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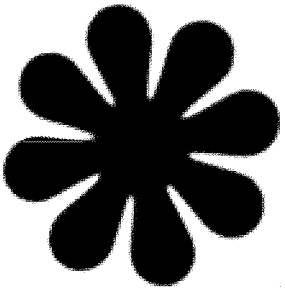

DEVELOPMENTS IN CROP SCIENCE (6)

TRACE ELEMENTS IN PLANTS

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"The physiological importance of mineral elements is all-embracing, and only after having learned more about the role they play was it possible in experimental studies to come closer to the most mysterious areas of life science."

(transl. from S.P. Kostychev, 1937, p. 247)

PREFACE

Almost 30 years have passed since the appearance of a monograph by the present author "The Role of Trace Elements in Plant Life and Agriculture" (Shkolnik, 1950), almost 25 years since "Biochemie der Spurenelemente" (Scharrer, 1955) and almost 20 years since the publication of "Trace Elements in Plants" (Stiles, 1961). Over this period, further insight has been gained into the physiological role of trace elements, significantly outdating these publications. Major achievements both in the study of metalloenzymes and in molecular biology, revealing the detailed mechanism of the transfer of genetic information and energy, the control of metabolism, and the structure and function of mitochondria, chloroplasts, ribosomes and cellular membranes, have shown that trace elements are omnipresent and are important constituents of living systems at all levels.

The study of metalloenzymes had already advanced sufficiently for Gurd and Wilcox (1956) to state that the study of metalloenzymes had taken a central position in the physiology and biochemistry of plants and animals.

A major discovery, announced in the mid-60s in the context of nitrogen fixation, revealed the role of molybdenum and iron in this process. These two trace elements have been shown to be components of the key enzyme in nitrogen fixation (nitrogenase). Understanding the biochemical mechanism of nitrogen fixation is also important for the control of nitrogen fixation and protein production in agriculture (Hardy et al., 1971a,b).

Of particular importance are investigations of the role of trace elements in physiological processes, through the study of the molecular mechanisms of these processes. In recent years a number of new aspects of the role of trace elements in the structure and the function of nucleic acids have been found.

The chief discoveries are those concerning the role of trace elements such as manganese, copper, iron and chlorine in photosynthesis - discoveries which are of great assistance in the study of the mechanisms of this complicated process.

Significant advances in the field of plant physiology have revealed the involvement of trace elements in the operation of growth regulators.

A specific feature of trace element research is also the expansion of investigation in all branches of botany. However, the

evidence available from the various disciplines has not yet been collected for review and analysis to make the interested reader aware of the extent to which trace element science has changed over recent years.

All these considerations have necessitated the writing of a new volume to summarize the information available on the principal trends in trace element research. The author has ventured to prepare a monograph which differs from those published earlier, in that the reader finds not only a critical analysis of various aspects of the physiological role of trace elements, but also a major emphasis on the botanical aspects of trace elements, as seen in botanical geography, taxonomy, phytocenology, geochemical ecology, morphology, anatomy, embryology and genetics.

Much attention has been given by the author to the evolutionary aspects of trace element metabolism, their role in the evolution of enzymes, photosynthesis and plants in the biosphere.

As far as the author is aware, this book makes a large amount of investigation performed by Russian and Soviet workers available for the first time.

The present English version of the Russian edition of "Trace Elements in the Life of Plants" (Shkolnik, 1974a) differs from the original in that much new material has been added based on the most important works on trace elements that have appeared in the period 1973-1981, and in that several chapters of the third part of the book have been altered. It contains the original chapters: Geochemical Ecology, Trace Elements and Genetics, and Trace Elements and the Adaptation of Plants. A new chapter has been added, entitled: The Role of Trace Elements in the Evolution of Plant Metabolism in the Biosphere.

It is apparent that the investigation of the physiological role of trace elements is not only of theoretical importance, but may also provide a basis for the development of a rational system of plant nutrition. Various aspects of the problems dealt with in the physiology of trace elements bear on practical issues in agriculture.

The author wishes to express his gratitude to Drs. V.M. Ponyatovskaya, T.I. Igoshina, without whom neither the Russian, nor the present English version of this book could have been compiled. While acknowledging the assistance of these and many other scientists, the author would wish to take full responsibility both for the material presented and for the views expressed.

PART I. GENERAL ASPECTS

Chapter 1

THE HISTORY OF THE STUDY OF TRACE ELEMENT REQUIREMENTS IN PLANTS

The science of trace elements, which has radically changed our ideas about the nutrition of living organisms, has a rather complex and interesting history of development. Pioneering investigations on trace elements were performed by a French scientist Raulin (1869, 1870), who was the first to discover the unexpectedly pronounced effect of supplying minor concentrations of zinc salts to a nutrient medium supporting a growth of Aspergillus niger. Still earlier, he had succeeded in detecting a positive effect of manganese upon the growth of slime molds. Raulin possessed an exceptional gift of insight: he supposed that zinc and other elements are not only useful growth stimulators of a non-essential nature, but, on the contrary, that they are indispensible for plants. It was to him that the following prophetic words were attributed: "To my mind, it is still unproven that nitrogen, phosphoric acid, potassium and lime are the only ingredients constituting a complete and adequate fertilizer" (Raulin, 1869, p. 634).

The problem of trace elements is a part of the more general problem of the mineral nutrition of plants, the recognition of which has heralded the beginning of the application of science to agriculture. The discovery made by Raulin, as pointed out by Arnon (1958), coincided with a wave of enthusiasm that was spreading in the middle of the XIX century among those working in agriculture. This wave had been initiated by the valuable investigations of Sosura, Bussenko, Looz, Gilbert and Liebig dealing with plant nutrition. These works served to provide the basis for a real revolution in agriculture, the significance of which has been showed by Arnon referring to the following statistical information: from the times of the Roman Empire until 1804, the year of publication of the classical work by Sosura on plant nutrition, an average wheat crop in Western Europe ranged from 4.0 to 6.7 centners per hectare, while a hundred years later the crop yield of these cultures was already three times larger. Such a significant increase in crop yield could only be obtained on the basis of the introduction into agriculture of new knowledge about plant nutrition.

The experiments of Raulin pointed to the existence of a new group of indispensible mineral elements which formerly had remained unnoticed; the small quantities of the essential elements which were found necessary had usually been supplied to plants together with the unpurified salts of nutrient media. However, it took almost 50 years progress from the formulation of this concept to the development of sophisticated methods for the purification of nutrient media in order to exclude even traces of the elements studied. With the development of the latter methods it became possible to demonstrate absolute requirements by plants for a whole group of trace elements. That was a difficult and uncertain phase that led to findings and fallacies, disenchantments and discoveries. This period we designate the first, initial phase of the study of the necessity of trace elements in plants.

Following the works of Raulin there appeared in 1872 a publication by K.A. Timiryazev, in which the significance of zinc in the development of higher plants was demonstrated for the first time. K.A. Timiryazev discovered the effects of zinc on the growth and development of maize and showed that the transformation of phylloxanthin into chlorophyllin@ proceeded more rapidly in the presence of zinc. He gave a description of his experiments, which had shown that chlorosis in plants can be cured by adding zinc.

During the following 25 years, after the discoveries of Raulin and Timiryazev, there were no further publications concerning the effects of trace elements on plants. However, between 1860 and 1890 dissertations were submitted to the Medico-Surgical Academy in St.Petersburg, which dealt with the effects of copper, iron, manganese, rubidium, caesium and nickel upon the functioning of certain organs and physiological processes in experimental animals and man (Zaikovsky, 1863; Zalessky, 1886). In 1895 there appeared a paper by Baumann on the role of iodine in animal nutrition. The publication of this paper almost coincided with the interesting investigations carried out by the prominent French biochemist Bertrand (1897a) concerning the essential nature of manganese for plants. All these publications made the general biological significance of trace elements fully evident.

Bertrand conducted extensive investigations in which he succeeded in elucidating the important functions of manganese - including its participation in the oxidative processes.

@ Terminology used by K.A. Timiryazev

Gabriel Bertrand

Bertrand drew conclusions about the specific nature of the manganese influence upon oxidases and called this element a co-enzyme of oxidases (Bertrand, 1897a); he also indicated that laccase was a manganese-containing enzyme (Bertrand, 1897b). The credit due to Bertrand is certainly very great since he was the first to suggest that trace elements may be constituent parts of enzymes. This has turned out to be a remarkable piece of scientific foresight of major significance, as was recognized later on when the studies of biochemists and enzymologists showed that many of the metallic trace elements are actually found either to be constituent parts of a large number of enzymes, or to serve as activators of enzymes. The science of metalloenzymes, which emerged from these findings, has played a major role in the elucidation of the physiological significance of trace elements. And despite the fact established at a later date that laccase was not a manganese-containing, but a copper-containing enzyme (Keilin and Mann, 1939), it has turned out, as had already been repeatedly shown earlier, that an erroneous hypothesis could still play a positive role. The interest it had aroused served to stimulate intensive investigation in this field, which eventually brought scientists to the realization of the truth as presently understood.

In 1905 Bertrand found that manganese deficiency resulted in a deterioration, or even a termination of plant growth and concluded that manganese was indispensible for the life of plants. He reported the results of his field experiments in which the fertilization of oats with manganese considerably increased the crop yield, and suggested that the substances useful for the growth of plants - the stimulating, or as he called them, catalytic fertilizers, should be used in agriculture in small amounts. In his later works Bertrand repeatedly advocated the idea of the application of such fertilizers.

Even before the appearance of Bertrand's work and especially after its publication, studies on the effects of manganese have been widespread in different countries in both laboratory and field experiments alike. Later, other trace elements attracted attention, although on a lesser scale - zinc, arsenic, aluminium, rubidium, boron - in the context of their influence on growth and crop yield in different plants (Loew, 1903, 1913, and others). Results from these investigations were summarized in great detail in the reviews of E.E. Uspensky (1915) and A.A. Khalizev (1934).

Furthermore, the work of E.E. Uspensky was the most significant physiological investigation since the appearance of Bertrand's works referred to above. The author developed the idea of the specific role attributed to manganese in maintaining the oxidation-reduction conditions in the organism. We shall deal with this work of Uspensky in greater below, when discussing the physiological role of this element.

In 1911, Bertrand and Javillier in their joint investigations succeeded in providing firm evidence for the necessity of manganese in the development of Aspergillus niger. By purifying the growth medium for the fungus and by substituting various salts by others displaying higher purification properties ($MgCO_3$ for $MgSO_4$, $FeSO_4$ for $FeNH_4 \cdot (SO_4)_2$), Bertrand and Javillier prepared a medium which contained no more than 0.0006 mg of manganese per 100 ml, and found that the fungus never produced conidia in such a medium. An addition of only one part of manganese per 1 milliard, or even 10 milliard parts of the medium promoted a severalfold increase in the weight of Aspergillus niger and the conidia were normally developed. In order to give an idea of the infinitesimal quantities of the element producing the effect described, it may be noted that the concentrations used are lower than the concentration of hydrogen ions in a solution of pH 7.0.

In 1913 there appeared a paper by F.V. Chirikov, who found that with an addition of manganese chloride to a nutrient medium for the sand culture of wheat, the crop yield was increased 4-fold. One might have expected this author to have drawn a conclusion concerning the necessity of manganese for the nutrition of higher plants; however, he has never stated such a conclusion, but only continued to regard manganese as a "stimulant", "an irritant of that plasm", in conformity with the views shared by other investigators.

At that time such an understanding of the role of trace elements was the prevailing one. In 1914 a monograph by Brenchley "Inorganic Plant Poisons and Stimulants" appeared, in which she summarized findings concerning the effects of manganese, copper, zinc and arsenic on plants. It was stated in this review that the elements mentioned, even when they lead to an acceleration of growth, are not indispensible. Brenchley divided trace elements into two categories: 1) poisons, which when strongly diluted become non-toxic, and 2) poisons, which, if taken in sufficiently low concentrations, produce a slight, but clearly pronounced acceleration of growth.

Such an attitude towards trace elements, in which they are viewed as irritants, has emerged following a trend in plant physiology that originated early in the XX century in Germany, where it was given the name Reizphysiologie (physiology of irritation). K.A. Timiryazev was a vehement opponent of this trend. A strong opposition was also raised within trace element science. Suffice it to mention Bertrand, who regarded manganese as an essential element in the nutrition not only of fungi, but also of the higher plants.

In 1914 there appeared an interesting publication reporting the investigations conducted by Maze. This author, working with water cultures under strictly controlled conditions, frequently introducing manganese, succeeded in providing evidence for the necessity of zinc for the normal growth of maize. In the absence of zinc manifestations of plants' lack of vigour could be noted, and in 37-44 days the plants died. Since the addition of zinc could not save the plants from death, Maze suggested that maize required some other elements and continued his investigations. Having tested for boron, aluminium, iodine and fluorine by supplementing nutrient media with these elements, Maze obtained a perfectly normal growth of maize plants.

Unfortunately, even these interesting studies did not inspire scientists to start investigating in the right direction, and as late as the second decade of this century trace element studies were confined to experiments conducted mostly in soil cultures or under field conditions, which resulted in a lack of any substantial progress in the study of trace elements. In addition, the prevailing views on trace elements as "stimulants", "irritants" and "promoters" had negative consequences on the development of the subject as a whole, and delayed the realization of the essentiality of trace elements for the life of plants. During the 50 years since the discovery by Raulin, who, as early as 1870, considered zinc and manganese (and probably other trace elements also) to be not only stimulants of plant growth, but also indispensible nutrients, albeit in minor quantities, there were no new ideas concerning the necessity of trace elements for plant growth, and scientists made no progress in understanding the physiological role of trace elements.

No less harm was done by these notions to the practical application of trace elements. In considering the action of trace elements to be a physiological irritation, promoting acceleration

of growth and enhancement of crop yields, investigators were looking out for such effects in all cases of the introduction of trace elements into the soil. An absence of the effects observed in some experiments led to disappointments and decisions to give up working on the problem as a whole. This, in fact, resulted in an almost complete termination of research on trace elements during the period from 1915 to 1922.

These facts may serve as an illustration of the situation in science and practice, which take a deplorably heavy toll where there is an absence of imaginative, progressive ideas leading to a slowdown in basic, theoretical investigation.

So, what was the reason for the prolonged interval during which erroneous beliefs about trace elements were held? This can be accounted for partly by the effects of the then prevailing view in physiology of the theory of irritation mentioned above, and partly by the contradictions observable in Nature, which complicated the finding of the correct solutions to the problems faced. It is instructive to study the analysis of separate periods in the history of biology from K.A. Timiryazev, who, in his monograph "An Historical Method in Biology", analyzed the problem of why the revolution made in biology by Darwin had not occurred earlier. K.A.Timiryazev wrote: "All of them - Jucier, de Candolle, Baer, Agassiz and others - either stubbornly ignored or even openly revolted against this apparently irresistable conclusion that could be drawn from their brilliant discoveries. However, once they had taken this stand they produced apparently logical grounds for maintaining it. Let us see where the obstacles lie for recognizing the fact of an historical development of the organic world ... At first glance, it seems there is nothing easier than to admit, as did Goethe in the Padua Botanical Gardens: "all plants have originated from a single plant", all organisms are linked by the uniformity of their origin, by the degree of similarity - or degree of relatedness. Nevertheless, the majority of scientists, including the greatest among them, avoided this conclusion, or even directly repudiated it. The source of this controversy lies, evidently, not only in the manner of thinking of these scientists, but also in the nature of the phenomena themselves. There could be observed a deep contradiction in the structure of the organic world, which accounted for the differences in view between scientists, or which at least gave them a debating point. The majority of scientists, however, following their intuition without inhibi-

tion, openly discussed this contradiction, while others on the contrary, made attempts to ignore it without being able to provide an adequate and satisfactory explanation for the contradiction (Timiryazev, 1939, p.64). K.A. Timiryazev exposes these controversies.

What were these contradictions of Nature which stood in the way of a correct understanding of the significance and functioning of trace elements in the mineral nutrition of plants? If trace elements are essential for plants, then in water culture the plants should show normal development only when these trace elements are present in the nutrient medium. In fact, working with water cultures, it was possible to obtain normal growth of almost all of the plants tested (except for the sugar beet, maize and flax) when only seven of the mineral elements formerly regarded as essential (now considered to be the macronutrients) had been introduced into the nutrient media. That created the impression that only these elements alone were required for plants to grow. Only later was it established that the normal growth of most plants in water culture without the addition of trace elements could be accounted for by the impurities of nutrient media and in water.

The second difficulty was seen in the practice of the fertilization of field crops. Above were mentioned some observations on the abnormal growth of certain plants in water culture, even when the full complement of trace elements had been introduced into the nutrient medium. Such observations could lead one to suggest the possibility of making use of trace elements as fertilizers in agricultural practice. One the other hand, the effects of trace elements were noticed when they were supplied in very small amounts so that an impression was created that they must be universally present in sufficient quantities required for plant growth; they are already present in soils in easily measurable concentrations. It has never been fully appreciated, however, that trace elements in the majority of cases are bound by the soil much more strongly than are macronutrients. Also neglected was the fact that the presence of macronutrients (nitrogen, phosphorus, potassium) also increased the requirements of plants for trace elements by several orders of magnitude; the use of fertilizers, however, is a prerequisite for obtaining high crop yields.

It is true that in some soils, despite the addition of adequate amounts of NPK fertilizer, good crops are never produced, or the plants are affected by various nonparasitic diseases such as

chlorosis, necroses, morphological abnormalities, deformities of the fruits, etc. However, investigators have not sought explanations for these effects in the shortage of some unknown essential mineral nutrient, but have tended to look for other reasons for the abnormalities - unfavourable proportions of mineral elements in the soil solution, acidity or alkalinity of the medium, etc. And only much later was it found that in the majority of cases the cause was a deficit of this or that trace element.

In this context it will be instructive to look at the history of the study of a typical disease caused by manganese deficiency, the so-called grey speck, and the history of the ideas that evolved about manganese as an element which exerts a positive influence on plant growth solely due to its indirect effects on certain processes going on in the soil.

Early in the nineteenth century in different countries grey speck was observed on oats. In other plants grown mostly in soils with a neutral and alkaline reaction (predominantly in chalky and heavily chalky soils), different chloroses have been detected. A number of investigators (Hudig, 1911; Hiltner and Korff, 1917) were able to eliminate this disease completely by introducing manganese or acidic fertilizers into the soil. In contrast to their initial expectations, however, these investigators arrived at the conclusion that the disease they had dealt with was not related to a shortage of manganese in the soil.

The lack of information regarding the necessity of manganese for higher plants resulted in further strengthening of the idea that the apparently indirect effect of manganese on plants is connected with its participation in oxidative processes in soils (Skinner and Sullivan, 1914). This property of manganese was used by these American scientists as the starting point for the development of an erroneous theory about so-called soil tiredness - the poor fertility of soil resulting from the appearance of soil poisons. According to Skinner, Sullivan and others, the essence of the action of manganese comes down to its ability to oxidize these poisons and thus to eliminate their adverse effects. Other hypotheses have also been advanced, in which manganese is reduced to having only an indirect influence on the processes going on in the soil, i.e. to participation in metabolic reactions and to having effects on the vitality of the soil microflora and the like.

The second period began in 1919. During the fifty years since this time, striking discoveries were made which revealed the essential nature of numerous trace elements for the nutrition of plants and animals as well as pointing to their physiological role. All this taken together served to provide a basis for trace element science as a fundamental scientific discipline which has been contributing significantly to the elucidation of the mechanisms underlying basic biological phenomena, including those encountered in the application of mineral nutrition to plants.

The visualization of trace elements as stimulants, however, proved to enjoy a lasting popularity. Thus, for instance, as late as 1934 A.A. Khalizev, greatly inspired by the widely acclaimed work of a Bulgarian scientist M. Popoff (1931) concerning the chemical stimulation of seeds, made an attempt to revive these old ideas in his valuable review on trace elements entitled "Chemical Stimulants".

The second period of the investigation and elucidation of the essentiality of trace elements in plant nutrition was a period of research projects which opened a new chapter in the science of plant mineral nutrition. During the first decade, the absolute necessity of four trace elements - manganese, boron, zinc and copper, and later, molybdenum and some others - for the life of higher and lower plants was established unequivocally. A decisive role in this research was taken by the methods of purification both of water and nutrient salts from traces of the elements under study.

In those years the interesting works of V.I. Vernadsky were published, demonstrating a relationship between the chemical composition of living organisms and the chemistry of the Earth's crust. In a series of papers V.I. Vernadsky (1922, 1926, 1931) has shown that a continuous exchange of substances occurs between the environment and all organisms. He also put forward the view that the environment and the organism are interrelated through the common history of the atoms of chemical elements. The geochemical processes occurring continuously in the Earth's crust, on the one hand, and the evolution of the molecular composition of the living substance, on the other one - are two conjugate processes. These ideas have contributed to the consolidation of the view of trace elements as essential consituents in plant nutrition, and we have good reasons for regarding V.I. Vernadsky as one of the founders of the science of trace elements.

In 1919 there appeared a publication by Steinberg, in which he described a method for purifying nutrient media by precipitating heavy metals as carbonates, hydroxides and phosphates. Working with solutions freed from traces of zinc, Steinberg was able to increase 2000-fold the dry weight of Aspergillus niger. That was an impressive result, which dealt a blow to the concept of trace elements as stimulants and irritants. This work of Steinberg was highly innovative and opened up new vistas in trace element research, while his method for purifying nutrient solutions provided a new technological basis for investigations on the essentiality of trace elements for higher plants.

In this way the dam, which precluded any rapid advancement of the science of the mineral nutrition of plants, has been eliminated and a succession of discoveries has significantly enriched our knowledge in this field.

In 1922, three years after the publication of the work of Steinberg, there appeared a report by McHarque who worked with purified salts and obtained unequivocal evidence for the absolute requirements of manganese as a nutrient element for the growth of wheat and peas in sand culture. A year later, Warington (1923) conducted experiments with water cultures of broad beans and other legumes, and clearly demonstrated that boron is an essential element for plant growth. Three years later, Sommer and Lipman (1926) provided conclusive evidence, by studying the growth of sunflower and barley in water culture under strictly controlled conditions, that zinc is essential for the growth of these plants (Fig. 1). In that year it was also shown that the "treatment disease" encountered in grasses growing on marsh soils is attributable to copper deficiency and can be cured by applications of copper (Hudig and Mayer, 1926). At a later date, the essential nature of copper for the growth of sunflowers (Sommer, 1931), flax and tomato plants has been demonstrated in water cultures of these plants. Even prior to the appearance of the above-quoted publications it had been established that copper is required for the growth of Aspergillus niger (Bortels, 1927).

