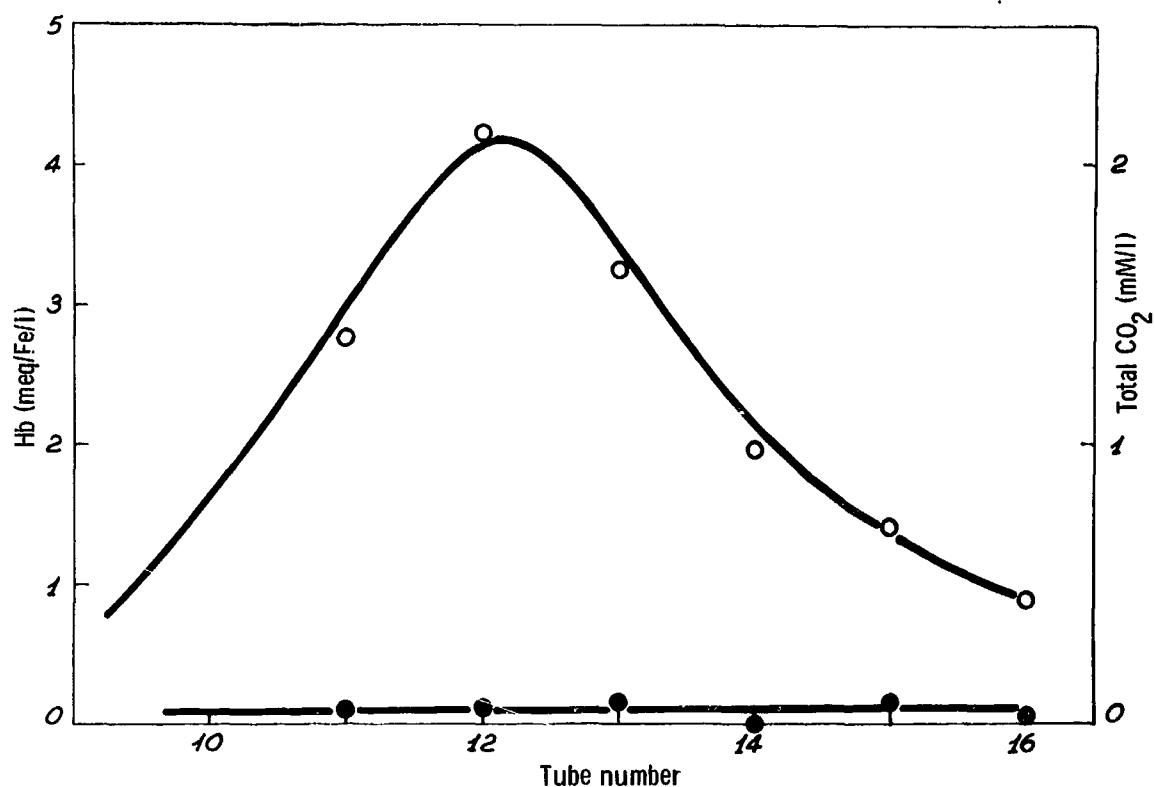


FIGURE 3.—The same mixture as in figure 2, except that the column contained both the Sephadex phase and the ion-exchange phase.

Hb, HbCO<sub>2</sub>, CO<sub>2</sub>, H<sub>2</sub>CO<sub>3</sub>, HCO<sub>3</sub><sup>-</sup>, and small amounts of CO<sub>3</sub><sup>2-</sup>, whereas the other syringe contained sufficient KOH to give the required pH of 11.7 to 12.0 on mixture of the two solutions. In figure 4 we show, for a typical experiment of this kind, the concentration of hemoglobin and total CO<sub>2</sub> in the samples eluted from the Sephadex +Dowex column. The total CO<sub>2</sub> content is presented as shaded rectangles and represents the amount of HbCO<sub>2</sub> originally present in the CO<sub>2</sub> equilibrated solution

plus (a): such fraction of the dissolved CO<sub>2</sub> originally present in the CO<sub>2</sub>-equilibrated Hb solution as combines to form additional carbamate during the addition of the alkali.

minus (b): such fraction of the HbCO<sub>2</sub> as dissociated into CO<sub>2</sub> (and amine groups) during the time (≤3 minutes) taken by the column process.

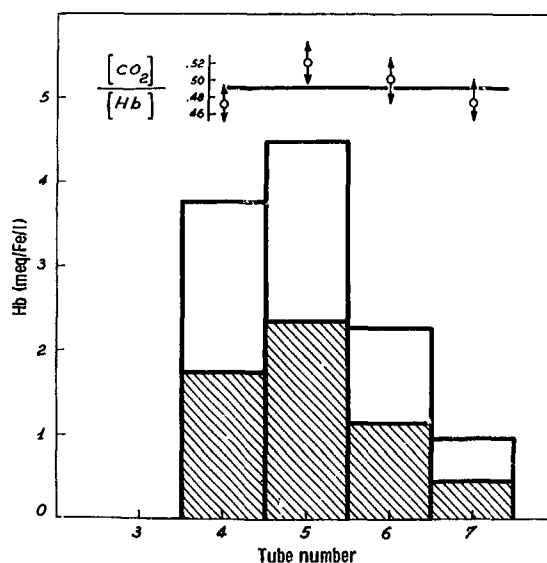


FIGURE 4.—Results obtained in an actual hemoglobin-carbamate determination. For details, see text.

In regard to (a), the controls of Ferguson and Roughton (1934) and of Ferguson (1936) show that about 10 percent of the original dissolved CO<sub>2</sub> gets turned into fresh hemoglobin carbamate during the estimation process. To reduce this proportion we replaced the 0.15 M KOH solution, with which the hemoglobin solution was mixed, by a solution of 0.25 M K-glycinate in 0.15 M KOH, the object of the glycinate being to "quench" the dissolved CO<sub>2</sub> by enabling it to combine rapidly and preferentially as glycinate-carbamate. The latter, like the other small ionic species present, should be retarded by the Sephadex gel particles. (Faurholt has similarly used dimethylamine as a quencher in his studies on the kinetics of hydration of CO<sub>2</sub> in aqueous solution.) In an experiment at 18°C on Hb solution in equilibrium with  $P_{\text{CO}_2}$  = 45 mm Hg, the addition of 0.25 M K-glycinate to the 0.15 M KOH solution was found to reduce the estimated value of the HbCO<sub>2</sub> by about 12 percent, which, on the basis of the controls used by Ferguson and Roughton, tallies closely with the effect to be expected if the glycinate is performing its quenching function properly. We hope to do further controls both with other concentrations of glycine and also with other potential CO<sub>2</sub> quenchers.

In regard to (b), i.e., the possibility of significant dissociation of the HbCO<sub>2</sub> during the passage through the column, approximate calculations indicate that under the prevailing conditions the loss should not have exceeded 1 to 2 percent of the total carbamate found.

That such was indeed the case is confirmed by the total CO<sub>2</sub>/Hb ratio, as calculated from the experimental data reported in figure 4. Because each fraction was collected at about 30-second intervals, any rapid dissociation of HbCO<sub>2</sub> should betray itself by a significant progressive decrease of the ratio in the later samples. The approximate constancy of the ratio, i.e.,  $0.5 \pm 0.02$ , implies that dissociation of HbCO<sub>2</sub> does not occur to a measurable extent under these conditions.

## DISCUSSION

In figure 5 we summarize the differences between the technique used by Ferguson and Roughton (1934) and that described in the present paper, which should be applicable, without signifi-

cant modifications, to other proteins besides hemoglobin. In particular it should be interesting to study with its aid the carbamate reactions of the blood plasma proteins, which Ferguson and Roughton found impossible to investigate by their method in view of their much greater "protective colloid" effect on the formation of the BaCO<sub>3</sub> precipitates and their separation by centrifugation. In the meantime, however, there would seem to be great opportunities for the further application of the method to the carbamate reactions of deoxygenated and oxygenated hemoglobin solutions—a very live subject at the present time.

## ACKNOWLEDGMENTS

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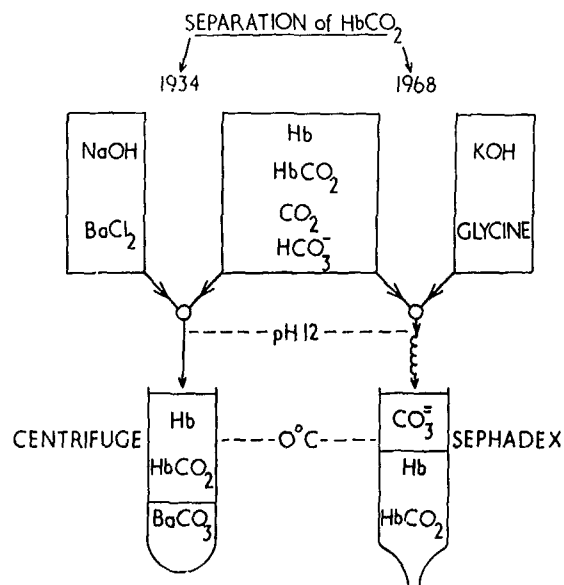


FIGURE 5.—Comparison of the earlier technique of Ferguson and Roughton (1934) with that described in this paper. The column is shown as containing the Sephadex phase only.

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## Some Properties of Horse Hemoglobin Specifically Modified at the $\alpha$ -Amino Groups

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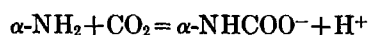
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Horse hemoglobin was specifically modified at the  $\alpha$ -amino groups by reaction with cyanate. These derivatives retained heme-heme interactions. When the  $\alpha$ -amino group of the  $\alpha$ -chain was chemically modified, the Bohr effect was reduced by 30 percent. When both  $\alpha$ -amino groups of both chains were modified, there was no oxygen-linked CO<sub>2</sub> binding and probably no CO<sub>2</sub> binding at all under physiological conditions, thus showing that the  $\alpha$ -amino groups are responsible for the binding of CO<sub>2</sub> by horse hemoglobin.

The carbamino reaction between CO<sub>2</sub> and hemoglobin accounts for a significant fraction of the total CO<sub>2</sub> exchanged by the blood during respiration (Rossi-Bernardi and Roughton, 1967b; Roughton, 1964). The combination between CO<sub>2</sub> and hemoglobin is "oxygen linked," i.e., at constant pH and  $P_{CO_2}$ , less CO<sub>2</sub> is bound to oxy than to deoxyhemoglobin (Rossi-Bernardi and Roughton, 1967b). The identification of the CO<sub>2</sub>-binding groups of hemoglobin will give a better understanding of the physiological function of hemoglobin in relation to its molecular structure. The most likely group in the hemoglobin molecule to react with CO<sub>2</sub> would be the  $\alpha$ -amino group because of its low  $pK$ . This reaction would occur in the following way:



To confirm this hypothesis, we have prepared derivatives of horse hemoglobin specifically modified at the  $\alpha$ -amino groups. If these chemically modified hemoglobins still show oxygen-linked CO<sub>2</sub> effects, then the  $\alpha$ -amino groups probably are not involved in such effects. If oxygen-linked CO<sub>2</sub> effects are not present, two interpretations

are possible: (1) that they are the groups responsible for CO<sub>2</sub> combination, or (2), that they are not, but that the chemical modification has caused a change in tertiary structure leading to a masking of the groups really responsible for CO<sub>2</sub> combination. It may be argued, by a somewhat similar line of reasoning, that the chemical modification of the protein may significantly lower the  $pK$  of some amino group and also make the CO<sub>2</sub> combination with such groups oxygen linked under physiological conditions of pH and  $P_{CO_2}$ . In this case the modified hemoglobin would still show oxygen-linked CO<sub>2</sub> effects even if the  $\alpha$ -amino groups are blocked. The possibility of this is, however, remote.

### PREPARATION AND CHARACTERIZATION OF THE MODIFIED HEMOGLOBINS

Neer and Konigsberg (1968) used fluorodinitrobenzene to modify specifically the  $\alpha$ -amino group of the  $\alpha$ -chain of human hemoglobin. After modification, however, the hemoglobin exhibited no Bohr effects and lost all heme-heme interaction. These facts would indicate a change in

tertiary structure; but the high oxygen affinity of this hemoglobin would increase the technical difficulties of physicochemical measurements.

To prepare a modified hemoglobin with more normal functional properties, we used a different reagent for the modification of the  $\alpha$ -amino groups. Stark (1965b) and Smyth (1967) have presented evidence that cyanate reacts with  $\alpha$ -amino groups in the following way:



Stark (1964; 1965a, b, c) and Smyth (1967) have also shown that the reaction of cyanate with tyrosine, histidine, and cysteine is reversible at alkaline pH, although its reaction with carboxyl groups is not.

In the preparation of a hemoglobin specifically modified at the  $\alpha$ -amino group with cyanate, it is necessary to insure that, under the reaction conditions used, the  $\alpha$ -amino group is most reactive toward cyanate and to limit the reaction so that this is the main reaction product. The hemoglobin reacted only at the  $\alpha$ -amino group can be separated from overreacted hemoglobin (i.e., reacted at both the  $\alpha$ -amino groups and lysines) and from unreacted hemoglobin on an ion-exchange column. An outline of the reaction scheme is shown in figure 1. Horse hemoglobin was used to study the structure of

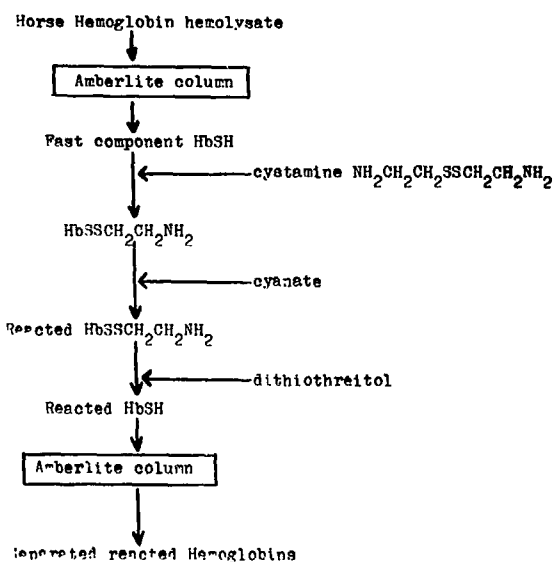


FIGURE 1.—Reaction scheme.

these derivatives by X-ray crystallography. Horse hemoglobin, however, consists of two components—fast and slow—present in approximately equal amounts (Bangham and Lehmann, 1958). Because it is necessary to separate out one of the components before chemically modifying the hemoglobin, the fast component was purified from the horse-hemoglobin hemolysate by ion-exchange chromatography on Amberlite CG-50. The band containing the fast component was eluted after two column-volumes had passed by increasing the temperature from 4 C to 25 C (Clegg and Schroeder, 1959). Practically all the non-heme proteins present in the erythrocyte should have been removed by this procedure.

The hemoglobin was then treated with cystamine (Taylor, Antonini, Brunori, and Wyman, 1966) to protect the reactive sulfhydryl group of the  $\beta$ -chain because it is difficult to remove all the cyanate that has reacted with this group after treatment at alkaline pH. After the reaction with cyanate, the cystamine was removed by treatment at pH 8.5 with the thiol reagent dithiothreitol. Experiments with S<sup>35</sup>-cystamine showed that less than 1 percent of the cystamine remained on the hemoglobin after this treatment. This treatment at pH 8.5 would also remove any cyanate reacted with histidine, tyrosine, and unreactive -SH groups. The reacted hemoglobin was then placed on another Amberlite column giving the elution pattern shown in figure 2. The number of moles of cyanate per mole of  $\alpha\beta$  dimer was measured in each of the numbered peaks by using radioactive cyanate (table I).

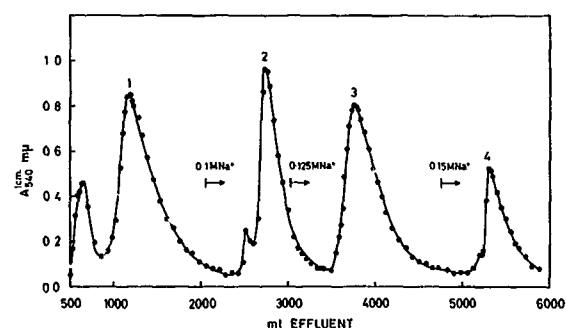


FIGURE 2.—Elution pattern of hemoglobin reacted with cyanate. The Amberlite column was equilibrated with 0.05 M sodium phosphate buffer and developed with a discontinuous salt gradient.

For each of the numbered peaks, the  $\alpha$ - and  $\beta$ -chains were separated by a modification of the method of Clegg, Naughton, and Weatherall (1966) (fig. 3). In the elution pattern of peaks 1 and 2, the small peaks marked  $\beta'$  are chromatographic artifacts and are identical with the large peaks marked  $\beta$  (they have the same fingerprint and the same radioactive peptide). Peak 1 has approximately equal amounts of radioactivity in both the  $\alpha$ - and  $\beta$ -chains; peak 2 has radioactivity in the  $\beta$ -chain only; peak 3, radioactivity in the

$\alpha$ -chain only; and peak 4, no radioactivity in either chain. Tryptic digestion and fingerprinting of these separated chains were carried out by the methods previously described (Clegg et al., 1966; Kilmartin and Clegg, 1967). In the case of the radioactive  $\beta$ -chains from peaks 1 and 2, a single radioactive peptide was isolated whose amino acid composition corresponded to the N-terminal tryptic peptide, residues 1-8. In the case of the radioactive  $\alpha$ -chains from both peaks 1 and 3, two radioactive peptides were isolated whose

TABLE I.—Chemical and Oxygen Equilibrium Data for Modified Hemoglobin Fractions

| Peak no. | Moles $C^{14}$ -cyanate per mole of $\alpha\beta$ dimer | Symbol                | Hill constant, $n$ | $P_{50}$ (mm $O_2$ )<br>pH 7.40; 25 C |
|----------|---|-----------------------|--------------------|---------------------------------------|
| 1.....   | 1.86  | $\alpha_2^c\beta_2^c$ | 2.5                | 4.2                                   |
| 2.....   | 1.03  | $\alpha_2\beta_2^c$   | 2.0                | 5.9                                   |
| 3.....   | .96   | $\alpha_2^c\beta_2$   | 2.6                | 3.9                                   |
| 4.....   | .08   | $\alpha_2\beta_2$     | 2.7                | 6.6                                   |

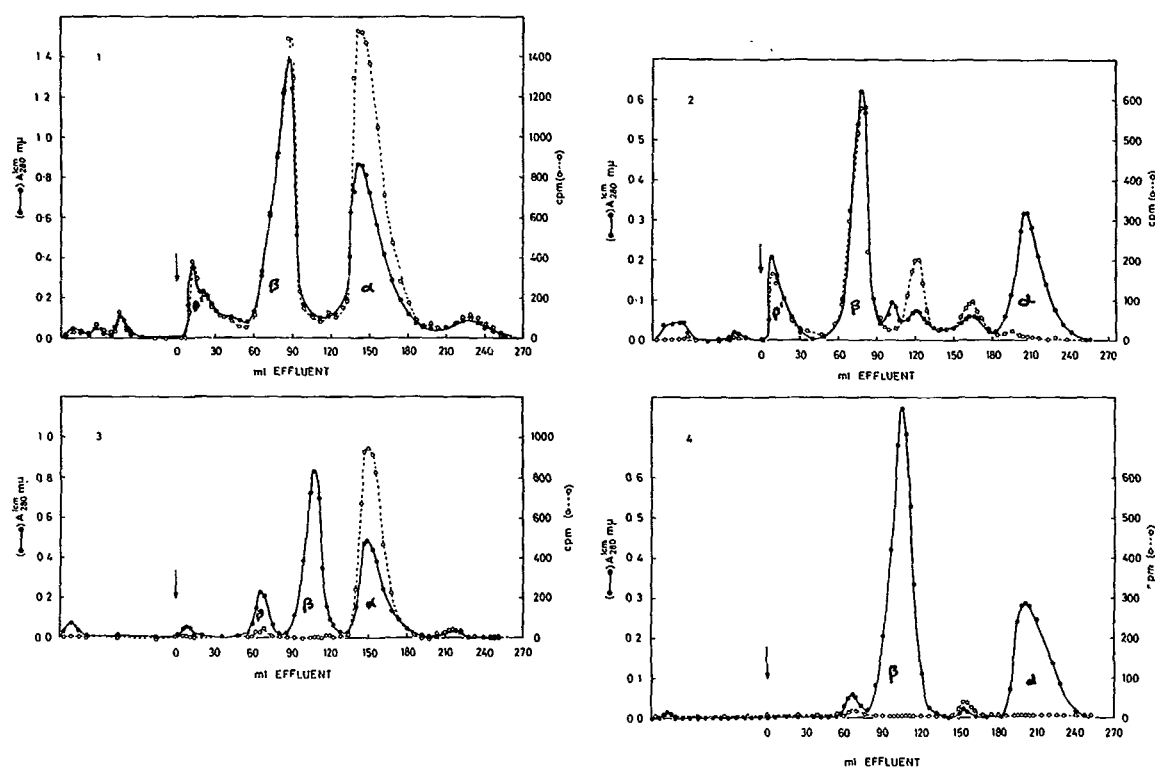


FIGURE 3.—Elution pattern of the separation of the  $\alpha$ - and  $\beta$ -chains of the reacted hemoglobins. The arrow marks the beginning of a linear salt gradient.

amino acid composition corresponded to residues 1-7 and residues 1-11.

These results would indicate that cyanate had reacted with the N-terminal  $\alpha$ -amino groups only. If this were correct, then all the radioactivity should be recovered as valine hydantoin if the protein was treated, as described by Stark and Smyth (1963) for the determination of the N-terminal amino acid of a protein. Preliminary results have shown that when this procedure is

applied to the radioactive hemoglobins in peaks 1, 2, and 3, 80 to 85 percent of the counts are released together with an equivalent amount of valine hydantoin, the latter being measured as valine after alkaline hydrolysis. Further experiments are in progress to determine whether this is the maximum yield of valine that can be released by this procedure.

Thus these experiments show that peak 1 is hemoglobin in which cyanate has reacted with

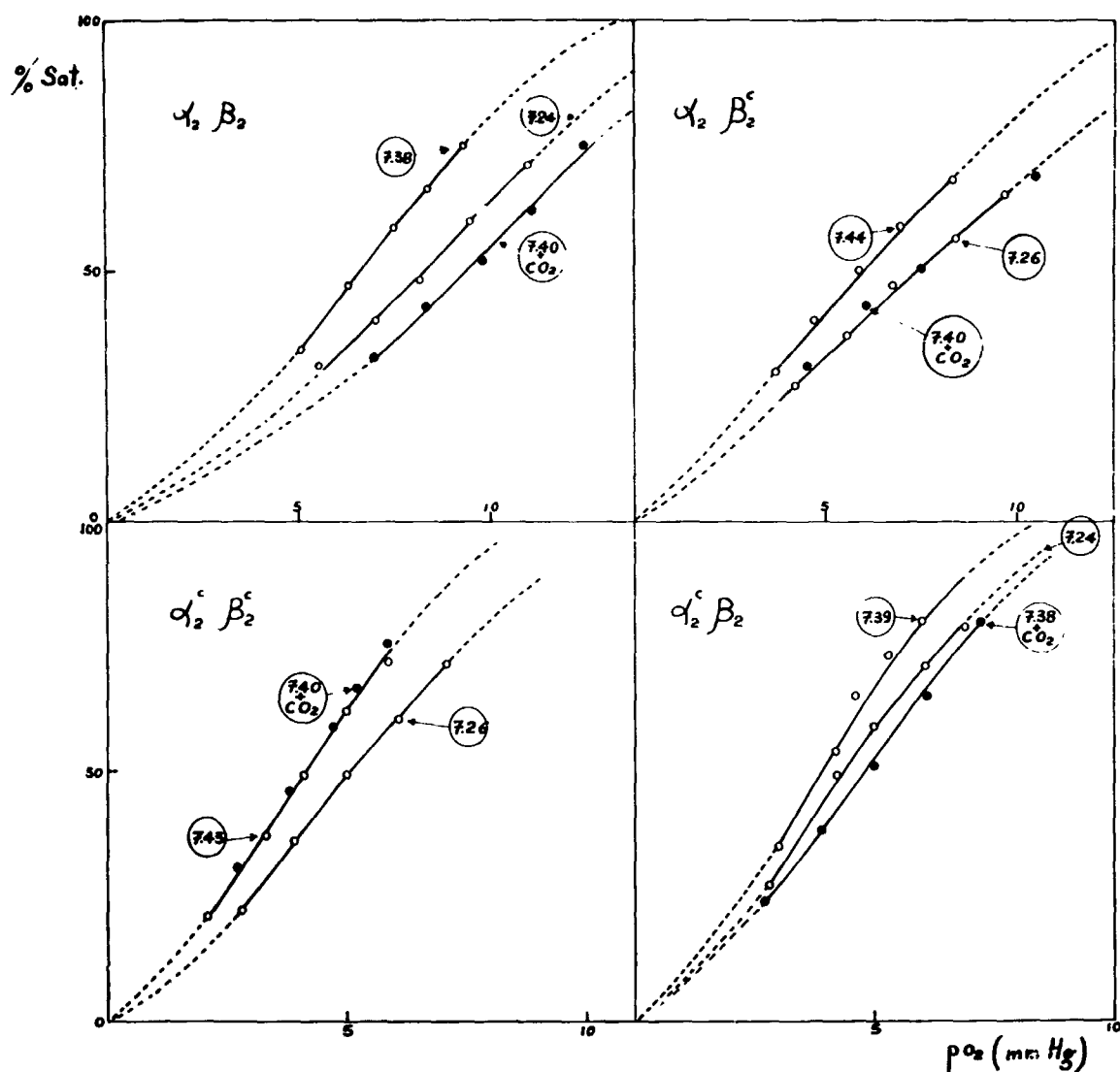


FIGURE 4.—Oxygen equilibrium curves of the derivatives. The conditions in the absence of CO<sub>2</sub> (○) were: 15 mM phosphate buffer, 0.2 M KCl, 0.05 percent hemoglobin concentration, temperature 25 C. For the curves in the presence of CO<sub>2</sub> (●), 50 mM bicarbonate, 15 mM phosphate, and 0.15 M KCl were used and equilibrated with 5 percent CO<sub>2</sub> and 95 percent N<sub>2</sub>. pH is indicated.

the  $\alpha$ -amino groups of both the  $\alpha$ - and  $\beta$ -chains. This modified hemoglobin is given the notation  $\alpha_2\beta_2^c$ ; peak 2 is hemoglobin with the cyanate reacted with the  $\alpha$ -amino group of the  $\beta$ -chain only,  $\alpha_2\beta_2^c$ ; peak 3 is cyanate reacted with the  $\alpha$ -amino group of the  $\alpha$ -chain only,  $\alpha_2^c\beta_2$ ; peak 4 is unreacted material,  $\alpha_2\beta_2$ .

### PHYSICOCHEMICAL PROPERTIES OF THE CARBAMYLATED HEMOGLOBINS

The major problem in attempting to carry out physicochemical studies on purified horse hemoglobin and its carbamylated derivatives has been their rapid oxidation to methemoglobin in the presence of even small traces of oxygen. At pH 7.0 and 4 C under the best "deoxygenated" conditions, about 15 percent oxidation occurs in 24 hours for  $\alpha_2\beta_2^c$  and 5 percent oxidation for  $\alpha_2\beta_2$  (i.e., the unreacted fast component), probably because the stabilizing factors (presumably proteins) present in the hemolysate are removed from the hemoglobin by the Amberlite column. There are also unknown factors (possibly differences in oxygen affinity) that influence this susceptibility to oxidation.

The oxidation can be prevented by removal of oxygen from the hemoglobin under vacuum followed by equilibration with nitrogen; this proce-

dures is repeated several times, followed by a final equilibration with carbon monoxide. All buffers used were similarly treated. The Amberlite columns were run with these CO-saturated buffers in an atmosphere of CO. Before each experiment the CO was removed from the hemoglobin by displacement with oxygen under strong light. The hemoglobin was then deoxygenated by several equilibrations with nitrogen. By standard van Slyke gas-analysis methods, the residual amounts of both CO and O<sub>2</sub> were less than 5 percent. The methemoglobin concentration was also less than 5 percent.

The study of the functional properties, that is, oxygen affinity, heme-heme interactions, and the Bohr effect of the various derivatives, is an essential step before any CO<sub>2</sub>-binding studies can be carried out.

### OXYGEN EQUILIBRIUM CURVES

The oxygen equilibrium curves were determined by the method of Riggs (1951). The conditions are shown in figure 4 and the results in table I. The Hill constant  $n$  in the case of  $\alpha_2\beta_2^c$  and  $\alpha_2^c\beta_2$  is essentially unchanged and, therefore, indicates normal heme-heme interactions. It is somewhat low for  $\alpha_2\beta_2^c$ . It is significant that  $\alpha_2\beta_2$  and  $\alpha_2\beta_2^c$ , on the one hand, and  $\alpha_2^c\beta_2$  and  $\alpha_2^c\beta_2^c$ , on the other (which have similar Bohr effects, see p. 78), also have similar oxygen affinities. However, a better comparison would be to measure the absolute oxygen affinities at pH 9.5 where no Bohr effect is present, although this experiment would prove difficult in view of the very high oxygen affinity of hemoglobin at this pH.

### THE BOHR EFFECT AND TITRATION CURVE STUDIES

The Bohr effect is defined here as the change in oxygen affinity with pH or as the difference in proton binding between oxy- and deoxyhemoglobin at constant pH in the absence of CO<sub>2</sub>. As buffers may affect the oxygen affinity, we have measured the Bohr effect for all derivatives by differential titration curves. The method used was essentially that of Rossi-Bernardi and Roughton (1967a). The Bohr effect, as measured by this method for all the derivatives, is shown in figure 5. It is

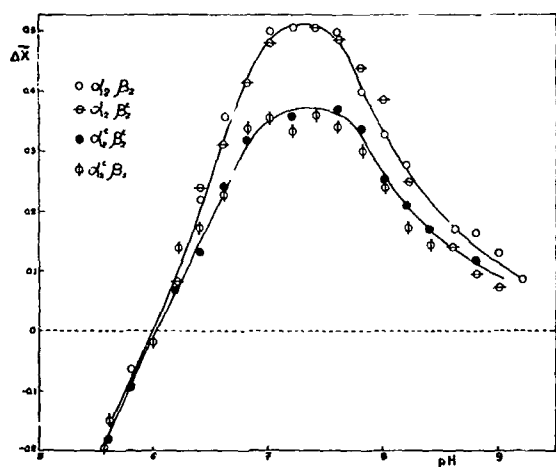


FIGURE 5.—The difference in negative charge per heme,  $\Delta\bar{X}$ , between oxy- and deoxyhemoglobin plotted against pH at 25 C. Hemoglobin concentration 1.0 to 1.5 meq in 0.2 M KCl.

interesting to see that  $\alpha_2\beta_2^c$  and  $\alpha_2\beta_2$ , on the one hand, and  $\alpha_2\beta_2^c$  and  $\alpha_2\beta_2$ , on the other, have similar Bohr effects. The diminished Bohr effect of  $\alpha_2\beta_2^c$  (more easily obtained in larger quantities than the others) was confirmed in four other differential titration experiments. It should be added that, because of shortage of material, the results for  $\alpha_2\beta_2^c$  and  $\alpha_2\beta_2$  are from a single experiment. Thus it would appear that the  $\alpha$ -amino group of the  $\alpha$ -chain is responsible for a part of the "alkaline" Bohr effect. However, as stated previously, the chemical modification of this group could cause a change in conformation to give a similar diminished Bohr effect. We hope that such changes, should they occur, will be seen by examination of the X-ray crystallographic structure of these derivatives.

Over 30 years ago, Ferguson and Roughton (1934b) put forward the idea that some amino groups of hemoglobin were oxygen linked not only in regard to carbamino formation but also in H<sup>+</sup> ion binding. This idea was confirmed by the more detailed results of Rossi-Bernardi and Roughton (1967). More recently, Hill and Davis (1967) stated that they have obtained evidence that the  $\alpha$ -amino groups of the  $\alpha$ -chain are two of the four groups responsible for the alkaline Bohr effect. Tanford and Nozaki (1966), by analyzing published titration curves, have shown that an  $\alpha$ -amino group with a low  $pK$  is present in oxyhemoglobin, and that in deoxyhemoglobin the  $pK$  of this group increases to that expected for a normal  $\alpha$ -amino group. An opposite view has been taken by Antonini (Antonini, Wyman, Brunori, Fronticelli, Bucci, and Rossi-Fanelli, 1965) from titration curve data and by Zito and Brunori (unpublished results quoted in Wyman, 1968).

If we assume that the chemical modification does indeed suppress the ionization of the two truly oxygen-linked  $\alpha$ -amino groups, then it would be interesting to estimate from our data the change in  $pK$  of these two groups on oxygenation. Thus, from figure 5, at pH 7.3, we obtain for  $\Delta\bar{X}_{\max}$  ( $\Delta\bar{X}_{\max}$  is the maximum value attained by this quantity at any pH) a value for  $\alpha_2\beta_2$  of 2.0 H<sup>+</sup> ion/mole hemoglobin released by oxygenation, and for  $\alpha_2\beta_2^c$ ,  $\Delta\bar{X}_{\max}=1.5$  H<sup>+</sup> ion/mole hemoglobin. The blocking of two amino groups thus contributes 2.0-1.5=0.5 H<sup>+</sup> ion, or

0.25 H<sup>+</sup> ion for each group. The maximum  $\Delta pK$  compatible with this value can now be estimated (if the contribution to  $\Delta\bar{X}_{\max}$  from the acid Bohr groups is assumed negligible). By substituting  $\Delta\bar{X}_{\max}=0.25$  into Wyman's formula (eq. (24), p. 463 (Wyman, 1948)), we obtain

$$\Delta pK = 2 \log (1+0.25)/(1-0.25) = 0.44$$

Therefore it seems that for the fast component of horse hemoglobin, a major part of the Bohr effect comes from groups other than the  $\alpha$ -amino groups. It would be of interest to extend these studies to human and bovine hemoglobin, which have recently been worked on intensively by Rossi-Bernardi and Roughton (1967a) and Chipperfield, Rossi-Bernardi, and Roughton (1967) with regard to the titration curves and total heat of ionization.

To see whether the ionization of any other groups has been affected by the chemical modification of the  $\alpha$ -amino groups, titration curves of the different modified hemoglobins were compared. These were measured as previously described (Rossi-Bernardi and Roughton, 1967a), except that the protein solutions were deionized before use by passage through a Dintzis column, as described by Nozaki and Tanford (1967).

These results are shown in figure 6. The tests were repeated several times and each gave the same result. The CO form was used to prevent possible methemoglobin formation. It can be seen from figure 6 that at alkaline pH, no differ-

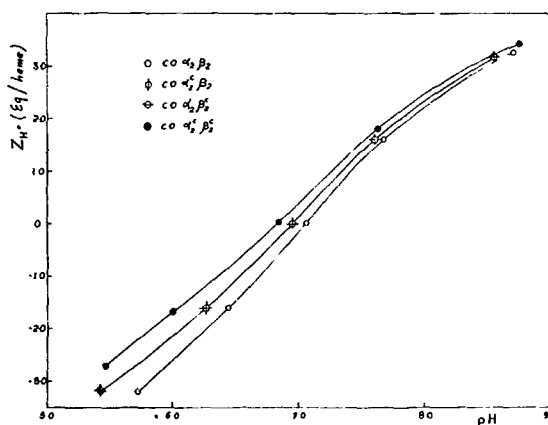


FIGURE 6.—Titration curves of the carbonmonoxy forms of the derivatives. Hemoglobin concentration 0.8 meq in 0.2 M KCl, temperature 25 C.

ence in charge is observed among any of the derivatives and  $\alpha_2\beta_2$ . This result would be expected because the  $\alpha$ -amino groups, whether reacted with cyanate or not, should be unprotonated at this pH. At acid pH, however,  $\alpha_2^c\beta_2^c$  differs by about 1.0 protonic charge per heme from  $\alpha_2\beta_2$ ; again, this result would be expected because the  $\alpha$ -amino groups would be in the  $-\text{NH}_3^+$  form at this pH, whereas  $\alpha_2^c\beta_2^c$  would not have any charge on its  $\alpha$ -amino groups. At neutral pH, the curve for  $\alpha_2\beta_2$  and  $\alpha_2\beta_2^c$  lies halfway between that of  $\alpha_2^c\beta_2^c$  and  $\alpha_2\beta_2$ ; this fact suggests that the  $pK$  of the  $\alpha$ -amino group of both the  $\alpha$ - and  $\beta$ -chains of HbCO is the same. Therefore  $pK$  can be estimated from figure 6 as the value of pH where  $Z_{\text{H}^+}(\alpha_2^c\beta_2^c) - Z_{\text{H}^+}(\alpha_2\beta_2) = 0.5$ , and is about  $7.1 \pm 0.1$ . This value for the apparent  $pK$  of the  $\alpha$ -amino groups of horse hemoglobin can be compared with a value of 6.72 for the  $\alpha$ -amino of the  $\alpha$ -chain of human CO-hemoglobin obtained by a different method (Hill and Davis, 1967). Thus the titration curves of HbCO indicate that the ionization of no other group has been affected by the chemical modification of the  $\alpha$ -amino groups.

The evidence collected thus far indicates that no gross conformational change has occurred in these derivatives. However, while it is easy to show that a conformational change has occurred, it is very difficult to prove, without X-ray analysis, that no major conformational change has occurred.

#### CO<sub>2</sub> AND O<sub>2</sub> EQUILIBRIA OF CARBAMYLATED HEMOGLOBINS

Because of the small amounts of each derivative available, no studies of the absolute amounts of bound CO<sub>2</sub> could be performed. In fact, using either the classical method of Ferguson and Roughton (1934a) or the more recent CO<sub>2</sub> electrode approach of Constantine, Rossi, and Roughton (1963) and Forster, Constantine, Craw, Rotman, and Klocke (1968), many times more material would be required than was ordinarily produced in a week.

Instead, we have studied the change in oxygen affinity produced by CO<sub>2</sub> at constant pH, which is thought to be caused by the formation of oxygen-linked carbamino compounds, and the

difference in the amounts of CO<sub>2</sub> bound by deoxy and carbonmonoxy hemoglobin. The oxygen equilibrium curves measuring this effect are given in figure 4. The equilibrium curve measured at lower pH in the absence of CO<sub>2</sub> was used to give an indication of the size of the CO<sub>2</sub> effect and not to measure the Bohr effect (which would be very inaccurate over such a small pH range). As already shown by Margaria and Green (1933), the effect of CO<sub>2</sub> at constant pH can be seen clearly in the case of  $\alpha_2\beta_2$ . In  $\alpha_2^c\beta_2^c$  there is no effect at all and, interestingly enough, there is an effect in both  $\alpha_2\beta_2$  and  $\alpha_2\beta_2^c$  indicating that all four  $\alpha$ -amino groups participate in CO<sub>2</sub> binding. Because interference between the specific effect of the phosphate ion and CO<sub>2</sub> may occur, we have also investigated the effect of carbon monoxide on CO<sub>2</sub> affinity at constant  $P_{\text{CO}_2}$  and pH, according to the method of Rossi-Bernardi and Roughton (1967b), except that HbCO was used instead of HbO<sub>2</sub> (because of the high rate of methemoglobin formation at 37 C in presence of oxygen). The results of the experiments for  $\alpha_2\beta_2$  and  $\alpha_2^c\beta_2^c$  are shown in figure 7. In the case of  $\alpha_2\beta_2$ , the usual difference in total CO<sub>2</sub> of 1.3 to 1.4 mM/l between Hb and HbCO is seen; however, with  $\alpha_2^c\beta_2^c$  no difference is seen, which is in agreement with the results obtained from the effect of CO<sub>2</sub> on the oxygen equilibrium curve. The dotted line in figure 7 has been calculated to correct for the slight difference in  $P_{\text{CO}_2}$  in the

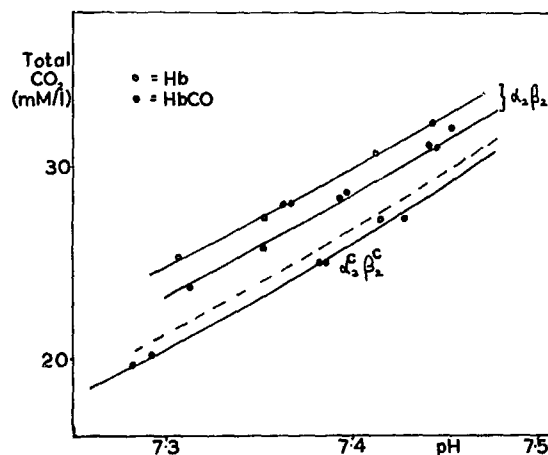


FIGURE 7.—Total CO<sub>2</sub> content at 37 C and  $P_{\text{CO}_2} = 46$  mm Hg, of 8.0 meq horse carbonmonoxy- and deoxyhemoglobin  $\alpha_2\beta_2$  and  $\alpha_2^c\beta_2^c$ .

case of  $\alpha_2^c\beta_2^c$ , because a cylinder containing a different gas mixture was used.

The difference between the dotted line of figure 7, that is, the total CO<sub>2</sub> of  $\alpha_2^c\beta_2^c$  (deoxy and carbonmonoxy forms) and of  $\alpha_2\beta_2$  (deoxy form) and  $\alpha_2\beta_2$  (carbonmonoxy form), should measure only the differences in carbamino content of these forms. Thus  $\alpha_2\beta_2$  (deoxy) has 3 mM carbamino compounds, and  $\alpha_2\beta_2$  (carbonmonoxy) has 1.5 mM under these conditions. These results agree with the results obtained, under comparable conditions, by Ferguson (1936), Ferguson and Roughton (1934a, b), and Stadie and O'Brien (1937).

Our results, indicating that  $\alpha_2^c\beta_2^c$  does not show any oxygen-linked carbamino formation and that it probably does not bind an appreciable amount of CO<sub>2</sub>, show that the  $\alpha$ -amino groups are the ones responsible for the binding of CO<sub>2</sub> by horse hemoglobin.

#### ACKNOWLEDGMENTS

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## DISCUSSION

**ROUGHTON:** The topics just discussed are of a controversial nature and I would very much like to hear any discussion, either of them or of the earlier papers.

**EDSALL:** This work looks very impressive and convincing. Zito, Wyman, and Antonini have obtained findings which will be very difficult to interpret in light of these findings. They blocked the terminal amino groups by transamination reactions and reported that the Bohr effect, as determined by protons released on oxygenation or by change in  $O_2$  pressure for half saturation of pH, is still unaltered in these forms of hemoglobin. This finding is perplexing in light of the findings of Kilmartin and Rossi-Bernardi. Zito's work is not yet published, although Wyman (1968) has referred to it in his recent review in the *Quarterly Review of Biophysics*. The work done with the cyanate derivatives seems to be clear-cut and convincing—the most clear-cut and decisive finding produced in this field—and points unmistakably to the  $\alpha$ -amino groups as being responsible for the  $CO_2$  binding. We thought so anyway, but it seems to me that this really proves it. I also find it difficult to believe that, if the  $\alpha$ -amino groups change their affinity for  $CO_2$  on oxygenation, they do not also change their affinity for protons on oxygenation. It is conceivable that they do not, but it is very hard for me to understand.

**FORSTER:** Dr. Rossi-Bernardi, how do you prevent reactions from continuing in the column?

**ROSSI-BERNARDI:** The pH was changed to about 11.7 in a msec, while the temperature was reduced to about zero in a second or so. The carbamate in solution dissociates very slowly under these conditions, with a probable half-time of the order of 1 hour, which is 20 times longer than the passage time through the column.

**ROUGHTON:** The column method made it feasible to introduce glycine as a "quencher" for the dissolved  $CO_2$  originally present in the  $CO_2$ -equilibrated hemoglobin solution. This modification was not appropriate in the old barium precipitation method of Ferguson and Roughton (1934a, b).

**EDSALL:** In the presence of the glycine, if allowed enough time, carbamate  $CO_2$  will be

removed from the hemoglobin and will become attached to the glycine—which is simply a matter of the rates of the reactions involved. This will not happen to any appreciable extent during the time the mixture is flowing through the column.

**ROSSI-BERNARDI:** Exactly. We found that by leaving the reactant mixture 10 minutes, you do not get an appreciable difference in the carbamate, which proves that the reaction is very slow.

**MAREN:** From the physiological point of view, when the carbamate reaction is mobilized, is any conformational change in the protein implied or is it completely explicable on the basis of the elevated  $P_{CO_2}$ ?

**ROSSI-BERNARDI:** We do not know whether the reaction of  $CO_2$  with the hemoglobin produces a configurational change—it is possible. We just know that  $CO_2$  reacts to a greater extent with deoxygenated than with oxygenated hemoglobin.

**MAREN:** Such experiments can be done.

**KILMARTIN:** The way to do it, of course, would be in a crystal, but that would be extremely difficult.

**HASTINGS:** How does the magnitude of the shift, with and without  $CO_2$  at constant pH, correspond to what I believe is Margaria's oxygenation dissociation curve shift?

**KILMARTIN:** We really did not attempt to develop this quantitatively.

**HASTINGS:** Has Neera done any additional work on this during the last 3 years?

**ROSSI-BERNARDI:** Neera did some work on the more physiological conditions. He found a  $CO_2$  effect independent of pH for the oxygen dissociation curve of whole blood, but he has not, as far as I know (I saw him 3 years ago), published any findings. When we measure the difference in total  $CO_2$  in  $CO_2$ -dissociation curves, we are working with solutions which are 10 to 15 percent concentrated hemoglobin. When we measure oxygen dissociation curves, we have to measure the Hb in 0.05 percent solution with the phosphate ion present, so the system is not exactly the same. We added the same solutes and observed roughly the same effects, but the phosphate ion may well have an effect on oxygen affinity that interferes with the  $CO_2$  effect. We are on much safer ground

when we measure the total CO<sub>2</sub> without any ions but those of NaCl and KCl present.

**HASTINGS:** These results are beautiful!

**EDSALL:** Have these derivatives been crystallized and studied by X-ray?

**KILMARTIN:** In collaboration with J. Greer, we have crystallized the  $\beta$ -blocked HbO<sub>2</sub> and have found it isomorphous with normal HbO<sub>2</sub>. We are doing the  $\alpha$ -blocked HbO<sub>2</sub>, which looks the same.

**PARTICIPANT:** Have you any comment on the fact that it seems that the CO<sub>2</sub> is bound to the valine, i.e., to the terminal-amino groups on both  $\alpha$ - and  $\beta$ -chains, yet both chains do not seem to participate in the Bohr effect?

**KILMARTIN:** The only evidence we have so far for the CO<sub>2</sub> effect is derived from the observed specific effects on the oxygen dissociation curves of the hemoglobin compounds. There is still an effect of CO<sub>2</sub> on the oxygen dissociation curve when only the  $\beta$ -chain terminal-amino groups are free; therefore, it does seem that all four terminal-amino groups might be involved in this effect. Unfortunately it is not, at present, feasible to do the more direct determinations on the CO<sub>2</sub> dissociation curves of the Hb compounds, because

of the relatively enormous quantities needed. For example, for a single point on the CO<sub>2</sub> dissociation curve, something like 200 milligrams of modified hemoglobin would be required; this amount would take several days to prepare.

**ROSSI-BERNARDI:** When we measure the Bohr effect in the presence of CO<sub>2</sub> in normal hemoglobin, the Bohr effect is reduced; but when the same experiment is repeated with the doubly blocked hemoglobin, the Bohr effect is unchanged. This observation clearly demonstrates the relation between the CO<sub>2</sub> binding and the Bohr effects associated with the terminal-amino groups.

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*SESSION III*

**Carbonic Anhydrase**

*Chairman: J. T. EDSALL*

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## Introductory Remarks

JOHN T. EDSALL

*Harvard University*

A discussion of the carbonic anhydrases, of necessity, will occupy a central position in a symposium on carbon dioxide in biology today. About 40 years ago, physiologists and biochemists began to recognize that the dehydration of bicarbonate ion to form  $\text{CO}_2$  in the lungs, and the hydration of  $\text{CO}_2$  as it flows into the blood from the tissues, must proceed with a speed that was inexplicable in the absence of a specific catalyst. In 1932, Meldrum and Roughton first reported this finding of that catalyst in red blood cells. In the following year they presented a masterly and detailed report of its preparation and properties and named it carbonic anhydrase. Keilin and Mann, in 1940, demonstrated that this enzyme contained zinc as an essential constituent and also discovered the specific inhibition of carbonic anhydrase by sulfonamides. It soon became apparent that carbonic anhydrases are not confined to red blood cells, but are widely distributed throughout many tissues of animals and plants. For nearly 20 years, however, although the physiologists and pharmacologists were active in studying the role of carbonic anhydrases, there was relatively little progress in their chemical characterization. From the point of view of the biochemist concerned with protein structure and function, carbonic anhydrase appeared for a time to be the forgotten enzyme.

This period of quiescence ended abruptly in 1960, when Lindskog reported the chromatographic fractionation of bovine erythrocyte carbonic anhydrase into at least two distinct isoenzymes; and a year later, Nyman, in the same laboratory at Uppsala, obtained three distinct human carbonic anhydrases. In our laboratory, Egon Rickli (independent of the work in Uppsala) had fractionated the human isoenzymes by a

different method; and in Marseille, Professor Derrien, Mme. Laurent, and their collaborators were also studying these isoenzymes and fractionating them by other distinctly different techniques.

During the last 5 years, the new work on carbonic anhydrases has poured forth in a flood which shows no sign of abating. Pocker and his collaborators have shown that the carbonic anhydrases of red cells are powerful catalysts for the hydration of aldehydes; Tashian, Malmström, Pocker, and others have shown that they function also as esterases. Pierre Henkart, Guido Guidotti, and I were recently surprised to find that they had catalyzed the hydrolysis of 2,4-dinitrofluorobenzene. Nobody doubts that the prime and perhaps sole physiological function of these enzymes is to promote the hydration of  $\text{CO}_2$  and the dehydration of bicarbonate; but it is important to know that as catalysts they are far more versatile than we had once supposed.

In optical rotatory dispersion and circular dichroism, the carbonic anhydrases show striking Cotton effects, arising from interactions of the aromatic side chains with asymmetric centers in the native molecule; these effects disappear on denaturation. They were first observed by Dirck Myers in our laboratory and independently by Andreas Rosenberg at the University of Minnesota. John Armstrong and Carole Lindblow in our laboratory studied these phenomena further, in close and indispensable collaboration with Sherman Beychok at Columbia University. Dr. Coleman has discovered a closely related effect, the induction of intense circular dichroism bands and Cotton effects by the interaction of optically inactive aromatic sulfonamides with carbonic anhydrases. Later in this session he will discuss

this and other phenomena in relation to the structure of these enzymes and their mechanism of action.

Our understanding of the mechanism of carbonic anhydrase action is still in its early stages; but we shall hear an important contribution to the subject by Dr. Wang during this session; and I look forward also to hearing the views of Dr. Pocker and others who have new insight to offer.

In Göteborg, Nyman and his collaborators have made much progress with the amino-acid sequence of human carbonic anhydrase B; and Louis Henderson in our laboratory has made more limited progress with carbonic anhydrase C. Both of them will present the current state of their work at this session.

In Uppsala, Strandberg, Liljas, and their collaborators have made tremendous progress in their X-ray measurements on human carbonic anhydrase C. More than a year ago they reported a three-dimensional structure for this enzyme, at 5.5 Å resolution. They are now making

steady progress toward a high-resolution structure that will be indispensable for understanding the mechanism of action of the enzyme. We are fortunate that Dr. Liljas is with us today to describe the current state of the work in the Uppsala laboratory.<sup>1</sup>

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<sup>1</sup> I have not provided any specific references for these introductory remarks. However, some readers of the published monograph might appreciate some general references to the literature, so I list four reviews, in chronological order:

ROUGHTON, F. J. W., AND A. M. CLARK, Carbonic Anhydrase. *The Enzymes*, I, First ed. (J. B. Sumner and K. Myrbäck, eds.), Academic Press, 1250-1265, 1951.

DAVIS, R. P., Carbonic Anhydrase. *The Enzymes*, V, 2d ed. (P. Boyer, H. Lardy, and K. Myrbäck, eds.), Academic Press, 545-562, 1961.

MAREN, T. H., Carbonic Anhydrase: Chemistry, Physiology and Inhibition. *Physiol. Rev.*, 47: 595-781, 1967.

EDSALL, J. T., The Carbonic Anhydrases of Erythrocytes. *Harvey Lectures*, Series 62, Academic Press, 191-230, 1968.

# X-ray Diffraction Studies on the Structure of Carbonic Anhydrase<sup>1</sup>

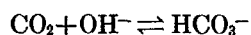
A. LILJAS, K. K. KANNAN, P. C. BERGSTÉN, K. FRIDBORG, L. JÄRUP, S. LÖVGREN,  
H. PARADIES, B. STRANDBERG, AND I. WAARA

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N70-23300

A number of complexes with human carbonic anhydrase form C (HCAC) was studied using X-ray diffraction methods in projections. It was found that all anionic inhibitors (such as sulfonamides, iodide, Roussin's salt, and negatively charged heavy metal complexes) occupy almost the same position. This phenomenon explains the competition observed among some for the active site. It was also found that all these groups, except the iodide, were within binding distance of the zinc atom of the enzyme. The position in projections of two cationic inhibitors, Ag<sup>+</sup> and Cu<sup>2+</sup>, was shown to be, with some probability, in the vicinity of the zinc atom.

Since the days of the discovery of carbonic anhydrase by Meldrum and Roughton (1933), the structure and function of this enzyme has been investigated extensively. Most of the work which has been done is discussed in the review by Maren (1967). The large amount of physiological and pharmacological data available, as well as the simplicity of the *in vivo* catalyzed reaction (Davis, 1961)



makes the X-ray crystallographic structure determination of carbonic anhydrase very significant.

At present the two human forms B (HCAB) and C (HCAC) (Nyman, 1961; Laurent, Charrel, Castay, Nahon, Marriq, and Derrien, 1962; Rickli and Edsall, 1962) are being investigated in this laboratory by X-ray diffraction methods. These two enzymes, having both similarities and

dissimilarities in the primary structures (Nyman, Strid, and Westermarck, 1966) as well as in the physical constants (Armstrong, Myers, Verpoorte, and Edsall, 1966), have affinities for the same substrates (Pocker and Dickerson, 1968; Henkart, Guidotti, and Edsall, 1968). The activity of the B-form is, however, much lower for most substrates, especially for CO<sub>2</sub> (Rickli, Ghazanfar, Gibbons, and Edsall, 1964). It will be of considerable interest to relate the three-dimensional structures of these two enzyme forms to the different activities and binding constants for the wide range of known substrates and inhibitors (Maren, 1967).

The low-resolution structure of HCAC was described by Fridborg, Kannan, Liljas, Lundin, Strandberg, Strandberg, Tilander, and Wirén (1967). The most important observed features of the molecule (fig. 1) are the molecular shape, which is ellipsoidal, and the position of the zinc atom in the center of the molecule and at the bottom of a crevice. (The zinc atom is essential for the activity (Lindskog and Malmström, 1962).) Part of the crevice constitutes the active site. Aromatic sulfonamides, known to be specific for carbonic anhydrase (Mann and Keilin, 1940), seemed to bind with the sulfonamide group to the zinc atom in a position above the zinc atom

<sup>1</sup>This work has been supported by grants from the Faculty of Science, University of Uppsala, from the Lennander Foundation, Sweden, from the National Institutes of Health, U.S. Public Health Service (AIGM 07382), from the Swedish Medical Research Council (11X-26) from the Swedish Natural Science Research Council (18-84 and 2142, as well as a postdoctoral research fellowship for B. Strandberg), and from the Knut and Alice Wallenberg Foundation, Stockholm.

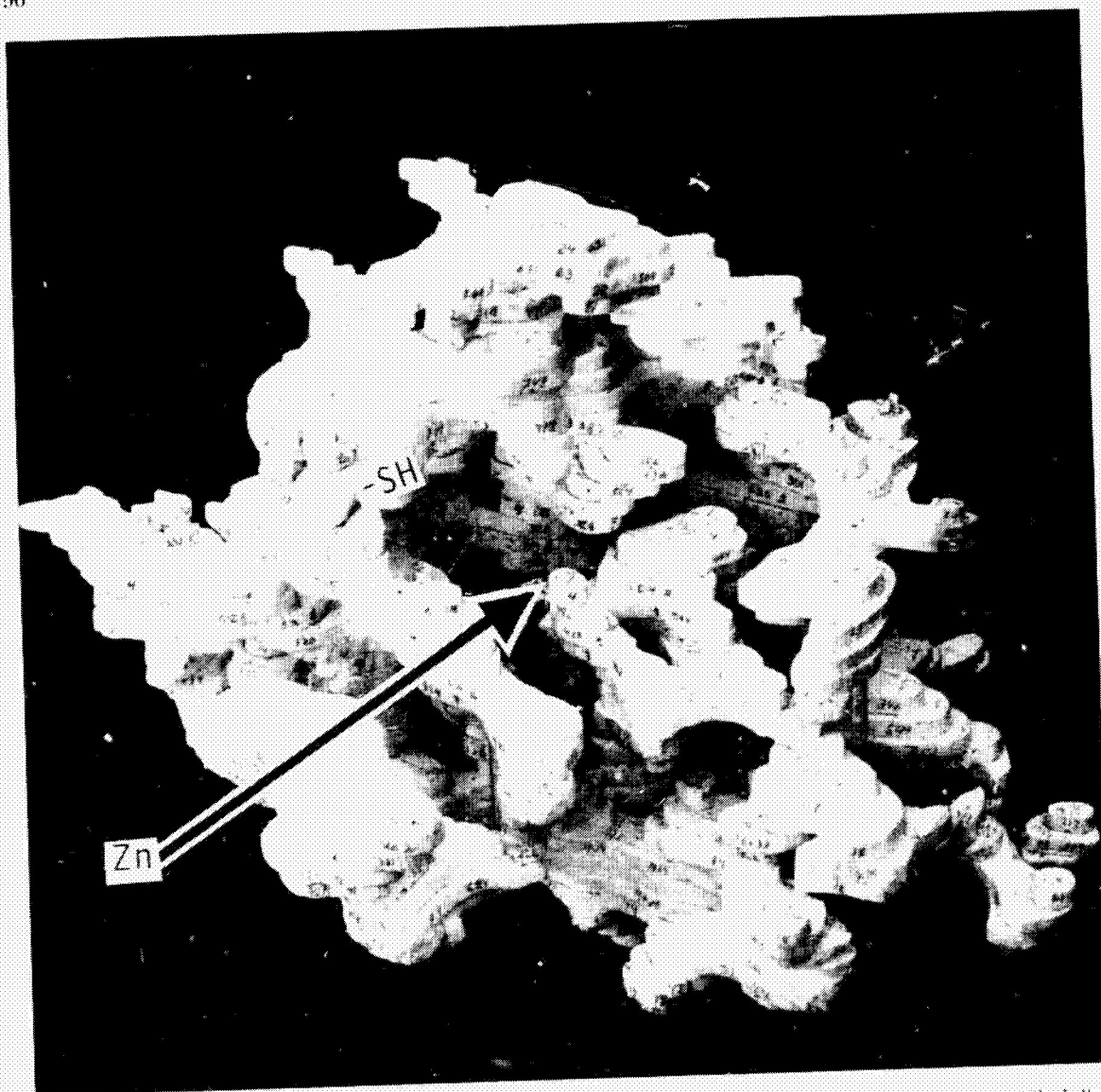


FIGURE 1.—A model of HCAC at low resolution. This side of the model shows the active site with the zinc atom at the bottom of a large crevice. The only SH-group is just outside the crevice about 14 Å from the zinc atom.

and inside the crevice. The position of the only cysteine residue was found to be on the surface of the molecule and just outside the crevice. It is, therefore, most probably not a part of the active site, the zinc-SH distance being about 14 Å.

This investigation presents a number of complexes between HCAC and smaller compounds, mainly inhibitors. Most of them will be more thoroughly discussed when the three-dimensional 2.0 Å resolution structure is obtained!

## MATERIALS AND METHODS

### Purification

Human carbonic anhydrases B and C were purified with a method similar to the one described by Armstrong et al. (1966). Compared with the old purification method using chloroform-ethanol treatment (Nyman, 1961), the quality of the crystals has improved considerably. Previously



it was possible to collect X-ray data to a resolution of about 2.8 Å, a figure which has now been decreased to less than 2.0 Å. This improvement is probably caused by the reduced damage to the molecules during purification. This increased resolution will simplify the detection of the lighter atoms or minor movements of the groups taking part in the catalytic mechanism.

### Crystallization

The crystallization of HCAC was performed using the method reported by Tilander, Strandberg, and Fridborg (1965). The enzyme was first complexed with methylmercurithioglycolic acid (MMTGA, prepared by Prof. A. Fredga, Uppsala) or with other mercurials. In the case of the complex with iodide, the crystals (obtained from Dr. P. O. Nyman, Göteborg) were prepared from native enzyme material. One difference from earlier published methods of crystallization was that some precipitate was removed by centrifugation at a  $(\text{NH}_4)_2\text{SO}_4$  concentration of 2.0 M. The crystallizations were then performed in 2.1–2.3 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 8.5. Dr. P. O. Nyman succeeded 2 years ago in obtaining good

HCAB crystals complexed with acetazolamide (Diamox). The crystallization method has now been improved both in Göteborg and in our laboratory and includes the native enzyme as well as a variety of complexes.

### Chemical Modifications

The different chemical modifications of HCAC discussed in this paper are listed in table I. Most of the compounds were studied to obtain better heavy atom derivatives, but others were studied because they were interesting inhibitors. In some cases, the salt medium in which the crystals were immersed was changed to  $\text{MgSO}_4$ . Especially for the investigation of the  $\text{Cu}^{2+}$ -complex, the  $\text{MgSO}_4$ -solution was changed three times before adding the  $\text{CuSO}_4$ .

In the soaking experiments, a few good HCAC crystals (native enzyme or enzyme crystals in complex with MMTGA) were dropped into a test tube containing 5 to 10 ml of the reaction solution. The crystals were not mounted for X-ray diffraction investigations earlier than 1 week after the complexing experiments were started.

TABLE I.—Investigated Complexes With Human Carbonic Anhydrase C (HCAC)

| Complexing compound concentration  | Complexing procedure                | Salt medium concentration            | X-ray data resolution    |
|--|-------------------------------------|--------------------------------------|--------------------------|
| MMTGA, <sup>a</sup> $10^{-3}$ M  | Crystn. as complex                  | $(\text{NH}_4)_2\text{SO}_4$ , 2.2 M | hol, 2.0 Å<br>3D, 5.5 Å  |
| AMSulf, <sup>b</sup> $10^{-3}$ M   | Crystn. as complex                  | $(\text{NH}_4)_2\text{SO}_4$ , 2.2 M | hol, 2.0 Å<br>3D, 5.5 Å  |
| AMAzoSulf, <sup>c</sup> $10^{-4}$ M  | Soaking of HCAC <sup>d</sup> cryst. | $(\text{NH}_4)_2\text{SO}_4$ , 2.3 M | hol, 2.0 Å               |
| KI, $6 \times 10^{-2}$ M   | Soaking of HCAC <sup>e</sup> cryst. | $(\text{NH}_4)_2\text{SO}_4$ , 2.1 M | hol, 2.0 Å               |
| $\text{KFe}_2\text{S}_3(\text{NO})_7$ , Roussin's salt; $5 \times 10^{-3}$ M | Soaking of MMTGA-HCAC cryst.        | $(\text{NH}_4)_2\text{SO}_4$ , 2.2 M | hol, 5.5 Å<br>hko, 5.5 Å |
| $\text{AgNO}_3$ , $5 \times 10^{-2}$ M                                       | Soaking of HCAC <sup>d</sup> cryst. | $(\text{NH}_4)_2\text{SO}_4$ , 2.2 M | hol, 3.0 Å               |
| $\text{CuSO}_4$ , $10^{-6}$ M  | Soaking of HCAC <sup>d</sup> cryst. | $\text{MgSO}_4$ , 2.0 M              | hol, 2.0 Å               |
| $\text{K}_2\text{Pt}(\text{CN})_4$ , $5 \times 10^{-3}$ M                    | Soaking of MMTGA-HCAC cryst.        | $\text{MgSO}_4$ , 2.0 M              | hol, 5.5 Å<br>hko, 5.5 Å |
| $\text{K}_2\text{PtCl}_6$ , $5 \times 10^{-3}$ M                             | Soaking of MMTGA-HCAC cryst.        | $\text{MgSO}_4$ , 2.0 M              | hol, 5.5 Å               |
| $\text{K}_2\text{PdCl}_4$ , $5 \times 10^{-3}$ M                             | Soaking of MMTGA-HCAC cryst.        | $(\text{NH}_4)_2\text{SO}_4$ , 2.2 M | hol, 5.5 Å               |
| $\text{KAuCl}_4$ , $5 \times 10^{-3}$ M                                      | Soaking of MMTGA-HCAC cryst.        | $\text{MgSO}_4$ , 2.0 M              | hol, 5.5 Å               |
| $\text{K}_2\text{HgI}_6$ , $5 \times 10^{-3}$ M                              | Soaking of MMTGA-HCAC cryst.        | $(\text{NH}_4)_2\text{SO}_4$ , 2.2 M | hol, 5.5 Å               |

<sup>a</sup> MMTGA = Methylmercurithioglycolic acid.

<sup>b</sup> AMSulf = 3-Acetoxymercuri-4-aminobenzenesulfonamide.

<sup>c</sup> AMAzoSulf = 3'-Acetoxymercuri-4'-hydroxy-4-azobenzenesulfonamide.

<sup>d</sup> HCAC = native enzyme. The crystals were prepared as previously reported (Tilander et al., 1965) by soaking MMTGA-HCAC crystals with cysteine.

<sup>e</sup> HCAC crystallized in native form.

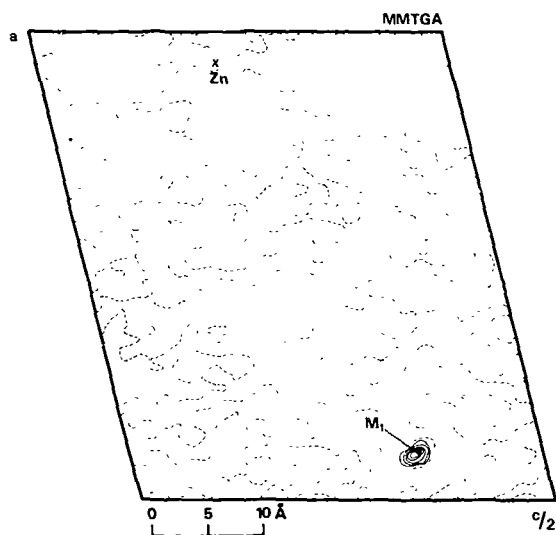


FIGURE 2.—High-resolution difference electron-density projection along the *b*-axis between the MMTGA derivative and native HCAC. The mercury at  $M_1$  is bound to the only SH group of the protein. In all the following maps the zero level is dotted and the contours are drawn at an interval of about 10 electrons.

### X-Ray Diffraction Methods

Data for the *h*0*l* and *h**k*0 layers were collected with Buerger-Supper precession cameras and CuK  $\alpha$ -radiation from Philips fine-focus tubes. The films were measured partly on a Joyce-Loebel microdensitometer made available by Dr. C.-I. Brändén, Royal Agricultural College of Sweden, Uppsala, and partly on a semiautomatic microdensitometer constructed in cooperation with Dr. Klimecki, Institute of Chemistry, University of Uppsala. The data were stored on magnetic tape by a Dymec tape recorder lent us by Drs. J. Ekstedt and E. Ståhlberg, Wallenberg Laboratory. These tapes can be used directly for computer calculations on the CD 3600 computer in Uppsala. The programs involve indexing the reflexions and selecting proper data (written by L. Järup and K. K. Kannan); calculating film factors, applying Lorenz and polarization corrections, scaling similar sets of data to each other (written by Dr. C.-I. Brändén); calculating phase angles (written by Dr. H. Muirhead, Cambridge, England; Muirhead, Cox, Mazzarella, and Perutz, 1967); and a Fourier summation program (written by Dr. A. Zalkin and modified by Drs. J. O.

Lundgren and R. Liminga, Uppsala). All the borrowed programs have been modified by L. Järup and K. K. Kannan.

## RESULTS AND DISCUSSION

### Human Carbonic Anhydrase B

The crystals of HCAB provided by Dr. Nyman, as well as those obtained by us, have the same cell dimensions:  $a = 81.5 \text{ \AA}$ ,  $b = 74.3 \text{ \AA}$ ,  $c = 37.1 \text{ \AA}$ , and the space group  $P2_12_12_1$ . The corresponding data for HCAC are:  $a = 43.1 \text{ \AA}$ ,  $b = 42.1 \text{ \AA}$ ,  $c = 73.6 \text{ \AA}$ ,  $\beta = 104.6^\circ$ , and the space group  $P2_1$  (Strandberg, Tilander, Fridborg, Lindskog, and Nyman, 1962). With the difference in primary structure, a complete structure determination would also have to be performed for HCAB.

### The Complex of Human Carbonic Anhydrase C and Methylmercurithioglycolic Acid (MMTGA)

The complex of HCAC and MMTGA, used as a heavy atom derivative at low resolution, has also proved to be of great value in the high-resolution structure determination. One clear position,  $M_1$ , of 65 electrons is at the only cysteine of the protein, as shown earlier (Fridborg et al., 1967). A high-resolution projection of the difference electron density is presented in figure 2. The position next in height has an occupancy of five electrons. The refined parameters are shown in table II.

### The Sulfonamide Complexes of Human Carbonic Anhydrase C

One of the heavy atom derivatives used at low resolution was the sulfonamide inhibitor AMSulf (acetoxymercurisulfanilamide). A high-resolution difference electron density projection is shown in figure 3. The four positions earlier reported deriving from two AMSulf-groups (Fridborg et al., 1967) are easily observed and now well resolved. Two new peaks were obtained and are possible to refine (see table II). The higher (25 electrons) of the two is probably the mercury of a third AMSulf-group. According to the low-resolution map, the position is in a narrow region between two molecules. The binding of this group certainly

TABLE II.—Heavy Atom Parameters Refined From High-Resolution Projection Data (for exceptions see footnotes) Using the Least-Squares Method (Program Written by Dr. H. Muirhead)

| Derivative                                | Position                    | Occupancy electrons | Fractional coordinates |  |                   |
|---|-----------------------------|---------------------|------------------------|--|-------------------|
|   |                             |                     | x(43.1 Å)              | y(42.1 Å)                                | z(73.6 Å)         |
| MMTGA                                     | M <sub>1</sub>              | 65.4                | 0.096                  | <sup>a</sup> 0.260                       | 0.346             |
| AMSulf                                    | A <sub>1</sub>              | 59.1                | .104                   | <sup>a</sup> .259                        | .356              |
|   | A <sub>2</sub>              | 16.2                | .178                   | <sup>a</sup> .276                        | .442              |
|   | A <sub>3</sub>              | 18.5                | -.038                  | <sup>a</sup> .041                        | .235              |
|   | A <sub>4</sub>              | 69.2                | .000                   | <sup>a</sup> .048                        | .171              |
|   | A <sub>5</sub>              | 24.0                | .415                   | <sup>a</sup> -.086                       | .178              |
| AMAzoSulf                                 | Z <sub>1</sub>              | 34.3                | .095                   | .258                                     | .350              |
|   | Z <sub>2</sub>              | 20.0                | -.035                  | .030                                     | .237              |
|   | Z <sub>3</sub>              | 11.2                | .020                   |  | .184              |
| I <sup>-</sup>                            | I <sub>1</sub> <sup>b</sup> | 31.0                | -.044                  | <sup>b</sup> .026                        | .248              |
|   | I <sub>2</sub>              | 11.9                | .263                   | .310                                     | .317              |
| MMTGA + Roussin's salt                    | R <sub>1</sub>              | <sup>c</sup> 65.0   | <sup>c</sup> .105      | <sup>c</sup> .258                        | <sup>c</sup> .345 |
|   | R <sub>2</sub>              | <sup>c</sup> 36.0   | <sup>c</sup> -.040     | <sup>c</sup> .050                        | <sup>c</sup> .248 |
| MMTGA + Pt(CN) <sub>4</sub> <sup>2-</sup> | P <sub>1</sub>              | <sup>d</sup> 61.4   | <sup>d</sup> .095      | <sup>d</sup> .253                        | <sup>d</sup> .347 |
|   | P <sub>2</sub>              | <sup>d</sup> 50.0   | <sup>d</sup> -.041     | <sup>d</sup> .040                        | <sup>d</sup> .235 |
| Cu <sup>2+</sup>                          | C <sub>1</sub>              | 14.2                | -.034                  |  | .215              |
|   | C <sub>2</sub>              | 11.6                | -.178                  |  | .214              |
| Ag <sup>+</sup>                           | S <sub>1</sub>              | 44.5                | .122                   |  | .444              |
|   | S <sub>4</sub>              | 12.4                | -.360                  |  | .201              |
|   | S <sub>5</sub>              | 11.5                | -.293                  |  | .278              |
| Zn  |                             |                     | <sup>e</sup> -.068     | <sup>e</sup> -.040<br><sup>d</sup> -.027 | <sup>e</sup> .218 |

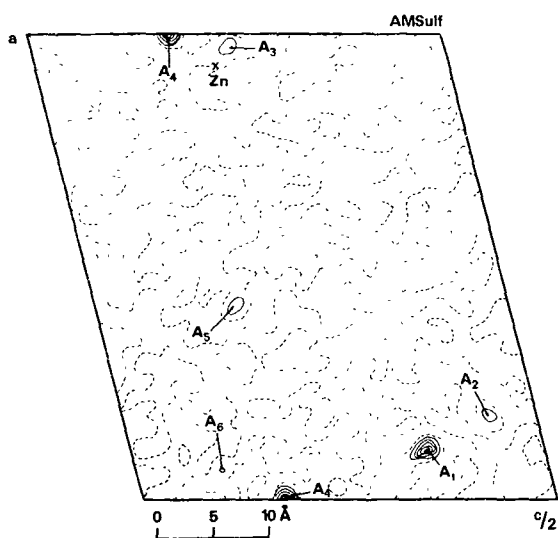
<sup>a</sup> Refined from low-resolution 3-dimensional data.<sup>b</sup> See note added in proof, p. 97.<sup>c</sup> Obtained from low-resolution difference electron density projections.<sup>d</sup> Refined from low-resolution projection data.<sup>e</sup> Obtained from low-resolution 3-dimensional electron density maps.

FIGURE 3.—High-resolution difference electron-density projection along the b-axis between the AMSulf derivative

cannot be because of the mercury alone, because the MMTGA derivative does not show any distinguishable occupancy at this position. It is almost impossible to find the corresponding sulfonamide group because of the number of peaks of less than 10 electrons. This extra sulfonamide binding site, far away from the zinc atom, is probably not related to the two zinc-dependent binding sites for bromoacetazolamide reported by Kandel, Wong, Kandel, and Gornall (1968).

Coleman (1967a) has investigated the binding between carbonic anhydrase and an azosulfonamide and suggested that the complex formed

and native HCAC. The mercury at A<sub>1</sub> is bound to the SH group, and A<sub>2</sub> indicates the corresponding sulfonamide group. A<sub>3</sub> is the position of the sulfonamide close to the zinc atom (marked by a cross) and the corresponding mercury is marked A<sub>4</sub>. A<sub>5</sub> is probably a mercury in a region between two molecules.

should be studied with X-rays. Dr. G. Fölch in Göteborg provided us with two mercuri-azo-sulfonamide derivatives. One of these, 3'-acetoxy-mercuri-4'-hydroxy-4'-azobenzene-sulfonamide (AMAzoSulf), gave the difference electron density projection presented in figure 4. During the complexing reaction, the yellow AMAzoSulf solution lost its color and the crystals turned a deep yellow. It would have been necessary to add more AMAzoSulf to get a more complete substitution (fig. 4 and table II). The fact that no mercury position corresponds to the sulfonamide group ( $Z_2$ ) at the zinc atom and that no sulfonamide peak corresponds to the mercury in position  $Z_1$  can be explained in several ways. It is as yet too early to draw any conclusions. One of the most important features of the sulfonamides is the inhibiting sulfonamide position close to the zinc atom.

#### The Iodide Complex of Human Carbonic Anhydrase C

Several monodentate anions have been reported as being carbonic anhydrase inhibitors (Coleman, 1967b; Kernohan, 1965; Lindskog, 1966). One of these, iodide, has now been investigated with X-ray techniques. The high-resolution difference electron-density projections show two clear peaks

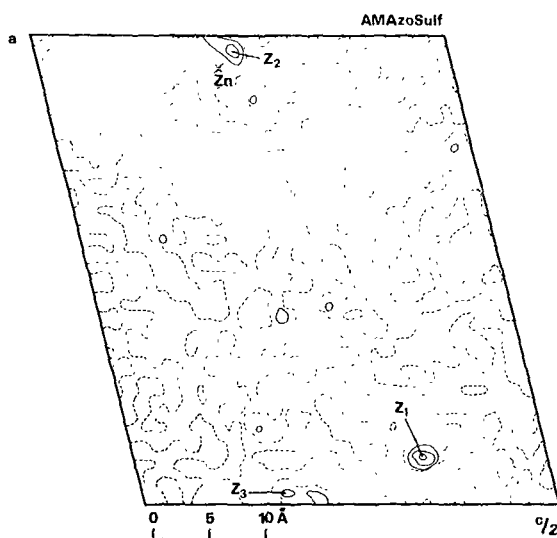


FIGURE 4.—High-resolution difference electron-density projection along the *b*-axis between the AMAzoSulf-HCAC complex and native HCAC.  $Z_1$  indicates a mercury at the SH group, and  $Z_2$ , the sulfonamide at the zinc atom.

(fig. 5), the higher of which occupies the inhibitor position  $I_1$  at the zinc atom. (The refined coordinates are given in table II.) Also, in this case, the direction from the zinc atom to the inhibitor is inward into the molecule.

#### The Enzyme—Roussin's Salt Complex

One carbonic anhydrase inhibitor that also can be regarded as an anionic inhibitor is Roussin's black salt,  $KFe_4S_3(NO)_7$ , which has been studied by Dobry-Duclaux (1966). The structure of this compound has been determined by Johansson and Lipscomb (1958). The low-resolution X-ray investigation of the complex between HCAC and Roussin's black salt (provided by Dr. Dobry-Duclaux) shows only one clear peak at the inhibitor site (fig. 6 and table II).

#### The Complexes With Heavy-Metal Salts

To search for better heavy atom derivatives, we have investigated the following commonly used negatively charged metal complexes:  $Pt(CN)_4^{2-}$ ,  $PtCl_6^{2-}$ ,  $PdCl_4^{2-}$ ,  $AuCl_4^-$ , and  $HgI_4^{2-}$ . All of them show clear differences and can be used as heavy atom derivatives, except for  $HgI_4^{2-}$ , or more probably  $HgI_3^-$  (Kretsinger, Watson, and Kendrew, 1968), which gave too many

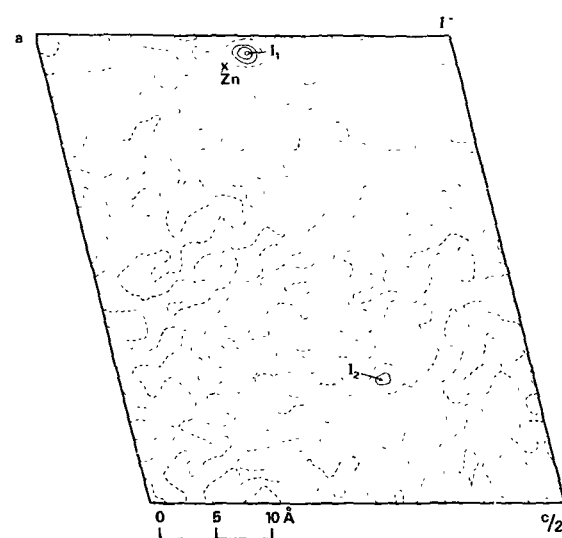


FIGURE 5.—High-resolution difference electron-density projection along the *b*-axis between the HCAC-iodide complex and native HCAC. The position marked  $I_1$  is an iodide bound close to the zinc atom.

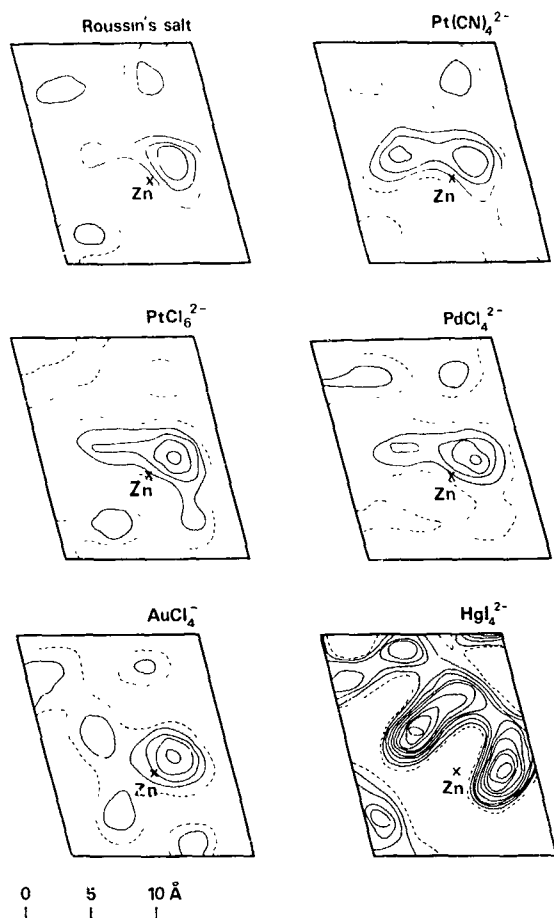


FIGURE 6.—The zinc region of low-resolution difference electron-density projections along the *b*-axis between the complex HCAC–MMTGA with various inorganic anions and native HCAC. In all cases one peak is close to the inhibitor position. In most of the cases there is an additional site close to the zinc atom. In the case of  $\text{Pt}(\text{CN})_4^{2-}$ , this position is far away from the zinc in the *b*-direction and situated in a region between two molecules.

peaks and almost denatured the protein crystals. In the projection along the *b*-axis, all those compounds show two strong peaks close to the zinc atom (fig. 6), with one at the inhibitor position. We have investigated the projection along the *c*-axis only in the case of  $\text{Pt}(\text{CN})_4^{2-}$ , which shows that one of the groups occupies the inhibitor site close to the zinc atom.

Some positively charged metal ions,  $\text{UO}_2^{2+}$  and  $\text{Tl}^+$ , as well as the uncharged complex  $\text{TlCN}$  (obtained together with a sample of  $\text{K}_2\text{Pt}(\text{CN})_4$

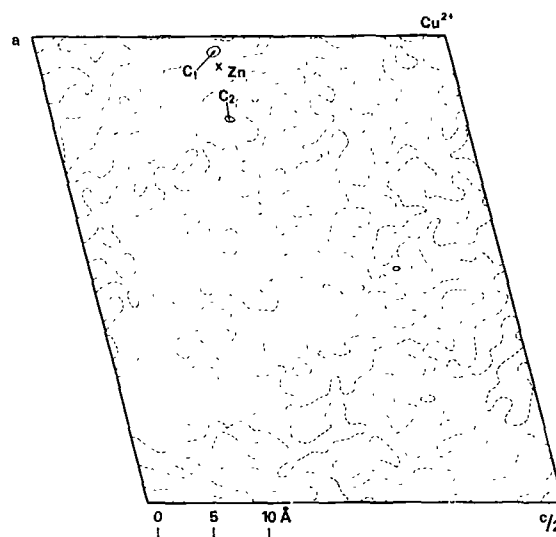


FIGURE 7.—High-resolution difference electron-density projection along the *b*-axis between the complex of HCAC with copper and native HCAC. One position ( $\text{C}_1$ ) is found at about the same *x*- and *z*-coordinates as the zinc atom.

from Dr. C.-I. Brändén), gave no visible differences in the X-ray intensities to 2.0 Å resolution.

#### The Enzyme Complex With Silver and Cupric Ions

Meldrum and Roughton (1933) report that several heavy atoms have an inhibitory effect on carbonic anhydrase. A thorough investigation of the effect of copper on the two human forms of the enzyme is presented by Magid (1967). He concludes that it is possible to inhibit the HCAC activity with  $\text{CuSO}_4$ , although the HCAB activity is not affected. Because the erythrocytes usually contain an amount of copper which is not accounted for by the copper-containing proteins, the cupric ion would seem to be an inhibitor of physiological interest.

A high-resolution difference electron-density projection of the copper complex with HCAC (fig. 7) shows some peaks of smaller magnitude that are possible to refine (table II). One of them,  $\text{C}_1$ , has about the same *x*- and *z*-coordinates as the zinc atom; i.e., not the usual inhibitor position. No occupancy is observed at the SH group of the protein.