The experiments reported at that time by I.V. Michurin (1947) are of interest, since he obtained striking results concerning the effects of manganese on the acceleration of the development of hybrid seedlings of almond. Manganese not only was effective in accelerating the growth of the seedlings, but also induced, according to I.V. Michurin, a "monstrous" jump in the development

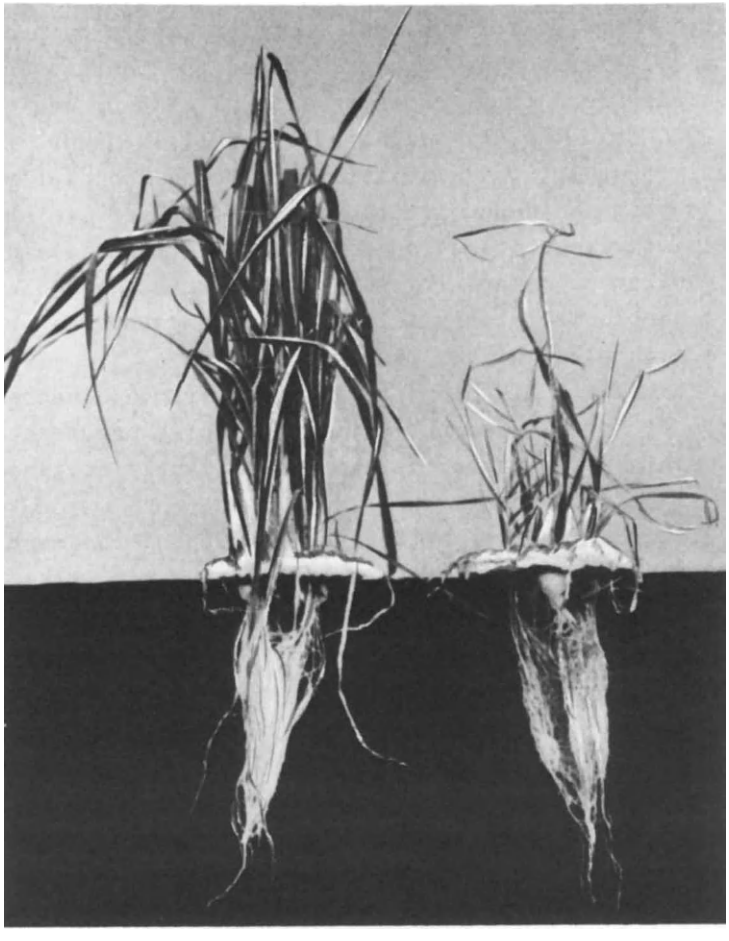


Fig. 1. Barley growth with and without zinc. Left, with zinc; right, without zinc (after Sommer, Lipman, 1926).

of the plant by shortening the period required for the onset of the first fruit-bearing stage by six years. During the first year the almond seedlings grew as tall as 180 cm with the flower buds formed, and they flowered and beared fruits the following year.

Shkolnik (1933, 1935) provided evidence that boron was an essential element for flax, and thereby the former view of the non-essentiality of boron for grasses were shown to be wrong (Brenchley and Warington, 1927). It was also shown that in wheat and Sudanese sorghum, contrary to what is observed in dicotyledonous plants, vegetative organs develop normally in the absence of boron and deficiency symptoms appear only at the stage of formation of

the reproductive organs. Later, complete sterility of the spike was demonstrated in barley grown in conditions of boron deficiency (Shkolnik et al., 1956).

In 1937 Bortels showed molybdenum to be essential for Aspergillus niger, and still earlier - for Azotobacter chroococcum (Bortels, 1940). Soon afterwards, Arnon and Stout (1939) indicated that molybdenum was essential for tomatoes. Then, requirements for molybdenum were demonstrated in legumes (Bobko and Savvina, 1940), in sugar beet (Fig. 2), in plums, oak seedlings, and other plants.

In some higher and some lower plants there has been shown to be a requirement for a number of trace elements such as vanadium, cobalt, aluminium, silicon, iodine, sodium, chlorine, lithium and selenium. Steinberg (1938) showed gallium to be an essential element for Lemna. The biological roles of all these elements (besides gallium) will be discussed in detail in the appropriate chapters of this book. Evidence concerning rubidium, fluorine and titanium will also be presented.

The discovery of the essentiality of boron, manganese, zinc, copper and molybdenum for plants provided a basis for explaining a number of plant diseases which were formerly ascribed to the deleterious action of fungi or bacteria; these are now known to be functional diseases, attributable to a deficiency of individual trace elements in the soil. Thus, for instance, "brown heart" of sugar beet has proved to be a disease caused by boron deficiency in the soil. And since the demonstration in 1931-1932 that the application of boron was an effective means for combating brown heart of sugar beet (Brandenburg, 1931; Belousov, 1932), the disease that was the plague of sugar beet production in Western Europe, boron compounds moved to the top of the league among reputable artificial fertilizers. A summary of the early findings concerning functional diseases attributable to trace element deficiency can be found in a number of publications (Shkolnik and Makarova, 1950; Wallace, 1953).

To date some 71 chemical elements have been detected in plants. A.P. Vinogradov (1935) speculated not only that all chemical elements can be found in living material, but also that these elements may have a physiological significance for plants. In the author's book "The Role and Significance of Boron and Other Trace Elements in the Life of Plants", it was indicated when dealing with the historical approach to the science of trace elements that the major significance of those investigations which have revealed

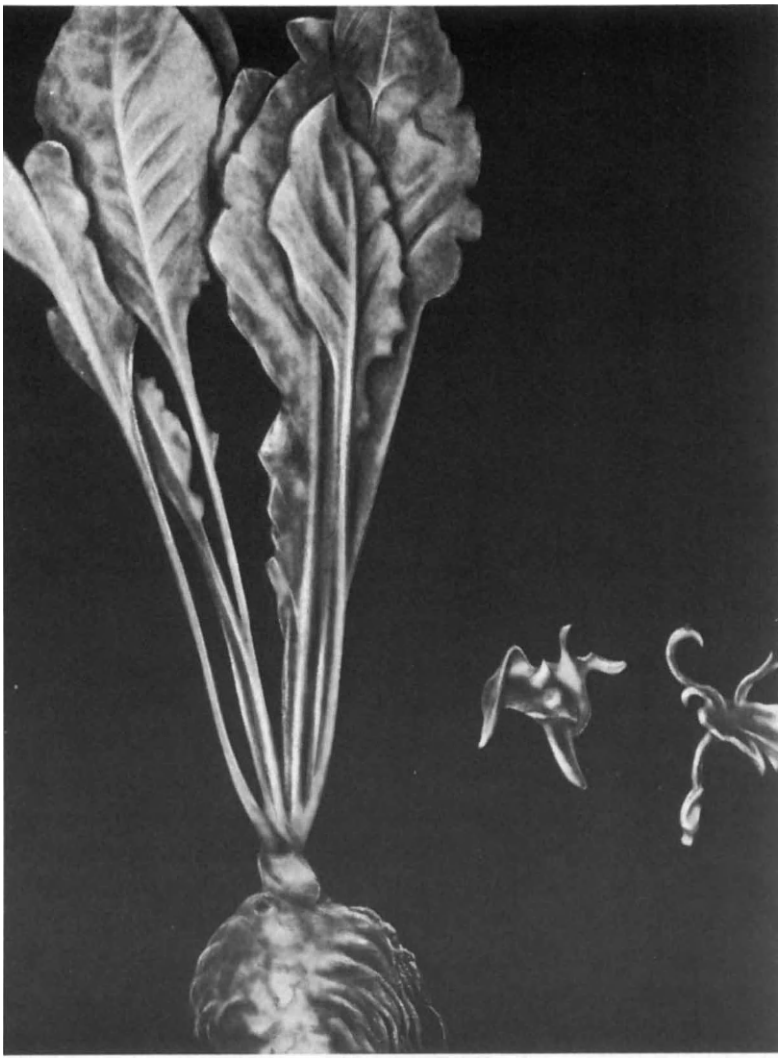


Fig. 2. Effect of molybdenum on the growth of sugar beet (after Borys and Childers, 1954). Left, with Mo; right, without Mo.

the essentiality of a number of trace elements for plants not only resides in the fact that such investigations have enriched our knowledge of the requirements for these elements (formerly regarded as unnecessary for plants), but also stems from their explicit demonstration of the extent to which the organic world depends on the majority, and very probably all the elements found in nature.

The evidence accumulated over the forty years since the discoveries mentioned above has indicated that the number of physiologically important elements is in fact considerably larger that that suspected earlier, and it is only the shortcomings of purification methods that precludes the demonstration of the essentiality of a number of trace elements. In order for such evidence to be provided, more effective means of purifying nutrient media are required, making use of air-tight chambers which are filled with sterile air freed from traces of dust, and which are equipped with automatic devices for dispensing nutrient media. In addition, new methods for the assessment of the purity of the materials used are also needed.

Investigations should be extended to include a wider range of organisms. Nicholas (1961), for instance, believes that in wild flora, and in particular in marine microflora, organisms may be found that display unusual requirements for metals, the essentiality of which is not suspected at present. Bowen (1966) reminds us that only 300 out of 0.5 million plant species and only 200 out of 3 million animal species have been studied with respect to their requirements for trace elements.

Arnon (1958) has indicated that, in contrast to higher plants which exhibit biochemical uniformity in their basic metabolic processes, photoautotrophic microorganisms such as some of the algae display a biochemical diversity which is manifested, for instance, in their pigment composition and their capacity to utilize molecular nitrogen. This is associated with a requirement for certain specific biocatalysts containing trace elements as well as the trace elements themselves. In fact, Arnon and other investigators proved that vanadium and cobalt are necessary for blue-green algae; the essentiality of trace elements for higher plants had been established previously. Arnon also succeeded in demonstrating the higher requirements of some of these organisms for specific trace elements: in particular, the blue-green algae have been shown to require 100 times more molybdenum than that required by green algae and green plants. It has also been found that certain transition metals such as vanadium, the essentiality of which has not been established for higher plants, are necessary for nitrogen-fixing microorganisms and blue-green algae as well as for the actual process of nitrogen fixation.

The search for those trace elements the essentiality of which for plants has not yet been established should be carried out in

plant-concentrators. The peculiarities of the process of concentrating nutrients may be so intimately linked to metabolism that a deficiency of certain elements may interfere with some physiological processes. It will be shown later that among the concentrators of iodine, aluminium and lithium species have been found for which these elements are essential.

Arnon and Stout (1939) suggested three criteria for the assessment of the essentiality of a trace element, and these have been widely accepted: 1) an organism cannot complete its life cycle without a specific trace element; 2) the action of a trace element is specific and it cannot be substituted for another element; 3) the action of the trace element on an organism is a direct one. Later investigations have established, however, that the second criterion is difficult to apply. Thus, for example, molybdenum is necessary for nitrogen fixation in Azotobacter, but in some Azotobacter species molybdenum may be substituted for vanadium (Bortels, 1936). Another case: chlorine is required by higher plants, but other halogens such as bromine may be used instead, being effective however, only when taken in higher concentrations. Thus, according to the criteria of Arnon, molybdenum and chlorine should not be regarded as essential elements.

To resolve these controversies, Nicholas (1961) suggested a wider criterion. He introduced the term "functional, or metabolic, nutrient element", which is applicable to any mineral element participating in metabolism, irrespective of the specificity of its action. This approach is based on the latest studies concerning the involvement of trace elements in plant metabolism and helps to avoid interpretation difficulties in those cases in which an element is essential only in the presence of specific substrates, as will be shown to be the case with molybdenum. This broader approach to the problem of the essentiality of trace elements for plants does not, however, take account of the fact that only a limited number of macro- and micronutrients may be absolutely essential for a particular organism. Certain elements are apparently quite unnecessary for living organisms. Bowen (1966) presents calculations showing that actinium, radon, polonium and radium may be found in concentrations of less than one atom per cell. Such elements, according to Bowen, should not be regarded as essential.

Interesting ideas have been formulated concerning the relationship between the functional significance of an element and its

position in the periodic system of Mendeleyev. A.P. Vinogradov (1933) has established that the chemical composition of living matter is a periodic function of the ordinal number of an element. Frey-Wyssling (1935) has made an attempt to relate the possible biological significance of elements on the basis of their positions in the periodic system of elements. He focussed his attention on the fact that all of the essential elements for plants are positioned in the periodic table on imaginary lines linking carbon and argon; these lines he called the "nutrient lines". The only exception that is not found on the "nutrient lines" is hydrogen. Sodium, aluminium, silicon, chlorine, fluorine, nickel, cobalt and titanium being located in the vicinity of the "nutrient line", were expected to be essential elements. From the data available on the physiological role of various trace elements it may be seen that the deductions of Frey-Wyssling have turned out to be true in many respects.

Interesting concepts about the relationship between plant requirements for trace elements and the atomic structure of the latter have been suggested by Steinberg (1938). According to his approach, Steinberg indicated that plants may show a requirement for scandium, and in fact it was found some time later that scandium is indeed an essential element for Aspergillus niger, for example, but only when glycerol is used as the sole carbon source. If, however, the nutrient medium contains dextrose, the fungus shows no requirement for scandium.

Dixon and Webb (1958) pointed out the following 15 cations which serve as activators for one or more enzymes: Na^{+}, K^{+}, Rb^{+}, Cs^{+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} Al^{3+}. The metals included in this list have atomic weights from 11 to 55, mostly within the range 19 to 30. And there is no case known in which a metal with an atomic number above 55 is an activator of an enzyme.

I will refer to some examples which show that a definite regular pattern exists in the mode of action of elements on the organism and their position in the periodic system. It has been established, for instance, that elements of the seventh main subgroup (halogens) show an increasing tendency towards the formation of biologically active organic compounds with increasing magnitude of the ordinal number. As for the elements in the sixth main subgroup, it may be noted that besides the well known interchangeability in a number of organic compounds between oxygen and sulfur,

there can be also observed a reversible displacement of sulfur from natural compounds by selenium and tellurium that results in the formation of substances displaying a high toxicity (Voinar, 1960).

Dobrolyubsky (1969) has arrived at the conclusion that the biological action of trace elements depends to a significant degree on the properties of atoms, such as, for example, the ionization potential, the electron affinity, the polarizability and the ionic potantial. The positions of a number of trace elements in the periodic table in the group of d-elements determines their high capacity to form complexes.

The recent studies of Lebedev (1970) have shed new light on the problem of the chemical bond and the dimensions of atoms. and have given us deeper insight into the problem of trace element action. These investigators concluded that, if account is taken of the results of the evaluation of similarities and differences between elements, a systematic search for biologically important metallo-organic complexes can be undertaken. At present, such an evaluation of the similarity and differences between elements is apparently feasible, taking into account the peculiarities of the structure of their electron shells, the specificity of the distribution of electron energy levels, and the possibility of the participation of excited and hybrid orbitals in chemical bonds.

A vivid illustration of the above is given by the findings of Borovik-Romanova (1969), which testify to different degrees of concentration of alkaline metals achieved by brown algae. She found that potassium, rubidium and caesium can be concentrated by marine algae in amounts several times greater than those characteristic of sea water, whereas lithium and sodium cannot be concentrated by these algae. The author made an attempt to relate the data she obtained with the characteristic of the atoms and ions of the elements concerned. In this connection Borovik-Romanova (1969) referred to the works of Cooper (1950) and Volobuev (1968) which consider the significance of the energy status of an element for its uptake and for the intensity of oxidation-reduction reactions in plants; also a relationship between the quantitative concentration of elements in plant tissues and their electrode potential and electronegativity is discussed. Volobuev has suggested that the known instability of certain energy levels might be the cause of the higher activity of such elements in the biochemical reactions taking place in plants, and that this might probably

account for the high potassium concentration found in plant tissues Borovik-Romanova (1969) noticed also that among other alkaline elements found in sea water in ionic form, rubidium and caesium, as well as potassium, possess unoccupied inner levels, namely (4d, 4f) and (4f, 5s, 5p, 5d), and these very elements, particularly potassium, are concentrated by marine algae. Lithium, which has all the electron shells surrounding the nucleus filled with electrons, cannot be concentrated by algae. The same holds true for the earth metals found in sea water in ionic form: magnesium, in which no inner electron filling takes place, is also poorly concentrated by brown algae, whereas calcium, strontium and barium, which are similar in their structure to potassium, rubidium and caesium, are concentrated by algae to a greater extent.

Let us look at some more examples. Since rubidium is close to potassium in terms of its atomic weight and atomic volume, various investigators have made attempts to substitute rubidium for potassium. As will be shown later, rubidium can indeed partially replace potassium. Similarly, the closeness of the properties of potassium and sodium atoms suggests the possibility of substituting sodium for potassium. Such an expectation is well founded. Thus, for instance, the requirement of spinach for potassium can be satisfactorily met by supplying 7/8 parts of sodium. The feasibility of a partial replacement of calcium by strontium has also been shown.

Numerous investigators have noticed that almost all trace elements essential for plants are found among the elements comprising the d-family of the 4-th group of the periodic table. Troitsky (1960) attaches great importance to trace elements as being capable of shifting reacting molecules into an excited state. Troitsky arrived at the interesting conclusion that the principal role of the trace elements of the d-family of the 4-th group as biocatalysts is attributable to their ability to induce excited states in intracomplex bioorganic systems by elevating the density of electrons around themselves (due to their negative-inducing effect). This accounts for a decrease in the activation energy of the system so that complex bioprocesses become possible at moderate temperatures, almost neutral pH, and normal atmospheric pressure.

Interesting concepts also arise from the work of Ermolenko (1960), who noticed that the metals found in organo-mineral compounds are often the metals with variable valence. Most of them are found within the lateral subgroups of the periodic table, and consequently are characterized by incomplete peripheral electron subshells. The following trace elements are among such metals: iron, manganese, copper, molybdenum, titanium, chromium, cobalt, nickel, bismuth, uranium, tellurium, mercury, platinum, iridium, tungsten, tantalum, osmium, lead, vanadium and others, many of which play an important role in the lives of plants and animals. In the ionic state these elements produce a strong polarizing effect on the addenda and therefore are good complex-forming agents.

The ability of trace elements to form organo-mineral complexes with organic compounds, as well as that of producing intracomplex compounds, is of the utmost importance for the processes of intracellular metabolism.

Chapter 2

THE CHEMICAL FORMS OF TRACE ELEMENTS IN PLANTS

One of the reasons for the long lifetime of the view that trace elements were stimulants and irritants, and the stubborn unwillingness to acknowledge their essentiality in the nutrition of plants, was the fact that formerly it was difficult to comprehend how such minute amounts as those implicated could exert so striking an effect on plant life. For instance, 10 g of molybdenum applied over one hectare, for the nutrition of 5-6 million individual plants, may increase the crop yield of legumes by 30-40%, and sometimes by 200%. However, the fact that such elements are necessary only in minute quantities proves their involvement in the most intimate processes of life.

Let us turn to the facts, which may clarify this point. The revolutionary discoveries in natural science made in the XX century (we are witnessing these as they take place) have been possible thanks to the development of microphysics, which studies the processes involving elementary material particles, and the progress made in the understanding of biological processes at the molecular level. Among these accomplishments are the advances that have been made in the physical and chemical study of molecular structures in the two most important classes of compounds - proteins and nucleic acids, and the understanding of the functional roles of the latter. All this taken together has made it possible to approach the solution to the mystery of living substance - protein. These successes brought us to the major event in biology - the deciphering of the genetic code that constituted one of the principal mysteries of Nature.

Now let us try to understand why trace elements may exert considerable influence in very minor quantities. Consider the following facts.

One gram of soil contains several millions of microorganisms, each microbial cell containing hundreds of enzymes. A multitude of transformations involving the synthesis and degradation of enormous amounts of organic material proceed ceaselessly in each cell. And one should not forget that trace elements are the co-factors or activators of almost 1/3 of the enzymes found in the microbial cell.

The number of mitochondria in the cell may vary with different plants from scores to several hundreds. Each mitochondrion in the words of the great biochemist Green(1961) is a real chemical factory, the power-station of the cell, in which the process of accumulation and transformation of energy takes place. As we now know, mitochondria are the sites of respiration and oxidative phosphorylation. The outstanding role of trace elements in the functioning of mitochondria has also been well established. Even more striking is the existence in one cell of millions of ribosomes - the sites of protein synthesis, the mechanism of which also requires trace elements.

The contents of some trace elements in plants can be expressed in 10^{-4}-10^{-5} per cent. However, in terms of absolute quantities of molecules, we are dealing with strikingly large numbers.

There are other important considerations which must be taken into account in order to understand the ability of trace elements to exert influences even when they are taken up in vanishingly small amounts. Thus there is ability of trace elements to enter into organo-mineral complexes with organic compounds to produce intracomplex compounds. When they are incorporated in organic complexes, the ability of trace elements is enhanced a hundred- or a thousand-fold, and frequently even a million-fold over the activity of the simple ionic state. Here is an example to illustrate the point. In studies of the difference between the catalytic action of free iron ions (which possess a weak oxidative capacity) and the iron within an organo-mineral complex, it has been established that the complex-bound iron linked to four pyrrole rings is 1000 times as active as the inorganic iron. Moreover, in the haem complex in which iron is associated not only with the pyrrole rings but also with a specific protein (as for example in the haem enzyme catalase), its activity increases by an order of magnitude of 10^7. One milligram of iron complexed in this way is equivalent, according to Oparin (1957), to the catalytic action of 10 tons of inorganic iron.

The first rational approach to solving the problem of the importance of organo-mineral compounds is seen in the work of the Russian chemist Gustavson (1937) and is reflected in his report "About the chemical role of mineral salts in organic nature" delivered at the Petrov Agricultural Academy in 1881.

Complex formation is characteristic, to a greater or lesser degree, of all the elements of the periodic system; however, large

differences do exist in the ability of certain metals to form complexes with different donor groups (ligands). The capacity of metals for complex formation is intimately related to the mode of their participation in metabolism. The increasing ability of each metal in the following sequence (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Fe^{3+}) to form stable complexes is matched by their increasing ability to participate as specific catalysts in important life processes (Williams, 1961).

Martell and Calvin (1952) believe that a general principle exists in the formation of complexes by metals: the greater the ease with which a donor group shares an electron pair, the stronger will be the covalent bond that is established between this group and the metal. The ability of metals to form coordinate bonds is more pronounced in reactions involving N-atoms as opposed to O-atoms, and only negatively charged oxygen atoms can compete for metal ions with neutral nitrogen atoms.

At this point we shall consider in greater detail the problems arising in the participation of certain trace elements in the formation of complexes.

The alkaline metals such as lithium, sodium, potassium and rubidium are typically found in the ionic state. They exhibit a reduced tendency to form complexes. The earth metals - magnesium, calcium, strontium and barium, in comparison with the alkaline metals, show a marked increase in their capacity to form complexes with ligands containing oxygen, more rarely with those containing nitrogen. Aluminium forms complexes with ligands containing oxygen. It displays the characteristic peculiarity of producing intra-complex compounds with hydroxyl-containing substances. Aluminium is found in plants in the form of salts of organic acids - acetic, lactic, pyruvic, and other acids. Typically, titanium forms complexes predominantly with oxygen-containing ligands, this also being the case with vanadium, which forms complexes preferentially with oxygen-containing, and more seldom, with nitrogen-containing ligands. From methanol extracts of the fly agaric has been isolated a compound - amavadine - that contains vanadium (Bayer and Kneifel, 1972). Its structure is unknown. Chromium, like molybdenum, shows a typical tendency to form polyacids; complexes with these metals usually involve oxygen-containing, and less frequently nitrogen-containing ligands. Magnesium, calcium, strontium, barium, aluminium and chromium, like other divalent metals, are capable of

forming complexes with nucleic acids. These elements have been found even in thoroughly purified RNA and DNA preparations.

Molybdenum produces ions of different valency and can easily enter into complexes - thus accounting for its good catalytic properties. The biochemical peculiarity of molybdenum, according to Ivchenko (1978), apparently lies in its ability to form coordinated compounds with a variety of different ligands. Existing in two (or probably more than two) oxidized states, molybdenum is involved in the in vivo reduction and modification of the reactive capacity of ligands taking part in various physiological mechanisms Mo^{6+} can form a variety of complexes with hydroxyl-containing compounds such as sugars and tartaric acid; the composition of these complexes, however, has been poorly studied.

The molybdate ion can produce complexes by interacting with amino acids, which possess a sufficient number of functional groups ($-NH_2$, =NH, =CO, -COOH, $=CH_2$, $-CH_3$, -SH, -SS-). These groups, acting as addenda, can enter into a coordinate bond with the molybdate ion. Numerous histidine complexes with di- and tri-valent metals have been obtained. Spence and Lee (1965) have shown that at pH 6.0 Mo^{6+} forms a complex with histidine. Melby (1969) found that Mo^{5+}, taken in a ratio of 1:2 forms a binucleate complex (containing an oxygen bridge) with the ethyl and methyl esters of cysteine. It has been reported that Mo^{5+} forms a complex with a flavoprotein at a pH of around 2 (Spence and Tokatlian, 1961).

Manganese has long been known to participate in a number of organic complexes involving the oxygen of alcohols, oxalates and derivatives of some oxyacids. It has been shown by numerous investigators that the stability of manganese-organic complexes, as well as the stability constant of manganese intra-complex compounds, is lower than that of other divalent metals. The ability of manganese to form complexes with hematoporphyrin has been shown in alkaline solution, where manganese is found in an oxidized state (Mn^{4+}).

Manganese forms complexes with glycylglycine and phosphatidylserine, fructose-1-phosphate and fructose-1,6-diphosphatase, and biotin and its derivatives. The nature of a complex of manganese with inosine-5-triphosphate has been disclosed (Kuntz and Kotowycz, 1975). This element also becomes bound to myosin at two sites which show essentially different association constants. These workers discuss the relation between ATPase and the cation-binding activity of myosin.

Iron and nickel are vigorous complex-forming agents which give rise to stable complexes with ligands containing sulfur, nitrogen and oxygen. Haem derivatives are known in which iron is associated with nitrogen-containing groups of porphyrins. Iron is a constituent of the active sites of various reductases and hydrogenases, most frequently being associated with sulful-containing ligands (King et al., 1965). The iron proteins - haemoglobin and ferrodoxin- play a central role in metabolism.

In contrast to iron, nickel does not as a rule form compounds with proteins (Williams, 1961).

Various organic Ni compounds occur in roots. Amounts of inorganic nickel in the nickel metabolic pool are small. The greater part of the root-formed nickel compounds is transported by the xylem flow and undergoes partial modification, eventually being deposited in the leaves (Cataldo et al., 1978).

It is of considerable interest that one of the most biologically active vitamins, B_{12}, has proved to be a typical complex containing cobalt. The latter can form complexes with amino acids and proteins. Two types of cobalt complex have been discovered: A - with proteins, and B - with polypeptides, in which cobalt is linked to the polypeptide through peptide bonds (Bello and Bello, 1962). It is supposed that type A complex contains Co^{2+}, whereas the type B complex contains Co^{3+}. Various proteins such as gelatin, pepsin and edestin, as well as glycine and poly-DL-alanine, are able to form such complexes with cobalt. Nothing is known about the types of linkage between cobalt and amino and other groups, cobalt complexes have been found in which there is a reversible association with molecular oxygen (Calvin et al., 1946). It has been established that oxygen can combine with complexes of cobalt and amino acids, so that the complex involving oxygen together with histidine-cobalt proves to be particularly stable (Michaelis, 1947), each oxygen molecule being associated with two molecules of the cobalt complex. The ability of cobalt within organic complexes to form compounds with hydrogen molecules has been demonstrated (Winfield, 1955). When it is bound to certain amino acids, cobalt forms complexes which are able to be reversibly oxygenated (i.e. they can accept oxygen without changing their valence), and which can be irreversibly oxidized.

Cobalt-cysteine has been found which is reduced by NAD-H_2-cytochrome reductase (Dickinson and Chien, 1974).

Considering the physico-chemical properties of copper ions, it might be supposed that copper would not occur in tissues as a free ion. Being a potent complex-forming agent, the copper ion interacts with a number of organic compounds. On the one hand, copper forms compounds with groups such as the carboxyl (-COOH), the enol (2(-OH)), the sulfono ($-SO_3H$) and the oxime (-NOH), through main valences by replacing the hydrogen ion; on the other hand it forms compounds with other groups such as primary, secondary and tertiary amines, the alcohol hydroxyl (-OH) group, the carnonyl group (=CO), the thiol ester (-S-) and others with the utilization of additional valences (Grinberg, 1961). The organic compounds containing such groups are amino acids, peptides, proteins and a number of other biologically active substances.

Copper forms complexes with many amino acids as well as with dipeptides: L-alanyglycine, glycyl-L-alanine, glycyl-D-alanine, L-alanyl-L-alanine, L-alanyl-D-alanine, glycyl-L-valine, L-valylglycine and L-glycylglycine.

The physiological activity of the copper-amino acid complexes was shown in the model experiments of Bakardjieva (1980). These complexes play an essential role in growth regulation. According to the data available in the literature, as well as original experimental results, the copper-amino acid complexes are involved in the redox processes of plant cells in close interaction with enzyme systems. They catalyse ascorbate oxidation by both the oxidase and peroxidase paths. The competition of these complexes with enzymes depends on their concentration and on the sort of ligand involved. A convincing example is the process of ascorbate oxidation, the rate of which is dependent on the amino acid to which the copper ion is bound. Other metal complexes are believed to produce the same effect, which may be one of the modes of their involvement in metabolic regulations.

Considering the ability of copper ions to form stable complexes, it may be assumed that most proteins should bind copper. Copper-containing proteins have long been known to display notable biological activity, although such proteins are not enzymes. Haemocyanin the principal respiratory pigment in the blood of many invertebrate species, is among these. Haemacuprein, haematocuprein and iron-copper-nucleoprotein complexes are the intermediate compounds involved in the synthesis of haemoglobin, cytochromes and the oxidases of higher animals and man. A large number of copper proteins which show no catalytic activity have been isolated from

mammalian tissues. These are the true metalloproteins, i.e. proteins in which copper is a structural component of the molecule and is not found in an association-dissociation equilibrium with copper ions in solution. The physiological significance of such proteins has not been elucidated so far. Balevska (1972) isolated another three colourless cuproproteins from liver tissue.

Yuferova and her co-workers (1969) have found considerable amounts of copper in a lipid fraction.

From chloroplasts a copper-containing protein - plastocyanin - has been isolated, this being one of the key factors in the process of photosynthesis. Low molecular weight copper proteins have also been detected in non-green plant parts: in the roots of horseradish, in etiolated seedlings of mung beans, in the latex of pokeweed. More recently, a number of plants have been found to contain copper compounds in which the metal is linked to acetylglucosamine and O-phenols (Yuferova and Udelnova, 1971). These compounds are usually found in the leaves, whereas complexes of copper with acetylglucosamine occur only in chloroplasts. Copper can also form stable complexes with flavonoids. Some plants, having a high content of flavonoids feature elevated copper contents (Grinkevich et al., 1970).

Besides non-enzymatic copper proteins a number of proteins also of non-enzymatic nature but containing iron have been discovered in animal tissues (haemoglobin, myoglobin, haemeretrin I, haemeretrin II, transferrin, ovotransferrin, lactotransferrin, agavain, ferritin, echinochrome). There are also proteins such as that containing cadmium - cadmium protein (in molluscs), metallothiotein, which contains cadmium and zinc, cobalt protein (in molluscs), and those proteins that contain manganese - pinnaglobin and transmanganin.

Zinc can form more-or-less stable complexes with various physiologically active substances: proteins, nucleic acids, ATP and ADP, amino and organic acids, sugars, vitamins, antibiotics and many others. Zinc has been found to be a constituent of some non-enzymatic animal proteins - metallothiotein, cytocuprein and α-2-macroglobulin, and of porphyrins (Parisi and Vallee, 1969).

To date little is known of the chemical form in which zinc occurs in plants; it has been established, however, that soluble forms of zinc in leaves are largely low molecular weight substances (Kositsin, 1965). Associations of zinc with specific protein fractions obtained in the course of a sequential extraction of plant

tissue homogenates are currently being studied. Zinc has been found only in alcohol-soluble and water-soluble fractions of total proteins. In the subcellular structures studied to date - mitochondria and chloroplasts - the bulk of the zinc found is incorporated in the high-molecular-weight compounds where it is bound to protein (Kositsin and Igoshina, 1970). Kositsin and Igoshina (1971) have found that in tomato chloroplasts zinc is associated with certain electrophoretic fractions of soluble proteins. The results obtained have been interpreted by these authors as indicating that there are several zinc proteins in the soluble protein moiety of chloroplasts.

The metals: Cd^{2+}, Pb^{2+}, Hg^{2+} and Ni^{2+} fail to produce complexes with proteins (Williams, 1961). The literature on the interaction of metals with proteins has been repeatedly summarized (Osterberg, 1974). Osterberg (1974) considered the physico-chemical aspects of the interaction of metal ions with proteins yielding labile or inert complexes. He pointed out the tendency of proteins to produce associations on becoming bound to a few ions of copper, zinc, calcium, mercury, lead and cadmium, which thus cause proteins to aggregate. In the greatest detail, this worker considers the interaction of Cu^{2+} and Zn^{2+} with individual side groups in proteins, in particular, imidazole groups. Many interesting observations of interactions between metals and proteins are presented in a report on a symposium held in 1973 in Chicago (Protein-metal interactions, 1974).

Silicic acid easily forms complexes with organic compounds, especially with native proteins. In the culm of rye has been found a silicogalactose complex. There are indications that silicon can be bound to cellulose structures. In many plants, silicon is present in the form of opal, whereas it is in the α-quartz form in Fragaria vesca and Lantana camara. In rice, up to 90% of silicon is found in the form of the biogenic opal; the rest of the element is in the ionic form and exists as traces of silicic acid (Yoshida et al., 1959).

In plants which concentrate selenium, the latter replaces sulfur. This element gives rise to selenium-containing cycteine, methionine, glutathione and other selenium-containing amino acids in legumes. Otherwise it occurs in the form of selenium-containing allylic oila, glucosides, thiocyanates and esters of sulfuric acid, etc. in the Cruciferae, Compositae and other plants (Trelease and Beath, 1964). In addition to the amino acids mentioned, the

presence of selenium and its derivatives has been detected in selenium-β-carboxypropyl, selenocysteine and γ-glutamyl-selenium-propenylselencysteine-3'. Phosphoadenosine-5-phosphoselenate is required for the enzymatic synthesis of selenate esters and selenium lipids.

Fluorine is found in some plants as fluorocemate and fluoroleninic acid.

In the most important oxidation-reduction processes - photosynthesis and respiration - a prominent role is given to complexes involving transition metals - iron, manganese and copper - in association with lipids. The complexes of some metals with lipids control the direction of electron transport in the cell. We shall discuss these topics in greater detail later. It is also known that cysteine and glutathione, which have a sulfhydryl group incorporated in their structure, are important intermediates in the processes of oxidation and reduction. Nickel, cobalt and ferric complexes of cysteine have been obtained, as well as stable arsenic complexes of cysteine.

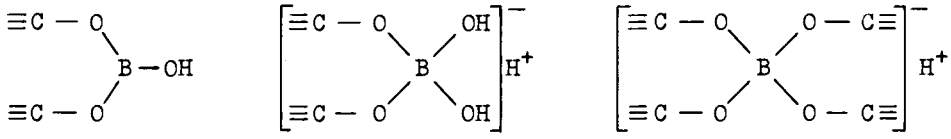
Titanium, like iron, manganese, vanadium, nickel, chromium and copper, is largely associated with cellular lipids (Gryzhankova and Boichenko, 1973). The latter workers were the first to isolate a titanium compound from plant cells which proved to be similar to 4-phosphopantatheine.

Those investigations which have revealed the tendency of various metallic trace elements to form complexes with nucleic acids are of great interest (Wacker and Vallee, 1959a, 1959b). This subject will be dealt with in detail in Chapter 4, Part I.

The non-metallic element, boron, can form complex compounds with a large number of organic substances. Boric acid easily forms complexes with alcohols, carbohydrates, and oxyacids (Gerrard, 1961). It has been shown that boric acid complexes with a number of molecules occurring in living cells: d-fructose, d-galactose, D-L-glucose, d-arabinose, d-mannose, L-rhamnose, d-ribose, d-ribitol d-mannitol, persitol, pyridoxine (vitamin B_6), dulcitol and others (Winfield, 1945). Boron can form complexes with riboflavin and ascorbic (Karabach, 1973) and dehydroascorbic acids. The formation of complexes involving boric acid and other organic acids is well known (Böeseken, 1949; Isbell et al., 1948). Borates form complexes with glucose-6-phosphate, ribulose-5-phosphate and other esters, the carbon in the fifth or sixth position being esterified.

Of outstanding interest are complexes involving boron and polyoxycompounds. A detailed review of the pertinent literature can be found in a monograph by Shwarz (1968). Chelate complexes are formed by the interaction of boric acid with polyols and phenols, aliphatic and aromatic oxyacids, carbohydrates, and other polyoxycompounds containing at least two hydroxyl groups in the alpha- or ortho-positions (Böeseken, 1949). The formation of these complexes can be visualized if boric acid is understood as a Lewis acid, the dissociation of which into ions is the result of the addition of a molecule of water.

The esterification of this acid leads to the formation of intracomplex (chelate) compounds; those that have more than two OH-groups show a considerably more vigorous interaction with boron, the rate of reaction increasing as the number of hydroxyl groups is increased.



Boron complexes with polyoxycompounds

Isbell and co-workers (1948) described three types of complex compound involving sugars and their alcohols with boron. It has been found (Mazurek and Perlini, 1963) that in addition to the complex compounds referred to above, solutions of polyols and borates may also produce more highly structured complex formations such as tridentate boron complexes which display properties characteristic of strong acids. Pentatriol 1,3,5, for instance, forms a tridentate complex with boric acid.

In the process of the formation of complexes the properties of the interacting compounds, and their chemical activity, undergo changes. The optical activity of polyols increases in solutions of boric acid and borates; their chromatographic and electrophoretic behaviour changes too. The formation of complexes markedly affects the solubility of the compounds formed and modifies the colour of the aqueous solutions of a number of dyes.

From the results of his investigations Neales (1960) states that in plants boron is found in complexes with diols.

A separate mention should be made of the ability of boron to form complexes with phenolic compounds, especially the flavonoids;

Wilson (1939) was the first to obtain boron complexes with flavonoids. As early as 1942 Taubök (1942a) suggested that there are natural products of the reaction of flavonols with boric and organic acids in plants, these products serving as easily mobilizable reserves of boron. Hörhammer and colaborators (1952) investigated a complex compound of quertzetrin in which boric acid and oxalic acid were present in a molar ratio of 1:1:1. The stabilizing action of the acid in complexes of flavonoids with boron, and the position and nature of its association within the complex are not yet understood, since isolation of the complex is rendered impossible by its considerable lability and sensitivity to solutes.

In his review concerning flavonoid derivatives in plants, Paech (1950) indicated that all flavonoids (except phytesin), as well as 5-oxyflavones, chalcones and certain isoflavones interact with boric acid, especially in the presence of oxalic or citric acids, producing yellow-coloured compounds. This reaction may be of some biological significance. The high susceptibility of some flavonoid-rich plants to diseases attributable to boron deficiency was explained by Paech as arising from the fact that in these plants boron is fixed through this type of interaction. The affinity of boric acid for o-hydroxyl configurations favours the formation of borate complexes, not only with flavonoids but also with a number of other phenolic compounds (Torssell, 1956). This may have important biological consequences. Detailed evidence concerning the formation of boron complexes with phenolic compounds is summarized in one of our papers (Shkolnik and Mayevskaya, 1970).

Interesting data are available concerning natural metalloantocyanin complexes, which include aluminium, iron, and sometimes other macro- and micronutrients. Investigations in this area were started in 1958 simultaneously and independently by Bayer (1958) and Hayashi and collaborators (1958), in the context of studies on the nature of the blue colour of petals. As soon as it became clear that the cell sap of petals is acidic whatever the colour of the petals (Shibata et al., 1949), the blue colour of petals attributable to antocyans was considered a mysterious phenomenon. No ready explanation was forthcoming for the fact that the antocyanic pigments, which show pronounced indicator properties <u>in vitro</u>, produced the blue colour of the petals in many plants inspite of the acidity of the petal cell sap. The suggestion that metalloantocyanin complexes might be involved in producing the blue colouring has been made (Chenery, 1948); however, the natu-

rally occurring complexes themselves have not been isolated.

By adding ethanol to sap squeezed from Centaurea cyanus petals, Bayer (1958) was able to precipitate the blue antocyan pigment, which he called protocyanin to distinguish it from the ordinary cyanin of red petals. Hayashi and his colleagues (1958) isolated a blue complex involving antocyan and metals - commelinin - from Commelina communis flowers. Spectral analysis of commelinin ash demonstrated that the compound contained magnesium, potassium, sodium, and traces of calcium, manganese, copper and titanium. In addition to protocyanin and commelinin, detailed studies have been carried out on the metalloantocyanin complex derived from blue and violet petals of Lupinus polyphyllus - protolupinin (Bayer, 1959). Bayer believes that protolupinin is a complex of delphinidine-monoglucoside and iron or aluminium. To date natural metalloantocyan complexes have been isolated from 5 plant species (Harborne, 1965). Almost all of these complexes, except for that isolated from Viola tricolor petals, contain trace elements.

The formation of chelate complexes of antocyans and trace elements markedly affects the properties of the organic part of the complex, increasing the resistance of the pigment to the action of various agents. The very fact of the preservation of the blue colour of the cell sap in an acidic environment points to considerable stability of the antocyans. In the form in which they occur in complexes, the latter differ from the usual form, not only in terms of a marked stability against acids, but also in such characteristics as behaviour during dialysis and electrophoretic mobility. Metalloantocyan complexes are not dialyzable. Such a drastic change in the properties of antocyans when they are found as part of a chelate compound supports the view that the complexing of antocyans with trace elements undoubtedly plays an important role in the metabolism of plants.

Intracomplex or chelate compounds are widespread in both the animal and plant kingdoms. Such important compounds as chlorophyll, haemins - the constituents of haemoglobin and iron-containing respiration enzymes as well as some other enzymes - are chelates in which iron, copper, magnesium and cobalt are tightly bound to organic moieties, and can only be released upon the decomposition of the organic part. Interestingly, the respiratory process in invertebrates are also mediated by intracomplex compounds - derivatives of the variable valence metals such as vanadium, copper, manganese, etc.

Chlorophyll and haemoglobin which play exceptionally important roles in the lives of plants and animals are typical intracomplex compounds. They are the prototypes or the structural analogues of a large number of other intracomplex and organomineral compounds in living organisms. They display structural features in common with vitamin B_{12}, which is also a chelate compound. Among the naturally occurring chelate forming compounds we find dicarbon amino acids, proteins, humic compounds and lichen acids.

Discoveries about the nature of chelates have made for a better understanding of the functions of metals in enzymic reactions. It has been demonstrated that the operation of a large number of enzymes, particularly oxidation-reduction enzymes, involves the participation of complex and intracomplex derivatives of various metals. The most active site of any molecule that is represented by a complex between an enzyme and a metal,as in the case of the enzyme-mediated catalytic activation of decarboxylation, has the structure of a specific chelate complex binding established between the metal and the carbonyl and carboxyl groups of the substrate. The binding occurs on account of the high mobility of electrons at exactly this site (Steinberg and Westheimer, 1951).

The activating properties of metals are manifested in the formation of intracomplex compounds containing pyrrole rings, as in the case of haem and chlorophyll, or in the formation of a chelate linkage, as is usually observed in enzymes. The effects of metals on enzymes, however, can also be realized through other types of intracomplex binding. Evidence for this may be seen in the fact that manganese (Mn^{2+}) and magnesium (Mg^{2+}) ions, although forming chelate bindings less readily, are sufficiently potent to activate hydrolytic enzymes.

The formation of a metal-binding chelate linking enzyme and substrate can be illustrated by the example of the binding of the pyrophosphate moiety of a co-enzyme with the enzyme through magnesium. According to Calvin (1954), magnesium chelates are formed during the association of ATP, NAD, NADP and other pyrophosphate structures with enzymes. This results in magnesium being linked through two principal bonds with neighbouring oxygen atoms and through two additional bonds with the protein moiety of the enzyme.

Chelates play a prominent role in enzyme-substrate reactions. Bridges can be formed through chelated metals between the enzyme and the substrate; these bridges can result in a displacement of the electron configuration.

It has been established (Martell and Calvin, 1952; McElroy and Nason, 1954) that metals that are not associated with proteins may react catalytically with the substrate, as, for example, in the enzymatic decarboxylation of α-ketoacids. In the decarboxylation of ketosuccinic acid in the presence of copper, copper chelate complexes are produced favouring the transfer of electrons from the carboxyl to the copper, and further to other electron acceptors.

Ermolenko (1960) suggested that trace elements - magnesium, iron, copper, manganese, vanadium and others - are bound in plant and animal tissues in the form of organo-mineral compounds of an intracomplex nature. It is exactly this type of linkage between these compounds and proteins that provides the most favourable conditions for the activation of the gases oxygen and CO_2 when they become bound by these metals. This is an important part of the oxidation-reduction processes of respiration and photosynthesis. Ermolenko believes that a metal acts as a catalyst and initiates the subsequent reactions of oxidation.

Considering the mechanism by which exceptionally low concentrations of trace elements influence physiological and biochemical processes, Troitsky (1960) admits that trace elements, acting as intracomplex compounds, initiate a chain of reactions with the involvement of free radicals. Only the first reaction is triggered by a trace element; while at later stages of the radical reactions the trace elements are not involved. He maintains that a chain reaction once started can itself create the conditions that are required for the subsequent reaction stages, so that it becomes unnecessary for a specific trace element to participate in each subsequent reaction stage. In this way, trace elements can trigger, or initiate chain reactions.

The subject of the action of chelates and their role in metabolism has been extensively covered in the relevant literature and has been summarized in several interesting reviews (Martell and Calvin, 1952; Ostrovskaya, 1970). This knowledge has heralded a new era not only in the physiology and biochemistry of plants, but also in soil science, animal physiology and biochemistry, analytic chemistry, and a number of other disciplines.

Important contributions that have stimulated the study of the significance of chelate compounds in living processes have been made by those investigations which established chelates as being useful agents for the treatment of chlorosis-diseases attributable

shortages of iron and some other trace elements. As is widely known, the problem of the supply of iron to plants is a difficult one. In the Soviet Union, particularly in the Crimea, Moldavia, Georgia, Kirgizia, some regions of the Ukraine, and along the Volga, chlorosis of the vine and some other fruit crops is widespread. Applications of iron and other trace elements to plant roots and above-ground parts frequently give no positive results. In Florida, chlorosis of citrus plants is a widespread disease. In this context scientists have long looked for methods of applying iron to plants in its complexed form, at the same time trying to avoid the ionogenic form.

In 1951 Jacobson drew attention to the intracomplex compounds (chelates) or complexons, which had long been known to chemists, although neglected by them. He used the disodium salt of ethylenediaminetetraacetic acid (Na-EDTA), which forms complexes with almost all metals including iron. These complexes are soluble and stable at extremes of pH. Ferrochelates are translocated within the plant without becoming dissociated: they facilitate the movement of iron in plants. Stewart and Leonard (1952) as well as other investigators continued the innovative studies of Jacobson, and after about a year, found a clue to the treatment of the serious ferric chloroses of Florida plants. During the subsequent five years similar experiments were commenced in numerous countries. In addition to Fe-EDTA, there also appeared Cu-, Zn-, Mn-, Mo-EDTA and other chelates containing trace elements. Other preparations such as diethylenetriaminopentaacetic acid (DTPA, or Chel-330) and ethylenediaminodioxyphenylacetic acid (EDDEA, or Chel-138) enjoyed widespread practical application.

In the Soviet Union, extensive research has been carried out by Ostrovskaya et al. (1965) on the elucidation of the physiological basis of the origin of chloroses, and on the application of chelates in fighting the disease.

Since the use of artificial chelate-forming agents has been made a practical possibility, the methodological approaches to various physiological problems have become a more active area of research, which has included the study of the physiological role of trace elements.

In conclusion it should be pointed out that transition metals occurring in cells mainly as complex compounds control the activity of several enzymes, are contained in vitamins, regulate redox

processes, participate in processes of absorption and transport, and have a role in the adaptive responses of organisms, etc.

At present a new field of science, bioinorganic chemistry, is very rapidly developing. One of its main goals is the study of structures and mechanisms in metal complexes with natural macromolecular ligands (nucleic acids, proteins, phospholipids, etc.).

An idea of the scope and significance of this science can be obtained from the monograph of Eichhorn (1975) which also gives an idea of the relationships between inorganic chemistry and biochemistry. It discusses the points of connection between the two subjects and their areas of overlap. Eichhorn limits the field of bioinorganic chemistry to the application of the principles of the coordinative chemistry of metals to biological problems.