In the case of the silver complex with HCAC, ammonium sulfate was used as the salt medium

and the complex  $\text{Ag}(\text{NH}_3)_2^+$  is formed. A great number of binding sites with negatively charged groups of the protein is formed (fig. 8). In this case an occupancy of 10 electrons is found at the  $x$ - and  $z$ -coordinates of the  $M_1$  position at the SH group. The highest occupancy in this derivative is 45 electrons. An interesting feature is that two peaks are observed having about the same  $x$ - and  $z$ -coordinates as the zinc atom.

#### The Zinc Position of Human Carbonic Anhydrase C

Thus far the zinc-free enzyme has not been studied at high resolution. The zinc position determined previously by Fridborg et al. (1967) may suffer from its being refined as a negative peak close to the mercury, and from substituting it in one of the derivatives at low resolution. This derivative has now been omitted from the refinement; and the phase angle and three-dimensional electron density calculations are performed. (The zinc coordinates obtained are given in table II.) A refinement in the projection along the  $c$ -axis at low resolution was also performed using the zinc-free enzyme, the native enzyme, and the derivatives used in the three-dimensional low-resolution study. In this case, we omitted the zinc-free mercury-containing derivative. The  $y$ -coordinate obtained in this refinement is given in table II. In both those cases there is considerable discrepancy with the previously published  $y$ -coordinate (Fridborg et al., 1967).

#### CONCLUSIONS

For a long time it was believed that the sulfonamide inhibitors were noncompetitive with CO<sub>2</sub> in the hydration of CO<sub>2</sub> catalyzed by carbonic anhydrase (Davis, 1959). But it has recently been shown by Lindskog and Thorslund (1968) that the sulfonamide inhibitors are competitive with respect to this substrate.

These authors, as well as Coleman (1967c), found that the sulfonamide inhibitors compete with the anionic ones. They also found, as did Kernohan (1966), that the ionized form of the inhibitor binds to the enzyme and that the  $pK$  of the sulfonamide group is related to the binding of the inhibitor. This finding is in agreement with our observation that the sulfonamide group of an

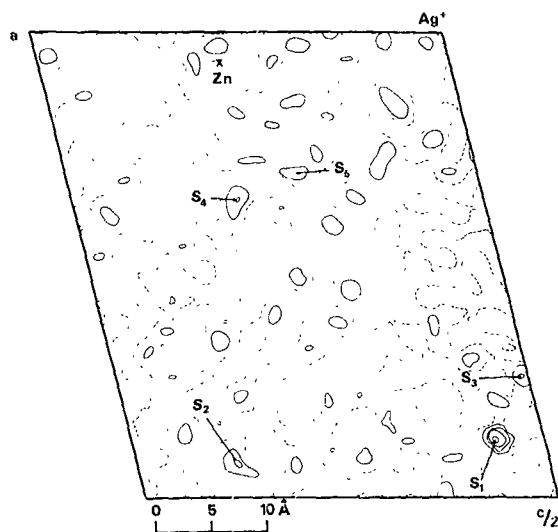


FIGURE 8.—High-resolution difference electron-density projection along the  $b$ -axis between the HCAC-silver complex and native HCAC. A number of peaks is observed, probably caused by binding between  $\text{Ag}(\text{NH}_3)_2^+$  and negatively charged groups of the protein.

inhibitor occupies almost the same position as, e.g., the iodide ion (tables II and III). There is evidence that the anionic inhibitors bind directly to the zinc atom (Riepe and Wang, 1968). Table III shows the observed and calculated distances from the zinc atom to the inhibitor. In all cases except for that of the distance between zinc and iodide, it is impossible to determine anything but maximal binding distances caused by the angular variability from zinc through lighter atoms, and to the center of gravity in the inhibitor groups; the calculated values agree with the observed distances in all the cases, but for the iodide (see note added in proof, p. 97).

The anionic heavy metal complexes seem to constitute a new group of inhibitors. The finding by Meldrum and Raughton (1933) that  $\text{Pt}(\text{CN})_4^{2-}$  does not inhibit carbonic anhydrase is not in agreement with the observed position of the heavy atom groups.

At present, the inhibitory power of  $\text{Cu}^{2+}$  and  $\text{Ag}^+$ , probably in complex with  $\text{H}_2\text{O}$  and  $\text{NH}_3$ , respectively, is not so easily explained. Their presence in the active site cavity is probable. The question of which groups are blocked by them must await the three-dimensional, high-resolution structure determination.

TABLE III.—Distances Between Certain Atoms in the Investigation of Human Carbonic Anhydrase C and Its Complexes

| Atoms                                | Observed distance, Å | Approximate calculated maximal distance, Å |
|--------------------------------------|----------------------|--|
| Zn-I <sup>-</sup> -----              | * 4.4                | 2.9  |
| Zn-A <sub>3</sub> -----              | 3.7                  | ~3.5                                       |
| Zn-P <sub>2</sub> -----              | 4.7                  | ~5.1                                       |
| Zn-R <sub>2</sub> -----              | 4.4                  | ~5.9                                       |
| A <sub>3</sub> -I <sup>-</sup> ----- | 1.1                  |  |

\* See note added in proof below.

#### ACKNOWLEDGMENTS

We wish to thank Profs. A. Fredga, G. Hägg, J. Porath, and A. Tiselius for their interest in our work. We are also grateful to Prof. B. Malmström and Drs. G. Fölch, S. Lindskog, and P. O. Nyman for valuable discussions and collaboration.

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#### NOTE ADDED IN PROOF

Calculations have now been performed using 85 percent of the three-dimensional 2.0-Å reflexions with the phase angles determined from three heavy atom derivatives. The derivatives are MMTGA, AMSulf, and as the third one we have partly used MMTGA+I<sup>-</sup> and partly I<sup>-</sup> alone. In one of the two cases, MMTGA+I<sup>-</sup>, the distance between I<sup>-</sup> and the zinc atom does not deviate significantly from the expected bond distance.

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## DISCUSSION

**EDSALL:** The unit cell of enzyme B is larger than that of enzyme C, is it not?

**LILJAS:** Yes; there are four molecules in the B cell and two molecules in the C cell.

**MAREN:** You made a comment on a Danish finding that copper is inhibitory to the C enzyme, and not to the B.

**LILJAS:** Yes; this was the work of Dr. Magid.

**NYMAN:** There is, apparently, a kind of specific binding of copper that takes place with a binding constant of approximately one micromolar which leads to total inhibition of activity of the C enzyme. Dr. Magid has used it in working on a method to determine the amount in various forms of the mixture.

**MAREN:** Is there any way in which the techniques of crystallography could be used to determine the mode of sulfonamide inhibition. It seems that the inhibition by sulfonamides for hydration is noncompetitive and the inhibition of dehydration is competitive.

**LILJAS:** I hope so, but it is not quite certain how much we can do. There are different positions of binding for different groups, and from that you might be able to throw light on such a complication.

**MAREN:** Also, it might be possible by dislodging experiments with an inhibitor. I think the confirmation of this by X-ray methods, if possible, would be exceedingly interesting.

**POCKER:** I was interested in your X-ray structure studies, and particularly in your data pertaining to the Zn-I distance. Anionic inhibition has been studied in our laboratories in some detail by Pocker and Dickerson (*Biochemistry*, 7: 1995, 1968) and Pocker and Stone (*Biochemistry*, 6: 668, 1967; 7: 2936, 1968), and it was noted that, in general, the order observed with the very strong anionic inhibitors ( $K_i \sim 10^{-6}$  to  $10^{-5}$  M) appears to parallel their association constants with zinc ions, whereas the order observed with moderate ( $K_i \sim 10^{-4}$  to  $10^{-3}$  M) and weak anionic inhibitors ( $K_i \sim 10^{-2}$  to  $10^{-1}$  M) appears to follow the Hofmeister lyotropic series.

These data, in conjunction with the dependence of  $K_i$  on pH, suggested to us that two distinct enzyme complexes exist: an ion-pair complex in which the anion is separated from zinc by a water molecule, and an inner sphere species in which the anion is in contact with the zinc. We have also noted that the ion-pair complexes would be stabilized in the enzyme by additional interaction with a  $BH^+$  group that lies close to the zinc and has an apparent  $pK_a$  value between 7 and 7.5. In short, the anionic inhibitors that follow the Hofmeister lyotropic series are, in our opinion, associated with the breakdown of the water structure at the active site and are separated by one or more water molecules from zinc; they could inhibit by interfering with the proton-transfer process required for the normal hydration and hydrolysis cycles. It is pleasing to us that the zinc-iodide distance is indeed large enough to accommodate a water molecule "bridge." We shall await your X-ray structure studies with great interest.

**EDSALL** (to Liljas): At present can you say anything more than you could in the paper of last year about the probable content of helix in the molecule?

**LILJAS:** No. It is still impossible.

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# On the Catalytic Mechanism of Carbonic Anhydrase<sup>1</sup>

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Previous infrared data have shown that in the carbonic anhydrase-catalyzed hydration of carbon dioxide, it must be the  $\text{OH}^-$  coordinated to the  $\text{Zn(II)}$  of the enzyme that attacks the bound  $\text{CO}_2$  and converts the latter to  $\text{HCO}_3^-$ . A comparison of the calculated pseudo-first-order hydration rate constant for a hypothetical system, in which an  $\text{OH}^-$  ion is placed in juxtaposition with a bound  $\text{CO}_2$  molecule, with the observed  $k_2$  of carbonic anhydrase, shows that the enzyme must have additional means of expediting the reaction. A possible mechanism for activating the  $\text{OH}^-$  of  $\text{Zn(II)}$  is proposed in which this  $\text{OH}^-$  group is assumed to be hydrogen bonded to a strategically located basic imidazole group for facilitated proton transfer. The proposed mechanism is supported by infrared absorption, cyanide titration, and reaction rate data on the native enzyme, by optical data on the  $\text{CO(II)}$ -enzyme, as well as by the results of labeling experiments from several laboratories.

The elucidation of the catalytic mechanism of carbonic anhydrase is of interest not only because the enzyme has multiple physiological functions but also because its mechanistic simplicity may serve as a useful model to guide our thinking on the problem of enzyme action in general.

## CONCLUSION FROM INFRARED STUDIES

Previous infrared absorption spectra studies in our laboratory (Riepe and Wang, 1967, 1968) showed that the  $\text{CO}_2$  molecule bound at the active site of carbonic anhydrase is neither coordinated to the  $\text{Zn(II)}$ -atom nor appreciably distorted, but that it is loosely bound, as in clathrate compounds, to a hydrophobic surface or cavity adjoining the  $\text{Zn(II)}$  as illustrated by structure I (fig. 1). It was also concluded from the infrared data that the bicarbonate ion is coordinated to the  $\text{Zn(II)}$  through its negatively charged oxygen atom in such a way that its relatively neutral oxygen atom and  $\text{OH}$  group are placed at the hydrophobic  $\text{CO}_2$  site, as illustrated by structure II (fig. 1).

<sup>1</sup> Some of the experimental work discussed in this article was supported by a research grant (GM 04483) from the U.S. Public Health Service.

Consequently in the dehydration reaction, proton transfer must accompany the breaking of the susceptible  $\text{C—O}$  bond, because it is already known that only  $\text{CO}_2$  will be left in the hydrophobic binding site. Conversely, because of microscopic reversibility, it must be the  $\text{OH}^-$  on the  $\text{Zn(II)}$  that attacks the bound  $\text{CO}_2$  and converts the latter to  $\text{HCO}_3^-$  in the reverse hydration reaction. Coleman (1967) recently showed that his titration data also support such a mechanism.

Although these conclusions are unambiguous, the explanation of the catalytic mechanism of carbonic anhydrase is still incomplete, because the infrared data yielded no information on the proton-transfer path. Specifically we want to know whether the proton transfer is facilitated by the participation of a certain functional group of the protein, and if so, what is the identity of this group and the nature of its participation.

## KINETIC CONSIDERATIONS

To gain further insight into this problem, let us make an order of magnitude estimate of the pseudo-first-order hydration-rate constant for a hypothetical system in which a given  $\text{OH}^-$  ion is

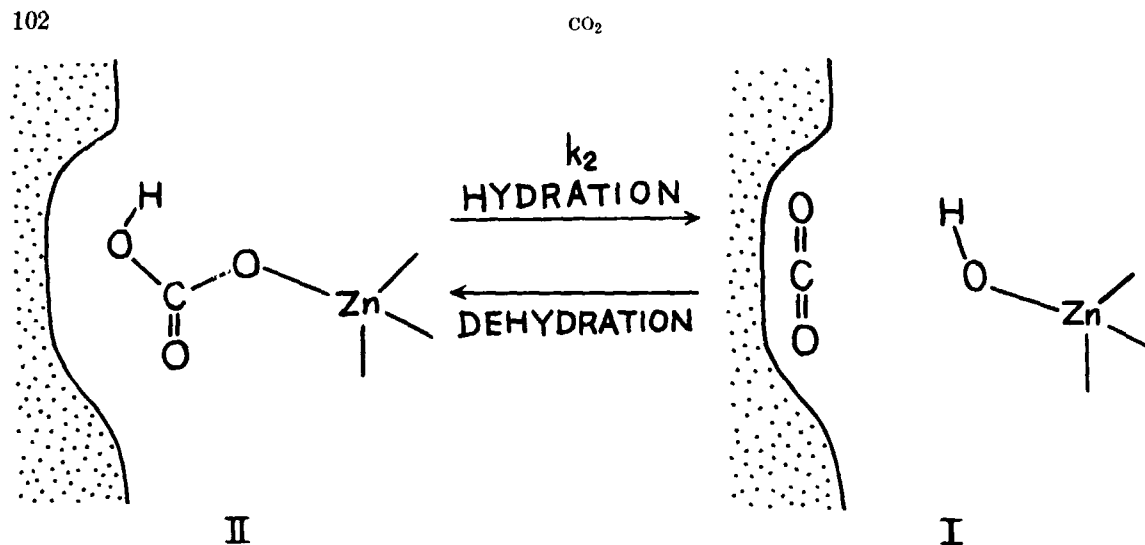


FIGURE 1.—The catalytic mechanism of carbonic anhydrase deduced from infrared data.  $k_2$  represents the first-order rate constant of the enzyme-substrate complex in the hydration reaction.

held in juxtaposition to a bound  $\text{CO}_2$  molecule (similar to the situation illustrated by structure I, in fig. 1, but without the  $\text{Zn(II)}$ ). The observed bimolecular rate constant for the reaction between  $\text{OH}^-$  and  $\text{CO}_2$  to form  $\text{HCO}_3^-$  at 25 C has been estimated to be  $\sim 8 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$  (Kern, 1960). Assuming that the  $\text{OH}^-$  ion in such a hypothetical system has a molar volume of  $\sim 10 \text{ ml}$ , we estimate the pseudo-first-order hydration-rate constant to be  $\sim 8 \times 10^3 (10^3/10) = 8 \times 10^5 \text{ sec}^{-1}$ . The observed first-order rate constant  $k_2$  for the bovine carbonic anhydrase-catalyzed hydration step in figure 1 is  $\sim 4 \times 10^8 \text{ sec}^{-1}$  at 25 C (Kernohan, 1965). In spite of the uncertainties of the above-estimated value (Bruice and Pandit, 1960), it is surprising indeed that the observed and calculated first-order hydration-rate constants are of the same order of magnitude, because the free  $\text{OH}^-$  (with  $K_a = 10^{-15.7}$  as the dissociation constant of its conjugate acid) is a much stronger base than the  $\text{OH}^-$  coordinated to the  $\text{Zn(II)}$  of carbonic anhydrase (with  $K_a = 10^{-7}$  for its conjugate acid) (Kernohan, 1965; Lindskog, 1963). Although the ratio of nucleophilic reactivities of free and coordinated  $\text{OH}^-$  need not be equal to the ratio of their basic strengths, these ratios are not expected to differ very much in order of magnitude for very similar reacting groups. In other words, the  $\text{OH}^-$  coordinated to the  $\text{Zn(II)}$  reacts faster by a factor of  $\sim 10^8$  or  $10^9$  than expected

from a simple activation entropy effect. Therefore the enzyme must have additional means of expediting the reaction, either by increasing the nucleophilic reactivity of the coordinated  $\text{OH}^-$  or by facilitating the necessary proton transfer, or both.

#### FACILITATED PROTON TRANSFER VIA A HYDROGEN-BONDED IMIDAZOLE GROUP

One conceivable way of incorporating the activation of the  $\text{OH}^-$  on the  $\text{Zn(II)}$  and the facilitation of proton transfer in a single molecular process consistent with the mechanism in figure 1 is to assume that the  $\text{OH}^-$  on the  $\text{Zn(II)}$  is hydrogen bonded to a strategically located basic imidazole group of the protein as illustrated by structure I (fig. 2). By analogy to the suggested activation of functional  $\text{OH}$  groups in  $\alpha$ -chymotrypsin (Wang and Parker, 1967; Parker and Wang, 1968) and ribonuclease (Wang, 1968) catalyses, we expect facilitated proton transfer along such an H-bond to make structure I be in rapid equilibrium with structure III, which transforms to structure II (fig. 2).

A quantitative treatment of this mechanism is not feasible at present, for not even an approximate value is known of the dissociation constant of the coordinated  $\text{OH}^-$  as an acid. However, because of the inductive effect of the  $\text{Zn(II)}$ ,

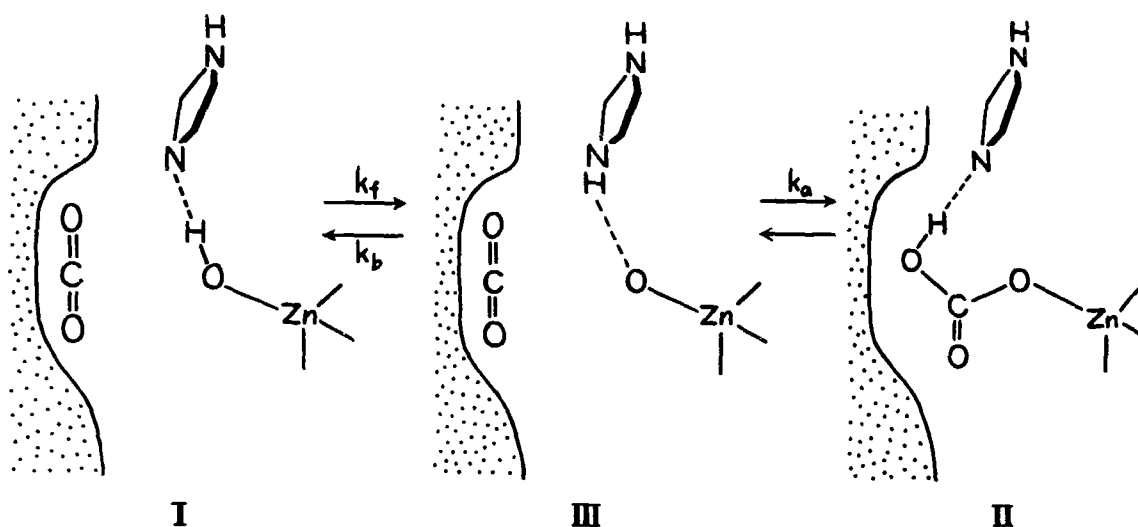


FIGURE 2.—Suggested participation of a basic imidazole group in the catalysis by carbonic anhydrase through facilitated proton transfer. The broken lines represent hydrogen bonds.  $k_f$ ,  $k_b$ , and  $k_a$  represent the first-order rate constants of the corresponding reaction steps.

this coordinated  $\text{OH}^-$  is expected to be a much stronger acid than a free  $\text{OH}^-$  ion—but presumably a somewhat weaker acid than the active OH group of serine-195 in  $\alpha$ -chymotrypsin. Thus if we consider this coordinated  $\text{OH}^-$  as an acid with  $K_a > 10^{-15}$  (Dietrich and Johnston, 1927) and use an estimated value of  $\sim 10^{14} \text{ sec}^{-1}$  (Wang, 1968) for  $k_b$  in figure 2, we may give an order of magnitude estimate of  $k_f$  as

$$k_f = \frac{(K_a)_{\text{ZnOH}}}{(K_a)_{\text{Imidazolium}}} \cdot k_b \geq 10^6 \text{ sec}^{-1}$$

Because this value is larger than the observed overall first-order rate constant  $k_2$  of figure 1, we may, as a tentative approximation, express the latter as

$$k_2 \approx \frac{(K_a)_{\text{ZnOH}}}{(K_a)_{\text{Imidazolium}}} \cdot k_a$$

and consider the transformation  $\text{III} \rightarrow \text{II}$  (fig. 2) as the rate-limiting step.

Because the  $\text{O}^{2-}$  coordinated to the  $\text{Zn(II)}$  in structure III is expected to be much more nucleophilic than the  $\text{OH}^-$  of the  $\text{Zn(II)}$  in structure I, we could regard the transformation  $\text{I} \rightarrow \text{III}$  as the mechanism through which a participating basic group of the protein facilitates the proton transfer

and increases the nucleophilic reactivity of the  $\text{OH}^-$  coordinated to the  $\text{Zn(II)}$ . An imidazole group appears to be the best candidate for such a role, because it is the strongest base among all functional groups of the protein that remain unprotonated at neutral pH. Recent labeling experiments at three different laboratories seem to show that there is indeed an essential imidazole group near the  $\text{Zn(II)}$  atom (Whitney, Fölsch, Nyman, and Malmström, 1967; Whitney, Nyman, and Malmström, 1967; Bradbury and Edsall, 1968; Kandel, Wong, Kandel, and Gornall, 1968).

#### INTERPRETATION OF THE pH DEPENDENCE

The catalytic mechanism illustrated in figure 2 requires that the basic imidazole group be held near the  $\text{Zn(II)}$  to form an H-bond with its ligand  $\text{OH}^-$ , but not close enough to form a coordination bond directly with the  $\text{Zn(II)}$  itself. This necessary assumption is supported both by the infrared data on azide binding (Riepe and Wang, 1968) and the titration data on cyanide binding (Coleman, 1967). It is also consistent with the observation that both kinetic data on the native enzyme (Kernohan, 1965) and optical data on the  $\text{Co(II)}$ -enzyme (Lindskog, 1963)

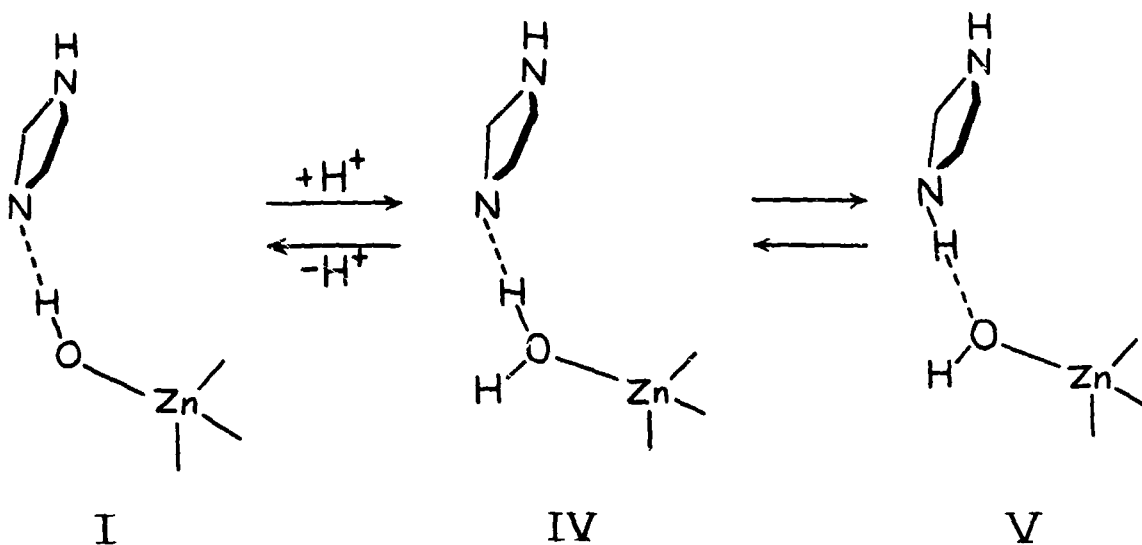


FIGURE 3.—A suggested mechanism of acid-base transformation at the active site of carbonic anhydrase.

require the existence of a single essential basic group with an apparent dissociation constant  $K_a' \approx 10^{-7}$  for its conjugate acid. If this essential imidazole group were directly coordinated to the Zn(II), the apparent dissociation constant of its conjugate acid would be

$$K_a' = \frac{K_a}{K_{Zn}}$$

where  $K_a$  is the true acid dissociation constant of the imidazolium group and  $K_{Zn}$  the dissociation constant of the imidazole—Zn(II) coordination bond in the enzyme; i.e.,  $pK_a' = pK_a - pK_{Zn}$ . Since  $pK_a \sim 7$  and since  $pK_{Zn}$  should be a positive number of comparable magnitude, we would expect  $pK_a'$  to be much less than 7, a deduction that contradicts both the kinetic (Kernohan, 1965) and the optical data (Lindskog, 1963).

On the other hand, protonation of the active center as illustrated in figure 3 changes the catalytically active form represented by structure I to the inactive forms represented by structures IV and V. Because of the rapid protonation equilibrium, the two forms IV and V should behave as a single acid group with  $pK_a' \approx 7$ , as has indeed been found experimentally. Pocker and Dickerson (1968) recently suggested a scheme for the carbonic anhydrase-catalyzed hydration of aldehydes which bears some resemblance to

the present mechanism, except that they consider IV and V rather than I and III as the active forms.

#### GENERALIZATION

From the standpoint of the present mechanism, the catalytic hydration and hydrolysis by carbonic anhydrase can be included as a particular case of a large group of enzyme catalyses in which the attacking OH group is activated by H-bonding to a strategically located basic imidazole group for facilitated proton transfer (Wang, 1968). In  $\alpha$ -chymotrypsin, trypsin and subtilopeptidase A, the attacking OH group belongs to a serine residue; in ribonuclease, the attacking group is the 2'-OH of a ribose residue; and in carbonic anhydrase, it is the OH<sup>-</sup> coordinated to the Zn(II).

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<sup>1</sup> Editor's note: A full report of this work has now appeared. See Bradbury, S. L., The Carboxymethylation of Human Carbonic Anhydrase B. I. The Nature of the Reaction. II. The Amino Acid Sequence Around a Reactive Histidine Residue. *J. Biol. Chem.*, **244**: 2002-2016, 1969.

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## DISCUSSION

**CAPLOW:** What  $pK$  do you assign to the  $ZnOH$  of carbonic anhydrase?

**WANG:** I have assumed  $(K_a)_{ZnOH} \geq 10^{-15}$  or  $(pK_a)_{ZnOH} \leq 15$ . Presumably this  $ZnOH$  group is a somewhat weaker acid than the active  $OH$  group of serine-195 in  $\alpha$ -chymotrypsin that has a  $pK_a \sim 13$ . But this is just an order-of-magnitude guess.

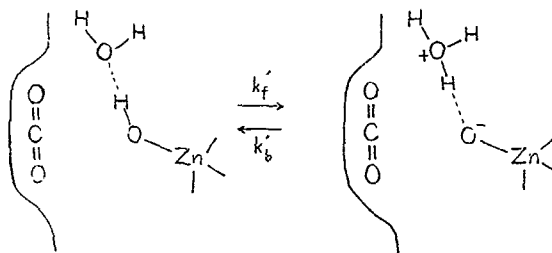
**CAPLOW:** If this assignment is made, I believe that it is possible to rule out the mechanism that you have proposed. A mechanism involving imidazole-catalyzed attack of a  $ZnOH$  species (structure I in your fig. 2) on carbon dioxide is kinetically indistinguishable from a reaction of a zinc oxide anion facilitated by imidazolium (structure III), because the stoichiometric composition of the two transition states is identical. If the former path holds, the rate constant for reaction of (I) with carbon dioxide is approximately  $10^5 \text{ sec}^{-1}$ ; but, if the latter path is the predominant one, the rate constant ( $k_a$  in fig. 2) must be about  $10^{13} \text{ sec}^{-1}$  to account for the observed rate. That is, if we are to assume that the fraction of the enzyme in the catalytically active form is only one part in  $10^8$  (i.e.,  $K = (III)/(I) = 10^{-8}$ ), the specific rate constant must be remarkably large. There is, however, no precedent for a rate constant of this magnitude in nonenzymatic intermolecular or intramolecular processes involving carbon-oxygen bond formation.

**WANG:** I agree wholeheartedly that ordinary organic reaction mechanisms are invaluable in helping us clarify enzyme action. But to insist that enzymes should not react faster than the reported rates for small molecules reflects a very limited point of view. Why is there not more enzyme action? After all, enzymes are remarkable catalysts; their highly organized, intricate structures may enable them to react along certain paths that are not as rapidly accessible to randomly distributed small molecules. The observation by Eigen's group that proton transfer in ice at  $-10 \text{ C}$  is 70 times faster than in water at  $25 \text{ C}$  is a good illustration of the effect of three-dimensional

structure on reaction rates. This effect has no precedent in solution chemistry.

According to my proposal, a strategically located basic imidazole group could facilitate the proton transfer  $I \rightarrow III$ , as illustrated in figure 2 of my paper, and III could then react with an unusually high speed,  $k_a$ , to form II.

In the absence of such a participating basic functional group, the corresponding proton transfer would be directed toward a water molecule:



Consequently,  $\frac{k_f'}{k_b'} = (K_a)_{ZnOH}$

and even by using the highest estimated value of  $k_b'$ , we would still obtain a  $k_f'$  that is lower by several orders of magnitude than the observed  $k_2$  for carbonic anhydrase. Therefore the participation by a basic functional group of the protein is necessary.

**CAPLOW:** I agree that by taking advantage of the facilitation of proton transfer along preformed hydrogen bonds, as you have in your mechanism, it is possible for proton transfers to occur as a preequilibrium process. In addition, in the absence of a special mechanism like this, the proton transfers probably would not be fast enough. Finally, for your mechanism to hold, two processes with little precedent in nonenzymatic systems must hold. Proton transfer must be exceedingly fast and  $C-O$  bond formation must occur with remarkable facility.

**WANG:** I do not think I need comment, because Dr. Caplow has essentially answered his own question.

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## Primary Structure Studies on Human Carbonic Anhydrase B and Its Active Site<sup>1</sup>

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The amino acid sequence of human erythrocyte carbonic anhydrase B has been investigated. The protein, which consists of a single polypeptide chain of approximately 260 amino acid residues, has been split with cyanogen bromide and by tryptic hydrolysis of arginyl peptide bonds after blocking of the lysine side chains by acetamidation. Fragments that together account for the complete sequence of the protein have been purified, and an arrangement of these fragments in order has been suggested. Sequence studies of the fragments have been carried out, and at present about one-third of the total sequence of the protein has been determined.

In parallel, studies have been carried out with the enzyme chemically modified with active-site-directed reagents. Bromoacetate and *N*-chloroacetylchlorothiazide have previously been shown to inactivate the enzyme with a concomitant formation of one equivalent of 3' derivatives of histidine. Studies of modified enzyme show that the two reagents react specifically with different histidines, which implies the presence of two histidine side chains in the active center region of human carbonic anhydrase B.

An understanding of the mechanisms of enzyme action is a central problem in biochemistry with implications that extend to other aspects of biological phenomena. In principle, such studies consist of considerations of the kinetic properties of the enzymic reaction in relation to the structures of enzymes and substrates. The progress in this field, as in many other fields of biochemistry, is often limited by an incomplete knowledge of the structure of the macromolecule involved. Developments during the last decade have shown that a detailed elucidation of enzyme structures can, in fact, be achieved, and for a few enzymes, it has been possible to bring the discussion of mechanism to the level of organic or inorganic chemistry. The detailed structure of an enzyme can be derived by combining the three-dimensional electron-density distribution obtained by

X-ray diffraction with knowledge of the amino acid sequence determination by chemical methods. In the case of carbonic anhydrase, X-ray studies are successfully progressing with form C of the human enzyme and studies of the human B form have been started recently (Liljas, Kannan, Bergstén, Fridborg, Järup, Lövgren, Paradies, Strandberg, and Waara, 1968). There is, however, insufficient knowledge of the amino acid sequences of these carbonic anhydrases.

The present paper contributes to the knowledge of the amino acid sequence of human carbonic anhydrase B and applies the results to enzymes chemically modified in the active-site region. It can be regarded as a progress report of a determination of the complete sequence of the enzyme which, besides providing data useful for the interpretation of X-ray results, should give a reference matrix for characterization of mutant forms of the enzyme and for studies of reactivity of individual amino acid side chains by chemical modification.

Human carbonic anhydrase B, similar to the bovine enzyme and the human C form, consists

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of a single polypeptide chain of approximately 260 residues. The protein is devoid of disulfide bridges, but contains one sulfhydryl group. Our investigation is carried out in the conventional manner by splitting the polypeptide chain into fragments that are investigated separately. We felt it desirable to get a low number of fragments from the initial degradations of the polypeptide chain as that would make it easier to establish a general survey of the sequence at an early stage in the investigation and to avoid the overlooking of peptides. This dictated the choice of methods for cleavage.

The first method tried for fragmentation was the cleavage of methionyl bonds with cyanogen bromide. Human carbonic anhydrase B contains two methionine residues, and it turned out that a carboxyl-terminal fragment of 19 residues could easily be purified from the reaction mixture. Table I shows the amino acid sequence of this fragment and the corresponding sequences for human enzyme C and the bovine enzyme that were derived in the same way (Nyman, Strid, and Westermarck, 1968). A comparison of the sequences shows considerable similarities between the proteins, which would suggest that sequence results obtained for one form of carbonic anhydrase would facilitate the studies of other forms of the enzyme. The data in table I conform excellently with the idea that polymorphism in the human carbonic anhydrase has arisen by a process of gene duplication followed by divergent evolution of the two genes (Tashian, Shreffler, and Shows, 1968; Nyman et al., 1968).

The second method used for the fragmentation of the polypeptide chain of human carbonic anhydrase B was tryptic hydrolysis of arginyl peptide bonds. The lysine side chains were blocked by acetamidination. The experimental conditions used for the acetamidination reaction and the tryptic hydrolysis of acetamidinated protein are described in Andersson, Göthe, Nilsson, Nyman, and Strid (1968). Table I shows that the carboxyl-terminal region of human carbonic anhydrase B is rich in arginine. In fact, three of

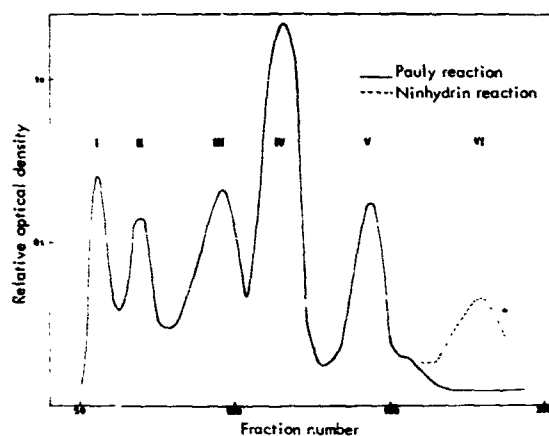


FIGURE 1.—Chromatography of tryptic digest of acetamidinated human carbonic anhydrase B on Sephadex G-75 in 1 M acetic acid. 565 mg were applied to a column with the dimensions 6 x 100 cm. room temperature. Fraction volume: 17 ml. Fractions I, III, IV, and V were rechromatographed, which led to the purification of the peptides with corresponding numbers listed in table I. Fraction VI contains two tripeptides from the carboxyl-terminal end of the enzyme.

TABLE I.—Carboxyl-Terminal Amino Acid Sequences for Species and Isozyme Variants of Carbonic Anhydrase

| Residue no.     | 20   | 19 | 18 | 17 | 16 | 15 | 14 | 13 | 12 | 11 | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 |
|-----------------|--|----|----|----|----|----|----|----|----|----|----|---|---|---|---|---|---|---|---|---|
| Human enzyme B  | -Glx-His-Asn-Asp-Arg-Pro-Thr-Gln-Ile-Leu-Lys-Gly-Arg-Thr-Val-Arg-Ala-Ser-Phe-    |    |    |    |    |    |    |    |    |    |    |   |   |   |   |   |   |   |   |   |
| Human enzyme C  | -Val-Asp-Asn-Trp-Arg-Pro-Ala-Gln-Pro-Leu-Lys-Asp-Arg-Gln-Ile-Lys-Ala-Ser-Phe-Lys |    |    |    |    |    |    |    |    |    |    |   |   |   |   |   |   |   |   |   |
| Bovine enzyme B | -Leu-Ala-Asp-Trp-Arg-Pro-Ala-Gln-Pro-Leu-Lys-Asp-Arg-Gln-Val-Arg-Gly-Phe-Pro-Lys |    |    |    |    |    |    |    |    |    |    |   |   |   |   |   |   |   |   |   |

NOTE.—The residues have been numbered from the carboxyl-terminal end, and the starting point for human enzyme B has been considered as residue no. 2. Concord-

ance between the three sequences is indicated by dashed lines. The experimental details of these sequences are given elsewhere by Nyman et al. (1968).

the seven residues of the whole molecule are located within the carboxyl-terminal cyanogenbromide fragment and, consequently, one would expect to obtain the rest of the molecule in the form of large fragments.

The fractionation of a tryptic digest of acetamidinated human carbonic anhydrase B is shown in figure 1. Fraction number VI in this chromatogram contains the two tripeptides Thr-Val-Arg and Ala-Ser-Phe from the carboxyl-terminal region (table I). Fractions I, III, IV, and V were rechromatographed in various ways (B. Anderson, P. O. Nyman, and L. Strid; to be published) which led to the purification of the peptides listed in table II. These peptides were regarded as pure because they appeared as homogeneous in chromatography under various conditions. For instance, acetamidyl groups on the lysine side chains were removed, and the peptides were reacted with

maleic anhydride as described by Butler, Harris, Hartley, and Leberman (1967). This reaction brings about an acylation of the amino groups and reverses the charge on the lysine side chains, often with a concomitant change in the solubility properties of the peptide and its tendency to aggregate. The maleylated peptides were run on DEAE-Sephadex in the presence of 8 M urea (Konigsberg, Weber, Notani, and Zinder, 1966). The appearances of a symmetric peak and an amino acid composition remaining unchanged during this procedure were taken as criteria of purity.

The next to the last column in table II shows the sum of the compositions of peptides I, III, IV, V, and the two tripeptides in fraction VI. The values obtained are in good agreement with the amino acid composition of the whole enzyme molecule (given in the last column of table II), which

TABLE II.—Amino Acid Composition of Peptides From Tryptic Digest of Acetamidinated Human Carbonic Anhydrase B<sup>a</sup>

| Amino acid         | Peptide I | Peptide III | Peptide IV        | Peptide V | Fraction VI | Sum of preceding columns | Composition of the enzyme |
|--------------------|-----------|-------------|-------------------|-----------|-------------|--------------------------|---------------------------|
| Lysine.....        | 1.00      | 10.61       | 5.70              | 1         | .....       | 19                       | 18                        |
| Histidine.....     | 1.01      | 5.90        | 3.02              | 1         | .....       | 11                       | 11                        |
| Arginine.....      | .99       | 1.89        | .99               | 2         | 1           | 7                        | 7                         |
| Tryptophan.....    | 1.74      | 2.14        | 2.05              | .....     | .....       | 6                        | 6                         |
| Aspartic acid..... | 3.06      | 7.92        | 15.84             | 5         | .....       | 32                       | 31                        |
| Threonine.....     | 5.18      | 3.35        | 3.96              | 1         | 1           | 14                       | 14                        |
| Serine.....        | 9.09      | 10.33       | 7.87              | 2         | 1           | 30                       | 30                        |
| Glutamic acid..... | 5.34      | 8.78        | 5.91              | 3         | .....       | 23                       | 24                        |
| Proline.....       | 5.98      | 2.34        | 5.84              | 3         | .....       | 17                       | 18                        |
| Glycine.....       | 1.19      | 9.29        | 4.03              | 2         | .....       | 16                       | 16                        |
| Alanine.....       | 1.92      | 11.86       | 4.12              | 1         | 1           | 20                       | 19                        |
| Valine.....        | 2.14      | 8.13        | 4.09              | 2         | 1           | 17                       | 17                        |
| Methionine.....    | .....     | 1.02        | .....             | 1         | .....       | 2                        | 2                         |
| Isoleucine.....    | 2.88      | 2.16        | <sup>b</sup> 5.00 | .....     | .....       | 10                       | 10                        |
| Leucine.....       | 6.06      | 9.15        | 2.22              | 3         | .....       | 20                       | 20                        |
| Tyrosine.....      | 1.98      | 2.97        | 2.94              | .....     | .....       | 8                        | 8                         |
| Phenylalanine..... | 3.86      | 4.00        | 2.09              | .....     | 1           | 11                       | 11                        |
| Cysteine.....      | .70       | .....       | .....             | .....     | .....       | 1                        | 1                         |
| Total.....         | 54        | 101         | 76                | 27        | 6           | 264                      | 263                       |

<sup>a</sup> The data, based upon 20- and 70-hour hydrolysates, are expressed as number of residues per molecule of peptide. The numbers of the peptides refer to the peaks in the chromatogram in figure 1 from which they have been derived by rechromatography. Fraction VI represents 2 tripeptides comprising the first 6 residues from the car-

boxyl-terminal end (see table III). The amino acid composition for the whole enzyme molecule given in the last column is that determined by Nyman and Lindskog (1964).

<sup>b</sup> 70 hours of hydrolysis gave 3.98 residues of isoleucine, but sequence studies on peptide IV suggest the presence of five residues.

implies that the whole sequence of the enzyme can be accounted for in terms of the peptides listed in table II.

Sequence studies have been carried out with some of the peptides in table II and the results are given in table III. Peptide V, which contains one of the two methionine residues of the whole protein, has been shown to bridge the carboxyl-terminal cyanogen-bromide fragment and the rest of the enzyme molecule (Andersson et al., 1968). Peptide IV has been subjected to tryptic and chymotryptic hydrolysis after deblocking the lysine side chains. From the digests, peptides have been obtained with amino acid compositions identical with those of peptides described by Laurent, Marriq, Garçon, Luccioni, and Derrien (1967) in their determination of the amino-

terminal sequence of human carbonic anhydrase B. This finding implies that peptide IV represents the amino-terminal portion of the enzyme. The two fragments that then remain to be ordered, namely peptides I and III, should originate from the interior of the polypeptide chain of the enzyme. Certain evidence, although not conclusive, has been obtained concerning the order of these two peptides. From peak II in the chromatogram in figure 1, a fraction has been obtained with an amino acid composition rather closely corresponding to the sum of peptides III and IV. This may be ascribed to an incomplete tryptic hydrolysis of the arginyl bond at the carboxyl-terminal end of peptide IV and would imply that peptide III comes next to the amino-terminal peptide IV. Table III is an attempt to show the

TABLE III.—A Tentative Survey of the Present Knowledge of the Amino Acid Sequence of Human Carbonic Anhydrase B

| Peptide IV  |                |
|---|----------------|
| 1   | 5 10 15        |
| Acetyl-Ala-Ser-Pro-Asp-Trp-Gly-Tyr-Asp-Asp-Lys-Asn-Gly-Gln-Pro-Glu-Trp-Ser-Lys(Lys <sub>2</sub> ,His,Asx <sub>3</sub> ,Thr <sub>2</sub> ,Ser <sub>2</sub> ,Pro <sub>2</sub> ,Ala <sub>2</sub> ,   |                |
| Peptide IV—Continued  |                |
| Val,Ile,Leu,Tyr)(Lys,Thr <sub>2</sub> ,Ser,Glu)(Lys,Asx <sub>4</sub> ,Ser,Glx,Pro <sub>2</sub> ,Gly,Ala,Val,Ile <sub>2</sub> ,Leu,Tyr)(His,Asn,Glu,Gly,Val,Ile <sub>2</sub> )   |                |
| Peptide III   |                |
| 65 to 76  |                |
| (His,Ser,Val,Phe <sub>2</sub> ,Asx <sub>4</sub> ,Glx,Arg)(Lys <sub>11</sub> ,His <sub>6</sub> ,Arg <sub>2</sub> ,Trp <sub>2</sub> ,Asx <sub>6</sub> ,Thr <sub>2</sub> ,Ser <sub>10</sub> ,Glx <sub>9</sub> ,Pro <sub>2</sub> ,Gly <sub>9</sub> ,Ala <sub>12</sub> ,Val <sub>8</sub> ,Met,Ile <sub>2</sub> ,Leu <sub>1</sub> ,Tyr <sub>3</sub> ,Phe <sub>2</sub> ) |                |
| Peptide I   |                |
| 85  | 80 75 70 65    |
| Ala-Pro-Phe-Thr-Asn-Phe-Asp-Pro-Ser-Thr-Leu-Leu-Pro-Ser-Ser-Leu-Asp-Phe-Trp-Thr-Tyr-(Pro,Gly,Ser)-Leu-Thr-Hi-   |                |
| Peptide I—Continued   |                |
| 60  | 55 50 45 40 35 |
| Pro-Pro-Leu-Tyr-Glu-Ser-Val-Thr-Trp-Ile-Ile-(Cys,Lys,Ser,Glu)-Ile-Ser-Val-(Glx,Ser,Ser,Glx)-Leu-Ala-Gln-Phe-Arg-  |                |
| Peptide V   |                |
| 30  | 25 20 15 10    |
| Ser-Leu-Leu-Ser-Asn-Val-Glu-Asp-Asn-Gly-Ala-Val-Pro-Met-Glx-His-Asn-Asn-Arg-Pro-Thr-Gln-Pro-Leu-Lys-Gly-Arg-  |                |
| Fraction VI   |                |
| 5   | 2              |
| Thr-Val-Arg-Ala-Ser-PheOH   |                |

NOTE.—The acetylated amino-terminal end of the protein is shown to the left in the upper part of the table, and the carboxyl-terminal end to the right in the lower part. The residues have been numbered from both ends of the polypeptide chain. The sequence given for the first 18 residues from the amino-terminal end is that determined by Laurent et al. (1967); the carboxyl-terminal sequence up to residue no. 34 is described by Nyman et al. (1968) and Andersson et al. (1968). The peptides indicated are those

derived by tryptic hydrolysis of acetaminated enzyme, and their arrangement in order is suggested by experimental evidence mentioned in the text. The residue preferentially modified in the irreversible inactivation of the enzyme with bromoacetate is the histidine belonging to peptide I (residue no. 62 from the carboxyl-terminal end). The histidine reacting with N-chloroacetylchlorothiazide is located in the region from residue nos. 55 to 76 from the amino-terminal end.

present status of the primary structure studies on human carbonic anhydrase B.

The methods of investigation described above have been applied to enzyme chemically modified in the active-site region with bromoacetate and *N*-chloroacetylchlorothiazide (i.e., 6-chloro-7-chloroacetylsulfamoyl-1,2,4-benzothiadiazine 1,1-dioxide (fig. 2)). Both of these reagents act as reversible inhibitors of the enzyme, and prolonged incubation leads to an irreversible loss of enzymic activity with a concomitant formation of one equivalent of modified histidine (Whitney, Föisch, Nyman, and Malmström, 1967; Whitney, Nyman, and Malmström, 1967). Amino acid analysis after acid hydrolysis of modified enzyme shows the presence of 3'-carboxymethyl histidine for both reagents; apparently the chlorothiazide moiety of the sulfonamide reagent is split off in the acid hydrolysis.

Studies of peptides from bromoacetylated enzyme showed that bromoacetate reacts with a pronounced specificity with a histidine residue belonging to peptide I. From a modified enzyme containing 0.8–0.9 equivalents of modified histidine, peptide I was obtained with a 3'-carboxymethyl histidine content of 0.87 residues. The contents in the other peptides were <0.07 residues. Peptide I contains only a single histidine and the modification reaction could be ascribed to residue no. 62 from the carboxyl-terminal end (see table III). This result is in agreement with the finding of Bradbury and Edsall (1968), who showed that the main reaction taking place during the irreversible inactivation of human carbonic anhydrase B with iodoacetate is the formation of a 3'-derivative of a histidine belonging to the sequence Thr-His-Pro-Pro-Leu.

With an enzyme inactivated with *N*-chloroacetylchlorothiazide (containing 0.8–0.9 equivalents of modified histidine), 0.83 equivalents of modified histidine were found in peptide IV; the contents in the other peptides were <0.05 residues. Peptide IV contains, in total, three histidine residues. Sequence studies showed that the modifiable histidine is located not far from the carboxyl-terminal end of peptide IV, and with the present knowledge it can be ascribed to the sequence region from residue nos. 65 to 76 from the amino-terminal end of the enzyme (see table III).

As discussed by other contributors to this sym-

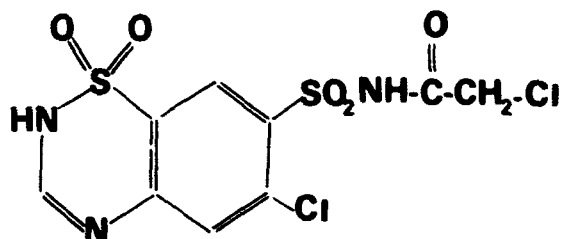


FIGURE 2.—*N*-Chloroacetylchlorothiazide.

posium, the zinc ion is an important element in the active site of carbonic anhydrase, perhaps due to a direct involvement in the catalytic reaction. Aromatic sulfonamides and monovalent anions inhibit the enzyme by binding close to the metal ion as shown by X-ray crystallography of the sulfonamide and iodide complexes of human enzyme C (Liljas et al., 1968) and by spectroscopic studies of the cobalt (II) derivative of carbonic anhydrase (Lindskog, 1966). Evidence has been obtained (Whitney, Nyman, and Malmström, 1967) which strongly suggests that the modifications with bromoacetate and *N*-chloroacetylchlorothiazide take place by an initial reversible binding at the ordinary inhibitor site followed by the formation of a covalent bond at or near the place of reversible binding. The finding that different histidines are labeled with these two reagents would, therefore, imply the presence of two histidine side chains in the active-site region of human carbonic anhydrase B.

#### ACKNOWLEDGMENT

The authors are very indebted to Dr. B. S. Hartley, Cambridge University, who has considerably enhanced the studies presented here by illustrating, in our laboratory, modern techniques in primary structure chemistry.

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# Investigations of the Structure of Human and Bovine Carbonic Anhydrases

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The N-terminal amino acid of human C(HCAC) and bovine B(BCAB) carbonic anhydrases was identified as N-acetyl-serine. An identical N-terminal hexapeptide, N-acetyl-Ala-Ser-Pro-Asp-Trp-Gly, has been isolated from the A and B human enzymes (HCAA and HCAB).

Alkaline treatment of HCAB in suitable conditions is followed by important modifications of the ultraviolet spectrum and a splitting into multiple electrophoretically different components; the first of these to be formed is probably identical to HCAA. Similar results have been obtained with BCAB. Ultraviolet spectrum modifications are reversible, whereas the splitting into multiple conformers is not reversible by neutralization. Of the A and B forms of HCA and BCA, only the B form is probably physiologically significant.

We shall summarize some data recently established in our laboratory<sup>1</sup> on the N-terminus of human and bovine carbonic anhydrases (EC 4.2.1.1) and on the multiple molecular forms of the B enzymes from both origins.

## STUDIES ON THE N-TERMINUS OF HUMAN AND BOVINE CARBONIC ANHYDRASES

The absence of a free-amino-terminal residue in human carbonic anhydrases was demonstrated in the B and C forms by Rickli, Ghazanfar, Gibbons, and Edsall (1964) and in the A form by our complementary investigations (Marriq, Gignoux, and Laurent, 1965). We reported later (Laurent, Marriq, Garçon, Luccioni, and Derrien, 1967b) that the estimation of the acetyl content of the B and C forms of the human enzyme according to Phillips (1963) yielded one N-acetyl group per mole of either protein, and that the B form is acetylated at its N-terminus. Thus, it seemed of interest to extend the investigations to the A and C human enzymes and to the A and B forms of bovine carbonic anhydrase.

Sanger's technique has been applied to the A

and B bovine isoenzymes that were highly purified from the ethanol-chloroform extract of the hemolysate by chromatography on Amberlite CG-50, which Luccioni (1966) described. No N-terminal amino acid could be detected with fluorodinitrobenzene in either enzyme even under conditions where uncoiling of the molecule by action of the sodium dodecylsulfate might be expected. Acetyl groups have been estimated by the method of Phillips, as in our previous work (Laurent et al., 1967b).

After 17 hours hydrazinolysis at 100 C, 0.85 mole of DNP-acetylhydrazide per mole of BCAB and 0.93 mole per mole of BCAA are formed. After 1 hour hydrazinolysis at 20 C, the amount of DNP-acetylhydrazide is lower than 0.1 mole per mole of protein. This amount implies that no O-acetyl group is present in BCAA and BCAB, and that the isoenzymes contain one N-acetyl group per molecule, as previously demonstrated for the B and C forms of human carbonic anhydrase.

These results strongly suggest that the N-terminal amino acid of the several forms of the human and bovine enzymes is blocked by an acetyl group. Such a situation was demonstrated for HCAB (Laurent et al., 1967b) by studying a N-acetyl hexapeptide isolated by Narita's (1958) procedure from a peptic digest of this protein.

<sup>1</sup> With the collaboration of M. Charrel and C. Marriq and the technical assistance of D. Gignoux.

TABLE I.—*N-Terminal End of the Human and Bovine Carbonic Anhydrases*

|      | 1  | 2 | 3 | 4 | 5 | 6                                    |
|------|--|---|---|---|---|--------------------------------------|
| HCAB | N-Acetyl—Ala-Ser-Pro-Asp-Trp-Gly-Tyr-Asp-Asp-Lys |   |   |   |   |                                      |
|      |  |   |   |   |   |                                      |
|      |  |   |   |   |   | .... Lys-Ser-Trp-Glu-Pro-Gln-Gly-Asn |
| HCAA | N-Acetyl—Ala-Ser-Pro-Asp-Trp-Gly....             |   |   |   |   |                                      |
| HCAC | N-Acetyl—Ser....                                 |   |   |   |   |                                      |
| BCAB | N-Acetyl—Ser....                                 |   |   |   |   |                                      |

Therefore we tried to isolate, in a similar way, an acetylpeptide from HCAC, BCAB, and HCAA.

Highly homogeneous HCAC (Laurent, Charrel, Luccioni, Autran, and Derrien, 1965) and BCAB preparations (Luccioni, 1966) have been digested with pepsin, chymotrypsin, and pronase under various experimental conditions. By screening these digests through Dowex-50 X-2 (H<sup>+</sup>) columns, acetylated material was found only in the pronasic digests, obtained at pH 8.0 with an enzyme/substrate ratio of 10 percent;<sup>2</sup> 0.4 mole acetylhydrazide per mole of hydrolyzed proteins was found. The constituents of these filtrates, which also contain pyrrolidone carboxylic acid and pyroglutamic peptides, are separated into four fractions by chromatography on Dowex-2 X-8 (H<sup>+</sup>) columns, using an HCl linear gradient as eluant. The second eluted fraction contains the whole acetylated material. When submitted to paper chromatography in *n*-butanol-acetic acid-water (4:1:5 v/v) followed by high-voltage electrophoresis at pH 6.5 in the second dimension, this fraction splits into six components. After elution from their spots and purification by rechromatography, the presence of an acetyl group in a single one of these, containing serine in nearly equimolecular amount as unique amino acid, has been established by Akabori's technique (Akabori, Ohno, and Narita, 1952) as modified by Narita (1958). Identification of *N*-acetylserine has been confirmed by paper chromatography and electrophoresis by reference to a pure synthetic marker. Using Phillips' technique, we established that, in both native proteins, the bond between the acetyl group and an amino acid residue is an amidic one; no O→*N*-acetyl

transfer during the analytical procedure can be taken into consideration. Thus the failure of FDNB to detect an *N*-terminal amino acid and the presence of one *N*-acetylserine residue strongly suggest that *N*-acetylserine is the *N*-terminal residue of both BCAB and HCAC. Only 40 percent of the *N*-acetyl group of BCAB and HCAC is recovered by screening their pronasic digest on Dowex-50 X-2 (H<sup>+</sup>); and since no acetyl peptide is detected in the same way in the other endopeptidasic digests tested, it may be that a basic amino acid residue is bound to the *N*-acetylserine. We failed to characterize lysine in such a position by the study of succinylated BCAB and HCAC; no acetylpeptide could be obtained from various endopeptidasic digests of these succinylated proteins. Thus, arginine or histidine can eventually be the second residue of the *N*-acetylterminal sequence in both proteins.

The *N*-acetyl hexapeptide previously isolated (Laurent et al., 1967b) from a peptic digest of HCAB (*vide supra*) by Narita's procedure was identified as *N*-acetyl-Ala-Ser-Pro-Asp-Trp-Gly. The same *N*-acetyl hexapeptide has recently been isolated by applying identical methods to nearly homogeneous HCAA preparations. Thus HCAA and HCAB contain an identical hexapeptidic *N*-acetylated end chain, as shown in table I.

*N*-acetylalanine is the *N*-terminal residue of the low-specific-activity human A and B isoenzymes and *N*-acetylserine, the *N*-terminal residue of the high-specific-activity enzymes HCAC and BCAB. The identity of the *N*-terminal residue of the two last enzymes can be connected with the results of Nyman, Strid, and Westermarck (1966), showing that the C-terminal sequences are more similar in HCAC and BCAB than in the human B and C forms.

Our results suggest that acetylation at the

<sup>2</sup> Using blanks as controls, we found that, under these conditions, no acetylated material is freed by the pronase itself.

amino terminus may be a structural feature common to various erythrocyte carbonic anhydrases. The presence of an N-acetyl radical and the absence of a free amino end group in BCAA and also the absence of a free amino end group in the carbonic anhydrase of the dog (Byvoet and Gotti, 1967) support our suggestion.

The identity of the primary structure of the A and B forms of human carbonic anhydrases is highly probable: an N-acetyl terminal hexapeptide of identical amino acid sequence has been isolated from both; their zinc content and activity are identical (Laurent, Charrel, Castay, Nahon, Marriq, and Derrien, 1962; Laurent et al., 1965; Nyman 1961), as are their amino acid composition (Laurent, Garçon, Marriq, Charrel, and Derrien, 1964b; Laurent, Charrel, Marriq, Garçon, and Derrien, 1966; Nyman and Lindskog, 1964); antigenic properties (Fine, Boffa, Charrel, Laurent, and Derrien, 1963); C-terminal amino acid residue (Marriq et al., 1965); and the fingerprints of their tryptic and chymotryptic digests (Laurent, Garçon, Charrel, Ardoino, and Derrien, 1967a). The difference of net charge that differentiates one enzyme from the other by electrophoresis and chromatography could be due to the absence in HCAA of some amide groups present in HCAB, or to conformational differences inducing charge differences by unmasking protonable groups. The first hypothesis is contradicted by the data of Nyman and Lindskog (1964) and by identical tryptic and chymotryptic fingerprints of HCAA and HCAB (Laurent et al., 1967a); the second agrees with the results described in the second part of the present paper.

#### MULTIPLE MOLECULAR FORMS OF HUMAN AND BOVINE CARBONIC ANHYDRASES B

Preparations of HCAB homogeneous in the ultracentrifuge and practically homogeneous to paper and cellulose acetate electrophoresis (0.025 M sodium barbiturate buffer, pH 8.6) split into four components by acrylamide gel electrophoresis (*tris*-glycine buffer, pH 8.6), as shown in figure 1.

We observed before (Laurent, Nahon-Garçon, Charrel, and Derrien, 1963) that HCAB submitted to pH 12.7 for 2 minutes was partly trans-

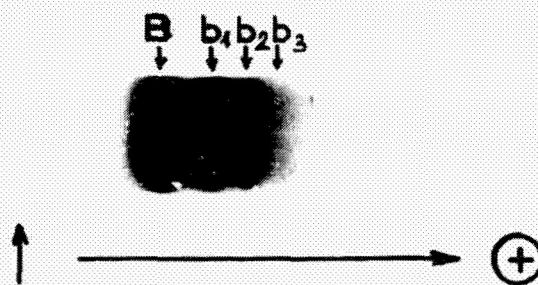


FIGURE 1.—Acrylamide gel electrophoresis of human carbonic anhydrase B performed with a Canaleo Model 6 disk electrophoresis apparatus. The gel, made according to the directions supplied by the manufacturer, stacks at pH 8.3 (*tris*-glycine buffer, pH 8.3) and runs at pH 9.5.

formed into a component of higher electrophoretic mobility; therefore, it seemed that the heterogeneity of HCAB induced by acrylamide gel electrophoresis could be related to the pH gradients raising the pH in the gel. To test this hypothesis, we studied the resistance to denaturation by alkali of 3 percent solutions of HCAB by the technique previously used to differentiate HCAB and HCAC (Laurent et al., 1963). The following procedure was applied: the pH of the sample was increased to a given value by addition of a previously checked volume of KOH N/10 or N at zero time. The mixture was neutralized after an indicated time by the addition of an equal volume of a 3.5 M potassium phosphate buffer, pH 6.8. The protein precipitate eventually formed was discarded by centrifugation. Comparison of absorbency of the supernatant at 280 millimicrons and of a blank in which KOH is omitted gives the percentage of precipitated protein. After elimination of salts by dialysis and lyophilization, the soluble fraction was submitted to electrophoresis on cellulose acetate. Figure 2 shows our most significant results.

At pH 11 and 12, the neutralization after 30 and 60 minutes is followed neither by precipitation nor by modification of the electrophoretic behavior of the protein. At pH 12.7, more than 90 percent of HCAB remained in solution after 30 or 60 minutes, but a minor component  $b_1$  was formed as Laurent et al. (1964b) had shown. At pH 13.0, 60 percent of the protein was precipitated by neutralization after 30 minutes. The 40-percent soluble fraction contained three electro-



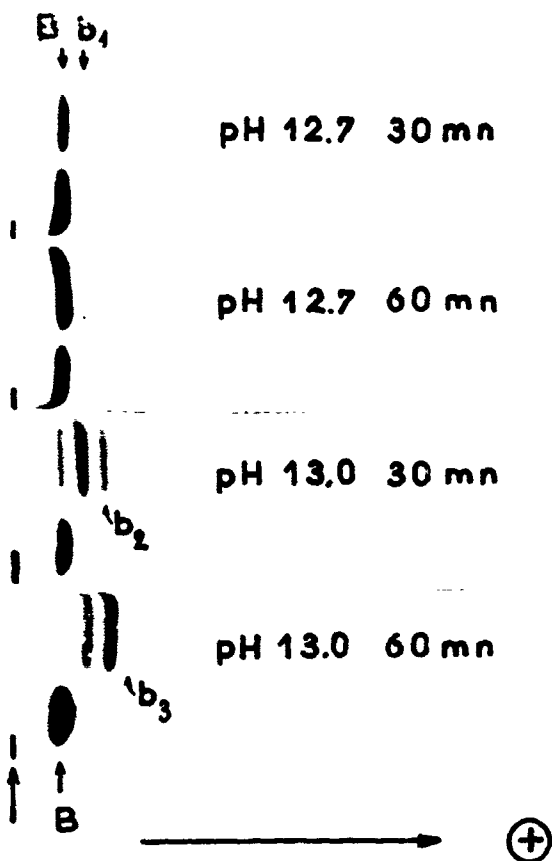


FIGURE 2.—Cellulose acetate electrophoresis patterns. From top to bottom, human carbonic anhydrase B allowed to remain for 30 or 60 minutes at pH 12.7 or 13.0. At the lower part of each strip is native enzyme B as reference; sodium barbiturate buffer 0.025 M, pH 8.6.

phoretically different constituents: unmodified HCAB and two components of increasing mobility,  $b_1$  and  $b_2$ . After 60 minutes, only 12 percent of the enzyme remained in solution when neutralized; a fourth component,  $b_3$ , appeared, and  $b_2$  replaced  $b_1$  as the major form; only traces of HCAB were present. The analogy of these results and of the behavior of the enzyme on acrylamide electrophoresis can be interpreted as the action of a high pH gradient in the gel being largely responsible for the observed heterogeneity.

Spectrophotometric study of the alkaline medium and of the neutralized supernatant separated by centrifugation and dialysis has also been performed on appropriate dilutions of the protein (about 0.05 percent). Figure 3 shows that

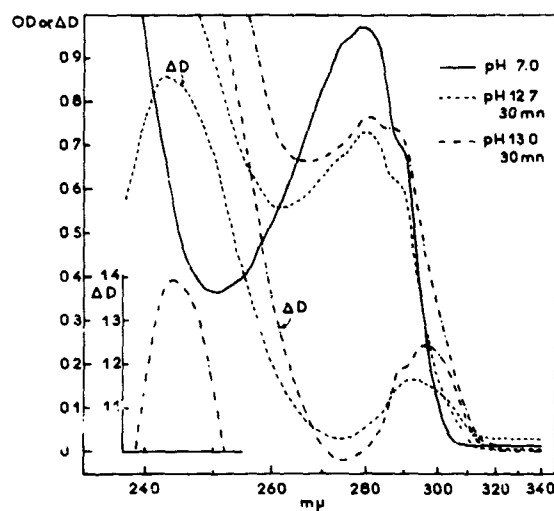


FIGURE 3.—Ultraviolet absorption spectra of human carbonic anhydrase B at pH 7.0 (solid line), pH 12.7 (dash line), and pH 13.0 (dot-dash line); Protein concentration,  $\sim 0.65$  g per 100 ml; temperature  $\sim 25$  C. In addition, spectra of alkaline difference ( $\Delta D$ ) at pH 12.7 (dash line) and pH 13.0 (dot-dash line) is read against the same protein solution at pH 7.0.

the ultraviolet spectrum of the native preparation of HCAB is modified when the pH is increased from pH 11.0 to 13.0. These modifications result in increased absorbency at 245 and 295 millimicrons, which is visible on the difference spectra. On the other hand, after neutralization and dialysis of the medium, a reversion of the modification of the spectrum is observed. These findings are in agreement with the more extended observations published by Riddiford, Stellwagen, Mehta, and Edsall (1965). It is remarkable that such a reversion does not take place with a renaturation of the protein. In fact, the multiple forms originating from the native enzyme at pH 13.0 are stable, and ultracentrifugation shows that they do not correspond to different degrees of aggregation of HCAB.

The isolation of  $b_1$  by chromatography on Amberlite CG-50 (Laurent, Charrel, Garçon, Castay, Marriq, and Derrien, 1964a) enabled us to establish: (1) that the transformation  $B \rightarrow b_1$  is accompanied neither by a modification of zinc content, enzyme activity, or amino acid composition nor by changes in fingerprints after digestion by trypsin; (2) that the enzyme of the form  $b_1$  can be differentiated from HCAA neither by

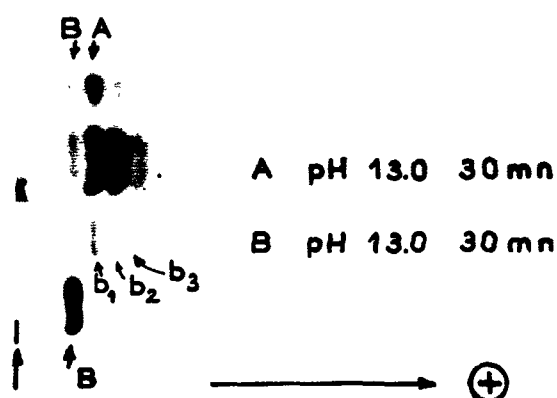


FIGURE 4.—Cellulose acetate electrophoresis patterns of native and alkali-treated human carbonic anhydrases A and B: Upper and lower: semipurified human carbonic anhydrase B. Middle: enzymes A and B treated for 30 minutes at pH 13.0.

its velocity of electrophoretic migration nor by its elution from Amberlite CG-50. Thus, by considering HCAA and HCAB as identical in their primary structure, we can also consider  $b_1$  and HCAA to be identical. If this hypothesis is true, one could expect that HCAA and HCAB would give the same multiple molecular forms at pH 13.0, the difference of net charge between HCAA and HCAB being due to a modification of the tridimensional structure only. Figure 4 shows that after 30 minutes at pH 13.0 and neutralization, the dialyzed supernatant of HCAA contains three components with the same relative migration velocity as  $b_1$ ,  $b_2$ , and  $b_3$ . Recent work of J. Reynaud in our laboratory established a number of comparative hydrodynamic data on HCAA and HCAB. The two enzymes have the same molecular weight, but show differences in molecular shape. The molecule of HCAB is practically spherical (Reynaud, Rametta, Savary, and Derrien, 1963; Reynaud, Savary, and Derrien, 1965), whereas that of HCAA is ellipsoidal with an  $a/b$  ratio of 2.9.

In conclusion, a working hypothesis drawn from our studies can be given: conformational modifications of HCAB without change in primary structure may involve as the initial step its transformation into HCAA as the first conformer of HCAB, in terms of the meaning given to this word by Kitto, Wassarman, and Kaplan (1966). Duff and Coleman (1966) expressed similar views

on the multiple forms of isoenzymes B and C of carbonic anhydrase of *Macaca mulatta* separated by gel electrophoresis in an 8 M urea buffer of pH 4.5. It would be important to verify this hypothesis by biochemical, hydrodynamic, and enzymatic comparative results on the  $b_1$ ,  $b_2$ , and  $b_3$  forms of HCAB. It must be pointed out that Luccioni (1966) established in our laboratory that BCAB shows a heterogeneity analogous to that of HCAB by acrylamide gel electrophoresis or by cellulose acetate electrophoresis after 2, 30, and 60 minutes at pH 10.0.<sup>3</sup> The first form, appearing within 2 minutes, has the same relative migration velocity on cellulose acetate and on paper as BCAA; the variability of proportions of BCAB and BCAA in chloroform ethanol extract and the absence of BCAA in the hemolysates have been pointed out by Lindskog (1960).

Such observations suggest that, of the A and B forms of human and bovine carbonic anhydrases, only the B form is physiologically significant. Research on the A and other conformers deriving successively from it would, however, eventually clarify the relationships existing between the activity and the tridimensional structure of the carbonic anhydrase.

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<sup>3</sup> At pH 12.7 the bovine enzyme is completely denatured in 2 minutes, as is HCAC; and similar to HCAC BCAB is much less stable than HCAB at high pH.

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# Primary Structure Studies on Human Carbonic Anhydrase C<sup>1</sup>

70-23304

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Two approaches have been used to obtain information relating to the primary structure of human carbonic anhydrase C. Peptides have been isolated from a tryptic digest of the whole protein. This straightforward approach did not yield a complete set of tryptic peptides.

The lysine  $\epsilon$ -amino groups of the protein were blocked with maleic anhydride and the protein digested with trypsin. Five large peptide fragments were isolated. After removal of the blocking agent, the isolated fragments were redigested with trypsin. The tryptic peptides derived from some of these large fragments have been isolated. These peptides, together with the tryptic peptides obtained from the first approach, comprise a nearly complete set of tryptic peptides from human carbonic anhydrase C.

The amino acid composition of the tryptic peptides is presented. The grouping of the tryptic peptides in the large fragments and a knowledge of the N- and C-terminal regions of the molecule permit the construction of an overall sketch of the amino acid sequence of human carbonic anhydrase C.

Human erythrocyte carbonic anhydrases B and C catalyze the reversible hydration of CO<sub>2</sub>. Human carbonic anhydrase C has the greater specific activity but it is present in lesser amounts; thus, they contribute about equally to the total catalytic activity of the cell. Each enzyme is composed of a single polypeptide chain of about 265 amino acids and 1 atom of zinc (Rickli, Ghazanfar, Gibbons, and Edsall, 1964; Nyman and Lindskog, 1964). The amino acid sequence

of carbonic anhydrase B is under study and has been reported by Nyman at this symposium (Andersson, Nyman, and Strid, 1969). The amino acid sequence of human erythrocyte carbonic anhydrase C is the subject of this report.

Human carbonic anhydrase C contains one residue of methionine located 20 residues from the C-terminal end of the molecule. Nyman and co-workers have cleaved the polypeptide chain at the methionine with cyanogen bromide, have isolated the 19 amino acid C-terminal peptide, and determined its amino acid sequence (Nyman, Strid, and Westermarck, 1968). G. Laurent and coworkers have determined that the N-terminal amino acid of the enzyme is N-acetyl serine (Marriq, Luccioni, and Laurent, 1965; Laurent, Marriq, Garçon, Luccioni, and Derrien, 1967). I would like to be able to report the sequence of the remaining 244 amino acids, but the work is not yet complete. Therefore, this communication will be in the nature of a progress report on the current state of the amino acid sequence work on human carbonic anhydrase C.

<sup>1</sup>The paper presented by Dr. Henderson at the Haverford conference gave only a very preliminary report of the status of his work on carbonic anhydrase C. Some of the data reported earlier have since been found to require revision and the scope of the work has been greatly extended in subsequent months.

These recent advances make it possible to give a more adequate account of the structural work on carbonic anhydrase C which provides a better basis of comparison with the work on carbonic anhydrase B as reported in a preceding paper by Andersson, Nyman, and Strid.

In view of these facts, the editors asked Dr. Henderson to prepare a new version of his paper reporting the status of his work as of March 1969. This revised manuscript was received on March 10, 1969.

The protein contains 25 residues of lysine and 7 residues of arginine, giving a total of 32 potential sites for attack by trypsin. However, the native enzyme is resistant to trypsin, and many denatured forms of the protein are only partly accessible to attack by trypsin. It is possible, however, to obtain an adequate digest by first denaturing the enzyme in 8 *M* urea and then diluting to 4 *M* urea with an ammonium bicarbonate buffer containing the trypsin. After 30 minutes, a second volume of buffer containing more trypsin is added and the digestion is allowed to continue for 8 hours at room temperature. It is sometimes necessary to lyophilize the mixture and repeat the process. The final trypsin-to-protein ratio is not more than 1:50 on a mole basis. This procedure results in cleavage of about 80 percent of the lysine bonds and 100 percent of the arginine bonds. The urea can be removed from the peptides by gel filtration. If a sufficiently long column is used, an initial separation of the peptides is also obtained. The peptides separate on the column according to their molecular weight and amino acid composition. Peptides containing tryptophan are retarded to a greater extent than one would expect from a simple consideration of their molecular weight.

A mixture of the tryptic peptides of human carbonic anhydrase C has been separated into eight distinct fractions on a column (2 by 200 cm) of Sephadex G-25 eluted with 0.2 *M* NH<sub>4</sub>HCO<sub>3</sub>. The peptide fractions obtained by this procedure have been separated into their major component peptides either by ion-exchange chromatography on Dowex-50 X-4 or by paper electrophoresis using a tank-type, high-voltage electrophoresis system. The electrophoresis system is faster and easier to use and is now being used exclusively.

The above approach to the sequence of human carbonic anhydrase C has been used successfully to obtain a number of the tryptic peptides, but it has not yet produced a complete set of tryptic peptides. The missing peptides may yet be found in unresolved peptide mixtures but, at present, it seems possible that some peptides may be lost during the initial separation on the gel filtration column. Aggregating peptides may spread over a large volume in the column and be present in only trace amounts in each fraction.

Such peptides would be very difficult to detect by the methods currently in use. Because of this inherent danger, a second approach to the problem has been attempted with very promising initial results.

The protein was allowed to react with maleic anhydride in the presence of 8 *M* urea. In this procedure the  $\epsilon$ -amino groups are modified such that lysine is no longer a suitable point of attack for trypsin (Butler, Harris, Hartley, and Leberman, 1967). Trypsin-catalyzed hydrolysis of the modified protein takes place only at the carboxyl bond of the arginine residues. The modified protein was easily digested with trypsin and the larger peptide fragments were separated by gel filtration on Sephadex G-75. After rechromatography, the fragments were obtained in greater than 80 percent purity. The approximate amino acid composition of these fragments is given in table I. The total amino acid composition of these fragments closely approximates the amino acid composition of human carbonic anhydrase C.

The blocking group was removed from the lysine  $\epsilon$ -amino groups (Butler et al., 1967), and the fragments were redigested with trypsin. The tryptic peptides derived from the fragments were separated directly by paper electrophoresis. Although this work is still in progress, it has already provided most of the tryptic peptides that were missing in the previous work. The amino acid compositions of the tryptic peptides that have been isolated by either one or both of these two approaches are listed in table I. The numbering of the tryptic peptides in table I is arbitrary but the postscript letters (-A, -B, etc.) indicate that the tryptic peptide is located in the fragment denoted by the same letter. The total amino acid composition of the tryptic peptides closely approximates the amino acid composition of human carbonic anhydrase C.

The amino acid sequence of a few of the tryptic peptides has been determined by subtractive Edman procedures. The large fragments give information concerning the grouping of the tryptic peptides. The arrangement of the large fragments in the whole molecule can be deduced by considering the known C-terminal sequence of human carbonic anhydrase C (Nyman et al., 1968) and by assuming a degree of homology between this enzyme and human carbonic anhydrase B

(Andersson et al., 1968). These assumptions permit the construction of an overall sketch of the structure of human carbonic anhydrase C, which is shown in table II.

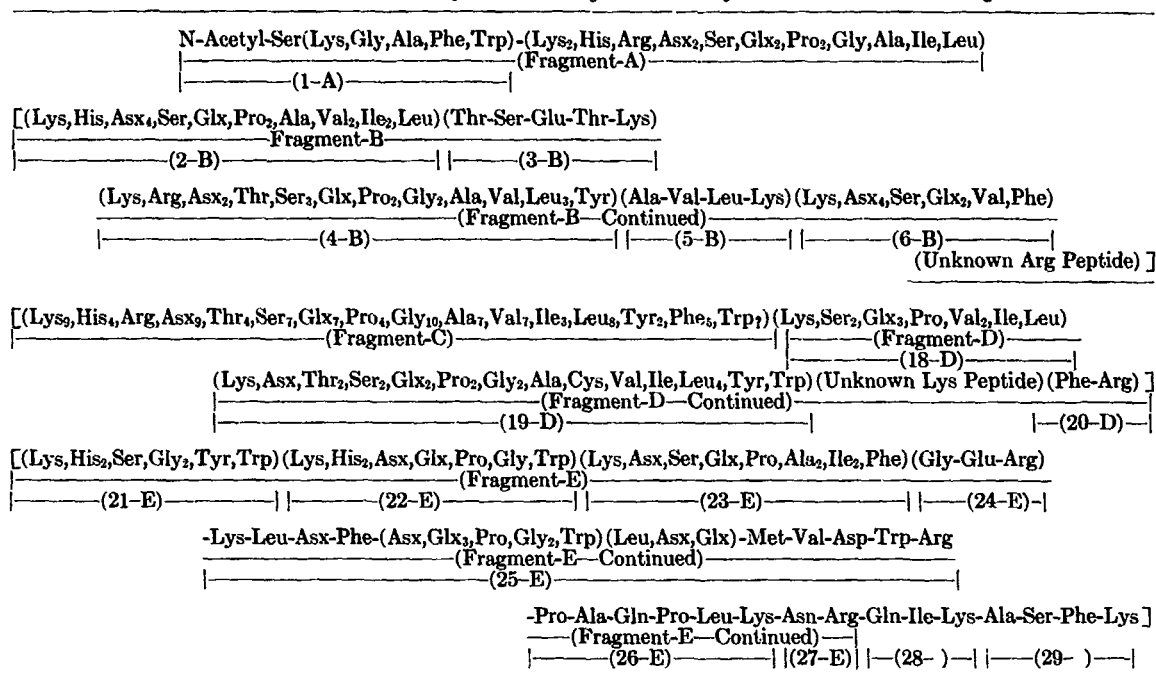
The sketch presented in table II must be regarded largely as a working hypothesis. Fragment-E can be definitely located as the C-terminal frag-

ment, but the arrangement of tryptic peptides 21-E through 24-E is unknown. Tryptic peptide 25-E can be definitely located, since it contains the single methionine residue and overlaps the known C-terminal sequence (Nyman et al., 1968). Fragment-B seems to be homologous with the N-terminal fragment of human carbonic anhy-

TABLE I.—Amino Acid Compositions of Human Carbonic Anhydrase C, Tryptic Peptides, and "Blocked Lysine" Fragments

| Peptide no. | Lys | His | Arg | Asx | Thr | Ser | Glx | Pro | Gly | Ala | Cys | Val | Met | Ile | Leu | Tyr | Phe | Trp |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1-A         | 1   |     |     |     |     | 1   |     |     | 1   | 1   |     |     |     |     |     |     | 1   | 1   |
| 2-B         | 1   | 1   |     | 4   |     | 1   | 1   | 2   |     | 1   |     | 2   |     | 2   | 1   |     |     |     |
| 3-B         | 1   |     |     |     | 2   | 1   | 1   |     |     |     |     |     |     |     |     |     |     |     |
| 4-B         | 1   |     | 1   | 2   | 1   | 3   | 1   | 2   | 2   | 1   |     | 1   |     |     | 3   | 1   |     |     |
| 5-B         | 1   |     |     |     |     |     |     |     |     | 1   |     |     |     |     | 1   |     |     |     |
| 6-B         | 1   |     |     | 4   |     | 1   | 2   |     |     |     |     | 1   |     |     |     |     |     | 1   |
| 7-          | 1   |     |     | 1   |     |     |     | 1   |     | 1   |     |     |     | 1   |     |     |     | 1   |
| 8-          | 1   |     |     | 1   |     |     |     |     | 2   |     |     |     |     |     |     | 1   |     | 1   |
| 9-          |     |     | 1   |     |     |     | 1   |     | 1   |     |     |     |     |     |     |     |     |     |
| 10-         | 1   |     |     |     |     |     | 1   |     |     |     |     |     |     | 1   |     |     |     |     |
| 11-         | 2   |     |     |     |     | 1   | 1   | 1   | 2   | 1   |     | 1   |     |     | 1   |     |     |     |
| 12-         | 1   |     |     |     | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 13-         | 1   |     |     |     |     |     |     |     | 1   |     |     |     |     |     |     |     |     |     |
| 14-         |     |     | 1   | 2   | 1   | 1   |     | 1   |     | 1   |     |     |     |     |     |     | 2   |     |
| 15-         | 1   | 2   |     | 1   | 1   |     | 1   |     |     | 2   |     | 1   |     |     | 2   | 1   |     | 1   |
| 16-         | 1   |     |     |     |     | 1   | 1   | 1   | 1   | 1   |     |     |     |     | 1   |     |     |     |
| 17-         | 2   | 2   |     | 5   | 2   | 3   | 3   | 3   | 5   | 1   |     | 5   |     | 1   | 5   | 1   | 3   |     |
| 18-D        | 1   |     |     |     |     | 2   | 3   | 1   |     |     |     | 2   |     | 1   | 1   |     |     |     |
| 19-D        | 1   |     |     | 1   | 2   | 2   | 2   | 2   | 2   | 1   | 1   | 1   |     | 1   | 4   | 1   |     | 1   |
| 20-D        |     |     | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     | 1   |     |
| 21-E        | 1   | 2   |     |     |     | 1   |     |     | 2   |     |     |     |     |     |     | 1   |     | 1   |
| 22-E        | 1   | 2   |     | 1   |     |     | 1   | 1   | 1   |     |     |     |     |     |     |     |     | 1   |
| 23-E        | 1   |     |     | 1   |     | 1   | 1   | 1   |     | 2   |     |     |     | 2   |     |     | 1   |     |
| 24-E        |     |     | 1   |     |     |     | 1   |     | 1   |     |     |     |     |     |     |     |     |     |
| 25-E        | 1   |     | 1   | 4   |     |     | 4   | 1   | 2   |     |     | 1   | 1   |     | 2   |     | 1   | 2   |
| 26-E        | 1   |     |     |     |     |     | 1   | 2   |     | 1   |     |     |     |     | 1   |     |     |     |
| 27-E        |     |     | 1   | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 28-         | 1   |     |     |     |     |     | 1   |     |     |     |     |     |     | 1   |     |     |     |     |
| 29-         | 1   |     |     |     |     | 1   |     |     |     | 1   |     |     |     |     |     |     | 1   |     |
| Total       | 26  | 9   | 7   | 28  | 10  | 20  | 27  | 19  | 23  | 16  | 1   | 16  | 1   | 10  | 22  | 6   | 13  | 7   |
| Fragment-A  | 3   | 1   | 1   | 2   |     | 2   | 2   | 2   | 2   | 2   |     |     |     | 1   | 1   |     | 1   | ?   |
| Fragment-B  | 5   | 2   | 2   | 12  | 4   | 6   | 5   | 5   | 5   | 5   |     | 4   |     | 2   | 6   | 2   | 3   |     |
| Fragment-C  | 9   | 4   | 1   | 9   | 4   | 7   | 7   | 4   | 10  | 7   |     | 7   |     | 3   | 8   | 2   | 5   | ?   |
| Fragment-D  | 3   |     | 1   | 1   | 4   | 3   | 8   | 7   | 2   |     | 1   | 4   |     | 2   | 9   | 2   | 1   | ?   |
| Fragment-E  | 4   | 4   | 2   | 6   |     | 2   | 7   | 4   | 5   | 2   |     | 1   | 1   | 1   | 3   | 1   | 2   | 2   |
| Total       | 24  | 11  | 7   | 30  | 12  | 20  | 29  | 22  | 24  | 16  | 1   | 16  | 1   | 9   | 27  | 7   | 12  | ?   |
| HCA-C       | 25  | 12  | 7   | 29  | 12  | 19  | 27  | 17  | 22  | 13  | 1   | 17  | 1   | 9   | 25  | 8   | 12  | 7   |

TABLE II.—A Tentative Sketch of the Primary Structure of Human Carbonic Anhydrase C



drase B (Andersson et al., 1968). However, the fragment from the C enzyme is smaller than the corresponding fragment in the B enzyme. The homology is greatest when fragment-B is located penultimate to the N-terminal fragment. Fragment-A is small enough to fit the requirements of the N-terminal fragment of the molecule. Moreover, its amino acid composition indicates that it may contain the tryptic peptide 1-A which is believed to be the N-terminal peptide on the basis of its electrophoretic mobility. There is no information available at this time about the arrangement of fragments C and D, except that they must be located somewhere in the middle of the polypeptide chain.