The major problems of bioinorganic chemistry as envisaged by Yatsimirsky (1976, 1977) are the study of the interactions of biometals and bioligands, and the investigation of the structure and properties of bioinorganic compounds thus formed as well as their biological functions at the molecular level. An important experimental approach is to model metal-binding centres with the use of simpler ligands, and to compare the properties of new artificial complexes with those of natural systems. An analysis has been carried out of the prospects for applying bioinorganic chemistry and using its results in medicine, agriculture and nature conservation (Yatsimirsky, 1974).

The structure and properties of a new group of biologically active peptides selectively binding the ions of alkaline and alkaline earth metals have been considered by Ovchinnikov and Ivanov (1976). The principles of the intersction between peptides and these metals in ionic form have been formulated.

The amide group $R\text{-}CONR_1$ (where R, R_1 and R_2=H, CH_3, C_2H_5 and other organic radicals) is the major structural unit of polyamides, polypeptides and proteins. Several hundred drugs and biostimulators contain the amide group. The formation of coordinative compounds of microelements by these ligands often improves their bioactive properties extending the spectrum of their pharmacological effect, reducing toxicity, etc. (Tsivadze, 1981).

It has been found that some coordinative compounds of copper such as copper-74 (bioparaaminocylicilatobisethylenediaminecopper (P), dihydrate) are more available and more active than their inorganic forms. It has been observed that copper-72, compared with copper sulfoxide and its other coordinative compounds, has

a more pronounced effect on the acceleration of phosphorus uptake and the transformation of phosphorus into organic forms in the tissues of plants. It has been shown that this compound significantly increases the content of oxygen-soluble phosphorus substances at the expense of organic forms. The content of RNA and DNA in cotton leaves notably increases, especially in the phases of flowering and fruiting. Under the effect of copper-74, the yield of cotton during vegetative growth increased by 12.8%, and in the field experiments - by 4.9 c/ha, whereas with the use of an inorganic copper salt the increase was 5.8% and 2.9 c/ha, respectively (Kadyrov, 1981).

Approximately the same differences in yield improvements of cotton have been obtained with coordinative compounds of copper-72, copper-48 and cobalt-46, comparing with the effects of the inorganic forms of these microelements. Relative to what is observed in controls and plants treated with inorganic salts of the microelements examined, the coordinative compounds stimulate growth, development and fruiting, and increase the rate of redox processes (Kadyrov, Azizov, 1981).

Let us consider in more detail the work of Batyr (1981) which provides some new evidence on the theory and practice of bioinorganic chemistry. Batyr points out that the discovery of the structure of myoglobin and haemoglobin, the Rh deciphering of a series of individual proteins, and the finding of an allosteric effect, have led to the study of living processes at the molecular level. Coordinative chemistry has progressed from simple inorganic complexes to the synthesis of highly complex compounds involving bimetals and organic ligands, and it has now become possible to speak in terms of making models of biological systems: haemoproteins, vitamin B_{12}, nitrogen-fixing enzymes. In the seventies this bilateral development of molecular biology and coordinative chemistry gave rise to a kind of a structural link, bioinorganic chemistry, which has at its disposal for the study of biological material reliable methods of chemical synthesis and physical investigation.

It is stated in the paper by Batyr (1981) that the aim of molecular biology is the interpretation of biological phenomena: the ability of haemoproteins to bind oxygen reversibly, the highly specific activity of enzymes (in terms of their molecular atructure), the specific interaction of macromolecules with low-molecular substances. In the course of many years, coordinative

chemistry has accumulated a wealth of knowledge on the interaction of ligands, on their physical properties (magnetic, optical, electrical), on their chemical behaviour including their catalytic properties, and on the physiological activity of substances with regard to their structural characteristics.

It is pointed out by Batyr that the subject of bioinorganic chemistry primarily involves the modelling of those biological systems and processes that require the participation of metal ions, the determination of the electron and geometrical structure of model complexes, as well as the investigation of natural coordinative compounds and the determination of their role in biological processes. The development of this field of science proceeds in parallel with that of certain aspects of molecular biology, facilitating the understanding of biological processes as represented by comparatively simple metal-complex models. Hence there is a certain degree of parallelism in approach which is important for maintaining progress in science and finding solutions to important industrial problems, such as obtaining new effective means of chemotherapy, the elucidation of the mechanisms of their effect, the synthesis of nitrogen-fixing enzymes, and arriving at optimal forms of microfertilizers and fodder additions for domestic animals.

It has been found that in spite of the negligible amount of metal in natural metal-complexes, the function of the metal atom is extremely important. Thus, by chemical methods, it is possible to extract the central atom of iron from the haemoprotein without disturbing the conformation of the organic part of the ligand, but the haemoprotein then loses its biological function. In more detail Batyr discusses haemoglobin and vitamin B_{12}, since these are the more intensively studied of the metal-bearing natural compounds.

Chapter 3

TRACE ELEMENTS AND ENZYMES

The discoveries which have made trace elements widely known as constituents of many enzymes have played a major role in disclosing the physiological significance of trace elements, and have transformed the science of trace elements into an active biological discipline. It has become evident that trace elements are involved in almost all known processes in which substances are synthesized and transformed with the aid of enzymes, and that the latter contain trace elements as their constituent parts. The activation of enzymes by metals has been reported for over 200 enzymes. Fifteen different metal cations with atomic weights ranging from 11 to 55 are capable of activating enzymes.

The science of metalloenzymes is at present a period of vigorous activity. In order to illustrate the intensity of research in this field, it may be recalled that particularly striking discoveries concerning the involvement of metals in flavoprotein catalysis were announced almost simultaneously and independently in a number of laboratories within the space of one year.

Recent years have witnessed the publication of a large number of studies on the involvement of trace elements in various enzyme systems, and the mechanism of their action. The science of metalloenzymes has become, as indicated long ago by Gurd and Wilcox (1956), a central issue in the physiology and biochemistry of plants and animals. The progress achieved in the study of metalloenzymes is important, not only for the elucidating the physiological significance of trace elements, but also for enzymology itself in the context of the problem of enzyme action - one of the focal points of research in modern biochemistry. The investigation of some metalloenzymes is of particular interest in connection with the intimate molecular interaction mechanisms between the catalytically active sites of enzymes and substrates, co-enzymes, and specific inhibitors. Understanding of the details of the molecular structure of the active sites of enzymes can also be gained from studies of their metal complexes. This is another reason for the considerable efforts made by biochemists in the study of metal complexes.

There are numerous interesting review papers on metalloenzymes and the mechanism of metal participation in enzyme catalysis (McElroy and Nason, 1954; Gurd and Wilcox, 1956; Peive, 1961; Nicholas, 1961; Gorkin, 1964; Singer, 1968; Parisi and Vallee,1969; Severin et al., 1970; Vallee and Wacker, 1970; Coleman, 1971; Eichhorn, 1973; Ganelin and Lvov, 1975; Frieden, 1976; Peive, 1976; Peive and Zhiznevskaya, 1976).

The classification proposed by Hoch and Vallee (1958) is now widely accepted. They subdivided metalloenzymes into metal-containing enzymes and metalloenzymic complexes (Table 1, 2).

Malmstrom et al. (1962) focused their attention on the difference between metalloenzymes, on the one hand, and enzymes that are activated by metals on the other. In metalloenzymes the metal ion performs its function in association with the protein moiety, while in enzymes activated by metal ions, the proper function of the metal is not dependent on the presence of a stable enzyme - metal complex. There are numerous enzymatic reactions in which a metal acts simply by being present in the reaction medium, and no compounds of the metal with the enzymes involved have ever been isolated in such reactions.

Every year the list of enzymes regarded as metalloenzymes is extended. The following were studied during the period 1973-1975: yeast alcohol dehydrogenase containing manganese as a prosthetic group (Coleman and Weiner, 1973); 8-aminolevulinate dehydratase from beef liver (Chen and Neilands, 1973), which has zinc as a co-factor; neutral proteinase I and II from Aspergillus sojal, a zinc enzyme (Sekine, 1973); endopeptidase-thermolysin, a zinc and copper enzyme (Bigbee and Dahlquist, 1974); NAD-dependent isocitrate dehydrogenase, a manganese-dependent enzyme (Morris, 1975); glutamate dehydrogenase, a zinc enzyme (Igoshina and Kositsyn, 1975; Polikarpochkina, 1975).

The dividing line between metal-containing enzymes and metalloenzymic complexes is, to a considerable degree, arbitrary. As indicated by Gorkin (1964), some enzymes may be regarded as true metalloenzymes in some of their properties (the strength of the bond between the metal and the enzyme), whereas in other characteristics (the possibility of the reactivation of apoenzymes by various metals) they resemble metalloenzymic complexes.

Recently α- and d-mannosidases, which can be activated by zinc, have been isolated from Canavalia ensiformis (Snaith and Levvy, 1968). These enzymes, unlike typical metalloenzyme complexes, are

TABLE 1 Zinc metalloenzymes and zinc metalloproteins (after Parisi and Vallee, 1969)

	Co-factor	Mol.wt.	In atoms per molecule	Source
Carbonic anhydrase	None	30,000	1 Zn	Bovine erythrocytes
Carbonic anhydrase	None	28,000	1 Zn	Human erythrocytes
Carbonic anhydrase	None	29,000	1 Zn	Maca mulata erythrocytes
Carboxypeptidase A	None	34,300	1 Zn	Bovine pancreas
Carboxypeptidase A	None	36,500	1 Zn	Pacific spiny dogfish pancreas
Carboxypeptidase B	None	34,300	1 Zn	Porcine pancreas
Carboxypeptidase B	None	34,300	1 Zn	Bovine pancreas
Neutral protease	None	44,700	1-2 Zn	Bacillus subtilis
Alcohol dehydrogenase	NAD	150,000	4 Zn 4 NAD	Yeast
Alcohol dehydrogenase	NAD	80,000	2-4 Zn 2 NAD	Horse liver
Glutamic dehydrogenase	NAD	1,000,000	2-6 Zn	Beef liver
d-glyceraldehyde-3-phosphate dehydrogenase	NAD	137,000	2-3 Zn	Swine muscle Beef muscle Yeast
Lactic dehydrogenase	NAD	Unknown	Unknown	Rabbit muscle
Malic dehydrogenase	NAD	40,000	1 Zn	Beef heart
d-lactic cytochrome reductase	FAD	50,000	4-6 Zn	Yeast
Alkaline phosphatase	None	89,000	4 Zn	Escherichia coli
Alkaline phosphatase	None	Unknown	Unknown	Swine kidney
Alkaline phosphatase	None	Unknown	Unknown	Human leucocytes
Aldolase	None	65-75,000	1 Zn	Yeast
Aldolase	None	50,000	1 Zn	Aspergillus niger
Aldolase	None	30,000	2 Zn	Escherichia coli
Phospholipase	None	Unknown	Unknown	Bacillus cereus
Mannose 6-phosphate isomerase	None	Unknown	Unknown	Brewer's yeast
Amylase	None	50,000	4 Ca 0.5 Zn	Bacillus subtilis
Proteinase	None	26,000	2 Ca 1 Zn	Snake venom
Metallothionein	None	9,000	9(Zn+Cd)	Horse kidney Human kidney Rabbit liver
Procarboxypeptidase A	None	90,000	1 Zn	Bovine pancreas
Procarboxypeptidase B	None	67,400	1 Zn	Bovine pancreas

TABLE 2 Metal-enzyme complexes activated by zinc[@] (after Parisi and Vallee, 1969)

Enzyme	Activating metals
Glycylglycine dipeptidase	Zn^{2+}
Arginase	Zn^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}
Dehydropeptidase	Zn^{2+}
Alanyl and leucyl glycine dipeptidase	Zn^{2+}, Pb^{2+}, Cu^{2+}, Mn^{2+}, Sn^{2+}, Cd^{2+}, Hg^{2+}
Tripeptidase	Zn^{2+}, Co^{2+}
Glycyl-l-leucine dipeptidase	Zn^{2+}, Mn^{2+}
Carnosinase	Zn^{2+}, Mn^{2+}
Amino peptidase	Zn^{2+}, Co^{2+}, Mn^{2+}
Histidine deaminase	Zn^{2+}, Hg^{2+}, Cd^{2+}
Lecithinase	Zn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}
Enolase	Zn^{2+}, Mg^{2+}, Mn^{2+}
Yeast and Clostridium aldolase	Zn^{2+}, Fe^{2+}, Co^{2+}
Oxaloacetic decarboxylase	Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, Ba^{2+}, Cu^{2+}
Dihydroorotase	Zn^{2+}
l-mannosidase	Zn^{2+}

[@] Other metals which are observed to increase the activity are also listed.

activated specifically by zinc and are inactivated by Co^{2+}, Ca^{2+} and Cu^{2+}. However, the zinc is only loosely bound with the protein moiety of the enzyme, in contrast to true metalloenzymes. Consequently, α- and d-mannosidases combine characteristics typical of both metalloenzymic complexes and metalloenzymes.

Gorkin believes that the subdivision of metalloenzymes into true metalloenzymes and metalloenzymic complexes is certainly justified, since it reflects the results of existing methods of isolation, purification and assessment of metalloenzymic properties better than any other classification.

Severin et al. (1970) maintain that the classification of metalloenzymes should not be based on differences in the strength of the association between the metal and the protein. They have proposed a classification which takes the functional role of the metal in enzymatic catalysis as a criterion. In this respect, they divide all metalloenzymes into two groups: (1) those in which the metal functions at the active site - either by influencing the structure of the substrate and, probably, that of the coenzyme or the enzyme's functional groups - or by facilitating interaction between the components of an enzyme reaction, and (2) those enzymes in which the metal is bound to the protein at a point other than the active site. In the latter case, the cation may affect the active site

allosterically or take part in the interaction of the subunits, resulting in the formation of an active aggregate.

Boichenko (1963) subdivides metalloenzymes into five categories based on the incorporation of the metal into prosthetic groups of enzymes. The first category comprises metalloenzyme complexes, and metalloenzymes are divided into four categories (Table 3).

TABLE 3 Classification of metalloenzymes (after Boichenko, 1963)

Enzymes	Type of association between metal and enzyme
1. Enzymes non specifically activated by the following metals: Na, Mg, Al, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Mo, Cd, Cs, Ba	Metal not tightly bound to enzyme
2. Proteins containing Cu	Metal located at active site of enzyme
3. Proteins containing Zn	Metal located at active site of enzyme, which contains pyridine nucleotides
4. Haemoproteins containing Fe	Metal located at active site of enzyme, and firmly bound to porphyrins
5. Metalloflavoproteins containing Mn, Fe, Cu, Mo	Metals located at active site of enzyme, which contains flavins

The involvement of metals in enzymatic catalysis is an important topic that has been reviewed by McElroy and Nason (1954) and by Nason (1958); these authors summarized the key findings and made significant generalizations which are still valid. Nason believes that the special role of a metal in the enhancement of an enzyme's catalytic activity is determined by the binding between the metal ion and the protein moiety of the enzyme. Enzyme proteins may produce metalloorganic complexes in association with trace elements. Many amino acids, for example, produce chelate complexes with metals through the carboxyl or the amino groups.

Considering the typical characteristics of the side chains in proteins, Klotz (1954) concluded that metal complexes are usually established with the imidazole, carboxyl, phenolic and sulfhydryl groups. Since most proteins have many side chains, they can be expected to produce essentially stable complexes with various metals.

Nason (1958) considers that the action of a metal as a catalyst in combination with a protein or a prosthetic group may take one of three general forms:

1. The primary effect of the protein on the properties of the metal. An illustration of such an effect is given by the catalytic oxidation of ascorbic acid by the copper incorporated in ascorbate oxidase, which accelerates the reaction approximately 1000-fold compared with the effect of free copper. Moreover, no hydrogen peroxide is formed in this reaction, indicating that the mechanism of oxidation by the copper protein is different. Studies of this enzyme, as well as of copper-phenolases, have revealed that copper ions show modified properties when they are bound to protein, enabling the metal to participate effectively in electron transport.

2. The primary effect of the metal on the enzyme protein. The association of the metal with the protein alters the protein's charge; this, in turn, modifies the enzyme-substrate interaction.

3. Combined activity of the metal and the protein. Metals and proteins may act together to enhance the enzyme's catalytic activity. The metal, in this case, plays the role of a bridge, permitting the enzyme to interact with the substrate. The chelate structure established between the metal ion and the substrate is very important in this respect. Figure 3 depicts manganese as a bridging link established on active intermediate substances participating in enzymatic reactions (Smith et al., 1954). The bond with manganese is established via carboxyl and amino groups.

Klotz (1954) offers another explanation for the mechanism of participation of metals, according to which the function of the metal is to establish and stabilize an intermediate linkage in an organo-mineral complex. Moreover, a complexly associated and positively charged metal may enhance the local concentration of OH-groups. An illustration of this type of binding role of metal in a multicomponent complex may be seen in flavoproteins, which interact with a protein and substrate via a metal - such as iron.

In his comprehensive review on metalloenzymes, Gorkin (1964) presented further findings, supplementing those summarized in the cited reviews of McElroy and Nason (see also Nason and McElroy, 1963) Gorkin was the originator of the following concepts concerning the mechanism of participation of metal cations in enzyme reactions:

a) the metal is a constituent of the enzyme's active site

b) the metal gives rise to or stabilizes a conformation of the

protein molecule, which is required for the catalytic activity of the enzyme

c) the metal affects the substrate by changing its electron distribution with the result that the substrate enters into enzymatic reaction

d) the metal provides for the association of the co-enzyme with the apoenzyme, or activates the co-enzyme

e) the metal serves as a bridge linking the enzyme and the substrate in an intermediate complex

f) the role of the metal involves a combination of two or more of the mechanisms listed above.

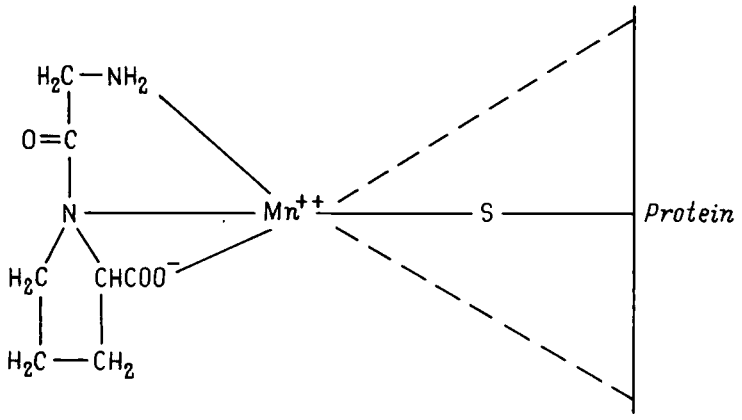


Fig. 3. Postulated coordination of glycyl-proline and prolidase (after Smith et al., 1954).

Gorkin offered confirmatory evidence to support these concepts of the mechanisms of action of metals in enzyme reactions. A typical case is that of zinc in carboxylase and carbonic anhydrase, where it is tightly bound in the active sites of the enzymes. The metal performs its catalytic functions without affecting the tertiary structure of the macromolecules.

The effects of metals on the activity of enzymes are not always attributable to their direct influence on the active site. Those metals which bond with functional groups of an enzyme protein, situated at a distance from the active site yet important for the maintenance of the enzyme's tertiary and quaternary structure, may appreciably affect the stability of the protein and may modify

the spatial configuration of the catalytic site and thus affecting the activity of the enzyme.

As an illustration, a reference may be made to the role of calcium in maintaining the conformation of α-amylase (Stein and Fischer, 1958). In the presence of calcium, the tertiary and quaternary structure of the protein macromolecule is stabilized. Treatment of α-amylase preparations with ligands binding calcium makes these preparations susceptible to proteolysis. Additions of calcium, as well as manganese, barium or magnesium, restore the initial resistance of α-amylase to proteolysis. Gorkin (1964) believes that calcium binds on to particular sites on the surface of the enzyme and thus stabilizes the enzyme by giving rise to a different conformation of the macromolecule.

Severin et al. (1970) reported that mono- and divalent metals act as allosteric activators. They referred to a series of studies carried out on glutamate synthetase, an enzyme which contains manganese. A molecule of native glutamate synthetase is composed of 10 subunits. Upon the removal of manganese, the compact, almost spherical molecule of the enzyme acquires a looser and somewhat more asymmetrical configuration in which the enzyme is inactive. This is not accompanied by dissociation of the enzyme into subunits. The reverse process - transformation into an active form - occurs when ions of divalent metals are added, this transition being essentially a two-stage process. The first, rapid phase is mediated by the divalent ions of many metals; no restoration of enzyme activity is observed at this stage. The active form of the enzyme emerges only after the second slow stage, carried out by incubating the enzyme with Mn^{2+}, Mg^{2+} or Ca^{2+}.

Reynolds and Schleisinger (1967) have shown that zinc is not only a component of the active site, but also supports the active conformation in alkaline phosphatase. A similar function is performed by metals in relation to other enzymes (Friedman and Epstein, 1967).

Interesting findings concerning the role of metals as associating links operating between enzymes and substrates have been reported in studies on the role of metals in phosphate transfer reactions. Fruton and Simmonds (1963) found that in the adenylate system the interaction of hexokinase with ATP is possible only if the latter contains one mole of bound magnesium per mole of the co-enzyme. Similar functions are performed also by alkaline metal ions.

For maximum enzymatic activation of the transphosphorylation reaction to occur it is necessary that, as well as divalent magnesium or manganese ions, monovalent metals, such as potassium, rubidium or the ammonium group, be present in the medium. In the presence of these ions the spatial distribution of reactive groups of the co-enzyme is modified so that these groups become particularly active during transphosphorylation. Lowenstein (1960) believes that ATP displays the highest activity in transphosphorylation reactions only in those cases when it is bound within a complex.

Hellerman and Stock (1938) and later Smith et al. (1954) also reported that a metal may serve as a bridge between enzyme and substrate.

L-arabinose isomerase isolated from Lactobacillus gayonii becomes inactive when the metal is removed by dialysis against EDTA and the unbound complex-forming agent is washed out (Nakamoto and Yamanaka, 1969). The enzyme activity can be restored by additions of Mn^{2+}, but a number of other mono- or divalent cations are not effective. Manganese lowers the Michaelis constant for arabinose, i.e., it facilitates the formation of the enzyme-substrate complex (for a review see Goudot, 1974).

Interesting data have been obtained in studies of the role of metals in the formation of an apoenzyme-co-enzyme complex. Reference will be made to some of the examples given in the paper of Severin et al. (1970) illustrating that metals are involved in co-enzyme binding.

Goudot (1974), in the context of metalloenzyme-substrate complexes, divides metals into two large groups: (1) metalloenzymes in which cations of a divalent metal are bound to the co-enzyme by two covalent bonds resulting in a coordinate 6-valence metalloenzyme-substrate complex; in this case the metal may be substituted for another as, for instance, in dipeptidases; (2) metalloenzyme complexes with 4 valencies and a planar configuration, the metal occupying the centre of the co-enzyme. In the centre of the transitional complex of the metalloenzyme-substrate, the metal acts not only as an intermediate link between the enzyme and the substrate, but also as an agent for the transfer of charges, delocalizing electron charges on ligands as well as on substrates. This is the result either of the metal's elevated cationic field (it attracts electrons from the ligands), or of its vacant internal orbits (in the case of the transition metals), which are able to accomodate

electrons from the ligand. In this case the transfer of electrons in a metal initiates chemical reactions.

Metals act as bridges in a number of NAD-dependent dehydrogenases (Vallee, 1960). The participation of the adenylate nucleus of NAD in the interaction with a metal is indicated in studies of the exchange of $^{65}Zn^{2+}$ ions in horse liver alcohol dehydrogenase with zinc in solution. This exchange occurs in the presence of the co-enzyme and substrates and their analogues. Evans and Rabin (1968) believe that the nicotinamide of the co-enzyme is involved in the association with zinc.

Schellenberger and Hübner (1967) have reported that purified pyruvate carboxylase requires magnesium and thiamine pyrophosphate for full catalytic activity. An active complex, which does not dissociate even after Sephadex chromatography, is formed when the apoenzyme is incubated with both co-factors. Binary complexes - enzyme-magnesium and enzyme-thiamine pyrophosphate - are produced rapidly and reversibly as a result of preincubation of the apoenzyme with magnesium or thiamine pyrophosphate. Such complexes, however, are unstable and disintegrate during gel filtration. Consequently, thiamine pyrophosphate can associate with the apoenzyme in the absence of magnesium, but this association is unstable. A complex arises only after the establishment of a second bond formation through a metal.

In xanthine oxidase which contains molybdenum, the metal associates at the same time with the protein, the substrate and flavin (Bayer and Voelter, 1966). Divalent metals ions, particularly zinc, easily form coordinate bonds with polar groups containing oxygen, nitrogen and sulfur, and also take part in the binding of nicotinamide co-enzymes to apoenzymes (Kosower, 1962).

A metal in an enzyme affects not only the enzyme's activity, but also the specificity of its action. Kretovich (1967) refers to carboxypeptidase A, an enzyme displaying two catalytic activities, one involving the hydrolysis of peptide bonds (the enzyme thus being a peptidase), the another, as in the case of other proteolytic enzymes, involving the hydrolysis of complex ester bonds (so that the enzyme is also an esterase). If the zinc in carboxypeptidase is replaced by cobalt or cadmium, the specificity of this enzyme changes dramatically. If, however, zinc is substituted for cobalt, the enzyme's peptidase activity sharply increases while the esterase activity is reduced. When, however, zinc is substituted for cadmium, the esterase activity of the enzyme rises consi-

derably, while its peptidase activity almost completely vanishes.

To illustrate the important role played by trace elements in the activation of a great variety of enzymes, a reference to tables compiled by McElroy and Nason (1954) is appropriate. Magnesium and manganese, and sometimes zinc, cobalt, chromium and rubidium, are involved in the activation of the enzymes of carbohydrate metabolism. Magnesium and manganese play a major role in the activation of the enzymes of the citric acid cycle together with cobalt, aluminum, chromium and potassium. Here again, magnesium and manganese, and occasionally calcium, are involved in the activation of phosphorylation enzymes.

The polyfunctional nature of metal ions in metal-containing enzymes deserves special emphasis. The same metal can perform several functions in the same enzyme. A metal ion may be essential for the catalytic process while it also stabilizes the tertiary or quaternary structure, as occurs, for example, in E. coli alcohol dehydrogenase or alkaline phosphatase where zinc is involved. Ions of various metals may perform various functions in the same enzyme, e.g. Ca^{2+} and Zn^{2+} in amylase from Bacillus subtilis.

An issue of major importance is that of the specificity of the function of the metal. Thus carbonic anhydrase, carboxypeptidase, alcohol dehydrogenase and alkaline phosphatase of E. coli are zinc-containing enzymes, and in each of them different amino acid residues are involved in the incorporation of zinc for enzymatic activity.

The majority of metalloenzymes have been discovered in animals. It may then be asked whether a priori these same metalloenzymes occur also in plants? Parisi and Vallee (1969) emphasize the necessity of a phylogenetic approach to this problem as illustrated by studies carried out on alcohol dehydrogenase and carboxypeptidase. These enzymes were shown to contain zinc and have been found in many animal species; Parisi and Vallee believe that in plants they may function in the absence of metals. Thus, carboxypeptidase isolated from citrus fruits and some other angiosperms is not inhibited by EDTA or orthophenanthroline and is apparently not a zinc metalloenzyme. This is also the case with aldolase. Warburg and Christian (1943) have shown that yeast aldolase is inhibited by chelating agents, whereas the enzyme isolated from animal muscles is not affected. It was found later that yeast aldolase from Aspergillus niger and Escherichia coli contains zinc. At the same time, aldolase isolated from muscles, liver and higher plants has no zinc

and is not inhibited by metal-complexing agents. Such findings, according to Parisi and Vallee, indicate that it is inadvisable to extrapolate evidence on the nature of an enzyme obtained from one species to that of another species, simply because in both species the enzyme concerned performs similar functions. Analogies drawn between phylogenetic types are only valid if they have been supported by experimental evidence.

Some metabolic reactions have been shown to occur non enzymatically and are realized with the aid of trace elements, this being another of the possible functions of trace elements in metabolism. The existence of such a mode of action of trace elements (Mn, Ni, Cu, Fe) has been established in the case of the oxidation of ascorbic acid (Bakardjieva, 1966). Under some conditions trace elements may act as plant oxidases. They change their valence in the course of the reaction they catalyse. This reaction has been studied both in vivo and in vitro for three elements with respect to their involvement in electron transport in isolated chloroplasts. In the reduced form, they are active donors of electrons.

Later Georgiev and Bakardjieva (1975) published a review of primary catalytic systems in biogenesis, and the structure-functional evolution of biocatalysts.

Boichenko (1966, 1968a, 1968b) puts forward original views on the involvement of metals in the evolution of oxidation-reduction processes in plants (see Part III, Chapter 1 "Trace Elements and the Evolution of Plants in the Biosphere").

Chapter 4

THE ROLE OF TRACE ELEMENTS IN NUCLEIC ACID METABOLISM AND PROTEIN SYNTHESIS

The progress achieved in the study of metalloenzymes has led some investigators to express strongly opposed views on the physiological role of metal trace elements. A tendency to conceive their role as being limited to enzymatic processes is evident. The major discoveries in molecular biology and the science of growth regulators, as well as the practical application of these findings in research on the physiological role of trace elements, have fully revealed the inadequacy of this point of view. Although trace elements do participate in the enzymatic reactions of nucleic acid metabolism and protein synthesis, this does not exhaustively cover their involvement in key metabolic processes. This chapter deals with the capacity of trace elements to produce complexes with nucleic acids, and to modify the physical properties, structure and biological functions of nucleic acids and ribosomes.

First their involvement in the function of the enzymes of nucleic acid metabolism will be discussed. Various metals - copper, molybdenum, lead, and especially zinc, have been found to inhibit plant ribonucleases (Holden and Pirie, 1955; Kessler and Monselise, 1959). Matsushita (1959) reported on the inhibiting effect of copper and zinc ions on the activity of 3-nucleotidase. A similar effect of cobalt ions has been noted, while divalent barium, calcium and molybdenum ions are found to enhance this enzyme's activity. Herman and Wright (1959) observed that 5-nucleotidase from Clostridium vinelandii required a reduced metal for its activity. Divalent iron has proved to be the most active ion, acting as both reducing agent and activator.

Intracellular ribonucleases from various sources have been found to be inhibited by EDTA, and to require Mo^{2+} and other cations for their activity (Matsushita, 1959; Maver and Greco,1962). Mo^{2+}, Hg^{2+}, Ag^{2+}, Co^{2+} and Ni^{2+} provided protection for m-RNA against a ribonuclease attack (Nishimura and Novelli. 1963).

Findings have been reported that metals may inactivate ribonuclease inhibitors. Thus Cu^{2+} and Zn^{2+} produced this effect, whereas Mo^{2+} failed to act likewise (Wojnar and Roth, 1964).

Breslow and Girotti (1966) discussed possible mechanisms for the inhibition of ribonuclease by copper and zinc ions. They

suggested that the inhibition observed is due to a modification of the enzymes's conformation by these elements or to alterations involving the active site. The inhibition of ribonucleases by zinc has been ascribed also to the ability of this metal to produce complexes with RNA that are resistant to ribonuclease attack (Wojnar and Roth, 1964). On the other hand, the inhibition of ribonuclease by copper is probably associated with the ability of copper to form complexes with the enzyme, which also may result in inactivation of the enzyme (Saundry and Stein, 1968). A considerable increase in ribonulcease activity under conditions of boron deficiency has been observed. These findings will be discussed later.

Metals have been shown both to inhibit and to activate deoxyribonucleases too. Wiberg (1958) reported an increase in the activity of these enzymes produced by Mo^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} and Co^{2+}. Stone and Burton (1962) corroborated this finding, having shown that Mo^{2+}, Mn^{2+} and Fe^{2+} activate DNase obtained from Streptococcus haemolyticus. The action of the latter ions is enhanced by Ca^{2+} and Sr^{2+}, whereas Co^{2+} and Zn^{2+} are potential inhibitors of this enzyme. Similar observations have been made in studies of the effect of streptococcal DNase on calf thymus DNA: the enzyme was activated by molybdenum, manganese, strontium, calcium, zinc and cobalt ions and completely inactivated by EDTA (Potter and Laskovski, 1959). Either molybdenum ions or manganese ions are required for activity of the exonuclease induced in Escherichia coli K-12 by bacteriophage 2 (Little et al., 1967a).

Sedykh et al. (1967), having studied the effect of divalent metal ions and EDTA on the activity of DNase from Bacillus amylozyma and Serratia marcescens, concluded that molybdenum, and to a lesser extent, manganese activate the enzyme from B. amylozyma, calcium is ineffective, whereas EDTA completely inactivates the DNase. The enzyme from S. marcescens could be activated only by molybdenum ions; manganese failed to stimulate, whereas calcium and EDTA inhibited the enzyme's activity. The observed elevation or inhibition of the enzymes from these organisms produced by the elements tested was found to closely correlate with the asymmetry of the enzymes' macromolecules.

Dvorak and Heppel (1968) have found that zinc is a component of 5-nucleotidase and cyclic phosphodiesterase. Cyclic phosphodiesterase from Pellicularia H-11 was active only in the presence of zinc ions.

Magnesium and manganese have been shown by numerous investigators to be essential for the activity of DNA and RNA polymerases (Pegg and Korner, 1967).

Manganese, in addition to magnesium, has been shown to be essential for the polymerization of ATP and UTP in polyadenylate and polyuridylate by RNA-polymerase (Smith et al., 1967). Hsieh and Buchanan (1967) observed that a manganese enzyme from Escherichia coli Q-13 was able to polymerize nucleosidediphosphates. The activity of RNA nucleotidyl transferase has been found to be manganese dependent (Klemperer and Haynes, 1967).

Changing the ionic composition of media used to incubate isolated nuclei has been found to bring about a switch-over to the synthesis of different types of RNA of different base composition. These phenomena were accounted for by a selective activation of different RNA-polymerases showing different preferences for magnesium and manganese ions (Pogo et al., 1967). Zylber and Penman (1971) incubated isolated HeLa nuclei with ^{3}H-nucleoside triphosphates. Nuclei incubated in the presence of 5 mM Mg^{2+} synthesized RNA with sedimentation constants ranging from 6 to 100 S. Substitution of magnesium ions for manganese ions resulted in suppression of nucleolar RNA synthesis and did not affect RNA synthesis on the extranucleolar DNA. DNA polymerase isolated from etiolated maize seedlings showed the same activity with magnesium and manganese ions alike at low ionic strength (Stout and Belva, 1975).

Steck et al. (1968) studied the effects of divalent cations on the activity of RNA-polymerase from Micrococcus lysodeikticus, using synthetic polynucleotides as templates. It was found that various combinations of manganese and magnesium influenced initiation efficiencies and the synthesis of RNase-made products in systems with poly-A, poly-U and poly-C. When poly-C was used as a template, the requirement of the system for divalent cations was not observed to be dependent upon the concentration of substrates. However, in the case of poly-A and poly-C, a narrow range of the optimum concentration of ions (approximately equimolar with the substrate concentrations) was found.

All polynucleotide phosphorylases (except for that isolated from yeast) are activated by magnesium ions, and to a lesser degree by other divalent ions (manganese, barium, calcium, zinc)(Steiner and Beers, 1961).

It has been shown in a number of studies that some nucleotidyl transferases are metalloenzymes and zinc has been identified as

the metal component. New evidence was reported indicating that tRNA nucleotidyl transferase from E.coli is a metalloenzyme (Williams and Schofield, 1975).

The exchange reaction between ^{3}H-nicotinamide mononucleotide and NAD catalysed by DNA-ligase from Escherichia coli has been shown to be dependent upon magnesium and manganese (Little et al., 1967b). Scarano et al. (1967) found that in the absence of magnesium ions, deoxycytidine triphosphate, deoxythymidine triphosphate and deoxy-5-methylcytidine triphosphate had no effects on the activity of deoxycytidylate aminohydrolase. The true substrates of the enzyme were complexes of these three compounds with magnesium, calcium or manganese, which are quoted here in descending order of effectiveness in forming substrates for the enzyme.

A cobalt-containing compound, cobaltmethylcorinoid, has been found to act as a donor of methyl groups for tRNA methylation (Walerych et al., 1966).

Manganese, molybdenum and calcium ions have proved to be indispensible for DNA synthesis (Mantsavinos and Cannelakia, 1959). Ca^{2+} has been shown to act synergistically with Mg^{2+} in the stimulation of DNA synthesis. Manganese by itself stimulates the process as efficiently as calcium and magnesium together; however, an addition of Mg^{2+} is found to enhance the stimulating activity of Mn^{2+}.

Nakamoto et al. (1964) reported that RNA synthesis required the presence of all four ribonucleoside triphosphates as well as Mn^{2+}, Mo^{2+} and Co^{2+}. Homopolymeric RNA could be synthesized with only one ribonucleoside-5-triphosphate together with Mn^{2+} in the incubation medium.

It was observed that for the synthesis of RNA from its ribonucleotide precursors, Mg^{2+}, Co^{2+} or Mn^{2+} is essential. Substitutions of other metals for these ions, or considerable deviation of the concentration from the optimal value, result in transcriptional errors. Metal ions can also interfere with the DNA structur in DNA-protein complexes of chromatin (Eihorn et al., 1977).

Zinc has been reported to be essential for DNA replication in rat liver cells (Fujioka and Liberman, 1964). EDTA was shown to inhibit DNA synthesis enhanced by partial hepatectomy, and only molybdenum ions were effective in counteracting this inhibition. Zinc stimulated DNA synthesis under post-operative conditions and this stimulation was concentration-dependent.

Turkington (1968) has shown that Li^+ and NH_4^+ suppress DNA synthesis whereas K^+, Mg^{2+} and Ca^{2+} do not. Lithium was found to prevent the stimulation of casein synthesis by insulin, hydrocortisone and prolactine. The author concluded that this effect involved the inhibition of DNA replication.

Magnesium at a concentration of 0.01 M markedly inhibited the hydrolysis of tRNA by exo- and endonucleases (Nishimura and Novelli, 1963). Ions of other divalent metals - Mn^{2+} and Co^{2+} - had similar effects. These workers suggested that magnesium may affect the physical properties of tRNA by giving rise to a more rigid secondary structure showing a higher resistance against ribonuclease attack. This assumption was supported by the finding that in the presence of 0.01 M magnesium chloride the melting temperature of tRNA was shifted by 5^oC.

Using nuclear magnetic resonance, Cohn (1963) showed that manganese activates enzymatic reactions involving nucleotides. Studying the interaction of four crystalline kinases with their substrates, he found that there were enzymes which formed a Mn^{2+}-enzyme complex, whereas other enzymes formed a Mn^{2+}-enzyme-substrate complex. In the case of creatine kinase, manganese does not act as a bridge between enzyme and substrate, but forms complexes with the nucleotides; only then does the metal-nucleotide complex bind to the enzyme.

The demonstration of the ability of metals to produce complexes with nucleic acids has significantly increased understanding of the involvement of trace elements in nucleic acid metabolism. DNA preparations isolated from microorganisms and animal tissues have been shown to produce complexes with calcium, manganese, copper and other metals (Dove and Davidson, 1962). Not only do metals produce complexes with nucleic acids in vitro, but such complexes have been detected involving nucleic acids in situ. Even highly purified preparations of nucleic acids isolated from various sources contain traces of one or more metals (Wacker and Vallee, 1959a, 1959b; Belokopylsky et al., 1968).

Wacker and Vallee (1959a, 1959b), in studies of highly purified RNA preparations isolated from various organs of mammals and from Euglena gracilis, invariably observed various amounts of magnesium, calcium, strontium, barium, aluminium, iron, copper, chromium, nickel, cobalt, manganese, zinc and vanadium bound to RNA molecules. These metals were present in bovine DNA preparations as well, but in concentrations three times lower. Some RNA preparations

also contained calcium and lead. The authors suggested that there might be a specific association between some metals and some specific RNA types.

Loring and Waritz (1957) showed that considerable amounts of metal remained in a viral RNA preparation after a 24-hour dialysis, and it took 7 days of dialysis markedly to reduce the amount of metal present initially; this reduction was accompanied by a loss of infectivity of the preparation. Kriss and Yatsimirsky (1966) also presented abundant evidence of associations between metals and nucleic acids; moreover, the compounds formed frequently displayed properties other than those of the original nucleic acids. Thus DNA has been shown to form complexes with copper, manganese, mercury and other metals.

In order better to understand the mechanism of association of metals with polynucleotides, the formation of metal-nucleotide complexes should first be considered.

Some purine derivatives may form various complex compounds with copper (Weiss, 1961). Zakharenko and Moshkovsky (1966) also reported that copper ions are able to form complexes with DNA and its degradation products.

Cohn and Hughes (1962), using nuclear magnetic resonance, were able to detect modifications in the structure of ATP and ADP as a result of their association with various divalent ions. Mg^{2+}, Cu^{2+} and Zn^{2+} produced complexes with the α- and β-phosphate groups of ATP, and with the α-and β-groups of ADP. Neither Mg^{2+} nor Ca^{2+}, but Zn^{2+} formed a complex with the adenyl ring of ATP. Ca^{2+} was found to interact exclusively with the α- and β-phosphate groups, and Mn^{2+} and Co^{2+} with the α-β and γ-phosphate groups of ATP.

Siegel (1975) showed that there is a strong interaction between the metal ion and the nucleic acid residue in nucleotide complexes with Mn^{2+}, Ni^{2+} and Zn^{2+}. This worker suggested a structure for the complexes involving intramolecular interaction with a transfer of charge between the purine and pyrimidine components.

Interestingly boron, which is not a metal, is also capable of producing complexes with nucleic acids. Employing potentiometry, Weser (1967) established that there is dependence of the ionization constants of boric acid upon the concentration of various ligands (ribose, inosite, uridine, d-galactose, d-glucose, d-ribose), and showed that boron chelates are produced in all cases with a ratio of 1:2 (one boron atom per 2 atoms of the ligand). The constants for the formation of boron chelates are of the same

order of magnitude as those pertaining to the formation of complexes between some earth metal ions and nucleotides. Numerous biochemical reactions are associated with nucleotides, and the latter are influenced by divalent metal ions which become associated with the phosphorus groups or the bases. These facts were the basis of the suggestion that borates may bind to the hydroxyl groups of the ribose residue at the 2' and 3' positions. Kúthy (1956) reported that boron produces a complex compound with ATP, which when illuminated releases one molecule of phosphoric acid with greater ease than does pure ATP. Apparently boron serves to enhance the photosensitivity of ATP.

According to Szer (1966), the monovalent cations Na^+, K^+ and Li^+ form weaker complexes with, for instance, polythymidylic acid, than do the divalent cations Mg^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+}.

Heath (1949) has found that zinc is firmly bound to deoxyribonucleoproteins. It has been reported that zinc exhibits a pronounced tendency to form complexes simultaneously with different regions of polynucleotides, however subsequent investigations have revealed that zinc ions bind largely to the phosphate groups in DNA molecules, and the association between this element and guanine is much weaker.

It has been shown that the formation of complexes of molybdenum and RNA (the latter isolated from pea leaves) is based on the phosphate groups and involves charge transfer, especially in an acid environment (pH 5). It has been found that the binding of the molybdate ion to the RNA structure is apparently more rigid than the binding to DNA; in the RNA complex there is a shift of the absorption bands in the UV spectrum of the RNA (Ivchenko, 1981).

Divalent copper ions form complexes with polynucleotides (synthetic poly-A, poly-U and poly-I) but do not interact with poly-U. Binding of the ions occurs because in the presence of Cu^{2+} no double helix is formed, and the addition of copper to a polynucleotide after the secondary structure is established results in the dissociation of poly(A-U) and poly(C-I) into separate chains (Eichhorn and Tarien, 1967).

Some information on the nature of the association between metals and nucleic acids is available. Employing the electron paramagnetic resonance technique, Eisinger et al. (1961) have shown that metals can bind to the phosphate groups of DNA. Eichhorn (1962) has shown that metals may enter a chelate binding with nucleic acids.

Dzierzewicz et al. (1976) investigated the binding of divalent metal ions to DNA, nucleotides and nucleosides. They examined the analytical methods used to locate the sites of ionic binding to the DNA molecule, and the methods used to evaluate changes in DNA structure induced by the binding of metal ions.

Eichhorn et al. (1966), using optical and radiospectroscopic techniques, studied the details of the complex formation between Cu^{2+} and DNA. The authors concluded that copper binds to bases and the phosphate groups of nucleotides. Yatsimirsky and Kriss (1966), having studied the kinetics of the complex involving DNA and Fe^{3+}, Cu^{2+} and Mn^{2+}, and having determined the association constants of the complexes formed, showed that the interaction of metals with DNA occurs largely through the phosphate groups. In the cases of copper and iron, however, evidence was presented that these also interact with DNA bases.

A conformational analysis performed with Stuard atomic models of the nucleotide-iron combination, has confirmed the possibility of simultaneous binding between a metal, a phosphate group and a base. Iron also interacts with the carbohydrate moiety of DNA, i.e. with deoxyribose (Goldstein et al., 1966).

Most probably the bases involved in the formation of complexes with metals are purines - adenine, and mostly guanine. The latter is very similar in its chemical structure and properties to a strong complex forming agent - 8-oxiquinoline. The constants of complex formation in the case of guanine are at least an order of magnitude higher than those pertaining to adenine complexes (Fiskin and Beer, 1965). In an examination of the interactions between copper and the following: guanosine, 2'-deoxyguanosine, 1-methylguanosine, 7-methylguanosine and guanosine-mono-phosphate, Maskos (1978) found that Cu^{2+} becomes bound to the guanosine residue, mainly to the seventh N-atom.

In the monograph of Eichhorn (1975), metal-nucleic acid interactions are discussed. Much attention is paid to the metal-nucleotide and metal-nucleoside complexes, and to the structure and biological function of metal-complexed polynucleotides.

Ivchenko (1981) points to the fact that purines have a conformation suitable for the formation of chelates: the seventh nitrogen atom in adenine and guanine molecules, as well as the heteroatom located outside the ring and associated with the sixth carbon atom are the most suitable sites for the formation of a 5-member chelate cycle with a highly stable conformation.

When Wacker and Vallee (1959a, 1959b) showed that metals are present even in highly purified preparations of nucleic acids, they suggested that metals probably play a role in maintaining the conformation of DNA molecules, by binding purine and pyrimidine bases through covalent bonds. They suggested also that metals are functionally involved in protein synthesis or the transfer of genetic information. According to these authors, a knowledge of how metals combine with nucleic acids may lead to a better understanding of the details of biological function and specificity of these compounds. They believed that the metals present in nucleic acids may have a significant biological role in stabilizing the nucleic acid structure, by forming intermolecular linkages through metal ions in a "sandwich" manner. A specific complex formation between protein and nucleic acid, according to Wacker and Vallee, depends upon the presence of metal ions.

Subsequent research carried out by numerous other investigators has provided support for some of the suggestions advanced by Wacker and Vallee.

The physico-chemical properties of nucleic acids are determined very much by the presence of metal ions. The rate of sedimentation of nucleic acids decreases in the presence of manganese, nickel, calcium and magnesium ions. Also, the viscosity of nucleic acids in the presence of these ions becomes less (Butler et al., 1954).