The tryptic peptides derived from fragments B and D are arbitrarily arranged. The amino acid compositions of these tryptic peptides do not total the amino acid composition of the parent fragments. Therefore, it is necessary to indicate the possible presence of an unidentified peptide in these fragments. Fragment-C has not yet been digested with trypsin. Presumably most of the tryptic peptides 7- through 17- will ultimately be found in this fragment.

This sketch of the molecule is highly tentative, but it is extremely useful in continuing the work on the amino acid sequence of the molecule. It would be very dangerous, at this time, to attempt to use this sketch as a basis for speculation about the relationship of the structure to the function of the molecule. It is equally dangerous to attempt to determine the homology between the B and C enzymes, because the sketch is constructed largely from homology considerations.

One point concerning the relationship of structure to function might be considered at this time. The human carbonic anhydrases B and C are strikingly different in their reactivities with some selective alkylating agents (Andersson et al., 1969; Bradbury, 1969; Whitney, Fölsch, Nyman, and Malmström, 1967; Whitney, Nyman, and Malmström, 1967). The B enzyme is labeled with these reagents by alkylating a specific histidine with the concomitant loss of enzymic activity. The actual histidine labeled depends upon the nature of the alkylating agent. One of the labeled histidine residues has been located in a tryptic peptide that also contains the single cysteine of the enzyme (Andersson et al., 1969). Human carbonic anhy-

drase C does not label with these reagents, and there is no histidine in the tryptic peptide that contains the single cysteine of the enzyme. In fact, there does not seem to be any histidine in the large fragment that contains the cysteine (Fragment D). This observation may account for the differences in the reactivity of the two enzymes toward the labeling reagents. There are considerable uncertainties about the location of the cysteine in both enzymes; therefore, it is impossible at this time to draw any further conclusions about this point.

The main body of the information presented in this report has been obtained within the last few months. The rate of obtaining information has greatly increased, and it is reasonable to expect that the sequence studies on this enzyme will be completed in the near future. When this information is added to the information provided by studies on the B enzyme and the X-ray data already available for both enzymes (Fridborg, Kannan, Liljas, Lundin, Strandberg, Strandberg, Tilander, and Wiren, 1967; Liljas, Kannan, Bergstén, Fridborg, Järup, Lögren, Paradies, Strandberg, and Waara, 1968; Liljas and Strandberg, personal communication), it should provide an interesting chapter in the study of the relationship between the structure and function of enzyme proteins.

#### NOTE ADDED IN PROOF

Since the writing of this report the work with the "blocked lysine" fragments has continued, and the data available at this time suggest that some corrections should be made in tables I and II. Fragment E has been found to be a mixture of two fragments. One fragment contains the tryptic peptides 25-E, 26-E, and 27-E, and may possibly contain 23-E. The other fragment contains the tryptic peptides 21-E, 22-E, and 24-E. This second fragment is most probably the N-terminal fragment with peptide 21-E being the N-terminal tryptic peptide. The position of the previous candidate for the N-terminal peptide (fragment A) in table II is unknown at this time.

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### DISCUSSION

**TASHIAN:** The ester substrates which are known to be hydrolyzed by carbonic anhydrase now include several naphthol (Tashian, Douglas, and Yu, 1964) and phenol (cf. Verpoorte, Mehta, and Edsall, 1967) esters and certain cyclic sulfonates (Lo and Kaiser, 1966); and, as with its CO<sub>2</sub> hydrase activity, this esterase activity has been demonstrated to be dependent on the single firmly bound zinc ion which is characteristic of carbonic anhydrase.

I would like to discuss briefly some preliminary findings on a genetically determined electrophoretic variant of human red cell carbonic anhydrase B(CAB) which is characterized by an unusual alteration in esterase activity. The normal and variant enzymes were isolated from hemolyzates of the same individual, and their esterase and hydrase activities were compared. In addition to the ester substrates  $\beta$ -naphthyl acetate ( $\beta$ -NA) and *p*-nitrophenyl acetate (*p*-NPA), the esterase activity toward 2-hydroxy-5-nitro- $\alpha$ -toluenesulfonic acid sultone was also measured. Under our experimental conditions, the relative specific esterase activities of normal CAB toward *p*-NPA and the benzyl sultone were about 10 times and 2000 times the activity toward  $\alpha$ -NA, respectively. No obvious differences in the esterase activity toward these three substrates were observed between the normal and mutant enzymes. The only significant change appears to be in the CO<sub>2</sub> hydrase activity which is reduced to about one-half that of the normal enzyme.

The unique feature of the variant carbonic anhydrase, however, is that its specific esterase activity toward  $\beta$ -NA, but not *p*-NPA or the benzyl sulfone, appears to be specifically activated by zinc ions. This specificity of metal-ion activation was demonstrated by the fact that when the esterase activity of the mutant enzyme toward  $\beta$ -NA was measured in the presence of five metal ions (Zn<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup>, and Ni<sup>2+</sup>) at a concentration of 10<sup>-4</sup> *M*, only zinc showed any activating effect on the esterase activity of the variant enzyme. These metal ions either inhibited or had no effect on the activity of the normal enzyme.

The substrate specificity of the mutant enzyme toward  $\beta$ -NA was shown by the fact that increasing concentrations of zinc activated only the esterase activity of the variant enzyme toward  $\beta$ -NA, while inhibiting the activity of the normal enzyme. At a zinc concentration of 10<sup>-4</sup> *M*, the esterase activity of the abnormal enzyme toward  $\beta$ -NA was about 275 percent above control levels, whereas the esterase activities toward *p*-NPA and the benzyl sultone, as well as the CO<sub>2</sub> hydrase activity, were all inhibited by this concentration of zinc in both the normal and variant enzymes.

The apparent values of  $K_m$  for the normal and mutant enzymes with  $\beta$ -NA as substrate, in both the presence and absence of zinc, were estimated from Lineweaver-Burk plots to have essentially similar values of about 5 × 10<sup>-3</sup> *M*.

The zinc content of the variant enzyme and its relative degree of binding was tested by dialyzing the normal and variant enzymes against *o*-phenanthroline at pH 6.5. The results indicate that only one zinc atom is bound to the abnormal enzyme, and it is bound as firmly as is the zinc of the normal carbonic anhydrase.

These findings suggest that the structural change brought about by this mutation does not seem to directly involve an alteration at the hydrolytic site, but rather that free zinc ions somehow specifically alter the binding position of  $\beta$ -NA, so that its hydrolysis is enhanced.

Whenever we get an electrophoretic variant of carbonic anhydrase, all the minor components migrate to the same electrophoretic positions. We get electrophoretic variants in carbonic anhydrase B, and the minor components *B*<sub>1</sub>, *B*<sub>2</sub>, and *B*<sub>3</sub> migrate to the same positions relative to that of the major component in the mutant as in the normal. This reaction suggests that the same conformational changes that take place in the normal variants have also taken place in the mutant form—indicating that the mother molecule has undergone a conformational change.

**EDSALL:** So, presumably, there is only one genetic source of information, whether it is in the normal or the mutant, and the different electrophoretic forms arise from that.

**MAREN:** It is still true, Dr. Tashian, that you do not see any mutant forms in enzyme C at all?

**TASHIAN:** Not in humans, but we have seen them in other primate species.

**EDSALL:** Could I ask whether anyone has crystallized carbonic anhydrase A? It would be of interest, in view of the suggested conformational difference between A and B, to know whether the crystals were distinguishable from each other, even without going into any detailed X-ray studies of enzyme A.

**MAREN:** Have they found any activity in any of the fragments? For instance, in the one with 101 residues?

**NYMAN:** The preparation of the fragments from human enzyme B starts with a heat denaturation of the enzyme molecule, and we have not tested the purified fragments for enzymic activity.

**LAURENT:** The conformation  $b_1$  has lately been isolated by us and has the same amino acid composition and activity as the B enzyme.

**EDSALL:** Did you say that  $b_2$  and  $b_3$  have full activity? As well as  $b_1$ ?

**LAURENT:** No. This point is under investigation.

**TASHIAN:** The minor components of carbonic anhydrase B in macaque species have a high esterase activity, so even when there is little protein, you can see these minor components clearly. I have detected up to eight—not only the four that move close to the main component, but four more beyond that which shift again with electrophoretic mutants of these forms. This makes a total of 10 minor components of some of these carbonic anhydrases.

**MAREN:** Has any of this work given you any inkling as to the physiological value of carbonic anhydrase B, even guessing?

**TASHIAN:** We found one mutant of the Japanese macaque which does not exhibit the same proportion of minor components. In other words, the electrophoretic mutant shows a reversal. What was a minor component in the normal molecule is now a major component. It is just possible that this shifting has been selected as a physiological advantage.

**EDSALL:** It has always been a great puzzle, but I think that the evolutionary biologists are convinced that when you have a phenomenon

like this with two or more isoenzymes, no matter how they originated (presumably from gene duplication and subsequent mutation), they would not persist indefinitely unless there is some selective advantage in having the two as compared with having only one.

**HASTINGS:** Is there any information about carbonic anhydrase from tissues other than the red cells?

**ROSSI-BERNARDI:** Dr. Cristiano Rossi of Milan has studied carbonic anhydrase of spinach in detail. [See the next paper in this volume.]

**EDSALL:** One of the students in our laboratory, Allan Tobin, has been working on carbonic anhydrase from parsley. The plant carbonic anhydrases have been reported by some people to be free of zinc and uninhibited by sulfonamides. Tobin's preparation is now more than 90 percent pure. It is a considerably larger molecule than that of the red-cell carbonic anhydrases, but it does seem definitely to contain zinc. There may be several subunits. The sulfonamide inhibition is present but weak. [A report by Tobin on this enzyme is also included in this volume.]

**POCKER:** I fully agree with this. We have been working with the enzyme from spinach. During 1965, Dr. Meany, working in our laboratories, has been able to show that plant carbonic anhydrase is not an absolutely specific catalyst for the reversible hydration of acetaldehyde. Recently, we have initiated more detailed studies which show that plant carbonic anhydrase exchanges its zinc with radioactive Zn<sup>2+</sup> in solution at rates significantly faster than the enzyme from bovine erythrocytes.

**MAREN:** Dr. Hastings was asking about mammalian tissues, with respect to the isoenzymes. There has not been a great deal of work in this area. I think Dr. Tashian has looked at a few kidneys and concluded that there was no B. What about C?

**TASHIAN:** We have done more extensive work on two carbonic anhydrase forms in other tissues using a radio-immuno-assay method to trace them. Apparently, the same two isoenzymes are present in those tissues but in different ratios. The brain, for example, has a markedly different ratio of B to C, after taking into account any contamination due to hemolysate.

**EDSALL:** Do they behave electrophoretically like the same enzymes as those in the red cells?

**TASHIAN:** They seem to be the same enzymes but in different proportions. In the brain we have even detected an extra band which may be a different carbonic anhydrase.

**MAREN:** You mean it is shifted so there is a different proportion of C in the tissues?

**TASHIAN:** It is shifted; the ratio of B to C, instead of being 6 to 1, is now 1 to 1.

**HASTINGS:** Do they all have the same amino acid composition?

**TASHIAN:** No; this is done by immunochemical means.

**MAREN:** You referred to the blood again, the blood contained in the tissues. That has always struck me as the chief problem involved in isolating the enzyme from tissues. You said you corrected for the hemolysate?

**TASHIAN:** We diluted and washed until we had virtually no hemoglobin content. Then, using a very sensitive radio-immuno-assay method, we could have detected any contamination that could ever be expected.

**ROSSI-BERNARDI:** As to the plant enzymes, Dr. Cristiano Rossi and his collaborators have isolated carbonic anhydrase from spinach. They have found a molecular weight of 140 000 by three different methods. It is present in the mitochondria and in the chloroplasts; of course, it is very important because CO<sub>2</sub> is produced. To have carbonic anhydrase inside the mitochondria is like having a good buffer, which otherwise would not be functioning when the enzyme is absent.

**EDSALL:** I know that there is a bacterial carbonic anhydrase, an interesting molecule.

**NYMAN:** We tried to purify the enzyme from *Neisseria sica* and I think we have obtained a fairly homogenous preparation; at least it appeared homogenous in the ultracentrifuge. The molecular weight we obtained was 28 000, which is close to that of the mammalian enzymes. The protein contains one zinc for approximately 30 000 molecular weight; the amino acid composition is notable; some features are the considerably lower content of proline; 12 residues compared with 17 in the mammalian; furthermore, the content of

tryptophan and tyrosine is much lower than in the red cell enzyme from mammals. There is probably room for a disulfide bridge.

**MAREN:** Kinetically, are the plant and bacterial enzymes rather like the mammalian?

**NYMAN:** The bacterial carbonic anhydrase resembles the human form in specific enzyme activity and sulfonamide inhibition.

**DAVIES:** Are there any other examples of two proteins with apparently identical primary structure, but different tertiary structure? The protein chemists seem to feel that this is not a thing they would expect, because, for the most part, they believe that primary structure determines tertiary structure. The tertiary structure is supposed to form spontaneously. Is it possible that the molecule is rather unstable and that one of these, when found, is partially denatured but still retains activity?

**EDSALL:** This is a serious question, of course. Nathan Kaplan argued for the existence of these conformational isomers (conformers), but as far as I know no one has produced any decisive evidence for the identity of primary structure in these isoenzymes. I think that the question is still open. Does anyone here know of a case where two proteins with proved identical primary structures have different tertiary structures? Certainly carbonic anhydrases A and B do look amazingly alike—I would not be surprised to see identical primary structure, except for minor variations, perhaps a slightly different number of amide groups or something of this sort, associated with their slightly different electrophoretic mobilities.

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## Studies on Carbonic Anhydrase From Spinach Leaves: Isolation and Properties<sup>1</sup>

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Within the last few years, renewed interest in plant carbonic anhydrase enzymes has arisen from the conflicting data obtained in different laboratories on the presence of a zinc atom in the protein molecule. More recently, Fellner (1963) has found that some of the most powerful inhibitors of the animal enzyme, such as acetazolamide and Cl 11366, were entirely inactive toward unpurified fractions of the plant enzyme. Confirmatory data arose from the results of Lindskog (1963), who studied the binding of acetazolamide to the active protein and the lack of such binding to the metal-free enzyme of animal origin.

These and other results, suggesting that the functional role of the zinc atom in animal carbonic anhydrase is as an essential agent for catalytic activity and as a part of the active site, have led us to undertake further studies on the plant enzyme because of its remarkable property of absolute specificity with respect to the reversible hydration of carbonic dioxide. These studies are intended to cover the purification of the isolated protein and to define some of its chemical properties.

### EXPERIMENTAL PROCEDURES

#### Materials

Buffer components, which included potassium dihydrogen phosphate, dipotassium hydrogen phosphate, imidazole, and 2-amino-2-(hydroxymethyl)-1,3 propanediol (*tris*), were analytical

grade reagents. Diamox (acetazolamide) was kindly supplied by Dr. L. Rossi-Bernardi.

*p*-Nitrophenol, other indicators, and *p*-nitrophenyl acetate (*p*-NPA) were obtained from BDH and used without further purification. Sephadex G-25 fine, G-75, and G-200 were purchased from Pharmacia, Uppsala.

Acetaldehyde, a generous gift from Dr. Carlo Cavenaghi, was distilled twice before use.

#### Carbonic Anhydrase (CA) Assay

For a study of the bicarbonate dehydration in the particulate enzyme, we used the manometric apparatus as described by Roughton and Booth (1938). The enzyme was placed with the buffer in one compartment of the special reaction vessel, and after temperature equilibration, it was rapidly mixed with the substrate. A rapid shaking was assured (about 300 oscillations per minute) in order not to limit the CO<sub>2</sub> diffusion rate. All the manometric experiments were done at atmospheric pressure and at the temperature of 0.0±0.1 C.

The purified enzyme fractions were assayed essentially by the method of Kernohan, Forrest, and Roughton (1963). A Gibson-type stopped-flow apparatus was used to follow the absorption changes of the buffered solution containing *p*-nitrophenol as indicator. An optical path length of 20 mm was used in all experiments. The hydrolysis of *p*-NPA was followed spectrophotometrically as described by Pocker and Stone (1967). Acetaldehyde hydration followed the method of Pocker and Jeany (1965). Zinc analyses were performed with the aid of an atomic absorption photometer constructed by Optica.

<sup>1</sup> This paper was not presented in full at the symposium.

### Ultracentrifugation

A Spinco model E analytical ultracentrifuge equipped with standard Schlieren and Rayleigh interference optical systems was used. Sedimentation velocities were determined in 0.05 *M* phosphate buffer and 0.05 *M* mercaptoethanol pH 7.2 at various protein concentrations. Molecular weight was determined by the sedimentation technique of Yphantis (1964). The partial specific volume of the protein was calculated (0.736 ml/g) from the amino acid composition.

### Electrophoresis

Polyacrylamide gel electrophoresis was performed with the Shannon apparatus in standard 7 percent polyacrylamide gel pH 9.5 at 4.5 mA per tube according to the method of Davis (1964).

Starch gel electrophoresis was done according to the general procedure of Smithies (1959) and Waller and Harris (1961). Runs were carried out at 0 C in a *tris*-buffer, pH 9.3, 0.05 *M*. Electrophoresis took place at a potential of 180 volts for 20 hours.

Cellulose acetate strip electrophoresis was carried out according to the method of Kohn (1958). The cellulose acetate was material commercially available from Chemetron. Runs were carried out at 4 C in a phosphate-borate buffer  $I=0.075$ , pH 8.5.

### Amino Acid Analyses

The hydrolysis of each sample to be analyzed was carried out in 6 *N* HCl under nitrogen at 110±1 C for 24 and 72 hours. The total cystine plus cysteine content was determined as cysteic acid after performic acid oxidation (Hirs, 1956). The procedure of Hamilton (1963), Piez and Morris (1960), and Hirs (1956) was used for the analyses with a Technicon analyzer. Modifications were adopted to allow accelerated analyses. Separate analyses were performed on the S-carboxymethyl derivative of the protein, according to Crestfield, Moore, and Stein (1963), after hydrolysis of the sample.

### Determination of Tryptophan

The tryptophan content was measured by the spectrophotometric method of Schmid and

Benceze (1957) and Beaven and Holiday (1952), or by the colorimetric method of Spies and Chambers (1949). Determination after alkaline hydrolysis was also carried out on the analyzer.

### Protein Determination

Protein was routinely determined by the biuret reaction or by the method of Lowry, Rosebrough, Farr, and Randall (1951).

## RESULTS

### Purification of Carbonic Anhydrase

#### *Extraction of the Enzyme*

In the routine large-scale preparation, 10 kilograms of fresh market spinach leaves were washed, drained, and homogenized for 2 minutes in a Waring Blendor with about 2 liters of 5 *mM* mercaptoethanol solution and 5 *mM* phosphate buffer adjusted to pH 7.2. The homogenate was then squeezed through cotton cheesecloth and precipitated in a Lourdes centrifuge at 4000 *g* for 15 minutes. All operations including the blending were carried out at 0 to 2 C. All solutions used throughout the preparation contained 5 *mM* mercaptoethanol.

#### *Acid Precipitation and Ammonium Sulfate Fractionation*

The extract (usually from 3 to 4 liters) was adjusted to pH 5.2 with acetic acid of sufficient concentration, and ammonium sulfate (solid) was added to bring the solution to 20 percent saturation (at 0 C). The mixture was kept at this pH and saturation for 30 minutes and the precipitate removed by centrifugation and discarded. The remaining supernatant was readjusted to pH 7.0 with *N*-ammonium hydroxide and brought to 65 percent saturation with ammonium sulfate. The solution was centrifuged and the precipitate was dissolved with 5 *mM* phosphate buffer pH 7.4 to obtain an (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation of 35 percent. The mixture was centrifuged and the precipitate discarded.

#### *Ethanol Fractionation*

The supernatant from (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation was placed in a 0 C bath with vigorous mechanical stirring. Ethanol, 96 percent (enough

to raise the final ethanol concentration to 55 percent), precooled to about  $-30^{\circ}\text{C}$ , was added. After stirring for approximately 30 minutes, the solution was centrifuged at 30 000 g for 10 minutes. The precipitate containing about 10 percent of the total activity was discarded. The supernatant solution was then brought to 65 percent ethanol concentration according to the procedure outlined above. After 30 minutes of stirring, the solution was centrifuged at 30 000 g for 10 minutes. The precipitate was then dissolved in 200 ml of 5 mM phosphate buffer pH 7.2.

#### Chromatography on Sephadex G-75

The enzyme solution was placed on a 4.0- by 80-cm column of Sephadex G-75 equilibrated with 5 mM phosphate buffer. Elution was done with the same buffer and the fraction corresponding to the second void volume was collected (fig. 1). The gel filtration step has proven particularly useful for achieving homogeneous fractions. In fact, it removes most of the yellow pigment that cannot be eliminated by other standard procedures such as adsorption on calcium phosphate, or a second ammonium sulfate fractionation. The enzyme, after passing through the column, was precipitated at 70-percent saturation

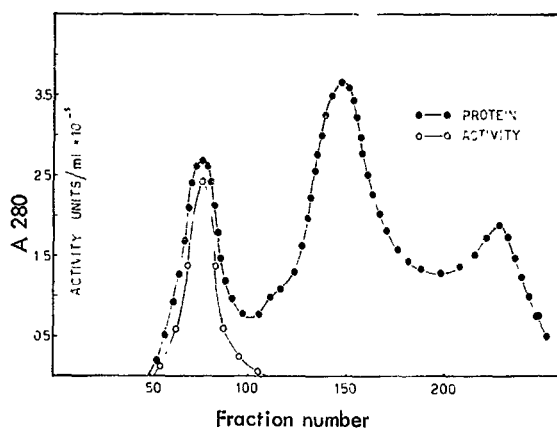


FIGURE 1.—Sephadex G-75 chromatography of spinach CA. A 4.0- by 80-cm column equilibrated with 5 mM phosphate buffer, pH 7.2, and 5 mM mercaptoethanol was used. The elution was carried out (60 ml/hr) with the same buffer. Fractions of 6.0 ml each were collected. The protein (full circles) and activity (open circles) were measured in each fraction.

with ammonium sulfate. The ammonium sulfate was removed by gel filtration on Sephadex G-25. A 3.0- by 25-cm column equilibrated with 0.3 M phosphate buffer, pH 7.2, was used.

#### Chromatography on DEAE-Cellulose

The enzyme solution was placed on a 2.6- by 20-cm column of DEAE-cellulose equilibrated with 30 mM phosphate buffer. Elution was started with a gradient of NaCl (0-0.15 M) in the same buffer (fig. 2). The enzyme fractions shown in the cross-hatched area (fig. 2) were pooled, omitting the earlier fractions in the peak that still contained a yellow protein. The pooled fractions were precipitated at 70-percent saturation with ammonium sulfate and desalted by filtering through a column of Sephadex G-25 fine. The results of the purification procedure are summarized in table I.

The purified enzyme remained stable for several months when stored at  $-20^{\circ}\text{C}$  in 30 mM phosphate buffer, pH 7.2, and 5 mM mercaptoethanol.

#### Physicochemical Properties of the Purified Enzyme

##### Criteria of Purity

The preparations appeared to be homogeneous on the basis of polyacrylamide, starch gel, and cellulose acetate electrophoresis. Sedimentation velocity runs in the analytical ultracentrifuge

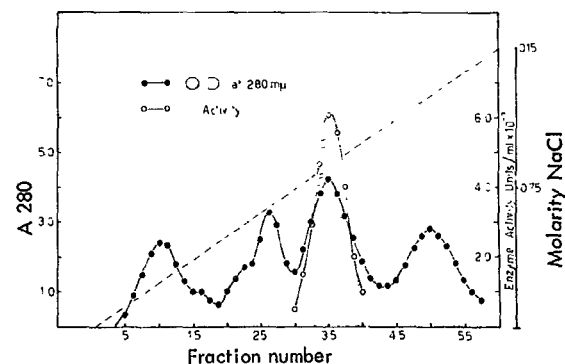


FIGURE 2.—DEAE cellulose chromatography of spinach CA. The enzyme was adsorbed with 0.3 M phosphate buffer, pH 7.2. Elution was started with a linear gradient of NaCl (0-0.15 M) when the unadsorbed proteins had come through. The flow rate was 30 ml/hr.

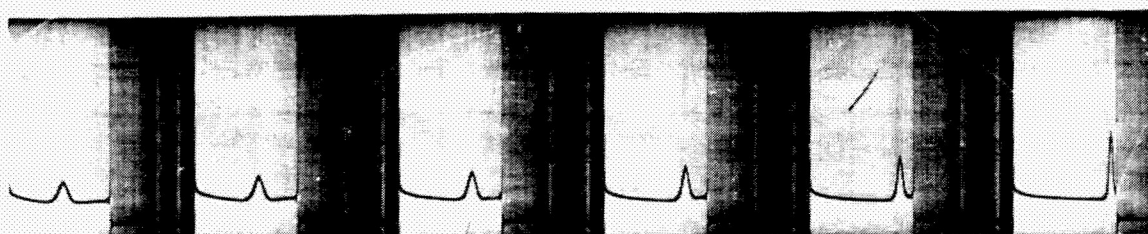


FIGURE 3.—Sedimentation velocity pattern of spinach CA. Runs were carried out in 0.05 M phosphate buffer and 0.05 M mercaptoethanol, pH 7.2, at a protein concentration of 5.6 mg/ml, speed 561 000 rev/min, and temperature 4 C. Photographs (left to right) were taken at 16-minute intervals.

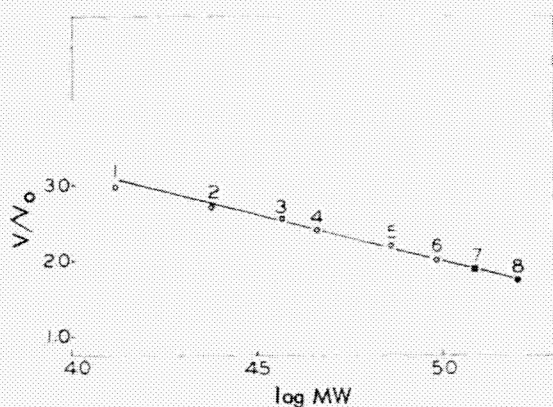


FIGURE 4.—Molecular-weight determination by gel filtration on Sephadex G-200. A 1.2- by 180-cm column was used; about 5.0 mg of each protein. The temperature was 4 C.

- |                             |                               |
|-----------------------------|-------------------------------|
| 1. CytC                     | 5. serum albumin              |
| 2. trypsin                  | 6. tropomyosin                |
| 3. human carbonic anhydrase | 7. spinach aldolase           |
| 4. ovalbumin                | 8. spinach carbonic anhydrase |

showed a single symmetrical peak with  $S_{20,w} = 6.8$  from which the molecular weight was calculated to be approximately 140 000 (fig. 3). Determination of the molecular weight by sedimentation equilibrium gave a value of 145 000. The gel filtration technique on Sephadex G-200 yielded a value of 148 000 (fig. 4).

As has been found for the animal enzyme (Rickli, Ghazanfar, Gibbons, and Edsall, 1964), a sharp transition in the behavior of the spinach enzyme occurs near pH 4.5. The enzyme was stable when protected with reducing agents from pH 5 to nearly 10, and no change in the sedimentation coefficient was observed. Below pH 4, the enzyme lost activity very rapidly and the sedimenting boundary became a diffuse, fast-

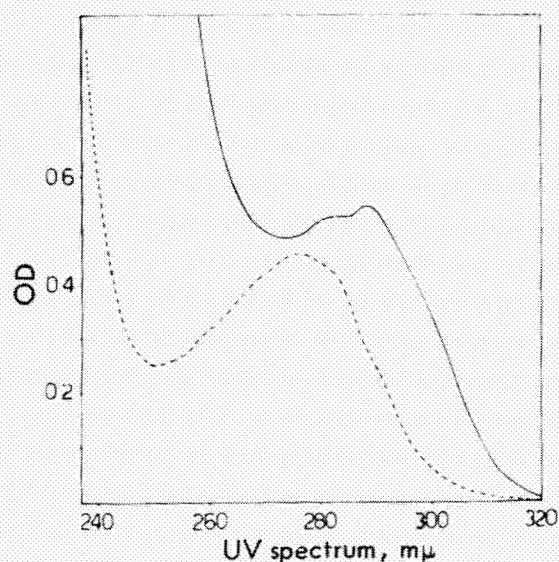


FIGURE 5.—Acid (dash) and alkaline (solid) absolute spectrum of spinach CA. The native enzyme was diluted with 0.1 N NaOH or with 0.1 N HCl to 3.0-ml final volume. Scanning of the spectra was carried out in a DB-G Beckman spectrophotometer.

moving peak. An almost identical figure was obtained upon exposure of the enzyme to 6 M urea or to 4 M guanidinium hydrochloride in the presence of 0.1 M mercaptoethanol. Under these conditions the enzyme immediately lost all activity. At pH near 4, the enzyme became insoluble and was irreversibly inactivated. At more acidic pH (around 2) and at extreme alkaline conditions, the enzyme could be brought again into solution.

The purified protein in solution was completely colorless. The ultraviolet spectrum showed two maximum peaks at 286 and 296 millimicrons. A

TABLE I.—Purification of Spinach Carbonic Anhydrase

| Fraction                                      | Total activity, units <sup>a</sup> | Specific activity, units/mg | Recovery, percent |
|---|------------------------------------|-----------------------------|-------------------|
| Extract.....                                  | 80 000                             | 1.2                         | 100               |
| pH 5.2 fraction.....                          | 80 000                             | 6.2                         | 100               |
| Ammonium sulfate: 35-65 percent fraction..... | 58 000                             | 12.4                        | 72                |
| Ethanol fraction: 55-70 percent fraction..... | 42 000                             | 76.5                        | 53                |
| Sephadex G-75.....                            | 40 000                             | 82.3                        | 50                |
| DEAE cellulose.....                           | 24 000                             | 160                         | 30                |

<sup>a</sup> Unit is defined as the amount of enzyme required to double the rate of the uncatalyzed reaction.

TABLE II.—Amino Acid Composition of Spinach Leaf Carbonic Anhydrase

| Amino acid         | Mean content, mole percentage |
|--------------------|-------------------------------|
| Aspartic acid..... | 8.85                          |
| Threonine.....     | .....                         |
| Serine.....        | <sup>a</sup> 11.85            |
| Glutamic acid..... | 9.50                          |
| Proline.....       | 4.70                          |
| Glycine.....       | 11.60                         |
| Alanine.....       | 8.25                          |
| Valine.....        | <sup>b</sup> 8.10             |
| Half cystine.....  | 4.0                           |
| Isoleucine.....    | 5.80                          |
| Leucine.....       | 5.90                          |
| Tyrosine.....      | 2.20                          |
| Phenylalanine..... | 4.20                          |
| Lysine.....        | 5.90                          |
| Histidine.....     | 2.90                          |
| Arginine.....      | 4.05                          |
| Tryptophan.....    | less than 1                   |

<sup>a</sup> Extrapolated value at zero hydrolysis time.

<sup>b</sup> Value at 72-hr hydrolysis time.

solution in 0.03 *M* phosphate buffer, pH 7.2, containing 1 mg/ml, gave an absorbance of 0.86 at 280 millimicrons. This result indicated that the protein had a low content of aromatic amino acids, a result that was confirmed by the composition as determined on the amino acid analyzer (table II). Figure 5 shows an absolute spectrum of the enzyme in alkaline and acid conditions. Compared to human erythrocyte CA (Armstrong, Myers, Verpoorte, and Edsall, 1966), the spinach enzyme shows a much higher content of glycine and phenylalanine and lower content of aspartic acid, threonine, serine, proline, and lysine residues.

#### Substrate Specificity

The enzyme was very active toward carbon dioxide and bicarbonate. The Michaelis constants for the two substrates were, respectively, 22.0 and 50 *mM* at Cl<sup>-</sup> concentration of 1.6 *mM*, at 19 C, in 20 *mM* imidazole buffer, pH 7.2. Increasing the chloride concentration decreased considerably the affinity of the enzyme for bicarbonate anion in a competitive manner.

The enzyme also catalyzes very strongly and reversibly the hydration of acetaldehyde. No clear kinetic data were obtained with this substrate because technical problems were encountered with a standard spectrophotometer at low temperature (our stopped-flow apparatus was not provided with a hydrogen lamp). The native enzyme did not possess any hydrolytic properties on any esters that we have been able to test.

#### Sulphydryl Group Determination

The importance of SH-groups in the spinach CA was pointed out by Kondo, Chiba, and Kawai (1952), who observed that cysteine prevented instability during the preparation of spinach CA fractions. Determination of SH groups was carried out spectrophotometrically according to Boyer (1954). The enzyme was filtered through a small column of Sephadex G-25 fine to remove mercaptoethanol and rapidly assayed with *p*-chloromercuribenzoate (PCMB) for SH groups. The total change in OD at 225 millimicrons due to the formation of enzyme PCMB complex corresponded to approximately 8 SH groups per molecule of enzyme. The value obtained with the spectrophotometric determination with



PCMB involves many uncertainties because of the rapid oxidation of the SH groups of the enzyme in solution. This uncertainty was increased by amino acid analyses from which a much higher value was obtained for the half-cystine residues. The complex formation between PCMB and the enzyme at pH 7.2 was rapid. The number of SH groups titratable and the time course of the reaction were not different in 8 *M* urea.

Rapid inactivation occurred when CA ( $10^{-6}$  *M*) was allowed to react at pH 7.2 in 0.05 *M* phosphate buffer at 25 C with iodoacetate, iodoacetamide or *N*-ethylmaleimide, each at a concentration of 5 mM. Under these conditions it was very difficult to establish a correct kinetic analysis of the reaction because the enzyme alone, without an SH reagent, lost activity at a rapid rate. Figure 6 shows the time course of the inactivation reaction and the complete recovery of the activity after addition of an excess of mercaptoethanol. The results in any case suggest the possibility that the SH groups may be associated with the active-site structures.

Among other substances, arsenite is a strong inhibitor of enzyme activity, giving a 50-percent inhibition at the concentration of 0.08 *M*. Sulfide, cyanide, and azide are poor inhibitors.

#### Zinc Analysis

Analysis for zinc was performed by the atomic absorption spectrometer in all fractions indicated in table III. When zinc was measured in the DEAE cellulose fraction, the amount of metal found was totally independent of the protein concentration. The figure for the experiment was constant at 0.0021  $\mu\text{g}/\text{ml}$ ; however, the proteins varied from 25 to 1 mg/ml.

#### Intracellular Distribution of CA Activity

Table IV shows the distribution of activity in the various fractions obtained by differential centrifugation from the total homogenate of spinach leaves. It can be seen that most of the activity occurs in the soluble part after centrifugation at 100 000 g. About 7 percent of the activity appears to be linked to the chloroplast fraction (prepared by the method of Arnon, Allen, and Whatley, 1956). Some 3 percent was sedimentable

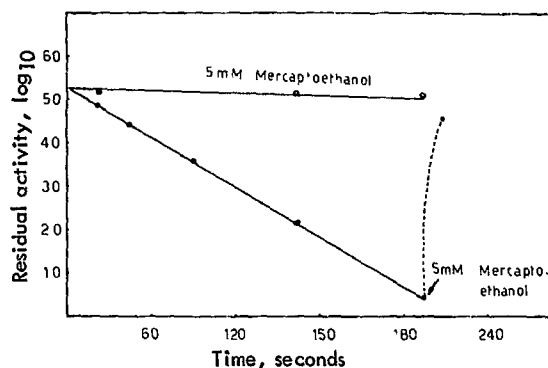


FIGURE 6.—The native enzyme passed through Sephadex G-25 was diluted in the syringe of the stopped-flow apparatus with 40 mM imidazole buffer and *p*-nitrophenol ( $10^{-5}$  *M*) at 25 C. After the interval indicated, it was mixed with a water solution equilibrated with 1 atm of CO<sub>2</sub>.

TABLE III.—Zinc Content of the Purification Fractions of Spinach Carbonic Anhydrase

| Fraction  | Zinc content, $\mu\text{g}/\text{mg}$ protein |
|---|---|
| Extract.....  | 0.7   |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> : 35-65 percent fraction..... | .002  |
| Ethanol: 55-70 percent fraction.....  | .0015   |
| Sephadex G-75.....  | .0016   |
| DEAE cellulose.....   | .0013   |

at 30 000 g. It has already been reported (Rossi, Chersi, and Pace, unpublished) that hypotonic treatment of chloroplasts, prepared according to the method of Arnon et al. (1956), releases activity from these particles. Recent experiments have shown that more careful preparations of chloroplasts contained up to 20 percent of the total activity. These experiments suggest that the enzyme was located in the double-membrane compartment and that it was easily released during the initial homogenization. It would be of interest to know more about this subject in view of the importance of the carbon dioxide transport through the chloroplastic membranes and its relation to the first step of photosynthesis.

TABLE IV.—*Intracellular Distribution of Carbonic Anhydrase in Spinach Leaves*

[Spinach chloroplasts were prepared according to the method of Arnon et al. (1956). Differential centrifugations were carried out on tissue homogenate from a small preparation of spinach leaves (50 g)]

| Fraction                 | Centrifugal force | Carbonic anhydrase activity |         |
|--------------------------|-------------------|-----------------------------|---------|
|                          |                   | Total units                 | Percent |
| Homogenate.....          |                   | 2000                        | 100     |
| Cell debris, nuclei..... | 300               |                             |         |
| Chloroplasts.....        | 1 000             | 150                         | 7.5     |
| Supernatant I.....       | 30 000            | 60                          | 3.0     |
| Supernatant II.....      | 100 000           | 1650                        | 83.0    |

### CONCLUSION

Although carbonic anhydrase activity has already been described in leaves of higher plants, incomplete data have been reported about the properties of the isolated protein. We attempted to isolate a homogeneous preparation of the enzyme. Spinach CA appears to possess properties different from those of the vertebrate enzyme. Considerable differences in the kinetic data were obtained between the spinach and the bovine enzyme.

The plant enzyme appears to possess less catalytic versatility than the erythrocyte carbonic anhydrase. No activity was found for the hydrolysis of *p*-nitrophenylacetate.

The molecular weight appears to be about five times that of the vertebrate enzyme. The amino acid composition shows some differences, particularly with respect to the tryptophan residues. The plant enzyme does not contain any metals as far as our studies could detect, and SH groups appear to be an essential part of the active site.

Work is currently in progress at this laboratory in an effort to define N- and C-terminal residues and other chemical properties, which may provide information concerning the mechanism of the reactions catalyzed by the plant enzyme.

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## Purification of Carbonic Anhydrase From Parsley Leaves<sup>1</sup>

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Carbonic anhydrase in plants was first observed by Neish in 1939. The characterization of partially purified enzymes from several plants has been reported by Waygood and Clendenning (1951); by Kondo, Yonezawa, and Chiba (1952); and by Fellner (1963). Fellner found that parsley carbonic anhydrase does not contain zinc and is not inhibited by sulfonamides, which indicates that the parsley enzyme must operate by a catalytic mechanism different from that proposed for the mammalian carbonic anhydrases. It was therefore of interest to investigate in more detail the structure of parsley carbonic anhydrase and its relationship to enzymatic function. The isolation procedure herein described is now being used for more detailed studies of enzyme kinetics and subunit structure.

Carbonic anhydrase was prepared from a blend of parsley leaves in 0.05 *M* sodium phosphate buffer, pH 7.0. This buffer also contained 0.001 *M* EDTA and 0.01 *M* 2-mercaptoethanol, as did all other buffers used in the preparation. The addition of 300 grams of ammonium sulfate per liter of filtered extract caused precipitation of the carbonic anhydrase activity. The precipitate was dialyzed against 0.1 *M* *N*-ethylmorpholine acetate, pH 7.0, and chromatographed on DEAE cellulose. The carbonic anhydrase activity was eluted after a step to 0.2 *M* *N*-ethylmorpholine acetate, pH 7.0. The active fractions were pooled, concentrated, and subjected to gel filtration on 8 percent agarose. Enzyme activity appeared in a single peak, which corresponded to a molecular weight of approximately 170 000. At this stage of purification the enzyme was 90 to 95 percent pure as judged from analytical disk gel electrophoresis.

It had a specific activity of 13 000 Wilbur-Anderson units, compared with the values of 16 000 and 80 000 for human enzymes B and C reported by Armstrong, Myers, Verpoorte, and Edsall (1966). Kinetic studies of the enzyme from this stage gave a Michaelis constant for CO<sub>2</sub> of 17 mM at pH 7.65. The enzyme showed no esterase activity toward *o*- or *p*-nitrophenyl acetate at pH 6 to 8, but it did catalyze the hydration of acetaldehyde. Acetazolamide and sodium azide were found to inhibit the enzyme under the conditions of the Wilbur-Anderson assay. For 50 percent inhibition 3 × 10<sup>-5</sup> *M* acetazolamide and 5 × 10<sup>-6</sup> *M* sodium azide were required.

Subsequent preparative disk gel electrophoresis at pH 8.7 yielded a less active enzyme, as shown in table I. The pooled enzyme from this step behaved as a single component in sedimentation velocity experiments and in analytic disk gel electrophoresis at pH 8.7 and pH 7.4. Sedimentation equilibrium experiments gave a molecular weight of approximately 160 000. Amino acid analysis showed lower mole fractions of histidine, arginine, aspartic acid, serine, phenylalanine, and tryptophan, and higher fractions of proline, glycine, alanine, and valine than in the human enzymes B and C.

The zinc content of individual and pooled fractions of column effluents was measured directly by atomic absorption spectrophotometry. At all stages of the purification, zinc content coincided with carbonic anhydrase activity. In the agarose chromatography step, the absorbance at 280 nanometers, enzymatic activity, and zinc content were found to have a constant ratio, within experimental error, through the carbonic anhydrase peak. The zinc content at that stage was 1 g atom zinc per 60 000 g protein. The pooled car-

<sup>1</sup> This paper was not presented in full at the symposium.

TABLE I.—*Protein Concentration and Specific Activity of Preparative Fractions*

| Preparative fraction                  | Protein, mg/ml | Specific activity, units/mg×10 |
|---------------------------------------|----------------|--------------------------------|
| Filtered blend of parsley leaves..... | 19.8           | 90                             |
| Ammonium sulfate fractionation.....   | 95.8           | 410                            |
| DFAE cellulose chromatography.....    | 73.8           | 5 350                          |
| Agarose chromatography.....           | .91            | 13 000                         |
| Preparative electrophoresis.....      | .29            | 5 340                          |

bonic anhydrase from the preparative disk gel electrophoresis contained 1 g atom zinc per 51 000 g protein. Because the concentrated fractions at each stage were dialyzed against buffers containing 0.001 M EDTA, zinc must be bound to the protein. It is not yet known whether the protein-associated zinc is necessary for enzymatic activity.

These results conflict with those of Fellner (1963) and of Konôo et al. (1952), who found no zinc by the dithizone method. The low yields of total carbonic anhydrase activity in this procedure do not rule out a second, zinc-free carbonic anhydrase in parsley, but this is considered unlikely in view of the evidence of only one electrophoretic component in the crude extract with carbonic anhydrase activity, as viewed by direct assay of analytical polyacrylamide gels at pH 7.4. The zinc content reported here is also higher than the values of 0.05 and 0.056 percent reported for spinach carbonic anhydrase by Waygood and Clendenning (1951) and by Wood and Sibly (1952). Because both of those analyses were done with incompletely purified enzymes, their results do not conflict with the present findings.

#### EDITOR'S NOTE, ADDED IN PROOF

Dr. Tobin's work is presented in detail in his Ph.D. thesis "Parsley Carbonic Anhydrase: Purifi-

cation and Properties" (Harvard University, 1969). Further purification of the parsley carbonic anhydrase gave a more active enzyme, containing 1 g atom zinc per 29 000 g protein. The maximum catalytic constant is approximately 10<sup>5</sup> per second at pH 7 and 25 C. Kinetic studies in a stopped flow apparatus, however, did not reveal significant inhibition by sulfonamides.

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# Carbonic Anhydrase: Conformation of the Active Center and the Mechanism of Action<sup>1</sup>

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7 N70-23306

Carbonic anhydrase combines with an optically inactive azosulfonamide to form a highly colored enzyme-inhibitor complex. Bathochromic-hypochromic shifts of the visible absorption bands ( $\epsilon_{500 \text{ m}\mu\text{molar}} \sim 25,000$ ) indicate the presence of a hydrophobic binding site for the sulfonamide. Binding is metal ion dependent and the protein environment induces large ellipticity in the previously optically inactive bands. Circular dichroism spectra of azosulfonamide complexes of five isozyme and species variants of carbonic anhydrase reveal that both sign and magnitude of the multiple large visible ellipticity bands ( $[\theta] = \pm 0.5 - 9.0 \times 10^3 \text{ deg cm}^2/\text{mole}$ ,  $R_s = \pm 0.4 - 19.7 \times 10^{-29} \text{ cgs units}$ ) are unique for each isozyme. Cyanide can displace the azosulfonamide from its binding site. Difference titration data show that below the  $pK_a$  values of  $\text{H}_2\text{S}$  and  $\text{HCN}$ , binding of these inhibitors to  $\text{Zn(II)}$  and  $\text{Co(II)}$  carbonic anhydrase is accompanied by the release of  $\text{H}^+$  ions. Over the pH range in which the inhibitors are in the form  $\text{CN}^-$  and  $\text{HS}^-$ , binding is accompanied by the appearance of  $^- \text{OH}$  ions. The data fit the theoretical curves constructed by assuming a single additional  $\text{H}^+$  dissociation associated with the protein, coupled with the metal ion, and described by a  $pK_a$  of 8.1. The latter coincides with the inflection point of the pH-rate profile for catalysis by human carbonic anhydrase B. A mechanism based on a mixed enzyme-zinc-hydroxide as the active form of the enzyme is proposed for the hydration of  $\text{CO}_2$  and the hydrolysis of *p*-nitrophenyl acetate. The multiple visible absorption bands introduced into human carbonic anhydrase B by replacement of  $\text{Zn(II)}$  with  $\text{Co(II)}$  are optically inactive. Sulfonamides, metal-binding anions, bicarbonate and acetate destroy this symmetry element in the human enzyme B complex and induce large, visible ellipticity bands. In contrast, the visible absorption bands of  $\text{Co(II)}$  bovine carbonic anhydrase B are all optically active. These are additionally altered by the sulfonamide and anion inhibitors. Comparative studies of the  $\text{Co(II)}$  human isozyme C show its coordination complex to be more closely related to that of the bovine enzyme. All the bands of the cobalt chromophore are initially optically active, and the magnitude and sign of the ellipticity bands are almost identical to those of the  $\text{Co(II)}$  bovine enzyme. These differences may relate to the positive charge of the C-terminal lysine present in both the bovine and human C enzyme and absent in the human B isozyme.

The isolation and characterization of carbonic anhydrases (CA) from the erythrocytes of several animal species has revealed significant differences in amino acid compositions and consequently in amino acid sequences of these proteins (Duff and Coleman, 1966; Laurent, Charrel, Luccioni, Autran, and Derrien, 1965; Lindskog, 1960; Nyman, 1961; Rickli, Ghazanfar, Gibbons, and Edsall,

1964). Such studies, however, have also emphasized the similarity in general physicochemical properties of this series of zinc metalloenzymes. Perhaps the most striking finding has been the demonstration of the simultaneous occurrence in primate red cells of two isozymes, B and C, which have significant differences in amino acid composition, especially the basic amino acids (Duff and Coleman, 1966; Rickli et al., 1964). While catalytic specificity appears to be similar for the series of carbonic anhydrases isolated thus far, there are

<sup>1</sup>This investigation was supported by grant AM-09070-04 from the National Institutes of Health, U.S. Public Health Service.

large differences in catalytic rate (Coleman, 1967b; Duff and Coleman, 1966; Rickli et al., 1964). The comparison of the gross physicochemical features of these isozyme and species variants does not reveal particularly striking functional or structural information about carbonic anhydrase. On the other hand, if techniques could be applied which reflected structural variations in small areas of the molecule, especially near the active center, comparisons between isozymes might reveal significant structural features influencing catalytic activity. Two spectroscopic methods and one titrimetric method, developed in our laboratory, have proved to be effective probes of the specific structure at the active center of carbonic anhydrase:

(1) The measurement of the changes in the energy levels, oscillator strengths, and optical activity of the  $d-d$  transitions of Co(II) when substituted for the native Zn(II) ion at the active center has served as an effective detector for structural changes in the vicinity of the metal ion.

(2) The use of an azosulfonamide inhibitor specifically bound at the active center has provided a set of intense visible transitions with energies and oscillator strengths that are highly sensitive to the local environment. Optical activity induced in this symmetrical chromophore by carbonic anhydrase provides an additional sensitive assay of structure at the active center.

(3) Precise measurement of the alterations in proton equilibria accompanying the binding of anions to the metal ion of carbonic anhydrase has revealed the probable function of a coordinated water molecule in the mechanism of catalysis.

Application of the three methods to a series of five isozymes has provided some significant new information arising from the different results observed with the several isozymes.

## MATERIALS AND METHODS

**Reagents.**—All chemicals were reagent grade. Buffer solutions, HCl, NaOH, substrates, and indicators were prepared metal free, as previously described (Coleman, 1967b). Spectrographically pure CoCl<sub>2</sub> (Johnson Mattney Co., Ltd.) was used in preparing the Co(II) enzyme.

**Sulfonamides.**—2-(4-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate

was purchased from Winthrop Laboratories. Ethoxzolamide (6-ethoxybenzothiazide-2-sulfonamide) and acetazolamide (2-acetamido-1,3,4-thiadiazole-5-sulfonamide, "Diamox") were supplied by Dr. Thomas H. Maren of the University of Florida and by Dr. Selby Davis of the Lederle Laboratories, respectively.

**Enzymes.**—Human carbonic anhydrases B and C were prepared as previously reported (Rickli et al., 1964). *Macaca mutata* carbonic anhydrases B and C were isolated in crystalline form as described in Duff and Coleman (1966). Bovine carbonic anhydrase B (BCAB) was prepared from whole bovine blood by the methods described for the preparation of human carbonic anhydrase using DEAE-Sephadex chromatography (Rickli et al., 1964). Cobalt(II) carbonic anhydrase was prepared by making the apoenzyme as previously described (Coleman, 1965) followed by dialysis against a buffered solution prepared from CoCl<sub>2</sub>. Protein concentrations were determined from measurements of the optical densities at 280 millimicrons using molar absorptivities of  $4.90 \times 10^4 M^{-1} \text{ cm}^{-1}$  for monkey B and  $5.35 \times 10^4 M^{-1} \text{ cm}^{-1}$  for human C (Rickli et al., 1964);  $4.88 \times 10^4 M^{-1} \text{ cm}^{-1}$  for monkey B and  $5.35 \times 10^4 M^{-1} \text{ cm}^{-1}$  for monkey C (Duff and Coleman, 1966); and  $5.70 \times 10^4 M^{-1} \text{ cm}^{-1}$  for bovine B (Lindskog and Nyman, 1964).

**Enzymatic activity.**—Esterase activities were determined using *p*-nitrophenyl acetate as substrate and following the absorbance change at 400 or 348 millimicrons (Coleman, 1965b). The reaction cuvette contained  $1 \times 10^{-3} M$  substrate in 0.025 *M* tris, 5 percent acetonitrile, 23 C. The pH was varied as shown in the text.

Circular dichroism (CD) measurements were made with a Durrum-Jasco ORD/UV-5 spectropolarimeter equipped with the CD attachment. Maximum deflection was  $\pm 0.002$  with a maximum deviation between runs of  $\pm 0.00005$  above 230 millimicrons and  $\pm 0.0001$  at 215 millimicrons. Calibration of the instrument was performed with an aqueous solution of *d*-10-camphorsulfonic acid (J. T. Baker Co.) with an  $(\epsilon_L - \epsilon_R)$  of 2.20 at 290 millimicrons. The slit width above 300 millimicrons was 0.3 mm or less. Path lengths varied from 0.2 to 1.0 cm. In the case of the highly colored azosulfonamide, baselines were run with the sulfonamide alone. Ellipticity is expressed as

molecular ellipticity,  $[\theta] = 2.303(4500/\pi)(\epsilon_L - \epsilon_R)$  with units of  $\text{deg cm}^2/\text{decimole}$ . Protein concentrations were expressed as moles per liter and molecular ellipticity  $[\theta]$  has been expressed per mole of protein rather than per mole of amino acid residue in view of the fact that the ellipticity bands of the complexes arise largely from the incorporation of 1 mole of azosulfonamide or 1 mole of Co(II). The values can be converted to approximate mean residue ellipticities by dividing by 260 for all isozymes. Solutions for CD measurements were contained in 0.025 M tris, pH 7.5, 25 C with the exception of solutions of the Co(II) enzyme that were at pH 8.5 or 9.0.

Absorption spectra were obtained with a Cary Model 15 recording spectrophotometer. All absorption spectra were recorded using the solutions employed for the corresponding CD measurements and represent the optical density of these solutions over the same path length used for the latter measurements.

## RESULTS AND DISCUSSION

### Far-Ultraviolet CD of Three Carbonic Anhydrase Isozymes

The far-ultraviolet CD would appear to be a reasonably sensitive criterion of similarities or differences in protein structure in solution. The ultraviolet CD spectra of three representative carbonic anhydrase isozymes—human B, monkey C, and bovine B—are shown in figure 1. All three spectra share certain features in common including the three positive ellipticity bands above 230 millimicrons and the large asymmetric negative band centered between 210 and 220 millimicrons. There are, however, significant differences in these spectra. What these differences mean in terms of structural changes in certain regions of the molecule cannot be deduced from the spectra which have contributions from a large number of individual chromophores. Even a minor variation in structure at the active center could have major functional consequences.

### Absorption and CD Spectra of Carbonic Anhydrase-Azosulfonamide Complexes

A set of electronic transitions that reflect much more localized features of carbonic anhydrase

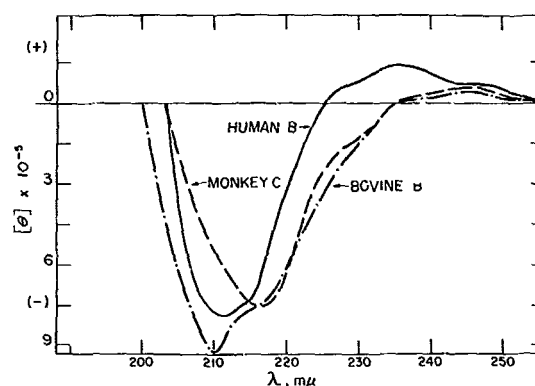
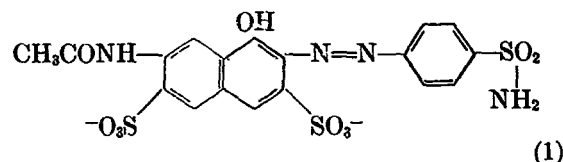


FIGURE 1.—Ultraviolet CD spectra of three carbonic anhydrase isozymes. Human isozyme B (—); monkey isozyme C (---); bovine isozyme B (- · -). Conditions: 0.025 M tris, pH 7.0, 23 C. Protein concentrations varied from  $1 \times 10^{-5}$  M to  $1 \times 10^{-4}$  M. Path lengths varied from 0.1 to 1 cm.

structure can be introduced into the enzyme by using 2-(4-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate (I) to form an enzyme-inhibitor complex:



The complex of this sulfonamide with carbonic anhydrase has a dissociation constant of  $< 10^{-6}$  M (Coleman, 1968b). Binding requires the presence of the metal ion at the active site and is limited to Zn(II) and Co(II) carbonic anhydrases, the only enzymatically active derivatives formed with the first transition and IIB series of divalent metal ions (Coleman, 1967a; Coleman, 1967c; Lindskog and Nyman, 1964). Thus a very firm 1:1 complex can be formed between this colored sulfonamide and carbonic anhydrase at protein concentrations as low as  $10^{-5}$  M (fig. 2(a)). All evidence indicates that the sulfonamide is placed at the active center in contact with the metal ion (Coleman, 1967a; Fridborg, Kannan, Liljas, Lundin, Strandberg, Strandberg, Tilander, and Wren, 1967; Lindskog and Nyman, 1964).

The first noticeable effect of complex formation is a major hypochromic-bathochromic shift in the visible transition ( $\epsilon_{500 \text{ m}\mu}$  initially  $\sim 25,000$ ) of



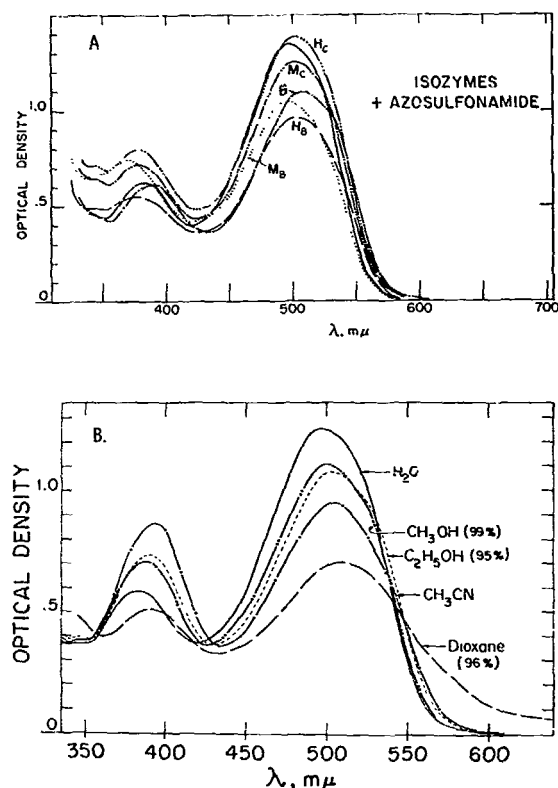


FIGURE 2.—Absorption spectra of carbonic anhydrase-azosulfonamide complexes. Solvent effects on the absorption spectrum of the azosulfonamide. (a) Visible absorption spectra of the 1:1 complexes of compound I with isozymes of carbonic anhydrase: free compound I (—); human isozyme B (— · —); monkey isozyme B (· · · · ·); human isozyme C (— · · · —); monkey isozyme C (— · · —); bovine isozyme B (— · —). All enzyme solutions contained  $5 \times 10^{-6}$  M enzyme,  $5 \times 10^{-5}$  M azosulfonamide, and 0.025 M tris, pH 7.5, 23 C. (b) Compound I,  $5 \times 10^{-5}$  M, was dissolved in H<sub>2</sub>O (0.025 M tris, pH 7.5, 23 C (—); methanol, 99 percent (— · · —); ethanol, 95 percent (— · —); acetonitrile, 98 percent (— · —); and dioxane, 96 percent (— · —).

the azosulfonamide (fig. 2(a)). A major color change from orange to red can be observed on the addition of compound I to the human isozyme B. The spectral shift is specific for each isozyme and thus implies the existence of a slightly different environment at the active center of each isozyme. The transitions of the phenylazonaphthols are very sensitive to the polarity of the immediate environment. This fact is illustrated by the spectra taken in solvents of increasing nonpolar character (fig. 2(b)). Nonpolar solvents induce hypochromic-

bathochromic shifts in the major absorption bands similar to those induced by carbonic anhydrase. By varying the percentage of dioxane in a dioxane-water mixture, the spectrum can be made to correspond closely to those observed for each isozyme complex. It would appear that the crevice in which the sulfonamide is bound (indicated by the X-ray diffraction studies (Fridborg et al., 1967)) must provide a rather nonpolar environment for the sulfonamide.

The most striking feature of the spectral studies of these azosulfonamide complexes is the very large optical activity induced in this initially symmetrical chromophore by the dissymmetric protein environment. The optically active visible transitions of the azosulfonamide complex with the bovine enzyme B are illustrated in figure 3(a). The resolution of this CD spectrum into a series of individual overlapping Gaussian ellipticity bands by means of a Du Pont 310 curve fitter (Coleman, 1968b; Myer, 1968) is shown in figure 3(b). The uniqueness of this fitting and the proof of its validity by using these ellipticity bands to generate the experimentally observed optical rotatory dispersion of the complex are discussed in detail elsewhere.

Calculations of rotatory strengths  $R_k$  (Moscowitz, 1960) of the individual bands from the data in figure 3(b) show that the rotatory strength of several of these bands approaches  $10^{-33}$  egs units. Rotatory strength values of this magnitude are more characteristic of intrinsically dissymmetric chromophores than of symmetrical chromophores perturbed by a dissymmetric environment (Moscowitz, 1967). Thus the protein environment, reflecting multiple asymmetric centers, appears to be a particularly effective inducer of optical activity.

Of more pertinence to the present discussion are the marked differences between isozymes in the conformation or the constellation of perturbing groups at the active center revealed by the induced optical activity of the bound sulfonamide. This is shown by a comparison of the CD spectrum of the bovine B isozyme complex with that of the human B isozyme complex (fig. 4). Not only are the induced ellipticity bands of different magnitude, but there are changes in sign as well. Thus the helical pathway for one of the azosulfonamide electronic transitions (band 3) has

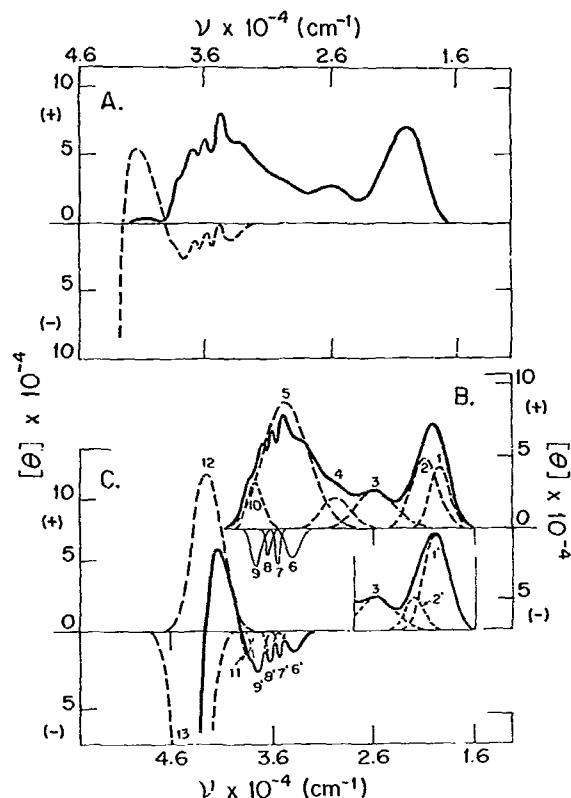


FIGURE 3.—CD spectra of bovine carbonic anhydrase B and its 1:1 complex with compound I. Resolution into a set of overlapping gaussian ellipticity bands. (a) (—) observed CD spectrum of the complex; (---) observed CD spectrum of the native enzyme. (b) (Complex) (—) resultant envelope for the 10 gaussian bands labeled from 1-10; (---) bands attributable to the sulfonamide; (—) bands attributable to the protein. The subset curve, bands 1' and 2', show an alternate fitting of the lowest energy region. (c) Native enzyme. (—) resultant envelope for the seven gaussian bands labeled on the figure. In parts B and C, the envelopes correspond within the limit of error to the experimental CD curves. Computer generation of the optical rotatory dispersion from the gaussian ellipticity bands shows the subset bands 1' and 2' to be the correct fitting of the CD spectrum. Reprinted from Coleman (1968b).

reversed its screw sense. Similar radical differences in the CD bands of the monkey carbonic anhydrase-azosulfonamide complexes are also observed (Coleman, 1968b). Thus this specific active center probe indicates that there are significant differences in conformation of the various isozyme and species variants involving the region of the active center. These differences presumably reflect

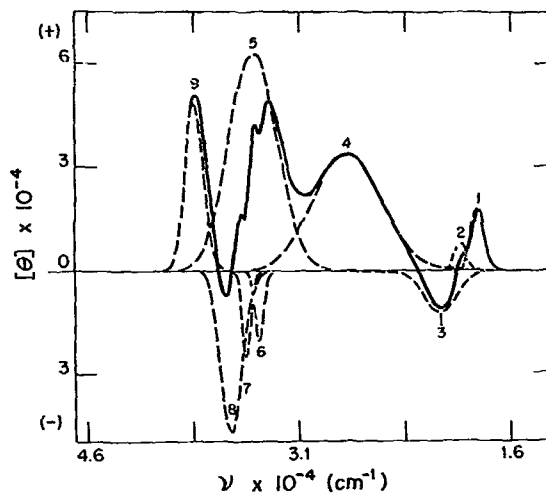


FIGURE 4.—Resolution of the CD spectrum of the 1:1 complex of Zn(II) human carbonic anhydrase B with compound I into a set of overlapping gaussian ellipticity bands. (—), resultant envelopes for the nine gaussian bands indicated by the dashed curves and labeled 1 to 9. The envelope corresponds within the limit of error of the measurement to the experimental CD spectrum of the complex. Reprinted from Coleman (1968b).

specific amino acid substitutions in the primary chain.

Much evidence including the major spectral shifts of the  $d-d$  transitions of Co(II) carbonic anhydrase induced by metal binding anions indicate that anions like cyanide, sulfide, cyanate, and azide combine with carbonic anhydrase by binding to the metal ion (Coleman, 1968a; Lindskog, 1966; Riepe and Wang, 1968). Infrared studies of the azide complex by Riepe and Wang (1968) provide strong support for this conclusion. Titration evidence indicates a single high affinity anion binding site per molecule (Lindskog, 1966). Compound I shares at least part of its binding site with the anions, because cyanide can apparently displace the bound azosulfonamide (fig. 5). Increasing concentrations of cyanide progressively abolish the induced optical activity of the azosulfonamide. The simplest explanation is that binding of the sulfonamide is being prevented by the anion. This mechanism has been demonstrated directly with  $^3\text{H}$ -acetazolamide showing that cyanide competes with the labeled inhibitor (Coleman, 1967a).

**Hydrogen Ion Equilibria Accompanying  
Reaction of Cyanide and Sulfide with  
Zn(II)-, Co(II)-, and Apo-Carbonic  
Anhydrase**

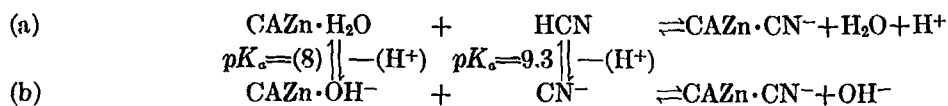
If the anions like CN<sup>-</sup> and HS<sup>-</sup> are inhibiting carbonic anhydrase by coordinating the metal ion, the reaction should be accompanied by the displacement of a proton from the inhibitors at pH values below the pH region for the dissociation of HCN and H<sub>2</sub>S; the former described by a *pK* of 9.3, the latter by a *pK<sub>a</sub>* of 6.9 at about 23 C (Sillén and Martell, 1964). The net hydrogen-ion release will depend on other hydrogen-ion equilibria altered by the binding of anions. To investigate the hydrogen-ion equilibria that accompany the reaction of carbonic anhydrase with cyanide and sulfide, a set of equilibrium measurements between pH 6 and 10 was made by the direct measurement of H<sup>+</sup> or -OH release with a difference titration method as previously described in detail (Coleman, 1967b). The method consists of adjusting both the concentrated anion solution and the protein solution to an identical pH, adding a small equimolar aliquot of the anion to the protein, and measuring the uptake or release of protons, on a pH stat at a precision of 1.0±0.02 μmole H<sup>+</sup>. The experiment can be done over a pH range from 6 to 10, because cyanide forms a firm 1:1 complex with carbonic anhydrase over this pH range at the concentration of proteins used (>10<sup>-4</sup> M) (Coleman, 1967b).

The results are best described by formulating one of the possible models that may apply to the anion-carbonic anhydrase reaction and comparing the predicted results with the observed findings. If a coordinated water molecule is displaced from the metal ion by the anions, the various proton equilibria may be pictured as in scheme I.

At low pH where the metal ion is in the hydrated form and the inhibitor is in the acid form, the reaction is described by the release of a water molecule and a proton (a). At very high pH, the inhibitor will be in the anion form, and if the

coordinated water molecule has a *pK<sub>a</sub>* within the experimental pH range, the metal ion will be in the hydroxide form (b). The reaction (b) at high pH will then be characterized by the release of a hydroxide ion. As seen in figure 6, the reaction of cyanide with the Zn(II) enzyme does show a biphasic titration curve; 1 mole H<sup>+</sup> is released at pH 6.0 while 0.7 mole OH<sup>-</sup> (H<sup>+</sup> uptake) is released at pH 10.0. These alterations in proton equilibria are a function of the metal ion, because the apoenzyme solution does not show any change in pH upon the addition of cyanide. The Co(II) enzyme shows a titration curve similar to that for the Zn(II) enzyme (fig. 6). The results can be explained if a single additional proton equilibrium associated with the protein, coupled to the metal ion, and described by a *pK<sub>a</sub>* of approximately 8, is added to the titration curve (dashed line, fig. 6). The results are compatible with scheme I if the coordinated water molecule is assumed to have a *pK<sub>a</sub>* of 8. As the pH rises, more enzyme is in the -OH form. The release of this -OH by cyanide progressively neutralizes the proton from HCN along the theoretical curve shown in the figure. Finally, the CN<sup>-</sup> species will displace only the -OH. An additional feature of the system predicted from scheme I is the dependence of the difference titration on the *pK<sub>a</sub>* of the inhibitor. This can be tested by using sulfide, since H<sub>2</sub>S has a *pK<sub>a</sub>* of 6.9. As expected, the biphasic titration curve shifts about 1.5 units toward acid pH (fig. 6). Once again a theoretical curve can be generated by the same additional ionization with a *pK<sub>a</sub>* of 8.

These results are compatible with the presence of a coordinated water molecule at the active center of carbonic anhydrase according to scheme I; they do not prove the identity of this ionization. An alternative is possible by assuming that the anion displaces a metal-bound protein ligand with a very high *pK<sub>a</sub>* that subsequently takes up a proton. This group cannot be initially bound to the metal ion, but must bind along a pH function



Scheme I

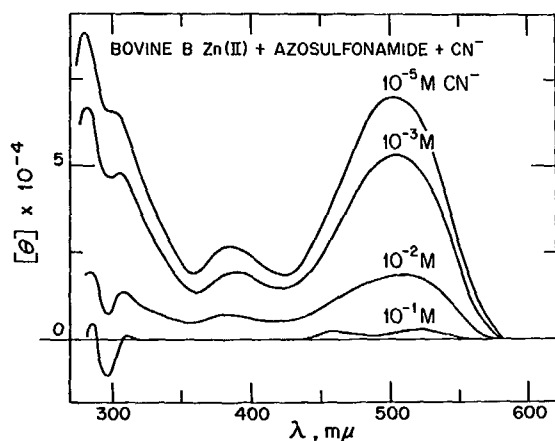


FIGURE 5.—Circular dichroic spectra of the bovine carbonic anhydrase B-azosulfonamide complex plus varying concentrations of cyanide. Conditions are as in figure 2.

with a midpoint of 8. The evidence is less convincing for this alternative (Coleman, 1967b).

The ionization described by the  $pK_a$  of 8 does occur in the region of the inflection point describing the esterase pH-rate profile for the enzyme. The esterase activity of human carbonic anhydrase B as a function of pH is plotted in figure 6 and follows the ionization revealed by the difference titration.

#### Suggested Mechanism of Action

If the ionization of a coordinated water molecule is the interpretation placed on the dissociation curve derived from the complexometric titration (fig. 6), then the form of the enzyme active in the hydration and hydrolysis reactions is a mixed enzyme-zinc hydroxide complex. This suggests a mechanism for the hydration reaction which is compatible with a large amount of other information presently available on carbonic anhydrase. The coordinated  $-OH$  may be visualized as attacking the  $CO_2$  carbon (scheme II) and giving intermediate (A). There may, of course, be additional interactions contributing to the binding of  $CO_2$ . Displacement of the intermediate would then liberate bicarbonate, regenerating the metal hydroxide at high pH, favoring hydration and the hydrated species at low pH at which the dehydration reaction is known to proceed best (Kernohan, 1965). An analogous mechanism can be formulated for the hydrolysis of *p*-nitrophenyl

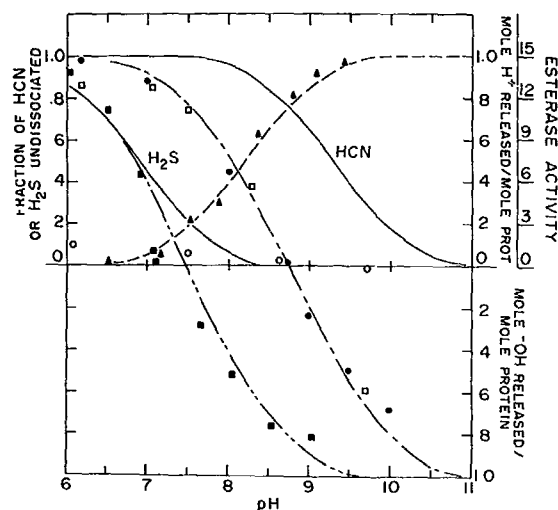
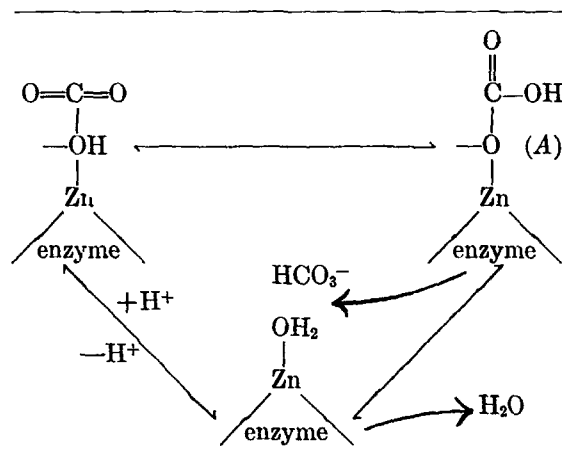


FIGURE 6.—Displacement of  $H^+$  and  $-OH$  from human carbonic anhydrase B by cyanide and sulfide. The solid lines represent the continuous titration curves for the dissociation of  $HCN$  to  $H^+$  and  $CN^-$  and  $H_2S$  to  $H^+$  and  $-SH$ , expressed as the fraction of inhibitor undissociated (left-hand ordinate). (— — —), theoretical titration curve for an acidic group of  $pK_a = 8.1$ , expressed as moles  $H^+$  dissociated/mole protein (upper right-hand ordinate).  $\blacktriangle$ , esterase activity of human carbonic anhydrase B,  $\mu$  moles substrate hydrolyzed/min/ $\mu$  mole enzyme. (— · — · —) and (— · — · —), theoretical difference titration curves expected for the displacement of  $H^+$  (upper right-hand ordinate) and  $-OH$  (lower right-hand ordinate) from  $Zn(II)$  carbonic anhydrase by cyanide and sulfide, respectively, as a function of pH according to scheme I. Moles of  $H^+$  or  $-OH$  released/mole protein on the addition of cyanide to  $Zn(II)$  carbonic anhydrase ( $\bullet$ ), apocarbonic anhydrase ( $\circ$ ), and  $Co(II)$  carbonic anhydrase ( $\square$ ). Maximum  $H^+$  and  $-OH$  release from the  $Co(II)$  enzyme was not reached until the addition of about 1.5 equivalents of cyanide.  $\blacksquare$ , Moles of  $H^+$  or  $-OH$  released/mole protein on the addition of sulfide to  $Zn(II)$  carbonic anhydrase.

acetate substituting an acetate intermediate. Both  $HCO_3^-$  and acetate can occupy the anion binding site (see below). Replacement of a  $Zn(II)$ -acetate intermediate by water could be reversible and acetate is observed to be an inhibitor of the forward reaction.

A mechanism as formulated in scheme II would explain the lack of inhibition by weakly binding anions at high pH (Kernohan, 1965; Lindskog, 1966), the reversion at high pH of the spectra of the cobalt enzyme-anion or sulfonamide complexes to that typical of the alkaline form of the unin-



Scheme II

hibited enzyme (Lindskog, 1966), and the displacement of the pH-rate profile to higher pH in the presence of anions (Kernohan, 1965; Lindskog, 1966). All can be related to competition with  $^-OH$ , which at high-enough concentration displaces the anions and generates the active enzyme. The above mechanism is compatible with the kinetic evidence which shows the anion binding site to be coupled to a group, the basic form of which is essential for the hydration of  $CO_2$  and the acidic form essential for the dehydration of bicarbonate (Kernohan, 1967; Lindskog, 1966).

The hydration reaction according to scheme II is accompanied by proton transfers which are written in the simplest form. These transfers may be more complicated and be kinetically significant steps in catalysis. Imidazole or other adjacent protein groups could participate in the hydrogen-ion equilibria and influence the catalytic step.

#### Optically Active $d-d$ Transitions of Co(II) Carbonic Anhydrase

A more localized series of transitions than those of the azosulfonamide are the  $d-d$  transitions of Co(II), when this ion is introduced at the active site instead of Zn(II). The latter possesses a filled  $d$ -shell and, hence, no low energy transitions are present. The energies and optical activity of these  $d-d$  transitions are likely to be highly sensitive to any changes in structure that occur in the immediate vicinity of the metal ion. The visible absorption spectra of Co(II) human and bovine

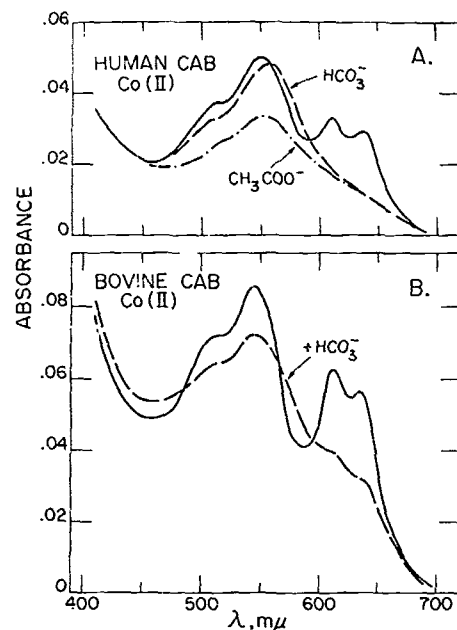


FIGURE 7.—(a) Visible absorption spectrum of Co(II) human carbonic anhydrase B (—), plus bicarbonate (---), plus acetate (- · -). (b) Visible absorption spectrum of Co(II) bovine carbonic anhydrase B (—), plus bicarbonate (---). The bicarbonate spectra were taken on solutions containing 0.025 M tris, pH 9.0, 23 C. Acetate was present at 0.2 M and enzyme concentrations were  $1.67 \times 10^{-4}$  M for the human enzyme and  $1.97 \times 10^{-4}$  M for the bovine enzyme.

carbonic anhydrases B are shown in figure 7. The unusual band structure (at least four bands between 450 and 650 millimicrons, see below) and the major spectral shifts induced by anionic and sulfonamide inhibitors have been discussed extensively (Coleman, 1968a; Lindskog, 1966). These spectral studies have provided significant evidence that these inhibitors add a donor atom to the first coordination sphere of the metal ion. This conclusion is supported by X-ray diffraction studies on the human C isozyme (Fridborg et al., 1967).

The band structure of the visible spectrum of the Co(II) enzyme is unlike most model Co(II) complexes with the possible exception of some five-coordinate complexes (Dennard and Williams, 1966). The band splitting would appear to argue for a distorted coordination geometry for the metal complex at the active site of carbonic anhydrase (Coleman, 1965; Dennard and Williams,

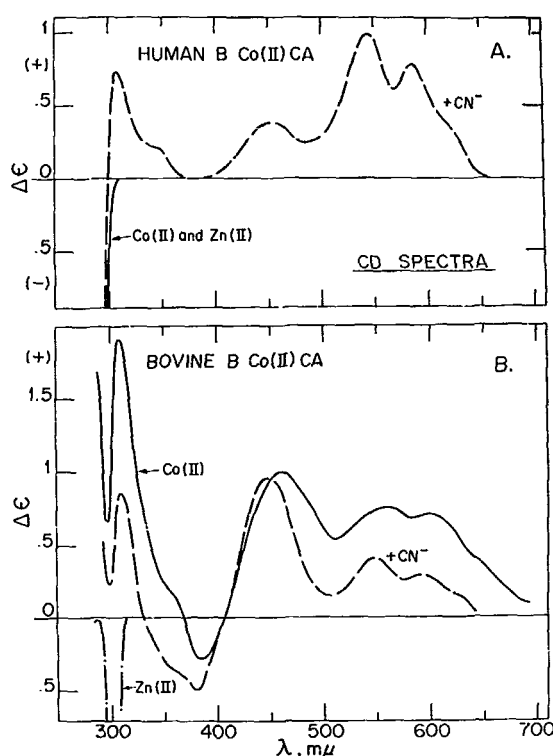


FIGURE 8.—Circular dichroic spectra of human and bovine carbonic anhydrases B and the cyanide complexes. (a) (—), Co(II) and Zn(II) human isozyme B; (---), Co(II) human isozyme B plus  $\text{CN}^-$ . (b) (—), Co(II) bovine isozyme B; (— · —), Zn(II) bovine isozyme B; (---), Co(II) bovine isozyme B plus  $\text{CN}^-$ . Conditions: 0.025 M tris, pH 9.0, 23 C. Enzyme concentrations were as in figure 7.

1966). The actual geometry may be controlled more by the three-dimensional structure of the protein ligands than by the preferred direction of the bonds which would be assumed if the ligands were entirely flexible.

A significant and unexpected property of the Co(II) derivative of the human isozyme B is that none of the visible  $d-d$  transitions show detectable optical activity (fig. 8(a)). This implies that the chromophore has a symmetry element, ordinarily a plane or center of symmetry, which prevents optical activity. How this is brought about in the context of a protein active site is an intriguing question. Even an intrinsically symmetrical chromophore is likely to be perturbed by the highly dissymmetric surroundings contributed by the protein. Such a perturbation does not appear to

occur in the human B isozyme. Suggestions as to the reason for the symmetry of the Co(II) chromophore itself have included distortion so that the major ligands are close to occupying a plane or that similar substituents, perhaps weak-water ligands, occupy positions above or below the plane.

Addition of anions like  $\text{CN}^-$  or sulfonamides destroys this symmetry element, because they induce strong optical activity in the  $d-d$  transitions of the Co(II) human B isozyme (fig. 8(a)) (Coleman, 1968a). At the same time, there are major shifts in band position and, hence,  $d$ -orbital energies (Coleman, 1968a; Lindskog, 1966). Both findings are compatible with the addition of a donor of strong ligand field to the coordination sphere. This addition alone may destroy the symmetry; however, there may also be shifts in some of the other metal-protein bond lengths or directions. The new ligands themselves, in addition, occupy a highly dissymmetric environment (figs. 2 and 3) which may add to the mechanism of induction of optical activity.

#### CD Spectra of Co(II) Bovine Carbonic Anhydrase

An unexpected finding that contributes significantly to further understanding of the structural factors affecting the metal complex is the surprising observation that the  $d-d$  transitions of the Co(II) bovine isozyme B all show large optical activity ( $\Delta\epsilon$  is generally larger than observed in many resolved chelate isomers). On the other hand, the similarity of the visible absorption spectra for all isozymes of Co(II) carbonic anhydrase has implied similar coordination geometries to exist in each isozyme. However, the symmetry elements affecting the  $d-d$  transitions are clearly not the same. The features of molecular structure responsible for this symmetry difference evidently do not alter the  $d$ -orbital energy level splitting, because band energies of all Co(II) isozyme spectra are similar. This is in marked contrast to the induction of optical activity by the coordination of anions and sulfonamides, which is accompanied by marked energy changes (Coleman, 1968a; Lindskog, 1966).

The anions and sulfonamides additionally alter the dissymmetry of the  $d-d$  transitions of the

Co(II) bovine isozyme. The separation of this mechanism from the one responsible for the initial optical activity of the Co(II) bovine isozyme B is best illustrated by the changes in ellipticity induced by a sulfonamide inhibitor, acetazolamide (fig. 9(a)). Major positive and negative ellipticity bands (or changes in rotatory strengths) are added to the original CD spectrum. The difference CD spectrum in figure 9(b) shows that

the band position and contour of this increment in the spectrum are similar to the change induced by the sulfonamides in the initially optically inactive bands of the Co(II) human isozyme B (fig. 9(c)). This finding apparently indicates that the two mechanisms of inducing or altering the symmetry around the Co(II) are independent and, hence, additive. In the case of the anionic inhibitors, however, this additive feature is not so clear as illustrated by the changes in the CD spectrum of the Co(II) bovine isozyme B upon the addition of CN<sup>-</sup> (fig. 8(b)). Charge distribution and sign over both the anion and the protein side groups in the immediate vicinity of the metal ion may be important factors influencing the rotatory strengths of the "d-d" transitions. Perturbation of an inherently symmetrical chromophore by a dissymmetric potential field induced by a constellation of charges on a dissymmetric molecule can be a powerful mechanism for inducing optical activity (Schellman, 1966, 1968).

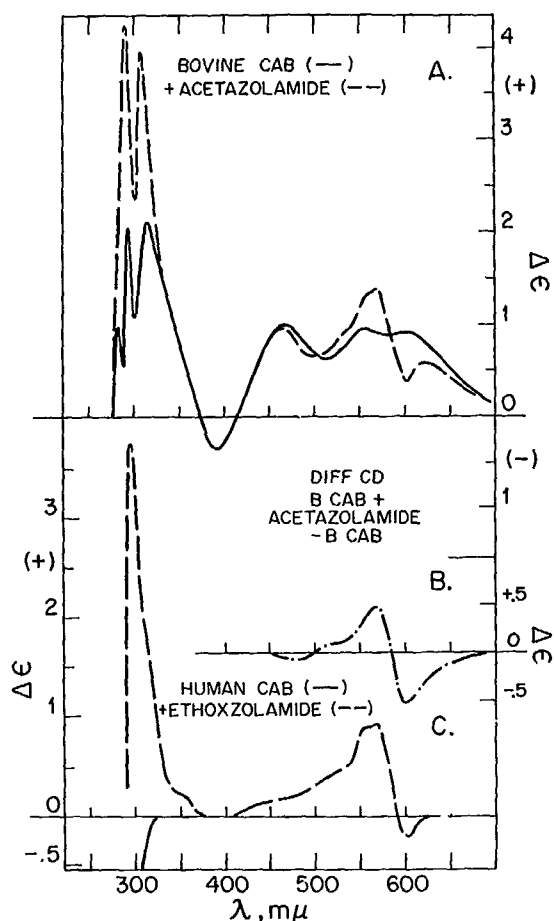


FIGURE 9.—Circular dichroic spectra of bovine carbonic anhydrase B and the complex with acetazolamide. Comparison to the spectrum of the human isozyme B ethoxzolamide complex. (a) (—), Co(II) bovine isozyme B; (---), Co(II) bovine isozyme B plus acetazolamide. (b) (— · —), Co(II) bovine isozyme B plus acetazolamide minus Co(II) bovine isozyme B. (c) (—), Co(II) human isozyme B; (---), Co(II) human isozyme B plus ethoxzolamide. Conditions: 0.025 M tris, pH 9.0, 23 C. Enzyme concentrations were as in figure 7.

#### CD Spectra of Anion, Substrate, and Product Complexes of Co(II) Human Carbonic Anhydrase B

The combination of all negatively charged metal binding anions with Co(II) carbonic anhydrase appears to induce the same symmetry change at the active center. If we compare the CD spectrum of the cyanide complex (fig. 8(a)) with analogous spectra for the complexes of cyanate, azide, and sulfide (fig. 10), the same set of at least three optically active transitions seems to be induced. Small variations in frequency and ellipticity may reflect ligand field strength and polarizability of the anion.

Among the possible anions interacting with the active center of carbonic anhydrase, bicarbonate and acetate are particularly interesting because the former is a substrate of the dehydration reaction and the latter a product of the esterase reaction. At pH 8.0, both of these anions form complexes with Co(II) carbonic anhydrase which bring about major shifts in the absorption spectrum (fig. 7). At pH 8.3, where the spectrum of the HCO<sub>3</sub><sup>-</sup> complex is observed, the equilibrium of the hydration-dehydration reaction is greatly in favor of bicarbonate (Gibbons and Edsall, 1963). The inhibition of esterase activity by ace-

tate has previously been observed (Coleman, 1967b). Whether these spectra represent those of intermediates on the reaction pathway is yet to be proved. These complexes do share the property, in common with the other anions, of inducing optical activity in the  $d-d$  transitions of the cobalt ion (fig. 11).

#### Gaussian Resolution of the CD and Absorption Spectra of Co(II) Human Carbonic Anhydrase B and the Cyanide and Ethoxzolamide Complexes

To form a better qualitative and quantitative picture of the band structure and shifts in band structure shown by the various Co(II) carbonic anhydrase spectra, the visible spectral envelopes

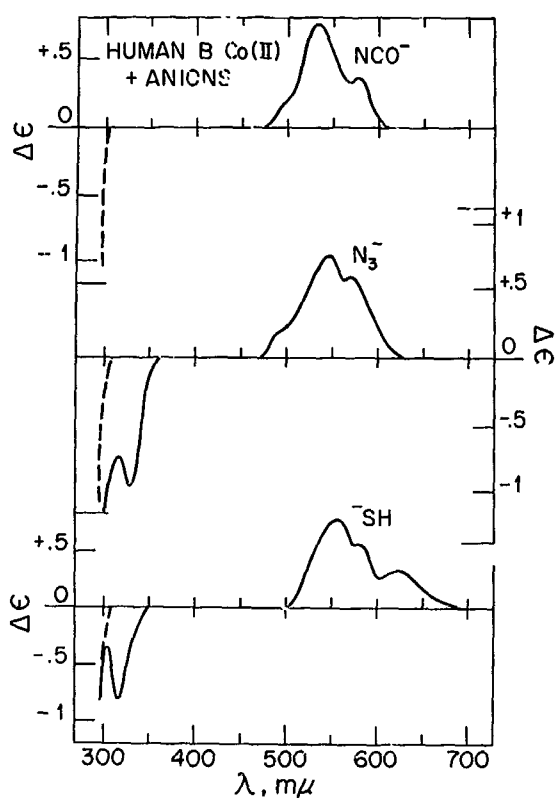


FIGURE 10.—Circular dichroic spectra of Co(II) human B carbonic anhydrase-anion complexes. (—), complexes as labeled  $\text{NCO}^-$ ,  $\text{N}_3^-$ , and  $\text{SH}^-$ . (---), native Co(II) enzyme in all cases. Conditions: 0.025 M tris, pH 9.0, 23 C,  $1.67 \times 10^{-4}$  M enzyme. Inhibitors were present in a *twofold* molar excess in each case.

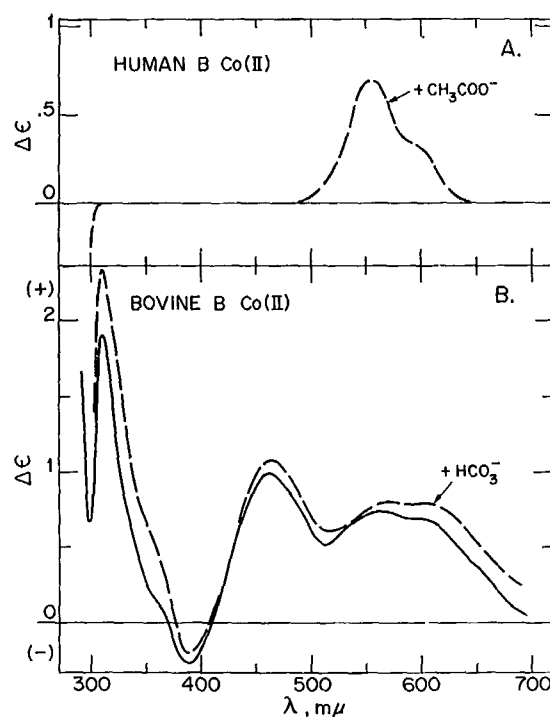


FIGURE 11.—Circular dichroic spectra of Co(II) carbonic anhydrase complexes with acetate and bicarbonate. (a) (---), Co(II) human B isozyme plus  $\text{CH}_3\text{COO}^-$ . (b) (—), Co(II) bovine B isozyme; (---), Co(II) bovine B isozyme plus  $\text{HCO}_3^-$ . Conditions and enzyme concentrations were as given in figure 7.

have been resolved into a series of overlapping gaussian bands with a Du Pont 310 curve fitter (Coleman, 1968b; Myer, 1968). The fitting reveals a complex band structure to exist for all the spectra. While such additional information does not as yet provide a precise determination of the geometry of the ligand field perturbing the  $d$ -orbitals, it does provide a quantitative basis for theoretical treatments. The absorption spectrum of the Co(II) human isozyme B in the visible region can be fitted by a minimum of five bands (fig. 12(a)). The analysis of the absorption spectrum of the ethoxzolamide complex (fig. 12(b)) shows that the sulfonamide causes the two low-energy bands to move to higher energy, but does not change the band multiplicity. The positions and bandwidths of bands 3, 4, and 5 are not changed, but there are major changes in relative oscillator strengths of all five bands. The CD spectrum of the ethoxzolamide complex can also be easily



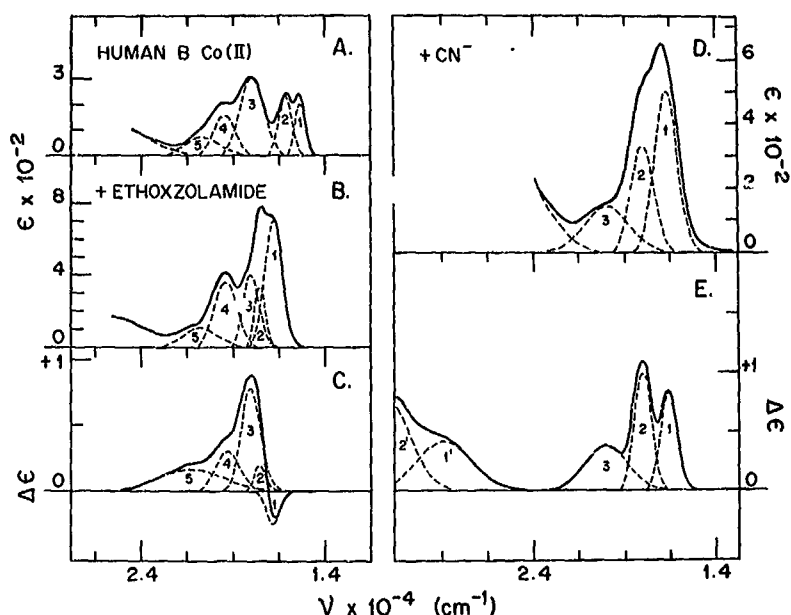


FIGURE 12.—Resolution of the visible absorption and CD spectra of Co(II) human carbonic anhydrase B and the complexes with ethoxzolamide and cyanide into a series of overlapping gaussian bands. In each part of the figure (—) represents the envelope for the sum of the constituent gaussian bands shown by the dashed lines and numbered consecutively from lower to higher energies. Each envelope corresponds within the error of the measurement to the experimental spectra: (a) absorption spectrum, enzyme alone; (b) absorption spectrum plus ethoxzolamide; (c) circular dichroism plus ethoxzolamide; (d) absorption spectrum plus CN<sup>-</sup>; (e) circular dichroism plus CN<sup>-</sup>.

fitted by five ellipticity bands at the same positions and of the same bandwidths as those present in the absorption spectrum (band 5 is slightly wider, which may reflect experimental error because the spectra were taken on two different instruments). Band 1 has negative ellipticity, while the rest show positive ellipticity. Band 2 in the CD spectrum appears hidden under band 3, but its presence is necessary for the proper fit because the steep profile of the CD spectrum where it shifts from positive to negative is not generated by any combination of two gaussian bands.

An independent fitting of the absorption and CD spectra of the cyanide complex shows that the visible region of both spectra can be fitted by three bands of the same position and bandwidth (fig. 12(d) and 12(e)). Bands 2 and 3 are in the same position and apparently correspond to bands 3 and 4 of the Co(II) enzyme alone. Band 1 may contain two bands that have moved so close together that they cannot be distinguished. Some of

the CD recordings suggest this (see fig. 8(a)). In addition, the anions induce two near-ultraviolet ellipticity bands, 1' and 2'. Thus, like the bovine Co(II) enzyme, there are near-ultraviolet bands associated with the Co(II) chromophore. The presence or absence and sign of these near-ultraviolet ellipticity bands depend on the character of the anion (fig. 10).

A precise interpretation of the absorption and CD bands of Co(II) carbonic anhydrase is not possible at present, partly because of the lack of any simple model Co(II) compounds that demonstrate the spectral properties shown by the protein complex. The oscillator strengths appear more characteristic of tetrahedral Co(II) complexes, but the band multiplicity and energies are not typical of tetrahedral models. The additional bands in the near ultraviolet associated with the Co(II) chromophore and revealed by the CD measurements further complicate the interpretation. If there is a significant contribution from ligand orbitals to the visible transitions of the



In addition to these findings, the determination of the structure of human carbonic anhydrase C by X-ray diffraction at 5-Å resolution shows that apparently the C-terminal lysine is located very near the metal ion. Thus, both the human C and bovine B isozymes appear to have an additional positive charge in the immediate vicinity of the active site. This fact could be responsible for inducing the observed optical activity in the *d-d* transitions of the Co(II) human C and bovine B isozymes. Such a charge could easily distort the potential field around the cobalt chromophore. This charge might also influence the catalytic mechanism as postulated in scheme II, because transfer of several charged species including protons are involved. Both human C and bovine B have somewhat higher specific activities than the human B enzyme (Coleman, 1967b; Rickli et al., 1964).

#### ACKNOWLEDGMENT

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## DISCUSSION

**EDSALL:** I notice that the cobalt enzyme has well-marked positive circular dichroism bands just a little above 300 millimicrons, which do not appear in the zinc enzyme. They are quite characteristic of the cobalt, just as the ones at longer wavelength are.

**COLEMAN:** It is very difficult to assign these to any well-known structure for a coordination complex. It may indicate that this complex is spectrally far more complicated than any of the models that have been looked at. One usually interprets these bands as charge transfer bands, but one would guess that they are relatively weak compared to the usual charge transfer bands, because no major change is seen in the absorption of the protein in the ultraviolet. Of course, the concentrations that one uses in the ultraviolet are relatively so much smaller that one may miss them. However, I think that some coordination chemists feel that it is not necessarily true to say that all charge transfer bands must have extinction coefficients as high as about a thousand. So these could be weak charge transfer bands.

**HASTINGS:** Can you define "Cotton effect"?

**COLEMAN:** In answer to Dr. Hastings' question, it is perhaps best to define first the two related phenomena, circular dichroism and optical rotatory dispersion, associated with an optically active absorption band. If an electronic transition occurs in a molecule having sufficient dissymmetry to render the transition optically active, the dot product of the electric ( $\mu_e$ ) and magnetic ( $\mu_m$ ) moments of that transition is not equal to zero ( $\mu_e \mu_m \cos \theta \neq 0$ , where  $\theta$  is the angle between the electric and magnetic moments). In more direct physical terms, the motions of the electrons involved in the transition are associated with both translational and rotational components. If plane polarized light of the frequency corresponding to the transition can be divided into its left and right circularly polarized components, the absorption band corresponding to the transition will demonstrate a greater or lesser absorption (molar extinction coefficient,  $\epsilon$ ) for the left-handed component,  $\epsilon_L$ , or the right-handed component,  $\epsilon_R$ , depending on whether the initial dissymmetry giving rise to the optical activity is of a left- or right-handed screw sense. If  $\epsilon_L - \epsilon_R$  is positive,

the absorption band is said to show positive circular dichroism; if  $\epsilon_L - \epsilon_R$  is negative, the band shows negative circular dichroism (CD) (see fig. D-1(a)). Circular dichroism is often expressed

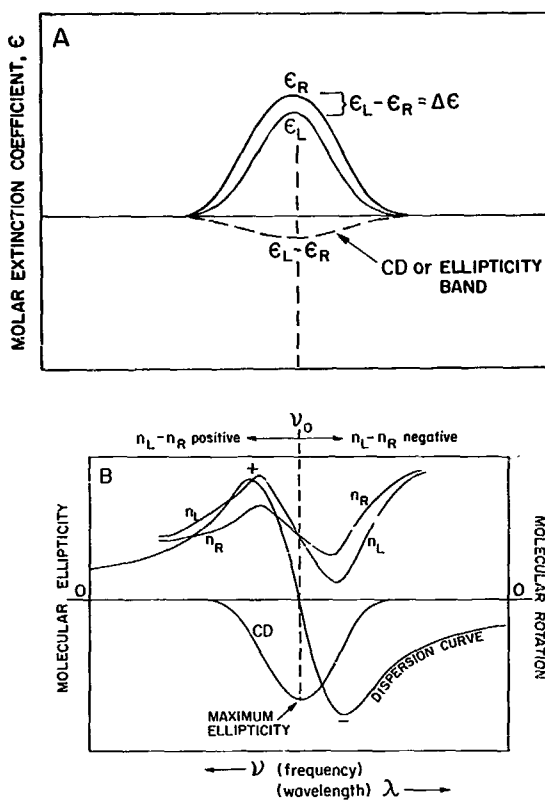


FIGURE D-1.—(a) Circular dichroism or differential absorption of the right and left circularly polarized components of plane polarized light produced by a single optically active electronic transition of frequency  $\nu_0$ .  $\epsilon_L$  = molar extinction coefficient for the left-handed component,  $\epsilon_R$  = molar extinction coefficient for right-handed component. The magnitude of the difference,  $\epsilon_L - \epsilon_R$ , has been exaggerated for graphical purposes. This difference is rarely greater than a few hundredths of the average molar extinction coefficient. (b) Optical rotatory dispersion (labeled dispersion curve) associated with a negative gaussian CD band as shown in (a). If circular dichroism is expressed as molecular ellipticity, the units readily convert in calculations to those of molecular rotation which are also  $\text{deg}\cdot\text{cm}^2/\text{decimole}$ . The wavelength dependence of the indices of refraction through the optically active absorption band for the two circularly polarized components of plane polarized light are given by the curves marked  $n_L$  and  $n_R$ . The conditions giving rise to a negative Cotton effect are pictured in this figure.

directly in units of  $\epsilon_L - \epsilon_R$  or  $\Delta\epsilon$ . In work with macromolecules, circular dichroism is most often expressed as molecular ellipticity,  $(\theta) = 2.303 (4500/\pi) (\epsilon_L - \epsilon_R)$ , which has units of deg cm<sup>2</sup>/decimole. It should be emphasized that the phenomenon of circular dichroism occurs within the wavelength range of the envelope of the ordinary absorption band (fig. D-1(a)).

On the other hand, a well-documented physical principle states that for every absorptive process there is an associated dispersive process. The general relation between absorption and dispersion is embodied in the mathematical relation expressed by the Kronig-Kramers transform which can be used to generate the dispersion curve (fig. D-1(b)) corresponding to an absorptive process like the gaussian CD band shown in figure D-1(a). For detailed discussions and development of the theory relating absorption and dispersion phenomena, the reader is referred to papers by Moscovitz (1960) and Schellman (1964). The dispersion phenomenon associated with circular dichroism is optical rotatory dispersion; namely, the rotation of the plane of polarization experienced by plane polarized light as it passes through the optically active substance measured as a function of the wavelength of the polarized light. The dispersion curve derived from the negative CD band in figure D-1(a) is illustrated in figure D-1(b). The optical rotatory dispersion curve for a single electronic transition follows a two-phase (positive and negative) symmetrical curve. The dispersion extends far outside the wavelength region of measurable absorption. If the original CD band is negative, the dispersion curve begins negative at low energy (long wavelength), reaches a negative minimum, and then becomes zero at the wavelength of maximum ellipticity, followed by a mirror-image positive curve. If the CD band is positive, the positive arm of the dispersion curve occurs at the low-energy side of the absorption band.

In the physical description of the dispersion phenomenon, it is helpful to point out that the index of refraction is a dispersion phenomenon. For a solution containing the optically active molecule, there are two different indices of refraction,  $n_L$  and  $n_R$ , one for the left and one for the right circularly polarized components of plane polarized light. The difference  $n_L - n_R$  undergoes a change as a function of wavelength following a dispersion curve that changes sign through the absorption band (fig. D-1(b)). Indeed this difference,  $n_L - n_R$ , is responsible for the rotation of the plane of polarization, because one circularly polarized component of the plane-polarized beam becomes retarded more or less than the other, depending on wavelength, hence rotating the plane of polarization in one direction and then the opposite direction as this difference changes sign. The resultant light in which the two circularly polarized components are unequally absorbed as well as unequally rotated is referred to as elliptically polarized, because the resultant electric vector traces an ellipse. It is the presence of elliptically polarized light that is associated with the Cotton effect.

In the American literature it has often been the practice to refer to the dispersion curve alone as a "Cotton effect," terming it positive or negative if the positive or negative arm, respectively, occurs at longer wavelengths. In the European literature, the designation "Cotton effect" has often included both the dispersion and associated circular dichroism.

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# On the Mechanism of Sulfonamide Inhibition of Carbonic Anhydrase<sup>1</sup>

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The evidence for the essential role of the metal ion in sulfonamide binding is reviewed. The pH-dependence of the inhibition of bovine carbonic anhydrase by acetazolamide has been studied and is interpreted in terms of the  $pK$  of the activity-linked group in the enzyme and the microscopic ionization constants of the inhibitor. It is shown that the ionization state of the sulfonamide group is of critical importance for the binding rather than the overall charge of the inhibitor molecule. A comparison of the inhibition constants and the apparent recombination rate constants for two closely related sulfonamides—benzene sulfonamide and sulfanilamide—suggests that the anionic form of the inhibitor is bound to the enzyme. It presumably competes with  $\text{OH}^-$  for the anion binding site, part of which is the metal ion. The inhibition constants for a large number of benzene sulfonamides with one additional ring substituent are discussed in terms of hydrophobic interactions between the inhibitors and the enzyme and suggest that the hydrophobic slot or surface on the enzyme is of considerable size. Apparent rate constants for the binding of various types of sulfonamides to bovine carbonic anhydrase are compatible with a direct combination of the ionized sulfonamide with the acidic form of the activity-linked group on the enzyme in an almost diffusion-controlled reaction, but may indicate a more complex mechanism. Preliminary results, indicating that acetazolamide does not compete with  $\text{CO}_2$  in human carbonic anhydrase B, are given.

As amply illustrated by the excellent and massive review by Maren (1967), sulfonamides are of great importance in the studies of the diverse physiological functions of carbonic anhydrase. The specificity of these inhibitors for carbonic anhydrase and their extremely strong interaction with the enzyme must be a function of peculiar features of the active site region of the enzyme molecule. Hence, biochemists interested in the catalytic mechanism of carbonic anhydrase have, to an increasing extent, employed sulfonamides as probes for the active site, be it in kinetic work or in various spectroscopic studies, as carriers of reactive groups in an effort to map the active site by chemical modification (Whitney, Fölsch, Nyman, and Malmström, 1967; Kandel, Wong,

Kandel, and Gornall, 1968), or as carriers of heavy metal atoms in X-ray crystallography (Fridborg, Kannan, Liljas, Lundin, Strandberg, Strandberg, Tilander, and Wiren, 1967). The effects of variations in sulfonamide structure on inhibitory power have been the subject of a recent and comprehensive review (Bar, 1963). The purpose of the present paper is to discuss the sulfonamide-enzyme interactions, their chemical nature, and their relation to the catalytic mechanism of carbonic anhydrase.

## THE INTERACTION BETWEEN THE METAL ION AND THE SULFONAMIDE GROUP

Some years ago we demonstrated in equilibrium dialysis experiments the importance of the metal ion for sulfonamide binding (Lindskog, 1963). These results have later been confirmed and extended by Coleman (1967a). He showed that

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only the activating metal ions, Zn(II) and Co(II), induce a strong binding in human carbonic anhydrase B, while the apoenzyme and inactive metallocarbonic anhydrases bind the inhibitor with a greatly reduced affinity. A close association between the metal ion and sulfonamide was further suggested by the effects of these inhibitors on the visible absorption spectrum of the cobalt enzyme—effects which are similar to those obtained with simple anionic inhibitors, such as cyanate (Lindskog, 1963). These effects can be reversed by an increase of pH. This result was interpreted as a competition between inhibitor and a basic metal ligand.

The spectrum of the cobalt enzyme itself varies with pH, and it could be shown that this spectral shift is directly related to the pH-rate profile of the enzyme (Lindskog, 1966a). The CO<sub>2</sub>-hydration activity as well as the esterase activity (Thorslund and Lindskog, 1967) requires the basic form of a group with a *pK* around 7. This group must be either a metal ligand or sufficiently close to the metal ion so as to trigger the spectral change. As was first shown on the bovine zinc enzyme by Kernohan (1965), the acidic form of the activity-linked group is required for strong binding of anions. One explanation of these phenomena is that a water molecule is coordinated to the metal ion and that the *pK* of 7 represents its ionization. The basic ligand that competes with inhibitors would consequently be OH<sup>-</sup>. The role of the metal ion in the catalytic mechanism of carbonic anhydrase has recently been reviewed (Lindskog, 1968).

Neither the absolute nor the relative magnitudes of the affinities of various anions to carbonic anhydrase correspond to those observed in simple metal complexes, but rather to those observed in some other proteins. (See Verpoorte, Mehta, and Edsall, 1967; Lindskog, 1966a.) These and other data force us to conclude that, in addition to the metal ion, the anion site comprises other elements of the protein structure, perhaps also other positive charges.

The evidence is now strong that the sulfonamide group interacts with this anion site. The competition between sulfonamides and anions has been demonstrated both kinetically (Lindskog and Thorslund, 1968), by various spectroscopic measurements (Coleman, 1967b; Riepe and Wang,

1968), and in direct binding studies (Coleman, 1967b). A great step forward in our understanding of this was provided by Kernohan (1966a), who showed that the binding of benzene sulfonamide to bovine zinc carbonic anhydrase is dependent on the ionization state of the sulfonamide as well as of the activity-linked group in the enzyme. An attractive interpretation of these results is that only the anionic form of the sulfonamide is reactive and that it competes with OH<sup>-</sup> for the anion site. The data, however, are also in accordance with the combination of the neutral sulfonamide with the basic form of the active site. Our studies on the bovine cobalt enzyme (Lindskog and Thorslund, 1968) indicated that the important factor is, indeed, the ionization of the sulfonamide group and not the overall charge of the inhibitor molecule.

Because acetazolamide particularly has been widely used in chemical and physiological studies, we investigated in detail the relation between its binding and acid dissociation constants. Potentiometric titration of acetazolamide yields two *pK*'s, 7.2 and 8.8 (table I) at 25 C and 0.1 ionic strength. The two protons are derived from the nitrogen atoms of the sulfonamide and the acetylamido groups, respectively. A spectrophotometric titration (fig. 1) showed that the absorption maximum of acetazolamide is shifted from 265 millimicrons in acid to 292 millimicrons in alkali. There are three spectral forms (note the isobestic points in fig. 1), but the intermediate form is obviously a mixture of chemical species. The spectral shift

TABLE I.—*The Acid Dissociation Constants of Acetazolamide and Chloroacetazolamide at 25 C and 0.1 Ionic Strength*

| Constant <sup>a</sup>         | Acetazolamide    | Chloroacetazolamide |
|-------------------------------|------------------|---------------------|
| <i>pK</i> <sub>1</sub> -----  | 7.2 <sub>0</sub> | 5.7                 |
| <i>pK</i> <sub>2</sub> -----  | 8.8 <sub>0</sub> | 8.4                 |
| <i>pK</i> <sub>12</sub> ----- | 7.4 <sub>6</sub> | <sup>b</sup> 7.4    |
| <i>pK</i> <sub>13</sub> ----- | 7.5 <sub>6</sub> | 5.7                 |
| <i>pK</i> <sub>24</sub> ----- | 8.5 <sub>4</sub> | <sup>b</sup> 6.7    |
| <i>pK</i> <sub>34</sub> ----- | 8.4 <sub>6</sub> | 8.4                 |

<sup>a</sup> As defined in fig. 3. The probable error in *pK* values are estimated to be less than 0.1.

<sup>b</sup> Based on the assumption that the electrostatic effect is the same as in acetazolamide.

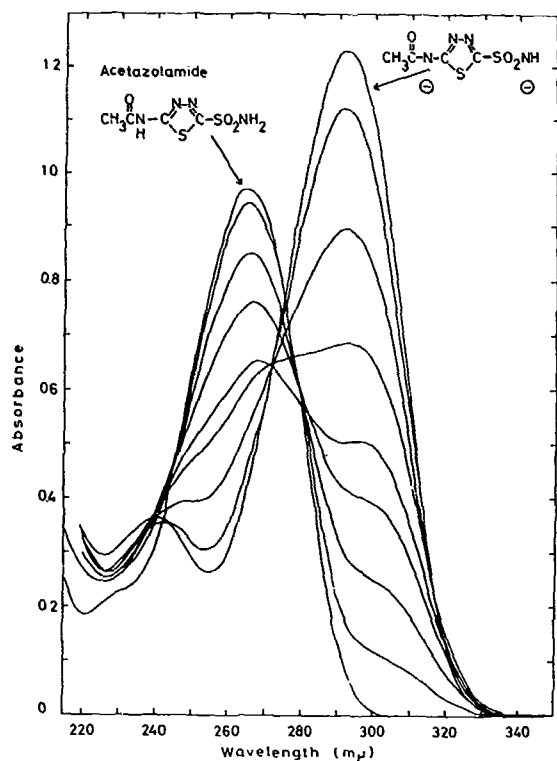


FIGURE 1.—Absorption spectra of  $10^{-4}$  M acetazolamide at different pH-values, 25 C, ionic strength 0.1. Phosphate, tris- $H_2SO_4$  or  $NaHCO_3$ - $Na_2CO_3$  buffers were used. The spectra were recorded with a Cary 15 spectrophotometer, a 1-cm cell and in 0.1 M HCl, at pH 6.58, 7.05, 7.50, 7.98, 8.46, 9.10, 9.49, and in 0.1 M NaOH, respectively.

is essentially due to the ionization of the acetyl-amido group. Figure 2 shows that in methazolamide,  $pK = 7.4$ , the sulfonamide ionization has a relatively small effect. In the evaluation of the microscopic ionization constants of acetazolamide (fig. 3), the spectrophotometric titration of chloroacetazolamide was very informative. The chloroacetyl-amido group has a  $pK$  of 5.7 in this compound, while that of the sulfonamide group is 8.4. As seen in figure 4, the ionization of the sulfonamide group results in a small blue shift and a small intensification of the peak (cf. fig. 2). At about 300 millimicrons there is an isobestic point for this process. Assuming that 300 millimicrons is an isobestic wavelength for the corresponding process in acetazolamide and that forms with protonated acetyl-amido groups have little absorption at this wavelength, we obtained the

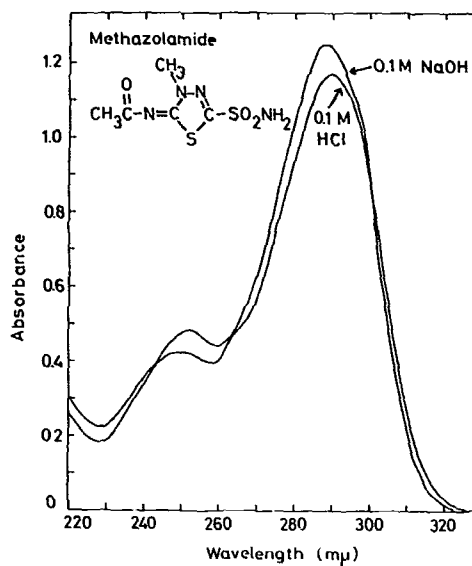


FIGURE 2.—Absorption spectra of  $10^{-4}$  M methazolamide in 0.1 M HCl and 0.1 M NaOH, respectively.

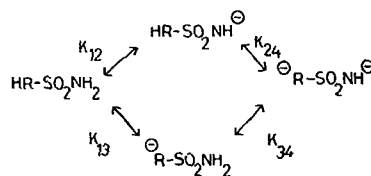


FIGURE 3.—Scheme defining the microscopic acid dissociation constants given in table I. The potentiometrically determined  $pK$ 's are related to these by the equations,  $K_1 = K_{12} + K_{13}$ ;  $1/K_2 = 1/K_{24} + 1/K_{34}$ . HR- represents  $CH_3CONH-$  in acetazolamide or  $ClCH_2CONH-$  in chloroacetazolamide, respectively, and the thiazazole ring.

degree of dissociation of this group as a function of pH, and the constants given in table I could be calculated. As shown in table I, the two ionizing groups have practically the same  $pK$  values, and the electrostatic effect corresponds to about 1 pH unit.

The pH-dependence of  $pK_i$  for acetazolamide (fig. 5) can easily be analyzed in terms of the  $pK$  of the activity-linked group in the enzyme ( $pK_E$ ) and the  $pK$  values of the inhibitor. As shown in figure 5, the apparent second-order rate constants for the formation of the enzyme-inhibitor complex have a similar pH-dependence, which shows that the observed  $pK$ 's are related to the free enzyme and free inhibitor, respectively. The



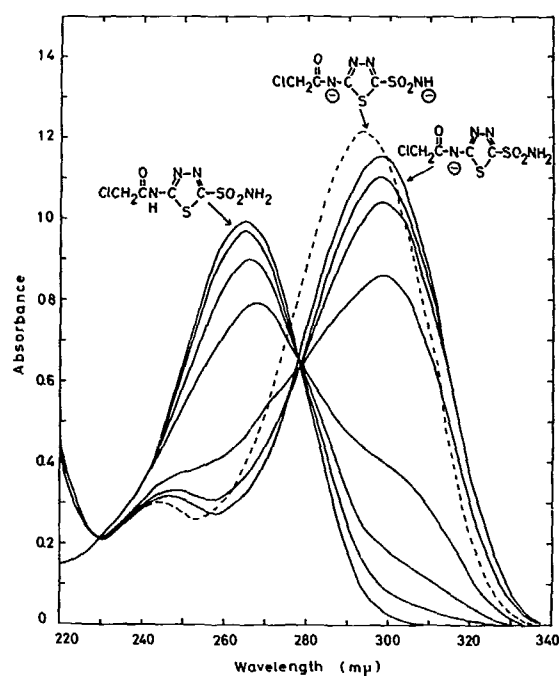


FIGURE 4.—Absorption spectra of  $10^{-4}$  M chloroacetazolamide at different pH-values, 25 C, ionic strength 0.1 tris- $H_2SO_4$ , phosphate or acetate buffers were used. The spectra were recorded with a Cary 15 spectrophotometer, a 1-cm cell, and in 0.1 M HCl, at pH 4.45, 4.95, 5.43, 6.09, 6.58, 7.05, 7.50, and in 0.1 M NaOH, respectively.

dissociation rate constant  $k_d$  appears to be independent of pH over the whole range investigated.

The inhibition by chloroacetazolamide has a rather complex pH-dependence, but  $pK_i$  does not decrease with a unit slope in the alkaline range until above the  $pK$  of the sulfonamide group, which clearly shows the critical importance of the proper ionization state of this group. To explain the complex pH-dependence, including the excessive decrease of  $pK_i$  for acetazolamide around pH 9, it must be assumed that the ionization of the side chain results in a somewhat weakened binding. As a consequence, the  $pK$  of the acetyl-amido group in the acetazolamide-enzyme complex must be shifted to 9.3. Preliminary spectrophotometric titrations in the wavelength region around 300 millimicrons indicated that this is, indeed, the case. The curves of figure 5 have been calculated according to equation (1):

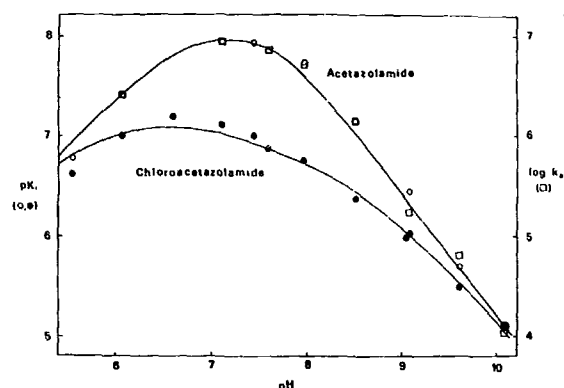


FIGURE 5.—pH-dependence of the negative logarithms of the inhibition constants ( $pK_i$ ) for bovine carbonic anhydrase B and acetazolamide ( $\circ$ ) and chloroacetazolamide ( $\bullet$ ), and the pH-dependence of the apparent second-order recombination rate constant ( $k_a$ ) for the reaction between bovine carbonic anhydrase B and acetazolamide ( $\square$ ). The  $pK_i$  values were estimated in phosphate or tris- $H_2SO_4$  buffers, ionic strength 0.1, 25 C with 0.4 mM p-nitrophenyl acetate as substrate as described by Lindskog and Thorstlund (1968). The  $k_a$ -values were determined from the initial phase of the reaction when substrate (0.4 mM ester at pH > 8, or 0.5 mM  $CO_2$  at pH < 8) containing inhibitor was mixed in a Durrum-Gibson stopped-flow spectrophotometer with buffer containing enzyme. The procedure and the equations used in the calculations are given by Lindskog and Thorstlund (1968). The curves were calculated according to equation (1) with  $K_1 = 1.3 \times 10^{-9}$  M, and the values of  $K_1$ ,  $K_2$ , and  $K_{12}$  indicated in table I. To obtain a good fit to the points,  $pK_E = 7.1$  rather than 6.9 found for the pH-rate profile (Thorstlund and Lindskog, 1967), was used.

$$K_i = \left( 1 + \frac{K_E}{[H^+]} \right) \frac{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}}{\frac{K_{12}}{K_1 K_1} + \frac{K_2}{K_1 [H^+]}} \quad (1)$$

which has been derived for the case of the combination of the ionized sulfonamide group with the acidic form of the active site (case 1).  $K_I$  and  $K_I'$ , which are the pH-independent, "intrinsic" dissociation constants for the enzyme-inhibitor complex with a neutral and ionized side chain, respectively, were estimated from the data on acetazolamide and assumed to be unchanged in the chloro derivative. As seen in figure 5, this assumption seems to be valid. The above treatment must, of course, be an oversimplification

TABLE II.—Apparent Equilibrium and Rate Constants for the Reaction of Various Sulfonamides With Bovine Carbonic Anhydrase at 25 C and 0.1 Ionic Strength

| Inhibitor <sup>a</sup>                    | $pK$ <sup>b</sup> | $K_i$ <sup>c</sup><br>$M \times 10^9$ | $k_a$ <sup>c</sup><br>$M^{-1} \text{sec}^{-1} \times 10^{-4}$ | $K_I$ <sup>d</sup><br>$M \times 10^{10}$ | $k_a$ <sup>e,d</sup><br>$M^{-1} \text{sec}^{-1} \times 10^{-8}$ | $k_a$ <sup>c</sup><br>$\text{sec}^{-1}$ |
|---|-------------------|---------------------------------------|---|--|---|---|
| Sulfanilamide.....                        | 10.7              | 3000                                  | 3.9   | 5  | 3   | 0.12                                    |
| <i>p</i> -Iodobenzene<br>sulfonamide..... | 9.9               | 34                                    | 100   | .3                                       | 11  | .03                                     |
| Acetazolamide.....                        |                   |                                       |   | 13<br>° 80                               | .8<br>° .13   | .10<br>° .10                            |
| CL 11,366.....                            | 8.25              | 9                                     | 590   | 2  | 2   | .05                                     |
| Ethoxzolamide.....                        | 8.05              | 1.6                                   | 2900  | .6                                       | 7   | .05                                     |
| Chlorothiazide.....                       | 9.35              | 540                                   | 4.5   | 17                                       | .14   | .02                                     |

<sup>a</sup> See Maren (1967) for the structure of these compounds.

<sup>b</sup> Refers to sulfonamide group as determined by titration. The value for *p*-iodobenzene sulfonamide was estimated on the basis of  $\sigma = +0.18$ , however.

<sup>c</sup> Determined at pH 8.0 in *tris*-H<sub>2</sub>SO<sub>4</sub> buffer, as described by Lindskog and Thorslund (1968), using the esterase

activity. The values for CL 11,366 and ethoxzolamide were estimated in phosphate buffer, pH 7.1, however, and the substrate was 0.5 mM CO<sub>2</sub>.

<sup>d</sup> As defined in the text for case 1.  $pK_E$  was assumed to be 6.9.

<sup>e</sup>  $K_I'$  and corresponding rate constants.

because, for example, variations in the overall charge of the protein have been neglected.

In the case of the combination of the neutral sulfonamide group with the basic form of the active site, case 2, an equation similar to equation (1) can be derived, but the two cases might be discriminated if two sulfonamides have the same "intrinsic"  $pK_I$  values but differ in acidity. Unfortunately, the microscopic ionization constants of the sulfonamide groups in acetazolamide and chloroacetazolamide are similar. Benzene sulfonamide and sulfanilamide, however, seem to constitute a pair where such a discrimination might be feasible. At pH 8 to 9, these inhibitors are essentially un-ionized and the activity-linked group of the enzyme is in the basic form, so if the  $K_I$  values can be considered the same, case 2 should give identical values of  $K_i$  (as well as of the apparent recombination rate constants,  $k_a$ ) whereas case 1 should give a ratio of  $K_i$  (and of  $k_a$ ) equal to the inverse ratio of acid dissociation constants. For the inhibition of bovine carbonic anhydrase by sulfanilamide ( $pK = 10.7$ ), we found (at 25 C, 0.1 ionic strength, and pH 8.0)  $pK_i = 5.51$  and  $k_a = 3.9 \times 10^4 M^{-1} \text{sec}^{-1}$ . These should be compared to Kernohan's (1966a) values for benzene sulfonamide:  $pK_i = 6.12$  and  $k_a = 2.1 \times 10^5 M^{-1} \text{sec}^{-1}$  (at pH 8.0) and  $pK = 10.1$ . These data favor case 1. Further support for case 1 was provided

by Chen and Kernohan (1967), who showed that the absorption maximum as well as the maximum of the fluorescence emission spectrum of dansylamide (5-dimethylaminonaphthalene-1-sulfonamide) were shifted on binding with bovine carbonic anhydrase to a degree compatible with a dissociation of a proton from the sulfonamide group.

Further, for the mechanism of case 1 to be feasible as a direct reaction of the ionized sulfonamide with the acidic form of the active site, the corresponding recombination rate constant must not exceed the value for a diffusion-controlled reaction, approximately  $1 \times 10^9 M^{-1} \text{sec}^{-1}$  (Eigen and Hammes, 1963). It can be estimated that the above values of  $k_a$  fulfill this requirement (see table II).

#### THE IMPORTANCE OF OTHER INTERACTIONS FOR SULFONAMIDE BINDING

Let us examine a series of benzene sulfonamides with one additional ring substituent. If the substituents were completely without effect on the binding, we should expect a plot of  $pK_i$  at neutral pH versus  $pK$  to yield a straight line with unit slope. Unfortunately, the  $pK$  values for most of these compounds are not known, but figure 6 instead shows  $pK_i$ -values found in the literature

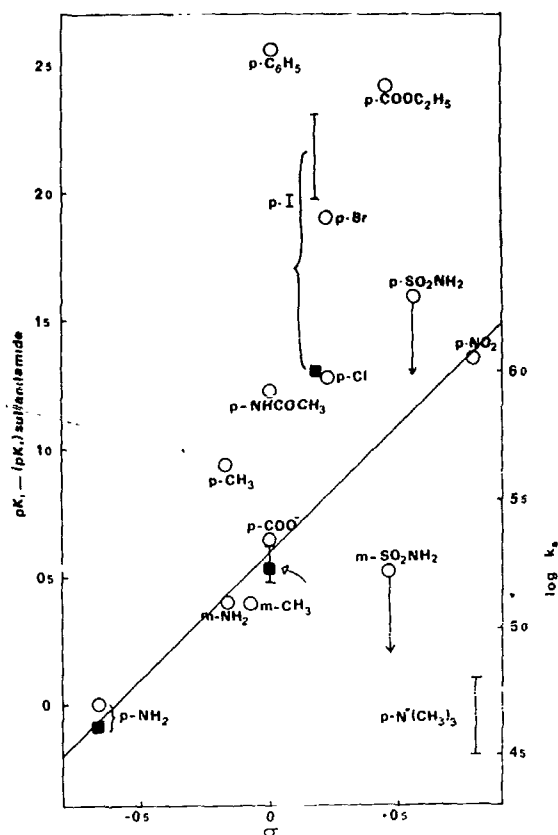


FIGURE 6.—Relative values of  $pK_i$  (○) for various benzene sulfonamides with one additional ring substituent versus Hammett's  $\sigma$ . Most of the  $pK_i$  values are those compiled by Bar (1963). The arrow in the center indicates the value for unsubstituted benzene sulfonamide. The vertical arrows going from the points representing  $p$ - and  $m$ - $\text{SO}_2\text{NH}_2$  indicate the direction of the correction due to the two sulfonamide groups in these inhibitors. The ranges indicated for benzene sulfonamide,  $p$ -iodobenzene sulfonamide, and  $p$ -trimethylammonium benzene sulfonamide include values of Kernohan (1966a), this study, and Maren and Wiley (1968) as well. For benzene sulfonamide (Kernohan, 1966a), sulfanilamide and  $p$ -iodobenzene sulfonamide, values of  $\log k_a$  for the reaction with bovine carbonic anhydrase B are given for comparison.

plotted against Hammett's  $\sigma$ . Although this procedure is not quite correct in this case, an approximate straightline relation with a slope close to +1 might again be expected. Figure 6 shows that only a few of these sulfonamides have  $pK_i$  values falling on the line going through the point representing benzene sulfonamide. If the above reasoning is correct, "innocent" substituents, in

addition to  $p$ - $\text{NH}_2$ , would be  $m$ - $\text{NH}_2$ ,  $p$ - $\text{COO}^-$ ,  $m$ - $\text{CH}_3$ ,  $p$ - $\text{NO}_2$ , and  $p$ - $\text{SO}_2\text{NH}_2$  (if the latter is corrected for a statistical factor due to the two sulfonamide groups). Most of the other substituents seem to give rise to increased inhibitory strengths. Exceptions are  $m$ - $\text{SO}_2\text{NH}_2$  and  $p$ - $\text{N}^+(\text{CH}_3)_3$ . The charge on the substituent appears to be of minor importance. Since  $p$ - $\text{COO}^-$  seems to be "innocent" and  $\text{CH}_3\text{CONH}^-$  in acetazolamide has a slight weakening effect, the effect of  $p$ - $\text{N}^+(\text{CH}_3)_3$  might be essentially steric. The enhanced binding, on the other hand, is very substantial in some cases. In addition to the compounds included in figure 6, it should be mentioned that the hexyl and benzyl esters of  $p$ -sulfamoyl benzoic acid are among the most powerful of all known carbonic anhydrase inhibitors (Beasley, Overell, Petrow, and Stephenson, 1958). Obviously, large hydrophobic substituents cause the greatest increase in inhibitory power. The series of  $p$ -halogen-substituted benzene sulfonamides are also of interest, and the effects of increasing size and polarizability of the substituents are striking.

The enhancement of inhibitory power must essentially be due to a decrease in the dissociation rate constant  $k_d$  or the mechanism involving a direct reaction of ionized inhibitor would not be possible. As indicated in figure 6 for  $p$ -iodobenzene sulfonamide, the enhancement of  $\log k_a$  is much less than that of  $pK_i$  (see also table II).

It has been clear for some time that the sulfonamide-metal interaction or, rather, the sulfonamide-anion site interaction is not sufficient to account for the high stability of these complexes. There has been accumulated recently a good deal of evidence for additional interactions. Optical rotatory dispersion and circular dichroism measurements on the cobalt and zinc enzymes (Lindskog, 1966b; Coleman, 1967c, 1968) have shown that not only are the  $d-d$  bands of the cobalt chromophore affected, but there are also Cotton effects due to the asymmetric binding of the ring system of the inhibitor. Chen and Kernohan (1967), in an elegant study of the fluorescence of bovine carbonic anhydrase and its complex with dansylamide, suggested that the sulfonamide is bound in a hydrophobic environment, possibly close to one or more tryptophanyl residues. Further support for the presence of tryptophan close

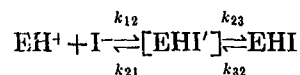
to the sulfonamide binding site has been obtained by means of phosphorescence measurements by Galley and Stryer (1968). Chen and Kernohan (1967) point out that compounds analogous to the inhibitors, but lacking the sulfonamide function, do not bind strongly, and it seems reasonable to assume that the major contribution to the stability is the "chelate effect" due to the multiple interactions with the enzyme.

The X-ray diffraction studies of Fridborg et al. (1967) have so far confirmed both the metal-sulfonamide bond and the ring-enzyme interaction. The size of the pocket or surface on the enzyme involved in this interaction must be considerable in view of the strong enhancement of the inhibition caused by the attachment of large hydrophobic side chains to a variety of sulfonamides.

#### ASPECTS OF THE MECHANISM OF THE REACTION BETWEEN SULFONAMIDES AND CARBONIC ANHYDRASE

As outlined in an earlier section, the pH-dependence and the magnitudes of the apparent second-order rate constants for the formation of carbonic anhydrase-sulfonamide complexes are compatible with a simple mechanism, namely a direct combination of  $\text{RSO}_2\text{NH}^-$  and the acidic form of the active site in an almost diffusion-controlled reaction. We have measured the recombination rates for a number of different types of sulfonamides, covering a large range of  $K_a$ -values, and the calculated second-order rate constants for this reaction  $k_a^o$  in no case significantly exceed  $1 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$  (table II). In view of the complex nature of the enzyme-inhibitor interaction, it is obvious that a large number of elementary chemical steps must be involved in the total reaction, and any scheme including only one step must be an oversimplification. So far, however, no direct or indirect evidence for intermediates has been obtained, and the detailed mechanism remains obscure. As the high stabilities are probably due to "chelate effects," it is likely that intermediate forms are never present in detectable amounts, but that the reaction will have to be described in terms of a rapid pre-equilibration or a steady state. Let us illustrate this point with an example. Assume that the ionized sulfonamide first reacts with the anion

binding site. The rate constants for this step should be independent of the rest of the molecule within reasonable limits set by steric and electrostatic effects. Stability is attained in a second, first-order step, in a reaction with the hydrophobic pocket as written schematically:



If the second step is very fast, the second-order step is rate limiting, and  $k_a^o = k_{12}$  and should vary only little as the sulfonamide is varied. The data in table II indicate that this is not the case. On the other hand, in a steady state,

$$k_a^o = k_{12}k_{23} / (k_{21} + k_{32}),$$

which might conceivably be compatible with the observed variations in  $k_a^o$ . It should be pointed out again that this is not the only possible mechanism (see Lindskog and Thorslund, 1968), and much more detailed kinetic measurements by means of rapid reaction techniques are required before a more complete description of the reaction can be made.

#### ASPECTS OF THE MECHANISM OF INHIBITION OF CARBONIC ANHYDRASE BY SULFONAMIDES

The essential feature of the inhibition by sulfonamides seems to be their binding to the metal ion, thus preventing the occurrence of the catalytic reaction. The details of how this is achieved are by no means established, but at least until firmer structural information has been obtained, the hypothesis that the inhibitor competes with an  $\text{OH}^-$  which participates in the catalytic process, is useful. Competition with anions, probably including  $\text{HCO}_3^-$  (Coleman, 1965; Leibman and Greene, 1967) seems well established. The question whether sulfonamides also interact with the  $\text{CO}_2$ -binding site has not been fully answered, however. Conventional inhibition studies have repeatedly given noncompetitive patterns with respect to  $\text{CO}_2$ . Kernohan's (1966a) results on the rates of sulfonamide inhibition suggested that a slow rate of equilibration between enzyme and inhibitor might be a complicating factor in this type of measurement. It was later shown that  $\text{CO}_2$  does influence the rate of binding of sulfonamides to

bovine zinc (Kernohan, 1966b) and cobalt (Lindskog and Thorslund, 1968) carbonic anhydrases to an extent which was compatible with a competition. The infrared studies of Riepe and Wang (1968) also showed a competitive effect. Competitive effects alone do not, of course, prove that there are common binding sites, and again there is a lack of structural information.

We have, however, done a few preliminary experiments to measure the effect of CO<sub>2</sub>-concentration on the rate of reaction of acetazolamide with carbonic anhydrase. In the bovine enzyme, there is definitely such an effect, but the results consistently indicate a mixed rather than a strictly competitive inhibition. Of more interest are the results on human carbonic anhydrase B where, at 25 C and pH 7.6, the apparent second-order rate constant was almost independent of the CO<sub>2</sub>-concentration in the range investigated (up to about 16 times the reported  $K_m$ ; Gibbons and Edsall, 1964). Acetazolamide binds much more weakly to this enzyme than to the bovine enzyme. At pH 7.6,  $K_i = 0.28 \mu M$ . The value of  $k_a = 6 \times 10^6 M^{-1} sec^{-1}$  is only slightly smaller than for the bovine enzyme, and, consequently, the dissociation rate constant  $k_d$  is larger, approximately  $1.8 sec^{-1}$ . This fact means that the time required for equilibration is smaller by a factor greater than 10, and it should be feasible to decide the type of inhibition by conventional means.

At present we may tentatively conclude that sulfonamides can perturb the binding of CO<sub>2</sub> more or less and that the binding sites are close but probably separate.

### CONCLUSION

Hopefully, it is clear from the above discussion that our knowledge of how sulfonamides inhibit and interact with carbonic anhydrase has increased greatly during recent years. Yet, many of the conclusions that we can draw now from the various types of experimental results must remain tentative until the detailed three-dimensional structure of the enzyme is known. With this structure available, however, sulfonamides will undoubtedly be even more useful as probes for the chemical nature of the active site of carbonic anhydrase.

### ACKNOWLEDGMENTS

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## DISCUSSION

**MAREN:** I would like to mention some preliminary experiments using ethoxzolamide and methazolamide. These have in common the fortunate fact that you do not have to worry about ionization outside the sulfonamide group. (Dr. Lindskog had a clever way of getting at the problem of the other proton in acetazolamide, but I think it is perhaps better to get away from the problem completely and have only to deal with the ionization of the sulfonamide group.) These two compounds have  $pK$ 's between 7 and 8, and so far we have not been able to see any difference in their  $K_i$ 's and esterase activity between the pH values of 6 and 9. At the moment I am unwilling to accept the idea that the drug has to be an anion to be active. (see note added in proof, p. 177.)

**COLEMAN:** I just might add that, of course, you can chelate it to the zinc atom, but in that case you may force off that proton. So that even if the drug starts out in neutral form, you may end up with a bound anion, in the same sense as in the formation of a coordination complex. It is probably much more complicated as far as the binding forces are concerned, but may result in displacement of a proton.

**MAREN:** You had to use a compleximetric method of titration?

**COLEMAN:** Yes. Because of the  $pK$  values of sulfonamides, titration becomes difficult. The inhibitor you are adding acts as a buffer itself, so that you end up being unable to do one of those difference titrations. I analyzed one or two points, and it looks as if the inhibitor displaces a proton or a hydroxide at high pH, but we really cannot be sure.

**MAREN:** What  $pK$  do you need to do this type of experiment?

**COLEMAN:** If the  $pK$  is anywhere within the region, you cannot do one of these titrations because you are essentially adding a buffer. You have to do it in a completely unbuffered solution and use a pH stat. It might work with one of your sulfonamides that does not have a second ionizing group, if it is tightly bound.

**CAPLOW:** I wonder if Dr. Lindskog could ex-

plain exactly what the reactive species are. Is the anionic form of the inhibitor combining with the  $ZnOH$  or the  $Zn(OH_2)$ ?

**LINDSKOG:** With the  $Zn(OH_2)$ .

**CAPLOW:** Then it should be pH independent?

**LINDSKOG:** No; not in the acidic region.

**CAPLOW:** According to Coleman, let us say from about pH 7.5 on.

**LINDSKOG:** Between the  $pK$  values; yes. It would be surprising if the  $pK$  of the sulfonamide group did not influence the binding at all, because we know it is bound very closely to the metal ions, so an absolutely flat curve would surprise me very much.

**POCKER:** The esterase activity of bovine carbonic anhydrase (BCA) rises continuously, but nonuniformly, with increasing pH. The pH rate profile appears to exhibit two sigmoid curves, one with an inflection around physiological pH (7-7.5) and the second, of much larger magnitude, with an inflection around pH 11 (fig. D-1). The dramatic increase in activity at high pH can also be documented in the enzymic hydration of  $CO_2$ , but not in that of acetaldehyde, because in the latter case the relative magnitude of the hydroxide ion term,  $k_{OH^-}(OH^-)$ , becomes dominant in comparison to the enzymic component. Dr. J. T.

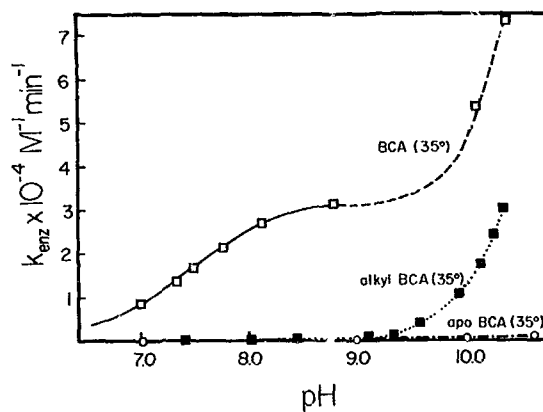


FIGURE D-1.—The bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate as a function of pH at 35°C; □, native enzyme (BCA); ■, carboxymethylated enzyme (alkyl BCA); ○, apoenzyme (apo BCA).

Stone, in our laboratories, has successfully carboxymethylated BCA with iodoacetate. His studies show that the carboxymethylation of a specific histidine residue in the enzyme is accompanied by an almost complete loss of enzymic activity around physiological pH and that the activity remaining at higher pH is, in fact, the threshold of the dramatic activity which occurs at pH values higher than 10. Apparently the  $pK_a$  of the group associated with the high pH inflection is but little affected in the carboxymethylated enzyme (referred to in the figure as "alkyl BCA"). The pH-rate profile for the apoenzyme is also given in figure D-1. It will be noticed that the apoenzyme has essentially no activity in the pH interval 6-10.5, although at a still higher pH some activity, but no turnover, was detected. The enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate was also investigated by Dr. Stone at four different temperatures ranging from 15 to 45 C (fig. D-2). By determining the formal Michaelis-Menten parameters at several pH values at each temperature, he was able to show that the turnover number  $k_2$  depends, at physiological pH, upon a group whose enthalpy of ionization is 6.9 kcal mole<sup>-1</sup> and that in D<sub>2</sub>O solvent the  $pK_a$  of this group is 0.5 unit higher than in H<sub>2</sub>O solvent (Pocker and Stone, 1968b). Dr. Stone has additionally shown that inhibition by moderate inhibitors ( $K_i \sim 10^{-4}$  to  $10^{-3}$  M at pH 7.5) decreases with pH as if dependent on two closely

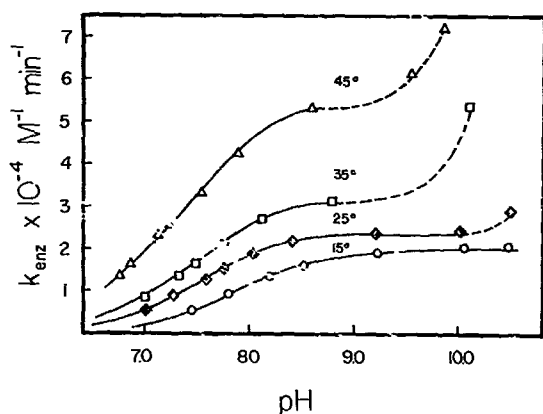


FIGURE D-2.—The bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate as a function of pH at four different temperatures; 10 percent (v/v) acetonitrile,  $\mu = 0.09$ ,  $[BCA] = 3 \times 10^{-5}$  M,  $[p-NPA] = 2 \times 10^{-4}$  M; O, at 15 C;  $\blacklozenge$ , at 25 C;  $\square$ , at 35 C;  $\triangle$ , at 45 C.

situated cationic centers in the enzyme, one of  $pK \sim 7.3$  and the other of  $pK_a \sim 11$ . Weaker inhibitors tend to reflect primarily the first inflection, whereas very potent inhibitors tend to emphasize principally the latter inflection (Pocker and Stone, 1968a). In publications from these laboratories over the last 4 years, we have provisionally ascribed a  $pK_a$  of about 11 to the transformation of  $-ZnOH_2$  to  $-ZnOH$  in the enzyme, and have attributed the inflection around neutral pH to the ionization of an imidazolium ion, the basic form of which promotes the hydrolysis process by removing a proton from the zinc-bound water. In this connection I might add that a number of model zinc-aquo complexes have  $pK_a$  values higher than 10, possibly around 10.5 or 11.

**BRADBURY:** I would like to describe a series of experiments in which iodoacetate has been used to study the active site of human erythrocyte carbonic anhydrase B. Iodoacetate reacts rapidly with the 3' nitrogen of only 1 out of 11 histidines of human enzyme B to produce a carboxymethyl enzyme that is almost completely devoid of activity. Several lines of evidence indicate that the reaction occurs at the active center:

- (1) Loss of catalytic activity occurs at the same rate as the appearance of carboxymethyl-histidine.
- (2) Iodoacetate inactivates the enzyme irreversibly only while bound as a reversible inhibitor.
- (3) Sulfanilamide, a potent inhibitor of the enzyme, protects against irreversible inactivation.
- (4) The native conformation of the enzyme is required for rapid alkylation. In the presence of 8 M urea the rapid reaction is no longer observed.

Furthermore, no gross conformational changes accompany carboxymethylation. The  $s_{20,w}$  value and the ORD are the same for the native and modified enzymes.

A study of papain peptides of <sup>14</sup>C-labeled carboxymethyl carbonic anhydrase revealed that at least 73 percent of the radioactivity lay in a single peptide, the sequence of which is Thr-3-CMHis-Pro-Pro-Leu. Therefore, the evidence strongly indicates that a histidine residue is indeed at the active site.

In an attempt to assign a function to the reactive histidine, I have studied both the reversible and irreversible interactions of carbonic anhydrase



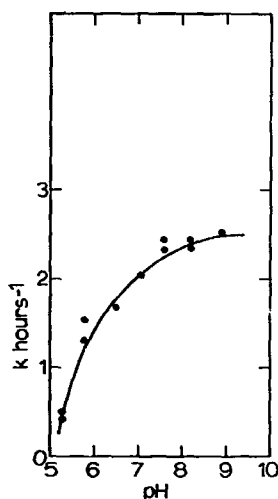


FIGURE D-3.—The pH dependence of  $k$ , the rate of the irreversible reaction. First-order rate constants were calculated from the half times of inactivation at iodoacetate concentrations that saturated the enzyme. At high pH, where saturation could not be achieved, the rates at saturation were calculated from the observed half times and the  $K_1$  values in figure D-2. Esterase activity assays were performed on diluted aliquots. Reactions performed at pH 5.3 to 7.1 were assayed at pH 8.2. The residual activity at this pH was 7 percent. Activity after reaction at pH 8.2 and 8.9 was assayed at pH 9.6. The residual activity at pH 9.6 was 11 percent. Inhibition by reversibly bound iodoacetate was less than 3 percent in all cases and was neglected. At pH 5.3, the protein was unstable and assays were performed after removal of precipitated protein by centrifugation. Activities relative to those at zero time were calculated accordingly. (Reprinted with permission from *J. Biol. Chem.*)

with iodoacetate as functions of pH. Figure D-3 shows a curve of the pH dependence of the irreversible reaction at saturation with iodoacetate as a function of pH. The data indicate that a group with a  $pK$  of 5.8 must be in the basic form for the reaction to occur. This  $pK$  refers to the group in the enzyme-inhibitor complex.

Figure D-4 shows the pH dependence of the reversible inhibition. It is clear from the data that the binding of iodoacetate is controlled by a group on the enzyme with a  $pK$  of 6.8–6.9. This group must be in the acid form for binding to occur. Enzyme to which iodoacetate is reversibly bound is completely inactive.

The interpretation of these results has been a matter of much concern. If the  $pK$  at 5.8 indicated by the irreversible reaction is that of the reactive histidine, then in all probability another group

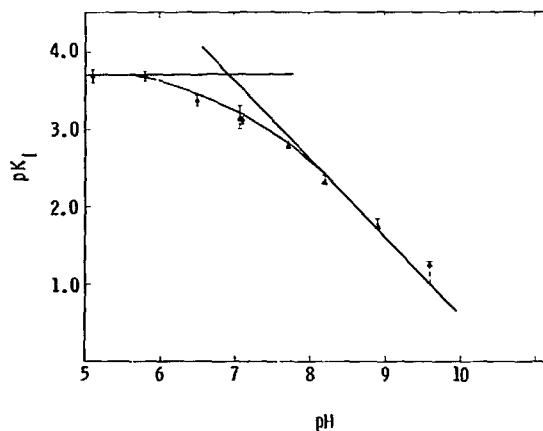


FIGURE D-4.—The pH dependence of binding.  $K_1$  values were calculated from the esterase activity observed at various concentrations of iodoacetate, assuming noncompetitive inhibition. All assays were performed at 25 C in 1 percent acetone. The buffers were as follows: O, 0.05 M bis-tris sulfate;  $\Delta$ , 0.05 M tris sulfate, with the exception of the experiment at pH 9.6 which was performed in 0.056 M veronal. The ionic strength of all buffer solutions was adjusted to 0.075 with  $K_2SO_4$ . The protein was stable throughout the entire pH range included in the diagram. (Reprinted with permission from *J. Biol. Chem.*)

controls binding. Because the  $pK$  of the group which controls binding is very nearly the same as the  $pK$  of the group which controls activity, determined from the pH-rate profile, and because the binding of iodoacetate occurs at the active site, it is tempting to postulate that one group controls both binding and activity. This group is probably not the reactive histidine and may very well be a zinc-bound water molecule at the active site.

Consequently, a specific function cannot yet be assigned to the reactive histidine. The presence of residual activity in the irreversibly inactivated enzyme but not in the reversibly inhibited enzyme indicates that the histidine does not play an essential role in catalysis. We must await the results of the crystallographic studies now in progress before we can visualize the events that occur at the active site during enzyme action.<sup>1</sup>

**COLEMAN:** We are worried about conformational changes in the molecule at such high pH values, because this may change things considerably.

<sup>1</sup> This work is described in detail in the thesis of S. L. Bradbury, "The Active Site of Carbonic Anhydrase" (Harvard, 1968), and in two papers in *J. Biol. Chem.*, **244**: 2002, 2010, 1969.

**POCKER:** I have no doubt, but still we find the increase in activity.

**COLEMAN:** Have you been able to reactivate that apoenzyme at high pH? One might think that the apoenzyme is a little more susceptible to conformational changes than the zinc enzyme, so it might be important to try to reactivate the latter and show this is possible.

**POCKER:** This is a good suggestion, but it is difficult to introduce zinc ions at high pH without forming a zinc hydroxide precipitate.

**EDSALL:** In connection with Laurent's beautiful studies on the effects of high pH, there is a tremendous difference in stability at high pH between human carbonic anhydrase B and the human C enzyme, which Laurent and her collaborators were the first to show is far less stable when you go to about pH 10.5. Riddiford and I extended these studies in more detail. I think that bovine B is like human C in this respect, although I am not sure.

**COLEMAN:** That is my impression also.

**CAPLOW:** How do you follow the reaction at a pH about 12?

**POCKER:** By stopped flow.

**CAPLOW:** How fast are the enzymatic reactions?

**POCKER:** The enzymatic reactions fortunately are much faster than the hydroxide-ion component—the OH<sup>-</sup> effect becomes terrific when you increase pH. We would never have been able to observe it if it were not for the fact that in the basic region the enzyme starts by being twentyfold better than an hydroxide ion and finishes by being 200-fold better.

**LINDSKOG:** I would like to comment on the zinc hydroxyl *pK*. It is true that the *pK* of the first ionization of inorganic zinc in water is around 9 or so, and that for a complex with more negative ligands, we would expect that the *pK* would be higher. Most of the complexes that have been studied are octahedral complexes in water solution. Of course, the zinc in the enzyme is definitely not octahedral. In the enzyme, the area around the zinc is different from pure water. The anions bind differently to this site, and a *pK* of 7 is not more abnormal than a *pK* of 11 or 12, so it might vary either way.

**POCKER:** I would agree with you if it were

not for the fact that we repeated your cobalt values, and the inflection at pH 7 is identical. It is a function of the substrate; for the same substrate, cobalt and zinc show the same inflection. The cobalt hydroxide and the zinc hydroxide, in a hydrophobic environment of any kind, could shift from a higher to a lower *pK* value. However, it would be surprising if they shifted to an identical point, would it not?

**LINDSKOG:** Considering the strange binding of other anions to this site, it seems that the binding of weak ligands is determined more by the protein, by the Hofmeister series, if you wish, than by metal ions. Or it is even possible that this same hydroxide that we are talking about all the time is not directly bound to the metal, that there may be another water molecule in between. I am not too sure it is necessary to expect different *pK* values for the zinc and cobalt enzymes.

**POCKER:** Of course, we believe that in the neighborhood of the zinc hydrate there is an imidazolium group which helps to bind ions—perhaps I should say a positively charged group which helps bind ions. This, I believe, is what causes the Hofmeister series and causes the weaker inhibitors to be pulled in.

**COLEMAN:** It is a difficult and complex problem to extrapolate from the data on the various metal hydroxides in aqueous solution because the number of species is infinite. To extrapolate from there to a protein complex that binds a single water molecule is a most uncertain business. The extrapolation from the model substance is likely to be very poor.

**POCKER:** Allow me just to say that had the extrapolation been easy, we would not have gone to the trouble of modifying the enzyme to prepare the alkylated BCA, and we would not have done the temperature studies in such great detail. Also, we would not have prepared the apoenzyme.

**EDSALL** (on behalf of Pierre Henkart, Biological Laboratories, Harvard University):

#### CHEMICAL MODIFICATION OF CARBONIC ANHYDRASE B BY FLUORODINITROBENZENE

In investigating the chemical modification of human carbonic anhydrase B by Sanger's reagent,

1-fluoro-2,4-dinitrobenzene (FDNB), we were surprised to find that this reagent is a substrate for the enzyme (Henkart, Guidotti, and Edsall, 1968). Although it is not as powerful a catalyst for this hydrolysis as for that of several other substrates, we continue to be impressed by this enzyme's versatility.

FDNB inactivates carbonic anhydrase B rapidly at neutral pH in a reaction that is partially blocked by inhibitors such as sulfanilamide, dinitrophenol, and  $\text{H}_2\text{PO}_4^-$ . In agreement with Whitney, Nyman, and Malmström (1967), we find that chloride ion does not block this inactivation although it does inhibit enzymatic activity.

The major product of a short incubation with FDNB is mono-DNP-carbonic anhydrase B, which can be separated from the unreacted enzyme on a BioRex-70 column. Amino acid analysis of this derivative shows the loss of one histidine and the appearance of one residue of Im-DNP-histidine. This histidine in carbonic anhydrase is about 100 times more reactive toward FDNB than  $\alpha$ -N-acetyl-histidine under similar conditions.

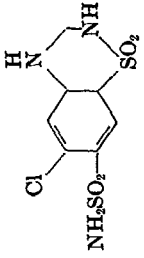
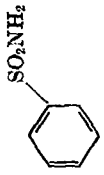
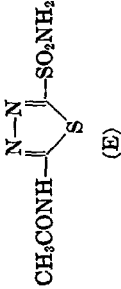
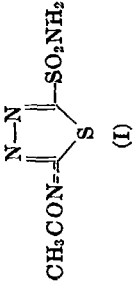
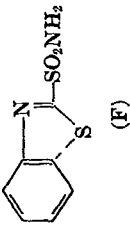
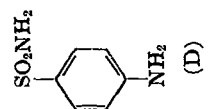
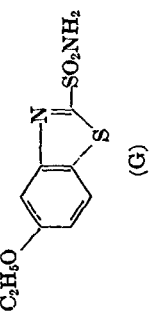
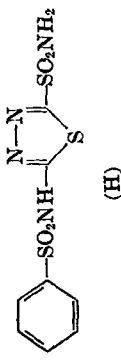
The DNP enzyme retains some of its activity. Measured by the Wilbur-Anderson assay for  $\text{CO}_2$  hydration, this activity is 2 to 4 percent of that of the unreacted enzyme. Experiments using the hydrolysis of *p*-nitrophenyl acetate show a retention of about 70 percent of the activity of the normal enzyme. Although we have not yet had a chance to examine the kinetic parameters of this residual activity, the dinitrophenylated enzyme appears quite different from other modified carbonic anhydrases in which loss of the two activities is more nearly parallel.

The DNP group can be removed from Im-DNP-histidine by thiolysis (Shaltiel, 1967). When incubated under mild conditions with  $\beta$ -mercaptoethanol, the DNP group is cleaved from the DNP enzyme, yielding the fully active enzyme which chromatographs normally.

Work is currently in progress to isolate a peptide containing this histidine, to see if it is the same one which is carboxymethylated rapidly. The latter was shown to be in the sequence Thr-His-Pro-Pro-Leu (Bradbury and Edsall, 1968). Initial results with DNP-carbonic anhydrase B suggest that the reactive histidines are not the same because the impure peptides we have isolated up to now contain little proline.

**MAREN:** I do not have any comprehensive theory to offer about the activity of the sulfonamide drugs—this will await further work. I only want to discuss what type of compound gives what type of activity, in a purely descriptive manner. The molecular bases for these differences are rather incompletely known. We have an enormous range of inhibitors, ranging from drugs with a  $K_i$  of about  $10^{-4}$  *M* to those of  $K_i$  of  $10^{-9}$  *M*. I will illustrate the characteristics and some of the properties of a few of the drugs you are using (table D-I). The weakest inhibitors are the aliphatic sulfonamides. The simplest compound would be something like (A); these are barely active. At one time people thought they were not active at all, but that the slight activity might be due to contamination; e.g., if there were a slight amount of benzene remaining from the synthesis. They are probably active in the  $10^{-3}$  to  $10^{-4}$  *M* range. Drugs with  $K_i$  on the order of  $10^{-5}$  *M* are represented by the thiazides, which have a structure of (B) (the only reason I am including these is that they have a different role in medicine). This is the structure of hydrochlorothiazide, an important diuretic. But its diuretic activity is due to a 1,3 configuration which has a special action on the kidney, more or less by accident. It has a sulfonamide group, which is necessary for high diuretic activity, so by chance it is a carbonic anhydrase inhibitor, although that is not its main pharmacological activity. In the  $10^{-6}$  *M* range, we have simple benzene sulfonamide (C). Dr. Lindskog, Dr. Kernohan and I agree that this is the simplest for us to use in this activity range. The drug that is most ordinarily used is sulfanilamide (D), but that amino group has nothing to do with this subject at all—it just makes it an antibacterial substance. Dr. Kernohan told me yesterday that you introduce certain problems simply because of the reactivity and insolubility conferred by the aryl amine. The important rule here is that all carbonic anhydrase inhibitors have this unsubstituted sulfonamide group; any alteration here utterly destroys activity. The simplest example: if this group becomes  $-\text{SO}_2\text{NHCH}_3$ , the activity falls immediately to zero. In the  $10^{-8}$  range we have acetazolamide (E). The big advance that was made in this field as far as drugs go was made by my former colleague, Richard O. Roblin,

TABLE D-I.—Sulfonamides Active Against Carbonic Anhydrase <sup>a</sup>

| $K_i, M$ | $10^{-4}$   | $10^{-5}$  | $10^{-6}$   | $10^{-8}$   | $10^{-9}$  |
|----------|---|--|---|---|--|
|          | <p>CH<sub>3</sub>SO<sub>2</sub>NH<sub>2</sub><br/>(A)</p> | <br>(B) | <br>(C)  | <p>CH<sub>3</sub>CONH-<br/>(E) </p> <p>CH<sub>3</sub>CON-<br/>(I) </p> | <br>(F)   |
|          |   |  | <br>(D) | <br>(G)   | <br>(H) |

CO<sub>2</sub><sup>a</sup> See Maren and Wiley, 1968.

of the American Cyanamid Co., when he discovered that heterocyclic molecules are much more active than homocyclic. There are many compounds studied, such as benzothiazole sulfonamide and/or thiadiazoles (the structure of the ring in acetazolamide). There is a curious aspect in the way groups influence activity. If there is an acetyl amino (acetamido) compound, activity is increased. This is true both in the sulfonamide series and in the acetazolamide series; acetylsulfanilamide is a much more active inhibitor than sulfanilamide, and acetazolamide is more active than des-acetyl-acetazolamide. It is as if activation of the proton in the former had an effect—acetylation causes it to be a stronger acid. In the latter, acetylation may have some effect on resonance in the ring, with a manifestation on the  $\text{SO}_2\text{NH}_2$  group. Going one log further, a somewhat different type of ring, the benzothiazole sulfonamide, is the most powerful. Benzothiazole-2-sulfonamide, which was another early Roblin compound, is shown (F). The drug that many of us have used, simply because it is commercially available, is the 6-ethoxy derivative ethoxylamide (G). Structure (H) is CL 11,366 (benzolamide) which is a relatively strong acid having interesting pharmacological properties because of differences in distribution from examples (E), (F), and (G).

It is evident then that any unsubstituted sulfonamide is active; this is one of the tightest rules in structure-action relation in pharmacology. Curiously enough, only sulfonamides are active. There is no other class of organic compounds that has activity against carbonic anhydrase. This activity extends to all mammalian carbonic anhydrases with the sole exception of rat-liver carbonic anhydrases. All other mammalian carbonic anhydrases and all vertebrate carbonic anhydrases are inhibited by these drugs; that of rat liver is a complete puzzle. As we heard yesterday, the bacterial enzyme in the *Neisseria* family is also inhibited. Plant enzymes are not inhibited, or to no significant extent. The difference in plant enzyme would be on the order of 10 000 times less inhibited.

**POCKER:** You state that when one alkylates the  $\text{SO}_2\text{NH}_2$  group, the inhibitor activity goes to zero. Our experience is that it falls by a factor of a thousand.

**MAREN:** But then you have to worry about residual, one-tenth of a percent, impurity. When you are dealing with such enormously active compounds, the problem of chemical purity becomes important.

**LINDSKOG:** Of course in the paper by Whitney, Fölsch, Nyman, and Malmström (1967) it was obvious that in human enzyme B, some of the modified chlorothiazides can act as inhibitors of that enzyme. The same modified inhibitor in the bovine enzyme was not an inhibitor. There was an inhibition, but you can distinguish kinetically if you have a small amount of unmodified inhibitor in the preparation or if you have weak inhibition by the modified inhibitor.

**MAREN:** I think it is important in that type of analysis to get very pure compounds.

**LINDSKOG:** I would also like to comment on the relation between structure and function. I did not go into that yesterday because there is a very good review I would like to draw attention to by Bar (1963), a French author, who covers this in great detail; e.g., CL 11,366 (compound (H) in table D-I). In view of hydrophobic interactions, its strong interaction is probably not due to the acidity of this group, but to the extra benzene ring that you add to the structure.

**COLEMAN:** I just might add that the extra benzene ring in CL 11,366 induces tremendous Cotton effects, apparently causing it to interact in some way with the surface of the enzyme.

**LINDSKOG:** In fact, the strong inhibition of acetazolamide is very much due to its acidity. It is not very hydrophobic.

**MAREN:** But ethoxzolamide is extremely hydrophobic and poorly acidic ( $pK_a=8.1$ ).

**LINDSKOG:** Chlorothiazide was the big exception in the table I showed yesterday. It was very slow to react with the enzyme, although it is a weak inhibitor. My guess is that there might be steric effects involved.

**MAREN:** That is quite true: the drugs with this thiazide configuration (B) are slow and they require a different setup in the laboratory to achieve equilibrium. You think that it is the hindering effect of the chlorine? Benzthiazide does the same thing—they all have the ring chlorine. I believe the congeners without the chlorine may be available from Merck, Sharp & Dohme. It would be interesting to test this idea.

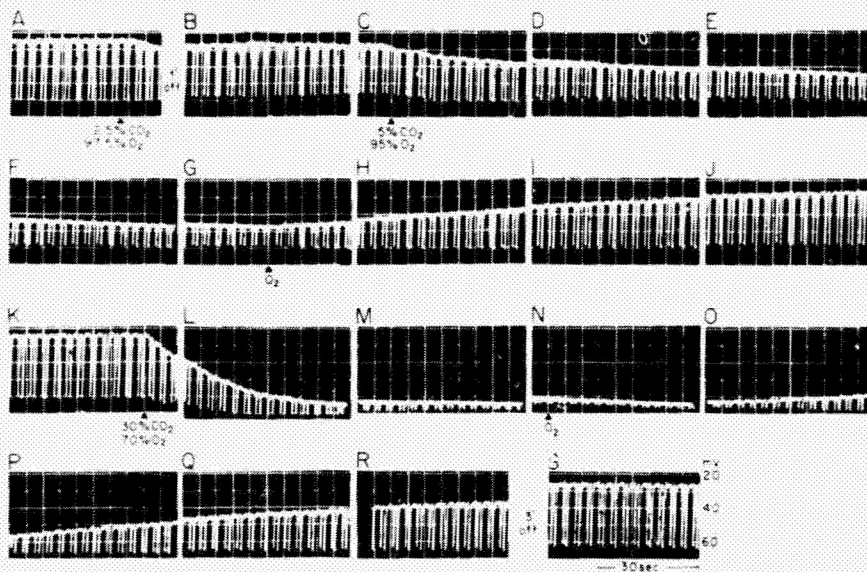


FIGURE D-5.—Effect of CO<sub>2</sub> on the horizontal cell membrane potential (isolated preparation of fish retina). Progressive increase of CO<sub>2</sub> concentrations in a moist chamber (2.5 percent CO<sub>2</sub> at A, 5 percent at C and 30 percent at K) produced fast hyperpolarizations of the resting potential accompanied by the corresponding amplitude reduction of the light-induced S-potentials (downward deflections). Readmission of pure O<sub>2</sub> (arrows at G and N) gave slow recovery. Membrane potential scale in mV and time scale are indicated at record S. (From Negishi and Svastichin, 1966.)

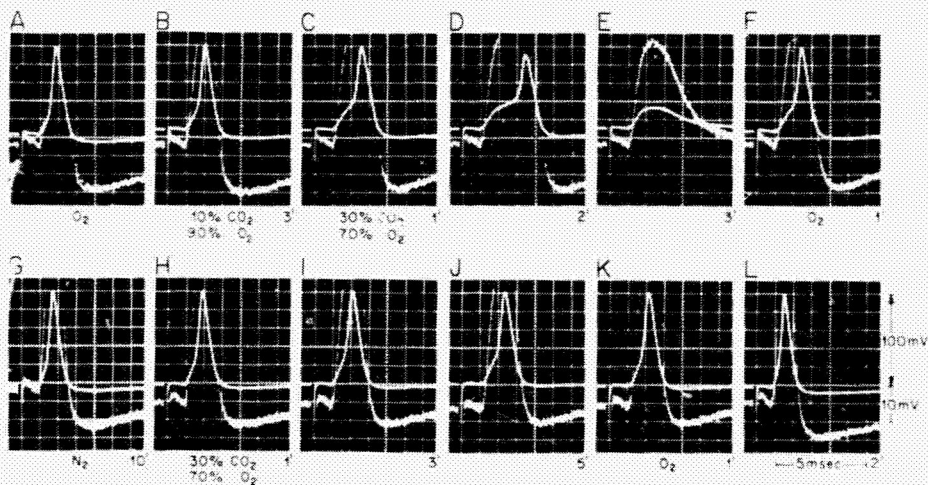


FIGURE D-6.—Effect of CO<sub>2</sub> on the neuronal membrane potential and its cancellation following long-term anoxia (isolated preparation of frog dorsal root ganglion). The action potential of the ganglion cell was evoked by sciatic nerve stimulation at a frequency of 1 hertz. The spike recordings shown were obtained by superimposing five sweep tracings. Upper tracing is a dc recording (calibration 100 mV at the right of record L). Lower tracing is an ac recording with higher amplification showing the hyperpolarizing afterpotential (calibration 10 mV also at the right of record L). Time given in minutes at the right corner below each record indicates time elapsed after change of gas medium in the moist chamber. Records A to F: Before anoxia the soma spike was blocked with a slight depolarization 3 minutes after exposure to 30 percent CO<sub>2</sub> (E). Records G to L: After 10 minutes anoxia (G), the same exposure did not block the spike potential and caused a negligible resting potential change. Similarly, the CO<sub>2</sub> effect was negligible in preparations dissected from Diamor-treated frogs. (From Negishi and Svastichin, 1966.)