Various investigators (Dove and Davidson, 1962) have found that the ionic environment affects the melting temperature of nucleic acids. With increasing sodium chloride concentration in a DNA solution one may observe a linear dependence of melting temperature on the logarithm of the ionic strength of the solution; this is attributable to stabilization of the DNA structure against thermal denaturation. It is supposed that the addition of salt brings about screening of the electric charges on the DNA, and thus the forces of electrostatic repulsion between charged phosphate groups become weaker; this promotes greater rigidity of the whole structure. Manganese, cobalt, zinc and nickel ions have been shown to elevate the melting temperature of DNA, whereas copper, cadmium and lead ions lower it. Under conditions favouring the disruption of hydrogen bonds (heating solutions of nucleic acids to 80^{o}C or dissolving them in 6 M urea), ions of nickel, manganese, zinc and chromium elevate the melting temperature, whereas alkaline and earth metal ions fail to produce any changes.

Vlasyuk and Ivchenko (1975) demonstrated the effect of molybdenum both on RNA heterogeneity and on the quantitative relationships among different fractions of nucleic acids from pea leaves (the greatest effect was on transport RNA and DNA). A rise in DNA hyperchromicity, stabilization of its inner structure, and reduction of nucleic acid hydration (because of the greater degree of complexing) were observed. The formation of DNA-K_2MnO_4 complexes results in elevation of the melting point of DNA. The molybdenum ion bonds with phosphorilose, purine and pyrimidine residues.

Venner and Zimmer (1966) have studied the effect of copper on the melting temperature of DNA isolated from various sources (Clostridium mirabilis, Escherichia coli, wheat embryos, etc). The destabilizing effect of copper ions was explained by the interaction of Cu^{2+} with the guanines in the DNA molecules.

Hiai (1965) has shown that copper markedly lowers the temperature of transition from double helical DNA into a coil. He offered evidence indicating that copper ions act to disrupt the hydrogen bonds between guanine and cytosine in DNA. Venner and Zimmer (1964) had earlier reported that copper influences the helix-coil transition in DNA.

When K_2MoO_4 forms complexes with DNA the concentration of molybdenum in the DNA increases by almost one order of magnitude, so that on average, one atom of molybdenum is bound to each nucleotide. This results in the elevation of the melting temperature of DNA by approximately $2^{o}C$ (Ivchenko, 1981).

Fuwa et al. (1960) have found that transition metal ions of the first order stabilize the structure of nucleic acids by establishing additional linkages between the metal and the bases. The influence of some divalent ions is not limited to screening the phosphate groups of DNA. Thus Eichhorn(1962) demonstrated that copper, cadmium and lead lower the thermal stability of DNA in solution, whereas magnesium, calcium, barium, manganese,cobalt, nickel and zinc elevate the melting temperature of DNA.

Monin and Bekker (1967) have found that association of manganese with tRNA resulted in destabilization of its structure as revealed by thermal denaturation profiles measured by the proton relaxation technique.

Mahler and Cordes (1968) point out that for nucleic acids, which are polyanions, to remain electrically neutral, they must be acreened by appropriate numbers of cations in their immediate vicinity. The degree of binding of cations varies over a wide range

The alkaline metal cations Na^+ and Li^+ serve only to neutralize the negative charges of the phosphate residues and thus allow the nucleic acid molecule to take up the normal conformation. The behaviour of divalent cations such as earth metals and some transition metals (Co^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}), or organic diamines usually accompanying DNA in vivo, differs in that they are bound to the phosphate residues in a stoichiometric manner. These divalent cations exert a much stronger influence on the molecular conformation than do monovalent ions. Thus, at the equivalence point, one Mg^{2+} ion is equivalent in effect to 10^2-10^3 sodium ions.

According to Goldstein and Gerasimova (1963) iron plays a specific role in the functioning of DNA - i.e. this metal is involved in the establishment of bonds between individual DNA helices. Therefore iron may regulate the processes of denaturation of DNA and bring about a non-enzymatic transverse disruption of DNA chains. According to an hypothesis of Ivanov (1965), DNA biosynthesis is associated with oxidation-reduction transformations of iron or copper during chelate complex formation of the latter with DNA. The oxidation of the metals makes the bonds between complementary pairs of DNA bases weaker, and the reduction of these metals brings about a stabilization of the double helical conformation of DNA.

These authors further demonstrated that iron and ascorbic acid exert opposite influences on the stability of the DNA structure, iron increasing its stability and ascorbic acid reducing it. Similar conclusions have been arrived at by these investigators in their later work (Goldstein et al., 1966). Conversely, Ivanov and Minchenkova (1965) reported that in their experiments iron and copper appeared to destabilize the DNA structure, and reducing agents such as ascorbic acid, hydroquinone, etc. added to DNA in solutions containing ionic copper and iron, stabilized the double helical DNA conformation. In this context, it is interesting to note that iron has long been found to be associated with nuclear structures in the plant cell.

Metals (manganese, cobalt, zinc, nickel) have also been reported to increase the stability of the tobacco mosaic virus (Huff et al., 1964), whereas cadmium was found to act as an inhibitor of TMV activity (Ulrichova-Zelnikova, 1959). Shin and Eichhorn (1968) reported successful experiments on the reversible denaturation of DNA in solutions containing zinc ions.

Reversibility of nucleic acid structure arising from an ability to form complexes with metals is, according to Kriss and Yatsimirsky

(1966), one of the most interesting features of the association of nucleic acids with metals. An assessment of such reversible reactions accompanied by profound changes in the physical properties of nucleic acids may be an important tool in gaining further understanding of nucleic acid function. A similar reversibility is typical of the TMV RNA structure modified by mercury chloride (Katz and Santilly, 1962). In this case, however, the changes produced by mercury ions are not fully reversible. Mercury ions block the infectivity of the viral RNA, but removal of the ions does not completely restore the infectivity of the RNA.

Yatsimirsky and Kriss (1966) believe that the association of DNA with iron reported by Goldstein and Gerasimova (1963) belongs to this same type of reversible reaction. Trivalent iron ions slow down the hydrolysis of DNA. The introduction of sodium pyrophosphate, which binds iron in a solution containing iron and DNA, results in a resumption of DNA hydrolysis.

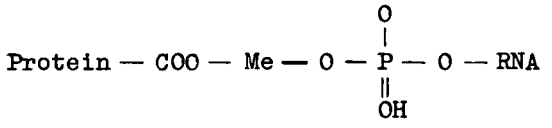
Magnesium has been found to influence the conformation of RNA (Staehelin, 1959). Poletaev (1973) reported the effects of various cations on the conformation of tRNA. According to his observations, tRNA in solutions of low ionic strength, and in the absence of divalent ions, is found in a partially disordered state. Additions of magnesium ions up to a final concentration of 10^{-4}M considerably increased the orderliness of the tRNA structure. Similar effects could be produced by earth metal ions, but these had to be added in concentrations 10,000 times higher.

Transition metals modify DNA conformation. By means of ultracentrifugation it has been shown that the ions of transition metals affect the hydrodynamic properties of DNA, so that additions of these metals suppress protonation at the sites of GC pairs. Earth metals failed to produce similar effects (Zimmer et al., 1974). Nanda and Govil (1975) carried out a theoretical analysis of metal ion binding to the phosphate group and the effects of metal ions on the conformational energy of the dimethylphosphate anion. These workers discussed the possibility of metals opening the double helix during replication. Williams and Loeb (1973) found that zinc was essential for the replication of DNA in phytohaemaglutinin-stimulated lymphocytes. Other divalent cations could not replace zinc in this function.

The significant effects of metals on the structure of nucleic acids should, according to Hershko et al. (1961), influence the function of nucleic acids in protein synthesis. Metal ions are

involved in maintaining the association between proteins and nucleic acids (Wacker and Vallee, 1959a). Kirby (1957) suggested that the association of nucleic acids, DNA in particular, with proteins is realized through the carboxyl groups of dicarbonic amino acids (aspartic and glutamic acids) with the involvement of a divalent metal. Such a linkage is a mobile one and operates like a "joint".

Bogdanov (1963) suggested that the binding of the carboxyl groups of proteins to phosphates in RNA molecules may be realized as follows:



The above evidence on complex formation between trace elements and nucleic acids, on the effects of metals on the physico-chemical properties and structure of nucleic acids, and on the synthesis and degradation of the latter, testifies to the major role played by trace elements in nucleic acid metabolism. In fact, recent years have witnessed the appearance of a series of studies concerning the role of trace elements in the nucleic acid metabolism of plant and animal cells. These studies will be dealt with in detail later in the book in the discussions on individual trace elements.

In the light of the important role of various metals in nucleic acid metabolism, their effects on protein synthesis are of great interest. This problem, however, has been given less attention hitherto, particularly as regards the effect of trace elements on the protein synthesizing machinery. Only limited information is available on the effects of metals on the activation of amino acids and their binding to transfer RNAs. Magnesium ions have been shown to be indispensible for the functioning of cell-free protein synthesizing systems. The transfer of amino acid residues from tRNA on to the protein in cell-free systems derived from higher plants has been reported to be significantly stimulated by the addition of manganese and GTP, provided that ribosomes were in excess (Mans, 1967). Hershko et al. (1961) have shown that manganese, calcium and spermine may substitute for one another in an in vitro protein synthesizing system. Calcium, manganese and cobalt were found to be essentially as effective as magnesium.

Elaev and Altaner (1964) reported on the effects of magnesium and cobalt on amino acid transfer to tRNA derived from cytoplasmic, mitochondrial and nuclear fractions from animal cells. The incor-

poration of labelled amino acids was considerably higher in systems based on cytoplasmic tRNA, compared with nuclear and mitochondrial tRNA systems. Incorporation in the cytoplasmic fraction was activated by magnesium, while cobalt activated incorporation in the nuclear and mitochondrial fractions.

Cobalt has also been implicated in a reduced accepting efficiency of tRNA in cases of vitamin B_{12} deficiency (Walerych et al., 1966). The positive role of cobalt in nitrogen fixation has been suggested to be linked with its involvement in the biosynthesis of nitrogenase, the most important enzyme in nitrogen fixation.

Borshchenko (1970) reported that boron deficiency did not result in the activity of aminoacyl-tRNA-synthetase in vitro. On the other hand, aminoacyl-tRNA complexes accumulate under conditions of boron deficiency in vivo. Hinde and Finch (1966), however, have reported that boron deficiency in beans results in a diminution of the activation of amino acids by root extracts as measured by ATP-^{32}P-pyrophosphate exchange.

Trace elements apparently play an important role in the biosynthesis of proteins on ribosomes. One of the probable reasons for this is their ability to stabilize the structure of ribosomes. Webster (1959) reported that the biological activity of isolated ribosomes depended on the supply of manganese, cobalt, cadmium and zinc, accumulated in plant cells. Trace elements, in particular magnesium, have been implicated in the formation of polyribosomes in cells (Moore and Asano, 1966). Boron deficiency results in a degradation of polysomes and ribosomal RNA (Borshchenko, 1970). Praske and Plocke (1971) observed a complete breakdown and disappearance of ribosomes in zinc-deficient Euglena gracilis cells.

In all types of cell, the association of ribosomal subunits is dependent on the presence of magnesium and calcium (Chao, 1957; Gordon and Lipmann, 1967); the removal of these ions from the medium brings about a dissociation of the ribosomes. Again, Webster (1959) reported that magnesium, manganese and calcium ions acting together have a more pronounced stabilizing effect on ribosomes isolated from wheat plants, pea plants and spinach chloroplasts, than that obtained with magnesium alone. Spirin and Gavrilova (1971) indicated that ions of other divalent metals (Be^{2+}, Sr^{2+}, Ba^{2+}, Ni^{2+} and Zn^{2+}) were apparently ineffective in maintaining the specific association of 70S ribosomes, whereas the stability of 50S and 30S subunits could be enhanced by NH_4^+, K^+, Rb^+ and Cs^+ (Belitsina et al., 1971). Sodium and lithium were shown to produce

a strong destabilizing effect on ribosomal association. Among divalent ions, calcium has the same stabilizing influence on the association of ribosomal subunits as that produced by magnesium, whereas manganese is a more potent stabilizing agent in this respect. Zinc, and particularly nickel, even in minor quantities, act as strong destabilizing agents.

Liautard et al. (1974) reported on the effects of alkaline and earth metals on the dissociation of ribosomes isolated from HeLa cells. Strom et al. (1975) studied the effect of the ionic composition of the incubation medium on the dissociation of 50S ribosomal subunits isolated from extreme halophiles. The removal of potassium and magnesium ions was shown to result in the splitting off of some ribosomal protein and 5S RNA.

Tal (1968, 1969) has shown that magnesium, manganese, zinc and nickel significantly enhance the thermal stability of ribosomes. Viscosimetric measurements have also confirmed the important role of trace elements in maintaining the optimum conformation of ribosomes. In re-association experiments nickel proved to be the most effective metal ion in promoting the restoration of ribosomal particles from their subunits. Similar effects were noted with manganese, zinc and cobalt, whereas calcium, strontium, barium and magnesium were less effective.

Backer et al. (1975) used nuclear paramagnetic resonance and electron spin resonance to study the screening of magnesium ions by aminoacyl-tRNA synthetase in the aminoacyl-tRNA-synthetase-tRNA complex at pH 7.5. This effect was not observed at pH 6. It was suggested that the ions interacted with some of the aminoacyl-tRNA synthetase groups that were protonated at a slightly acid pH. The authors discussed the role of manganese and magnesium ions in the formation of a functionally active tRNA-aminoacyl-tRNA-synthetase complex.

The competition of aminoacyl-tRNA-synthetase and low molecular weight compounds for the same sites as those binding magnesium ions, coordinated by tRNA at pH 7.5, may indicate that the specific coordinating sites of the ions are screened at the sites of contact of tRNA with aminoacyl-tRNA synthetase. Ions coordinated by tRNA interact with aminoacyl-tRNA synthetase only within a narrow pH range.

Calcium and manganese are able to substitute for magnesium in cell-free protein synthesizing systems (Hershko et al., 1961).

Evidence has been obtained on the involvement of metals in the formation of the peptide bond on ribosomes. Thus magnesium and potassium have been found to be necessary for peptide bond formation, whereas berillium inhibited the process. Peptidyl-transferase involved in polypeptide formation may be activated by ions of some mono- and divalent metals. Monovalent cations in high concentrations may dislodge magnesium from the ribosomes thereby dissociating ribosomal proteins and neutralizing the phosphate and hydroxyl groups of these proteins. In experiments on the effects of copper sulphate on the activation and incorporation of amino acids in a cell-free protein synthesizing system derived from seed lobes and leaves of tomato plants, it has been shown that copper ions inhibit the activity of ribonuclease and thus promote the incorporation of amino acids into proteins (Hall and Cocking, 1966). Magnesium also stimulated amino acid incorporation; zinc inactivated ribonuclease and in this way increased the incorporation rate.

A shortage of manganese has been implicated in cases of reduced protein synthesis, since manganese and magnesium have repeatedly been shown to stabilize polysomes (Vielemeyer, 1971).

Working with isolated pea roots, Abbot (1966) has shown that cell elongation as well as differentiation processes are disturbed, when there is a manganese deficiency; the cells contained less protein and RNA than normal cells, suggesting that manganese is concerned with maintaining the association of protein with RNA.

In Tetrahymena pyriformis cells, lithium inhibited protein synthesis (Volk et al., 1958). In a cell-free system derived from Escherichia coli a study was made of the effect of lithium on the association of phenylalanyl-tRNA with polyribosomes on poly-Y templates. Additions of lithium chloride to the system suppressed the binding of aminoacyl-tRNA with the polysomes, but did not affect the activity of aminoacyl-tRNA synthetases. These effects were apparently specific for lithium, since neither potassium nor ammonium chloride produced a similar effect. Moreover, lithium chloride caused the dissociation of ribosomes into subunits.

An effect of lithium on DNA conformation has been discovered, the conformation being an indicator of DNA metabolic activity. A specific effect is produced by lithium on the protein synthesis reaction, involving the process of aminoacyllation of tRNA by different aminoacids (Vlasyuk et al., 1979).

Primack (1975) found that protein synthesis in rat mitochondria could be stimulated in vitro by 1-10 M Cu^{2+}, Ag^{+} and Au^{3+}. Copper

and silver caused the mitochondria to swell. The results were interpreted as indicating that copper plays a definite role in the control of protein synthesis in mitochondria.

The effects of trace elements on protein biosynthesis may be attributable to their stabilizing influence on the ribosomal structure, and to the inactivation of ribonucleases. Vlasyuk and Kuznetsova (1972) observed an inhibition of ribonuclease activity by molybdenum and vanadium in a ribosomal fraction from germinating peas; the metals maintained the ribonuclease in a latent state. The accumulation of vanadium and molybdenum ions in ribonucleoprotein particles, and in particular in nuclei, as observed earlier by these authors, has been ascribed to the biological function of molybdenum and vanadium as protectors against the ribonucleases present in those structures.

Berlinguet and Normand (1968) reported that metals may interfere with histone function.

Trace elements influence the conformation of protein molecules. Since the realization of the biological function of proteins involves changes in their conformation, flexibility of their structure is a prerequisite to the normal functioning of protein macromolecules. According to Vasilyeva (1966), zinc enhances the flexibility of proteins isolated from winter wheat. Vasilyeva and Estrina (1970) concluded that zinc may arfect the secondary and tertiary structure of protein macromolecules by substituting for hydrogen in the imidazole ring of histidine, and in the side groups of lysine and aspartic acid. According to viscosity measurements made on native proteins and proteins with impaired secondary and tertiary structures, to quantitative assessments of free and screened sulfhydryl and disulfide groups, and to ultraviolet spectra for these proteins, differences exist in the properties and structure of proteins isolated from zinc deficient and normal plants.

As reported by Vlasyuk (1971), a shortage of manganese in plants results in a considerable decrease in the amounts of "open" and "hidden" sulfhydryl groups in their proteins, together with a slight increase in the numbers of S-S bonds. Changes in the relative proportions of these local structures under conditions of manganese deficiency may result in modifications of the protein macrostructure Such modifications were demonstrated by measuring the relative viscosity of proteins isolated from manganese-deficient plants.

Summarizing the material presented in this chapter, it may be concluded that the investigation of the role of trace elements in nucleic acid and protein metabolism is a promising line of research.

PART II. THE PHYSIOLOGICAL ROLE OF TRACE ELEMENTS

Chapter 1

BORON

Boron is one of the essential trace elements for plants. A boron deficiency in dicotyledons results in degeneration of the plants from the very beginning. Many plant diseases, especially those occurring in fruit plants are attributable to boron deficiency. Dicotyledons contain more boron than monocotyledons. Of the cell organelles the richest in boron are the ribosomes, the chromatin and the endoplasmic reticulum membranes (Sherstnev and Artemyeva, 1968). Large amounts of boron are present in cell walls (Mayevskaya et al., 1970).

Intensive research on the physiological role of boron has been under way for over half a century in many countries. Until recently, however, no significant progress has been made with the investigation of boron, there being two major sources of difficulty. First, in contrast to other essential trace elements, boron is neither a consituent of enzymes nor an activator. Second, too many points concerning the physiological significance of this element remain unexplained. Some of these are as follows.

1. Boron is not an essential element for animals (Voinar, 1960) or fungi (Bowen and Gauch, 1966)

2. Some algae show a requirement for boron - Diatomaceae (Neales, 1964; Lewin, 1966a); for others boron is not essential, e.g. the green algae, Scenedesmus obliquus and Chlorella vulgaris (Dear and Aronoff, 1968; McBride et al., 1971)

3. There is a strong specificity of the symptoms of boron deficiency in dicotyledons - inhibition of root growth and deterioration of the growing points (Fig. 4)(First group on sensitiveness to boron deficiency)

4. In some boron-deficient dicotyledons (sunflowers, tomatoes, etc.), inhibition of root growth and degeneration of the growing points appear simultaneously early in development, whereas in other dicotyledons (peas, soybeans, lupins, etc.) there is late degeneration of the stem growing points accompanied by an inhibition of root growth (Fig. 5)(Second group on sensitiveness to boron deficiency)

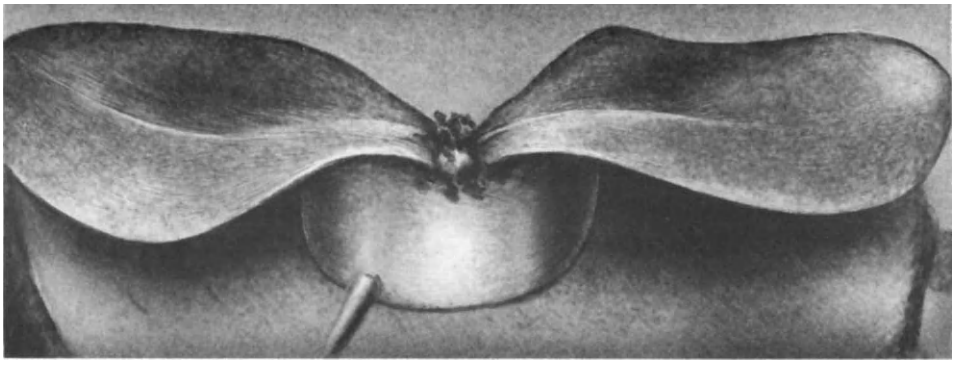


Fig. 4. Degeneration of the growing point in boron-deficient sunflower.

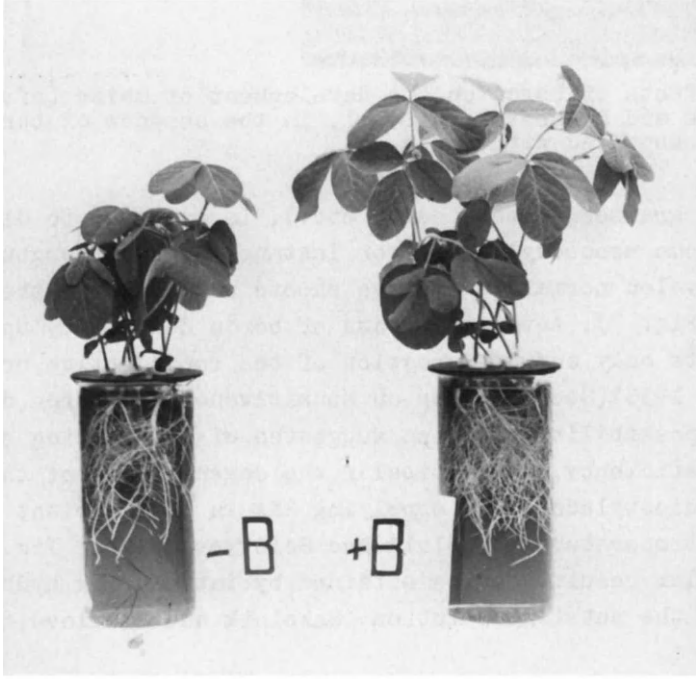


Fig. 5. Effects of boron on the growth of peas. Left, without boron; right, with boron.

5. Maize, sorghum and other plants, in contrast to other cereals, are able to maintain normal growth of the vegetative shoots under conditions of boron deficiency and retain the growing points (Fig. 6, 28)(First group on sensitiveness to boron deficiency)

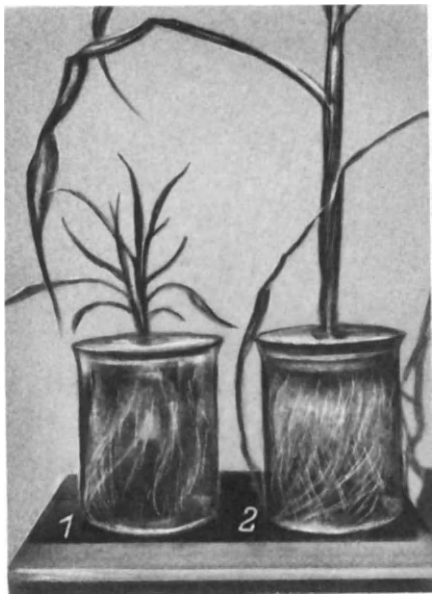


Fig. 6. Effects of boron on the development of maize (after Shkolnik and Bozhenko, 1960). 1, in the absence of boron; 2, supplied with boron.