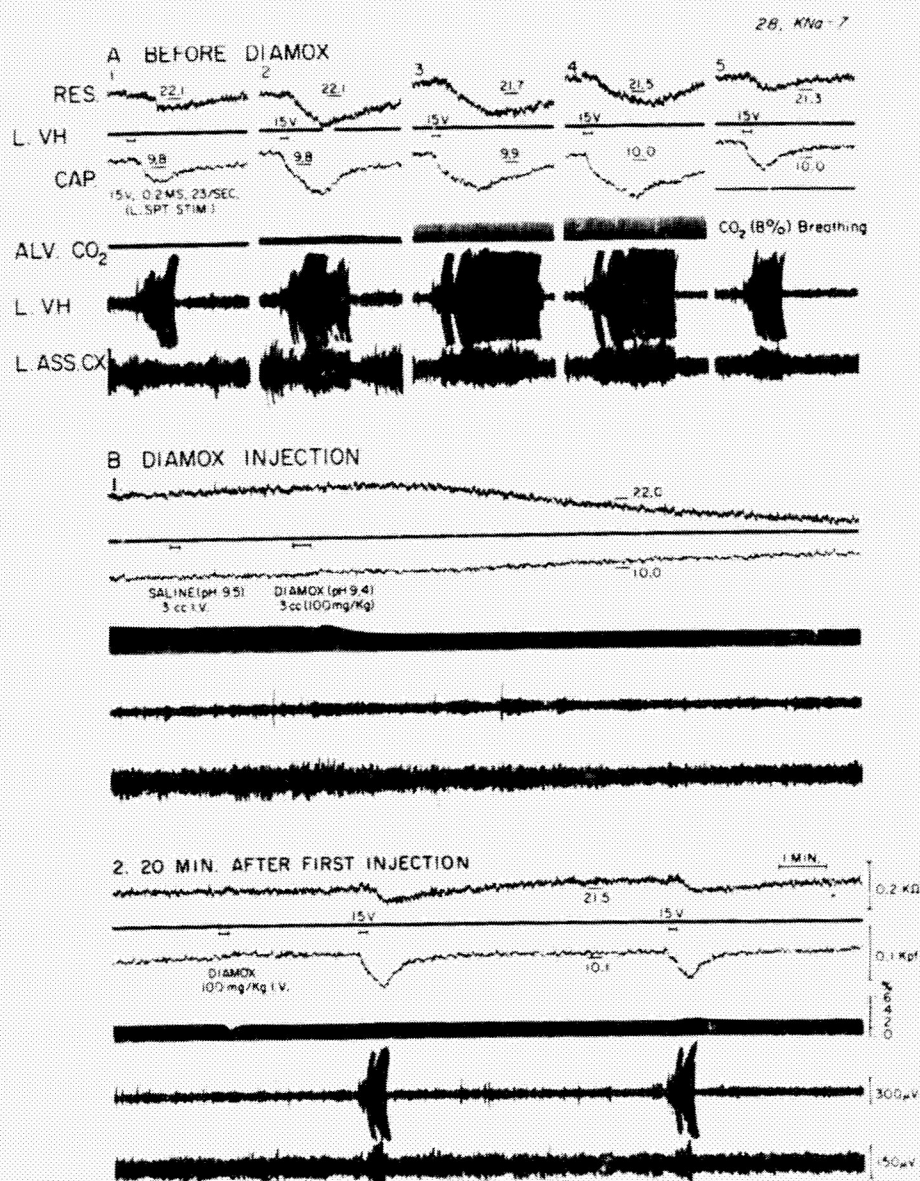


FIGURE D-7.—Simultaneous recordings of subcortical impedance, alveolar  $P_{CO_2}$  and electrical activity, showing the dependence of cerebral excitability on  $P_{CO_2}$  (acute rat preparation with artificial breathing). Record A<sub>1-5</sub>: Before Diamox, subcortical impedance changes associated with seizure discharges at different alveolar  $P_{CO_2}$  levels. Record B<sub>1</sub> and 2: Impedance changes following intravenous injections of Diamox (100 mg/kg each time) and reduced duration of seizure bursts after Diamox. The alveolar  $P_{CO_2}$  (ALV.  $CO_2$ ) was altered by manipulating the respirator volume and by breathing 8 percent  $CO_2$  in the air. Seizure discharges were elicited by electric stimulation to the left septal region (L. SPT) with a constant parameter and recorded from the left association cortex (L. ASS. CX) and the left ventral hippocampus (L. VH) simultaneously with impedance changes (RES: resistive compartment of impedance; CAP: capacitive compartment of impedance). The duration of induced seizure burst was longest at a ALV.  $CO_2$  of about 2.5 percent (A<sub>3</sub>), indicating that the cerebral excitability was maximal at this  $P_{CO_2}$ . Calibrations are given at the end of record B<sub>2</sub>. (From Negishi, Kado, and Adey, unpublished results.)

**NEGISHI:** Thank you, Dr. Edsall, for giving me the time to comment, from the neurophysiological aspect, on the effects of CO<sub>2</sub> and Diamox on nervous activity.

Carbonic anhydrase (CA) is present in high concentration in neuroglial cells and practically absent from neurons (Giacobini, 1961; Korhonen and Korhonen, 1965; and Parthe, personal communication). In the fish retina, Parthe (personal communication) has shown the presence of CA in the Müller fiber which is a glial cell and in the horizontal cells which are classed as "short-axon cells." The above facts have to be considered in the interpretation of the electro-physiological findings presented below.

Figure D-5 shows a drastic hyperpolarizing effect of CO<sub>2</sub> on the membrane potential, recorded from a horizontal cell in isolated fish retina which was kept in a moist chamber (see fig. 4 of Negishi and Svaetichin, 1966). Downward potentials are responses (*S*-potentials) to light flashes. As CO<sub>2</sub> tension in the chamber is increased, the membrane potential is markedly hyperpolarized; this is accompanied by a diminution of light-induced potentials.

This CO<sub>2</sub> effect is almost negligible in retinæ isolated from Diamox-treated fish. Further, the membrane potential of the horizontal and amacrine cells is highly aerobic dependent (Negishi and Svaetichin, 1966a); their function stops immediately without O<sub>2</sub>.

Figure D-6 shows the small depolarizing effect of CO<sub>2</sub> on the membrane potential recorded from a neuron (frog dorsal root ganglion cell) (Negishi and Svaetichin, 1966a and b). High concentrations of CO<sub>2</sub> block the action potential evoked by electric stimulation of the sciatic nerve. The neuronal membrane potential is resistant to anoxia, and the action potential could be evoked without O<sub>2</sub> for at least 30 to 40 minutes. However, the depolarizing and action potential-blocking effect of CO<sub>2</sub> was abolished in the asphyxiated preparation. This was also true for preparations isolated from Diamox-treated frogs.

The neurons of the dorsal-root ganglion are surrounded by satellite glial cells, which contain carbonic anhydrase. The results, presented in figure D-6, can be understood in the following manner: In the aerobic state, CO<sub>2</sub> may reduce

the neuronal excitability through the glial CA. If the CA activity is inhibited by Diamox, or the glial function is terminated by anoxia, the CO<sub>2</sub> effect is no longer observed on the neuronal membrane potential.

Furthermore, it was found in cat brain experiments that changes in the alveolar P<sub>CO<sub>2</sub></sub> and intravenous administration of Diamox rapidly influenced brain tissue impedance and cerebral excitability (fig. D-7). These results are possibly related to the function of CA-containing cells in the grey matter.

The idea concerning the glial control on neuronal excitability was suggested by Svaetichin, Laufer, Mitarai, Fatehchand, Vallecalle, and Villegas (1961) and Galambos (1961); possibly short-axon cells, which contain CA in the fish retina, might be involved in similar control mechanisms.

#### CARBONIC ANHYDRASE FROM THE CORN EAR WORM

**WHITNEY:** Following the suggestion of Dr. Lawrence Edwards at the Agricultural Research Service in Tifton, Ga., some of us in Professor Edsall's laboratory attempted to purify and characterize carbonic anhydrase from the moth, *Heliothis zea*, of the corn ear worm. Although these attempts were not very successful, we did learn a few things of interest.

We obtained a generous supply of male pupae from Dr. Edwards and tried to isolate carbonic anhydrase from the testes. The activity in a crude homogenate of testes from 5000 pupae corresponds to 1 to 10 milligrams of enzyme assuming a specific activity similar to other carbonic anhydrases.

The first step in purifying the crude homogenate was an extraction with toluene. After centrifugation, most of the activity was recovered in the toluene layer. This result suggested that the enzyme might be associated with membranes or with other hydrophobic material. In the second step we tried to remove the enzyme from the toluene layer. The use of dilute buffer resulted in turbid solutions of low activity, whereas 0.33 *M* potassium sulfate dissolved most of the enzyme in a clear solution.

Both fractions were chromatographed on Sepha-



dex G-100. Enzyme that had been extracted in dilute buffer and run in 0.05 *M* sodium phosphate, pH 6.9, was completely excluded from the resin. When 0.33 *M* potassium sulfate was included during extraction and chromatography, the fraction with CO<sub>2</sub>-hydrating activity eluted in a region consistent with a molecular weight of 80 000 to 90 000. If the enzyme is normally made up of subunits, then it may be dissociating in potassium sulfate to give a molecular weight which is lower than the true value. Alternatively, potassium sulfate may lower the apparent molecular weight by promoting dissociation from other cellular material. There is a strong possibility that the enzyme is still associating with some cellular material in potassium sulfate, in which case the true molecular weight will be less than 80 000.

Acetazolamide and azide are good inhibitors of the CO<sub>2</sub>-hydrating activity of the enzyme measured by the Wilbur-Anderson technique. Acetazolamide has a *K<sub>I</sub>* of about 0.03  $\mu$ *M* compared to *K<sub>I</sub>* values of 0.01 and 0.4  $\mu$ *M* for human C and B carbonic anhydrases. The *K<sub>I</sub>* for azide inhibition of the moth enzyme is about 60  $\mu$ *M*, compared to about 30  $\mu$ *M* for human carbonic anhydrase B. These results indicate that the enzyme from *Helethis zea* is a typical carbonic anhydrase with the ability to bind sulfonamides tightly. The inhibition by azide strongly suggests that, like other carbonic anhydrases, it contains a metal atom at the active site.

#### NOTE ADDED IN PROOF

Since the conference, we have studied catalytic dehydration of HCO<sub>3</sub><sup>-</sup> by stop-flow, and its inhibition by methazolamide (I, table D-1) whose *pK<sub>a</sub>* = 7.6. *K<sub>i</sub>* was the same at pH 6.6 as at 7.6. This, together with the maximal activity of ethoxzolamide (G; *pK<sub>a</sub>* = 8.1) and CL 11,366 (H; *pK<sub>a</sub>* = 3.2) lead me to conclude that ionic charge does not affect activity in this system. (Booher, K. R., and T. H. Maren, *Mol. Pharmacology*, in press, 1970.)

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*SESSION IV*

**Metabolic Processes Involving  $\text{CO}_2$  or  $\text{HCO}_3^-$**

*Chairman: A. B. HASTINGS*

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## Introductory Remarks

A. BAIRD HASTINGS

*University of California, San Diego*

I am a poor substitute for Joseph Priestley, who should be here to preside over this meeting. However, I take the liberty of conveying his greetings and of quoting from his observations on fixed air, published in 1774, in "Experiments and Observations on Different Kinds of Air." I understand that this conference is devoted to "Fixed Air"—and that this particular session is on "Fixation of Fixed Air"—or in the 20th-century vernacular, "Metabolic Processes Involving  $\text{CO}_2$  or  $\text{HCO}_3^-$ ."

On page 49, Mr. Priestley reports:

I have been so happy, as by accident to have hit upon a method of restoring air, which has been injured by the

burning of candles, and to have discovered at least one of the restorations which nature employs for this purpose. It is *vegetation*.

He then relates the details of the large number of experiments that he carried out to validate this conclusion. I shall quote one of these experiments:

... I took a quantity of air, made thoroughly noxious, by mice breathing and dying in it, and divided it into two parts: one of which I put into a phial immersed in water; and to the other [which was contained in a glass jar, standing in water] I put a sprig of mint. This was about the beginning of August 1771, and after eight or nine days, I found that a mouse lived perfectly well in that part of the air, in which the sprig of mint had grown, but died the moment it was put into the other part of the same original quantity of air. . . .

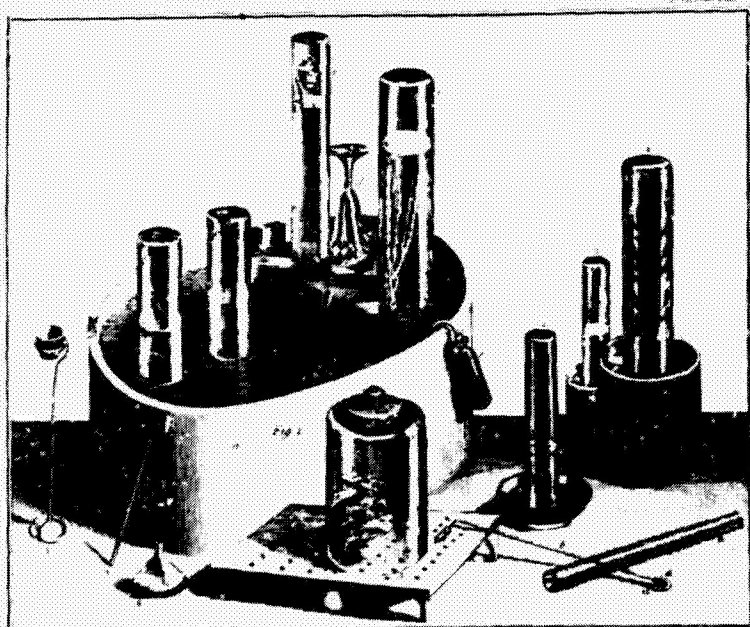


FIGURE 1.—Frontispiece of a work by Joseph Priestley showing his apparatus.

EXPERIMENTS  
AND  
OBSERVATIONS  
ON DIFFERENT KINDS OF  
A I R.

By JOSEPH PRIESTLEY, LL. D. F. R. S.

Printed and Sold by J. JOHNSON, No. 72, in St. Paul's Church-yard, LONDON.

L O N D O N :

Printed for J. JOHNSON, No. 72, in St. Paul's Church-yard.

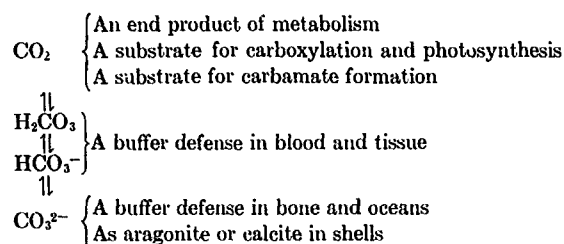
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In his Harvey lecture, Dr. Ephraim Racker immortalized this experiment as the first of the cycles by naming it "the Priestley cycle." Figure 1 portrays the apparatus that Mr. Priestley used in his experiments, including his mice.

We have traveled far in the last 200 years in pursuit of carbon dioxide—both inside and outside the body—but there are still exciting things to be learned about its adventures in its several chemical disguises.

I have had much pleasure following CO<sub>2</sub> for half a century: Its solubility, its hydration, its ionizations, its reactions, both organic and inorganic, have, at one time or another, been of interest to me and my laboratory colleagues. If I had another 50 years of lab work ahead of me, I would still be learning new things about CO<sub>2</sub>.

The following is the case for carbon dioxide:



Joseph Priestley said it so well in his preface:

As for myself, I find it absolutely impossible to produce a work . . . that shall be anything like complete. . . . In completing one discovery we never fail to get an imperfect knowledge of others, of which we could have no idea before; so that we cannot solve one doubt without creating several new ones.

Let us see what old doubts are solved and new ones created today.

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## The Species of "CO<sub>2</sub>" Utilized in the Carboxylation of P-enolpyruvate and Pyruvate<sup>1, 2</sup>

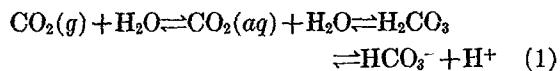
T. G. COOPER, T. T. TCHEN, H. G. WOOD, C. R. BENEDICT, AND D. L. FILMER

*Purdue University, Wayne State University, Case Western Reserve University,  
and Texas A&M University*

Previous studies with propionyl-CoA carboxylase and with P-enolpyruvate carboxylase using <sup>18</sup>O-labeled bicarbonate have indicated that bicarbonate is the reactive species in these fixations of "CO<sub>2</sub>." We have investigated the species of "CO<sub>2</sub>" in reactions catalyzed by pyruvate carboxylase, P-enolpyruvate carboxykinase, and P-enolpyruvate carboxytransphosphorylase. Because propionyl-CoA carboxylase and pyruvate carboxylase are biotin enzymes, they would be expected to have similar mechanisms. Likewise the reactions catalyzed by P-enolpyruvate carboxykinase and carboxytransphosphorylase are in some respects similar to that of P-enolpyruvate carboxylase, and it has been suggested that bicarbonate might be the reactant in each case. By means of radiochemical and spectrophotometric techniques, we have obtained evidence that the active species in the carboxykinase and carboxytransphosphorylase reactions is CO<sub>2</sub> and not bicarbonate. Bicarbonate appears to be the active species in the pyruvate carboxylase reaction, in conformity with the results obtained with propionyl-CoA carboxylase.

It is important in considerations of the mechanism of CO<sub>2</sub> fixation or decarboxylation to know whether or not the reactant is CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>). Two methods have been used to obtain such information. The first involves the use of carbonic anhydrase as exemplified by the studies of Krebs and Roughton (1948). They presented evidence that CO<sub>2</sub> is the product of decarboxylation of pyruvate as catalyzed by pyruvate decarboxylase (EC 4.1.1.1). The evidence consisted of the demonstration, by manometric methods, of an "overshoot" in CO<sub>2</sub> pressure during the decarboxylation which was eliminated in the presence of carbonic anhydrase. This observation could be explained if CO<sub>2</sub> is the product and if the rate of hydration of CO<sub>2</sub> is limiting. The CO<sub>2</sub> under these conditions escapes into the gas phase;

but as the substrate becomes limiting and the rate of the reaction decreases, the CO<sub>2</sub> is reabsorbed to attain the equilibrium shown below:



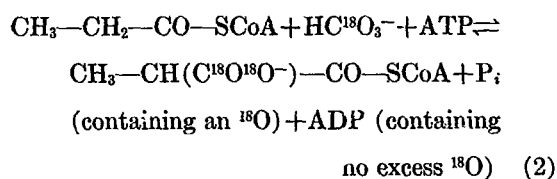
In the presence of carbonic anhydrase, the hydration of CO<sub>2</sub> is so rapid that equilibrium is maintained throughout the decarboxylation. In contrast, if bicarbonate were the initial product, the evolution of CO<sub>2</sub> into the gas phase would be faster in the presence of carbonic anhydrase than in its absence and there would be no "overshoot" of CO<sub>2</sub> pressure. Hansl and Waygood (1952), by use of this method and a heavy suspension of disintegrated cells of *Chlorella*, or extracts from plants, presented evidence that CO<sub>2</sub> is the primary product of decarboxylation of pyruvate, oxalacetate, glutamate, and α-ketoglutarate.

The second method of determining CO<sub>2</sub> species has involved the use of <sup>18</sup>O-bicarbonate. Kaziro, Hass, Boyer, and Ochoa (1962), and Maruyama,

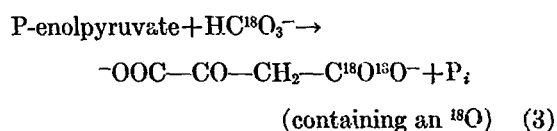
<sup>1</sup> This study was assisted by grants CA-08669, AM-05384, AEC contract AT-(30-1)-1320, PHS grant FR06013-02, and NSF grants GB-2766 and GB-2599.

<sup>2</sup> This paper is modified from that published by T. G. Cooper, T. T. Tchen, H. G. Wood, and C. R. Benedict, *J. Biol. Chem.*, **243**: 3857-3863, 1968.

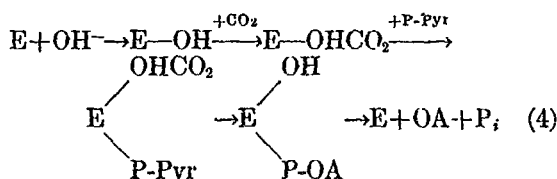
Esterday, Chang, and Lane (1966) have presented evidence that HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>) is the reactant in certain CO<sub>2</sub> fixations. They showed that all three oxygens of HC<sup>18</sup>O<sub>3</sub><sup>-</sup> were incorporated into the products, organic acids and phosphate, whereas if CO<sub>2</sub> were the reactant, only two would be expected to be utilized. Kaziro et al. (1962) used the biotin enzyme, propionyl-CoA carboxylase (EC 6.5.1.3) and found the equivalent of one <sup>18</sup>O in the orthophosphate and 2 in the 3-carboxyl of the methylmalonyl-CoA. The results are illustrated in reaction (2).



Maruyama et al. (1966) used P-enolpyruvate carboxylase (EC 4.1.1.31) and observed that <sup>18</sup>O was incorporated into the orthophosphate and oxalacetate in a ratio of 1:2 which is in accord with reaction (3).



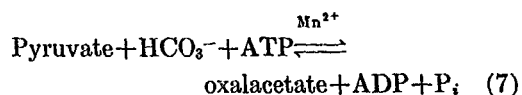
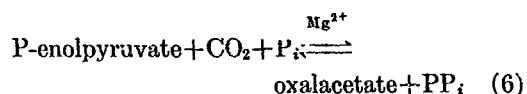
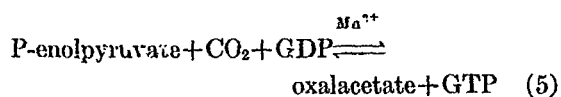
Although the <sup>18</sup>O results appear unequivocal, the method does involve one basic assumption. It is that OH<sup>-</sup> or water at the active site of the enzyme is in equilibrium with the solvent water. The reaction could occur as illustrated in reaction (4), where E is the enzyme, P-Pyr is P-enolpyruvate, OA is oxalacetate, and P-OA is P-enol-oxalacetate.



The reactions were conducted at pH 8.0; and, therefore, practically all the CO<sub>2</sub> was in the form of bicarbonate. On reaching the site, HC<sup>18</sup>O<sub>3</sub><sup>-</sup> could conceivably be converted to C<sup>18</sup>O<sub>2</sub> and <sup>18</sup>OH<sup>-</sup>; and then the C<sup>18</sup>O<sub>2</sub> could react to form P-enoloxalacetate which is subsequently cleaved to oxalacetate by the reaction involving the OH<sup>-</sup>.

If this occurred and the OH<sup>-</sup> at the site were protein bound and equilibrated slowly with the solvent water, the bound OH<sup>-</sup> by turnover would acquire <sup>18</sup>O equal to that of the bicarbonate. If the reaction were conducted at pH 6 or lower, possibly not as much <sup>18</sup>O would have been converted to the phosphate. Kaziro et al. (1962) did conduct a control using H<sub>2</sub><sup>18</sup>O and HC<sup>16</sup>O<sub>3</sub><sup>-</sup>. They found incorporation of <sup>18</sup>O into the orthophosphate, but they also noted an exchange of <sup>18</sup>O from the H<sub>2</sub><sup>18</sup>O with the HC<sup>16</sup>O<sub>3</sub><sup>-</sup>, and they estimated that all the <sup>18</sup>O entered the phosphate via the resulting HC<sup>18</sup>O<sub>3</sub><sup>-</sup> and none via H<sub>2</sub><sup>18</sup>O. However, as noted above, if the <sup>18</sup>O at the site did not equilibrate with the solvent water, a hydrolytic or hydroxyl cleavage would not necessarily reflect the <sup>18</sup>O of the solvent H<sub>2</sub><sup>18</sup>O as they assumed.

In view of these considerations and because we had available three highly purified enzymes that catalyzed CO<sub>2</sub> fixations, it seemed worthwhile to investigate further the problem of CO<sub>2</sub> species. The enzymes studied were P-enolpyruvate carboxykinase (EC 4.1.1.32) catalyzing reaction (5), P-enolpyruvate carboxytransphosphorylase (EC 4.1.1.38) catalyzing reaction (6), and pyruvate carboxylase (EC 6.5.1.1) catalyzing reaction (7).



Reactions (5) and (6) resemble reaction (3) except that, instead of cleavage of the phosphate group of P-enolpyruvate to inorganic phosphate, the high-energy phosphoryl group is preserved in GTP or PP<sub>i</sub>. Because of this similarity, Chang, Maruyama, Miller, and Lane (1966) and Maruyama et al. (1966) have considered that HCO<sub>3</sub><sup>-</sup> may be the functional species in reactions (5) and (6) as well as in reaction (3). Likewise following the studies by Kaziro et al. (1962), it has generally been considered that all fixations of "CO<sub>2</sub>" by biotin enzymes occur with HCO<sub>3</sub><sup>-</sup>.

It was therefore of interest to investigate a second biotin enzyme, pyruvate carboxylase.

This report presents two types of experiments that were used to determine the species of CO<sub>2</sub> utilized in the reactions. The results indicate that CO<sub>2</sub> is the reactive species in reactions (5) and (6) and HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>), in reaction (7).

### THEORETICAL AND EXPERIMENTAL RESULTS

The details of methodology employed in the following experiments have recently appeared in the literature (Cooper, Tehen, Wood, and Benedict, 1968), and, therefore, will not be discussed here.

#### Spectrophotometric Assays

The equilibrium between CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, and H<sub>2</sub>CO<sub>3</sub> may be represented as follows:

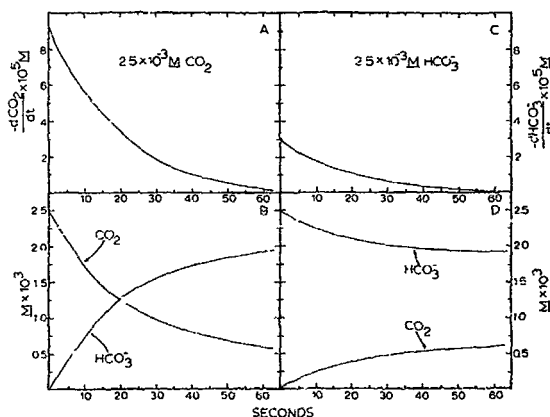
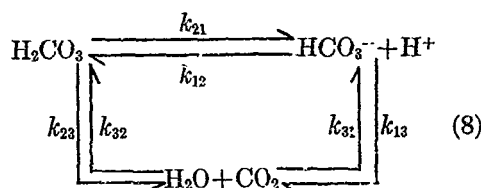


FIGURE 1.—(a) Estimated rate of change of concentration of CO<sub>2</sub> after addition of  $2.5 \times 10^{-3}$  M CO<sub>2</sub>. (b) The variation of the concentrations of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> following addition of  $2.5 \times 10^{-3}$  M CO<sub>2</sub>. (c) The rate of change of concentration of HCO<sub>3</sub><sup>-</sup> after addition of  $2.5 \times 10^{-3}$  M HCO<sub>3</sub><sup>-</sup>. (d) Variation of the concentrations of HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> after addition of  $2.5 \times 10^{-3}$  M HCO<sub>3</sub><sup>-</sup>. Calculations were made using the constants of Gibbons and Edsall (1963) and Ho and Sturtevant (1963) for a system at 25°C, pH 7.0 and low ionic strength.

The rate of hydration of CO<sub>2</sub> may be written:

$$-\frac{d[\text{CO}_2]}{dt} = k_{31}[\text{CO}_2] - k_{13}[\text{H}^+][\text{HCO}_3^-] \quad (9)$$

where

$$k_{31}' = k_{31} + k_{32} \quad \text{and} \quad k_{13}' = k_{13} + \frac{k_{23}}{K_{\text{H}_2\text{CO}_3}}$$

To calculate the concentration of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> at any given time, it is necessary to solve the above differential equation (9). If one assumes at  $t=0$

$$[\text{CO}_2]_{t=0} + [\text{HCO}_3^-]_{t=0} = [\text{CO}_2]_t + [\text{HCO}_3^-]_t \quad (10)$$

and by definition

$$k_{13}'' = [\text{H}^+]k_{13}' \quad A = [\text{CO}_2] \quad \text{and} \quad B = [\text{HCO}_3^-]$$

then the solution of the equation is

$$\begin{aligned}
 [\text{CO}_2] = & \frac{k_{13}''(A_0 + B_0)}{(k_{31}' + k_{13}'')} \\
 & + \left[ [\text{CO}_2]_0 - \frac{k_{13}''(A_0 + B_0)}{(k_{31}' + k_{13}'')} \right] e^{-(k_{13}'' + k_{31}')t} \quad (11)
 \end{aligned}$$

The variation in concentration of HCO<sub>3</sub><sup>-</sup> may then be determined by substitution of this value into equation (10). Using values for the constants ( $k_{31}' = 0.0375 \text{ sec}^{-1}$ ,  $k_{13}' = 5.5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ ) that have been determined by Gibbons and Edsall (1963) and Ho and Sturtevant (1963), one can calculate the hydration rate of  $2.5 \times 10^{-3} \text{ M}$  CO<sub>2</sub> or the rate of dehydration of  $2.5 \times 10^{-3} \text{ M}$  HCO<sub>3</sub><sup>-</sup> with respect to time (fig. 1(a) and 1(c)). From these rates, the variations of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> can be determined (fig. 1(b) and 1(d)).

When these considerations are applied to the enzymatic reactions, the theoretical rates of formation of oxalacetate may be calculated by assuming that the rate of fixation is directly proportional to the concentration of the active species of "CO<sub>2</sub>." This assumption is valid if the concentrations of the "CO<sub>2</sub>" species are below the  $K_m$  values. The  $K_m$  for HCO<sub>3</sub><sup>-</sup> plus CO<sub>2</sub> with carboxytransphosphorylase is 9.5 mM at pH 7.8 and 4.0 mM at pH 6.5 (Lochmüller, Wood, and Davis, 1966), that of P-enolpyruvate carboxykinase is 20 mM at pH 7.5 (Cooper and Benedict, 1968),

and that of pyruvate carboxylase 1 mM at pH 7.4 (Keech and Utter, 1963). Thus if the  $K_m$  is calculated on the basis of either species, it is 2 mM or above except for pyruvate carboxylase. The fact that the concentration of the added species was, in fact, greater than that of the  $K_m$  with pyruvate carboxylase does not invalidate the procedure. The results will in general be the same, but the inflection of the curves, and thus the sensitivity of the method, will be somewhat less.

The curves of figure 2(a) have been derived for the situation when CO<sub>2</sub> is the species utilized and those of figure 2(b) for when HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>) is the active species.<sup>3</sup> In each figure the curve designated CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> represents the case when the reaction is initiated with this species, and the curve labeled CA, the case when carbonic anhydrase is present. In the absence of carbonic anhydrase, if CO<sub>2</sub> is the active species, the reaction proceeds with a high initial rate upon addition of CO<sub>2</sub> and at a low initial rate when HCO<sub>3</sub><sup>-</sup> is added (fig. 2(a)). After approximately 50 seconds, when equilibrium between the three species of CO<sub>2</sub> is reached (reaction (8)), the rate becomes linear and identical regardless of the species of CO<sub>2</sub> added. At that time the rate is the same as that observed in the presence of carbonic anhydrase, with which equilibrium is reached almost instantaneously.

When HCO<sub>3</sub><sup>-</sup> is the active species, the situation is reversed (fig. 2(b)); the initial rate is more rapid when HCO<sub>3</sub><sup>-</sup> is added and slower when CO<sub>2</sub> is added. In figures 2(a) and 2(b), the rate observed when carbonic anhydrase is present is greater when the reaction is initiated with CO<sub>2</sub> than when initiated with HCO<sub>3</sub><sup>-</sup>. This occurs because there is a much greater change in concentration of both the CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> when CO<sub>2</sub> is added (fig. 1(b)) than when HCO<sub>3</sub><sup>-</sup> is added (fig. 1(d)). Thus initiation of the reaction with CO<sub>2</sub> provides the best indicator regardless of whether CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> is the reactive species.

It should be mentioned that the curves of figure 1 were obtained with rate constants for 25°C and low ionic strength and assumed a direct

<sup>3</sup> Although these curves were obtained by approximating the solution of equation (9), they closely agree (the error is below the resolution of the graph) with curves obtained from the exact solution (11).

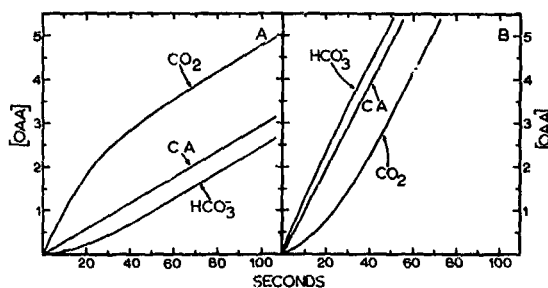


FIGURE 2.—Estimated theoretical formation of oxalacetate for the spectrophotometric assay: (a) If the active species used in the fixation is CO<sub>2</sub>, and (b) if it is HCO<sub>3</sub><sup>-</sup>. The lines designated CO<sub>2</sub> represent the situation when CO<sub>2</sub> is the initially added species, those designated HCO<sub>3</sub><sup>-</sup>, when it is HCO<sub>3</sub><sup>-</sup>, and those designated CA, when either CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> is added in the presence of carbonic anhydrase. The calculations have been made on the basis that the rate of fixation is directly proportional to the concentration of the active species using the values shown in figure 1. OAA equals oxalacetic acid. Absolute values are not given for the concentration of OAA, because the amount is proportional to the concentration of the active species but not necessarily equal to it.

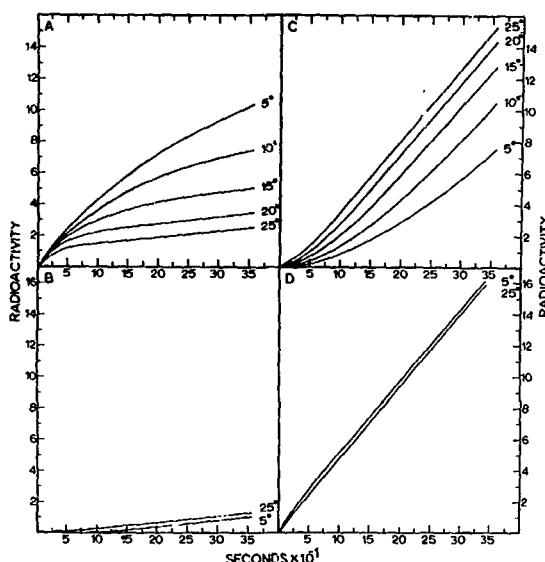


FIGURE 3.—Theoretical formation of oxalacetate at various temperatures for the spectrophotometric assay: Figure 3 (a), (b), if the active species used in the fixation is CO<sub>2</sub> and figure 3 (c), (d), if it is HCO<sub>3</sub><sup>-</sup>. Figure 3(a) and (c) represent the situation when 2.5 × 10<sup>-3</sup> M of <sup>14</sup>CO<sub>2</sub> is the initially added species and figure 3(b) and (d), the alternate situation when 2.5 × 10<sup>-3</sup> M H<sup>14</sup>CO<sub>3</sub><sup>-</sup> is the initially added species.



proportionality between the rate of oxalacetate formation and the concentration of the active species of "CO<sub>2</sub>." The experiments actually were performed at 10 C, which would slow down the attainment of equilibrium between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>, and at higher ionic strength, which would speed up the attainment of this equilibrium. Although Cooper and Filmer (1968) have constructed the theoretically predicted behavior of enzyme systems utilizing CO<sub>2</sub> (figs. 3(a), (b) and 4(a), (b)) and HCO<sub>3</sub><sup>-</sup> (figs. 3(c), (d) and 4(c), (d)) at various temperatures and pH's, their calculations were also based upon rate constants obtained at low ionic strength. Figures 3 and 4 have been included so that the effect of temperature and pH may be visualized. The temperature and pH, however, were constant in the present experiments. Until it is possible to assess quantitatively the effect of ionic strength on the CO<sub>2</sub> hydration:dehydration reactions, no exact theoretical curves can be drawn for these reactions. Nevertheless, the curves in figures 1 and 2 should qualitatively resemble the exact theoretical curves

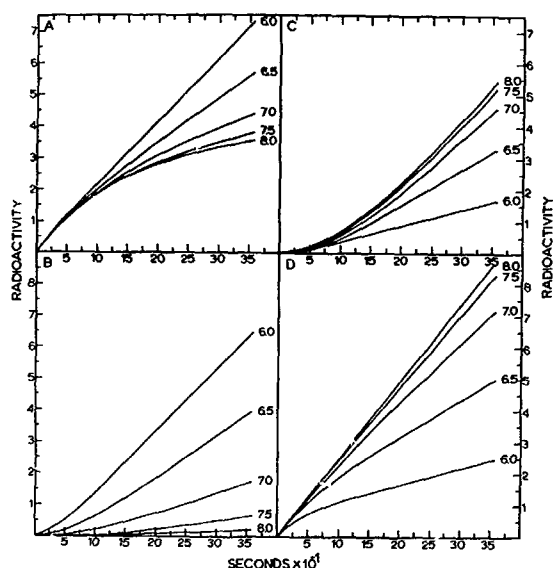


FIGURE 4.—Theoretical formation of oxalacetate at various hydrogen ion concentrations for the radiochemical assay: (a), (b), if the active species used in the fixation is CO<sub>2</sub>, and (c), (d), if it is HCO<sub>3</sub><sup>-</sup>. (a) and (c) represent the situation when  $2.5 \times 10^{-3}$  M of <sup>14</sup>CO<sub>2</sub> is the initially added species, and (b) and (d) represent the alternate situation when  $2.5 \times 10^{-3}$  M H<sup>14</sup>CO<sub>3</sub><sup>-</sup> is the initially added species.

and may serve as models for examining the experimental results.

The experimental results obtained with P-enolpyruvate carboxykinase and P-enolpyruvate carboxytransphosphorylase by the spectrophotometric method are shown in figures 5 and 6. This method was not used with pyruvate carboxylase. It is a biotin enzyme and it seemed likely that the active species would be HCO<sub>3</sub><sup>-</sup>. The radiochemical assay (to be described later) was considered to be the most reliable one for the HCO<sub>3</sub><sup>-</sup> species. With P-enolpyruvate carboxykinase, a constant and identical rate was obtained when HCO<sub>3</sub><sup>-</sup> was used to initiate the reaction whether or not the mixture contained carbonic anhydrase (fig. 5). The same rate was also observed when CO<sub>2</sub> was used to initiate the reaction in the presence of carbonic anhydrase. When CO<sub>2</sub> was

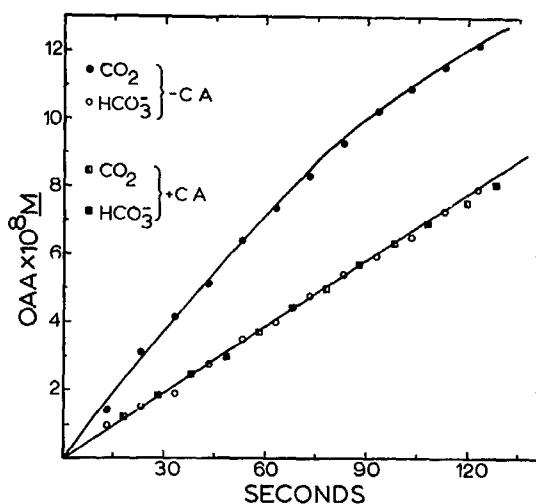


FIGURE 5.—Spectrophotometric assay of P-enolpyruvate carboxykinase activity when either CO<sub>2</sub> (—●—●—) or HCO<sub>3</sub><sup>-</sup> (—○—○—) is added initially. Complete reaction mixtures contained in a volume of 1.39 ml:  $1.44 \times 10^{-1}$  M trisbuffer, pH 7.60;  $3.6 \times 10^{-3}$  M GSH;  $5.05 \times 10^{-3}$  M GDP;  $5.05 \times 10^{-3}$  M P-enolpyruvate;  $2.6 \times 10^{-4}$  M NADH;  $1.44 \times 10^{-3}$  M MnCl<sub>2</sub>;  $1.8 \times 10^{-3}$  M HCO<sub>3</sub><sup>-</sup> or  $1.8 \times 10^{-3}$  M HCO<sub>3</sub><sup>-</sup> plus  $1.8 \times 10^{-3}$  M HCl (when CO<sub>2</sub> was the desired species); 100 micrograms of malate dehydrogenase; and 1.5 mg of carboxykinase preparation. Temperature was 10 C. The same experiments were done in the presence of 100 micrograms of carbonic anhydrase (CO<sub>2</sub>+CA —■—■— or HCO<sub>3</sub><sup>-</sup>+CA. —□—□—). In these experiments, and in the experiment of figure 6, the progress of the reactions was recorded with the Bristol recorder and smooth curves (or lines) were obtained. The values were taken from these curves and replotted.

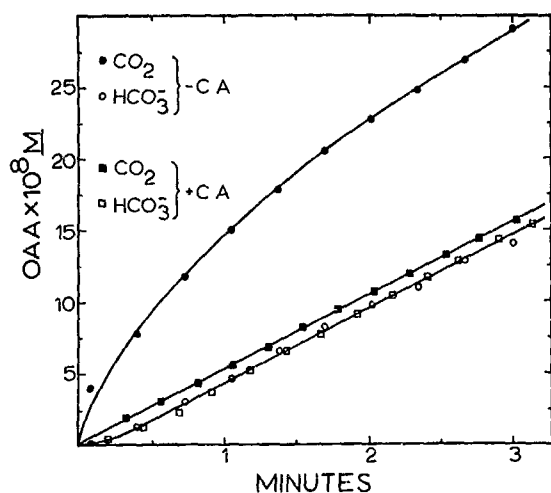


FIGURE 6.—Spectrophotometric assay of *P*-enolpyruvate carboxytransphosphorylase activity when either CO<sub>2</sub> (●-●-) or HCO<sub>3</sub><sup>-</sup> (○-○-) is added initially. The complete mixture contained in a volume of 1.25 ml:  $2.4 \times 10^{-2}$  M tris-buffer, pH 7.60;  $1.6 \times 10^{-2}$  M MgCl<sub>2</sub>;  $1.2 \times 10^{-2}$  M phosphate buffer, pH 7.60;  $4.0 \times 10^{-3}$  M *P*-enolpyruvate;  $2.0 \times 10^{-3}$  M HCO<sub>3</sub><sup>-</sup> or  $2.0 \times 10^{-3}$  HCO<sub>3</sub><sup>-</sup> plus  $2.0 \times 10^{-3}$  M HCl (when CO<sub>2</sub> was the desired species);  $2.6 \times 10^{-4}$  M NADH; 75 micrograms of malate dehydrogenase; and 0.10 mg of carboxytransphosphorylase preparation. Temperature was 10 C. The same experiments were done in the presence of 100 micrograms of carbonic anhydrase (CO<sub>2</sub>+CA -■-■-) or (HCO<sub>3</sub><sup>-</sup>+CA. -□-□-).

used to initiate the reaction in the absence of carbonic anhydrase, the initial rate was higher than in the other experiments, and after about 90 seconds, the rate decreased to that observed when carbonic anhydrase was present. Thus the experimental curves are similar to the theoretical curves produced when CO<sub>2</sub> is the active species (fig. 2(a)), except that the expected initial slow rate was not observed when HCO<sub>3</sub><sup>-</sup> was the initiating species. However, the experimental curves are more similar to those of figure 2(a) than they are to those of figure 2(b) and indicate that CO<sub>2</sub> is the active species for *P*-enolpyruvate carboxykinase. The cause of the deviation from the theoretical curve when HCO<sub>3</sub><sup>-</sup> was the initiating species is not known. The concentration of the species added was  $1.8 \times 10^{-3}$  M instead of the  $2.5 \times 10^{-3}$  M used for the calculations, but this does not alter the relative shapes of the curves to be expected.

The experimental results with *P*-enolpyruvate

carboxytransphosphorylase are shown in figure 6. Here again the initial rate was high, but it decreased when CO<sub>2</sub> was the species used to initiate the reaction. With HCO<sub>3</sub><sup>-</sup> there was an indication that the initial reaction was slower and then increased, but the results were the same whether carbonic anhydrase was present or not. Again the results are more similar to the theoretical curves of figure 2(a) than to those of figure 2(b), and they indicate that CO<sub>2</sub> is the active species for carboxytransphosphorylase.

#### Radiochemical Assay

Another approach to determine the active species is to use a mixture of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> with only one of the pair containing <sup>14</sup>C. The addition of the nonradioactive species of CO<sub>2</sub> assures that prior to isotopic equilibrium, one member of the pair will have a higher specific activity than the other. The <sup>14</sup>C incorporated into oxalacetate is converted to malate with malate dehydrogenase.

The theoretical results are shown in figures 7 and 8. The changes in the concentration of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> are shown in figure 7(a) and have been calculated using the constants of Edsall and Sturtevant and equation (11). In addition, by making the assumption that the initial specific activity of one of the species was 1 and the other 0 and by use of equation (9), the amount of <sup>14</sup>C transferred from one pool to the other in a given interval has been estimated. The change in the specific activity of the CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> with time was then calculated. The change when CO<sub>2</sub> is the initially labeled species is shown in figure 7(b), and when it is HCO<sub>3</sub><sup>-</sup>, in figure 7(c).

When these values are applied to the enzymatic reactions, the theoretical incorporation of <sup>14</sup>C into oxalacetate (malate) may be calculated, assuming that CO<sub>2</sub> is the active species as shown in figure 8(a) and assuming that HCO<sub>3</sub><sup>-</sup> is the active species in figure 8(b). For the calculations of figure 8(a), it was assumed that the fixation is directly proportional to the CO<sub>2</sub> concentration shown in figure 7(a) and that the CO<sub>2</sub> at any given time had the specific activity shown in figure 7(b) or 7(c). The CO<sub>2</sub> line of figure 8(a) represents the situation in which CO<sub>2</sub> is the source of the label and the HCO<sub>3</sub><sup>-</sup> line, when HCO<sub>3</sub><sup>-</sup> is the source. The specific activities of CO<sub>2</sub> of figure 7(b) were used for the former calculations, and

those of the CO<sub>2</sub> of figure 7(c), for the latter. Line CA of figure 8(a) is for the case when carbonic anhydrase is present and either species is labeled. The results are the same with either labeled species, because isotopic equilibrium is

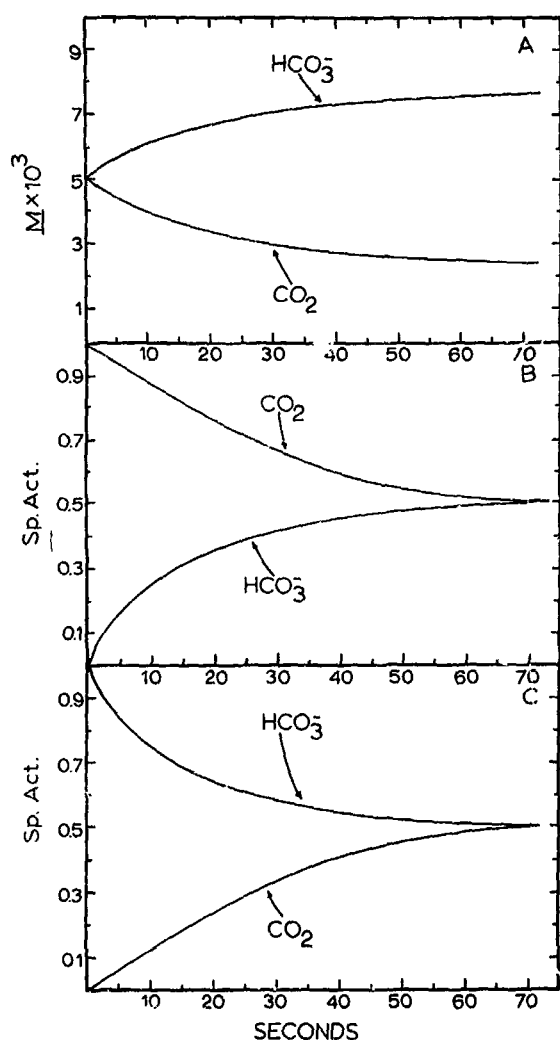


FIGURE 7.—(a) Estimated change in the concentration of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> when 0.01 M of each is added at 0 time. (b) Estimated change in specific activity of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> when the initial CO<sub>2</sub> is radioactive with a specific activity of 1.0 and the HCO<sub>3</sub><sup>-</sup> is unlabeled. (c) Estimated change in specific activity of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> when the initial HCO<sub>3</sub><sup>-</sup> is radioactive with a specific activity of 1.0 and the CO<sub>2</sub> is unlabeled. Calculations were made using the constants of Gibbons and Edsall (1963) and Ho and Sturtevant (1963) for a system at 25 C, pH 7.0 and low ionic strength.

attained almost instantly in the presence of carbonic anhydrase. Similar calculations were made for figure 8(b) where HCO<sub>3</sub><sup>-</sup> is the active species by use of the HCO<sub>3</sub><sup>-</sup> concentrations of figure 7(a) and the specific activities of the HCO<sub>3</sub><sup>-</sup> of figure 7(b) and (c), respectively.

When CO<sub>2</sub> is the active species and CO<sub>2</sub> is the source of <sup>14</sup>C, the incorporation of <sup>14</sup>C into the oxalacetate proceeds rapidly during the first 50 seconds and then levels off to a rate similar to that when carbonic anhydrase is present (CA of fig. 8(a)). When HCO<sub>3</sub><sup>-</sup> is added (HCO<sub>3</sub><sup>-</sup> of fig. 8(a)), the rate of <sup>14</sup>C incorporation is slower for about 30 seconds than it is with carbonic anhydrase present, but the difference is not as great as when CO<sub>2</sub> is the source of the label. Figure 8(b) shows the results when HCO<sub>3</sub><sup>-</sup> is the active species. The rate of <sup>14</sup>C incorporation is faster when H<sup>14</sup>CO<sub>3</sub><sup>-</sup> is the initial labeled species than when the initial labeled species is CO<sub>2</sub> and carbonic anhydrase is present. In both figure 8(a) and 8(b) the difference from that with carbonic anhydrase is greater with CO<sub>2</sub> as the labeled species than with HCO<sub>3</sub><sup>-</sup>; thus, labeled CO<sub>2</sub> is theoretically the best indicator, no matter which species is utilized in the reaction.

Experimentally, the three enzymes—P-enolpyruvate carboxykinase, carboxytransphosphorylase, and pyruvate carboxylase—behaved differ-

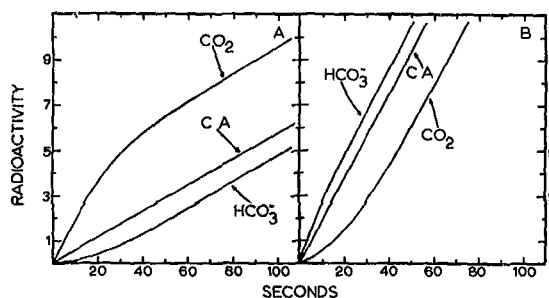


FIGURE 8.—Estimated theoretical radioactivity of oxalacetate for the radiochemical assay: (a) If the active species used in the fixation is CO<sub>2</sub> and (b) if it is HCO<sub>3</sub><sup>-</sup>. The lines designated CO<sub>2</sub> represent the situation when the added CO<sub>2</sub> is radioactive and the HCO<sub>3</sub><sup>-</sup> is unlabeled, those designated CA, when either member is radioactive and the other, unlabeled, and carbonic anhydrase is present. The calculations have been made on the basis that the rate of fixation is directly proportional to the concentration of the active species and with the specific activities shown in figure 7.

ently. With the first two enzymes (figs. 9 and 10), a high initial rate of formation of oxalacetate-<sup>14</sup>C was observed when H<sup>12</sup>CO<sub>3</sub><sup>-</sup> and <sup>14</sup>CO<sub>2</sub> were added. In contrast, with pyruvate carboxylase (fig. 11), a high initial rate of formation of oxalacetate-<sup>14</sup>C was observed when H<sup>14</sup>CO<sub>3</sub><sup>-</sup> and <sup>12</sup>CO<sub>2</sub> were added. The latter results indicate HCO<sub>3</sub><sup>-</sup> is the reactant (compare figs. 11 and 8(b)). These results are in agreement with the conclusions of Kaziro et al. (1962) from their studies with <sup>18</sup>O and the biotin-containing enzyme propionyl-CoA carboxylase. Thus there are two lines of evidence that HCO<sub>3</sub><sup>-</sup> is the active species for the enzymes of CO<sub>2</sub> fixation that contain biotin. Note that the equilibration time of the pyruvate carboxylase system was approximately 4.5 times shorter than that observed in the case of the P-enolpyruvate carboxykinase and carboxytransphosphorylase. This is close to the predicted behavior, because the cold lability of the avian enzyme preparation

necessitated that the analysis be carried out at 25 C rather than 10 C. Experiments with carbonic anhydrase were not done with pyruvate carboxylase because of the limited amount of pyruvate carboxylase available at that time.

With P-enolpyruvate carboxykinase (fig. 9) and carboxytransphosphorylase (fig. 10), the high initial rate of incorporation of <sup>14</sup>C into oxalacetate occurred when <sup>14</sup>CO<sub>2</sub> was the labeled species, indicating that CO<sub>2</sub> is the active species for these two enzymes. The results are in agreement with those of the spectrophotometric assay described in the preceding section. However, there was some discrepancy from the theoretical curves of figure 8(a). With P-enolpyruvate carboxykinase (fig. 9), the rate of <sup>14</sup>C incorporation with H<sup>14</sup>CO<sub>3</sub><sup>-</sup> was linear from time 0, whereas it was expected to be slow at first and then increase to a constant rate. However, with carboxytransphosphorylase (fig. 10) the slow initial rate was observed in

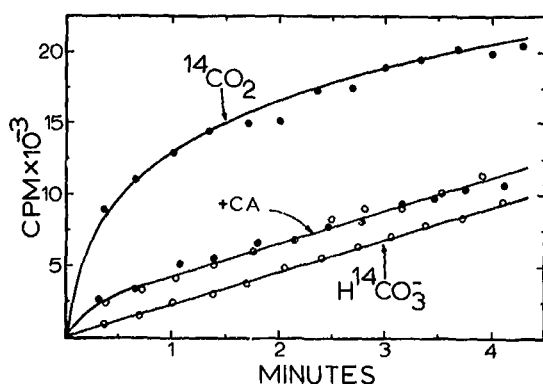


FIGURE 9.—Radiochemical assay of P-enolpyruvate carboxykinase activity when either H<sup>12</sup>CO<sub>3</sub><sup>-</sup> plus <sup>14</sup>CO<sub>2</sub> (—●—●) or H<sup>14</sup>CO<sub>3</sub><sup>-</sup> plus <sup>12</sup>CO<sub>2</sub> (—○—○) were added initially. The complete mixtures in a volume of 2.05 ml contained: 9.75 × 10<sup>-2</sup> M tris-buffer, pH 7.60; 2.44 × 10<sup>-3</sup> M GSH; 3.26 × 10<sup>-3</sup> M P-enolpyruvate; 7.35 × 10<sup>-4</sup> M NADH; 9.75 × 10<sup>-4</sup> M MnCl<sub>2</sub>; 2.44 × 10<sup>-3</sup> M HCO<sub>3</sub><sup>-</sup> plus 2.44 × 10<sup>-3</sup> M HCl (for the CO<sub>2</sub> species) or 2.44 × 10<sup>-3</sup> M HCO<sub>3</sub><sup>-</sup> (the indicated radioactive species contained 20 μC per μmole); 100 micrograms malate dehydrogenase; and 2.0 mg of carboxykinase preparation. Temperature was 10 C. The reaction was initiated as described in the text. The same experiments were done in the presence of 100 micrograms of carbonic anhydrase along with <sup>14</sup>CO<sub>2</sub> plus H<sup>12</sup>CO<sub>3</sub><sup>-</sup> or <sup>12</sup>CO<sub>2</sub> plus H<sup>14</sup>CO<sub>3</sub><sup>-</sup> and are indicated as +CA.

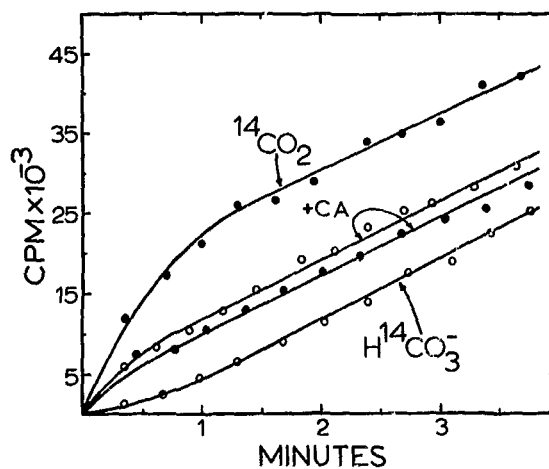


FIGURE 10.—Radiochemical assay of P-enolpyruvate carboxytransphosphorylase activity when either H<sup>12</sup>CO<sub>3</sub><sup>-</sup> plus <sup>14</sup>CO<sub>2</sub> (—●—●) or H<sup>14</sup>CO<sub>3</sub><sup>-</sup> plus <sup>12</sup>CO<sub>2</sub> (—○—○) were added initially. The complete mixtures in a volume of 1.88 ml contained: 1.6 × 10<sup>-2</sup> M tris-buffer, pH 7.60; 1.06 × 10<sup>-2</sup> M MgCl<sub>2</sub>; 8.0 × 10<sup>-3</sup> M phosphate buffer, pH 7.60; 2.67 × 10<sup>-3</sup> M P-enolpyruvate; 2.6 × 10<sup>-3</sup> M NADH; CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> as described in figure 9 with a specific activity of the labeled compound of 20 μC/μmole; 75 micrograms of malate dehydrogenase; 100 micrograms of carboxytransphosphorylase preparation. Temperature was 10 C. The reaction was initiated as described in the text. The same experiments were done in the presence of 100 micrograms of carbonic anhydrase and are indicated as +CA.

accordance with expectations. A partial explanation of the discrepancy may be that HCO<sub>3</sub><sup>-</sup> was always added prior to the CO<sub>2</sub>. This fact may mask the expected slow rate with H<sup>14</sup>CO<sub>3</sub><sup>-</sup>, because highly active <sup>14</sup>CO<sub>2</sub> would be formed from the H<sup>14</sup>CO<sub>3</sub> prior to the addition of the <sup>12</sup>CO<sub>2</sub>; time about 6 seconds. A further discrepancy is noted with carbonic anhydrase. The initial value in both figures 9 and 10 was somewhat greater than the subsequent constant rate. Again the reason for this divergence from the theoretical curves of figure 8(a) is not known.

### DISCUSSION

The present results indicate that CO<sub>2</sub> rather than HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>) is the active species of the reactions catalyzed by P-enolpyruvate carboxykinase and by P-enolpyruvate carboxytransphosphorylase. Apparently the mechanisms of these fixation reactions differ significantly from those catalyzed by P-enolpyruvate carboxylase

which, according to the <sup>18</sup>O labeling observed by Maruyama et al. (1966), involve HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>) as a reactant. It would be interesting to check the carboxykinase and carboxytransphosphorylase reactions by the <sup>18</sup>O method and the carboxylase by the spectrophotometric and radiochemical procedures. It is conceivable that the latter two methods are not a reflection of the mechanism per se, but rather a reflection of the rate of binding at the active site. For example, the charged HCO<sub>3</sub><sup>-</sup> might be hindered from approaching the active site and CO<sub>2</sub> might more readily approach the site. At the active site, CO<sub>2</sub> might react with H<sub>2</sub>O to form H<sub>2</sub>CO<sub>3</sub> and in this form undergo the actual chemical reaction. In this case, only two <sup>18</sup>O would enter the products from HC<sup>18</sup>O<sub>3</sub><sup>-</sup>, but <sup>18</sup>O from H<sub>2</sub><sup>18</sup>O should enter the product very effectively.

It is also possible that either form, i.e., CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup>, binds to the enzyme as shown in reaction (13):

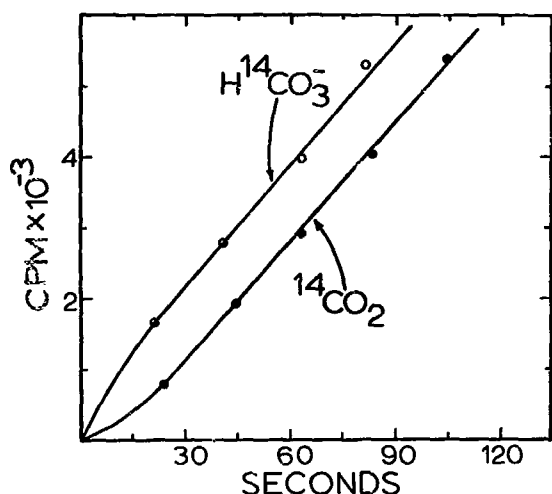
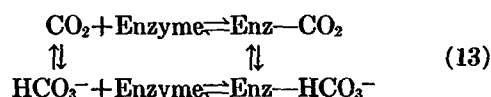
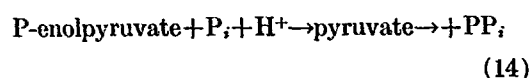


FIGURE 11.—Radiochemical assay of pyruvate carboxylase activity when either H<sup>14</sup>CO<sub>3</sub><sup>-</sup> plus <sup>14</sup>CO<sub>2</sub> (—●—●) or H<sup>14</sup>CO<sub>3</sub><sup>-</sup> plus <sup>12</sup>CO<sub>2</sub> (—○—○) were added initially. The complete mixtures in a volume of 1.92 ml contained: 1.04 × 10<sup>-1</sup> M tris-buffer, pH 7.60; 5.2 × 10<sup>-3</sup> M pyruvate; 2.6 × 10<sup>-3</sup> M NADH; 4.0 × 10<sup>-5</sup> M acetyl CoA; 2.6 × 10<sup>-3</sup> M ATP; 5.2 × 10<sup>-3</sup> M MgCl<sub>2</sub>; CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> as described in figure 9 with one of the two labeled; 100 micrograms of malate dehydrogenase; and 100 micrograms of pyruvate carboxylase preparation.

This binding would probably be followed by the reaction of only one of the enzyme-“CO<sub>2</sub>” complexes with bound P-enolpyruvate to yield oxalacetate. In this case, it would be difficult to predict the results of the above experiments except for the situation in which both forms of CO<sub>2</sub> have the same binding constants and the interconversion of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> is not limiting. Such limitations would lead to a linear reaction regardless of the species of CO<sub>2</sub> initially presented to the system.

Neither the <sup>18</sup>O studies nor the spectrophotometric and radiochemical assays provide conclusive evidence that the species involved is CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup>. If, however, both approaches are employed, it is possible to obtain a strong indication as to which species is active. With P-enolpyruvate carboxytransphosphorylase, there were indications prior to the present studies that bicarbonate per se need not be involved in a nucleophilic attack on the enolphosphoryl-phosphorus atom, thus leading to cleavage of the P—O bond as proposed by Maruyama et al. (1966) and Chang et al. (1966). Lochmüller et al. (1966) have shown that carboxytransphosphorylase catalyzes cleavage of the

enolphosphoryl bond in the absence of CO<sub>2</sub> as indicated in reaction (14).



Thus CO<sub>2</sub> is required neither for the cleavage of the bond nor for the combination of the phosphate with the orthophosphate to form inorganic pyrophosphate.

With the biotin enzyme, pyruvate carboxylase, the results from our experiments are in agreement with those of Kaziro et al. (1962) and indicate that HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>) is in general the reactant in CO<sub>2</sub> fixation by biotin enzymes. This confirmation is significant, because our methods did not involve the same assumption as did those of the experiments of Kaziro et al. (1962) using <sup>18</sup>O. Although we have noted that the results of the methods cannot be considered conclusive, it is reasonable to assume that agreement between the methods is fairly strong evidence that HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>) is the reactant with the biotin enzymes.

It is to be noted that the manometric method of Krebs and Roughton (1948) gives only an "overshoot" if CO<sub>2</sub> is the reactant. If HCO<sub>3</sub><sup>-</sup> (or H<sub>2</sub>CO<sub>3</sub>) is the reactant, carbonic anhydrase will have little effect in the manometric measurement, whereas in the spectrophotometric or radiochemical assay, carbonic anhydrase has an effect no matter which species is involved.

#### ACKNOWLEDGMENT

We are grateful to Dr. M. F. Utter and his colleagues for their gift of pyruvate carboxylase and to Drs. John T. Edsall and R. Khalifah for providing, prior to publication, the rate constants for the hydration and dehydration of CO<sub>2</sub> and the activation energies of these two reactions.

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## DISCUSSION

**ROSSI-BERNARDI:** It has been proposed to study the system of CO<sub>2</sub>-bicarbonate by an infrared stop-flow machine. If you dissolve your enzyme in aerated water, you can then mix it with CO<sub>2</sub> and you can, by using the proper wavelengths, know the concentration of dissolved CO<sub>2</sub> and bicarbonate, at any time. That I think would be a good confirmation of your point.

**COOPER:** I agree. Another thing that would be interesting would be to repeat the <sup>18</sup>O experiments on the PEP-carboxykinase and PEP-carboxytransphosphorylase. We have data from both approaches on the biotin-containing enzyme. It would be interesting to have it from both approaches on the PEP-carboxylating enzyme.

**HASTINGS:** I expect that Dr. Lane's paper will have something on that.

**LANE:** The  $K_m$  for bicarbonate is so low for the PEP-carboxylase—it is about 0.2 mM; I am not sure this technique would get you down that far. If you start with 2.5 mM, can you pick it up if the  $K_m$  for bicarbonate is 0.2 mM?

**COOPER:** Yes; these just happen to be the concentrations I selected. The same thing will apply regardless.

**LANE:** The problem is, it is difficult to reduce the concentration of CO<sub>2</sub> and bicarbonate in your media much below that level, in a buffered system.

**COOPER:** You would have to work in an inert atmosphere.

**LANE:** We did this kind of experiment when we were determining the  $K_m$  for that enzyme. We

found that the lowest we could get, within our capabilities, was about 0.2 mM; that is, about at the  $K_m$ . It is a tough problem.

**HASTINGS:** That is for total CO<sub>2</sub> or for bicarbonate?

**LANE:** In this case it is primarily bicarbonate, because the reactions were around pH 8.

**COOPER:** There is a way to get around this problem. You cannot use the spectrophotometric assay, if what you say is true, but you could use the radiochemical assay method. If the total amount of cold CO<sub>2</sub> in various forms in the solution is small and if you have high specific-activity radioactive CO<sub>2</sub> or bicarbonate, the concentration of the cold species will not make much difference. In the experiments I just reported, we used both <sup>14</sup>CO<sub>2</sub> plus cold bicarbonate, or cold CO<sub>2</sub> plus H<sup>14</sup>CO<sub>3</sub>. We computerized the analysis so we could also calculate the results if we used only the radioactive species and eliminated the cold species. The results are identical to those employing one radioactive and one nonradioactive species. We still had to do two sets of experiments: one with <sup>14</sup>CO<sub>2</sub> and one with H<sup>14</sup>CO<sub>3</sub>.

**HASTINGS:** Ochoa was delighted when our CO<sub>2</sub> effect on long-chain fatty-acid production from acetate by the liver was identified as being caused by bicarbonate ion. He had done this before.

However, we were unable to determine whether it was the effect of CO<sub>2</sub> molecules or bicarbonate ions on carbohydrate metabolism.

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## Comparison of Enzymatic Carboxylation Mechanisms<sup>1</sup>

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During the past two decades an impressive number and variety of enzymatic carboxylation reactions have been discovered (Calvin and Pon, 1959; Ochoa and Kaziro, 1965). These carboxylase-catalyzed reactions serve diverse metabolic functions ranging from photosynthesis (Calvin, 1962; Hatch and Slack, 1966; Hatch, Slack, and Johnson, 1967), amino acid catabolism (Knappe, Schlegel, and Lynen, 1961a; Rilling and Coon, 1960), and urea formation (Grisolia and Cohen, 1952; Jones, Spector, and Lipmann, 1955) to the energy coupling role of malonyl CoA formation (Wakil, 1958; Formica and Brady, 1959) in fatty acid biosynthesis (Lynen, Kessel, and Eggerer, 1964).<sup>2</sup> Despite these differences in metabolic function, there are basic chemical similarities among the enzymatic carboxylations. In several instances, namely, the carboxylations of biotin and PEP,<sup>3</sup> it is evident that the evolution of a productive enzymatic system in nature was followed by further refinement and hybridization to accomplish specific metabolic tasks.

The objectives of this paper are to review the basic chemical problem of carboxylation, to compare the different types of enzymatic carbox-

ylation, and finally to consider in more detail the mechanisms of carboxylation of biotin and PEP.

### The Basic Chemical Problem

The fundamental chemical problem in any carboxylation reaction is the activation of the, relatively stable carbon dioxide molecule or its hydration products, the activation of the acceptor molecule, or both. The few examples of purely chemical carboxylation reactions suggest two modes of activation (Kosower, 1962): electrophilic activation of CO<sub>2</sub>, in which the electrophilic character of the carbon atom is enhanced by a Lewis acid, thereby increasing its susceptibility to nucleophilic attack and nucleophilic activation of the CO<sub>2</sub> acceptor. The ease with which a C—C or C—N bond is formed between CO<sub>2</sub> and an acceptor molecule would be expected to depend upon the strength of the nucleophile. When the attacking nucleophile is a carbanion, its stabilization (hence nucleophilicity) may be promoted by the presence of a strong Lewis acid or of an adjacent electron-withdrawing substituent such as a keto or thioester carbonyl group.

Most nonenzymatic carboxylation reactions probably involve both attack by a moderately strong nucleophile and concomitant electrophilic activation (Stiles, 1960; Kosower, 1962). This point is illustrated in figure 1 with two chemical carboxylations; i.e., the carboxylation of an alkyl magnesium halide (Fieser and Fieser, 1962; Hine, 1962) and a nitroalkane (Stiles, 1960; Finkbeiner and Stiles, 1963). In the first example (fig. 1(a)), prior reaction of an alkane or alkyl halide to form its alkyl magnesium halide produces a strongly nucleophilic species which is

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<sup>2</sup> The carboxylation of acetyl CoA at the expense of 1 mole of ATP leads to the formation of malonyl CoA. The subsequent condensation of malonyl CoA with the fatty acyl CoA being elongated is energetically favored by the simultaneous decarboxylation of malonyl CoA.

<sup>3</sup> Abbreviations: PEP, phosphoenolpyruvate; RuDP, D-ribulose-1,5-diphosphate; Me<sup>2+</sup>, divalent metal cation; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase; and PEPCT, PEP carboxytransphosphorylase.





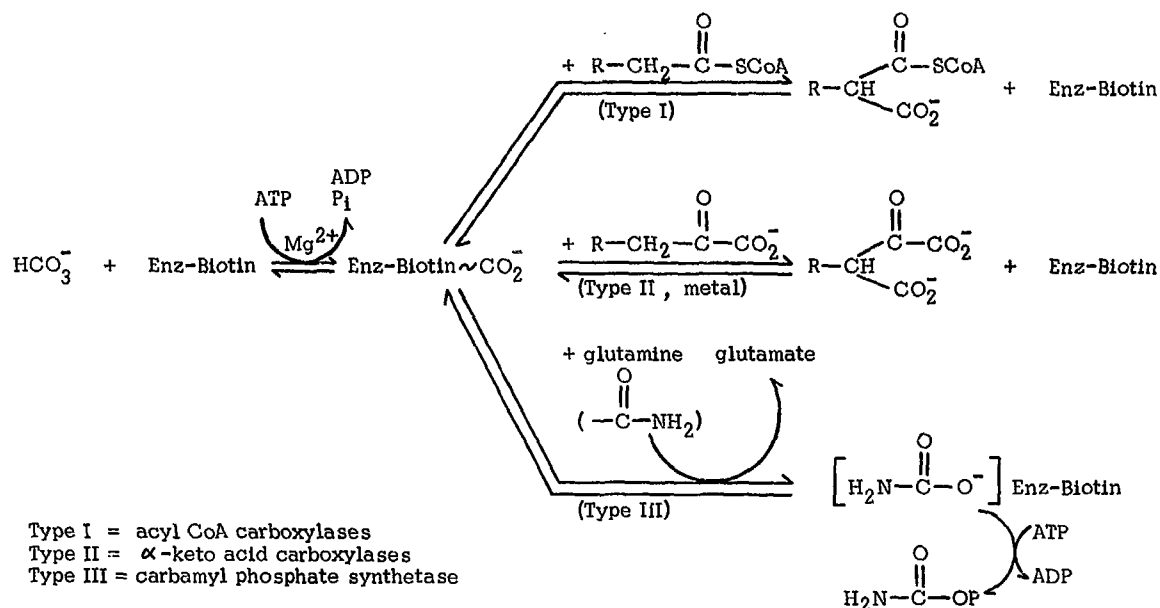


FIGURE 2.—Carboxylations coupled to ATP- $\gamma$ -phosphoryl group cleavage (biotin-containing carboxylases).

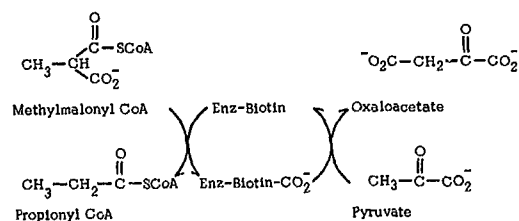
fer shifted to the  $\gamma$ -position because of a double bond at the  $\alpha$ - $\beta$  position.

The type II carboxylases, of which pyruvate carboxylase is the only known example, catalyze an analogous carboxylation at the site of  $\alpha$ -hydrogen activation. Unlike the type I acyl CoA carboxylases, pyruvate carboxylase is a manganese metalloenzyme (Scrutton, Utter, and Mildvan, 1966). The role of the metal in the reaction mechanism will be discussed by Dr. Scrutton (Scrutton and Mildvan, 1968).

A third type (type III) of biotin-dependent carboxylase that utilizes amide nitrogen or ammonia as the ultimate carboxyl acceptor was recently recognized. Wellner, Santos, and Meister (1968) have demonstrated that carbamyl phosphate synthetase from *E. coli* is a biotin enzyme. As illustrated in figure 2, the reaction catalyzed is more complex than those of the other biotin enzymes in that an additional step is involved which requires the expenditure of a second mole of ATP (Anderson and Meister, 1965). With the exception of the second step of the carbamyl phosphate synthetase-catalyzed reaction (Anderson and Meister, 1966), all other steps in the reactions shown are reversible. The active species of " $\text{CO}_2$ " involved in the carboxylation mechanism is apparently bicarbonate (Jones and Spector, 1960).

There is another type of biotin enzyme found in Propionibacteria which is a hybrid of the type I and type II carboxylases minus their initial ATP-dependent steps. This enzyme, methylmalonyl CoA-pyruvate transcarboxylase is incapable of  $\text{CO}_2$  fixation (Stjernholm and Wood, 1961; Wood, Allen, Stjernholm, and Jacobson, 1963), but has the ability to catalyze reversible carboxyl transfer from methylmalonyl CoA to pyruvate through a carboxy biotinyl enzyme intermediate (fig. 3(a)).

A. Methylmalonyl CoA-pyruvate transcarboxylase



B. Propionyl CoA carboxylase

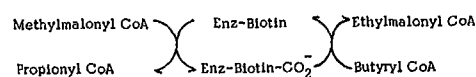


FIGURE 3.—Transcarboxylations catalyzed by methylmalonyl CoA-pyruvate transcarboxylase and by propionyl CoA carboxylase.



### Carboxylation Coupled to C—H Bond Cleavage (Reductive Carboxylation)

The reductive carboxylases, which include malic enzyme and isocitric dehydrogenase (Calvin and Pon, 1959; Ochoa and Kaziro, 1965), utilize the high electron transfer potential of NADH or NADPH to drive their respective carboxylation reactions. These carboxylation reactions can be visualized (Boyer, 1960) as hydride ion transfers from NADH or NADPH (C—H bond cleavage) to the enolate form of the substrate as illustrated for malic enzyme in figure 5(b). The  $\alpha$ -keto acid substrate is presumably bound to the enzyme by chelation to the metal (Hsu, Lardy, and Cleland, 1967). This would promote enolization of the  $\alpha$ -keto acid and facilitate simultaneous hydrogenation and carboxylation.

Having discussed the distinguishing characteristics of the various types of enzymatic carboxylation, I would like to consider in more detail: the carboxylation of biotin, i.e., the initial step common to all biotin-containing carboxylases, and the carboxylation of phosphoenolpyruvate. Dr. Scrutton, in the following paper, discusses in detail the second, or carboxyl transfer step, carried out by the biotin-containing carboxylases.

### THE ENZYMIC CARBOXYLATION OF BIOTIN

By 1958, compelling evidence had accumulated which implicated biotin in the enzymatic carboxylation of pyruvate, propionyl CoA, and acetyl CoA (Kaziro and Ochoa, 1964). It was demonstrated shortly thereafter that several highly purified acyl CoA carboxylases contained tightly bound biotin and that this prosthetic group was covalently bound in amide linkage to a lysyl  $\epsilon$ -amino group on the apoenzyme (Kosow and Lane, 1962; Kosow, Huang, and Lane, 1962). The available experimental evidence indicates that the enzymatic carboxylations of acetyl CoA, propionyl CoA,  $\beta$ -methylcrotonyl CoA, and pyruvate proceed by the two partial reactions shown in figure 6. Although the propionyl CoA carboxylase-catalyzed reaction has been used for illustrative purposes, the same basic reaction sequence applies for the other carboxylases. The evidence that supports the component steps of the reaction is also indicated in figure 6. The occurrence of step

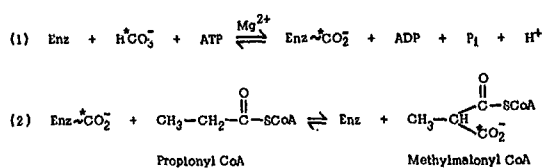


FIGURE 6.—Minimal reaction sequence for the propionyl CoA carboxylase-catalyzed reaction.

Evidence supporting step (1):

- a. ATP- $^{32}\text{P}_i$  exchange dependent on ADP,  $\text{Mg}^{2+}$ , and  $\text{HCO}_3^-$
- b. ATP- $^{14}\text{C}$ -ADP exchange dependent on  $\text{P}_i$ ,  $\text{Mg}^{2+}$ , and  $\text{HCO}_3^-$
- c.  $\text{Enz}\sim\text{CO}_2^-$  decarboxylation dependent on ADP,  $\text{P}_i$ , and  $\text{Mg}^{2+}$

Evidence supporting step (2):

- a. Methylmalonyl CoA- $^{14}\text{C}$ -propionyl CoA exchange independent of ATP, ADP,  $\text{P}_i$ ,  $\text{Mg}^{2+}$ , or  $\text{HCO}_3^-$
- b. Carboxyl transfer from  $\text{Enz}\sim\text{CO}_2^-$  to propionyl CoA

1 is supported by the finding (Kaziro, Leone, and Ochoa, 1960; Lynen, Knappe, Lorch, Jütting, Ringelmann, and Lachance, 1961) that the carboxylase catalyzes ATP- $^{32}\text{P}_i$  exchange and that the maximal rate of exchange is achieved only in the presence of ADP,  $\text{Mg}^{2+}$  and bicarbonate. Furthermore, the reciprocal ATP- $^{14}\text{C}$ -ADP exchange exhibits a requirement for  $\text{P}_i$ ,  $\text{Mg}^{2+}$ , and bicarbonate. Neither exchange reaction is dependent upon the presence of propionyl CoA. The simplest interpretation of these results is the concerted partial reaction shown as step 1. Strong support for the second partial reaction (step 2) was provided by the observation (Halenz and Lane, 1961b; Halenz, Feng, Hegre, and Lane, 1962) that propionyl CoA carboxylase catalyzes a rapid methylmalonyl CoA- $^{14}\text{C}$ -propionyl CoA exchange reaction which is independent of ATP, ADP,  $\text{Mg}^{2+}$ , or  $\text{P}_i$ . Under similar conditions, a methylmalonyl CoA- $^{14}\text{C}$ -bicarbonate exchange reaction does not occur (Halenz and Lane, 1961a). Collectively, these results suggested the participation of a carboxylated enzyme intermediate ( $\text{enzyme}\sim\text{CO}_2^-$ ). Kaziro and Ochoa (1961) successfully isolated the carboxylated enzyme intermediate ( $\text{enzyme}\sim\text{CO}_2^-$ ) following an incubation of the carboxylase with bicarbonate, ATP, and  $\text{Mg}^{2+}$ . They were then able to demonstrate stoichiometric carboxyl transfer from  $\text{enzyme}\sim\text{CO}_2^-$  to propionyl CoA to form methylmalonyl CoA (step 2) independently of ATP,  $\text{Mg}^{2+}$ , or  $\text{P}_i$ .

and the rapid decarboxylation of enzyme-CO<sub>2</sub><sup>-</sup> (reversal of step 1) dependent upon ADP, Mg<sup>2+</sup>, and P<sub>i</sub>. The fact that all of the above-mentioned reactions were inhibited by avidin, the specific biotin-binding protein from egg white, indicates that the biotin prosthetic group participates in the partial reactions.

The first insight into the chemical role of biotin in the carboxylation mechanism per se was provided by Lynen and his colleagues in 1959 (Lynen, Knappe, Lorch, Jütting, and Ringelmann, 1959) in their studies on a microbial  $\beta$ -methylcrotonyl CoA carboxylase. They made the rather startling observation that the carboxylation of free (+)-biotin was catalyzed by the enzyme (Lynen et al., 1961). The carboxylase-catalyzed reaction with  $\beta$ -methylcrotonyl CoA as substrate is shown in figure 7(a). Substitution of free (+)-biotin for the acyl CoA derivative led to the formation of an unstable carboxylated biotin derivative. This carboxylation product, after stabilization by conversion to its dimethyl ester, was identified as 1'-N-carboxy-(+)-biotin (fig. 7(b)) (Knappe, Ringelmann, and Lynen, 1961). On the basis of the results of these and of exchange experiments analogous to those cited earlier, it was proposed that the biotin prosthetic group of the enzyme is similarly carboxylated in the initial step of the overall carboxylation reaction. Final proof that the site of carboxylation on the biotinyl prosthetic group is the same as it is on free biotin was subsequently obtained with several acyl CoA carboxylases (Knappe, Wenger, and Wiegand, 1963; Lane and Lynen, 1963; Numa, Ringelmann, and Lynen, 1964) and with transcarboxylase (Wood, Lochmüller, Riepertinger, and Lynen, 1963). The structure of the carboxylated active site is shown in figure 8. Although it is commonly believed that the carboxylation of free biotin is a unique property of the microbial  $\beta$ -methylcrotonyl CoA carboxylase, it was recently demonstrated in our laboratory (Stoll, Ryder, Edwards, and Lane, 1968) that liver acetyl CoA carboxylase catalyzes a similar reaction. This carboxylation reaction exhibits a high degree of structural and stereochemical specificity; the effects of certain alterations of the biotin molecule on carboxylation rate are shown in table I. This high degree of specificity, as well as the high  $K_m$  value for free (+)-biotin ( $K_m = 40 \text{ mM}$ ), supports the view that

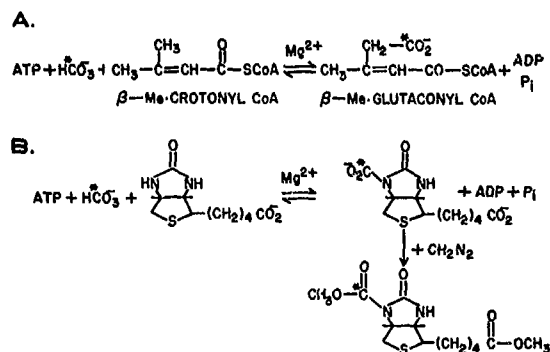


FIGURE 7.—The enzymatic carboxylation of  $\beta$ -methylcrotonyl CoA (a) and free (+)-biotin, (b) catalyzed by  $\beta$ -methylcrotonyl CoA carboxylase.

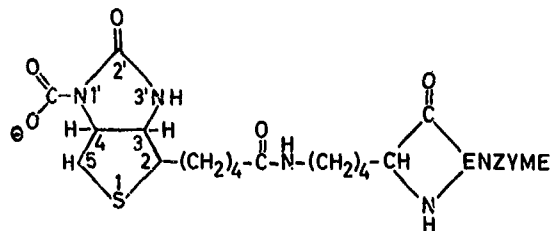


FIGURE 8.—The structure of the carboxylated active site ( $\epsilon$ -N-(1'-N-carboxy-(+)-biotinyl)-lysyl-enzyme) of acyl CoA carboxylases and transcarboxylase.

free biotin and certain of its derivatives bind and are carboxylated at the site normally occupied by the bicyclic ring of the biotinyl prosthetic group.