6. Some grasses (wheat, oats, etc.), in contrast to dicotyledons and some monocotyledons (for instance, maize, sorghum), are able to develop normal vegetative shoots and roots in the absence of boron (Fig. 7); severe symptoms of boron deficiency appear in these plants only during formation of the reproductive organs (Shkolnik, 1935)(Second group on sensitiveness to boron deficiency)

7. The possibility has been suggested of eliminating symptoms of boron deficiency, in particular the degeneration of the growing points in dicotyledons, by supplying RNA in the nutrient solution at normal temperature (Shkolnik and Solovyeva, 1961; Fig. 8)

8. Similar results can be obtained by introducing hydrogen peroxide into the nutrient solution (Shkolnik and Steklova, 1951; Fig. 9)

9. There is a possibility of temporarily eliminating the symptoms of boron deficiency by supplying germanium to the plants (Skok, 1957)

10. Inhibition of root growth under conditions of boron deficiency can be eliminated by supplying nucleotide bases (Johnson and Albert, 1967)

Fig. 7. Effects of boron on the development of wheat. 1, in the absence of boron; 2, with 0.3 mg boron per litre.

11. The boron requirement increases at elevated temperatures and high illumination intensities (Shkolnik, 1935; Thellier, 1959; Bozhenko et al., 1973; Fig. 10)

12. The boron requirement also increases with daylength (Warington, 1933)

13. In all boron-deficient higher plants the formation of the reproductive organs is impaired, resulting in sterility of the flowers (Shkolnik, 1939a; Nelyubova and Pryanishnikova, 1954). Boron is essential for the formation of flowers in rosette long-day plants of Rudbeckia spec. without roots (Chailakhyan, 1951).

14. On the removal of boron from the nutrient medium, the geotropic reaction of the roots rapidly disappears (Skok, 1958)

15. In boron-deficient dicotyledons teratogenic alterations are observed (Shkolnik and Mayevskaya, 1961; Fig. 11)

16. Dramatic alterations of cell walls develop well before the symptoms of boron deficiency appear (Lee and Aronoff, 1966; Alekseyeva, 1971)

In spite of the wealth of observations on disturbances to physiological and biochemical processes under conditions of boron deficiency, and a host of hypotheses concerning its physiological

role, none of the enigmas of the function of boron has ever been resolved.

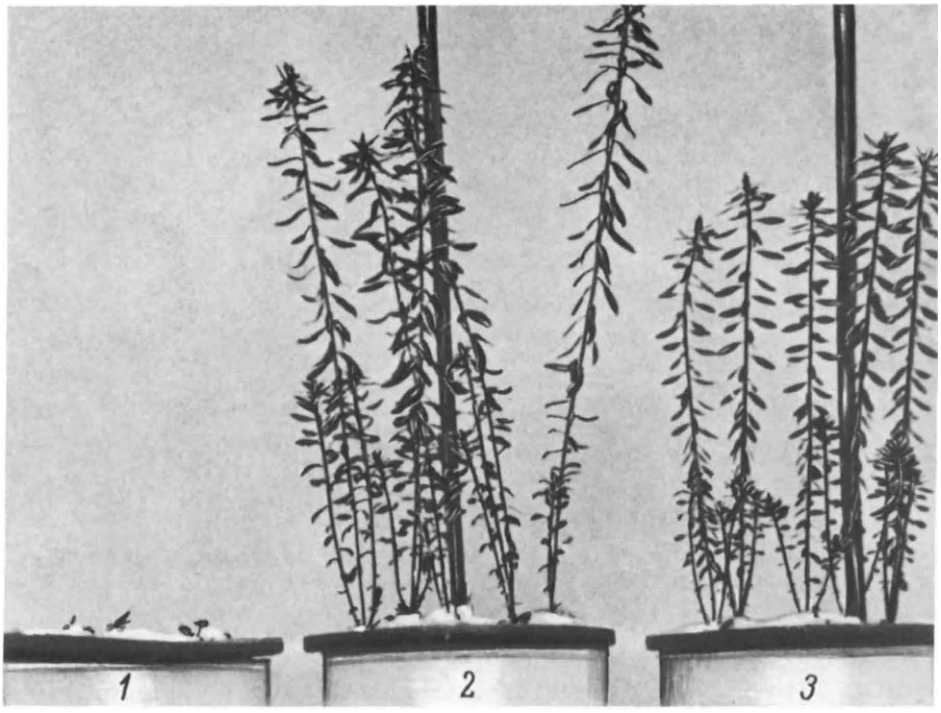


Fig. 8. Elimination of boron deficiency in flax by supplying free RNA to the culture solution (after Shkolnik and Solovyeva, 1961). 1, in the absence of boron; 2, supplied with boron; 3, in the absence of boron, but supplied with RNA.

Typically, in trying to explain the characteristic symptom of boron deficiency - degradation of the growing points - investigators have never attempted to provide answers to some of the other unsolved problems of boron deficiency. They have taken due account of the principal difference of behaviour between dicotyledons and monocotyledons suffering boron deficiency, namely, the degeneration of the growing points in all of the former, and in only some of the latter.

Before discussing critically current hypotheses, it will be necessary to touch on a challenging problem of great theoretical and practical consequence, i.e., that of the special function of boron in the development of reproductive organs.

Agarwala, Sharma, Chatterjee and Sharma (1981) found disturbances of pollen development in boron-deficient maize. The stamens of

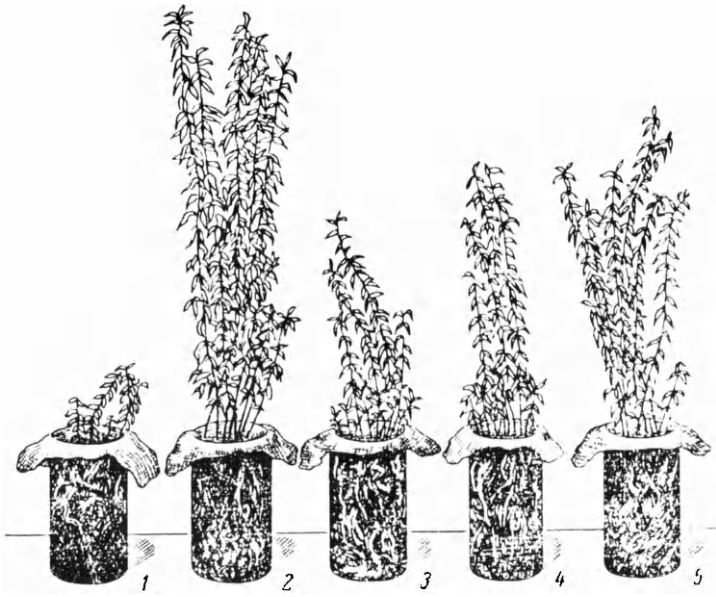


Fig. 9. Elimination of boron deficiency in flax by supplying hydrogen peroxide to the culture solution (after Shkolnik and Steklova, 1951). 1, in the absence of boron; 2, supplied with boron; 3-5, in the absence of boron, but supplied with hydrogen peroxide.

most boron-deficient plants lack sporogenic tissue, and the normal stamens of such plants show depressed pollen production.

Flower sterility is a general phenomenon in all plants, dicotyledons included. The provision of plants with small quantities of boron may prevent the decay of the growing points, and the plant itself may be brought to flowering. The flower sterility found in such plants is ascribed not to damage of the sporogenic tissue, as is the case in cereals, but to total decay of all parts of the flower (Shestakov, Nelyubova and Pryanishnikova, 1956).

Piland et al. (1944) reported a 600-fold increase in the crop yield of lucerne when boron fertilizers were supplied to the soil, while the yield of hay increased by only 3%. Midley and Dunclee (1937) reported a 35-fold increase in the seed crop yield of lucerne on supplying boron fertilizers, and only a 2% increase in hay. The role of boron in the process of reproduction has been increasingly shown to be highly specific and of wide-ranging influence. No anthers are formed, for example, in deficient plants (Fig. 12), whereas pollen is either nonviable or does not develop at all (Shkolnik and Solovyeva-Troitskaya, 1962). Schmucker (1932) found that boron plays an important role in pollen germination and the

Fig. 10. Effects of boron deficiency on the development of sunflower at elevated temperatures (after Bozhenko et al., 1973); a plant suffering from boron deficiency for six days at normal temperature and two days at +47°C.

growth of pollen tubes. Numerous interesting findings relevant to this problem were reported subsequently (Bobko and Tserling, 1938; Visser, 1955). According to Visser (1955), the temperature limits for the germination of pollen increase in the presence of boron.

During the period from the appearance of the staminate tubercles in the middle of the rudimentary spike to the formation of tetrads in cereals (wheat, oats, barley), there is a maximum sensitivity

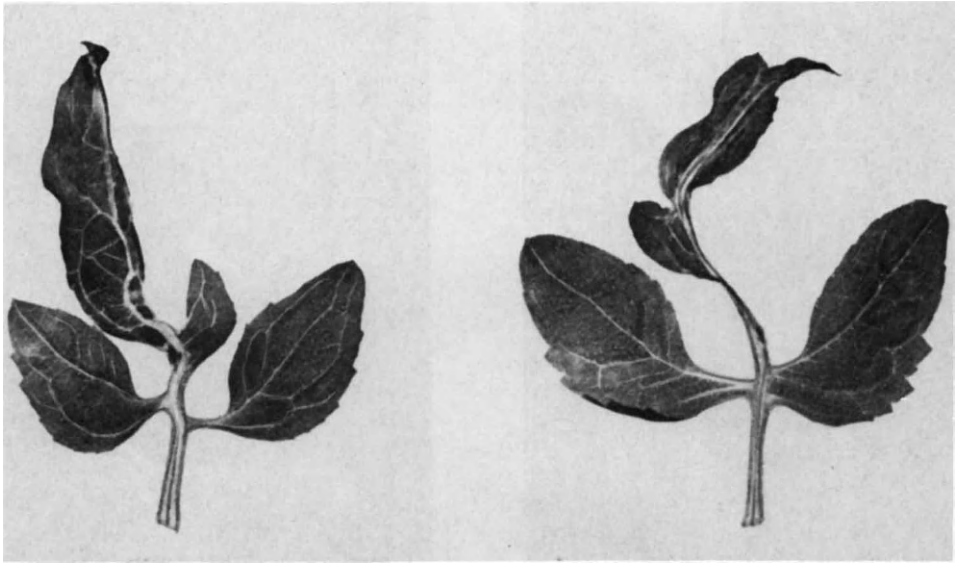


Fig. 11. Teratological alterations of sunflower leaves caused by a deficiency of boron (after Shkolnik and Mayevskaya, 1971). The plant had been grown for eight days in the absence of boron, and was then supplied with the element.

to a shortage of boron (Novikov, 1967). Deterioration of the growing points in barley occurs at this time. Tserling (1941) and Shestakov et al. (1956) have shown that boron deficiency during the flowering of soybean plants impairs the formation of the ovary more strongly than it impairs the development of the pollen. The reverse holds true for wheat (Troitskaya and Batygina, 1970), whereas the pistil, stigma and pollen develop abnormally in boron-deficient grapes (Scott, 1944). During the growth of the pollen tube, instead of a hollow rachis 1.6–2.0 mm in length, a club-like formation develops which prevents penetration of the male sexual cell and thus interferes with fertilization. Scott found that some grape cultivars produced parthenocarpic fruits when grown in the absence of boron. Jacob et al. (1972) found abnormalities in flower development in Cola nitida. Troitskaya and Batygina (1970) conducted more detailed studies on the effect of boron on macro- and microsporogenesis, and revealed marked alterations in the sporogenic tissues of anthers as a result of boron deficiency (Fig.13).

Boron is essential not only during the formation of the pollen and the ovary, but also for the subsequent development of the seed. If boron is in short supply prior to flowering or before the

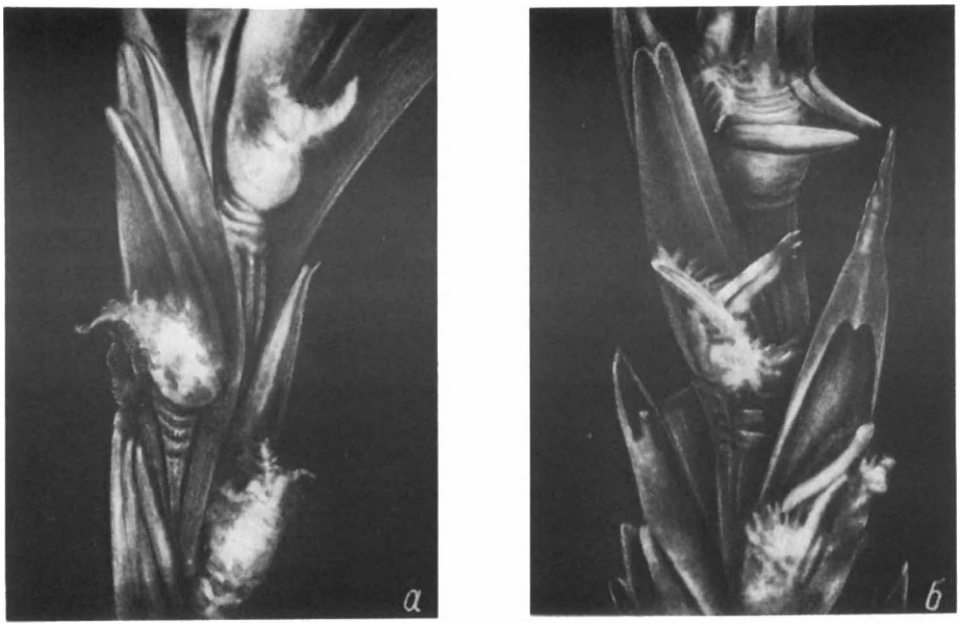


Fig. 12. Effects of boron on the development of the generative organs of wheat (after Shkolnik and Solovyeva-Troitskaya, 1962). 1, in the absence of boron (stamens absent); 2, in the presence of boron (stamens fully developed).

formation of the seed, the ovary dies (Bobko and Tserling, 1938). Maturation of seeds is also impaired in the absence of boron.

Interestingly, Moewus (1950) found that the flavon glycosides - rutin and quercitrin - present in the pollen of Forsythia intermedia inhibit self-pollination. Cross-pollination is only possible if the inhibitors of pollination are hydrolysed. In the pollen of the staminate flowers with long columnae is an enzyme which breaks down quercitrin, whereas pollen from flowers with short columnae contains an enzyme hydrolysing rutin. The inhibition produced by rutin and quercitrin can be counteracted by boron, so that both types of Forsythia intermedia plants become self-pollinating.

Kühn et al. (Kühn et al., 1942; Kühn, 1943; Kühn and Löw, 1947) studied the effect of boric acid on the control of sexual reproduction in Chlamydomonas. Non differentiated Chlamydomonas gametes acquire the properties of male gametes when treated with boric acids. These workers suggest the following mechanism to explain their findings. Non differentiated gametes contain both a "female" substance - gynotermon (represented by the flavone - isochamnetic)

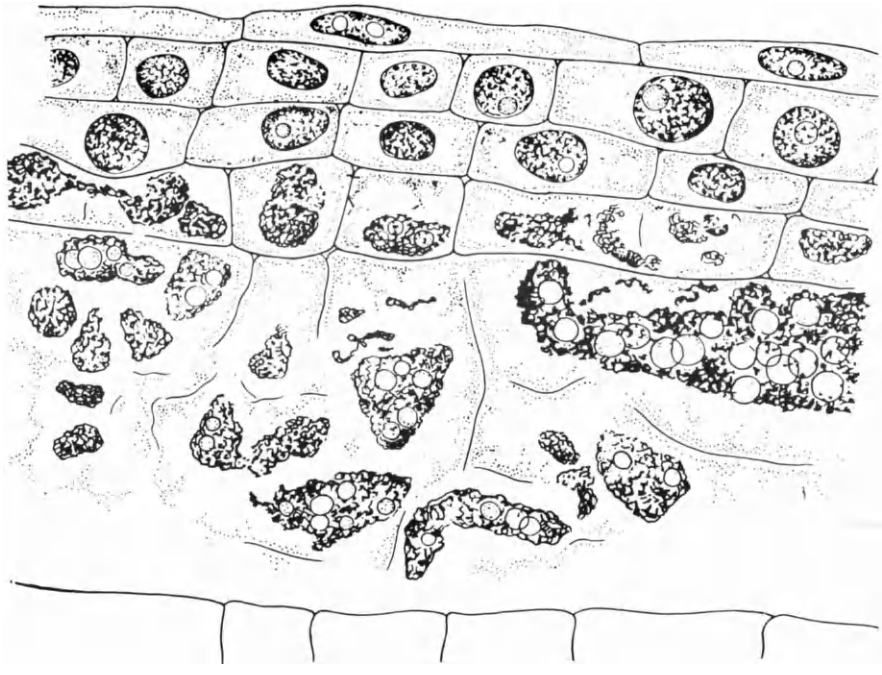


Fig. 13. Longitudinal section of the microsporangium of boron-deficient spring wheat at the stage of the formation of the sporogenic tissue. Chromatin irregularities are clearly seen (after Troitskaya and Batygina, 1970.

and a "male" substance - androtermon (an oxyaldehyde). A shift in the relative concentrations of the female and male substances is easily obtained by placing the gamete in a solution of one of these substances, enabling sexual activity in this organism to be controlled. Supplying boric acid, which produces complex compounds with isochamnetin, may also result in an excess of the male substance, and thus boron may influence the sexual process.

The significance of boron for function of the generative organs is not only of theoretical, but also of practical interest. This has been well demonstrated by Pisarev and Zhilkina (1954), who solved the problem of the partial infertility in wheat amphiploids by introducing boron, thus facilitating the use of hybrid varieties obtained by plant breeders (Table 4). Shcherbenev (1973) successfully hybridized quinces and apples by supplying boron and gibberellic acid.

The metabolism of calcium and boron in sterile hybrids is impaired and nucleoprotein synthesis is inhibited at the stage of sporo- and gametogenesis. Fursov (1965) concluded that mineral

elements bound to nucleoproteins were responsible for the vitality of the plant's generative organs. Calcium and boron were entirely absent from the scutella and the aleurone layer of the wheat-rye grain from which plants of the first sterile hybrid develop. This was not the case with the karyopsis of a fertile hybrid.

TABLE 4 **Effect of boron on the formation and grain content of the spike in low-fertility wheat hybrids** (after Pisarev and Zhilkina, 1954)

Hybrid	Treatment	Number of spikelets (per spike)	Number of grains (per spike)
Triticum timococum	Control	12.5	4.8
	+ boron	15.7	9.2
Triple hybrid	Control	15.7	1.4
	+ boron	22.9	41.3
AD 20/1	Control	18.2	8.4
	+ boron	18.9	13.9
I h AD	Control	17.4	2.5
	+ boron	18.7	17.1
AD 74	Control	14.2	6.5
	+ boron	16.0	13.5

The effects of boron on the chromosomal apparatus have been only poorly studied. Whittington (1957) observed an abnormal chromosome separation in dividing cells from boron-deficient plants.

Summarizing the above evidence it can be stated that reproduction does not occur in the absence of boron.

A vast body of observations has accumulated in the literature concerning the physiological role of boron (Gauch and Dugger, 1954; Skok, 1958; Hewitt, 1963; Shkolnik, 1952, 1967, 1970, 1974 ; Augsten and Eichhorn, 1976). Only the most important findings and hypotheses will be dealt with here including those obtained in the author's laboratory.

Over a relatively long period a hypothesis of Gauch and Dugger (1954) enjoyed great popularity. These workers assigned the principal role of boron to the facilitation of sugar translocation, explained by the ability of sugars to produce complex compounds with boron, which, according to these workers, possesses a high degree of mobility and easily permeates membranes. The deterioration of the growing points in the absence of boron is attributable to a shortage of carbohydrates.

Eighteen years before the formulation of the above hypothesis, Belousov (1932) suggested a similar hypothesis, which differed from that of Gauch and Dugger in proposing an inhibition of the translocation of sugars linked with changes in sugar metabolism that occurred under boron deficiency; according to Gauch and Dugger, the enhanced sugar transport attributable to boron could be accounted for by the ability of this element to produce compounds with sugars of high mobility. Experimental findings have not corroborated the views expressed by Belousov; according to the author's observations (Shkolnik, 1939a), sugars accumulate in all the organs of boron-deficient plants and not only in the leaves, indicating retardation of the growth processes.

Numerous findings may be cited to indicate that the hypothesis advanced by Gauch and Dugger is no longer valid. Thus, Ziegler (1956) found that in a wide variety of woody plants, the boron content of the sieve tubes was usually very low and only small amounts of boron and sugar complex compounds could be found. Neither spraying leaves with sugars and organic acids nor supplying sugars to the roots could eliminate the symptoms of boron deficiency (McIlrath and Palser, 1956; Skok, 1957; Albert and Wilson, 1961). Skok (1958) found also that sugars enter plant cells in the absence of boron, which would not occur if boron were essential for the transport of sugars across cell membranes.

Recently Dugger (1974) presented a unified scheme to account for the role of boron in carbohydrate metabolism. He referred to both in vitro and in vivo studies in order to clarify the role of this element in controlling the synthesis and use of starch and sugars. This scheme is certainly of major interest.

Various workers (Skok, 1957; Spurr, 1957) arrived at the conclusion suggested by the present author more than 40 years ago (Shkolnik, 1939a), namely, that the effects of boron on the translocation of sugars are indirect, and are related to growth processes rather than to the transport of sugars across membranes based upon the formation of sugar - borate complexes, as visualized by Gauch and Dugger.

Kibalenko (1966a) reported positive effects of boron on photosynthetic phosphorylation. Somewhat later, Timashov (1967, 1968) found that boron positively influences photosynthetic and oxidative phosphorylation. He suggested that the deterioration of the growing points caused by boron deficiency is a result of the exhaustion of the energy reserves of the cell; ATP synthesis is delayed rela-

tive to phosphorylation, this delay being the consequence of alterations in mitochondrial structure. That no exhaustion of energy reserves occurs under conditions of boron deficiency has been shown by Shkolnik and Kopmane (1970a), although a decrease in the amounts of all organic phosphorus compounds, including the energy-rich compounds, was noted. A study of the mitochondrial structure in boron-deficient plants revealed no alterations, and certainly no irreversible modifications of this organelle's structure (Alekseyeva and Shkolnik, 1970).

An investigation of the effects of boron on the swelling of mitochondria isolated from the apices and roots of peas and from sunflower roots, using various agents promoting swelling or contraction of mitochondria, demonstrated that boron does not modify mitochondrial membranes. Judging from findings on the extensive damage done to chloroplast and nucleolar structures under conditions of boron deficiency, mitochondria display the greatest resistance among cell organelles to the destructive effects of boron deficiency. Mayevskaya and Alekseyeva (1966) found no changes in the P:O ratio in the initial stages of boron deficiency in sunflower plants. Such changes could only be observed under conditions of advanced boron deficiency.

As early as 1932, Schmucker suggested that boron plays a role in the formation of cell walls. Other workers advanced similar views (Winfield, 1945; Bobko, 1949; Torsell, 1956; Odhnoff, 1957; Spurr, 1957; Starck, 1963). The hypothesis that boron was involved in the formation of cell wall components was based on the well-known ability of boron to produce complexes with sugars and pectin substances. The bulk of boron in plants is known to be concentrated in the cell walls, and in boron-deficient tissues the parenchyma and other thin-walled cells disintegrate; fragility of the stem is also frequently observed (Winfield, 1945; Mayevskaya et al., 1970). According to Skok (1958), the accumulation of boron in cell walls may be related to the high concentration of polyoxycompounds there. These compounds form complexes with boron, and may not have any bearing on the involvement of boron in the formation of cell wall components. Other workers (Torsell, 1956; Starck, 1963) believe that boron influences the elasticity of cell walls, and more specifically the arrangement of microfibrils, which largely determine this elasticity.