Further insight into the mechanism of carboxybiotin formation was gained by an investigation of the active species of "CO<sub>2</sub>" involved and the fate of its oxygen atoms. It was demonstrated (Kaziro, Hass, Boyer, and Ochoa, 1962) in experiments with <sup>18</sup>O-labeled bicarbonate that in the course of propionyl CoA carboxylation, one bicarbonate oxygen atom was incorporated into orthophosphate for every two bicarbonate oxygen atoms incorporated into the carboxyl group of methylmalonyl CoA. Similar experiments with carbamyl phosphate synthetase, a type III biotin enzyme, also showed that bicarbonate oxygen was incorporated into orthophosphate during the course of the overall reaction (Jones and Spector, 1960). The simplest interpretation of these results is that bicarbonate is the active species of "CO<sub>2</sub>" in these carboxylations. It is interesting to note

TABLE I.—*The Specificity of the Carboxylation of Free Biotin by Liver Acetyl CoA Carboxylase*

| Derivative <sup>a</sup>              | Concentration, mM | Carboxylation rate relative to <i>d</i> -biotin, percent |
|--------------------------------------|-------------------|--|
| <i>d</i> -Biotin.....                | 20                | 100  |
| <i>l</i> -Biotin.....                | 20                | 0  |
| <i>d</i> -Biocytin.....              | 20                | 121  |
| <i>d</i> -Homobiotin.....            | 20                | 36   |
| <i>d</i> -Norbiotin.....             | 20                | 33   |
| <i>dl</i> -O-Heterobiotin.....       | 40                | 29   |
| <i>dl</i> -Desthiobiotin.....        | 40                | 0  |
| 2-Imidazolidone (ethylene urea)..... | 20-100            | 0  |

<sup>a</sup> In these *d*- and *l*-compounds, the configuration differs about the 2 position (point of juncture of the side chain to the thiophane ring) or its equivalent.

that the same conclusion was reached for still another biotin enzyme, i.e., pyruvate carboxylase, using an independent method (Cooper, Tchen, Wood, and Benedict, 1968; Cooper, Tchen, Wood, Benedict, and Filmer, 1968). In view of these results and the ATP-<sup>32</sup>P<sub>i</sub> and ATP-<sup>14</sup>C-ADP exchange data discussed earlier that suggested a concerted reaction between enzyme-bound biotin, HCO<sub>3</sub><sup>-</sup>, and the  $\gamma$ -phosphoryl group of ATP (step 1, fig. 6), the mechanism shown in figure 9(a) was proposed (Kaziro et al., 1962). The bicarbonate anion is visualized as a nucleophilic reagent that attacks the  $\gamma$ -phosphoryl phosphorus atom displacing ADP in concert with the nucleophilic attack on bi-carbonate by the 1'-nitrogen of biotin. A parallel to this mechanism exists in the PEP carboxylase-catalyzed carboxylation reaction as illustrated in figure 9(b) (Maruyama et al., 1966). In the latter reaction, which will be discussed in more detail later, the phosphoryl phosphorus atom of PEP undergoes nucleophilic attack by bicarbonate.

Although a concerted reaction between ATP, HCO<sub>3</sub><sup>-</sup>, and the biotinyl prosthetic group (fig. 9(a)) is the simplest interpretation of most of the experimental facts, a stepwise mechanism has not been ruled out. The observation by Scrutton and Utter (1965) that pyruvate carboxylase, unlike propionyl CoA and  $\beta$ -methylcrotonyl CoA carboxylases, catalyzes a slow P<sub>i</sub> and bicarbonate independent ATP-<sup>14</sup>C-ADP exchange reaction is difficult to reconcile in terms of the concerted mechanism shown above. Because

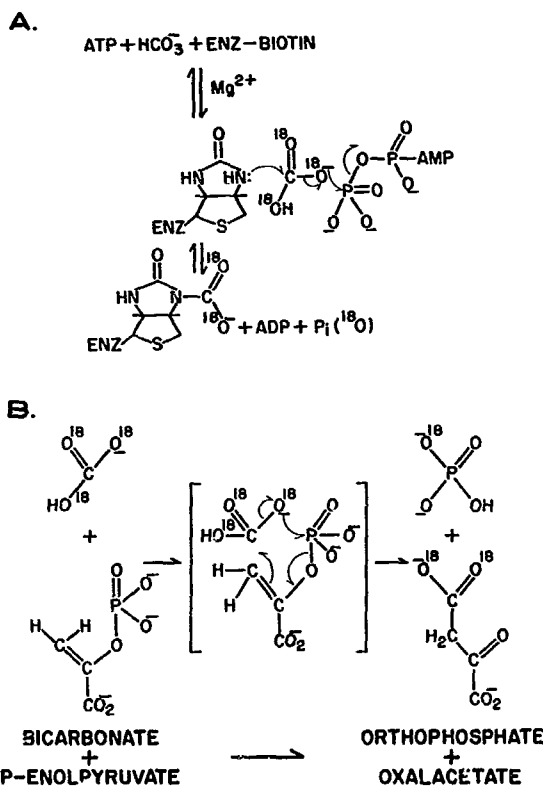
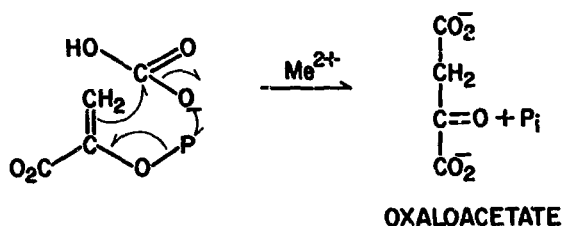


FIGURE 9.—Possible concerted mechanisms for the carboxylation of biotin (a) and phosphoenolpyruvate (b).

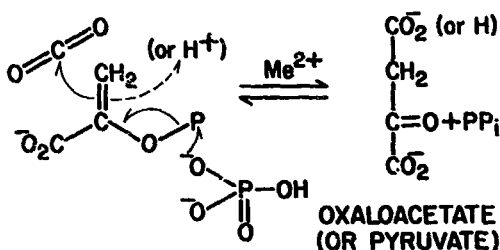
this exchange reaction is not inhibited by avidin, the participation of the biotin prosthetic group in the exchange mechanism appears unlikely. These results indicate either a more complex carboxylation mechanism or an abortive pathway.



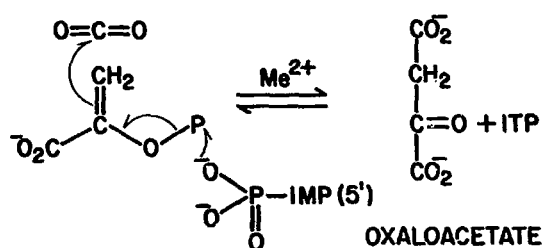
## PEP CARBOXYLASE:



## PEP CARBOXYTRANSPHOSPHORYLASE:



## PEP CARBOXYKINASE:



## PYRUVATE KINASE:

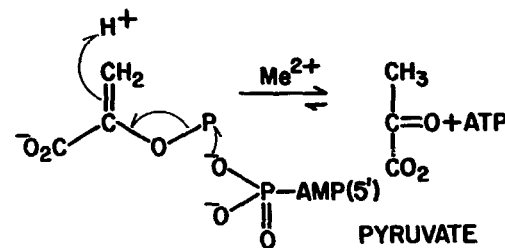


FIGURE 12.—Homology of mechanisms for the PEP carboxylating enzymes and pyruvate kinase.

the case of the PEP carboxykinase- and pyruvate kinase-catalyzed reactions is a nucleoside diphosphate (GDP or IDP in the former case, ADP in the latter). On the other hand, the nucleophile in the case of the PEP carboxylase-catalyzed reaction also appears to be the carboxylating electrophile; i.e., bicarbonate. The latter point requires further explanation. The possibility that bicarbonate rather than  $\text{CO}_2$  is the active carboxylating species was investigated by using  $^{18}\text{O}$ -bicarbonate as substrate (Maruyama et al., 1966). It was found that 1 atom of  $^{18}\text{O}$  from substrate  $^{18}\text{O}$ -bicarbonate was incorporated into  $\text{P}_i$  for every 2 atoms of  $^{18}\text{O}$  incorporated into oxaloacetate. Nucleophilic attack by a bicarbonate oxygen on the enol phosphoryl phosphorus atom is visualized as initiating the cyclic concerted reaction shown in figure 9(b). Simultaneous displacements at the bicarbonate carbon and phosphoryl phosphorus atoms lead to the initial formation of the keto form of oxaloacetate. This phenomenon is consistent with the finding of Tchen et al. (1955) that the keto and not the enol form of oxaloacetate is the primary carboxylation product of this reaction. Note, however, that this mechanism involving attack by bicarbonate on a phosphoryl phosphorus atom is formally analogous to that already described for

the propionyl CoA carboxylase-catalyzed reaction (Kaziro et al., 1962).

As discussed earlier, there is a striking similarity between the probable structures of the ternary enzyme-metal-PEP complexes of PEP carboxykinase and PEP carboxylase (Miller et al., 1968) and that proposed for pyruvate kinase (Mildvan and Cohn, 1966). This similarity in structure of the ternary complexes is not surprising, because the three enzymes catalyze homologous reactions of PEP. Although the nature of the complexes of PEP carboxytransphosphorylase (Siu and Wood, 1962) with metal and substrate have not been described, this carboxylase would appear to fit into this category. As illustrated in figure 12, in the reactions catalyzed by each of these enzymes, PEP undergoes nucleophilic attack on its phosphoryl group, tautomerizes and accepts a positively charged group on its no. 3 carbon atom (the carbonium form of bicarbonate with PEP carboxylase, the carbonium form of  $\text{CO}_2$  with PEP carboxykinase and PEP carboxytransphosphorylase, and a proton with pyruvate kinase). The essential difference in these reactions is the nature of the phosphoryl group acceptor and the electrophile which adds to PEP. The notion of homology of mechanisms (Miller et al., 1968) for pyruvate kinase and for the enzymes that



carboxylate PEP is strongly supported by the presence of mechanistic features of both in the reactions catalyzed by PEP carboxytransphosphorylase. The latter enzyme catalyzes the transfer of a phosphoryl group from PEP to orthophosphate (fig. 12) and, depending upon conditions, can either protonate or carboxylate carbon atom no. 3 of PEP (Davis and Wood, 1966).

While our understanding of this and certain other types of enzymatic carboxylations has improved markedly, we are still unable to write anything but tentative mechanisms. Nevertheless, it has been possible to compare the carboxylation reactions themselves and to demonstrate common mechanistic features both within and between the major groups. Within the next few years we can expect better definition of the individual carboxylation mechanisms.

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1 N70-23310

## The Role of Bound Metal Ions in CO<sub>2</sub> Fixation by the Biotin Carboxylases<sup>1</sup>

M. C. SCRUTTON

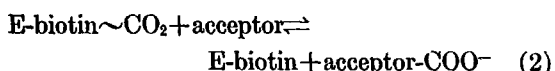
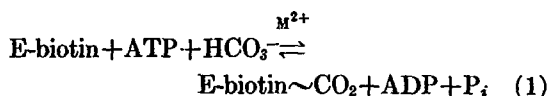
*Rutgers Medical School*

AND

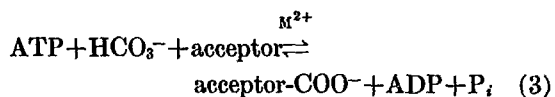
A. S. MILDVAN

*University of Pennsylvania*

The studies discussed by Dr. Lane in the preceding presentation have resulted in the formulation of a general minimal mechanism to describe the reactions catalyzed by most biotin carboxylases (reactions (1)-(2)).<sup>2</sup>



Sum:



The major differences between these enzymes are observed with respect to (1) their requirements for activation by metabolic effectors, and (2) the nature of the substrate that acts as the carboxyl acceptor. This latter difference provides a basis for classification of the biotin carboxylases (see fig. 2 of Lane's paper, this symposium).

<sup>1</sup> The data presented were obtained from research supported in part by contract AT(11-1)1242 of the Atomic Energy Commission; U.S. Public Health Service grants AM-09657, AM-09760, AM-11712, and GM-08320; and National Science Foundation grant G-23384.

<sup>2</sup> The mechanism of carbamyl phosphate synthetase purified from *Escherichia coli* is not adequately described by reactions (1)-(2) because an additional partial reaction is involved (Anderson and Meister, 1965).

Because the only example of a type III carboxylase has been described very recently (Wellner, Santos, and Meister, 1968), I will be concerned here with types I and II. Examination of acceptor specificity for both of these types of biotin carboxylase has indicated that even minor modification of acceptor structure causes greatly decreased catalytic activity (Hegre and Lane, 1966; Stoll, Ryder, Edwards, and Lane, 1968; D. S. Kerr and M. F. Utter, unpublished observations quoted in Mildvan, Scrutton, and Utter, 1966). This specificity may be reflected both in the differing extents of activation of adjacent carbon-bound protons (p. 216) and in the differing spatial requirements for the acceptor site in the two carboxylase types. The latter consideration implies that the environment of the biotin ring which is adjacent to the acceptor site may also differ in type I and II carboxylases. A difference in the environment of the biotin ring is suggested also by a comparison of properties of acetyl-CoA carboxylase (type I) and pyruvate carboxylase (type II) which indicates that the biotin residue of acetyl CoA carboxylase (type I) is more exposed to interaction with solvent or avidin than are the corresponding residues in pyruvate carboxylase (type II) (cf. Scrutton and Mildvan, 1968). The suggested difference is in the direction expected from a consideration of the relative size of the acceptors for these two enzymes.

Our studies have been concerned with the

mechanism of the second partial reaction (reaction (2)) in pyruvate carboxylase, the only known example of a type II carboxylase. These studies were initiated as an attempt to elucidate the role of the freely dissociable divalent metal ion in the first partial reaction (reaction (1)), using the nuclear magnetic resonance techniques developed by Dr. Mildred Cohn and her coworkers (cf. Cohn, 1963). However, the initial examination of pyruvate carboxylase purified from chicken liver revealed the presence of a nondissociable paramagnetic component in the enzyme preparation that we presumed to be a metal ion. The paramagnetic species was not removed by treatment of the enzyme preparation with 10 mM EDTA and had properties consistent with its participation in catalysis. Our subsequent studies on this aspect of reaction (2) have proved so informative that we have not yet returned to the original objective of these studies.

#### IDENTIFICATION OF THE BOUND METAL ION AS MANGANESE

Following the initial observations, manganese has been detected as a constituent of pyruvate carboxylase purified from chicken liver by several methods including emission, atomic absorption, and electron paramagnetic resonance<sup>3</sup> spectroscopy; and neutron activation and wet chemical analysis. The EPR spectrum of a perchloric acid extract of pyruvate carboxylase (fig. 1) provides the most convincing qualitative demonstration of the presence of significant amounts of manganese, because this spectrum is highly characteristic of Mn(II). Iron is also present in purified preparations of pyruvate carboxylase at a concentration approaching 1 g atom/mole enzyme (0.25 g atom/mole biotin), but other metal ions are absent. Although it is paramagnetic, the iron present cannot account for the effects observed in the NMR experiments.

Several lines of evidence support our conclusion that the bound manganese detected in pyruvate carboxylase purified from chicken liver is a consti-

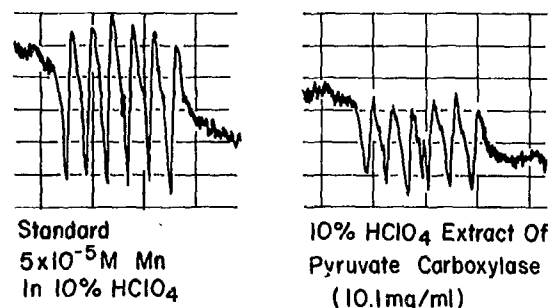


FIGURE 1.—EPR spectra of standard  $Mn^{2+}$  and a perchloric acid extract of pyruvate carboxylase. Pyruvate carboxylase (10.1 mg/ml; specific activity 19.0) was extracted with 10 percent perchloric acid and the denatured protein was removed by centrifugation. EPR spectra were taken on this extract and the standard  $Mn^{2+}$  solution in a Varian V-4280A EPR spectrometer at  $9.5 \times 10^9$  hertz as described previously (Mildvan and Cohn, 1963).

tutive component of this enzyme (Scrutton, Utter, and Mildvan, 1966):

(1) The 20–30 preparations of pyruvate carboxylase that have been assayed for manganese have all contained significant concentrations of this metal ion. No active or inactive preparation that is devoid of manganese has been obtained.

(2) Administration of  $^{54}Mn$  to chickens *in vivo* results in incorporation of radioactivity into pyruvate carboxylase. Both the radioactivity and total manganese purify in approximately constant ratio with the enzymic activity during the final stages of the isolation procedure.

(3) Quantitative analysis of purified preparations by five methods has indicated manganese contents in the range of 2.5–4.3 g-atoms/mole enzyme as compared with a biotin content of 3–4 moles/mole enzyme (Scrutton and Utter, 1965a). Assay for both of these cofactors in the same preparation has given a mean manganese:biotin ratio of 0.91 (Scrutton and Mildvan, 1968).

These data indicate that pyruvate carboxylase is a manganese metallobiotin enzyme. The presence and role of bound metal ions in other biotin enzymes will be discussed below. However, pyruvate carboxylase is also the only manganese metalloprotein described thus far. Although Mn(II) can be exchanged into the active sites of such enzymes as carboxypeptidase and carbonic anhydrase (Coleman and Vallee, 1960; Coleman, 1967), these enzymes are isolated as zinc metallo-

<sup>3</sup> The abbreviations used are PRR, proton relaxation rate ( $1/T_1$ ); EPR, electron paramagnetic resonance; NMR, nuclear magnetic relaxation.

proteins. Exchange of Mn(II) for Zn(II) at the active site causes either altered catalytic properties (carboxypeptidase) or loss of catalytic activity (carbonic anhydrase) (Coleman and Vallee, 1961; Coleman, 1967).

The constitutive manganese of pyruvate carboxylase is tightly bound to the protein (Scrutton et al., 1966) and is therefore clearly distinguished from the freely dissociable divalent metal ion, e.g., Mg(II) or Mn(II), which is required for activation of all kinases and synthetases (cf. Cohn, 1963). The first partial reaction of the biotin carboxylases (reaction (1)) is a synthetase reaction and requires such activation. Hence pyruvate carboxylase both requires activation by a dissociable divalent metal ion and also contains bound manganese. These two metal ions have, however, entirely different roles in catalysis.

### THE ENHANCEMENT PHENOMENON

Measurement of the longitudinal nuclear magnetic relaxation (NMR)<sup>3</sup> rate ( $1/T_1$ ) of the protons of water (PRR)<sup>3</sup> in solutions of pyruvate carboxylase indicates that the bound manganese has an enhanced effect on this relaxation rate as compared with the effect of  $\text{Mn}(\text{H}_2\text{O})_6^{2+}$  (table I).

TABLE I.—*The Enhanced Effect of the Bound Manganese of Pyruvate Carboxylase on the PRR*<sup>a</sup>

| System   | PRR (sec <sup>-1</sup> ) <sup>b</sup> |
|--|---------------------------------------|
| Buffer ( <i>tris</i> -Cl+KCl).....                           | 0.35–0.42                             |
| Pyruvate carboxylase (9.04 mg/ml).....                       | 1.92                                  |
| Pyruvate carboxylase (9.04 mg/ml) + 14% perchloric acid..... | 0.68                                  |

<sup>a</sup> From Scrutton et al., 1966.

<sup>b</sup> From these data,  $\epsilon_b$  may be calculated as 4.2, using eq. (4).

This enhancement phenomenon, which was discovered for proteins by Cohn and Leigh (1962) and for nucleic acids by Eisinger, Shulman, and Szymanski (1962), is considered here briefly because it is fundamental to our studies on the role of the bound manganese in catalysis. The en-

hancement factor ( $\epsilon^*$ ) is defined by

$$\epsilon^* = \frac{\frac{1}{T_1^*} - \frac{1}{T_1^0}}{\frac{1}{T_1} - \frac{1}{T_1^0}} \quad (4)$$

where  $1/T_1^*$  is the PRR of a solution of the complexed metal ion;  $1/T_1$ , the PRR of a solution containing an equivalent concentration of the metal ion in the uncomplexed state (as provided in table I by extraction of the pyruvate carboxylase preparation with perchloric acid); and  $1/T_1^0$ , the PRR of the buffer system. Since, for pyruvate carboxylase, free manganese is absent,  $\epsilon^*$  is equal to  $\epsilon_b$ , the enhancement of the complexed metal.

In solutions containing a complex of a paramagnetic ion, the PRR, and hence  $\epsilon^*$  (eq. (4)), is directly proportional to the number of water molecules in the coordination sphere  $p^*$  and to a correlation time  $\tau_c^*$ , if the exchange of water molecules into the hydration sphere of the metal ion  $1/\tau_m$  is rapid. For ions with a slow electron spin relaxation time,  $\tau_c^*$  is dominated by  $\tau_r^*$ , a correlation time describing the rotation of the ion relative to its hydration sphere. A slow electron spin relaxation time is characteristic only of Mn(II), Cu(II), and Cr(III) among the ions of the first transition series elements (Eisinger et al., 1962; Cohn and Leigh, 1962). If the exchange rate  $1/\tau_m$  is slow as compared with the relaxation rate of coordinated water molecules, then the PRR—and hence  $\epsilon^*$ —is proportional to  $p^*$  and to  $1/\tau_m$  (Luz and Meiboom, 1964).

In complexes involving small molecules as ligands, the primary effect on the PRR results from a reduction in the number of coordinated water molecules, because the rate of water exchange is rapid and the effects on molecular rotation times  $\tau_r$  are small. Hence for these complexes,  $\epsilon^*$  either approximates 1.0 (the enhancement of  $\text{Mn}(\text{H}_2\text{O})_6^{2+}$ ) or decreases below this value. However, in a complex involving a macromolecule, the relative rotation time ( $\tau_r^*$ ) may be greatly increased and may contribute to  $\epsilon^*$  for ions for which  $\tau_c$  is dominated by  $\tau_r$ ; e.g., Mn(II). Thus, if this increase in  $\tau_c^*$  outweighs the decrease in the number of coordinated water molecules  $p^*$ , an increase in  $\epsilon^*$  will result. This effect probably results from hindrance of the rotation of the

hydration sphere by the surrounding structure of the macromolecule (Cohn, 1963). The observation of an enhanced effect on the PRR is clearly dependent on measurement of a property of the bulk water. Hence, exchange of H<sub>2</sub>O on the complexed metal ion must occur at a rate that averages the contributions from the coordinated and non-coordinated states. Hence, if  $\epsilon^*$  exceeds 1.0, the site containing the metal ion must be accessible to solvent, and therefore, potentially, to the substrates.

Examination of the effect of substrates and inhibitors on the PRR (expressed as  $\epsilon^*$ ) provides a sensitive probe for interaction of these ligands with the metal ion or modification of its immediate environment due to complex formation, because the paramagnetic effect decreases as the sixth power of the distance and therefore is very weak beyond the first coordination sphere (Cohn, 1963). If ligands, e.g., substrates, interact with a metal ion complexed to a macromolecule,  $\epsilon^*$  will decrease as additional water molecules are displaced from the coordination sphere. The enhancement  $\epsilon_c$  of the metal ion in the macromolecule-ligand complex and the dissociation constant  $K_d$  of this complex may then be obtained by measurement of the decrease in  $\epsilon^*$  as a function of ligand concentration (Mildvan and Cohn, 1963, 1965, 1966). From the above discussion it is apparent that the enhancement factor may contain contributions from the rate of exchange of coordinated water molecules and the relative rotation rate, as well as from the number of coordinated water molecules. The ratio of enhancement factors observed for different complexes cannot therefore be used to determine relative solvation numbers for these complexes unless these other contributions are also evaluated.

#### THE LOCUS OF ACTION OF THE BOUND MANGANESE IN THE PYRUVATE CARBOXYLASE REACTION

For the pyruvate carboxylase reaction, components and inhibitors that provide ligands to the bound manganese may therefore be tentatively identified by examining their effects on  $\epsilon^*$  (table II). With the exception of HCO<sub>3</sub><sup>-</sup> (cf. table II, footnote b), the components of the first partial reaction (reaction (1)), added either singly or in combination, have no significant effect. Marked

TABLE II.—*The Effect of Substrates, Cofactors, and Inhibitors on the Enhancement of the Bound Manganese*<sup>a</sup>

| Reaction components added   | $\epsilon^*$ |
|---|--------------|
| None.....   | 4.2          |
| A. +1 mM A <sub>1</sub> P, 0.45 mM acetyl-CoA, or 20 mM MgCl <sub>2</sub> ..... | 4.0-4.2      |
| +20 mM KHCO <sub>3</sub> <sup>b</sup> .....                                     | 3.9          |
| B. +17 mM K pyruvate.....   | 1.8          |
| +3.3 mM Na oxalacetate <sup>c</sup> .....                                       | 2.6          |
| +0.25 mM K oxalate.....   | .4           |
| C. +ATP, acetyl-CoA, MgCl <sub>2</sub> and KHCO <sub>3</sub> .....              | 4.1          |
| +21 mM K pyruvate.....  | 1.8          |
| +3.6 mM Na oxalacetate <sup>c</sup> .....                                       | 1.8          |
| +0.16 mM K oxalate.....   | .5           |

<sup>a</sup> Modified from Mildvan et al., 1966.

<sup>b</sup> Further examination shows that HCO<sub>3</sub><sup>-</sup> interacts with the bound manganese forming a complex ( $K_d=0.45$  M,  $\epsilon_c=1.4$ ) that appears to have no catalytic significance.

<sup>c</sup> Addition of oxalacetate in the presence or absence of other reaction components causes a rapid decrease in  $\epsilon^*$  followed by a slower return toward the original value. The values for  $\epsilon^*$  given here are extrapolated to zero time.

decreases in  $\epsilon^*$ , however, are observed on addition of the substrates and inhibitors of reaction (2) (table IIB). The relationship of these effects to events at the catalytic site is established by comparison of dissociation constants obtained by titration measuring the decrease in  $\epsilon^*$  with (1) kinetic constants ( $K_i$ , apparent  $K_m$ ) obtained from initial rate studies of the overall reaction; and (2) dissociation constants obtained from the effect of these substrates and inhibitors on the rate of inactivation of pyruvate carboxylase by avidin. Inactivation by avidin results from interaction of this protein with the biotin residues of pyruvate carboxylase. Thus alterations in the inactivation rate induced by reaction components or inhibitors reflect binding of these ligands at the catalytic site. For the inhibitors of reaction (2), all three constants agree reasonably well (table IIIB). For the substrates the dissociation constants obtained by the avidin inactivation and PRR methods are in agreement but differ by one to two orders of magnitude from the apparent  $K_m$  determined in the overall or second partial re-

TABLE III.—Comparison of Kinetic and Dissociation Constants for Substrates and Inhibitors of the Second Partial Reaction of Pyruvate Carboxylase<sup>a</sup>

| Substrate (A) or inhibitor (B) | Initial rate studies |                       | Avidin inactivation  | PRR                   |              |
|--------------------------------|----------------------|-----------------------|----------------------|-----------------------|--------------|
|                                | $K_m$ (app.), M      | $K_i$ , M             | $K_d$ , M            | $K_d$ , M             | $\epsilon_c$ |
| A. Pyruvate.....               | $4.4 \times 10^{-4}$ | -----                 | $4.8 \times 10^{-3}$ | $4.5 \times 10^{-3}$  | 1.7          |
| Oxalacetate.....               | $5.0 \times 10^{-6}$ | -----                 | $2.4 \times 10^{-3}$ | $0.9 \times 10^{-3}$  | 1.9          |
| B. Oxalate.....                | -----                | $12.5 \times 10^{-6}$ | $8.9 \times 10^{-6}$ | $2.9 \times 10^{-6}$  | <.3          |
| Fluoropyruvate.....            | -----                | $1.7 \times 10^{-4}$  | $1.3 \times 10^{-4}$ | $2.3 \times 10^{-4}$  | <.3          |
| Phenylpyruvate.....            | -----                | $2.5 \times 10^{-4}$  | $4.0 \times 10^{-4}$ | $5.3 \times 10^{-4}$  | <.14         |
| Mesoxalate.....                | -----                | $2.1 \times 10^{-3}$  | $1.5 \times 10^{-3}$ | $0.96 \times 10^{-3}$ | <.31         |
| Tartronate.....                | -----                | $9.1 \times 10^{-4}$  | $5.0 \times 10^{-4}$ | $6.9 \times 10^{-4}$  | <.28         |
| L-Malate.....                  | -----                | $6.5 \times 10^{-3}$  | $2.3 \times 10^{-3}$ | $3.0 \times 10^{-3}$  | .32          |

<sup>a</sup> Modified from Mildvan et al., 1966.

actions (table IIIA; and Scrutton, Keech, and Utter, 1965).

In a multisubstrate reaction, the apparent  $K_m$  for a substrate is, however, seldom equal to its dissociation constant, and the discrepancy noted in table IIIA is therefore not surprising.

#### STRUCTURES PROPOSED FOR THE ENZYME-SUBSTRATE AND ENZYME-INHIBITOR COMPLEXES

A model may then be proposed to describe the interaction of substrates and inhibitors of reaction (2) with the bound manganese which is consistent with most of the data presented in tables II and III. In this model the metal ion receives three *cis* ligands from the protein and has therefore three additional *cis* ligand positions which are accessible to solvent. These accessible ligand positions are proposed to have defined specificities for interaction with substrates and inhibitors of reaction

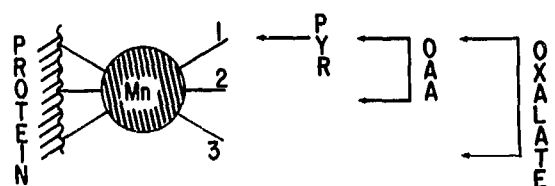


FIGURE 2.—Proposed structures for the complexes of pyruvate (PYR), oxalacetate (OAA) and oxalate with the bound manganese of pyruvate carboxylase.

(2) as a consequence of their distribution in space. This distribution results in differing environments with respect to the surrounding tertiary structure of the protein. If the accessible ligand positions are numbered 1, 2, and 3, the interactions proposed are pyruvate at 1; oxalacetate at 1 and 2; and the inhibitors, e.g., oxalate, at 1 and 3 (fig. 2). These assignments represent the simplest explanation that is consistent with our data and do not exclude more complex explanations. The evidence that supports the proposed interactions is summarized below.

#### THE ENZYME-INHIBITOR COMPLEX

The effect of the bound manganese on the PRR is profoundly de-enhanced in the enzyme-inhibitor complex ( $\epsilon_c \leq 0.14-0.32$ ) (table IIIB). This observation indicates that the inhibitors displace water molecules from the coordination sphere of the bound metal because a decrease in  $\tau$ , resulting, for example, from a change in the conformation of the protein, can reduce this enhancement only to a value approximating 1.0. Because many of the inhibitors examined can provide at most two ligands to the bound manganese, the effect on  $\epsilon^*$  may result, in part, from the displacement of two coordinated water molecules. Although the effect of the enzyme-inhibitor complex on the PRR is weak, residual coordinated water molecules must be present on the bound manganese in this complex because  $1/T_{1p}$  is not equal to  $1/T_{2p}$ , and an



exchange-limited region is observed when  $1/T_{1p}$  is measured as a function of temperature (cf. Luz and Meiboom, 1964). The enhancement observed for the enzyme-inhibitor complex is similar to that found for the Mn(II)-EDTA complex (King and Davidson, 1958). Because crystallographic studies indicate the presence of a single coordinated water molecule in the Mn(II)-EDTA complex (Hoard, Pederson, Richards, and Silverton, 1961), we propose that one water molecule also remains in the coordination sphere of the bound manganese on formation of the enzyme-inhibitor complex. These comparisons also support the suggestion that the protein provides three ligands to the bound manganese.

The relationship of the ligand positions used by the inhibitors with that used by pyruvate is established by the demonstration of a simple competitive relationship between these ligands (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1968), indicating that one of the positions used by the inhibitors is identical with that used by pyruvate (1 in fig. 2). Studies with the pyruvate carboxylase-avidin complex indicate that the other position (3) is used only by the inhibitors (Scrutton and Mildvan, 1968).

#### THE ENZYME-PYRUVATE COMPLEX

The enhancement observed for the enzyme-pyruvate complex ( $\epsilon_c = 1.7$ ) (table IIIA) is consistent with that expected for a complex involving a monodentate ligand, but does not prove direct coordination of this substrate because the decrease in the enhancement could result from a decrease in  $\tau_r$ . Evidence for direct coordination, however, is provided by analysis of the effect of pyruvate carboxylase on the methyl resonance in the NMR spectrum of pyruvate (fig. 3). Addition of either MnCl<sub>2</sub> or manganese bound to pyruvate carboxylase causes marked increases in the radio-frequency power required to saturate the resonance and the line width at half-line height (fig. 3). These parameters may be used, respectively, to calculate  $1/T_{1p}$  and  $1/T_{2p}$ , the paramagnetic contributions to the longitudinal and transverse relaxation rates of the pyruvate methyl protons. Such studies indicate that, in the presence of 25 mM pyruvate, the effect of the bound manganese is enhanced approximately twentyfold on  $1/T_1$  and

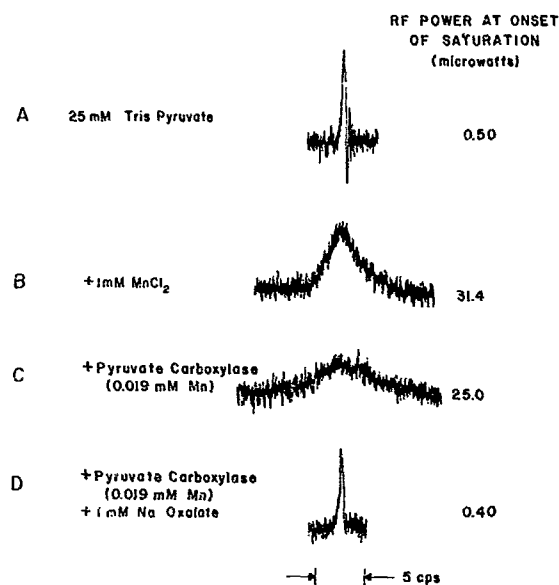


FIGURE 3.—Effect of MnCl<sub>2</sub> and of pyruvate carboxylase on the methyl resonance in the nuclear magnetic resonance spectrum of pyruvate. The solvent was 95 percent D<sub>2</sub>O containing 0.1 M tris-DCI (pD = 8.4) and 0.27 M KCl in a total volume of 0.5 ml; temperature, 28 C. (Reproduced from Mildvan and Scrutton (1967) by permission of the editor of Biochemistry.)

hundredfold on  $1/T_2$  as compared with the effect of Mn<sup>2+</sup> (Mildvan and Scrutton, 1967).

Because binding of small molecule ligands to proteins in the presence or absence of diamagnetic ions causes much smaller increases in the relaxation rates of ligand protons (Jardetsky, 1964; Mildvan and Scrutton, 1967), the enhanced effects observed for  $1/T_1$  and  $1/T_2$  of pyruvate methyl protons on addition of pyruvate carboxylase are consistent with formation of a pyruvate carboxylase-manganese-pyruvate bridge complex. This conclusion is supported by calculation of  $r$ , the distance between the bound manganese and the methyl protons of pyruvate, to be  $3.5 \pm 1.0$  Å from these data as compared with measurements of  $r$  in Dreiding molecular models of the Mn(II)-pyruvate complexes involving a carbonyl ( $r = 3.7 \pm 1.1$  Å) or a carboxyl ( $r = 4.0 \pm 1.7$  Å) ligand. Calculation of the hyperfine coupling constant, which measures the interaction operating through chemical bonds, gives a value more consistent with coordination involving a carbonyl ligand (Mildvan and Scrutton, 1967).

Two lines of evidence support the postulate that the observed complex participates in catalysis. First, addition of oxalate abolishes the effects of pyruvate carboxylase on the NMR spectrum of pyruvate (fig. 3(d)). A linear relationship is observed between the line width and reciprocal oxalate concentration; and the  $K_d$  for oxalate calculated from these data ( $5.4 \mu M$ ) agrees with other measurements of this constant ( $2.9$ – $12.5 \mu M$ ) (table IIIB). Second, the exchange rates for pyruvate in this complex are two to four orders of magnitude faster than the maximal turnover number for CO<sub>2</sub> fixation on pyruvate (table IV). Hence, the complex forms and dissociates at a rate consistent with catalytic function (Mildvan and Scrutton, 1967).

TABLE IV.—Some Properties of the Pyruvate Carboxylase-Manganese-Pyruvate Complex<sup>a</sup>

| Parameter  | Units                             | Observed value            |
|--|-----------------------------------|---------------------------|
| $k_{on}^b$   | M <sup>-1</sup> sec <sup>-1</sup> | $4.5 \times 10^6$         |
| $k_{off}^b$  | sec <sup>-1</sup>                 | $2.1 \times 10^4$         |
| Maximal turnover number per biotin residue (CO <sub>2</sub> fixation) <sup>d</sup> | sec <sup>-1</sup>                 | $0.9$ – $1.0 \times 10^2$ |
| $\Delta H$ (pyruvate exchange) <sup>b</sup>  | Kcal/mole                         | 2.5                       |
| $\Delta S$ (pyruvate exchange) <sup>b</sup>  | eu                                | -30.3                     |
| $r$ , (Mn, methyl distance) <sup>c</sup>   | Å                                 | $3.5 \pm 1.0$             |

<sup>a</sup> From Mildvan and Scrutton (1967).

<sup>b</sup> From  $1/pT_{2p}$ , which is dominated by exchange of pyruvate on the bound manganese over the temperature range examined (2 C–40 C) (Mildvan and Scrutton, 1967).

<sup>c</sup> From  $1/pT_{1p}$ , which is dominated by relaxation of coordinated pyruvate methyl protons over the temperature range examined (2 C–40 C) (Mildvan and Scrutton, 1967).

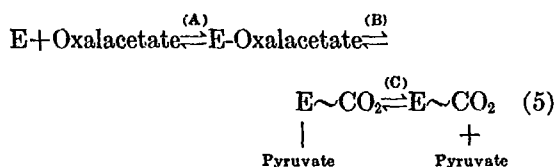
<sup>d</sup> Scrutton and Utter (1965a).

The exchange rates for both H<sub>2</sub>O and pyruvate, which may be equated with  $k_{off}$  for these ligands (table IV, Scrutton and Mildvan, 1968), are two orders of magnitude slower than the exchange rate for H<sub>2</sub>O on Mn(H<sub>2</sub>O)<sub>6</sub><sup>2+</sup> (Swift and Connick, 1962). Furthermore, the kinetic barrier to the departure of H<sub>2</sub>O and pyruvate from the coordi-

nation sphere of the bound manganese is primarily entropic (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1968) in contrast to the primarily enthalpic barrier observed for departure of H<sub>2</sub>O from Mn(H<sub>2</sub>O)<sub>6</sub><sup>2+</sup> (Swift and Connick, 1962). From these comparisons, we may tentatively conclude that departure of both these ligands from the coordination sphere of the bound manganese is restricted as a consequence of shielding by the surrounding tertiary structure of the protein. Formation of the pyruvate carboxylase-manganese-pyruvate complex appears to occur by the mechanism proposed by Eigen and Tamm (1962), because the rate of binding of pyruvate is limited by the rate of departure of a coordinated water molecule (Mildvan and Scrutton, 1967).

#### THE ENZYME-OXALACETATE COMPLEX

The proposed bidentate structure for the enzyme-oxalacetate complex has, until recently, received little support from our experimental observations, although it is a required feature of the mechanism proposed for the catalytic role of the bound manganese (Mildvan et al., 1966) (fig. 5). The enhancement observed for this complex ( $\epsilon_c = 1.9$ ) is similar to that observed for the enzyme-pyruvate complex ( $\epsilon_c = 1.7$ ) and might be expected for a monodentate ligand. Although pyruvate carboxylase reacts with oxalacetate, as shown in reaction (5),



the suggestion that this apparent anomaly results from the contribution of other complexes is not supported by the observation of a similar enhancement ( $\epsilon_c = 2.0$ ) for the avidin-pyruvate carboxylase-oxalacetate complex in which reaction (5) is blocked at step (B) (Scrutton and Mildvan, 1968).

Some support for the bidentate structure proposed for the enzyme-oxalacetate complex is provided by the finding that the enhancement of the bound manganese in the avidin-pyruvate carboxylase-oxalacetate complex ( $\epsilon_c = 2.0$ ) is consistently lower than the enhancement in the

avidin-pyruvate carboxylase-pyruvate complex ( $\epsilon_c = 2.3$ ) (Scrutton and Mildvan, 1968). Although this difference is small, it is consistent with the proposed bidentate structure, if the avidin-pyruvate carboxylase-oxalacetate structure differs from the structure of the bidentate enzyme-inhibitor complex ( $\epsilon_c \leq 0.3$ ) (cf. fig. 3).

Recently we have examined the effect of pyruvate carboxylase on the NMR spectrum of oxalacetate. These studies, which are analogous to those described for pyruvate (see preceding section), are more readily interpreted than studies utilizing the relaxation rate of water protons, because measurements made on resonance lines arising from substrate protons are specific for complexes of that substrate and do not contain contributions from other complexes. For example, the methylene resonance of oxalacetate is clearly separated from the methyl resonance of pyruvate, the product of reaction (5). Preliminary analysis of data obtained in studies on the methylene resonance of oxalacetate permits the following conclusions:

(1) The effect of the bound manganese of pyruvate carboxylase on  $1/T_1$  and  $1/T_2$  of these methylene protons is enhanced as compared with the effect of  $Mn^{2+}$ .

(2) The distance between the bound manganese and the methylene protons calculated from the NMR data is consistent with measurements of this distance obtained using Dreiding molecular models of both the mono- and bidentate complexes of  $Mn(II)$ -oxalacetate.

(3) The rate of departure of ligand ( $k_{off}$ ) is slower for oxalacetate as compared with pyruvate, and  $\Delta H^{2+}$  for oxalacetate exchange is approximately fivefold greater than for pyruvate exchange (cf. table IV).

The decreased exchange rate and increased  $\Delta H^{2+}$  are predicted for a bidentate ligand and support the structure proposed for the enzyme-oxalacetate complex (fig. 2).

The enhancement observed for the enzyme-oxalacetate complex in the PRR studies ( $\epsilon_c = 1.9$ ) may be reconciled with the proposed bidentate structure, if binding of oxalacetate at positions 1 and 2 causes the water molecule at position 3 to interact more strongly with the bound manganese. Such an effect would be analogous to that invoked

to explain the increase in  $\epsilon_c$  that occurs on formation of the pyruvate-carboxylase-avidin complex (Scrutton and Mildvan, 1968), although the underlying mechanism may differ. An alteration in the properties of position 3 on formation of the enzyme-oxalacetate complex is suggested because, in contrast to the oxalate-pyruvate system previously discussed, oxalacetate and oxalate do not exhibit a simple competitive relationship for interaction with the bound manganese. Instead, addition of oxalacetate causes an increase in the  $K_d$  for oxalate which is several orders of magnitude greater than that predicted by simple competition; but the  $K_d$  for oxalacetate does not appear to be affected by addition of oxalate. This increase may be compared with the increase of four orders of magnitude in the  $K_d$  for oxalate on formation of the pyruvate-carboxylase-avidin complex (Scrutton and Mildvan, 1968). The proposed difference in the effects of pyruvate and oxalacetate on the conformation of the protein is supported by the observation that these substrates have opposite effects on the rate of inactivation at 2°C (J. J. Irias and M. F. Utter, personal communication).

#### THE ROLE OF THE BOUND MANGANESE IN CARBOXYL TRANSFER BY PYRUVATE CARBOXYLASE

As indicated by Lane, the basic chemical problem in any carboxylation reaction is the activation of either the relatively stable CO<sub>2</sub> molecule (or HCO<sub>3</sub><sup>-</sup> ion) or the acceptor, or both. For the biotin carboxylases, this problem is partially solved by the formation of a C-N bond (as 1'-N-carboxybiotin) in the first partial reaction. Calculation of  $\Delta F^{70}$  for decarboxylation of the 1'-N-carboxybiotinyl intermediate of methylmalonyl-CoA-oxalacetate transcarboxylase, however, gives a value of -4.74 kcal/mole (Wood, Lochmüller, Riepertinger, and Lynen, 1963b), which suggests that this intermediate is at the low end of the spectrum of "energy-rich" compounds. In addition, for type II carboxylases, the acceptor is a weak nucleophile. Before the discovery of bound manganese in pyruvate carboxylase, metal-ion catalysis of carboxylation and decarboxylation reactions related to the second partial reaction of a type II carboxylase had been

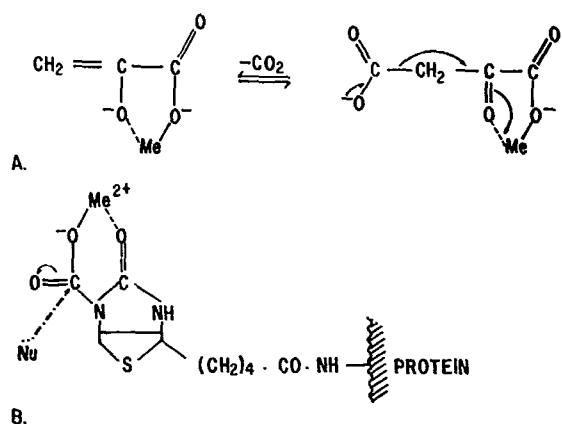


FIGURE 4.—Proposed roles for metal ions in the catalysis of model carboxylation (decarboxylation) reactions involving  $\alpha$ -keto acids (Steinberger and Westheimer, 1951) (a) or 1'-N-Carboxybiotin (Stiles, 1960; Caplow, 1965) (b).

examined in model systems, and several mechanisms had been proposed (fig. 4).

Metal-ion catalysis of decarboxylation of keto-dicarboxylic acids was first demonstrated for dimethylmalonate by Steinberger and Westheimer (1951) following the earlier spectrophotometric demonstration of complexes of divalent metal ions with these ligands by Kornberg, Ochoa, and Mehler (1948). Although trivalent ions, e.g., Al<sup>3+</sup>, are most effective in the model decarboxylation reaction, the isolation of oxalacetate decarboxylases which were specifically activated by divalent metal ions, e.g., Mn<sup>2+</sup> (Plaut and Lardy, 1949; Herbert, 1951), provided support for a biological role for divalent metal-ion catalysis. The mechanism proposed by Steinberger and Westheimer (1951), illustrated for pyruvate and oxalacetate in figure 4(a), requires formation of a chelate complex between the metal ion and the carbonyl and carboxyl groups of the  $\alpha$ -keto acid. Decarboxylation then proceeds, leaving the enol form of the product. Two observations suggest, however, that the mechanism shown in figure 5(a) does not adequately describe the catalytic role of the bound manganese in the pyruvate carboxylase reaction. First, pyruvate appears to be a monodentate ligand for the bound manganese. Second, incubation of pyruvate carboxylase with tritiated pyruvate provides no evidence for a complex involving an enol form of this substrate, because a

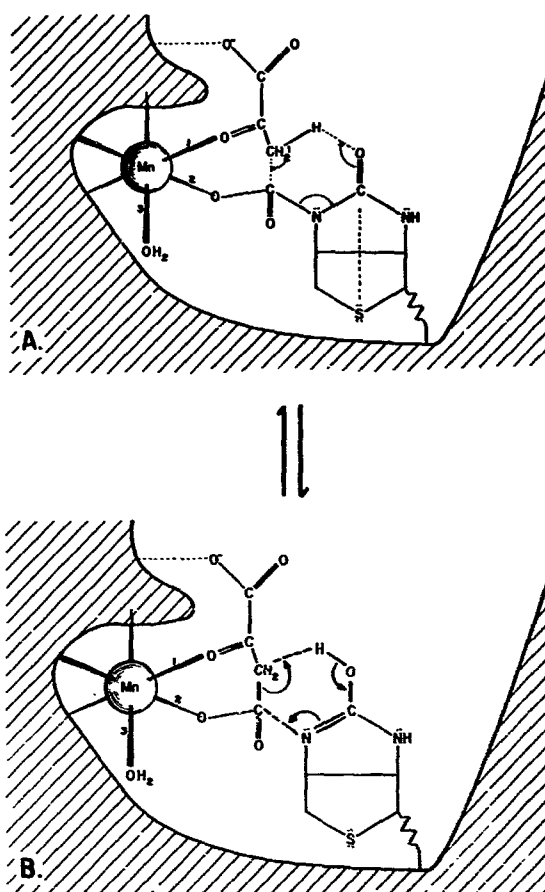


FIGURE 5.—Proposed role for the bound manganese in carboxyl transfer by pyruvate carboxylase. (a) E-CO<sub>2</sub>-pyruvate; (b) E-oxalacetate. Modified from Mildvan et al. (1966) and Mildvan and Scrutton (1967).

significant rate of tritium release is only observed in the presence of the E-biotin CO<sub>2</sub> intermediate (Mildvan et al., 1966). Moreover, the NMR studies (cf. fig. 3) indicate a very slow rate of exchange of deuterons into the methyl group of pyruvate in the pyruvate carboxylase-pyruvate complex. These data are, however, not conclusive evidence against the presence of an enol complex because the proton released on enolate formation may be retained by the enzyme. The phenomenon of "proton retention" has been demonstrated for several enzymic reactions; e.g., phosphoglucose isomerase (O'Connell and Rose, 1961).

Alternatively, catalysis by the metal ion might involve interaction with the carboxybiotin residue

rather than the  $\alpha$ -keto acid. Formation of a chelate complex between the metal ion, and the ureido carbonyl and 1'-N-carboxyl groups of carboxybiotin (fig. 4(b)) should activate the 1'-N-carboxyl group to nucleophilic attack (Stiles, 1960). This effect has been demonstrated by Caplow (1965) in model studies on esters of N-carboxy-2-imidazolone, an analog of the ureido ring of biotin. The rate of reaction of these esters with nucleophiles, (e.g., hydroxylamine) at alkaline pH is accelerated up to fortyfold by addition of 0.8 M CaCl<sub>2</sub>, whereas CaCl<sub>2</sub> seems to have little effect on the rate of reaction of acetate esters under similar conditions (Caplow, 1965). Additionally the rate of decarboxylation of N-carboxy-2-imidazolone is markedly decreased by addition of divalent metal ions; e.g., Mn<sup>2+</sup>, Cu<sup>2+</sup> (Caplow and Yager, 1967). If decarboxylation of N-carboxy-2-imidazolone is truly unimolecular as suggested by Caplow (1965), such stabilization by divalent metal ions might provide an additional driving force for bimolecular carboxyl transfer. This mechanism is, however, also an inadequate explanation of the catalytic role of the bound manganese, because formation of catalytically significant pyruvate carboxylase-manganese-pyruvate and -oxalacetate complexes has been demonstrated. In addition, the 1'-N-carboxybiotinyl residue does not interact with the bound manganese when it is formed from ATP and HCO<sub>3</sub><sup>-</sup> in reaction (1) (table IIC).

Our data are satisfied by a mechanism (fig. 5) which combines features from both those shown in figure 4 and which provides a dual role for the bound manganese. Formation of the E-biotin~CO<sub>2</sub> intermediate from ATP and HCO<sub>3</sub><sup>-</sup> occurs at a site that is not adjacent to the metal ion. The subsequent binding of pyruvate causes an alteration of the conformation of the protein, which brings the 1'-N-carboxyl group to the metal, forming the E~CO<sub>2</sub>-pyruvate complex (fig. 5(a)). Carboxyl transfer from the 1'-N-carboxybiotinyl residue to the methyl group of pyruvate then occurs simultaneously with departure of a proton; the E-oxalacetate complex results (fig. 5(b)). In this mechanism the electrophilic character of the metal ion facilitates the departure of a proton from the methyl group of pyruvate and assists incipient carbonium ion formation from 1'-N-carboxybiotin. The structures of the enzyme-sub-

strate complexes required by this mechanism are supported by the evidence summarized in the foregoing sections: "The Enzyme-Pyruvate Complex" and "The Enzyme-Oxalacetate Complex."

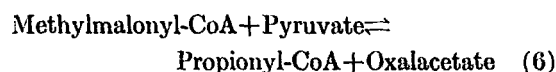
Although we still lack proof that the mechanism shown in figure 5 adequately describes the role of the bound manganese in catalysis, the proposed alteration in protein conformation resulting from the binding of pyruvate is supported by the differing effects of various reaction components on the rate of inactivation of pyruvate carboxylase by avidin. This rate is decreased in the presence of ATP (Scrutton and Utter, 1965b), but is increased by addition of pyruvate or oxalacetate (Mildvan et al., 1966). The biotin residues may therefore exist in at least two environments that differ in their accessibility to avidin. If these environments represent spatially separated parts of the catalytic site, movement between them may be facilitated by the 14 Å flexible arm on which the biotin residue is mounted in the biotin carboxylases (Green, 1963). Interaction of inhibitors (e.g., oxalate) with the bound manganese does not appear to cause movement of the 1'' N-carboxybiotinyl residue into the vicinity of the metal ion because both  $K_d$  and  $\epsilon$  for the enzyme-oxalate complex are unaffected by formation of E-biotin~CO<sub>2</sub> from ATP and HCO<sub>3</sub><sup>-</sup> (table IIC).

#### THE PRESENCE AND POSSIBLE ROLE OF BOUND METAL IONS IN OTHER BIOTIN ENZYMES

Following the discovery of bound manganese in pyruvate carboxylase purified from chicken liver, both pyruvate carboxylases purified from other sources and other biotin enzymes have been examined for bound metal ions. All pyruvate carboxylases described thus far are inhibited by low concentration of oxalate (less than 0.1 mM) (Ruiz-Amil, de Torrontegui, Palacian, Catalina, and Losada, 1965; Seubert and Weicker, 1969; M. C. Scrutton, unpublished observations), suggesting that bound metal ions may be present. Bound manganese has been detected in purified preparations of pyruvate carboxylases from turkey and calf liver at levels similar to those observed for chicken liver (Utter, Scrutton, Young, Tolbert, Wallace, Irias, and Valentine, 1967). However,

pyruvate carboxylase purified from baker's yeast does not contain significant concentrations of manganese, and the metal present in this enzyme, if any, has not been identified (Young, Tolbert, Valentine, Wallace, and Utter, 1968).

Although other mechanisms, e.g., formation of a Schiff's base intermediate, could provide the required activation of the methyl group of pyruvate, recent observations on methylmalonyl-CoA-oxalacetate transcarboxylase support the proposal that bound metal ions provide the activation required for carboxyl transfer to this  $\alpha$ -keto acid. This latter enzyme which catalyzes reaction (6) (Swick and Wood, 1960; Wood, Allen, Stjernholm, and Jacobson, 1963)



is inhibited by oxalate ( $K_i = 1.8 \mu M$ ) and contains bound cobalt and zinc. The combined content of these two metals approximates the biotin content of the purified enzyme (Northrop and Wood, 1967; Wood, Jacobson, Gerwin, and Northrop, 1969). In preliminary studies analogous to those described for pyruvate carboxylase (fig. 3), addition of methylmalonyl-CoA-oxalacetate transcarboxylase broadens the methyl resonance in the NMR spectrum of pyruvate. This effect is reversed by addition of oxalate (Northrop, 1969). An enzyme-cobalt-pyruvate bridge complex may therefore be formed with a role in the reaction catalyzed by methylmalonyl-CoA-oxalacetate transcarboxylase which resembles that proposed for the pyruvate carboxylase-manganese-pyruvate complex (fig. 5).

Studies on type I carboxylases have thus far been more limited in scope. However, significant concentrations of manganese, cobalt, or zinc are not present in purified preparations of acetyl-CoA carboxylase from chicken liver and propionyl-CoA carboxylase from pig heart (A. S. Mildvan, C. Gregolin, M. D. Lane, D. Prescott, and J. Rabinowitz; unpublished observations quoted in Mildvan and Scrutton (1967)). These data do not exclude the presence of another bound metal ion. It is also possible that bound metal might not be required, for example, if the adjacent thioester provides the required activation of the methyl (or

methylene) group on which fixation of CO<sub>2</sub> occurs. In derivatives of acetoacetic acid, the introduction of a thioester group reduces the  $pK_a$  of the protons on the vicinal carbon by approximately 2 pH units (Lynen, 1953; Lienhard and Jencks, 1965). However, the effectiveness of activation of adjacent carbon-bound protons by a thioester as opposed to an  $\alpha$ -keto acid group does not appear to have been compared directly.

### CONCLUSION

The studies described here confirm in some measure earlier speculation based on theoretical considerations and model studies that metal ions may play an important role in enzymic carboxylation and decarboxylation reactions. The recognition of manganese as the metal ion involved in carboxylation of pyruvate in avian liver has been of special interest because it has provided the first example of a manganese metalloprotein and, by virtue of the paramagnetic properties of this metal ion, has permitted us to use nuclear magnetic resonance techniques in our studies of its role in catalysis. We have devoted most of our effort thus far to this latter end, but it is apparent that other directions of investigation may prove fruitful. For example, pyruvate carboxylase is suggested as a key enzyme in gluconeogenesis from three-carbon precursors; e.g., lactate (Utter, 1963). If a sufficiently severe manganese deficiency can be induced, a direct test of the role of pyruvate carboxylase in gluconeogenesis should be feasible. It may also be possible to test the postulate that the gluconeogenic flux from lactate is limited by the capacity of this enzyme and to gain some understanding of the metabolic roles of nondialyzable manganese in avian liver.

### ACKNOWLEDGMENTS

We are most grateful to Dr. Mildred Cohn and Dr. Merton Utter for their interest and encouragement throughout these studies.

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DISCUSSION

**POCKER:** Do you know of any examples of chemical reactions in which bicarbonate is a carboxylating agent? According to chemical models, I know that enzymes are wonderful things which we are all trying to analyze.  $\text{HCO}_3^-$  has one saturated double bond and is a good molecule to be attacked by a nucleophile. You can write bicarbonate with one double bond and one single bond, but if you are honest, it is rather stable and requires a terrific amount of energy for activation. There should be some such reactions that are biochemical; then it would be easier for chemists to accept the fact that bicarbonate is involved. Otherwise, they say  $\text{CO}_2$  is involved; the enzymes carboxylate it, and you never know it.

**LANE:** I really know of no chemical reaction other than enzymic, where bicarbonate is involved. I have been discussing this with Dr. Caplow. There is even some question whether methylmagnesium carbonate is the active species in the carboxylation described by Stiles (1960). It was reported as such, but in a footnote, Stiles said he had not strictly ruled out  $\text{CO}_2$  as the carboxylating species, so I really cannot throw any light on that; perhaps Dr. Caplow can.

**HASTINGS:** Do not photosynthetic people really think it is bicarbonate?

**POCKER:** They do, but I do not see any mechanisms written down, except ADP, ATP, and those arrows.

**LANE:** I do not think it has been established what the active species is in the case of the ribulose diphosphate carboxylation. I think that enzyme should be very susceptible to Dr. Cooper's approach; carbonic anhydrase, particularly, because it has such a very high  $K_m$  for bicarbonate or  $\text{CO}_2$ . In fact, the  $K_m$  is unphysiologically high unless the chloroplast has the ability to concentrate  $\text{CO}_2$ .

**POCKER:** A question to Dr. Scrutton. I was very interested in the pyruvate-enzyme complex. We are concerned about it from another point of view. What is the real evidence that manganese binds pyruvate at one place rather than at two places? I am delighted you do activate the carbonyl, because you do have to do something with the hydrogen of the  $-\text{CH}_3$ ; but why do you not also bind the  $\text{COO}^-$ ?

**SCRUTTON:** Our evidence for the structure of the enzyme-manganese-pyruvate complex consists of the relative enhancement values for the free enzyme and its various complexes obtained in the PRR studies and on measurement of the hyperfine coupling constant in the studies on the pyruvate methyl protons. This evidence is all somewhat equivocal and the proposed structure is a best fit to our data. However, we hope soon to study the enzyme-pyruvate complex further using either  $\text{C}^{13}$  or  $\text{O}^{17}$  resonance, and these studies should permit an unequivocal assignment of the structure of this complex.

**BERGER:** Do you feel that the six-line EPR spectrum is influenced by its environment? We have been able to shift it all the way from one line to six.

**SCRUTTON:** The EPR spectrum shown was taken on a perchloric acid extract of pyruvate carboxylase and is valid as evidence for the presence of manganese, because a similar six-line spectrum is obtained for a solution of an  $\text{Mn}^{2+}$  salt in perchloric acid. Our EPR studies on the native enzyme have thus far been uninformative, in part because of technical difficulties in obtaining reproducible results. Nothing significant appears at room temperature, but at about 100 K we obtained a spectrum showing some structure. With the eye of faith, six "major" lines can be detected but as many as 10 or 12 lines are discernible if one looks with care. We also think we can see differences in the 100 K spectrum given by the enzyme alone as compared with that given by the enzyme-oxalate complex. However, interpretation of manganese EPR spectra is very complex, and because we have been able to get so much information from NMR, we have not yet tried very hard with EPR.

**HASTINGS:** There is a manganous porphyrin compound that Cotzias has gotten out of the red cell. It may be an enzyme seeking a substrate.

**SCRUTTON:** That is interesting because our evidence from the *in vivo* studies with  $^{54}\text{Mn}$  indicates that there is incorporation into other proteins in chicken-liver mitochondria in addition to pyruvate carboxylase. Very preliminary studies suggest that  $^{54}\text{Mn}$  is present in a fraction that exhibits a heme-type spectrum, but we do not

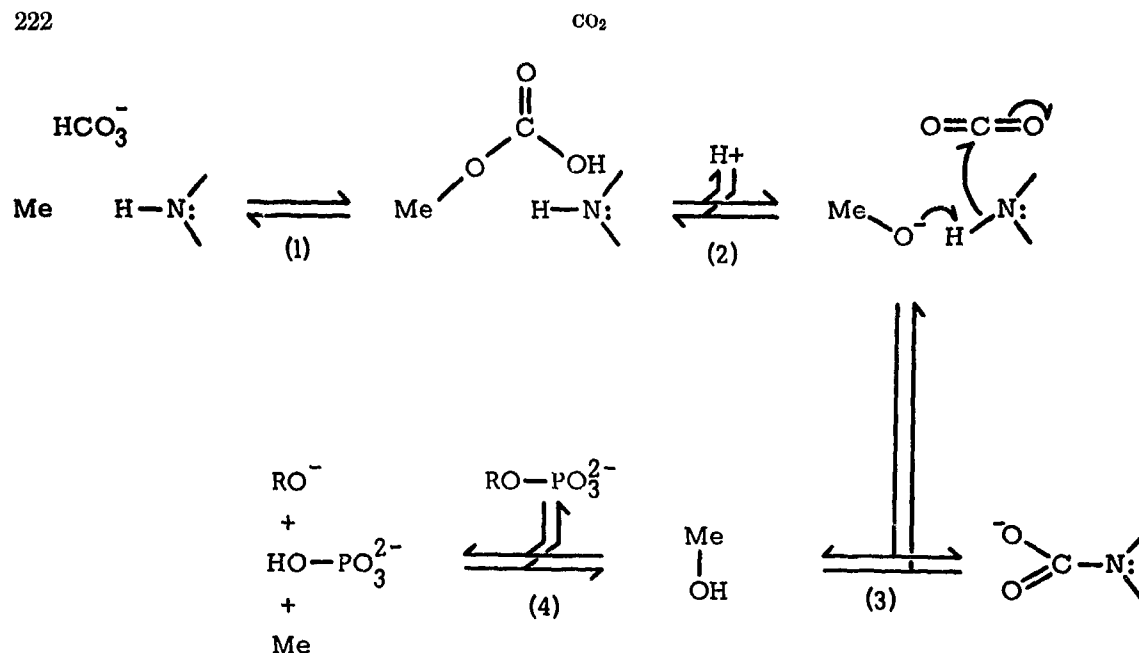


FIGURE D-1.—Proposed mechanism for the carboxylation of biotin. Abbreviations are: Me, divalent metal; H—N:, 1'-nitrogen of biotin; RO—PO<sub>3</sub><sup>2-</sup>, ATP; and RO<sup>-</sup>, ADP.

know how many components this fraction contains. I am just about to start further studies on this problem.

**SCRUTTON and LANE** (submitted later): Kaziro et al. (1962) have proposed that formation of the carboxybiotin intermediate from ATP and bicarbonate in a biotin carboxylase reaction is a concerted process that involves the HCO<sub>3</sub><sup>-</sup> ion as the carboxylating species (cf. Lane, this symposium, p. 195). However, chemical considerations that are amply documented in the proceedings of this symposium indicate that CO<sub>2</sub> is a superior electrophile as compared with HCO<sub>3</sub><sup>-</sup> and hence a better carboxylating species. On the other hand, the bicarbonate anion is a more attractive ligand for interaction with an enzyme than the uncharged CO<sub>2</sub> molecule. We would therefore like to suggest an alternate mechanism for formation of the carboxybiotin intermediate in which HCO<sub>3</sub><sup>-</sup> initially bound to the enzyme is converted to CO<sub>2</sub> by interaction with the dissociable divalent metal ion that is required for activation of the carboxylation reaction. This alternate mechanism, which is shown in figure D-1, represents an adaptation of the mechanism proposed by Wang for the carbonic anhydrase

reaction (cf. Wang, this symposium, p. 101), and therefore invokes the participation of a Me-O<sup>-</sup> species. This alternate mechanism for the biotin carboxylases has two advantages over the concerted process. First, although an E-HCO<sub>3</sub><sup>-</sup> complex is formed initially (step 1), subsequent reaction with the metal ion leads to formation of CO<sub>2</sub> as the true carboxylating species (step 2). Second, the participation of the Me-O<sup>-</sup> species provides a feasible mechanism for abstraction of a proton from the 1'-nitrogen of the ureido biotin, thereby increasing the nucleophilic character of this nitrogen atom. The studies of Caplow (1965) have indicated that biotin is a very weak nucleophile.

The final step in the scheme shown (step 4) involves attack of the metal-coordinated hydroxyl group on the  $\gamma$ -phosphoryl group of ATP. Although reversal of step 4 seems somewhat problematical, it should be noted that Lowenstein (1958) has demonstrated metal ion catalysis of transphosphorylation in model systems.

The mechanism proposed here is compatible with the studies of Cooper and his coworkers (cf. Cooper, this symposium, p. 183) and also with earlier O<sup>18</sup> studies (cf. Lane, this symposium,

p. 195) provided that the metal-bound hydroxyl ion generated in step 3 does not exchange rapidly with the solvent. Furthermore, unpublished studies indicate the formation of an E-Me<sup>2+</sup> complex by pyruvate carboxylase which is distinct from both the bound manganese present in this enzyme (cf. Scrutton and Mildvan, this symposium, p. 207) and the Me<sup>2+</sup>-ATP complex.

The only other enzyme of CO<sub>2</sub> metabolism that has been reported thus far to utilize HCO<sub>3</sub><sup>-</sup> rather than CO<sub>2</sub> is PEP carboxylase. The mechanism proposed in figure D-1 is also applicable to the reaction catalyzed by PEP carboxylase if

step 4 is omitted, and steps 2 and 3 involve the attack of Me-O<sup>-</sup> on the phosphoryl phosphorus of PEP.

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*SESSION V*

**CO<sub>2</sub> Exchange Rates in the Body**

*Chairman: A. B. OTIS*

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## Introductory Remarks

A. B. OTIS

*University of Florida*

We turn now from the realm of more or less pure chemistry with its clearly defined problems and decisive solutions to the world of physiology where the questions are frequently fuzzy and the answers are often muddled.

The problem that forms the theme of this afternoon's program can be traced back a century to the days of Ludwig and Pfüger and the first attempts to measure gas tensions in arterial and venous blood. The conclusion reached at that time was that in the lungs,  $\text{CO}_2$  is given off and  $\text{O}_2$  is absorbed by simple diffusion.

In the intervening years, this point of view has been challenged from time to time, most notably by Bohr and Haldane, who concluded that active secretion of these gases might occur. Krogh, however, confirmed the earlier notion and for many years respiratory physiologists have generally considered that diffusion equilibrium of  $\text{CO}_2$  takes place in the lungs and have regarded  $P_{ACO_2}$  and  $P_{aCO_2}$  to be identical.

Recently, however, reports from Jones and Campbell in London and from Gurtner and Farhi in Buffalo would lead us to believe that under some conditions, appreciable differences may occur between the  $P_{CO_2}$  in pulmonary blood and that in the alveolar gas. The first two papers on the program will provide us with first-hand information from the London and Buffalo laboratories. In the next pair of papers, Dr. Chinard and Dr. Dubois will tell us something about the barrier that exists in the lung between the blood and gas phase.

Finally, Dr. Piiper and Dr. Forster will remind us that the blood is itself a two-phase system and that the rates of exchange between cells and plasma as well as those between blood and gas must be considered in the analysis of the problem at hand.

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## Alveolar-Blood $P_{CO_2}$ Differences During Rebreathing<sup>1</sup>

N. L. JONES AND E. J. M. CAMPBELL

Royal Postgraduate Medical School, London

We have confirmed our previous finding of a difference in  $P_{CO_2}$  between alveolar gas and blood during rebreathing at rest and on exercise under conditions of negligible net  $CO_2$  movement. The difference is slightly reduced when net movement of  $O_2$  as well as  $CO_2$  is avoided by rebreathing  $CO_2$  in  $N_2$ , but remains as large as 10 mm Hg in heavy exercise.

When mixtures of  $CO_2$  in  $O_2$  are rebreathed from a small bag and the  $CO_2$  concentration in the gas passing to and fro between the lungs and bag is measured rapidly, one of four patterns may be seen, depending on the initial concentration of  $CO_2$  in the bag (fig. 1). In the first pattern, the  $CO_2$  in the bag is so low that  $CO_2$  is added at once from the lungs; in the second, a transient equilibrium is achieved between the bag and lung gas, but addition of  $CO_2$  from venous blood leads to a rapid rise in  $CO_2$ ; in the third, equilibrium between the bag, lungs, and venous blood is achieved, the  $P_{CO_2}$  is unchanged over several breaths, and the subsequent rise in  $CO_2$  occurs after recirculation has raised the  $CO_2$  in venous blood. In the last record the initial  $CO_2$  concentration is so high that equilibration is not achieved before recirculation.

Consider the situation in the third record in which sustained equilibrium with no net movement of  $CO_2$  implies equilibration of  $P_{CO_2}$  between alveolar gas and pulmonary capillary blood; the  $P_{CO_2}$  should be that of oxygenated mixed venous blood. However, during our investigation of rebreathing during exercise, we found that the implied venous  $CO_2$  content calculated from the equilibrium  $P_{CO_2}$  and a standard dissociation curve was higher than that predicted from a knowledge of the cardiac output and the arterial  $CO_2$  (Jones,

Campbell, McHardy, Higgs, and Clode, 1967). To look into this, we sampled arterial blood during rebreathing (fig. 2). The gas passing to and fro between the bag and lungs was rapidly analyzed, and after equilibrium was achieved, several samples of blood were rapidly taken via a percutaneous catheter using a manifold of taps. Each sample was taken over 3 seconds and analyzed within a few minutes by electrodes. The blood  $P_{CO_2}$  was related to the  $P_{CO_2}$  of the gas 5 seconds previously (fig. 3). The assumption of 5 seconds for lung-to-arm circulation time is not critical, for the change in gas and blood  $P_{CO_2}$  during the procedure is small. The gas  $P_{CO_2}$  was higher than the  $P_{CO_2}$  in arterial blood. This was found consistently in exercise and the difference increased with increasing workloads, being related to  $CO_2$  output. We did not find any relation to the initial bag  $P_{CO_2}$  as long as equilibrium was obtained, nor with time; the difference was still present up to 40 seconds after the onset of rebreathing.

We postulated that the rapid oxygenation of hemoglobin releases  $CO_2$  which cannot be excreted during rebreathing, so that the  $P_{CO_2}$  in the pulmonary capillaries is transiently higher than would be predicted from the *in vitro*  $CO_2$  dissociation curve; this suggestion implied that under these conditions, equilibration between the various forms of combined  $CO_2$  in plasma and red cells occurs only after the blood has left the lung. The remainder of this paper describes the testing of this hypothesis. If rapid oxygenation of hemo-

<sup>1</sup>This work was supported by the Medical Research Council.

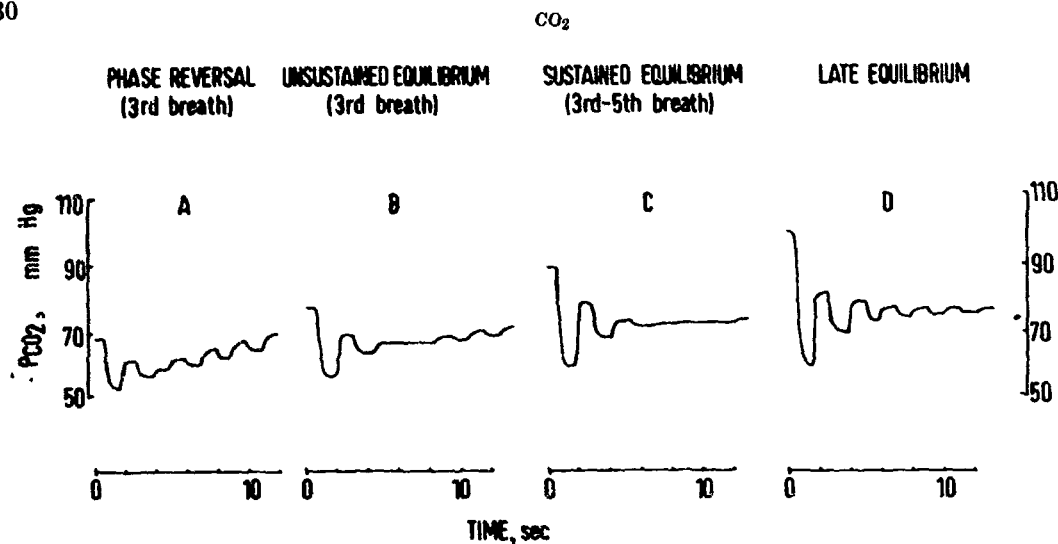


FIGURE 1.—Records of  $P_{CO_2}$  during rebreathing. Four different patterns are shown, obtained with successively increasing initial bag  $CO_2$  concentrations in a normal subject exercising at a workload of 600 kpm/min ( $CO_2$  output 1.5 l/min). Pattern (C) shows a sustained equilibrium; the unsustained, phase reversal, patterns (A) and (B) occur at a lower  $P_{CO_2}$  and the delayed equilibrium (D) at a higher  $P_{CO_2}$ . (Reproduced by permission of Clinical Science.)

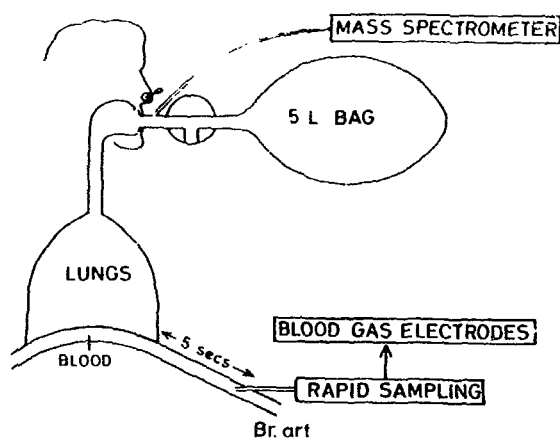


FIGURE 2.—Experimental procedure.

globin were an important cause, the gas-to-blood difference should be abolished during rebreathing of mixtures containing no oxygen. The subjects exercised on a cycle ergometer; when steady-state conditions were achieved, expired gas was collected over a minute to give  $CO_2$  output and  $O_2$  intake; a mixture of  $CO_2$  in  $O_2$ , previously shown to give an equilibrium pattern of  $CO_2$ , was re-breathed and serial arterial blood samples rapidly taken. This procedure was followed by rebreathing a  $CO_2$  in  $N_2$  mixture.  $CO_2$  and  $O_2$  in the gas were continuously analyzed with a mass spectrometer.

The difference in  $P_{CO_2}$  was on the average 1.2 mm Hg ( $SD \pm 3.7$ ,  $n=19$ ) less during the low  $O_2$  rebreathings, but an appreciable difference still remained (fig. 4). We have been unable to account for these findings on technical grounds. Moreover, there was no significant alveolar-arterial  $P_{O_2}$  difference during the low  $O_2$  rebreathings, which suggests that neither failure to attain equilibrium because of shunts or ventilation:perfusion problems nor a net uptake of  $CO_2$  because of bag shrinkage consequent upon  $O_2$  uptake are responsible.

## DISCUSSION

We do not know the extent to which the  $P_{CO_2}$  difference is present when  $CO_2$  is allowed to leave the lung, but we would guess that it is less important than in the rebreathing situation. However, we should face the possibility that it may exist during normal breathing in exercise, and we should consider the implications that it might have for gas exchange. A transient increase in  $P_{CO_2}$  of the blood passing through the lungs may facilitate the removal of  $CO_2$ , acting in a way similar to the Christiansen-Douglas-Haldane effect.

Secondly, the end-tidal  $P_{CO_2}$  during exercise is almost invariably higher than arterial  $P_{CO_2}$ ; this fact has been explained previously by the magni-

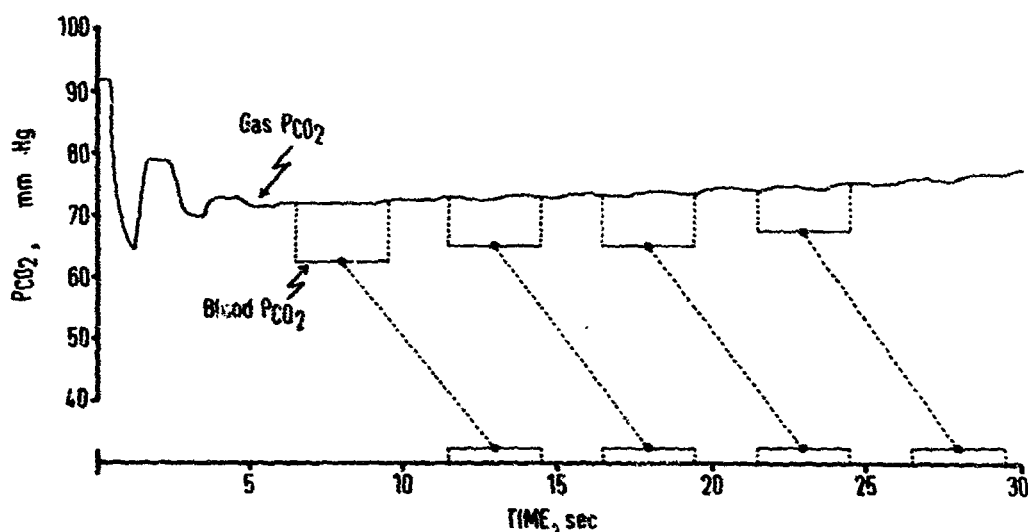


FIGURE 3.—Comparison of  $P_{CO_2}$  in the rebreathing system and in arterial blood samples. Subject exercising at 600 kpm/min. The blood samples were drawn during the periods shown by the lower bars; the  $P_{CO_2}$ 's of these samples are indicated by the upper bars which are offset by the lung-arm circulation time (5 seconds). Upper continuous record is  $P_{CO_2}$  of gas sampled at the mouth. The difference in  $P_{CO_2}$  between the end-tidal gas and the corresponding blood varies between 7 and 10 mm Hg. (Reproduced by permission of Clinical Science.)

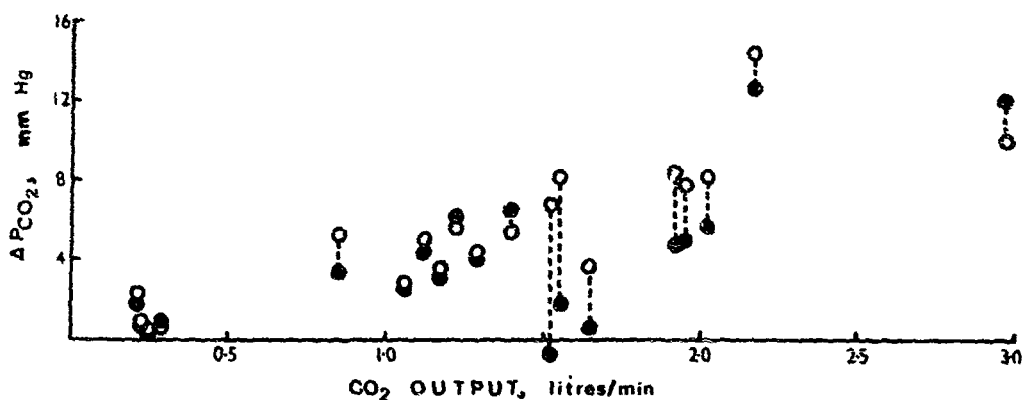


FIGURE 4.—Gas-blood  $P_{CO_2}$  difference during the rebreathing of  $CO_2$  mixtures in  $O_2$  (open symbols) and  $CO_2$  mixtures in  $N_2$  (closed symbols).

fied oscillations in alveolar  $CO_2$  because of the increase in  $CO_2$  excretion, but it could be partly caused by this alveolar-to-blood difference. We have tried to investigate this with the technique used in the present studies, but the difficulties of prolonged expiration and sufficiently rapid sampling did not permit us to obtain any reliable data. Finally, if alveolar  $P_{CO_2}$  were higher than the arterial  $P_{CO_2}$ , the validity of certain calcu-

lations that are based on the assumption that they are equal, such as that for physiological dead space, might need modification.

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## Alveolar to Mixed Venous $P_{CO_2}$ Difference Under Conditions of No Gas Exchange<sup>1</sup>

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During prolonged breathholding or rebreathing,  $CO_2$  transfer from the blood into the alveolar spaces diminishes and eventually ceases, the  $CO_2$  content of end capillary blood becoming equal to that of the pulmonary artery. Because of the  $O_2$ - $CO_2$  interaction, if  $O_2$  uptake by the blood is maintained, the  $P_{CO_2}$  of capillary blood must rise above that of the mixed venous blood; and it has generally been accepted that the alveolar  $P_{CO_2}$  is then equal to the arterial and represents "oxygenated mixed venous  $P_{CO_2}$ " (Collier, 1956; Fenn and Dejours, 1954; Butler, 1965). More recently, Campbell and coworkers noted that in human subjects rebreathing oxygen, the difference between alveolar  $P_{CO_2}$  and that of mixed venous blood exceeded what could be explained on the basis of the Haldane effect (Jones, Campbell, McHardy, Higgs, and Clode, 1967). They found that during prolonged rebreathing of 100 percent  $O_2$ ,  $P_{ACO_2}$  also exceeded arterial  $P_{CO_2}$ . One of the possible explanations presented by these authors was that equilibrium across the alveolar membrane was indeed achieved, but that some slow reactions involving the coupling between  $O_2$  and  $CO_2$  transport by hemoglobin were still taking place in the blood after it had left the lungs, lowering the  $P_{CO_2}$ . This "downstream effect," as it was called by the Hammersmith group, left the concept of alveolar capillary  $CO_2$  equilibrium unchallenged.

Cerretelli, Cruz, Farhi, and Rahn (1966) have described a rebreathing technique in which proper

selection of gas volume and composition resulted in a plateau for both alveolar  $O_2$  and  $CO_2$ . This means that because there is no  $O_2$  uptake, there should be no  $O_2$ - $CO_2$  effect and that the alveolar and arterial gas tensions should be equal and reflect "true mixed venous" pressure. We have found that this was true for  $O_2$ , but not for  $CO_2$  (Gurtner, Song, and Farhi, 1967). The alveolar  $CO_2$  tension exceeded mixed venous and arterial values. In the following presentation we shall first review briefly our experimental results and set forth a tentative explanation from our data. The second part of this paper will deal with additional evidence supporting our theory.

### $CO_2$ DIFFERENCES ACROSS THE ALVEOLAR MEMBRANE

#### Methods and Results

All the experiments were performed on mongrel dogs anesthetized with pentobarbital. Some animals were rebreathed manually, with a large syringe containing initially a  $CO_2$ - $N_2$  gas mixture. If the proper volume and composition of gas were used, steady values for both  $P_{AO_2}$  and  $P_{ACO_2}$  (monitored continuously with a mass spectrometer) were encountered. Following this short procedure, normal breathing was allowed to resume. To obtain longer lasting plateaus, a different type of experiment was performed in which a lobar bronchus was cannulated and used for rebreathing while ventilation was maintained in the rest of the lung. In this case, rebreathing was monitored for periods of 15 to 20 minutes. In a similar experiment, the cannulated lobe was first rendered

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air free, and then filled with a 10-percent sucrose solution which was used as the rebreathing medium. Experiments with fluid-filled lobes lasted for 4 to 5 hours. In all of these experiments, alveolar  $P_{CO_2}$  was measured and compared to mixed venous  $P_{CO_2}$  obtained by sampling blood from the pulmonary artery. The results were similar in all types of experiments.  $P_{ACO_2}$  exceeded  $P_{VCO_2}$  in 51 of the 55 determinations in which blood pH was between 7.3 and 7.5. The mean value for  $\Delta P_{CO_2}$  was 7.2, with a standard deviation of 3.3.

### Discussion

Because in our experiments no O<sub>2</sub> was exchanged at the alveolar membrane, a "downstream effect" for the lung could not explain our results. It has been suggested, however, that the equilibrium in terms of CO<sub>2</sub> is not achieved in the systemic circulation between the time the blood leaves the tissues and reaches the lung. If this were so, one would encounter an apparent gradient similar to that observed by Cain and Otis (1961) with carbonic anhydrase inhibition. However, the carbonic-anhydrase-catalyzed reactions are so fast that it seems likely that under normal conditions distribution of CO<sub>2</sub> between plasma and red cells is complete by the time the blood reaches the lung. This contention is supported by the work of Chinard, Enns, and Nolan (1960), who found that the volume of distribution of <sup>14</sup>C-labeled CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> were similar when injected into the right heart. After carbonic anhydrase inhibition, tagged HCO<sub>3</sub><sup>-</sup> had a volume of distribution similar to that of a dye which did not leave the circulation.

Because the gas in the lung is in contact with alveolar capillary membrane and not with the capillary blood itself, it follows that the gas in the lung might reflect the  $P_{CO_2}$  of the membrane rather than that of capillary blood. Our hypothesis is based on the fact that in biological systems, local CO<sub>2</sub> tension is affected by local H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> concentration as well as by the CO<sub>2</sub> tension of surrounding tissues. If for any reason the concentration of H<sup>+</sup> increases in the vicinity of the membrane or in the membrane itself, an increase in the association reaction would occur and CO<sub>2</sub> tension would become higher than in the bulk

phase of the blood. There are several possibilities for this increase in hydrogen-ion concentration.

The source of this hydrogen ion might be in the membrane itself if acids were produced metabolically in lung tissue. It is known that lactic acid is generated by the lung (Glaviano, Shiuntetsu, and Masters, 1967). The site of production within the lung is unknown, but if this were to be in the membrane and if this membrane had a high permeability to HCO<sub>3</sub><sup>-</sup>, it is possible that a steady shift of bicarbonate to CO<sub>2</sub> could occur, resulting in the gradient that we have described.

A more tempting hypothesis is one in which the source of hydrogen ion is the blood itself, a difference in hydrogen-ion concentration existing between the major part of the blood in the capillaries and the outermost layer of this fluid. Most biological membranes appear to carry negative surface charges (Weiss, 1967; Weiss and Woodbridge, 1967) and blood vessels seem to carry a higher density of negative surface charges than other cells (Sawyer and Hummelfarb, 1965). It is well known that because of the electrostatic attraction of cations by the negatively charged groups, the pH near a negatively charged membrane is lower than that of the bulk phase of solution (Hartley and Roe, 1940; Danielli, 1934). If the HCO<sub>3</sub><sup>-</sup> concentration near the charges is similar to that in the bulk phase, then for the equilibrium condition

$$\frac{[H^+][HCO_3^-]}{[CO_2]} = K$$

to be satisfied:

$$\Delta[CO_2] = \Delta[H^+] \cdot \frac{[HCO_3^-]}{K} \quad (1)$$

The increase in hydrogen-ion concentration at the membrane level could be caused not only by attraction of the H<sup>+</sup> normally present in the plasma but also by generation of additional H<sup>+</sup>. It is known that weak acids increase their dissociation under the influence of electrical fields. This effect is known as the Wien effect (Hartley and Roe, 1940) and the magnitude of the effect is related to differences between mobilities of central and counter ions (Onsager, 1934). Large synthetic polyelectrolyte ions show a marked Wien effect even with low field strengths (1-2 V/cm) (Wali, Terayama, and Techakumpuch, 1956). It

seems possible that protein molecules would also exhibit a large Wien effect. This might then increase the hydrogen-ion concentration almost instantaneously which in association with bicarbonate could lead to the production of  $CO_2$  and explain our findings. Because one  $H^+$  ion is required to associate with each  $HCO_3^-$  ion to form one  $H_2CO_3$  molecule, we feel that the major source of  $H^+$  is the dissociation of protein.

### EVIDENCE SUPPORTING POSTULATED THEORY

To test the validity of this explanation, a certain number of additional experiments was also performed. In the first to be described below, we tried to alter the magnitude of  $\Delta P_{CO_2}$  by changing blood composition or by varying the rate of blood flow in the pulmonary capillaries. In a second type of experiment, the transport of other molecules which can be expected to behave like carbonic acid was studied. Finally, we attempted to demonstrate the presence of a  $CO_2$  gradient across a commercial negatively charged membrane.

### FACTORS AFFECTING $\Delta P_{CO_2}$

#### Levels of $[H^+]$ and $[HCO_3^-]$

Equation (1) indicates that if our tentative explanation is correct, there should be a relationship between  $\Delta P_{CO_2}$  and  $\Delta[H^+]$ . If the distribution of  $H^+$  around a negative charge can be described by Boltzmann's distribution law (Hartley and Roe, 1940), then

$$H_w = H_B e^{+(\zeta F)/RT} \quad (2)$$

$w$  indicates ionic concentration near capillary wall

$B$  indicates ionic concentration in bulk phase

$\zeta$  indicates potential difference between wall and bulk phase

Because  $\Delta H = H_B - H_w = H_B(1 - e^{+(\zeta F)/RT})$ ,  $\Delta H^+$  at constant  $\zeta$  is a constant multiple of  $H_B$ , and therefore  $\Delta P_{CO_2}$  should be related to blood pH. Figure 1(a) indicates that there is a trend to higher values of  $\Delta P_{CO_2}$  when pH decreases, and that at any given pH the value of  $\Delta P_{CO_2}$  is related to the  $HCO_3^-$  concentrations predicted by equation (2). In figure 1(b) we have plotted

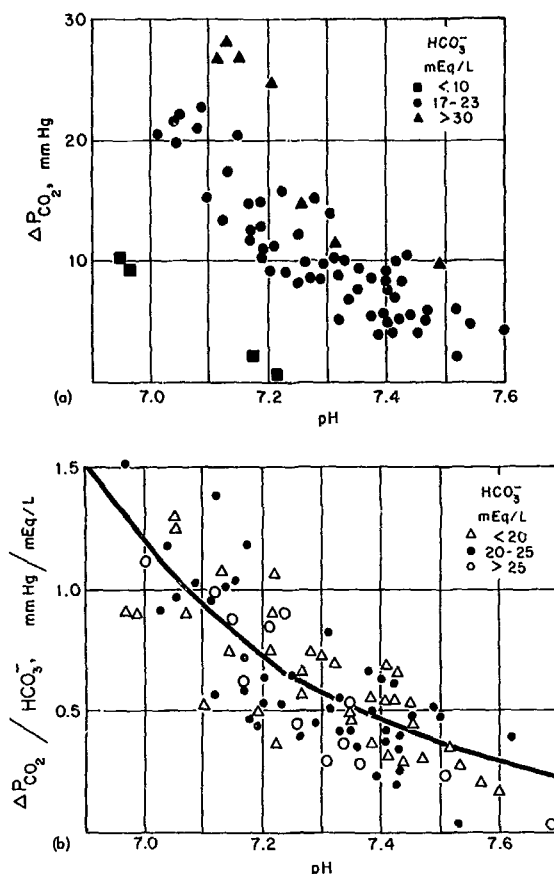


FIGURE 1.—(a) Relationship between  $\Delta P_{CO_2}$  and the pH of mixed venous blood. Three different  $HCO_3^-$  contents are shown. 1.—(b) Relationship between the normalized  $P_{CO_2}$  gradients ( $\Delta P_{CO_2}/HCO_3^-$ ) and mixed venous pH. The solid line indicates the slope of the predicted relationship (see text).

$\Delta P_{CO_2}/[HCO_3^-]$  versus pH. The close grouping of the points indicates that our experimental data justify the concept that the  $P_{CO_2}$  differences may be attributed to attraction of hydrogen ions by the negative charges near the membrane. The slope of the solid line gives the slope of the predicted relationship.

#### Effect of Cardiac Output

If the increase in  $P_{CO_2}$  is indeed a result of an increased association of bicarbonate and hydrogen ion, the assumption is that the factors that lead to a rise in  $H^+$  do not affect the bicarbonate concentration in the opposite direction. Obviously,

the electrostatic charges that attract hydrogen ion will repel bicarbonate until at equilibrium the effect of the decrease in  $[\text{HCO}_3^-]$  would balance exactly that of the increase in  $[\text{H}^+]$  and  $P_{\text{CO}_2}$  would be the same throughout. We must therefore postulate that to obtain a  $\Delta P_{\text{CO}_2}$ , it is necessary that contact time between blood and membrane be such that the average  $P_{\text{CO}_2}$  of the membrane be higher than the  $P_{\text{CO}_2}$  of the blood. This could occur if the rate of redistribution of  $\text{HCO}_3^-$  ion were slower than the rate of increase of  $\text{H}^+$  ions (because of the Wien effect) and if the redistribution of  $\text{HCO}_3^-$  ions were not complete until the blood had spent a relatively large proportion of its transit time in the capillary. A theoretical analysis of these assumptions in light of what is known about the rates of  $\text{CO}_2$  reactions and diffusion in blood is given in the appendix.

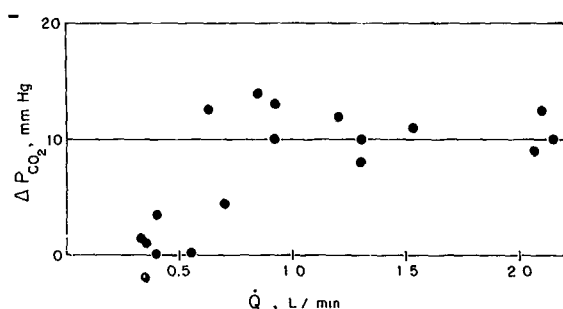


FIGURE 2.—Relationship between  $\Delta P_{\text{CO}_2}$  and pulmonary blood flow rate  $\dot{Q}$ .

To test this hypothesis, the cardiac output of five dogs was changed by hemorrhage and subsequent reinfusion of the blood. Simultaneous measurements of  $\Delta P_{\text{CO}_2}$  and cardiac output were obtained, and the relationship between these two values appears in figure 2 which clearly demonstrates that when cardiac output falls,  $\Delta P_{\text{CO}_2}$  starts to decrease and eventually disappears.

#### ALVEOLAR-CAPILLARY DMO DIFFERENCES

The mechanism presented above is not specific for  $\text{CO}_2$ . Any weak acid with a  $pK$  similar to the effective  $pK$  of carbonic acid should show similar gradients. 5,5-dimethyl oxyzolidinedione

(DMO) is a weak acid with a  $pK$  of 6.12 at 37 C which is neither metabolized nor bound to protein, and has been widely used to investigate intracellular pH (Butler, 1953; Waddell and Butler, 1959).

Using this material, we performed a number of experiments in which a single lobe was isolated with a cuffed catheter and filled with a 10-percent sucrose solution. After a control period, 0.5 to 1.0 gram of DMO was infused into the animal's pulmonary artery and paired alveolar and mixed venous samples were withdrawn at half-hour intervals. Total DMO was chemically analyzed by the method of Butler (1953). Undissociated DMO was calculated from the measured pH using a  $pK$  of 6.12.

#### RESULTS

DMO appears to move rapidly across the alveolar capillary membrane, and a steady state was apparent after 1 hour. In most samples a gradient of undissociated DMO was present when a steady state had been reached. Figure 3 shows the

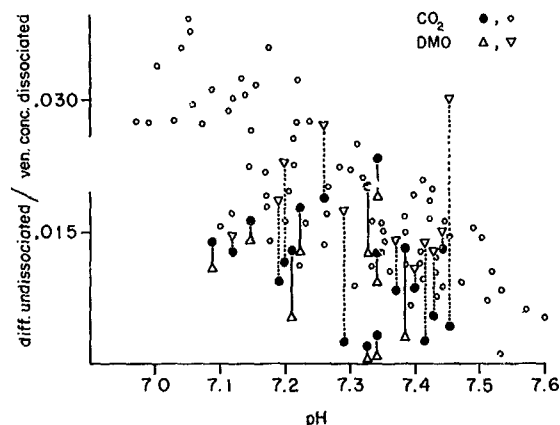


FIGURE 3.—Concentration gradients of undissociated weak acid ( $\text{CO}_2$  or DMO) divided by the venous concentration of the dissociated form. As a function of mixed venous pH, symbols connected by lines indicate simultaneously taken samples (see text).

Dotted lines indicate:  $\frac{\Delta \text{DMO}}{\text{DMO}^-} > \frac{\Delta \text{CO}_2}{\text{HCO}_3^-}$

Solid lines indicate:  $\frac{\Delta \text{DMO}}{\text{DMO}^-} > \frac{\Delta \text{CO}_2}{\text{HCO}_3^-}$

Open circles are previously measured  $\text{CO}_2$  gradients.

normalized  $CO_2$  and DMO gradients observed in four experiments plotted against mixed venous pH. The magnitude of the gradient in the undissociated weak acid ( $CO_2$  or DMO) is divided by the mixed venous concentration of the dissociated form of the weak acid ( $HCO_3^-$  or  $DMO^-$ ). Because both gradient and mixed venous content are expressed in the same units, the ratio is a dimensionless number. The magnitude of the previously measured  $P_{CO_2}$  gradients is given by the open circles. The DMO gradients observed are of a magnitude similar to the  $CO_2$ , although no apparent relationship is seen between the value of the DMO gradients and the mixed venous pH.

Demonstration of the steady-state gradient of DMO is not a proof that our hypothesis is true, but shows, nevertheless, that whatever the mechanism may be, it is not specific for  $CO_2$  and probably involves a source of strong acid that shifts the ratio of dissociated to undissociated weak acid. It is important to mention here that if differences in  $CO_2$  and DMO exist across cell membranes, the measurements of intracellular pH by these methods must contain systematic errors. However, if the gradients of undissociated weak acid were of the same magnitude as the gradients across the alveolar capillary membrane (10–30 percent of the venous concentration), the error would be small. As an example, at a pH of 7.0, this error would be approximately 0.1 to 0.2 pH units.

#### EXPERIMENTS WITH ARTIFICIAL MEMBRANES

In order to differentiate between  $CO_2$  differences caused by charged membranes and differences that could result from biological transport mechanisms, experiments were conducted on artificial membranes known to have a considerable charge density. Unfortunately these membranes are thick and have a low permeability, so that long experiments were required.

The membranes were mounted in a frog skin chamber. The liquid on both sides of the membrane had been preequilibrated to an identical  $P_{CO_2}$ . The liquid on one side of the membrane was not stirred, and was only withdrawn periodically for measurement of  $P_{CO_2}$ . Following this, it was reinjected into the chamber. On the other

side, the volume of which was 1.5 ml, the liquid could be drained by gravity, pumped up into a bag through which the gas used for preequilibration was bubbled, defoamed by passage through a siliconed sponge, and returned to the chamber. All experiments were performed at room temperature (21 to 25 C).

When a cation-exchange membrane, made of sulfonated copolymers of vinyl compounds (membrane 61 AZL 183, Nepton CR, made by Ionics, Inc.), was used, if blood was caused to flow at a rate of  $500 \text{ ml min}^{-1}$ , a  $\Delta P_{CO_2}$  of 10 to 15 mm was established within 1 hour (fig. 4). When the chamber was filled with blood, but the pump stopped, no difference developed, presumably because in this case movement of  $HCO_3^-$  away from the membrane was allowed to occur. Similarly, when flowing blood was replaced by a flowing solution of  $NaHCO_3$ , the difference remained zero, a fact that we explain on the basis of the absence of large polyelectrolyte molecules having a marked Wien effect.

Experiments were also performed on cation-exchange membranes (membrane 111 B2L 183, manufactured by Ionics, Inc.). This membrane is packaged in a fluid of low pH—approximately 1.5 to 2.0. In this case, all flowing solutions containing bicarbonate gave rise to a  $P_{CO_2}$  difference; a fact we explain by the attraction of bicarbonate

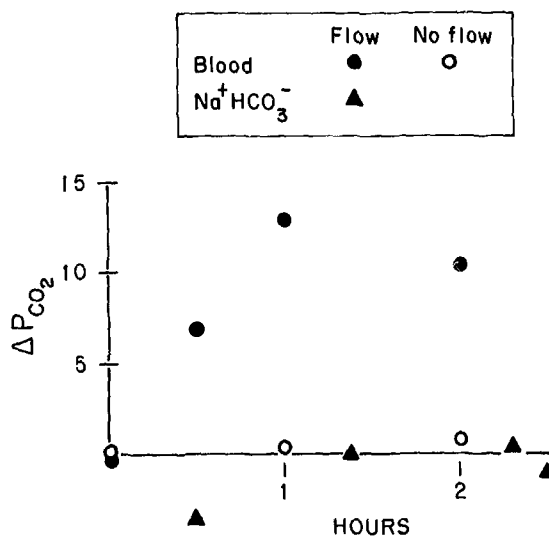


FIGURE 4.— $\Delta P_{CO_2}$  across fresh anion-exchange membranes as a function of time.

by the positive charges, a plentiful supply of hydrogen ions being available in the membranes.

### APPENDIX

In this hypothetical model we will consider the rates of change of concentration of H<sup>+</sup>, HCO<sub>3</sub><sup>-</sup>, and CO<sub>2</sub> in a volume of blood that is passing through a pulmonary capillary under the influence of the charges on the wall. At time=0, the volume of blood enters the capillary and is acutely exposed to an electrical field. This last assumption is realistic because the surface to volume ratio of the capillary bed is much larger than that of the arterial bed and the probability is therefore small that the volume of blood we are considering was recently close to a charged vascular wall. The rate of change of number of molecules in an arbitrary volume can be given by the following equation:

$$-\frac{dn_i}{dt} = -\int_v \frac{\partial c_i}{\partial t} dV = \int_a J_{EC_i} da + \int_v J_{CHEM} dV$$

(Katchalsky and Curran, 1965)

where

- $n$  represents number of molecules
- $V$  represents volume
- $a$  represents the surface area across which exchange is occurring
- $i$  represents each different molecular species
- $J_{EC_i}$  represents the flux across the surface caused by difference in electrochemical potential
- $J_{CHEM}$  represents the rate of chemical change per unit volume

If volume, area, and concentration within the volume are constant, the above equation reduces

to

$$-\frac{dc_i}{dt} = \frac{a}{V} J_{EC_i} + J_{CHEM}$$

If the surface area-to-volume ratio is 1/1, such as with a cubical compartment exchanging matter on one side, the equation is further simplified.

In one dimension the flux for each molecular species exposed to an electrical field is given by the Nernst-Planck equation

$$J_{EC} = -D \left[ \frac{dc_i}{dx} + \frac{zFc_i}{RT} \frac{d\psi}{dx} \right] \quad (3)$$

where

- $J_{EC}$  flux caused by electrochemical potential gradient
- $D$  is the diffusion coefficient
- $\frac{dc_i}{dx}$  and  $\frac{d\psi}{dx}$  are the gradients of concentration and potential with respect to distance
- $dx$  from the charged surface
- $z$  is the charge of the species
- $\frac{F}{RT}$  is a constant = 38.4 volt<sup>-1</sup> at 37 C

Our representation of the flux of the chemical reaction using the law of mass action is as follows:

$$J_{CHEM} = K_A [H^+] [HCO_3^-] - [CO_2] K_B$$

The letters in brackets represent the concentration of molecular species. The values taken for  $K_A$  and  $K_B$ , the forward and backward rate constants respectively, were calculated from the uncatalyzed rates given by Roughton (1964).

Because we are interested in the simultaneous rates of change of H<sup>+</sup>, HCO<sub>3</sub><sup>-</sup>, and CO<sub>2</sub>, we need three simultaneous differential equations describing the fluxes.

$$\frac{d[H^+]}{dt} = -D \left[ \frac{d[H^+]}{dx} + \frac{F[H^+]}{RT} \frac{d\psi}{dx} \right] - K_A [H^+] [HCO_3^-] + K_B [CO_2] + [H \text{ from the Wien effect}]$$

$$\frac{d[HCO_3^-]}{dt} = -D \left[ \frac{d[HCO_3^-]}{dx} - \frac{F[HCO_3^-]}{RT} \frac{d\psi}{dx} \right] - K_A [H^+] [HCO_3^-] + K_B [CO_2]$$

$$\frac{d[CO_2]}{dt} = -D \left[ \frac{d[CO_2]}{dx} \right] + K_A [H^+] [HCO_3^-] - K_B [CO_2]$$

Although the above equations are not analytically solvable, it can be seen that at chemical equilibrium when the net fluxes of all chemical reactions equal zero and when

$$\frac{d[H]}{dt} = \frac{d[\text{HCO}_3^-]}{dt} = \frac{d[\text{CO}_2]}{dt} = 0,$$

that there will be a chemical gradient  $dC/dx$  for  $\text{H}^+$  and  $\text{HCO}_3^-$  but not for  $\text{CO}_2$ , because it is not charged. We can calculate the equilibrium  $\text{H}^+$  and  $\text{HCO}_3^-$  concentrations from the equation inside the bracket of equation (3) (Nernst equation).

It is our hypothesis that because of the dissociation of protein (plasma protein and protein of red cells) in blood (a monomolecular reaction) under the influence of the charge,  $\text{H}^+$  comes to equilibrium almost instantaneously, leaving an excess of  $\text{HCO}_3^-$  able to react with the  $\text{H}^+$  ion producing  $\text{CO}_2$ . If the  $\text{H}^+$  ion reaches equilibrium instantaneously, we can eliminate the first equation and make  $[\text{H}^+]$  in the other equations a constant.

The concentration gradient

$$\frac{d[\text{CO}_2]}{dx} \quad \text{or} \quad \frac{d[\text{HCO}_3^-]}{dx}$$

is proportional to the thickness of the electrical double layer. This, by the Debye-Hückel theory, is inversely proportional to the ionic strength and directly proportional to the dielectric constant of the medium. Because little is known about the dielectric constant of water near charged groups of proteins and because the Debye-Hückel theory does not hold for high ionic strengths (such as plasma), the thickness of the double layer is unknown.

We have looked on this layer as a shell through which the  $\text{CO}_2$  produced in the region in closest proximity to the charged wall as well as the  $\text{HCO}_3^-$  must diffuse.  $\text{CO}_2$  is also probably produced throughout the shell, as the largest dissociation of polyelectrolytes takes place at low field strengths (Wall et al., 1956).

We have substituted for the gradients the difference in concentration between the final equilibrium state and the instantaneous  $\text{CO}_2$  or  $\text{HCO}_3^-$  concentration. We take the permeability of this shell to be equal to the diffusion coefficient for  $\text{CO}_2$  and  $\text{HCO}_3^-$  divided by the thickness of the

shell. Because part of this shell may be made up of red-cell membranes, the diffusion coefficient may be less than those already assumed. If we make a change of variable (Burton, 1939) from  $\text{HCO}_3^-$  and  $\text{CO}_2$  to

$$x_1 = [\text{HCO}_3^-] - (\text{HCO}_3^- \text{ at equilibrium})$$

$$x_2 = [\text{CO}_2] - (\text{CO}_2 \text{ at equilibrium})$$

we get rid of all constant terms and we are left with two equations of the form:

$$\frac{dx_1}{dt} = - \left[ K_A (\text{H}_{\text{eq}}) + \frac{D}{dx} \right] x_1 + (K_B) x_2$$

$$\frac{dx_2}{dt} = [K_A (\text{H}_{\text{eq}})] x_1 - \left( K_B + \frac{D}{dx} \right) x_2$$

With initial conditions

$$X_1(0) = \text{HCO}_3^-_{\text{bulk}} (1 - \exp(-F\psi/RT)) = 0.0101 \text{ ml}$$

$$X_2(0) = 0$$

$$K_A = k_u \text{ of Roughton (7)} = 0.13 \text{ second}^{-1}$$

$$K_B = k_r/K_1 \text{ of Roughton} = 163\,660 \text{ second}^{-1}$$

$K_1$ , the true dissociation constant of carbonic acid, was calculated using the  $k_u$  value of Roughton of  $89 \text{ sec}^{-1}$ , and taking the effective dissociation constant of carbonic acid to be equal to  $10^{-6.12}$

$(\text{H}_{\text{eq}}) = \text{equilibrium } [\text{H}^+]$

$$\approx [\text{H}^+]_{\text{bulk}} \exp + (F\psi/RT) = 10^{-7.192} \text{ mole/liter}$$

$$[\text{H}^+]_{\text{bulk}} = 10^{-7.4} \text{ mole/liter}$$

$$[\text{HCO}_3^-]_{\text{bulk}} = 0.0276 \text{ mole/liter}$$

$$dx \text{ varied from } 10^{-6} \text{ cm to } 5 \times 10^{-6} \text{ cm}$$

$$D = 1.5 \times 10^{-5} \text{ cm}^2/\text{sec}$$

$$\psi = 0.0125 \text{ volt}$$

The solution for  $x_2$  (which is by definition the instantaneous  $\Delta P_{\text{CO}_2}$ ) is

$$x_2(t) = \Delta P_{\text{CO}_2}(t) = A e^{-\lambda_1 t} - A e^{-\lambda_2 t}$$

$$A = \frac{x_1(0) (K_A) (\text{H}_{\text{eq}})}{\lambda_1 - \lambda_2}$$

$$\lambda_1 = 2 \left[ K_B + \frac{D}{dx} + (K_A) (\text{H}_{\text{eq}}) \right]$$

$$\lambda_2 = 2 \frac{D}{dx}$$

It is clear that  $P_{\text{CO}_2}$  gradients predicted by this model are proportional to the bulk phase  $\text{H}^+$  and  $\text{HCO}_3^-$  concentration because the coefficient  $A$  is proportional to these concentrations. In this aspect the theory fits the experimental results well. The model also predicts that the  $P_{\text{CO}_2}$  gradients would be related to the blood-flow rate through the lungs, because blood-flow rate is inversely related to the transit time of blood through the lungs. Solutions for the equations for two different thicknesses of the electrical double layer and for different carbonic acid reaction rates, multiples of the uncatalyzed rates given by Roughton (1964), are plotted in figure 5. If the

thickness of the layer truly is, it seems that 100 to 500 Å is too thick.

However, because the capillary diameter is close to the diameter of a red cell, it seems likely that part of this layer under the influence of the electric fields will be made up of red-cell membranes. Roughton (1959) has estimated that the diffusion coefficients for  $\text{CO}_2$  in red-cell membranes is 1/10 to 1/100 of the diffusion coefficient of  $\text{CO}_2$  in water. If the diffusion coefficient of the layer was close to that of the red-cell membrane, the thickness of the double layer could approach that of simple salt solutions, 10 to 30 Å (Robinson and Stokes, 1955).

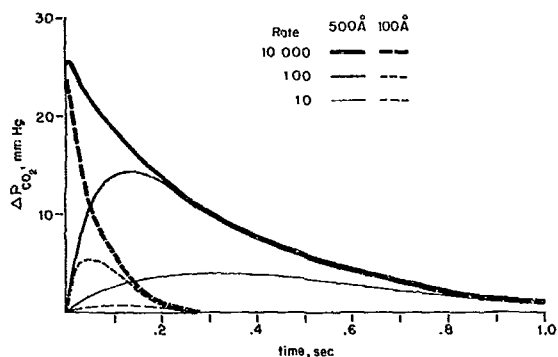


FIGURE 5.—Theoretical curves of  $\Delta P_{\text{CO}_2}$  in a small volume of blood exposed to an electrical field. Different diffusion shell thickness and reaction rates (multiples of uncatalyzed rate) are shown (see text).

net  $P_{\text{CO}_2}$  gradients are the time-averaged instantaneous  $P_{\text{CO}_2}$  gradients, it is clear that the net  $P_{\text{CO}_2}$  gradients would decrease as transit time was increased. In this respect, the theory also agrees with the experimental results. The theory predicts that the carbonic acid reaction rates must be faster than the uncatalyzed rates for any gradients to exist. However, it seems possible that the reaction rates need not be as fast as the carbonic-anhydrase-catalyzed rates in blood for  $P_{\text{CO}_2}$  gradients to exist.

Using the diffusion coefficients for  $\text{CO}_2$  and  $\text{HCO}_3^-$  in water (taken to be  $1.5 \times 10^{-5}$  cm<sup>2</sup> second<sup>-1</sup>), it seems that, according to this mechanism, significant gradients could exist only if the thickness of the double layer were from 100 to 500 Å. Although we have little idea what the

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### DISCUSSION

**LASZLO:** The simplest preparation in which the absence of gas exchange can be guaranteed is the excised lung or lung lobe ventilated with a constant gas mixture through which blood is recirculated by means of a pump. Dr. Caldini, Mr. Bane, and I, working in Dr. Riley's department in Baltimore, have tested Dr. Gurtner's hypothesis using this preparation. We have failed to demonstrate any difference between the mean  $P_{CO_2}$  in the gas and the  $P_{CO_2}$  of the blood sampled at the same time from the pulmonary artery and vein (fig. D-1). There is some scatter, but the mean difference is zero, and the standard error of the mean is less than 1 mm Hg. We have carefully controlled the temperature and have used physiological blood flows. Most of the experiments were carried out at  $P_{O_2}$  values near 150 mm Hg, but hypoxia did not influence the result. Since Dr. Gurtner's model requires only blood flow and not gas exchange, these results are inconsistent with this hypothesis.

We have been able to demonstrate alveolar-arterial  $P_{CO_2}$  differences during short-lived *in vivo* plateaus in anesthetized dogs rebreathing mixtures of  $CO_2$  in nitrogen. Figure D-2 shows the blood-gas differences when blood was sampled during perfectly horizontal plateaus. There is a strong suggestion that gas-blood differences are largest when the plateau appears after a recir-

ulation time between 20 and 40 seconds following the onset of rebreathing. The plateau values of alveolar  $P_{CO_2}$  were in agreement with the  $P_{CO_2}$  of pulmonary arterial blood when the gas mixture chosen resulted in a plateau appearing before 20 seconds in the anesthetized animals. Average values of  $P_{CO_2}$  and pH were the same in PA and aortic samples.

It is possible that the hydrogen and bicarbonate ions of red cells and plasma are imperfectly equilibrated when the blood enters the pulmonary capillaries so that  $P_{CO_2}$  continues to fall in the syringe after sampling. Such a mechanism could account for both alveolar-arterial and alveolar-pulmonary artery  $P_{CO_2}$  differences *in vivo* when there were large differences among various components of mixed venous blood which would be absent in the isolated lung preparation. Alternatively there may be exchange of bicarbonate or hydrogen ion between plasma and lung

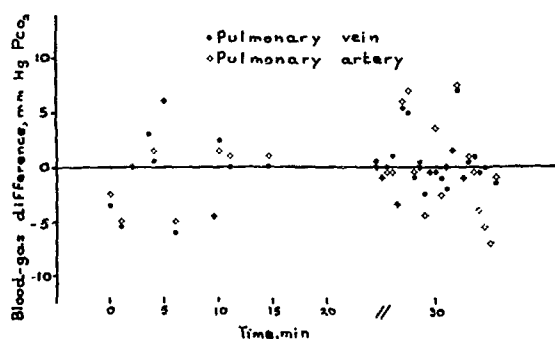


FIGURE D-1.—Difference in  $P_{CO_2}$  between aortic blood and alveolar gas (dots) and pulmonary arterial blood and alveolar gas (open symbols) in the isolated lung preparation. The time scale refers to the time after the disappearance of the inspired-expired  $P_{CO_2}$  difference following a step change in  $P_{CO_2}$  of one gas mixture.

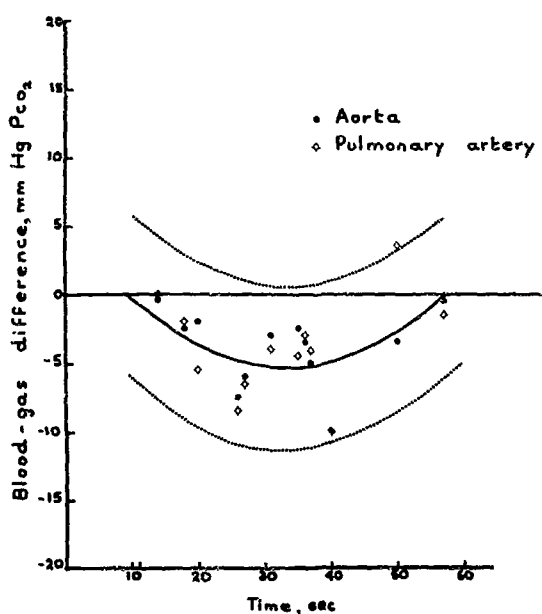


FIGURE D-2.—Blood gas  $P_{CO_2}$  differences during rebreathing experiments *in vivo* in anesthetized dogs. The time scale refers to the time at which the samples were drawn after the exchange from normal respiration to rebreathing. The blood and gas samples were drawn simultaneously when continuous rapid analysis showed no inspired-expired difference in  $P_{CO_2}$ .

tissue, final equilibration taking place after the blood has left the lungs. This explanation, however, requires a difference in acid-base state between PA and arterial blood which we have not so far been able to demonstrate.

**GURTNER:** I was not quite clear on the experimental conclusion of Dr. Laszlo. I could see that this sort of gradient could occur if there was not any sort of equilibrium with the CO<sub>2</sub> produced in the tissue and if it did not come to equilibrium between cells and plasma. Is that what you are proposing?

**LASZLO:** Yes; I think that if you had blood coming up from various parts of the body, of various acid-base compositions which were not fully equilibrated in respect to HCO<sub>3</sub><sup>-</sup>, CO<sub>2</sub>, and H<sup>+</sup>, the P<sub>CO<sub>2</sub></sub> might subsequently fall.

**GURTNER:** That is possible, but I certainly do not think it is likely. The experiments of Enns and Nolan indicate that the H<sup>+</sup>, CO<sub>2</sub>, and HCO<sub>3</sub><sup>-</sup> have reached equilibrium in the time it takes blood to flow from the right ventricle to the pulmonary artery.

**GUYATT:** I am the second refugee from Hammersmith, now in Florida, and we have just started to work on this problem. We also agree that we find this gradient. In our work with the Cain-Otis preparation, we take one lung and continuously rebreathe it from a bag. We keep the animal rebreathing all day, so that we have a continuous mixture of gas. We get this gradient (we have studied nearly 50 dogs) of from about 2 to 8 millimeters, an average of 4. We find that when you raise the P<sub>CO<sub>2</sub></sub>, the gradient increases. We have done this by letting the dog breathe a mixture with CO<sub>2</sub> in it and keeping the mixture stable; we find, again, that the gradient increases. We looked very quickly at the question of altering pH at a constant P<sub>CO<sub>2</sub></sub>. We do not have many data on this. It looks as though there might be a slight fall in gradient with decreasing pH. When we looked at Diamox again, we found considerable variation among four dogs, but in all, the gradient increased to some degree. In a study of why the blood does not come into equilibrium, we divided the pulmonary artery by putting a tube into it, thus increasing its length, and thus increasing the transit time from the heart to the lungs. In this case we delayed the blood three-quarters of a minute from the heart to the lungs and we found the

gradient hardly altered; there is a very slight fall that I attribute to the fact that blood was slightly cooled. There is no real change in the gradient in these conditions. Therefore I would doubt very strongly if this is an effect of a lack in equilibrium. Using the same basic preparation, we altered the flow rate, this time with a short tube altering the flow rate through the lung with a screw clamp, leaving the cardiac output more or less the same and, again, the gradient hardly altered. If we cut the flow rate completely, i.e., leave the lung without any pulmonary flow, the gradient rises. This must be an effect of bronchial circulation and lung metabolism. It is well to bear in mind that there is a certain amount of CO<sub>2</sub> being produced by the tissue. The final thing that we wondered was whether we could show that the CO<sub>2</sub> was coming from the blood or was coming from the lung tissue itself. We tried this by altering the position of the dog, turning it over to increase the area of the blood/gas interface, but this did not seem to affect it. In summary, I will say that the only thing that seems to change the gradient is increasing the P<sub>CO<sub>2</sub></sub>, which we are doing independent of exercise. I think that this may be an explanation for the results with exercise: you are simply increasing the P<sub>CO<sub>2</sub></sub>. If you increase the flow, you do not seem to change the gradient much, and if you increase the time it takes for the blood to get from mixing to passing into the lungs, again there seems to be very little change. The bronchial circulation is a very slight complication; I do not think it is very important here.

**FORSTER:** That delay you produced was not after the lung to your electrode, but before the lung?

**GUYATT:** The transit time through the lung itself is probably the same, but there is an imposed delay between the heart and the lung of about three-quarters of a minute.

**CAMPBELL:** The idea is that the blood is mixed up in the right ventricle and then, by delaying the time it takes to get from the right ventricle to the lung, you are giving it more time to mix and reach chemical equilibrium.

**KREUZER:** We have reasons to be interested in fluctuations of the P<sub>O<sub>2</sub></sub>. We have not got to P<sub>CO<sub>2</sub></sub> yet, but doubtless the same argument may hold in the pulmonary artery and in the right heart with respiration and heartbeat. We have

found considerable fluctuations of  $P_{O_2}$  with respiration and heartbeat. I think the arguments of Afonso and coworkers, who found that temperature differences also fluctuated, could only explain less than 10 percent of this phenomenon (Afonso, Rowe, Castillo, and Crumpton, 1962; Afonso, Herrick, Youmans, Rowe, and Crumpton, 1962). Our interpretation is that the blood from the vena cava superior and inferior is mixed in different proportions according to respiration and heartbeat. I think this argument of delaying the time between the right heart and the lung might not hold, because as long as the flow remains laminar in the pulmonary artery, these various portions might still be separated and might not mix even if the delay is quite considerable.

**GUYATT:** The blood would still not be homogeneous?

**KREUZER:** Yes; that is what I mean. In other words, stream lines would still be maintained.

**GUYATT:** Our tube was narrow, becoming wider, and then narrowing again.

**KREUZER:** You had turbulence?

**GUYATT:** The flow of blood in the external tube appeared to be laminar, but we assumed that the blood was previously well mixed during its passage through the right heart.

**KREUZER:** It might be interesting to cause turbulence in the tube by inserting a mesh or something.

**GUYATT:** You could very easily do that.

**CAMPBELL:** Could I just remind you that when we rebreathed a  $CO_2$  in  $N_2$  mixture, we found no difference for oxygen. I think that makes differences due to inhomogeneities in the blood less likely.

**FORSTER:** If I could compare myself to Dr. Whitehead, which is presumptuous, my prejudices have all been stated. I can now rearrange them. First, I find this all very fascinating, and there must be some reasonable explanation. Second, there are probably several red herrings. Dr. Laszlo's work, I think, demonstrates that there is exchange of  $CO_2$  going on; under most of the circumstances when you eliminate it, you eliminate the factor producing the gradient. By a delay in the blood, I mean a delay from the lung capillaries to where one measures  $P_{CO_2}$ , not from the point of mixing. We have measured the rates of exchange between the plasma and the red

cell, and they have half times from 0.1 to 0.2 second under very rapid conditions. Thus, there is a delay that is proportional to the total amount of  $CO_2$ , the absolute value. In Dr. Campbell's terms, his "error" was proportional to the total amount of  $CO_2$  involved, and a rough qualitative check would make me suspect that that is also true of Dr. Gurtner's. If you take the proportion of the "error" over the absolute  $P_{CO_2}$ , it is roughly constant. Though we may not be able to explain it completely, I do not see anything entirely incompatible with the idea that you reach equilibrium for  $CO_2$  in the lungs, and there is then a readjustment within the blood. I had not appreciated how slow this could be until we were working with Dr. Steen recently. You can get extraordinary delays depending on how you shift the balance. If you add acid to the external fluid, for example, things can be very slow, because the process then becomes limited by the rate of uncatalyzed dehydration of  $H_2CO_3$  from the plasma. The whole process—the shifts of hydroxyl ion then may be very slow—may take a number of seconds before completion. We may just have increased our accuracy in  $P_{CO_2}$  determination enough so that we are beginning to pick this up. I do have one worry about your argument regarding the Donnan effect near the capillary wall. The  $[H^+]$  would increase by precisely the same ratio as the  $[HCO_3^-]$  would decrease, giving you no  $[H_2CO_3]$  gradient.

**COLLIER:** We tried to repeat Dr. Gurtner's experiments this spring. Dr. Hackney has two slides.

**HACKNEY:** Recent reports have suggested that a positive  $P_{CO_2}$  arterial-mixed venous difference ( $A-\bar{V}$ ) can exist during rebreathing. We have investigated this in a series of experiments on five dogs.

The animals were anesthetized with Nembutal and paralyzed with Anectine. After a tracheostomy and thoracotomy, a lobe was cannulated (usually the left lower lobe), tied securely, and tested for leaks. The cannulated lobe, a pump, and a rebreathing bag formed a closed rebreathing circuit. The remainder of the lung was ventilated with a second pump at various selected levels of ventilation and end-tidal  $CO_2$  monitored with a rapid infrared analyzer. Mixed venous blood was sampled through a catheter in the right

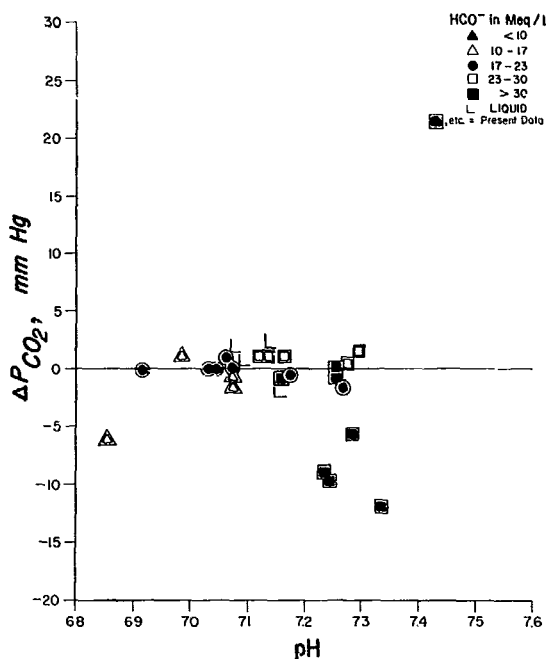


FIGURE D-3.—(Alveolar  $P_{CO_2}$ -mixed venous  $P_{CO_2}$ ) plotted against the pH of the blood for the one lobe of a dog's lung which was rebreathing.

ventricle or pulmonary artery. An arterial cannula served for sampling and pressure monitoring. The metabolic state of the animals was changed by infusing HCl or NaHCO<sub>3</sub>. Blood was analyzed for  $P_{CO_2}$ ,  $P_{O_2}$ , and pH by a thermostated (37 C) electrode system. Gas from the rebreathing bag was analyzed by the same electrodes or by an infrared analyzer, or by both methods. The same standard gases were used to calibrate both systems. In four runs in two animals, the isolated lobe was equilibrated with 10 percent sucrose. Temperature corrections were not made for either air- or liquid-filled airways.

The results are shown in figure D-3. A significant positive A- $\bar{V}$   $P_{CO_2}$  gradient was not found. A consistent negative gradient was observed (the three most negative points) in one animal that

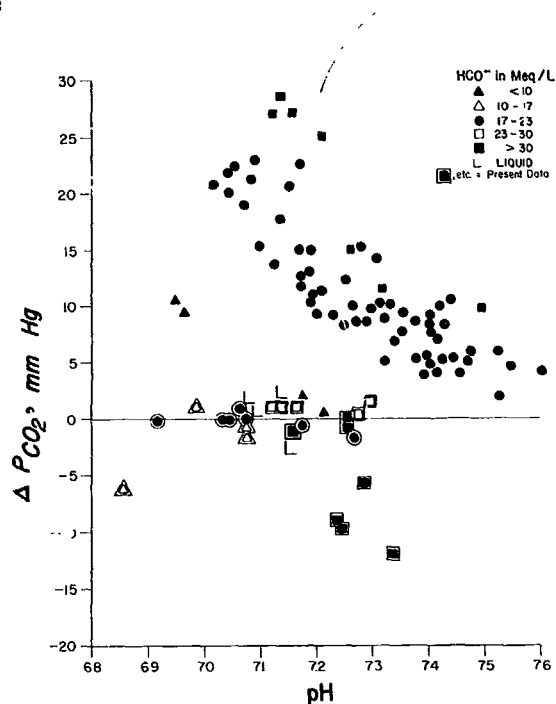


FIGURE D-4.—The same graph as for figure D-1 with the addition of the data of Gurtner and Farhi.

was in poor condition because of blood loss. In figure D-4 our data are plotted along with that of Gurtner and Farhi. The reasons for the marked difference are not apparent.

**OTIS:** It appears that not everybody can do the same thing in this type of experiment.

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# Permeability of the Alveolar-Capillary Barrier to Dissolved Carbon Dioxide and to Bicarbonate Ion<sup>1</sup>

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N70-23313

The multiple indicator dilution technique has been applied to an investigation of the permeability of the alveolar-capillary barrier to  $\text{H}^{14}\text{CO}_3^-$  and to dissolved  $^{14}\text{CO}_2$  ( $\text{CO}_2$  *diss*) with T-1824 or  $^{22}\text{Na}$  as vascular indicators and DHO or THO as indicators for the aqueous compartment. Under control conditions the outflow patterns of  $\text{HCO}_3^-$  and  $\text{CO}_2$  *diss* cannot be distinguished from each other; the mean transit times are equal to or larger than the mean transit times of simultaneously injected water, and there is invariably prolonged tailing. Recoveries in the blood are about 95 percent for both species. These results are taken as indication of distribution of injected carbon dioxide into the gas phase and randomization of the labeled carbon dioxide among the several chemical species present.

After inhibition of carbonic anhydrase by acetazolamide, the curves for  $\text{HCO}_3^-$  and for  $\text{CO}_2$  *diss* are very close to those for the vascular indicator; the mean transit times are essentially equal to those of the vascular indicators, but the recoveries of  $\text{CO}_2$  *diss* are less than of  $\text{HCO}_3^-$  in blood and about three times larger in expired gas. These facts are taken as evidence of restriction of  $\text{HCO}_3^-$  to a volume essentially the same as the vascular compartment, in common with other anions such as  $\text{Cl}^-$  and  $\text{SCN}^-$  and with the cations  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Li}^+$ . They indicate also that  $\text{CO}_2$  *diss* crosses the barrier readily. In another type of experiment,  $\text{H}^{14}\text{CO}_3^-$  in distilled water with THO was introduced onto the gas-phase side of the barrier through a tracheostomy and a fine catheter. The  $^{14}\text{C}$  outflow pattern in blood is almost identical with that of THO under control conditions but becomes intermediate to those of  $^{36}\text{Cl}^-$  and of THO after administration of acetazolamide. Such results are compatible with the presence of carbonic anhydrase in lung tissue. The content of lung tissue with respect to this enzyme is one-tenth to one-third that of kidney cortex. The multiple indicator-dilution technique has also been extended to provide *in vivo* values for intact organ or tissue pH. For lung tissue, with nicotine as the indicator (5,5'-dimethyl-2,4-oxazolidinedione or DMO is not satisfactory because it does not leave the vascular compartment), the pH value is about  $6.7 \pm 0.1$ , a bit less than the  $6.9 \pm 0.1$  reported by Hyde, Puy, Raub, and Forster (1968). With tissue pH of 6.7,  $P_{\text{CO}_2}$  of 45 mm Hg, and  $pK_1'$  of 6.1, total carbon dioxide content of lung tissue from the Henderson-Hasselbalch relationship is 0.10 to 0.16 ml per ml tissue, again lower than the value of about 0.33 ml per ml tissue reported by Hyde et al. (1968).

The determination of the permeability of a given biological barrier to given substances *in vivo* and *in situ* presents certain technical difficulties. It

would be highly desirable to obtain values for the permeability coefficient of the barrier for a permeating species in terms of the number of molecules crossing a unit surface of the barrier per unit time for a unit concentration difference of the species across the barrier. Unfortunately, in the lung, although both sides of the alveolar capillary are accessible to the investigator, the anatomical and physiological factors preclude obtaining values for such permeability coefficients in the normally perfused and ventilated lung.

The multiple-indicator dilution experiments de-

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scribed below provide qualitative information on the permeability of the alveolar-capillary barrier. Numerical values can be obtained but are not presented here. This is relatively unimportant because nature has designed the system nearly on a yes-or-no basis. For most substances of biological significance so far examined, we have found that these either remain in the bloodstream or equilibrate in a volume equal to that accessible to water (or in a larger volume) in the transit time of blood through the pulmonary vasculature. Thus, we have evidence that ions such as sodium, chloride, short chain fatty acids, and bicarbonate are restricted, in effect, to the vascular compartment (Chinard, 1966a). In contrast, monohydric normal alcohols, inert gases and carbon dioxide, the higher amides, and the higher alkane diols all distribute themselves in volumes equal to or greater than the volumes available to labeled water (Chinard, 1966a, b; Chinard, Effros, Perl, and Silverman, 1967; and unpublished data). Complications enter into the interpretation of data involving some of these and of other substances because of differences of pH between blood (plasma) and extravascular (cellular) compartments (Effros and Chinard, 1968), because of lipid: water partition coefficients (Chinard et al., 1967), and because of distribution into the alveolar gas phase and beyond (Chinard, Enns, and Nolan, 1961).

#### GENERAL PRINCIPLES APPLIED IN THESE STUDIES

As indicated above, the multiple-indicator dilution, sudden-injection technique has been used in these studies. In brief, a known volume of a solution containing various indicators is injected into the input of an organ system, and timed samples are obtained from the outflow from the system. The concentration or recovery of each indicator is determined in each sample and is plotted on the ordinate (logarithmic scale) against time on the abscissa (linear scale) to establish a curve called an outflow pattern. It is assumed that the system is stationary, linear, conservative, and that the distribution of the several indicators is homogeneous in a constant infusion experiment. It is further assumed that appropriate corrections can be made for recirculation or recycling of an

indicator in the system and that appropriate corrections for catheter delay have been applied (see Chinard et al. (1967) for a discussion of these assumptions).

The flow of blood  $F$  through the system can be calculated from the recovery-time curve of any indicator that meets the conditions just indicated. In addition, its volume of distribution  $V$  can be calculated from the product of its mean transit time  $\bar{t}$  through the system and  $F$ . Thus,

$$V_1 = F \cdot \bar{t}_1 \quad (1)$$

Let the subscript 1 denote a vascular indicator, that is, an indicator that is limited in its distribution to the vascular compartment, then  $V_1$  is the volume of blood contained between the site of injection and the site of sampling. For another indicator, for example, labeled water, use the subscript 2. Then, similarly,

$$V_2 = F \cdot \bar{t}_2 \quad (2)$$

where  $V_2$  is the volume of distribution of water in the system. Now we can obtain a value for the extravascular volume of distribution of water in the system by simple subtraction:

$$V_2 - V_1 = \Delta V_{2,1} = F \cdot (\bar{t}_2 - \bar{t}_1) = F \cdot \Delta \bar{t}_{2,1} \quad (3)$$

In the case of the lungs, the site of injection is between the right jugular and the branching of the pulmonary artery, and the site of sampling is the carotid artery. The only intervening capillary bed in series is that of the pulmonary parenchyma. Thus,  $V_{2,1}$  is the extravascular volume of distribution of water in lung tissue. As indicated elsewhere, there is substantial reason to believe that the volume of distribution of labeled water in the lung is indeed an anatomical volume surrounding perfused capillaries (Chinard, 1966a; Chinard et al., 1967). Obviously, unperfused pulmonary tissue is not seen or recognized in this kind of experiment. Thus, by comparing the volumes of distribution of various indicators with the volume of the vascular compartment, one can determine whether or not these indicators were distributed in a volume equal to or larger than the vascular compartment. In the latter case, they must have crossed the blood-tissue barrier, because the barrier is permeable to such indicators. If the volume of distribution and the outflow pattern of a particular indicator are the same as those

for water, then we can reasonably conclude that it is distributed in the same anatomical volume as water.

For substances that have transit times and outflow patterns intermediate to those of labeled water and of vascular indicators and substances that have longer mean transit times than water and outflow patterns delayed relative to its outflow patterns, a somewhat more complicated approach must be used. In brief, such patterns are interpreted as indicating that the distribution of these indicators may not be homogeneous: there may be accumulation in a lipid compartment; there may be distribution in a gas phase; there may be differences of distribution because of a Gibbs-Donnan-type equilibrium of an ionized species. In these circumstances additional information is required to permit calculation of the volume of distribution of the nonhomogeneously distributed indicator. Specifically, the partition coefficient is required. There are alternate ways of approaching the problem of nonhomogeneously distributed indicators. A particularly fruitful one indicated in Chinard et al. (1967) and developed by one of my associates, Dr. Richard M. Effros (Effros and Chinard, 1968), has permitted the calculation of the pH of the extravascular tissues of the lungs *in vivo* and *in situ*.

The general principle for such calculations with nonhomogeneously distributed indicators is that, in a steady-state, continuous-infusion experiment, the distributions be homogeneous in each of the several compartments accessible to the indicator considered. Thus, we have for a single compartment

$$M = I\bar{t} \quad (4)$$

where  $M$  is the mass of the indicator in the compartment at equilibrium,  $I$  is the steady-state input flux, and  $\bar{t}$  is the mean transit time of the indicator through the system, as obtained by whatever means are available (e.g., a sudden-injection experiment). If the indicator is distributed homogeneously in the single compartment, we have:

$$M = I\bar{t} = C_{\infty}V \quad (5)$$

where  $C_{\infty}$  is the concentration of the indicator at infinite time and  $V$  is its volume of distribution.

If there is more than one compartment and if

the indicator is distributed homogeneously in each of these compartments, then we must write

$$M = I\bar{t} = C_1V_1 + C_2V_2 + C_3V_3 + \dots \quad (6)$$

Consider now the simple two-compartment system, with volumes  $V_1$ , vascular, and  $V_2$ , extravascular. Our aim is to determine the value of  $C_2$ , which is the concentration of the indicator in the extravascular compartment. A single indicator dilution experiment is of no value because there are too many unknowns. But with multiple indicators in a given experiment, the problem becomes solvable. Thus, for the vascular compartment  $V_1$  we use T-1824 or  $^{22}\text{Na}$ , and for the extravascular compartment  $V_2$  we use labeled water. The concentration ratio  $C_1/C_2$  of the nonhomogeneously distributed indicator can then be calculated because  $\bar{t}$ ,  $V_1$ ,  $V_2$ , and  $I$  (or  $M$ ) are known. Conversely, if the ratio  $C_1/C_2$  is known from other considerations (for example, as an oil:water partition coefficient), then  $V_2$  could be calculated.

#### EXPERIMENTAL METHODS AND PROCEDURES

These have been described in detail previously (Chinard, Enns, and Nolan, 1960; Chinard, 1966a). In brief, mongrel dogs anesthetized with pentobarbital (initial dose, 25 to 30 mg/kg) were used as experimental subjects. A catheter was introduced into the right jugular, right atrium, right ventricle, or into the pulmonary artery for injections, while another catheter was inserted into the left carotid artery to provide for sampling of arterial blood. In another group of experiments, the test substances were dissolved in distilled water and introduced into the left lower lobe by means of a fine polyethylene catheter introduced by way of a tracheostomy. Regulation of blood-sampling rate was accomplished by means of a peristaltic pump. Samples in the carbon dioxide studies were collected by means of an anaerobic collector (Enns, Chinard, Shepard, and Armant, 1958) and in studies of nonvolatile indicators by means of a simple linear collector (Chinard, Vosburgh, and Enns, 1955). In the earlier studies, the dogs were allowed to breathe spontaneously. In later studies, the dogs were paralyzed with succinylcholine and ventilated mechanically. Sup-



TABLE I.—Summary of Results Obtained Following Intravenous Injection of Test Substances<sup>a</sup>

| Expt. no. | Dog weight, kg | Conditions    | Form of <sup>14</sup> C injected | Flow, ml sec <sup>-1</sup> kg <sup>-1</sup> | Mean transit time, sec |                 |       | Fractional recovery |      |
|-----------|----------------|---------------|----------------------------------|---|------------------------|-----------------|-------|---------------------|------|
|           |                |               |                                  |   | Vascular indicator     | <sup>14</sup> C | DHO   | <sup>14</sup> C     | DHO  |
| 1a        | 11.4           | Control.....  | HCO <sub>3</sub> <sup>-</sup>    | 2.52  | 7.06                   | 9.08            | 8.49  | 1.08                | 0.94 |
| b         |                | CA inhib..... | HCO <sub>3</sub> <sup>-</sup>    | 1.84  | 7.22                   | 6.69            | 8.77  | .98                 | .98  |
| 2a        | 9.5            | Control.....  | HCO <sub>3</sub> <sup>-</sup>    | 2.43  | 5.14                   | 7.37            | 6.46  | .96                 | .95  |
| b         |                | CA inhib..... | HCO <sub>3</sub> <sup>-</sup>    | 2.05  | 4.43                   | 4.26            | 5.90  | .93                 | .94  |
| 3a        | 14.1           | Control.....  | CO <sub>2</sub> diss             | 2.91  | 7.13                   | 8.12            | 8.20  | .91                 | .90  |
| b         |                | CA inhib..... | CO <sub>2</sub> diss             | 2.56  | 8.74                   | 9.11            | 10.48 | .89                 | 1.11 |
| 4a        | 16.4           | Control.....  | CO <sub>2</sub> diss             | 2.54  | 7.25                   | 9.68            | 8.48  | .97                 | .99  |
| b         |                | CA inhib..... | CO <sub>2</sub> diss             | 2.41  | 7.85                   | 7.24            | 9.46  | 1.00                | 1.03 |
| 5a        | 10.5           | CA inhib..... | CO <sub>2</sub> diss             | 2.54  | 7.40                   | 8.05            | 9.29  | .99                 | .99  |
| b         |                | CA inhib..... | HCO <sub>3</sub> <sup>-</sup>    | 1.91  | 9.04                   | 8.91            | 11.37 | 1.01                | 1.03 |

<sup>a</sup> "CA inhib" indicates that the carbonic anhydrase inhibitor had been administered. T-1824 was used as vascular indicator in experiments 1 and 3, while <sup>22</sup>Na was used

as vascular indicator in the other experiments. The data are from previously reported experiments (Chinard et al., 1960).

plementary doses of succinylcholine were given as required, but were always preceded by a supplementary dose of anesthetic of about one-tenth the initial dose. In general, two sample collections were made from each dog; in some, three were made. Mean arterial pressures were continuously monitored and generally ranged between 100 and 135 mm Hg. Cardiac outputs, calculated from vascular indicator data (T-1824 or <sup>22</sup>Na), were between 1.8 and 4.5 ml/sec/kg. At the completion of the experiment, the dogs were killed with a large dose of pentobarbital.

Analytical procedures have been reported previously (Chinard et al., 1960), except for the specific points mentioned in the text.

Acetazolamide, when used, was injected in doses of about 100 mg/kg.

In the experiments in which test solutions were injected into the bloodstream, the volume injected was between 2 and 4 ml and was roughly related to the weight of the dog, but in the tracheal injection experiments, a volume of 5 ml was injected.

## RESULTS AND INTERPRETATIONS

Results of representative experiments in which injections of the test materials indicated were made into the bloodstream are given in table I

and are illustrated in figures 1 through 4. In the control experiments, there is no feature that provides a basis for separating the results obtained with bicarbonate from those obtained with dissolved carbon dioxide. The mean transit times are always greater than those of the vascular indicators and in general are greater than those

FIGURE 1.—Outflow patterns of T-1824, DHO, and <sup>14</sup>CO<sub>2</sub> in arterial blood following sudden injection into pulmonary artery. Control conditions. <sup>14</sup>C was injected as bicarbonate. Ordinates are fractional recoveries in each sample. Abscissae are times after injection.

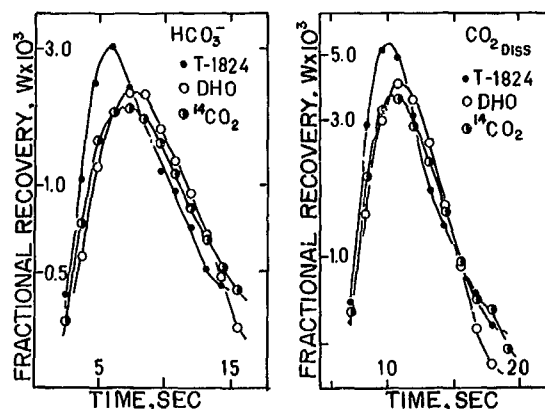


FIGURE 2.—Same type of experiment as illustrated in figure 1, except that <sup>14</sup>C was injected as dissolved carbon dioxide.

of labeled water. The recoveries do not show any differences. Further, as shown in figures 1 and 2, the outflow patterns do not appear to be different: with both forms of carbon dioxide, the peak time is the same or earlier than that of labeled water while the tail of the curve is always prolonged compared to the tail for the labeled-water curve. These results are to be expected in view of the rapid interconversion of the several forms of carbon dioxide in the presence of active carbonic anhydrase and in view of the distribution of carbon dioxide into a larger volume of distribution than water in the gas phase of the lungs.

These results are, of course, indicative of the permeability of the alveolar-capillary barrier to carbon dioxide and are substantiated by experiments reported previously on the time course and on the recovery of labeled carbon dioxide in the expired gas (Chinard et al., 1960). However, there is no indication as to which (if not all) species of carbon dioxide crosses the barrier. To obtain information on this question, large amounts of acetazolamide were administered to inhibit the carbonic anhydrase of the red cells and to limit the extent of the interconversion of the several forms of carbon dioxide. In the control experiments, carbon dioxide injected as dissolved CO<sub>2</sub> would exist mainly as bicarbonate ion because of the preponderance of this latter form. However, after blockage of the carbonic anhydrase, we could

hope that bicarbonate would be, as before, mainly restricted to the bicarbonate pool, whereas dissolved carbon dioxide would behave more as that species. These expectations were in large part realized. As shown in table I, the mean transit times of the injected <sup>14</sup>C now are equal to or slightly less than the mean transit times of the simultaneously injected vascular indicators. In addition, the outflow patterns no longer show the peak displacement and the prolonged tails, but are quite close to the outflow patterns of the vascular indicators (figs. 3 and 4).

In the case of the bicarbonate injection, we have interpreted these results to indicate that the alveolar-capillary barrier was impermeable to bicarbonate ion as such and that such passage as did occur under control conditions of <sup>14</sup>C injected as bicarbonate was caused by its conversion to dissolved carbon dioxide. Why then did the carbon dioxide injected as dissolved carbon dioxide have patterns so similar to those of bicarbonate ion? In fact, although the patterns are similar, the recoveries of the dissolved carbon dioxide are in general less than those of the bicarbonate relative to the recovery of labeled water, an indication of irreversible loss. Thus under control conditions the air:water partition of carbon dioxide favors water (or blood) because of the large bicarbonate pool. But after inhibition of carbonic anhydrase, the air:water partition of dissolved carbon dioxide is shifted toward air (or the gas phase) because the bicarbonate pool is no longer accessible. This interpretation is supported by results of experiments in which the expired gases were collected and analyzed for <sup>14</sup>CO<sub>2</sub> after injection of bicarbonate ion and of dissolved carbon dioxide under control conditions and after administration of the carbonic anhydrase inhibitor (table II). Recoveries of expired <sup>14</sup>CO<sub>2</sub> were similar for the two species under control conditions, but were considerably larger after injection of dissolved carbon dioxide following the administration of acetazolamide. Thus, a much larger contribution came from dissolved carbon dioxide than from bicarbonate ion relative to the concentrations present in blood at pH numbers between 7.30 and 7.45.

These findings support the contention that the alveolar-capillary barrier is permeable to dissolved carbon dioxide but relatively impermeable to bi-

FIGURE 3.—Same type of experiment as illustrated in figure 1, but after administration of acetazolamide.

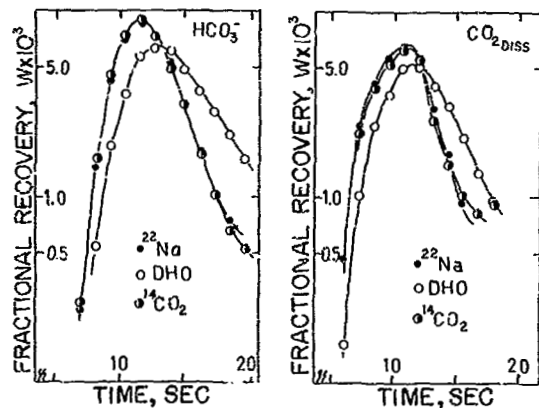


FIGURE 4.—Same type of experiment as illustrated in figure 2, but after administration of acetazolamide.

TABLE II.—Expired Gas Recoveries of <sup>14</sup>CO<sub>2</sub><sup>a</sup>

| Percentage recovery in expired gas                    |  | Ratio of recoveries   |
|---|--|---|
| H <sup>14</sup> CO <sub>3</sub> <sup>-</sup> injected | <sup>14</sup> CO <sub>2</sub> injected | <sup>14</sup> CO <sub>2</sub> diss/H <sup>14</sup> CO <sub>3</sub> <sup>-</sup> |
| Control experiments                                   |  |   |
| 5.3   | 4.3                                    | 0.81  |
| 3.9   | 4.2                                    | 1.08  |
| 10.5  | 8.0                                    | .76   |
| 2.2   | 3.6                                    | 1.12  |
| After acetazolamide (10 to 100 mg/kg)                 |  |   |
| 7.6   | 16.4                                   | 2.16  |
| 3.2   | 18.7                                   | 5.84  |
| 7.6   | 14.2                                   | 1.87  |
| 3.3   | 9.6                                    | 2.91  |

<sup>a</sup> Data from Chinard et al., 1960.

carbonate ion. In this regard, one of the features of the outflow patterns illustrated in figures 1 and 2 is of importance: the precession of the <sup>14</sup>CO<sub>2</sub> curve over the labeled-water curve. This result is found consistently in the control experiments. It is, I believe, a reflection of the fact that we are dealing with at least two species of carbon dioxide, of which one, the bicarbonate ion, is the most abundant and is restricted to the vascular compartment. Since, in such control experiments, there is full carbonic anhydrase activity, distribution of the <sup>14</sup>C among its various forms in the several compartments must be quasi-instantaneous. That, in these circumstances, there is still precession of the <sup>14</sup>CO<sub>2</sub> over the labeled water implies a nonhomogeneous distribution of the <sup>14</sup>C and, accordingly, a lower total carbon dioxide concentration in the extravascular compartment (tissue and gas phase) than in the vascular compartment. The selective permeability of the alveolar-capillary barrier to one species of carbon dioxide, dissolved CO<sub>2</sub>, and the impermeability to bicarbonate ion is quite similar to that reported earlier for the nephron in the dog (Chinard, Nolan, and Enns, 1964). In those studies, the indicator dilution technique was applied to the kidney, and precession of dissolved carbon dioxide

and of bicarbonate over creatinine was found in the urine. Following inhibition of the renal carbonic anhydrase with acetazolamide, precession of <sup>14</sup>CO<sub>2</sub> diss was unchanged; but now the excretion pattern of H<sup>14</sup>CO<sub>3</sub><sup>-</sup> was symmetrical to that of creatinine. A diffusion bypass was available to dissolved carbon dioxide but not to bicarbonate ion.

To test further the hypothesis of the impermeability of the barrier to bicarbonate ion, experiments were carried out in which H<sup>14</sup>CO<sub>3</sub><sup>-</sup> was introduced, dissolved in distilled water, by way of a catheter threaded through a tracheostomy on the gas-phase side of the alveolar-capillary barrier (Chinard, Enns, and Nolan, 1962). With the bicarbonate, there were included labeled water, known now to have a flow limited distribution in the lung, and chloride ion, as <sup>36</sup>Cl, known to cross the barrier very slowly and, indeed, only to a negligible extent during a single pass of blood

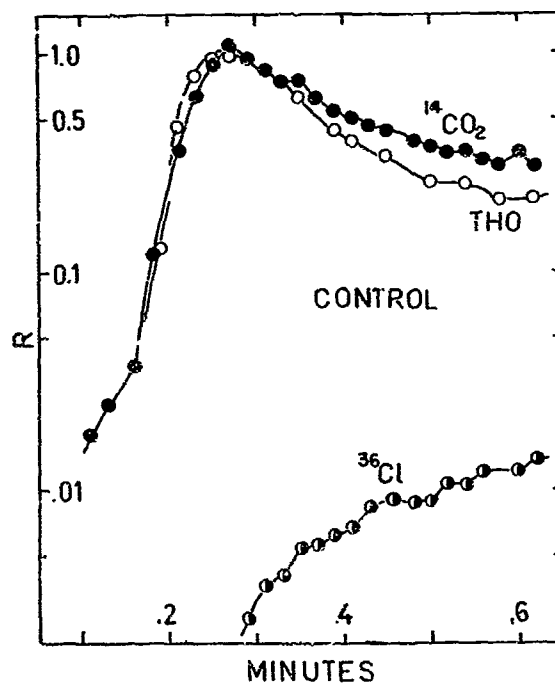


FIGURE 5.—Outflow patterns in arterial blood of <sup>14</sup>C, THO, and <sup>36</sup>Cl after introduction of 5 ml of solution containing these substances on the gas-phase side of the alveolar-capillary barrier by way of a catheter in trachea. Control conditions. R is the concentration of the different substances in the outflowing blood relative to an arbitrary standard and plotted on a logarithmic scale.

through the lungs. The results were surprising (see fig. 5) in that the curve for the recovered  $^{14}\text{CO}_2$  was almost identical to that for water. This made no sense unless there was carbonic anhydrase in the lung tissue. As a first check on this, we administered acetazolamide and found that the recovered  $^{14}\text{CO}_2$  curve was now between the curve for labeled water and that for  $^{36}\text{Cl}$  (fig. 6). This effect could not be the result of the inhibition of carbonic anhydrase of red cells, because carbonic anhydrase localized in these could hardly affect the interconversion of the different forms of carbon dioxide on the other side of the barrier. Further, the bicarbonate pool, or sink, in plasma would still be accessible to bicarbonate on the alveolar side if the  $^{14}\text{C}$  crossed as bicarbonate ion.

The simplest explanation was that there was carbonic anhydrase in lung tissue. The only report to this effect was by Berfenstam (1952) some years previously with regard to the fetal lung. Using a modification of Maren's method (1960) for determination of carbonic anhydrase activity, we found lung-tissue activity to be about one-tenth

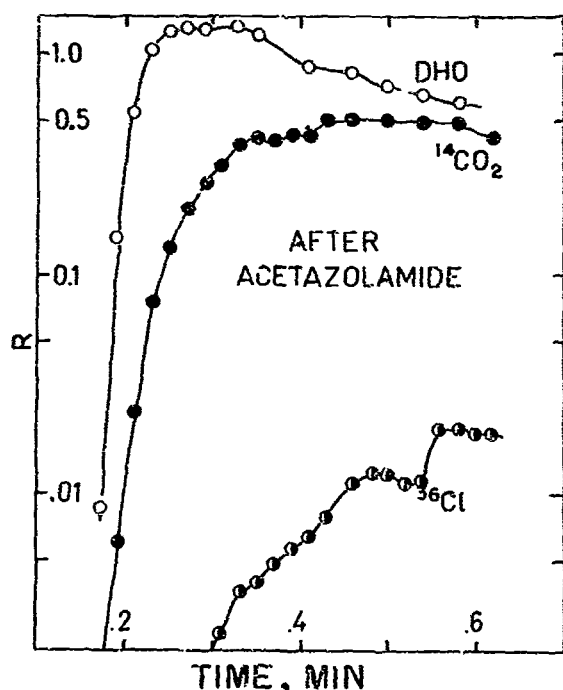


FIGURE 6.—Same type of experiment as illustrated in figure 5, but after administration of acetazolamide.  $R$  is defined for figure 5.

TABLE III.—*Tissue Carbonic Anhydrase Activity*<sup>a</sup>

| Tissue             | mg tissue required to halve standard reaction time |
|--------------------|--|
| Pancreas.....      | 71 (equivalent to blank)                           |
| Kidney:            |  |
| Papilla.....       | 2.6  |
| Outer medulla..... | 2.3  |
| Cortex.....        | 0.66 to 1.32                                       |
| Lung.....          | 7.1 to 13.1  |

<sup>a</sup> Tissue weights were obtained by drying for 16 hours at 120 C. Lungs were perfused and ventilated for 15 to 30 minutes with NaCl to remove blood. Drained lungs perfused for 2 to 3 minutes had activities about 30 percent higher than the values reported in table III. All tissues were homogenized in a Waring Blendor. Activities were determined in diluted homogenates.

to one-third that of kidney cortex (table III). Thus, under control conditions the exchanges of carbon dioxide between the gas phase and the blood appear to be mainly the result of exchanges of dissolved carbon dioxide, rather than of any other species of carbon dioxide, between blood and the alveolar-capillary barrier itself. In addition, the presence of carbonic anhydrase in lung tissue indicates that the total carbon dioxide in the lung tissue may play a role in the moderation of the transients of the concentration of carbon dioxide in blood and in expired gas that may occur following sudden changes of the partial pressure of carbon dioxide.

#### DISCUSSION

The impermeability of the alveolar-capillary barrier to bicarbonate follows the pattern found earlier for other ions. In brief, the cations sodium, lithium, and potassium and the anions chloride, phosphate, sulfate, *p*-aminohippurate, iodide, and thiocyanate, as well as the monocarboxylic acids from  $\text{C}_1$  through  $\text{C}_9$ , are restricted in the lungs to a volume of distribution little larger than that available to vascular indicators. This impermeability to bicarbonate ion contrasts with the permeability to dissolved carbon dioxide that follows, as well as we can judge, the pattern found for the inert gases and for the monohydric alcohols (Chinard et al., 1967). Except for the distribution into the gas phase, this is also the pattern followed

by labeled water. Whether water and the other substances that cross the barrier have similar pathways is another matter. The conventional hypothesis is that "gases" and lipid-soluble substances dissolve in lipid, which makes up most of the surface of the barrier, and that water and water-soluble materials cross the barrier through pores of definite radius. There is no anatomical evidence that such pores exist. Further, in studying the homologous series of the normal terminal and of the normal alkane amides, permeation of the barrier is slight for the shorter chain compounds and increases with molecular weight. It is possible to explain such data on the basis of a combined model of pores and of a lipid barrier with increasing lipid:water distribution coefficients as the molecular weight increases. But the retention of the pore hypothesis is not essential.

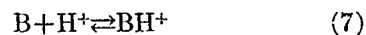
As suggested elsewhere (Chinard, 1966a), an alternative is possible, based on the flickering cluster model of water structure introduced by Frank and Wen (1957) and elaborated by Nemethy and Scheraga (1962a, b). In this hypothesis, water molecules form an integral part of the barrier and play a determining role in the permeability of the barrier. The water molecules are considered to be associated with the nonpolar parts of the barrier matrix and to be structured on this matrix in tetra-coordinated, hydrogen-bonded clusters as in normal water. Substances that enhance the normal cluster formation of water molecules or do not alter it (for example, the inert gases, hydrocarbons, and hydrocarbon parts of molecules) can enter the barrier because they find the same arrangement of water molecules about them in the barrier as in blood. For contrast, ions (and particularly cations) disrupt the normal structure of water, break the hydrogen-bonded clusters, and force the orientation of individual water molecules as layered shells surrounding the central charge. Normally such structuring is postulated to be found but rarely in the barrier. A third group of substances must be considered, namely, those that form hydrogen bonds themselves, such as the glycols, polyols, and carbohydrates. These, too, are excluded from the barrier, possibly because they become attached to, or caught on, the membrane matrix.

The main advantage of this hypothesis is that

it is unitarian, that it allows certain predictions to be made (e.g., a sufficient increase of ionic strength should disrupt the barrier or at least increase its permeability to ions), and that it does not require anatomically defined pores. Molecular permeability is the result of molecular organization and not of a structural or supra-molecular organization. The membrane matrix is merely a framework on which the water molecules and perhaps other substances are organized—much as a braided-wire outer covering can prevent a relatively thin flexible tube from bursting. Finally, we need not even have a fixed geometry of the membrane. Thus, the permeability to sodium ion is small but real. The sodium ion can cross the barrier only when a site is encountered that is complementary to it. Such sites may not be simply distributed sparsely over the surface of the barrier; the structure of the barrier may itself be changing with time at any one geometrically defined locus. Impermeability to sodium ion is then the result of the low probability of a given locus of the membrane or barrier having a conformation that will accept sodium. This concept of a flickering structure for the barrier is, of course, compatible with the flickering cluster concept for water structure.

It will be noted that no attempt has been made here to calculate volumes of distribution for the carbon dioxide system. One limitation in the validity of such calculations is the constraints implied in expression (6). Although we have measures of  $V_1$  and  $V_2$ , we have no concomitant measure of  $V_3$ , the volume of the gas phase in which the carbon dioxide can distribute itself. Nor do we have values for the partition coefficient of carbon dioxide between tissue and gas phases, because this will vary with tissue pH. Further, one of the basic conditions for application of the fundamental relationship (4) is not met; namely, that of conservation.

We can, however, arrive at values for the tissue content of carbon dioxide based on *in vivo* estimates of pulmonary tissue pH. It can be shown (Effros and Chinard, 1968; and unpublished) by extension of expression (6), that with an appropriate base of the type



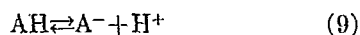
the hydrogen ion concentration of the tissue,  $[\text{H}^+]_e$ , is given by

$$[\text{H}^+]_e = \rho'([\text{H}^+]_p + K_a) - K_a \quad (8)$$

In this,  $\rho' = (\bar{i}_i - \bar{i}_v) / (\bar{i}_w - \bar{i}_v)$  in which the subscript  $i$  denotes the pH indicator;  $v$ , the vascular indicator; and  $w$ , labeled water. The subscript  $p$  denotes plasma and  $K_a$  is the dissociation coefficient of the base.

The conventionally used substance for intracellular pH, 5,5'-dimethyl-2,4-oxazolidinedione or DMO, is not suitable because it does not enter the barrier within the time of transit of blood through the pulmonary capillaries. However, nicotine does enter the barrier, and we have used this substance extensively. We have found values for pulmonary tissue pH averaging  $6.71 \pm 0.09$  at normal arterial pH values. Hyde et al. (1968) have recently reported values of  $6.97 \pm 0.09$  obtained by means of a different technique. With respect to the carbon dioxide content of lung tissue, agreement is not so good. Dubois, Fenn, and Britt (1952) found in dog lungs perfused with Ringer's solution, about 0.35 ml  $\text{CO}_2$  (STPD) per milliliter of tissue, while Hyde and his associates obtained a value of about 0.33 milliliter  $\text{CO}_2$  per milliliter of tissue. With the lower tissue pH, we calculate a bicarbonate ion concentration between 4 and 6 mmoles per liter and a total  $\text{CO}_2$  content corresponding to 0.10 to about 0.16 ml  $\text{CO}_2$  per milliliter of tissue. It is possible that all the carbon dioxide of lung tissue may not be in the aqueous compartment but may be, in part, in a more slowly equilibrating lipid compartment. It is also possible that the pH value we have obtained is a composite value for the compartments accessible to the indicator dilution experiments and is not the same as for the compartments accessible in the experiments of Dubois and of Hyde and their collaborators. Finally, it is possible that forms of carbon dioxide other than those conventionally recognized are present.

With respect to the use of labeled carbon dioxide to provide estimates of extravascular pH, we can only report that the values obtained are completely out of line with those obtained with other indicators. For an indicator of the type



the expression to be used is of the form:

$$[\text{H}^+]_e = \frac{K_1'}{\rho' \left( \frac{K_2'}{[\text{H}^+]_p} + 1 \right) - 1} \quad (10)$$

where  $K_1'$  is the apparent dissociation coefficient of the carbon dioxide system and the other symbols have the same significance as above. The values for  $\rho'$  average about 2.0 for experiments 1, 2, and 4 of table I. Corresponding values for  $[\text{H}^+]_e$  are well below those for  $[\text{H}^+]_p$ , because the values for  $\rho'$  are too large. This is in accord with expectations, because the mean transit times are affected by distribution in the gas phase.

In conclusion, I wish to emphasize that I have made no attempt to review the literature on indicator-dilution theory and practice. Those who wish further information on these matters will find material in the references cited. Of those mentioned, I would like to single out the contributions of Drs. Kenneth L. Zierler, Per-Erik Bergner, and Carl A. Goresky.

#### ACKNOWLEDGMENTS

The studies with radioactive carbon dioxide were carried out in collaboration with Dr. Theodore Enns at the Johns Hopkins School of Medicine and with the help of Miss Mary F. Nolan. The studies on tissue pH are largely the work of Dr. Richard M. Effros. Miss Anne Delea collaborated in the other permeability studies. Dr. William Peil and Dr. Melvin Silverman have made major contributions to some of the theoretical and experimental developments mentioned in the text.

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## Significance of Carbonic Anhydrase in Lung Tissue

ARTHUR B. DuBois

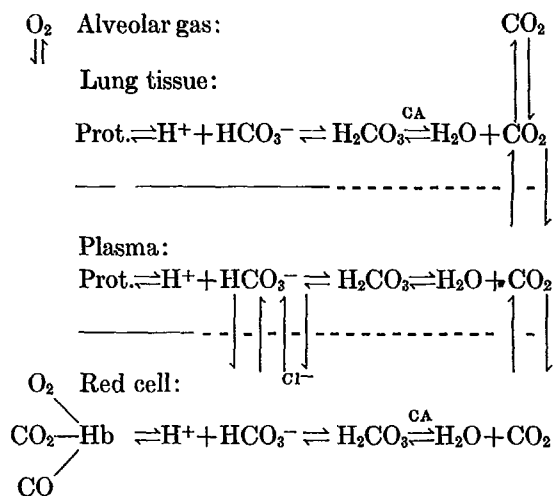
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### DEMONSTRATION OF PRESENCE AND ACTION OF CARBONIC ANHYDRASE IN LUNG TISSUE

Although it was Dr. Chinard (Chinard, Enns, and Nolan, 1962) who first called my attention to the fact that analysis of lung tissue by Berfentstam (1952) had shown the presence of carbonic anhydrase, even before that, there had been reason to suspect its presence. Experiments on alveolar CO<sub>2</sub> (DuBois, 1952) had shown that the rate of increase in the concentration during breathholding and the extent of its decrease in concentration during inspiration of fresh air seemed somewhat damped in degree compared to the output expected from pulmonary blood flow. The interpretation at that time was that the lung tissues were storing or releasing CO<sub>2</sub>, thereby acting as a reservoir having a capacity for CO<sub>2</sub>. Indeed, a dissociation curve, carried out on dogs' lungs freed of blood (DuBois, Fenn, and Britt, 1952) demonstrated that lung-tissue capacity for CO<sub>2</sub> was much like that of plasma, or muscle tissue. Fenn and Dejours (1954) further extended the experiments on breathholding and deep inspiration.

### PATHWAYS OF REACTION IN THE LUNGS

The various sites of CO<sub>2</sub> reaction in the lungs, as we now conceive them, are shown in the diagram below indicating the apparent pathways of CO<sub>2</sub> exchange in lung tissue, blood, and alveolar air. The horizontal lines indicate relative impermeability, whereas the dashed lines show permeability. CA stands for carbonic anhydrase, Cl<sup>-</sup> for the chloride ion that shifts, and prot. for protein. Water shifts are not shown.



### DILUTION SPACE OF CO<sub>2</sub> AND BICARBONATE IN THE LUNGS

Chinard et al. (1962) found that bicarbonate, injected in a pulmonary artery, stayed within the circulation, provided that a carbonic anhydrase inhibitor had been given, but that its CO<sub>2</sub> was spread in the lung tissue pool if the inhibitor was not given. The question was whether the inhibitor was acting on the red cells or on the lung tissue to block the CO<sub>2</sub> transfer during a single passage of blood through the lung capillaries.

Meanwhile, Sackner, Feisal, and DuBois (1964), using a body plethysmograph to study the absorption or release of gases in the lung, found a lung tissue space for CO<sub>2</sub> similar to that which would exist if the dissociation curve of lung tissue were like that of plasma and if the reaction were completed within a fraction of a second. About the same time, Hyde, Puy, Raub, and Forster (1968) measured the dilution pool in the lung for <sup>13</sup>CO<sub>2</sub> with a mass spectrometer and measured the total amount of bicarbonate in the lung tissue. There-



fore, it seemed that there was sufficient enzyme present in lung tissue to rapidly hydrate or dehydrate CO<sub>2</sub> brought into diffusion equilibrium with the tissue.

Feisal, Sackner, and DuBois (1963) injected bicarbonate into the pulmonary artery and found that it gave up CO<sub>2</sub>, which began to appear as the slug entered the lung capillaries and disappeared when the slug left the lung capillaries. But the CO<sub>2</sub> slug stayed in the capillaries several times longer than did the impermeable substances. The implication was that the CO<sub>2</sub> reacted with the extravascular tissues, thereby creating a larger pool and longer transit time for this transient bolus to pass through.

#### TRANSIENT VERSUS STEADY-STATE DILUTION SPACE OF CO<sub>2</sub> IN THE LUNGS

So far, however, these phenomena have all reflected the capacity of lung tissue to interact with a surge of CO<sub>2</sub>, accommodating part of the load by means of a reaction requiring carbonic anhydrase to possess sufficient speed.

The next question is whether the presence of carbonic anhydrase in the lung tissue and the lung's capacity to store or release CO<sub>2</sub> acts in any way to modify the steady-state transfer of CO<sub>2</sub> between the blood and alveolar air. Is the role analogous to or parallel to that of carbonic anhydrase in the blood? Presumably not, because reactions in the blood cannot be affected by events outside the blood, so carbonic anhydrase in lung tissue cannot help unload CO<sub>2</sub> from the blood in the lung capillary.

The alveolar CO<sub>2</sub> in man acts as such a large reservoir of CO<sub>2</sub> that inspiration dilutes and expiration removes some of the CO<sub>2</sub> gas with each breath. In this process, the lung tissue is not needed to supply CO<sub>2</sub> to the fresh air entering the lung, because the alveolar air is a sufficient reservoir. The tissue CO<sub>2</sub> only dampens the degree of the fluctuation by 10 or 20 percent.

On passage of blood through the pulmonary capillary bed, each molecule of CO<sub>2</sub> has a statistical chance of entering the various dilution pools, including those of the lung tissue. But the lung tissue, in the steady state, gives back as many molecules to the blood as it receives from the

blood, statistically speaking, and therefore the blood receives no net gain or loss as a result of this transfer.

#### DAMPING EFFECT OF CO<sub>2</sub> CAPACITY OF LUNGS OR GILLS ON SWINGS OF ARTERIAL P<sub>CO<sub>2</sub></sub>

In deep breathing, the damping effect of lung tissue on CO<sub>2</sub> excursions (Severinghaus and Stupfel, 1957) may be of some use in that alveolar CO<sub>2</sub> and arterial CO<sub>2</sub> would not fall to such a low level as they would without this damping effect.

The possibility exists that the damping of swings in arterial CO<sub>2</sub> may be more important in fish than in man. In man, the alveolar air acts as a buffer between the environmental CO<sub>2</sub> and the blood. In the fish, the water in the gills is less of a buffer. Thus, some rapidly reacting capacity of gill tissue in fish might act as a protection against a sudden change in CO<sub>2</sub> tension in the water, by "buffering" this change at the gill tissue level before the alteration reached the blood. This possibility is purely speculative. However, work on the role of carbonic anhydrase in fish-gill gas exchange (Hodler, Heinemann, Fishman, and Smith, 1955) reveals that Diamox prevented excretion of a bicarbonate load injected in the dogfish. This action is presumably at the blood level, rather than in the gill tissue.

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## Influence of Carbonic Anhydrase Activity on the Exchange of CO<sub>2</sub> Across the Alveolar-Capillary Membrane<sup>1</sup>

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We have studied the dynamics of CO<sub>2</sub> exchange between alveolar gas, pulmonary tissues, and capillary blood by inspiring and breathholding with gas mixtures enriched with the stable isotope of CO<sub>2</sub> labeled with carbon-13 (<sup>13</sup>CO<sub>2</sub>) (Hyde, Puy, Raub, and Forster, 1968; Hyde and Forster, 1962).

Figure 1 is a mass spectrometer record taken during collection of the data. The subject first rebreathed 1.5 liters of a gas mixture containing 12 percent CO<sub>2</sub> to make alveolar P<sub>CO<sub>2</sub></sub> equal to mixed venous CO<sub>2</sub>. He then inspired a gas mixture enriched with <sup>13</sup>CO<sub>2</sub>, acetylene, and neon. Three seconds later he exhaled, an alveolar gas sample was collected, and the gas concentrations were determined. Note that even though the total alveolar CO<sub>2</sub> was relatively constant before, during, and after breathholding, the <sup>13</sup>CO<sub>2</sub> concentration fell from 5.5 mm Hg in the inspired mixture to 2.2 mm Hg in the expired alveolar sample.

This procedure was repeated for breathholding times of 6, 8, and 12 seconds; the percent <sup>13</sup>CO<sub>2</sub> in excess of the natural abundance, \*CO<sub>2</sub>, and the percent acetylene left in the alveolar gas were plotted against time of breathholding (fig. 2). The line of least mean squares was drawn through the experimental points. The initial rapid disappearance of acetylene (determined by extrapolating back to time zero) permits calculation of the pulmonary tissue volume, V<sub>tis</sub>, and from the slope of the line, pulmonary capillary blood flow,

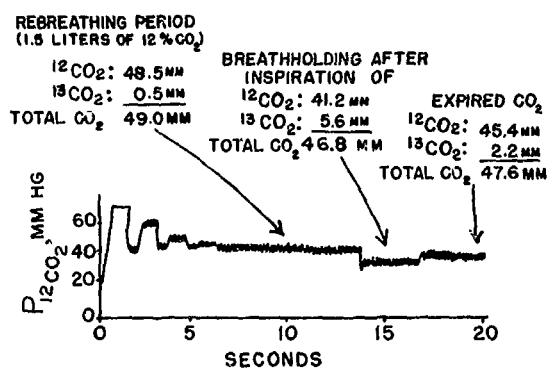


FIGURE 1.—Mass spectrometer tracing recording mass 44 (<sup>12</sup>CO<sub>2</sub>) before, during, and after breathholding with a mixture enriched with <sup>13</sup>CO<sub>2</sub>.

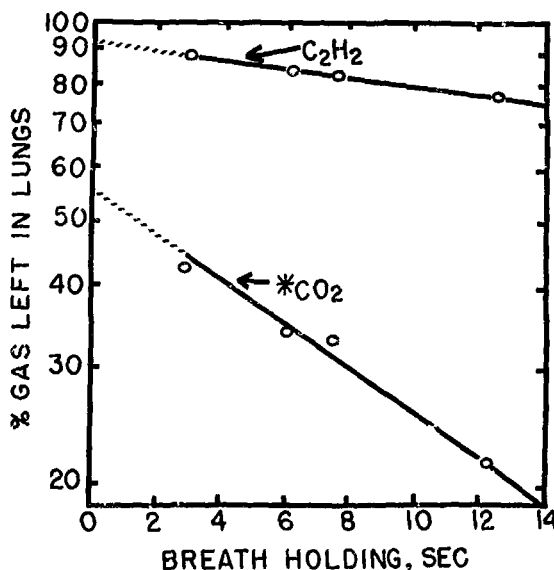


FIGURE 2.—The rate of disappearance of acetylene (C<sub>2</sub>H<sub>2</sub>) and \*CO<sub>2</sub> from the alveolar gas during breathholding.

<sup>1</sup>This work was supported in part by research grants HE-10324 and HE-4108 from the National Heart Institute, U.S. Public Health Service. Dr. Hyde is an established investigator of the American Heart Association.

$\dot{Q}_c$ , can also be determined (Cander and Forster, 1959). Similarly, for  $^*\text{CO}_2$ , the initial rapid disappearance of the isotope must be caused by exchange with  $\text{CO}_2$  in  $V_{tis}$ . The data allow calculation of this volume of  $\text{CO}_2$ , and in five adult humans we found the average volume of  $\text{CO}_2$  in  $V_{tis}$  to be 200 ml STPD. The slope of the disappearance curve for  $^*\text{CO}_2$  is a function of the uptake of  $^*\text{CO}_2$  by the capillary blood. From this slope,  $\dot{Q}_c$  can be calculated. We found  $\dot{Q}_c$  determined with acetylene and  $^*\text{CO}_2$  to be almost identical, meaning that there must be little or no alveolar to end-capillary gradient for  $\text{CO}_2$ .

Figure 3 illustrates the alterations that take place after the administration of a very large amount of carbonic anhydrase inhibitor (acetazolamide 100 mg/Kg). Note that the initial disappearance of  $^*\text{CO}_2$  is reduced from 45 to 18 percent and the subsequent disappearance is much slower. Even though  $\dot{Q}_c$  measured with acetylene increased from 6.4 to 10.9 liters/min after the administration of acetazolamide,  $\dot{Q}_c$  calculated from the  $^*\text{CO}_2$  data fell from 6.4 to 3.0 liters/min.

Some examples of the information that can be obtained from this type of data follow.

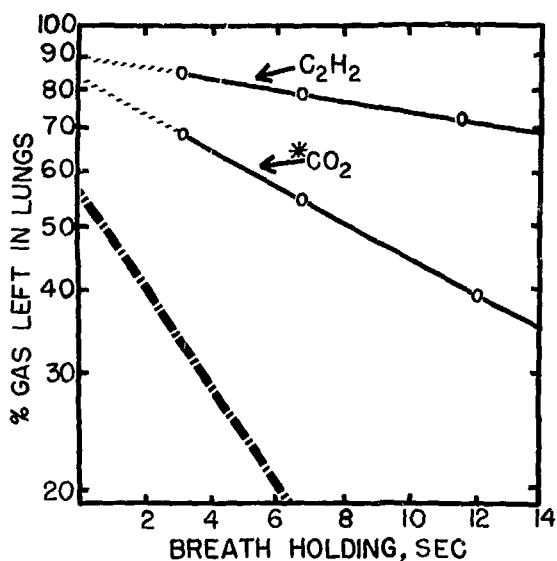


FIGURE 3.—The rate of disappearance of  $\text{C}_2\text{H}_2$  and  $^*\text{CO}_2$  from the alveolar gas after the administration of a large amount of the carbonic anhydrase inhibitor acetazolamide. The heavy broken line represents the rate of disappearance of  $^*\text{CO}_2$  expected if there was no inhibition of carbonic anhydrase activity.

### THE *IN VIVO* CO<sub>2</sub> DISSOCIATION CURVE OF LUNG TISSUE

From the initial disappearance of  $^*\text{CO}_2$ , the  $\text{CO}_2$  content of the pulmonary tissues  $V_{tis}$  at mixed venous  $\text{CO}_2$  can be determined, resulting in the middle point of the  $\text{CO}_2$  dissociation curve shown in figure 4. The slope of the dissociation curve above and below this point can be determined by collecting alveolar gas samples after rapidly inhaling and exhaling gas concentrations containing 12 percent  $\text{CO}_2$  or 0 percent  $\text{CO}_2$ , respectively (DuBois, 1952; Fenn and Dejours, 1954; Hyde et al., 1968, app. I). The  $\text{CO}_2$  dissociation curve for lung tissue (fig. 4) is similar to the curves published for plasma, brain, and muscle (Rahn, 1962). At resting lung volume, these curves indicate that approximately 2 ml of  $\text{CO}_2$  move into or out of the tissues for each mm Hg change in alveolar  $P_{\text{CO}_2}$ . Because this movement takes place in a second or less (Sackner, Feisal, and DuBois, 1964), it will produce approximately a 30-percent reduction in the swings in alveolar  $P_{\text{CO}_2}$  secondary to pulsatile pulmonary capillary blood flow and tidal ventilation.

### FORMS OF CO<sub>2</sub> PRESENT IN LUNG TISSUE AND PULMONARY CAPILLARY BLOOD

After administration of acetazolamide, the  $\text{CO}_2$  space in the lung tissues (initial  $\text{CO}_2$  loss) and the exchange with capillary  $\text{CO}_2$  (subsequent loss over time of breatholding) was reduced to one-quarter of the level measured without inhibition of carbonic anhydrase. Because the inhibitor presumably only interferes with the exchange of  $^*\text{CO}_2$  with bicarbonate, the difference in the size of the  $\text{CO}_2$  space before and during carbonic anhydrase inhibition must represent the bicarbonate space in the tissues and capillary blood. The fractionalization of total  $\text{CO}_2$  into bicarbonate, physically-dissolved  $\text{CO}_2$ , and other forms of  $\text{CO}_2$  is shown in figure 5. The fraction of  $\text{CO}_2$  in the form of physically dissolved  $\text{CO}_2$  was calculated from measurements of the size of  $V_{tis}$  (Cander and Forster, 1959), capillary blood volume  $V_c$ , the mixed venous  $P_{\text{CO}_2}$ , and solubility of  $\text{CO}_2$  in water at 37 C. Note that 11 to 21 percent of the total  $\text{CO}_2$  cannot be accounted for as bicarbonate or physically dissolved  $\text{CO}_2$ . In the case of blood, this fraction

can be ascribed to carbamate. In the tissues this may represent a CO<sub>2</sub>-protein complex such as the barium insoluble CO<sub>2</sub> fraction in rat muscle (Conway and Fearon, 1944).

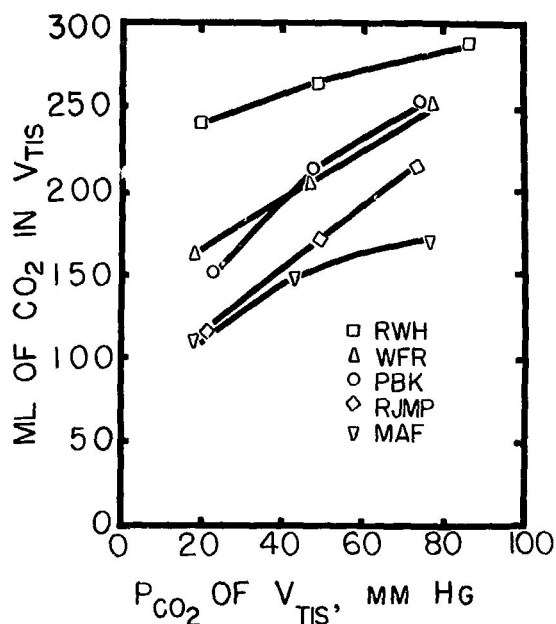


FIGURE 4.—CO<sub>2</sub> dissociation curves of pulmonary parenchymal tissue ( $V_{tis}$ ) in five human subjects.

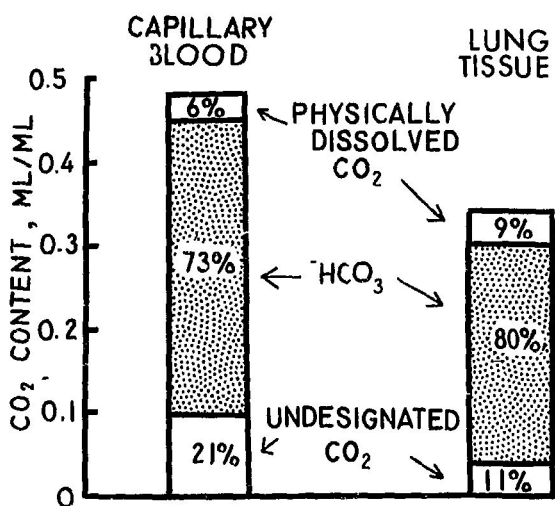


FIGURE 5.—Forms of CO<sub>2</sub> present in capillary blood and lung tissue.

**CALCULATION OF pH OF LUNG TISSUE**

Since both the bicarbonate fraction and physically dissolved CO<sub>2</sub> in the lung tissues are known, the pH of lung tissue can be calculated with the Henderson-Hasselbalch equation. At mixed venous  $P_{CO_2}$ , mean pH in five subjects was  $6.92 \pm 0.07$ . This value is slightly higher than recently reported values using an indicator dilution method (Effros and Chinard, 1968).

**PULMONARY DIFFUSING CAPACITY FOR CO<sub>2</sub>**

In the presence of active carbonic anhydrase, blood flows determined from the rate of disappearance of acetylene and <sup>14</sup>C<sup>18</sup>O<sub>2</sub> were almost identical, indicating there was little or no alveolar to end-capillary gradient for CO<sub>2</sub>. All we can conclude is that within the accuracies of our analytical methods, diffusing capacity for CO<sub>2</sub>, ( $D_{LCO_2}$ ), must be a value in excess of 200 ml/(min × mm Hg). However, after the administration of a large dose of carbonic anhydrase inhibitor, an alveolar to end-capillary CO<sub>2</sub> gradient is produced and  $D_{LCO_2}$  can be measured accurately. Figure 6 illustrates the changes in  $P_{CO_2}$  taking place along the pulmonary capillary during marked inhibition of carbonic anhydrase. The  $P_{CO_2}$  at the start of the capillary was assumed to

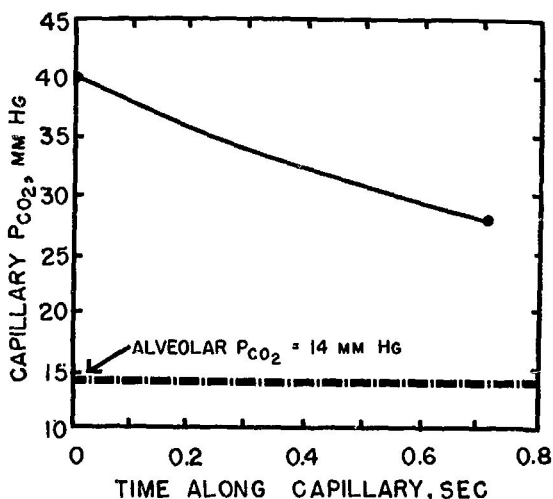


FIGURE 6.—Rate of fall in capillary  $P_{CO_2}$  along the pulmonary capillary during marked inhibition of carbonic anhydrase activity.

be equal to the mixed venous  $P_{CO_2}$  and was measured by the rebreathing method (Hackney, Sears, and Collier, 1958). Alveolar  $P_{CO_2}$  was estimated from the end expiratory  $P_{CO_2}$  measured with the mass spectrometer. The end capillary  $P_{CO_2}$  was calculated from the diffusing capacity for CO<sub>2</sub>, measured by the isotope breathholding method (Hyde, Forster, Power, Nairn, and Rynes, 1966), and the mean capillary transit time determined from  $\dot{Q}_c$  and an estimation of the pulmonary capillary blood volume. Because  $D_{LCO_2}$  had fallen from a value over 220 ml/(min×mm Hg) to 42 ml/(min×mm Hg), there is an alveolar to end-capillary CO<sub>2</sub> gradient of 14 mm Hg. The hyperventilation resulting in the low alveolar  $P_{CO_2}$  was probably the result of high levels of  $P_{CO_2}$  in the respiratory centers secondary to carbonic anhydrase inhibition (Mithoefer and Davis, 1958).

Figure 7 illustrates the importance of carbonic

anhydrase activity for CO<sub>2</sub> exchange in the lungs when the alveolar  $P_{CO_2}$  is maintained at 40 mm Hg. Curve A represents the fall in capillary  $P_{CO_2}$  as blood traverses the lungs in a man with a  $D_{LCO_2}$  of 250 ml/(min×mm Hg). Alveolar-capillary equilibration is reached at about one-third the distance along the capillary. Curve B represents what would happen if the subject lost the carbonic anhydrase activity in his blood. There would then be an alveolar to end-capillary gradient for CO<sub>2</sub> of 4 mm Hg.

Curve C describes the events along the capillary during carbonic anhydrase inhibition in a subject with a disease that reduced his capillary blood volume and pulmonary membrane diffusing capacity to one-third of normal. In this case there is an alveolar to end-capillary CO<sub>2</sub> gradient of 24 mm Hg. A similar gradient would result in a normal person with severe carbonic anhydrase inhibition during heavy exercise.

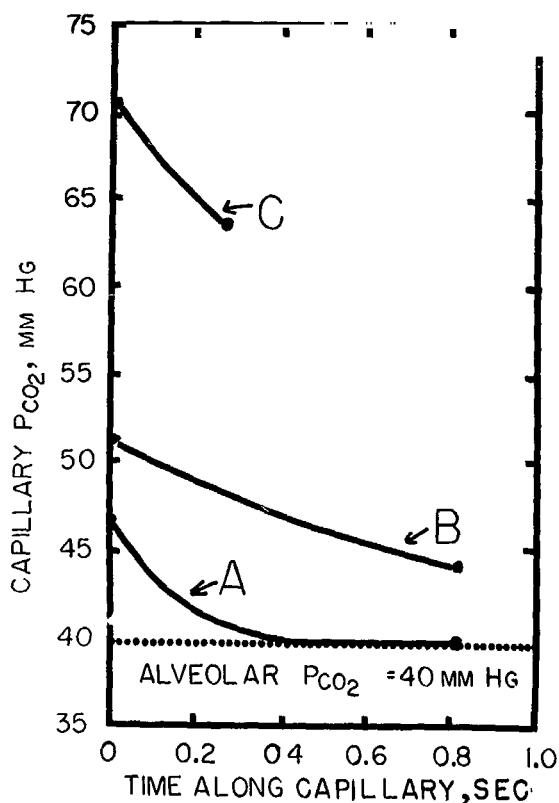


FIGURE 7.—Hypothetical rates of fall in capillary  $P_{CO_2}$  along the pulmonary capillary.

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## Rates of Chloride-Bicarbonate Exchange Between Red Cells and Plasma

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Attempts to determine the rate of processes involved in the equilibration of  $\text{CO}_2$  between red cells and plasma (hydration/dehydration of  $\text{CO}_2/\text{H}_2\text{CO}_3$ , transfer of  $\text{HCO}_3^-$  and  $\text{Cl}^-$ ) are reviewed. Measurements using either a thin layer of blood (Luckner, 1939) or the rapid reaction technique with filtration (Dirken and Mook, 1931; Tosteson, 1959; Piiper, 1964) have shown that at 37 C the processes are 90 percent complete in 0.13 to 1.1 seconds. Values estimated for pulmonary contact time (pulmonary capillary transit time) vary from 0.1 to 2.0 seconds. Comparison of pulmonary contact time values derived from measurement of  $D_{\text{CO}}$  at varied oxygenation levels (Roughton and Forster, 1957) with the kinetics of  $\text{CO}_2$  transfer between red cells and plasma indicate that the alveolar-capillary  $\text{CO}_2$  exchange is probably not limited by these processes at rest, but might be during exercise.

The process of  $\text{CO}_2$  exchange between blood and alveolar gas or tissue is complex, involving translocations and chemical reactions in series and in parallel (fig. 1). As diffusion of molecular  $\text{CO}_2$  in a  $\text{CO}_2$  pressure gradient is believed to be relatively fast because of the high solubility of  $\text{CO}_2$ , the rate-limiting steps could be one or several of the following component processes (in parentheses, fractional contribution to total  $\text{CO}_2$  exchanged or transported, according to Roughton, 1964):

(1) The hydration-dehydration reaction,  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ , accelerated by carbonic anhydrase in the red cells (60 percent)

(2) The transfer of bicarbonate ions across the red-cell membrane and the accompanying opposite transfer of chloride ions (45 percent)

(3) The formation-decomposition of carbamino-hemoglobin (30 percent)

Several investigators have studied the kinetics of  $\text{CO}_2$  exchange between red cells and plasma by disturbing the equilibrium between these two media and by determining the time course of the following reequilibration, measuring changes of chloride, total  $\text{CO}_2$ , and its components in the

plasma or in its substitute. As substantial changes in the  $\text{O}_2$  saturation of hemoglobin were avoided, the role of carbamino reactions must have been unimportant (the kinetics of these reactions is treated by others in this symposium). The hy-

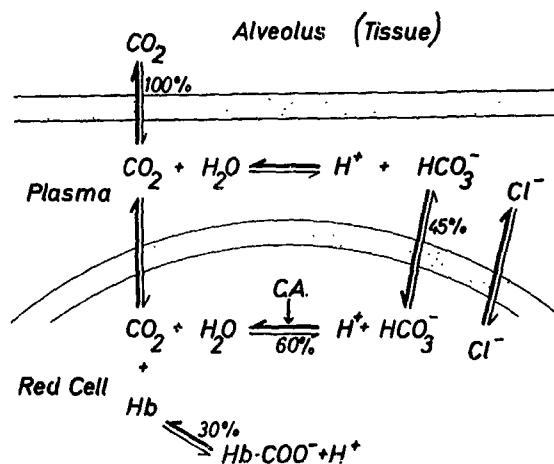


FIGURE 1.—Schema of the processes involved in exchange of  $\text{CO}_2$  between alveolar gas or tissue and blood. The percentage values (Roughton, 1964) indicate the relative contribution of a component process in the overall  $\text{CO}_2$  exchange.



dration-dehydration reaction and the transfer of bicarbonate and chloride took place to a variable extent, depending on the conditions of the experiment. Most authors have used a modification of the rapid reaction apparatus of Hartridge and Roughton (1923); only Luckner (1939) has applied a basically different method.

#### THIN-LAYER TECHNIQUE (LUCKNER)

Luckner (1939) developed an ingenious technique for measuring the kinetics of the chloride shift accompanying CO<sub>2</sub> exchange of the blood (fig. 2). A thin layer of blood, optimally about 10 microns thick, was spread out on a platinum surface that had been coated with silver chloride and, thus, in connection with a calomel electrode, functioned as a concentration cell for measurement of chloride concentration in the plasma of blood. The CO<sub>2</sub> partial pressure in the gas over the blood layer could be changed abruptly by lifting off the inner chamber of the apparatus. A typical record is illustrated in figure 3. On the average, the chloride concentration was found to approach the new equilibrium exponentially, with a half time of 0.11 second at 37 C in the blood of man, cow, and pig, both when  $P_{CO_2}$  was decreased or increased.

The most critical condition in the experiments of Luckner appears to have been the thickness of the blood layer. He considered the shortest half times of exchange, which were observed with

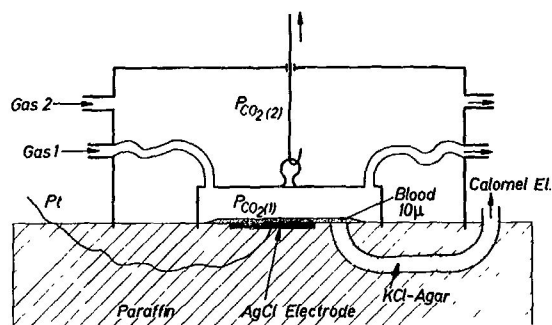


FIGURE 2.—Schema of the thin-layer technique of Luckner. A thin layer of blood (10 microns) is equilibrated with gas 1. By lifting the inner cover, the blood is abruptly exposed to gas 2, which has a different CO<sub>2</sub> content. The potential change measured by means of the AgCl electrode indicates the change in chloride concentration of blood plasma (Luckner, 1939).

layers of 8- to 12-micron thickness, as best values. In layers less than 8 microns thick, the exchange seemed to slow again, probably because of limited availability of bicarbonate and chloride in the thinned layer of plasma.

The principal advantage of Luckner's method is its excellent imitation of the CO<sub>2</sub> exchange in lungs or in tissues, because both the blood layer in the apparatus and the blood in the pulmonary or peripheral capillaries are open systems in respect to CO<sub>2</sub>, the CO<sub>2</sub> exchange occurring at approximately constant ambient  $P_{CO_2}$ .

#### RAPID REACTION TECHNIQUE WITH FILTRATION

Dirken and Mook (1931) first applied the rapid-reaction technique of Hartridge and Roughton (1923) to study the exchange of CO<sub>2</sub> between red cells and plasma. Later this technique was further elaborated by Paganelli and Solomon

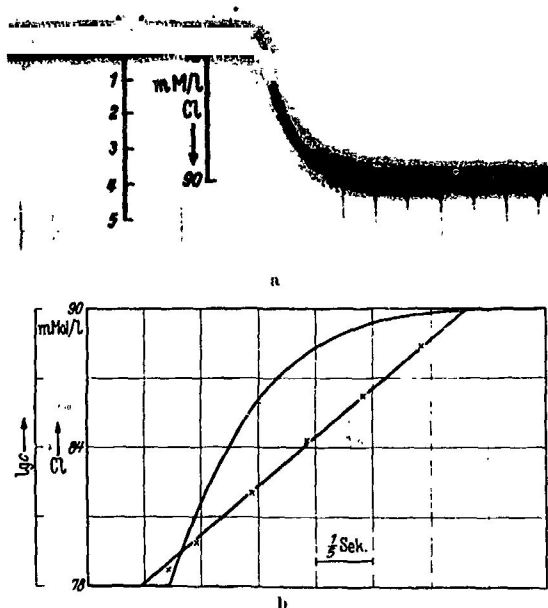


FIGURE 3.—Change of chloride concentration in human blood plasma upon decrease of CO<sub>2</sub> partial pressure. (a) Original tracing (MV = millivolts; time marks are at 0.2-second intervals). (b) Analysis of the change in chloride concentration derived from the voltage change. Crosses and straight line: chloride change on logarithmic scale. Curve: chloride change on linear scale (Luckner, 1939).

(1957), Tosteson (1959), and Piiper (1964). In principle, the stream of mixed red cells and plasma (or its substitute) is led past one or more membrane filters that retain the red cells but allow passage of plasma by filtration, aided by elevated pressure in the flow system (fig. 4). The time available for cell-plasma exchanges is equal to the volume between the sites of mixing and filtration divided by the flow rate.

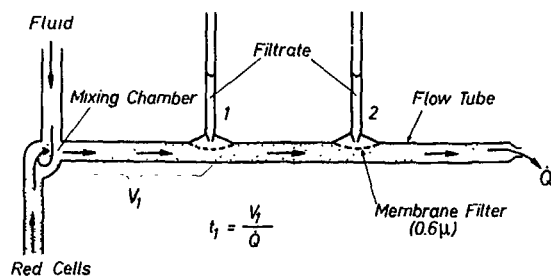


FIGURE 4.—Schema of the Hartridge-Roughton rapid reaction apparatus adapted for measurement of CO<sub>2</sub> exchange by using filters impermeable to red cells. The relationship between volume V between site of mixing and site of filtration, flow rate Q, and exchange time t is indicated.

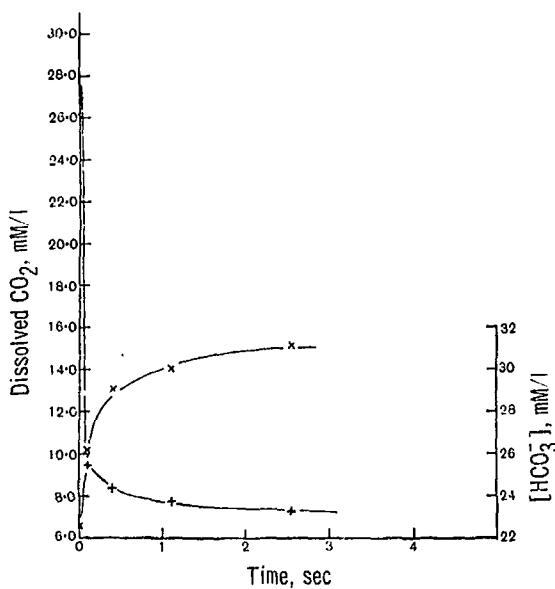


FIGURE 5.—Measurement of the change of physically dissolved CO<sub>2</sub> (+) and of bicarbonate (x) in plasma after mixing red cells of low P<sub>CO<sub>2</sub></sub> and separated plasma of high P<sub>CO<sub>2</sub></sub> (from Dirken and Mook, 1931).

Dirken and Mook (1931) report the data of one experiment only (fig. 5) in which red blood cells equilibrated with room air were mixed at room temperature with separated plasma equilibrated with pure CO<sub>2</sub> gas. The pH and total CO<sub>2</sub> content were measured in the filtrate, and bicarbonate and physically dissolved CO<sub>2</sub> concentrations were calculated. While initially the physically dissolved CO<sub>2</sub> decreased very rapidly because of equalization of CO<sub>2</sub> pressure between cells and plasma, it fell more slowly thereafter, indicating conversion of CO<sub>2</sub> into bicarbonate and transfer of bicarbonate from cells to plasma. The record shows that the transfer of bicarbonate was half completed in 0.16 second, with 90 percent completion in 1.4 seconds. Determination of chloride in the filtrate gave highly scattered values.

Using radioactive isotopes, Tosteson (1959) measured the transfer rate of chloride (and also of fluoride, bromide, and iodide) out of red cells into a chloride-phosphate medium. The outflux of chloride was found to be exponential, the half time averaging 0.22 second for human and bovine red cells at room temperature (fig. 6). The rate constants for the outflux of fluoride, bromide, and iodide were slower. Furthermore the outflux of chloride label was slowed down considerably when a chloride-free medium was used. In contrast to all other studies reviewed here, these measurements were performed in electrochemical equilibrium; i.e., without net exchange of chloride (or of other halides or bicarbonate). Because phosphate buffers probably in equilibrium with

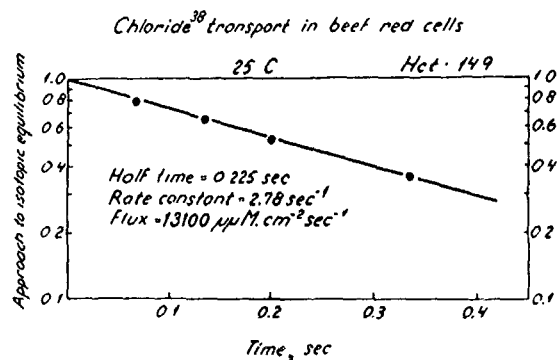


FIGURE 6.—Efflux of chloride<sup>38</sup> from bovine red cells into a chloride-phosphate buffer solution at 25 C (from Tosteson, 1959).

room air were used, the bicarbonate concentration must have been low.

Piiper (1964) measured the net influx and outflux rates of total CO<sub>2</sub> exchanged between erythrocytes and true plasma or NaCl solution, or NaCl solution with 30 meq/l NaHCO<sub>3</sub>, at 37 C. The time course of the exchange did not differ significantly from the exponential (fig. 7). For the red cells of all species studied (cow, pig, and dog) and for both inward and outward movements of CO<sub>2</sub>, the half time averaged 0.04 second; the 90-percent exchange time, 0.13 second. These rate values are much higher than those found by other investigators using similar techniques, but a reason for the discrepancy has not been found. In these experiments the amounts of physically dissolved CO<sub>2</sub> exchanged between red cells and plasma were relatively small, from 5 to 20 percent of the total amount of CO<sub>2</sub> exchanged because, unlike the experiments of Dirken and Mook (1931), high  $P_{CO_2}$ 's were avoided. Also, in most cases the transfer of physically dissolved CO<sub>2</sub> and bicarbonate occurred in the same direction. Therefore, the hydration-dehydration reaction  $CO_2 + H_2O \rightleftharpoons H_2CO_3$  must have been involved to a quantitatively lesser degree than in physiological conditions.

### CRITICISMS

The Hartridge-Roughton rapid-reaction technique is fraught with various sources of error. Thus it is evident that too slow or incomplete mixing of the red cells with the fluid would result in too long exchange times.

However, the most significant source of error (related not to the rapid-reaction technique in general, but specifically to the use of filtration in connection with the technique) seems to be that caused by an axial to marginal flow velocity gradient. When the marginal flow from which the samples are filtered is slower, the effective transit time for fluid elements in the marginal stream is longer than that computed from the mean flow rate and the volume of the tube between the sites of mixing and filtration. Thus the exchange rate is overestimated.

The radial velocity gradient is expected to be particularly important in the case of laminar flow and less in turbulent flow. From a consideration of the Reynolds number, Dirken and Mook con-

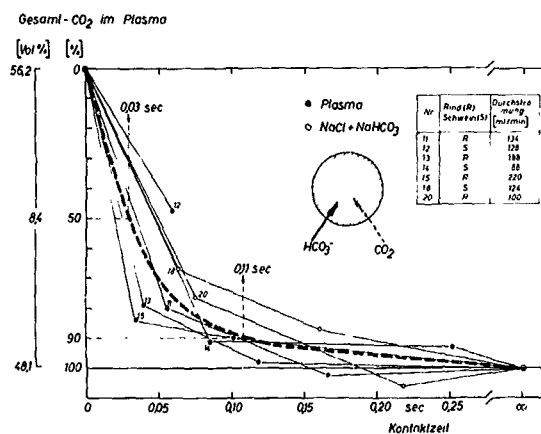


FIGURE 7.—CO<sub>2</sub> uptake by red cells (equilibrated with room air) from true plasma or from a solution containing 30 meq/l NaHCO<sub>3</sub>, at 37 C (from Piiper, 1964).

cluded that the flow under their experimental conditions must have been turbulent. Paganelli and Solomon (1957), as well as Piiper (1964), found that the flow rate was proportional to the square root of the driving pressure, which is the characteristic behavior of turbulent flow. Still, such a possibility cannot be excluded with certainty.

In this connection, it should be considered that the same streamline flow effect will influence determinations of the kinetics of O<sub>2</sub> and CO<sub>2</sub> exchange between cells and plasma by measurement of O<sub>2</sub> or CO<sub>2</sub> partial pressure in plasma with electrodes positioned in the flow tube. In fact, the rate of O<sub>2</sub> uptake by red cells was found to be higher when  $P_{O_2}$  in plasma was measured than in experiments in which the O<sub>2</sub> saturation of hemoglobin was determined photometrically (Staub, Bishop, and Forster, 1961).

### LIMITING SINGLE PROCESSES IN THE ERYTHROCYTE-PLASMA CO<sub>2</sub> EXCHANGE KINETICS

The component processes of hydration-dehydration reaction, bicarbonate transfer, and chloride transfer are functionally arranged in series. It will be attempted to find evidence for any one of these to constitute the main limiting step in the whole process of CO<sub>2</sub> exchange between red cells and plasma.

In the experiments of Tosteson, the time constant for isolated chloride exchange was similar

to the time constant for the whole exchange process involving both the hydration-dehydration reaction and bicarbonate-chloride transfer as measured by Luckner (1939) and by Dirken and Mook (1931). This may be interpreted to indicate that the hydration-dehydration reaction is fast enough not to limit the whole  $\text{CO}_2$  exchange process and that the limiting step is the exchange of bicarbonate or chloride across the red cell membrane. Luckner presented the following arguments in support of the same conclusion: the rates of  $\text{CO}_2$  uptake and release by red cells were found to be equal; and the temperature coefficient  $Q_{10}$  of the process was from 1.2 to 1.4, close to the temperature coefficients of diffusion in other systems.

That the hydration-dehydration reaction does not limit the  $\text{CO}_2$  exchange in the lungs is further supported by the results of Feisal, Sackner, and DuBois (1963) in anesthetized dogs. Although a slight inhibition of the carbonic anhydrase produced a small but measurable delay in the output of  $\text{CO}_2$  in the lungs, it did reduce the amount of  $\text{CO}_2$  output. However, the shorter exchange times measured by Piiper in experiments in which the hydration-dehydration reaction played a minor role may be explained by this reaction being a significant delaying factor.

Because, in the experiments of Tosteson, the exchange of chloride alone was not faster than that of the chloride-bicarbonate exchange found by Luckner and by Dirken and Mook, it should be inferred either that the transfer of chloride is intrinsically slower than that of bicarbonate or that their rates are about equal. The faster rates reported by Piiper for experiments in which both bicarbonate and chloride were exchanged cannot be made to fit the picture.

### RED-CELL PLASMA $\text{CO}_2$ EXCHANGE KINETICS AND PULMONARY CONTACT TIME

Whatever the limiting process in the erythrocyte-plasma  $\text{CO}_2$  equilibration may be, the physiological significance of  $\text{CO}_2$  equilibration kinetics is derived from its relationship to the capillary transit time or contact time; i.e., the average time spent by red cells in pulmonary or peripheral capillaries. The question to be examined

is whether or not  $\text{CO}_2$  equilibration is fast enough to assure practically full equilibration (i.e., higher than 90 percent) during the pulmonary contact time.

Since its first estimation by Roughton (1945), based on pulmonary uptake of  $\text{CO}$ , several authors have attempted to determine the pulmonary contact time using various methods. The scatter of their values, however, is considerable, exceeding that found for 90 percent  $\text{CO}_2$  equilibration (fig. 8).

The lowest values of the pulmonary contact time may be seriously questioned. The cinematographic observation of injected india-ink particles by Vogel (1947) possibly did not include the total pathway through the pulmonary capillary network, therefore resulting in transit times that were too short. Mochizuki and Fukuoka (1958) calculated their value, 0.23 second, from the alveolar-arterial  $P_{\text{O}_2}$  difference measured in man during hypoxia, applying their theoretical analysis of  $\text{O}_2$  uptake by red cells. They might have underestimated the contact time for two reasons. First, they probably overestimated the effect of diffusion limitation on the alveolar  $\text{O}_2$  exchange by neglecting effects of unequal distribution in the lungs. Second, assuming that all diffusion resistance was inside the red cells and none in an alveolar membrane, they probably underestimated the total resistance to  $\text{O}_2$  uptake in the lungs.

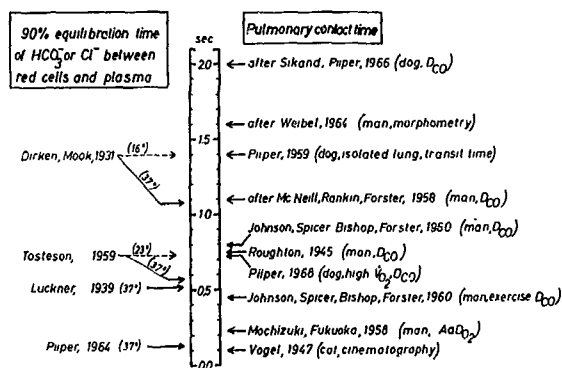


FIGURE 8.—Comparison of pulmonary contact time with 90 percent equilibration time of bicarbonate or chloride between red cells and plasma. The values of Dirken and Mook and of Tosteson have been adapted to 37 C by use of the temperature coefficients according to Luckner (1948).

The highest value of pulmonary contact time, 2.0 seconds, was determined from measurements of  $D_{CO}$  at different levels of oxygenation according to the method of Roughton and Forster (1957) in anesthetized dogs (Sikand and Piiper, 1966). The  $D_{CO}$  values were corrected for effects of inhomogeneity of the lungs, applying certain theoretical considerations (Piiper and Sikand, 1966) that may be questioned. If the uncorrected  $D_{CO}$  value obtained for the conventional 10-second apnea had been used, a contact time of 1.0 second would have resulted. The value of 1.4 seconds was estimated by Piiper (1959) using a method based on transit of O<sub>2</sub> saturation signals in isolated dog-lung lobes that possibly were not in physiological conditions.

Measurements performed by several groups using the  $D_{CO}$  method of varied oxygenation as introduced by Roughton and Forster (1957) have yielded pulmonary capillary volume values for resting man corresponding to contact time values in the range of 0.7 to 1.1 seconds. These values are clearly higher than most of those found for 90 percent CO<sub>2</sub> equilibration (fig. 8). During muscular exercise, the situation becomes more critical. Because the increase of cardiac output surpasses that of  $D_{CO}$  and of the pulmonary capillary volume, the contact time is decreased. According to the extensive study by Johnson, Spicer, Bishop, and Forster (1960), the pulmonary contact time during heavy exercise was reduced to about 0.45 second. This value is lower than three of the four values for 90 percent CO<sub>2</sub> equilibration (fig. 8). It should be mentioned, however, that there is a large variation in the degree of increase of  $D_{CO}$  during muscular exercise as determined by several authors, and the contact time derived therefrom should vary accordingly.

From the scatter of the values shown in figure 8, it is evident that any conclusions must remain tentative. However, consideration of pulmonary contact time values based on capillary volume determinations by the CO method in man seems to indicate that CO<sub>2</sub> exchange processes between red cells and plasma do not limit the CO<sub>2</sub> output in the lungs at rest, while during heavy exercise they must be taken into account as a potential limiting factor because of reduced pulmonary contact time.

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## The Rate of CO<sub>2</sub> Equilibration Between Red Cells and Plasma

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Any consideration of CO<sub>2</sub> exchange between red cells and plasma must distinguish between the movement of CO<sub>2</sub> per se and the movement of HCO<sub>3</sub><sup>-</sup>, possibly CO<sub>3</sub><sup>2-</sup>, H<sup>+</sup>, and H<sub>2</sub>CO<sub>3</sub>. However, it is difficult to separate the movement of the latter ions and molecules from that of the gas, because chemical reaction can rapidly convert one into the other. An exploratory approach to the question of the speed of CO<sub>2</sub> exchange of the red cell is to calculate the time required for this gas to achieve diffusion equilibrium in a layer of a watery solution approximating the erythrocyte in dimensions and composition. If we make this calculation, assuming that the membrane-diffusion resistance is negligible (which is equivalent to considering the diffusion coefficient to be the same for CO<sub>2</sub> as for water), that the equivalent thickness of the red cell is twice 0.8 micron (Forster, 1964), that the diffusion coefficient of CO<sub>2</sub> in concentrated hemoglobin solution at 37 C is  $6.6 \times 10^{-6}$  cm<sup>2</sup>/sec (calculated from the results of Longmuir and Roughton (1952), and that the diffusion coefficient is proportional to (molecular weight)<sup>1/2</sup>, then the average [CO<sub>2</sub>] inside the water layer will be 90 percent of its final equilibrium value in 0.0007 second. (See appendix.) A similar calculation for the case of O<sub>2</sub> by Roughton in 1932 gave a 90-percent equilibration time of only 0.0003 second, the slightly lower numerical value resulting from the lower estimate of the diffusion coefficient and the smaller half thickness used (Roughton, 1932).

However, the assumption that CO<sub>2</sub> diffuses through the cell membrane as easily as it diffuses through protein solution is probably not justified. The difficulty is to decide what the permeability

of the membrane to CO<sub>2</sub> is. A logical approach would be to measure the diffusion coefficient for the materials comprising the membrane. Because there are lipids in the membrane and CO<sub>2</sub> is generally more soluble in fats than in water (Radford, 1964), we would expect the permeability of the membrane to be greater than that assumed above; namely, that of a water solution. On this basis, CO<sub>2</sub> should equilibrate between the cell and its environment in less than 0.0007 second. However, Blank and Roughton (1960) found 8 years ago that the diffusion coefficient of lipid monolayers was considerably less than expected from measurements on the same molecules in nonoriented solutions, from which I drew the conclusion that it is not safe to assume that we can predict the permeability of a membrane from a knowledge of its chemical composition alone.

A minimal value for the time required for CO<sub>2</sub> to equilibrate between red cells and plasma can be obtained by assuming that the gas passes through the membrane only via paths available to water, that is, dissolving in water in "pores," and that it does not pass through the remainder of the membrane; it does not, for example, move through the lipids on the basis of its solubility in them. The permeability of the red-cell membrane to water (by diffusion, not hydraulic flow) is reported to be 0.0053 cm/sec (Paganelli and Solomon, 1957), which for a membrane of 100-Å thickness (Stein, 1967) corresponds to an effective diffusion coefficient  $d$  in the membrane of  $5.3 \times 10^{-9}$  cm<sup>2</sup>/sec. The self-diffusion coefficient  $d_{H_2O}$  in liquid water at 37 C is  $2.4 \times 10^{-5}$  cm<sup>2</sup>/sec (Dick, 1966; Wang, Anfinsen, and Polestra,

1954). Thus the red-cell membrane acts as though only  $5.3 \times 10^{-9} / 2.4 \times 10^{-5}$  or  $2.2 \times 10^{-4}$  of the surface were available for the diffusion of water. One could as well consider that the  $d_{\text{H}_2\text{O}}$  in the membrane was decreased by this factor, and that all the surface was available for exchange, although it is intuitively less likely. The  $d_{\text{CO}_2}$  in the membrane, on the basis of the original assumption, would also be decreased by this factor, becoming  $1.45 \times 10^{-9}$  cm<sup>2</sup>/sec.

The red cell can be approximated as an infinite sheet of watery solution of thickness twice 0.8 micron with a surface membrane layer 100 Å thick (Forster, 1964). If the amount of dissolved CO<sub>2</sub> in the membrane can be neglected, the mathematical solution of the diffusion equation is much simpler. Because the membrane is only one-eighth of the thickness of the cell, this approximation appeared justified. The increase in the average [CO<sub>2</sub>] inside the layer after an initial instantaneous increase in the [CO<sub>2</sub>] on each surface is described by the equation (Carslaw and Jaeger, 1959, eq. 12, p. 122)

$$1 - \frac{[\text{CO}_2]}{[\text{CO}_2]_0} = \sum_{n=1}^{\infty} \frac{2\lambda^2}{\alpha_n^2 [\lambda(\lambda+1) + \alpha_n^2]} e^{-\alpha_n^2 T} \quad (1)$$

where

[CO<sub>2</sub>] is the concentration of dissolved CO<sub>2</sub> at any time  $t$  and [CO<sub>2</sub>]<sub>0</sub> is the concentration of CO<sub>2</sub> at the surfaces of the layer.

$$\lambda = \frac{d_{\text{CO}_2, \text{ membrane}}}{d_{\text{CO}_2, \text{ layer}}} \times \frac{\text{thickness layer}}{\text{thickness membrane}}$$

(identical with  $\lambda$  of Nicolson and Roughton, 1951)

$\alpha_n$ , where  $n=1, 2, \dots$ , are the positive roots of  $\alpha \tan \alpha = \lambda$ .

$$T = \frac{\text{diffusion coefficient in cell interior}}{(\text{membrane thickness})^2} \times t \text{ in seconds}$$

It is very fortunate that graphical solutions for this formidable equation are given in Carslaw and Jaeger. I only give the equation here because, to my knowledge, this type of calculation of red-cell gas exchange has not been reported before. Using the numerical values given above

$$\lambda = \frac{2.2 \times 10^{-4} \times 0.8 \times 10^{-4}}{0.3 \times 10^{-6}} = 0.059$$

The factor 0.3 is the diffusion coefficient of N<sub>2</sub> in 30 percent hemoglobin solution divided by the diffusion coefficient of N<sub>2</sub> in water (Longmuir and Roughton, 1952) and is required because  $2.2 \times 10^{-4}$  is the ratio of the effective diffusion coefficient of water in the red-cell membrane to its value in water, not hemoglobin solution. From Carslaw and Jaeger:

$$\frac{d_{\text{CO}_2, \text{ interior } t_{90\%}}}{(\text{thickness layer})^2} = 40.0$$

and the time for 90 percent equilibration is 0.038 second, a relatively slow process. The value of the permeability of the membrane is so low that the interior can be considered completely mixed. In fact, this permits one to make a much simpler calculation based on this assumption whence the 90 percent time also becomes 0.038 second, affording a good check on the more complicated computations above.

Thus it turns out that the calculation of the rate of CO<sub>2</sub> exchange between the red cell and the plasma is critically dependent on the value used for the permeability of the membrane and that experimental measurements of the speed of CO<sub>2</sub> exchange itself may be required even to provide a rough answer to the question as to whether this process can be rate limiting in blood-gas exchange. Unfortunately, this appears too difficult a problem for our present rapid-reaction apparatus. The difficulty can be seen in the lower

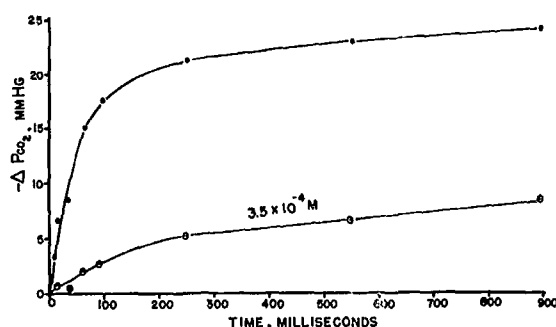


FIGURE 1.—The decrease in  $P_{\text{CO}_2}$  with time after mixing a 1/10 suspension of normal human blood at a  $P_{\text{CO}_2}$  of nearly zero mm Hg with a solution of 30 mM NaHCO<sub>3</sub> at 37 C in a continuous-flow rapid-reaction apparatus using a  $P_{\text{CO}_2}$  electrode. In the lower curve, 0.35 mM acetazolamide has been added to inhibit the carbon anhydrase. (Adapted from Constantine et al., 1965.)



graph in figure 1 which depicts the change in  $P_{\text{CO}_2}$  following the mixture of a 1:10 suspension of normal blood in saline-bicarbonate buffer at a  $P_{\text{CO}_2}$  near zero, with saline-bicarbonate buffer at a  $P_{\text{CO}_2}$  of 70 mm Hg. Both solutions contained  $3.5 \times 10^{-4} M$  acetazolamide. The ordinate is the difference between the measured  $P_{\text{CO}_2}$  at a given time and the initial  $P_{\text{CO}_2}$  in the mixture before any chemical reactions took place. The latter is calculated assuming complete mixing of the two solutions and equilibration of the gas between the suspending fluid and the cell contents. If CO<sub>2</sub> did not enter the cell at all, the actual initial  $P_{\text{CO}_2}$  would be higher than the calculated value because the volume of dilution was less than assumed. The volume of red cells was only 1:40 of the volume of the suspension, so that if no CO<sub>2</sub> entered the cells, the initial  $P_{\text{CO}_2}$  would be but one-fortieth higher than expected; this is  $1/40 \times 35$ , which is approximately 1 mm Hg. Inspection of figure 1 demonstrates that we cannot measure the intercept to this level of accuracy, even if gas equilibration had not proceeded at all by the time of the first  $P_{\text{CO}_2}$  measurement (0.007 second).

The sensitivity of the experiment could be increased by increasing the concentration of cells in the mixture, but it seemed unlikely that we would be able to detect an average  $P_{\text{CO}_2}$  difference between the interior of the cells and the suspension of less than that corresponding to half equilibration, and this not until 0.005 second. Therefore we have not pursued this approach further.

The difficulty in measuring the gas permeability of the red-cell membrane arises in designing an experiment in which the rate of cell-gas uptake is largely limited by the gradient across the membrane as contrasted with gradients within the cell. As seen above, if the diffusion resistance of the membrane is the same as that of the cell contents, CO<sub>2</sub> equilibration between the cell and its ambient will be complete in 0.001 second or less. The speed of this process is limited by gas diffusion within the red cell. If the diffusion resistance of the cell membrane is not several magnitudes less than that of water, it is necessary to provide a sink for the gas inside the cell to produce a relative reduction in the intracellular diffusion gradients. Hemoglobin is present in high

concentration in the red cell and does just this for O<sub>2</sub> and CO, but even so the bound gas cannot diffuse readily within the cell because of the large molecular weight and small diffusing coefficient of the protein. The measured rates of O<sub>2</sub> uptake by red cells are generally slower than are calculated assuming the erythrocyte can be approximated by an infinite layer of hemoglobin solution of about twice 0.8 micron (Forster, 1964). This discrepancy can be explained if we assume the diffusion resistance through the membrane partially limits the rate of the process. On this basis, the apparent average value of the ratio,  $d_{\text{membrane}}/d_{\text{interior}}$ , for O<sub>2</sub> in human cells at 37 C is 0.0175. Choosing the value of 100 Å again for the membrane thickness,  $\lambda$  in equation (1) is 1.5, and the time for 90 percent equilibration is 0.0024 second. Once again, however, we are not sure of the relation between the permeability of the membrane for O<sub>2</sub> and CO<sub>2</sub>. If these gases move through the watery parts of the membrane, its permeability will be almost the same for both, and the 90-percent equilibration time for CO<sub>2</sub> would approximate that for O<sub>2</sub> as above. On the other hand, if the gases diffuse across the membrane on the basis of their oil (cottonseed) solubility, then the apparent  $d_{\text{membrane}}/d_{\text{interior}}$  for CO<sub>2</sub> would be  $0.0175 \times 1.6/5 = 0.0035$  because the oil/water partition coefficient<sup>1</sup> of O<sub>2</sub> is 5, while that for CO<sub>2</sub> is only 1.6 (Lawrence, Loomis, Tobias, and Turpin, 1946). The corresponding value for  $\lambda$  would be 0.28, and  $t$  for 90 percent equilibration is 0.010 second, a process that is within reach of our instruments.

It might occur to you that the relative importance of the diffusion resistance of the cell membrane could be exaggerated to make the measurement easier by using a ligand gas with a

<sup>1</sup>The permeability of the red-cell membrane is calculated experimentally from the difference in concentrations in the two phases on either side of the membrane. It is not possible to measure the concentration difference in the membrane itself. The two phases are considered to be well mixed and the resistance to diffusion through the membrane is assumed to be rate limiting. The actual concentration difference within the membrane will be equal to the difference between the concentrations in the two external phases times the individual partition coefficients between the membrane and the respective external liquid phase.

faster chemical reaction with hemoglobin. Unfortunately, the velocity of the chemical reaction of hemoglobin with O<sub>2</sub>, and certainly that with NO (Gibson and Roughton, 1957), approaches the "advancing front" condition (Hill, 1929), and further unlimited increases in the reaction velocity would not eliminate intracellular gas concentration gradients during the exchange process.

It should be possible to calculate the diffusion resistance of the red-cell membrane from measurements of the rate of CO<sub>2</sub> exchange of the cells and from knowledge of the chemical reaction velocity for CO<sub>2</sub> within the cell contents, an analogous approach to that just discussed for O<sub>2</sub>. Constantine, Craw, and Forster (1965) did measure the rate of CO<sub>2</sub> uptake by a human red-cell suspension at 37 C using a continuous-flow rapid-reaction apparatus with a  $P_{\text{CO}_2}$  electrode and found that the initial rate was only one-sixth to one-half that expected from the known concentration of carbonic anhydrase in cell hemolysates (Kernohan, Forrest, and Roughton, 1963) on the assumption that it is the rate of intracellular hydration of CO<sub>2</sub> that limits the overall speed of the process. This discrepancy has several explanations, the most important of which is that diffusion is slowing the rate of exchange. Calculation of the diffusion gradients within the cell indicates that they should not represent more than 24 percent of the total  $P_{\text{CO}_2}$  difference (Constantine et al., 1965). On the other hand, Kernohan and Roughton (1968) have reported that, using a continuous-flow rapid-reaction apparatus to follow thermal changes, they have been able to ascertain that the catalytic action of the enzyme in intact bovine erythrocytes at 25 C is within 30 percent of that expected from the activity in hemolysates. The conclusions of Constantine et al. depend on a series of calculations and several assumptions and were based on cells from a different species, so there are numerous explanations for this disagreement. However, there are residual queries.

Klocke, Rotman, Andersson, and I thought that NH<sub>3</sub> was an ideal gas for the measurement of membrane permeability.<sup>2</sup> The rate of the reac-

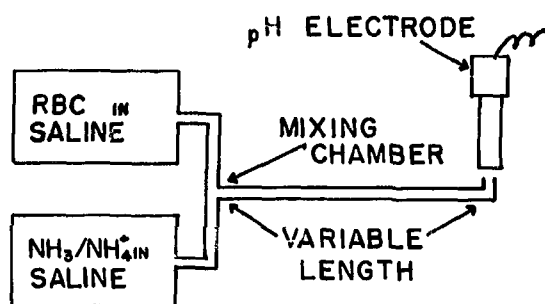


FIGURE 2.—The continuous-flow rapid-reaction apparatus using a pH electrode. The reactant mixtures were forced by motor-driven syringes into the mixing chamber. The observation tube was replaced to vary the reaction time observed. The syringes, mixing chamber, observation tube, and pH cuvette and assembly were maintained at constant temperature in a water bath.

tion of NH<sub>3</sub> with water is of the order of microseconds and can be considered instantaneous in comparison with overall red-cell exchanges (Eigen and DeMaeyer, 1963). The hemoglobin has a large buffer capacity, providing a sink for the OH<sup>-</sup> produced by the reaction. More significant is the fact that the products of the reaction, NH<sub>4</sub><sup>+</sup> and OH<sup>-</sup>, should diffuse approximately as rapidly within the red cell as NH<sub>3</sub> itself. Therefore, the rate of NH<sub>3</sub> exchange of the red cell had a good chance of being limited by the permeability of the membrane. Experiments were carried out by mixing a suspension of washed human erythrocytes at 37 C with a solution of NH<sub>3</sub> in a continuous-flow rapid-reaction apparatus and using a glass pH electrode to follow the [H<sup>+</sup>] changes in the mixture with time.

Figure 2 is the schematic diagram of the rapid-reaction apparatus showing the position of the glass electrode, which had a complete response time of less than 10 seconds, including that of the strip-chart recorder. The pH was measured to an accuracy of better than 0.01 pH unit. The time elapsed between mixing and the measurement of pH was varied by inserting glass observation tubes of different lengths.

In figure 3 is shown pH as a function of time in a typical reaction. There was no buffer present in the suspending fluid except  $\text{H}^+ + \text{NH}_3 \rightleftharpoons \text{NH}_4^+$ , the cells having been washed three times to remove the plasma protein. Therefore, the extracellular

<sup>2</sup> This work was supported in part by the Life Insurance Medical Research Fund.

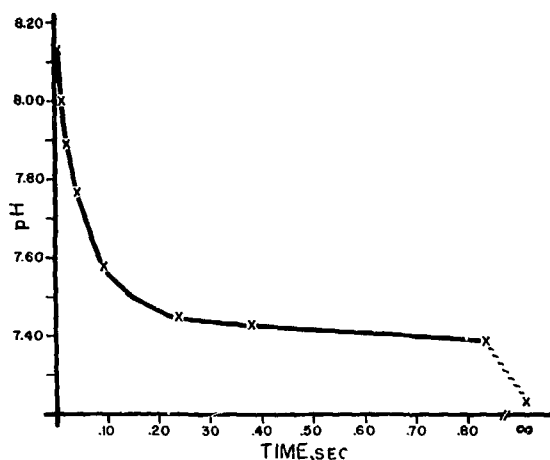


FIGURE 3.—The fall in pH with time after mixing a 2 mM solution of NH<sub>3</sub> in isotonic saline and a suspension of normal human red cells with a hematocrit of 21 percent.

fluid [NH<sub>3</sub>] can be calculated from the pH, knowing the original total [NH<sub>3</sub>+NH<sub>4</sub><sup>+</sup>] and the pK; the [NH<sub>3</sub>] inside the cell can also be calculated. The results for this type of calculation for the experiment shown in figure 3 are given in figure 4. The rate of movement of NH<sub>3</sub> into the cell can be calculated from the change in [NH<sub>3</sub>], and the permeability can be calculated by dividing by the gradient. A value of 0.0119 cm/sec was obtained. We believe that there are negligible intracellular [NH<sub>3</sub>] gradients during these experiments by

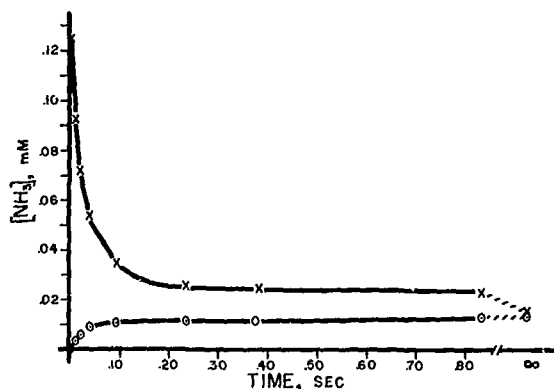


FIGURE 4.—The calculated [NH<sub>3</sub>] in the suspending fluid (X) and the interior of the red cells (O) from the experiment of figure 3. The point farthest to the right is the final equilibrium value.

reason of calculations of the speed of diffusion of NH<sub>3</sub> and NH<sub>4</sub><sup>+</sup> within the cell. Therefore, this permeability, given in terms of cm/sec (flux of moles/sec per concentration difference in moles/cm<sup>3</sup> per cm<sup>2</sup> of surface area), approximates that of the membrane alone. Although NH<sub>3</sub> and CO<sub>2</sub> are both uncharged small molecules (gases), we cannot calculate the ratio of their permeabilities in the membrane without knowledge of its composition, at least of that portion of the membrane through which these gases permeate. If CO<sub>2</sub> and NH<sub>3</sub> pass through the red-cell membrane primarily on the basis of their water solubility in it ("pore" mechanism), then their membrane permeabilities should be in the ratio of (mol wt NH<sub>3</sub>/mol wt CO<sub>2</sub>)<sup>1/2</sup>, which equals  $\sqrt{17/44}=0.63$ .  $P_{\text{memb, CO}_2}$  would equal  $0.63 \times 0.0119$  cm/sec, which approximates the value for water, and thus results in a calculated 90 percent equilibration time of the order of 0.03 second, as computed above.

If, on the other hand, CO<sub>2</sub> and NH<sub>3</sub> move through the red-cell membrane by dissolving physically in the membrane lipids, as is accepted classically (Pappenheimer, 1953), then the ratio of the membrane permeabilities of CO<sub>2</sub> and NH<sub>3</sub> should be equal to the ratio of their oil to water partition coefficients<sup>3</sup> in the membrane times the reciprocal of the square root of their molecular weights, namely

$$\frac{\beta_{\text{CO}_2, \text{lipid}}/\beta_{\text{CO}_2, \text{water}}}{\beta_{\text{NH}_3, \text{lipid}}/\beta_{\text{NH}_3, \text{water}}} \times \frac{[\text{mol wt NH}_3]^{1/2}}{[\text{mol wt CO}_2]} \quad (2)$$

where  $\beta$  is the respective solubility in ml (STPD) per ml fluid at 1-atmosphere pressure. The partition coefficient of NH<sub>3</sub> between olive oil and water has been determined by Klocke and Andersson (personal communication) as 0.02. Therefore, the permeability for CO<sub>2</sub> in the membrane should be equal to  $0.0119 \text{ cm/sec} \times 1.6/0.02 \times 0.63 = 0.58$  cm/sec. Again taking the thickness of the membrane as 100 Å, the diffusion coefficient for CO<sub>2</sub> in the membrane is  $5.8 \times 10^{-7}$  cm<sup>2</sup>/sec. The dimensionless coefficient  $\lambda$  of equation (1) is therefore

$$5.8 \times 10^{-7} \times 0.5 \times 10^{-4} / (6.6 \times 10^{-6} \times 10^{-6}) = 7.1$$

<sup>3</sup> See footnote 1, p. 277.

TABLE I.—Permeability of Human Red Cell Membrane to Ions and Nonelectrolytes at 37 C

| Chemical species      | Permeability, cm/sec × 10 <sup>2</sup> | Type of rapid-reaction apparatus and source  |
|-----------------------|--|--|
| NH <sub>3</sub> ..... | 1.19                                   | Continuous flow: pH electrode (Klocke, Forster, Rotman, and Andersson; unpublished). |
| CO <sub>2</sub> ..... | .75                                    | Calculated from NH <sub>3</sub> measurements; from pore theory.                      |
| H <sub>2</sub> O..... | <sup>a</sup> 1.67                      | Calculated from NH <sub>3</sub> measurements; from oil/water partition.              |
|                       | <sup>b</sup> .53                       | Stopped-flow: light scattering (Blum and Forster; unpublished).                      |
| Formic acid.....      | .12                                    | Continuous-flow: Millipore filtration (Paganelli and Solomon, 1957).                 |
| OH <sup>-</sup> ..... | .094                                   | Continuous flow: pH electrode (Klocke, Forster, Rotman, and Andersson; unpublished). |
| Lactic acid.....      | .056                                   | Stirred vessel: pH electrode (Klocke, Forster, Rotman, and Andersson; unpublished).  |
| Cl <sup>-</sup> ..... | .026                                   | Continuous-flow: pH electrode (Klocke, Forster, Rotman, and Andersson; unpublished). |
|                       |  | Continuous-flow: Millipore filtration (Tosteson, 1959).                              |

<sup>a</sup> Osmotic.<sup>b</sup> Diffusion.

Referring to the graphical solutions in Carslaw and Jaeger (1959), we find that  $Dt/L^2 = 1.26$  for 90 percent completion. Inserting the values for the diffusion coefficient within the red cell and for the thickness of the red cell of  $6.6 \times 10^{-6}$  and  $0.8 \times 10^{-4}$ , respectively, the time required is 0.001 second. This limit for the equilibration process is so rapid that it would not be rate determining in any of the exchanges we have measured in the red cell and should not affect the speed of CO<sub>2</sub> exchanges.

All these different approaches to the question of the permeability of the red-cell membrane to CO<sub>2</sub>, which is the basic fact in determining the rate of exchange of the whole erythrocyte to CO<sub>2</sub>, founder on the problem of the mechanism by which it diffuses through the membrane, because without this knowledge we cannot relate the permeability of CO<sub>2</sub> to that of any other gases. However, it seems most likely that CO<sub>2</sub> diffusion through the red-cell membrane does not limit physiological processes.

I have discussed the movements of CO<sub>2</sub> independent of its chemical reactions with buffers, but in actual fact we cannot separate the exchanges of CO<sub>2</sub> from the exchanges and reactions of bicarbonate and carbamates. Chemical analyses of blood are made under conditions of chemical equilibrium, but the CO<sub>2</sub> may not have been in chemical equilibrium with buffers when the blood

was in the tissue. Any exchange of CO<sub>2</sub> between the red cell and its surroundings disturbs, to some finite extent, the equilibrium of all permeable ions and ionized impermeable molecules, leading to the movement of many ionic species. Table I presents a summary of the permeability of the red-cell membrane to some pertinent molecules and ions. CO<sub>2</sub> appears to be more permeable than most, and we would intuitively expect that CO<sub>2</sub> equilibration would take place before the ions in general are equilibrated.

The rate of displacement of O<sub>2</sub> from HbO<sub>2</sub> in the red cell by an increase in [H<sup>+</sup>] can also give some index of the rate of CO<sub>2</sub> movement across the cell membrane. Figure 5 shows the change with time in [HbO<sub>2</sub>] of human red cells exposed to a sudden increase in external  $P_{CO_2}$  and a decrease in external pH. This decrement in [HbO<sub>2</sub>] was followed in the reacting mixture of a continuous-flow rapid-reaction apparatus using a  $P_{O_2}$  electrode. The overall process presumably consists of the diffusion of CO<sub>2</sub> into the red cell; its hydration there, catalyzed by carbonic anhydrase; the reaction of the H<sup>+</sup> produced with hemoglobin, leading to an increase in the dissociation velocity constant; and the dissociation of O<sub>2</sub> from the pigment and its diffusion into the suspending fluid. The reaction of H<sup>+</sup> with hemoglobin (step 3) is, presumably, extremely fast and can be considered instantaneous in com-

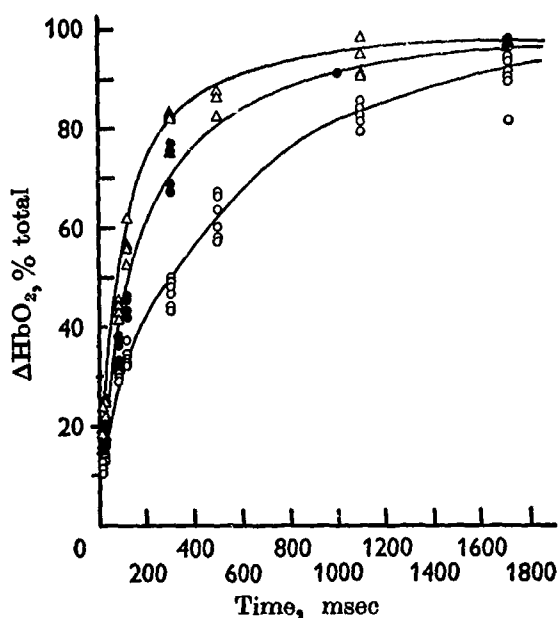


FIGURE 5.—The time course of the Bohr shift of human red cells. The  $P_{CO_2}$  was suddenly increased from 8 to about 70 mm Hg. The external pH was decreased from an average of 7.95 to an average of 7.28. The temperature was maintained at three different levels: 42.5 C ( $\Delta$ ); 31 C ( $\bullet$ ); and 23 C ( $\circ$ ).

The ordinate is the change in  $[O_2Hb]$  as a fraction of the total change that occurred at chemical equilibrium. In these experiments the change is a decrement. (Reproduced from Forster and Steen (1968) with permission of the Journal of Physiology.)

parison with the overall process.<sup>4</sup> Even the diffusion-dissociation reaction of  $O_2$  within the cell (step 4) has a half time of the order of 0.030 second. If we assume that steps 3 and 4 are instantaneous and that the rate of hydration of  $CO_2$ , step 2, can be calculated from knowledge of the carbonic anhydrase activity of cell hemolysates, the data of Craw, Constantine, Morello, and Forster (1963) give a rate only one-tenth that expected from the measurements of Kernohan et al. (1963) on bovine hemolysates. In other words, these measurements on the rate of the Bohr shift in human red cells give much the same answer concerning the possible diffusion resist-

<sup>4</sup> R. E. Forster, M. Kokowsky, R. A. Klocke, and H. Rotman (unpublished observations) have found that neutralization of  $H^+$  by hemoglobin solutions is completed before the continuous-flow rapid-reaction apparatus can measure it; less than 0.002 second.

ance of the membrane to  $CO_2$  as do the more direct measurements of the rate of  $CO_2$  uptake by red cells. Although it appears most likely that the diffusion resistance of  $CO_2$  through the membrane is not rate limiting, resolution of these disagreements should be sought in further experiments.

*In vivo*, the rate of  $CO_2$  exchange between the red cell and plasma may not be limited by the movement of  $CO_2$  itself, nor even its rate of intercellular hydration. The red cell is relatively impermeable to cations, so  $H^+$  moves into it minimally. The permeability to anions and simple acids is much less than that to  $CO_2$  and  $NH_3$ . The "catalyzed diffusion" of Jacobs and Stewart (1942) must often be important in the transfer of  $H^+$  across the cell membrane. This mechanism consists of uncatalyzed dehydration of extracellular  $H_2CO_3$ , the intracellular diffusion of the resulting  $CO_2$ , and the hydration of the  $CO_2$  with the formation of  $H^+$  and  $HCO_3^-$ , the latter

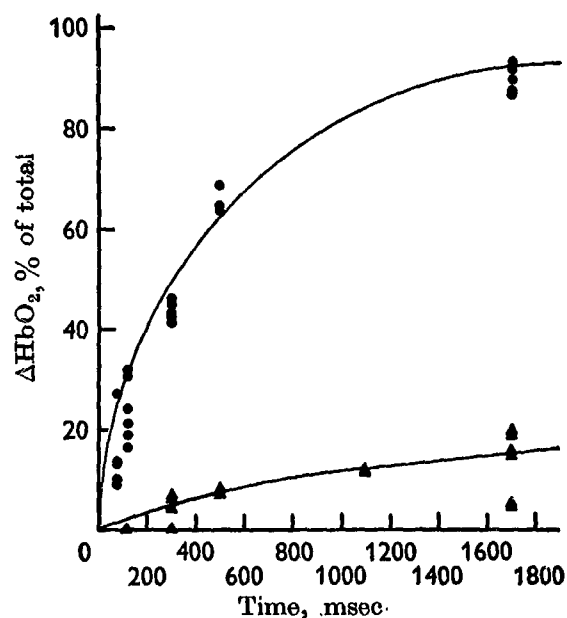


FIGURE 6.—Time course of the Bohr shift produced by adding lactic acid to a suspension of normal human red cells while keeping the  $P_{CO_2}$  constant at 8 mm Hg. The extracellular pH changed on mixing from 8.12 to 5.2. In the lower curve 1 mM acetazolamide was added to inhibit the carbonic anhydrase. The ordinate was calculated in the same way as in figure 5. (Reproduced from Forster and Steen (1968) with permission of the Journal of Physiology.)

then exchanging across the membrane for extracellular Cl<sup>-</sup>. The net result is the movement of H<sup>+</sup> into the cell. In the experiment described in figure 5, a gradient for dissolved CO<sub>2</sub> from the external fluid to the interior of the cell exists from the start, and the overall process is largely completed by the diffusion of existing CO<sub>2</sub>. The process is rapid, with a half time of 0.120 second (see Piiper, 1964). Figure 6, on the other hand, shows the results of experiments in which lactic acid was added to the suspending medium in the rapid-reaction apparatus, but in which the  $P_{CO_2}$  was initially unchanged; the  $P_{CO_2}$  of the cell suspension and the buffer with which it was mixed was 8 mm Hg. The half time of this process was 0.31 second. A large part of the transfer of H<sup>+</sup> into the cell had to take place via the dehydration of external H<sub>2</sub>CO<sub>3</sub>. At the start of the reaction, [H<sub>2</sub>CO<sub>3</sub>] in the mixed extracellular fluid was equivalent to a partial pressure of dissolved CO<sub>2</sub> of 5000 mm Hg while that inside the cell was equivalent to only 8 mm Hg. Thus, although no  $P_{CO_2}$  gradient originally existed, CO<sub>2</sub> was formed in the extracellular fluid and then diffused into the cell. This dehydration was uncatalyzed (at least relatively) and its rate should have been determined by the equation (Roughton, 1964)

$$\frac{d[CO_2]}{dt} = -0.12[CO_2] + \frac{89[H^+][HCO_3^-]}{3.5 \times 10^{-4}} \quad (3)$$

[CO<sub>2</sub>], [H<sup>+</sup>], and [HCO<sub>3</sub><sup>-</sup>] are the molar concentrations of dissolved CO<sub>2</sub>, hydrogen ion, and bicarbonate ion, respectively;  $t$  is the time in seconds. The uncatalyzed rate constant is 0.12 for the hydration of CO<sub>2</sub> and 89 for the dehydration reaction, both at 37 C.  $3.5 \times 10^{-4}$  is the acid dissociation constant for H<sub>2</sub>CO<sub>3</sub>. If we assume that [H<sup>+</sup>] is constant for the duration of the reaction, this equation can be solved, giving an exponential relationship in which the exponent is

$$0.12 + 89[H^+]/3.5 \times 10^{-4} \quad (4)$$

This is a convenient form in which to calculate the expected rate of the reaction. Although [H<sup>+</sup>] is only rarely constant, the limits still can be calculated with expression (4).

To return to the experiment shown in figure 6, at the start of the process the extracellular pH was 5.2; at the final equilibrium it was 6.8. The

original cell suspension at pH 8.12 had been mixed with a buffer solution at pH 2.8. If the whole reaction takes place at pH 5.2, expression (4) gives

$$0.12 + 89 \times 6.3 \times 10^{-6} / 3.5 \times 10^{-4} = 1.71$$

The half time, assuming an exponential process, is  $0.693/1.71 = 0.4$  second. It should be remarked that the calculated rate is very dependent on the [H<sup>+</sup>] of the external fluid, and this proton concentration will fall during the exchanges, slowing the process. If the reaction took place at the final pH, 6.8, the half time would be calculated as 4.3 seconds. Rigorously, we can only say that the average half time should lie between these two extremes. However, these Bohr shifts are not simple exponentials and the half time is an improper, although convenient and simple, index of its speed. In figure 6, the process slows down much more rapidly than a true exponential, and the measured half time should correspond more closely to the initial than to the later rates. We do not consider the discrepancy between the initial calculated half time of 0.4 second and the measured half time of 0.31 second unreasonable, particularly in view of the many assumptions involved. I conclude that, in these circumstances, the Bohr shift appears limited by extracellular H<sub>2</sub>CO<sub>3</sub> dehydration.

This phenomenon is not a test-tube curiosity, because the same conditions should be obtained in the venous blood of exercising muscle, to which has been added lactic acid (Harris, Bateman, and Gloster, 1962). Because lactic acid cannot permeate the cell membrane readily (table I), the "catalyzed diffusion" mechanism must apply. During exercise, venous pH can be as acid as 7 (Mitchell, Sproule, and Chapman, 1958), and because these measurements were not made instantaneously and there can be a significant arteriovenous lactate difference (Harris et al., 1962), the plasma pH actually in the vein during exercise may be still lower. Because the final equilibrated pH of the blood is greater than 7, the time required for completion of the "catalyzed diffusion" could be of the order of seconds, rather than of tenths of seconds.

When acetazolamide is added, the process is slowed (fig. 6, lower curve) because the hydration of CO<sub>2</sub> inside the cell becomes rate limiting. This

would not, however, be expected in whole blood. In the illustrative experiment the cells were only 1/200th of the total volume, while in normal blood they would represent one-half. Therefore, we can expect the uncatalyzed hydration of CO<sub>2</sub> within the cells in whole blood to be about 100 times as great per unit volume of suspension, and not rate limiting.

In conclusion, although there are no reported direct measurements of the permeability of the red cell to CO<sub>2</sub>, the diffusion of the dissolved gas is presumably very rapid and should not become rate limiting in the red cell. The *in vivo* rate of CO<sub>2</sub> equilibration between the red cell and its ambient medium, plus associated readjustments such as the Bohr shift, depend on chemical reactions and the exchanges of molecules and ions in addition to CO<sub>2</sub>, and may well take as long as several seconds for completion under extreme circumstances.

#### APPENDIX

The average concentration of CO<sub>2</sub>,  $\bar{x}_t$ , within a semi-infinite layer of water of thickness  $2b$  varies with time according to the following equation

$$\bar{x}_t/x_0 = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp \left[ - \left( \frac{(2N+1)\pi}{2b} \right)^2 dt \right]$$

where  $x_0$  is the concentration of CO<sub>2</sub> at the surfaces of the layer,  $d$  is the diffusion coefficient of CO<sub>2</sub> in the layer in cm<sup>2</sup>/sec, and  $t$  is the time in seconds (Jost, 1952, p. 37, eq. 1.139).

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## DISCUSSION

**SIESJO:** I wonder if Dr. Forster has done any calculations himself on the following or if he has seen any other calculations? I remember in Kety's (1951) review of inert gases in tissue, he quoted Copperman's calculations which used the intercapillary distances in tissues and the solubility. From these it was possible to calculate that 95-percent equilibration between the capillary blood and a tissue in the brain took around one or two seconds. Is that a reasonable figure?

**FORSTER:** I went over these calculations again for a book Dr. Papper edited (Forster, 1963). For inert gases this is purely a matter of what you choose for intercapillary distances. You can write many papers because all you have to do is choose a different distance and get a different answer. With some of the moderately extreme values of intercapillary distance that people have obtained, such as in fat and even in brain, there might be an end-capillary-tissue gradient. Kety tested the  $N_2O$  method by taking samples of brain and comparing simultaneous venous and brain  $N_2O$  concentrations. They did not check, though; I do not know that anyone else has ever done it. This does not invalidate the blood-flow method, because for this you simply want the integral of all the  $N_2O$  that has gone in. Kety's data certainly raise a doubt as to whether there is equilibration of inert gases. His alternative explanation, which I think is a more likely one, is uneven blood-flow tissue volume relationships. I do not know whether this answers your question. Pappenheimer has a very nice graph on the capillary diffusion exchange (Landis and Pappenheimer, 1963). You have a logarithmic relationship, and if you move it just a little you can get almost anything you want.

My guess is that for the brain the difference between 20- and 30-micron intercapillary distance could make a tremendous difference in equilibration, so it becomes critical.

**SIESJO:** It is critical for the intercapillary distances.

**FORSTER:** Kety's value is down around 18-micron radius (cortex) as I remember. We are talking about a rough, average, effective value. Normally, we would calculate that effective value by determining whether there was a gradient and then work backward.

**SIESJO:** We calculated a gradient of about 0.5 mm Hg between the mean tissue and the mean capillary  $P_{CO_2}$  using Krogh's model which, of course, is an oversimplification.

**FORSTER:** I think that we have gone about as far as possible with these calculations.

**CAMPBELL:** I would like to make some further comments on alveolar-arterial  $P_{CO_2}$  difference during rebreathing in the light of the subsequent presentations. The balance of the evidence suggests to my prejudice that incompleteness of the  $CO_2$  reaction in the blood in the pulmonary capillaries is the most likely explanation.

I think we should note that whatever the explanation under these special conditions, the  $P_{CO_2}$  of blood drawn from a systemic artery and subsequently analyzed can apparently be several mm Hg different from alveolar  $P_{CO_2}$ . If this observation is confirmed and if the mechanism is operative under ordinary conditions, it will have uncomfortable implications for much classical physiology which takes arterial  $P_{CO_2}$  = alveolar  $P_{CO_2}$  in studies of alveolar composition, dead space, some diffusing capacities, and so forth.

**DAVIES:** As I understood Dr. Forster's last calculations, it would require several seconds for equilibration of blood coming from an active muscle that was producing much lactic acid, because the  $CO_2$  dehydration would occur in the plasma where there was no carbonic anhydrase. If this is so, then it should not be too difficult to observe a transient in the pH of blood coming from a working muscle. Perhaps the following experiment has already been done—you put a glass electrode in the venous outflow and measure blood pH as it goes by. You should see a change in pH as measured by that system, if you vary the time of transit from the muscle to the electrode.

**FORSTER:** I do not believe anybody has done this. I applaud this as one thing to do, but it has been done in the eel swim bladder. Steen has looked at the venous blood coming back from the rete. The pH changes as it readjusts. There are very large amounts of lactic acid produced in the rete.

**DAVIES:** I have not seen this work, unfortunately. Where is it published?

**FORSTER:** This work is by Berg and Steen (1968). The lactic acid that is produced in the gas gland flows out in the venous blood through the rete and diffuses across into the incoming arterial blood, raising its  $P_{O_2}$  by means of the Root shift. Steen realized there was a logical problem because for the countercurrent exchanger to function,  $O_2$  should move from the venous to arterial blood; this means the venous  $P_{O_2}$  must be higher, and this leads to a possible paradox in net exchange of the organ if the venous blood actually had a higher  $P_{O_2}$  than the arterial blood. However, Berg and Steen measured the outflowing venous blood  $P_{O_2}$  at different distances (times) from the rete and found the  $P_{O_2}$  fell. Eel red cells are more impermeable to water (Blum and Forster). Therefore, by analogy we would expect periods of at least tenths of a second required for chemical readjustments in venous blood from an exercising muscle.

**DAVIES:** Well, it would be very nice for someone to measure it. I always tell students that all the measurements you get using the Henderson-Hasselbach equation cannot be accurate by the very fact that the  $CO_2$  is coming into the blood in the tissue and out of the blood in the lungs, so it is not an equilibrium system and the value you get assuming equilibrium cannot be accurate.

The problem is how far away from the resting equilibrium value is the real value in the tissue. I acknowledge that in a working muscle there is a lot of lactic acid being formed and somehow this seems nicer to me than this problem in the rete of an eel. Your explanation did not make direct sense to me, but that may be because I did not understand it.

**FORSTER:** It works very well for the eel though.

**DAVIES:** It should be possible to measure it rather simply and directly.

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