Boron deficiency may have different effects on the thickness of cell walls, depending on the type of tissue: the cell wall may

grow thicker in one tissue, while in others it becomes very thin. Spurr (1957) studied the cell wall structure in celery leaf stalks. The thickness of the collenchyma cell walls decreased in boron-deficient plants in proportion to the boron supply, whereas the cell wall thickness was markedly enhanced in the phloem and basic parenchyma. The dissimilar reactions of these tissues are thought by Spurr to be the result of an elevated carbohydrate content of the phloem and basic parenchyma cells, and a shortage of these substances in the more distant collenchyma cells.

As yet, however, no evidence of a decrease in the content of individual cell wall components - pectins, celluloses and hemicelluloses - has been provided. More often than not, an increase in their quantities has been noted. Bobko (1949) could not find any dependence of the pectin content upon boron supply. The activity of pectinase and pectin esterase, an enzyme responsible for the hydrolysis of pectin compounds, was higher in boron-supplied plants.

Slack and Whittington (1964) studied the rate of incorporation of ^{14}C into pectin compounds, hemicelluloses, lignin, sugars and acids in bean root tips. They found that a stimulation, rather than an inhibition, of the incorporation of labelled carbon takes place in conditions of boron deficiency. No amelioration of the symptoms of boron deficiency were noted by Skok (1958) when he supplied boron-deficient plants with D-galacturonic acid, D-(+)-galactose and L-(+)-arabinose. Some workers, however, have indicated that the lignin content can be lowered in boron-deficient plants. Palaveyeva-Kovachevskaya (1957) examined safron-stained sections of boron-deficient plants and found that in the dying-off growth points, and in a rather extensive region under them, the walls of the sclerenchyma cells were ligninless.

Dutta and McIlrath (1964), by quantifying the lignin in boron-deficient sunflowers, obtained unequivocal evidence of reduced lignification in the cell walls of both stem callus and roots. The total contents of lignin and the enzyme peroxidase which catalyzes lignin synthesis were reduced in boron-deficient tissues. The authors were able to explain why other workers (Neales, 1960; Odhnoff, 1957) had arrived at the opposite conclusion, i.e. that lignification is enhanced by boron deficiency: in this case the plants analyzed contained a comparatively greater quantity of old, and therefore more lignified tissues.

In his review of 1967 (Shkolnik, 1967) the author suggested that one of the major reasons for boron being non-essential for

man and animals is that, in contrast to plants, the latter have neither cell walls nor lignification processes. Lewis (1980) supports this view. The author also mentioned that fungi, in which boron dependence has not been demonstrated, do not synthesize lignin, the only exception being wood-attacking fungi (Shivrina, 1965).

The investigators are not, however, unanimous. Acerbo et al. (1973) having traced the metabolism of labelled lignin precursors concluded that boron deficiency has no appreciable effect on the transformation of phenols into lignin, but that boron seems involved in the synthesis of aromatic compounds from glucose. Also noteworthy is the research of Krosing (1978), attracting attention to the fine cellular structure of the xylem, to the elongation of internodes, and to lignin deposits in the xylem tissue of the third internode, which appear late under conditions of boron deficiency. The effect of boron deficiency on lignification needs further research.

In a recent paper, Lewis (1980a)develops the idea that the principal functions of boron are connected with the metabolism of phenolic acids and lignin biosynthesis, and with the mechanism of auxin action in the processes of xylem development and differentiation. Lewis thinks that borate regulates the hydrolytic and oxidative functions of phenolases, contributing to the biosynthesis of lignin precursors, i.e. of caffeic and hydroxypherulic acids.

According to Lewis, boron, with rare exceptions, is essential for vascular plants, i.e. plants with a well lignified xylem, while non-vascular plants do not need it. When boron is scarce incomplete lignification and differentiation of the xylem should result in an accumulation of lignin precursors, or of compounds metabolically related to lignin. This expectation is borne out by evidence for the accumulation of coffeic acid, chlorogenic acid, and scopoletin under conditions of boron deficiency.

One cannot but entirely accept the evidence. One cannot, however, accept it as the only reason for phenol accumulation when boron is deficient. As will be shown later the author's original experiments and the studies of other workers have proved that under conditions of boron deficiency the content of many phenolic compounds is increased, these compounds being neither lignin precursors, nor metabolically related to lignin. Consequently, a disturbance of lignin synthesis is not the only cause of the accumulation of phenols when boron is in short supply.

Special investigations into the role of boron in lignification, and elucidation of the effect of boron-deficit-induced disturbances to lignification on phenol accumulation are needed.

An electron microscopic study of the effect of boron deficiency on the state of subcellular organelles has revealed that the Golgo-apparatus, which according to current knowledge is involved in polysacharide synthesis and the formation of cell walls, is in an active state in cases of boron starvation (Alekseyeva, 1971). Nevertheless, severe alterations in the structures of cell walls appear, even before the manifestation of other symptoms of boron deficiency; numerous vesicles and tubules appear at the periphery of the cell in the area of the middle lamella, and the cell wall swells and splits in places (Alekseyeva, 1971, Fig. 14). Besides giving rise to cell wall alterations, boron deficiency is responsible for a number of morbid changes in subcellular structures: degradation of the nucleolus (Fig. 14), loss of cell membranes, alterations in chloroplast structure (Fig. 92-1,2), and the disappearance of peroxisomes (Alekseyeva, 1971).

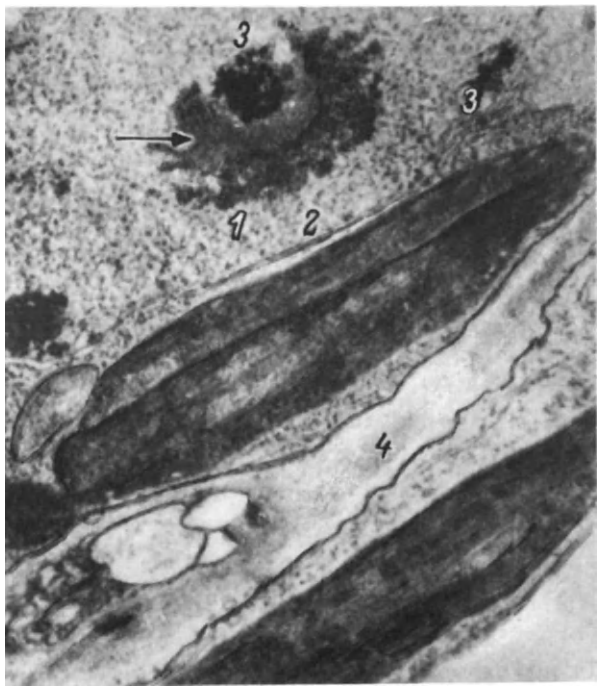


Fig. 14. Alterations in the structures of the nucleolus and cell walls in mesophyll cells of boron deficient flax plants (after Alekseyeva, 1971). 1, nucleus; 2, chloroplast; 3, chromatin in contact with the nucleolus; 4, cell wall.

Recently Hirsh and Torrey (1980) found severe changes in the ultrastructure of root cells.

During the first 6 hours of boron deficiency an intense formation of vesicles and lamellas in the periplasmalemma space, a thickening of the cell walls, and interruptions of membrane continuity became apparent. In 20 hours the alterations to the root cells were considerably aggravated, involving mitochondria and the membranes of other organelles, and creating the impression that there may have been an accumulation of toxic compounds as a result of impaired metabolism. Support for the validity of this view comes from the fact of the exceptionally specific symptoms of boron deficiency in dicotyledons - deterioration of the growing points and the preceding rapid browning of the tissues.

In 1961 the author advanced a hypothesis relating boron to nucleic acid metabolism, this being based on a number of observations. First, a decrease in the amount of RNA and DNA in various organs of boron-deficient dicotyledons has been observed (Shkolnik and Mayevskaya, 1961; Fig. 15). Second, it was found that deterioration of the growing points could be prevented in cooler weather by supplying RNA (Shkolnik and Solovyeva-Troitskaya, 1961; Shkolnik et al., 1961; Shkolnik and Mayevskaya, 1962a; Troitskaya, 1962). Boron enhanced the incorporation of ^{14}C-adenine into sunflower leaf RNA, and labelled phosphorus into RNA and DNA in the leaves and roots of sunflower (Sherstnev and Kurilyenok, 1962). The reverse was true in the case of the stem and cotyledonous leaves (Shkolnik and Kositsyn, 1962). Later, a decrease in r-RNA (Timashov, 1966), the appearance of DNA-like RNA (Sherstnev, 1967) and the breakdown of tRNA (Timashov, 1966) were observed.

A decrease in the amount of nucleic acids in boron-deficient plant cells is apparently not related to any impairment of the synthesis process. Thus, Mamedova and Sherstnev (1964) demonstrated that a shortage of boron did not affect the biosynthesis of free ribonucleotides, and that boron did not therefore affect the synthesis of nucleic acids, at least not at the stage of the formation of their precursors - the nucleoside triphosphates. It should be noted, however, that a decrease in AMP, ADP and ATP content was reported in some experiments (Shkolnik and Mayevskaya, 1962b; Mayevskaya and Alekseyeva, 1964; Timashov, 1963). A decrease in the concentration of ATP was paralleled by enhancement of ATPase activity (Mayevskaya and Alekseyeva, 1964).

It is conceivable that a decrease in the content of various RNA fractions in boron-deficient plant cells is related to a steep increase in the activity of ribonucleases (Timashov, 1963; Shkolnik et al., 1964a; Sherstnev and Rasumova, 1965). A particularly striking increase in ribonuclease activity occurs when there is a combination of boron deficiency and high temperature (Smirnov, 1971).

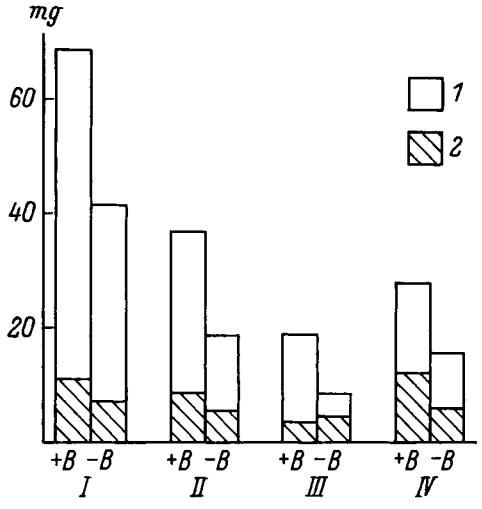


Fig. 15. Effects of boron on RNA content and DNA content (mg per kg dry wt.) in 11-day-old sunflower seedlings (after Shkolnik and Mayevskaya, 1962). 1, in the presence of boron; 2, in the absence of boron; I, growth points; II, leaves; III, stems; IV, roots.

The action of boron on ribonucleases is highly specific: the observed increase in their activities produced by shortages of other trace elements (zinc and copper) is certainly not comparable to that produced by boron deficiency. According to the author's assumptions, the increase in the activity of ribonucleases in the latter case is related to changes in the membrane-associated mechanisms by which the enzyme is released, so that the transition of the enzyme from a latent to an active form is affected. This view is reinforced by an observed decrease in the content of phospholipids and galactolipids (Shkolnik and Kopmane, 1970b; Mamedova, 1970; Alekseyeva and Shkolnik, 1971) in boron-deficient plants. The structure of chloroplasts in such plants undergoes considerable modifications (Kibalenko, 1966b; Lee and Aronoff, 1966;

Alekseyeva, 1971). Alekseyeva and Shkolnik (1971) found that boron deficiency resulted in a decrease in the contents of phosphatidyl choline (lecithin), phosphatidyl glycerine, phosphatidyl ethanolamine (cephalin), and phosphatidyl diglycerine (cardiolipin) in flax leaves (Fig. 16). In the leaves of maize, a plant displaying a lower sensitivity to boron than that shown by flax, decreases in the levels of onle the first three of these phospholipids could be noted, and the decreases were less than those occurring in flax.

Alterations to chloroplast structure in boron-deficient plants accompanied by a decrease in galactolipid content could explain the dramatic decrease in the rate of photosynthesis in boron-deficient plants (Shkolnik and Saakov, 1964; Kibalenko, 1973). The inhibition of photosynthesis associated with boron deficiency may also be accounted for by the disappearance of peroxisomes in boron-deficient plants.

The nucleotide composition of the total RNA content has been found to be altered in boron-deficient plants (Kibalenko et al., 1970; Bozhenko et al., 1973). With a combination of boron deficiency

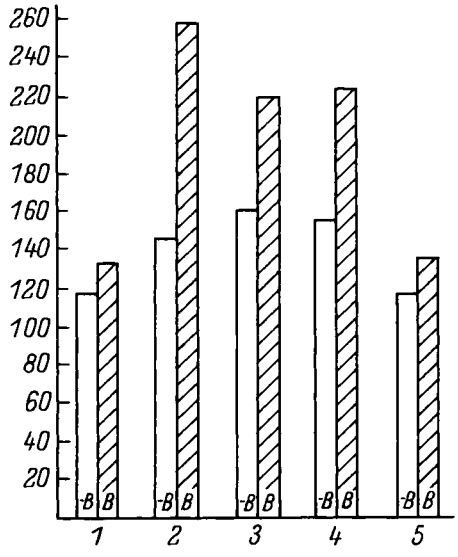


Fig. 16. Effect of boron deficiency on the phospholipid content of maize leaf (μg per g dry wt.). 1, phosphatidyl inosite; 2, phosphatidyl choline; 3, phosphatidyl glycerine; 4, phosphatidyl ethanolamine; 5, phosphatidyl (after Alekseyeva and Shkolnik, 1971).

and high temperature, which augments the symptoms of boron deficiency, the nucleotide composition of DNA may also show a decrease in the G-C pair content and an almost twofold decrease in the methylated cytosine residue (Bozhenko et al., 1972). An impairment of the physical characteristics of RNA involving destruction of the secondary structure has been observed in boron-deficient plant cells (Kibalenko et al., 1970).

Boron deficiency interferes with protein synthesis on the ribosomes, but does not disturb the activation of amino acids and the association of these with RNA. Sedimentation analysis has shown that ribosomal RNA, which constitutes the bulk of the cellular RNA, is largely degraded in boron-deficient plant cells. Ribosomal preparations obtained from root cells of boron-supplied plants showed four peaks of sedimentation corresponding to 79S, 117S, 146S and 179S (Fig. 17). Their counterparts from boron-deficient plants displayed only two peaks - 79S and 117S. Polysomes were absent from the preparations (Borshchenko, 1970), the breakdown of polysomes under conditions of boron deficiency apparently being the result of exceedingly high ribonuclease activity. Kalichava and Sherstnev (1974) studied the amino acid composition and N-terminal groups of ribosomal proteins from boron-deficient and normal pea roots. A shortage of boron produced some abnormalities with respect to both these aspects of protein structure: only traces of proline could be detected in boron-deficient ribosomal protein, whereas its content was considerably higher in normal, boron-supplied plants. The concentrations of almost all amino acids in cases of boron deficiency were slightly lower than in the controls, while the amount of alanine was 30% lower.

The effects on nucleic acid metabolism and protein synthesis discussed above are apparently typical only of dicotyledons. In a monocotyledonous plant such as barley, which shows normal development of the vegetative organs in the absence of boron, no increase in the activity of ribonuclease is found to result from boron deficiency (Smirnov, 1971).

The evidence presented demonstrates that alterations occur in the nucleic acid metabolism of boron-deficient dicotyledons. These alterations are obviously unrelated to the role of the element itself in the metabolism of nucleic acids, but rather may arise from some unknown toxic influence of substances produced as a result of metabolic disbalance under conditions of boron deficiency, by analogy with the modifications to cell walls discussed

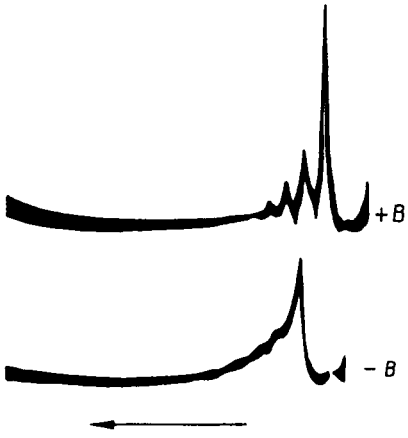


Fig. 17. Sedimentation pattern of ribosomal components from pea root preparations (after Borshchenko, 1970).

above. The view that boron itself is not concerned in nucleic acid metabolism finds support in the observation that this element is not required by animals, fungi, nor to any great degree by cereals during the formation of the vegetative shoots.

Cohen and Albert (1974) observed a cessation of mitosis and DNA synthesis 20 hours after boron deprivation; they suggest that boron is essential for continuous DNA synthesis and mitotic activity in root cells.

In the experiments of Smirnov and Shkolnik (1978), boron deficiency considerably reduced the mitotic index and shifted the proportions of cells in the different stages of division, significantly depressing the rate of progress through anaphase.

Ilyushchenko et al. (1978) found experimentally that it is not a disturbance of nucleic acid synthesis that is responsible for the inhibition of mitotic division.

Birnbaum et al. (1977) obtained unequivocal evidence that the dying back of boron-deficient plants is not attributable to reduced nucleic acid synthesis.

Sherstnev and Shneyer (1970) observed an increase in the template activity of chromatic under conditions of boron deficiency, and this was accompanied by structural disorganization of chromatin. Khudzhanazarov et al. (1973) found that the yield of histones from boron-deficient plants was considerably lower than that from control plant cells. Moreover, a notable degradation of the histones

was revealed by electrophoresis.

Alterations to the cell wall structure in boron-deficient plants, the occurrence of these alterations well before the appearance of other symptoms of boron deficiency, deformations of the nucleoli and the chloroplasts, the breakdown of ribosomes, disruption of the chromatin structure accompanied by impaired template activity, the degradation of histones - all these facts are interpreted by the author as providing further corroborative evidence for the view that plant poisoning by unknown products of impaired metabolism occurs when boron is in deficiency. This view has found support from morphological (deterioration of the growing points, Fig. 14), anatomical (decay of tissues, Fig. 90) and embryological (decay of all the organs of the flower, Fig. 18) observations of boron-deficient plants. The question of what these products could be still remains.

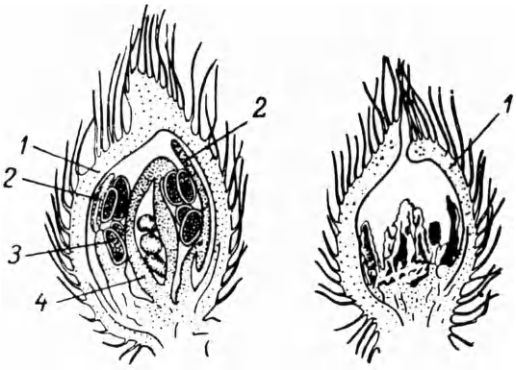


Fig. 18. Longitudinal section of soybean flower. 1, calyx; 2, petals; 3, anthers; 4, pistil with ovules. Left, with optimum supply of boron; right, with low supply of boron; the generative organs are completely degenerated by boron deficiency (after Shestakov et al., 1956).

Coke and Whittington (1968) suggested that auxins accumulate in high concentrations, producing a toxic effect within boron-deficient plants. They found that extracts of boron-deficient plant roots were more toxic to bean roots than those of control plants. The toxicity was found to be caused by indoleacetic acid. Employing bioassay methods they found that the auxin content of the roots of boron-deficient plants was roughly twice as high as that of normal roots. Such an increase in auxin content could hardly be sufficient to produce toxic effects in plant roots. Experiments with labeled indoleacetic acid revealed that it was taken up more

slowly by the roots of boron-deficient plants compared with normal plants. Moreover, the decarboxylation of indoleacetic acid was reduced in boron-deficient plants, which pointed to a lower activity of auxin oxidase. Coke and Whittington concluded that borates played a protective role in the auxin oxidase system by producing complexes with, and thereby paralyzing the activity of, the inhibitors of auxin oxidase.

Why, having obtained direct evidence that auxin oxidase was severely inhibited in boron-deficient sunflower plants well before Coke and Whittington advanced their hypothesis, did the author not conclude that auxins may accumulate when boron is deficient? The reason was the finding (Shkolnik et al., 1964b; Smirnov et al., 1977) that a decrease in the content of free auxins occurred.

In addition, Krupnikova (1967) found that maize plants, which exhibit lower boron requirements than sunflower, displayed a higher auxin oxidase activity under conditions of boron deficiency, in contrast to the expected lower activity of the enzyme in this plant. This might be explained by the fact that the concentrations of auxin oxidase inhibitors differ in maize and sunflower; such inhibitors are probably fewer in number in maize, which accounts for the higher activity of the enzyme and enhanced breakdown of the auxins.

A decrease in the activity of auxin oxidase occurs in boron-deficient dicotyledons (e.g. sunflower; Fig. 19). Then Krupnikova et al. (1975) showed that boron deficiency is accompanied by an increase in the activity of auxin oxidase in both wheat and maize leaves, the effect being even more pronounced in wheat than in maize. No difference in the activity of the enzyme could be found in boron-deficient and boron-supplied wheat roots, in contrast to the situation in maize. These findings testified to differences in the effects of boron deficiency on the activity of auxin oxidase in mono- and dicotyledons; the enzyme activity is lowered in the latter, whereas an increase or no change is observed in the former.

A year before the appearance of the publication of Coke and Whittington (1968), Jaweed and Scott (1967) noted an increase in the concentration of IAA in the roots and shoot apices of sunflower seedlings during the first 12 days of boron deficiency. In the apices of 16-day-old boron-deficient seedlings the reverse situation was found - in IAA concentration was 1.5 times lower. This experiment was performed in plants from which the cotyledons had been removed prior to planting.

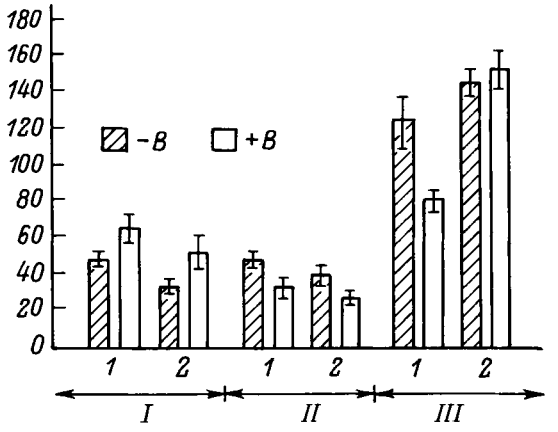


Fig. 19. Effect of boron deficiency on the activity of IAA-oxidase; /ug IAA degraded per 100 mg fresh wt. (after Krupnikova et al. (1975). I, sunflower; II, maize; III, wheat; 1, leaves; 2, roots.

The discrepancies in the available data on the effects of boron deficiency on IAA concentration and the limitations of the bioassay technique employed in all the investigations cited, led Smirnov et al. (1977) to assess quantitatively the IAA concentration in various organs of a number of plants with differing sensitivity to boron deficiency - sunflower, beans, maize and wheat (Fig. 20). The free IAA concentration in the shoot apices of the first three of these species was considerably reduced by boron deficiency. In wheat, a plant with a very low sensitivity to a shortage of boron, no difference in the amount of free IAA in boron(-) and boron(+) plants could be observed. Protein-bound IAA was also less abundant in sunflower, maize and wheat, and was only elevated in amount in bean plants. In maize, the amount of free auxin was reduced in roots, while that of bound auxin was elevated. In boron-deficient bean and wheat plants, the concentration of free auxin in the roots was elevated, being three times as high as the concentration in control wheat plants. The fact that in the shoot apices and growing points of sunflower and bean plants the concentration of both free and bound auxins is decreased by boron deficiency indicates that the degeneration of the growing points in boron-deficient plants cannot be attributed to an accumulation of auxins there. This contention is further supported by the observation that the highest

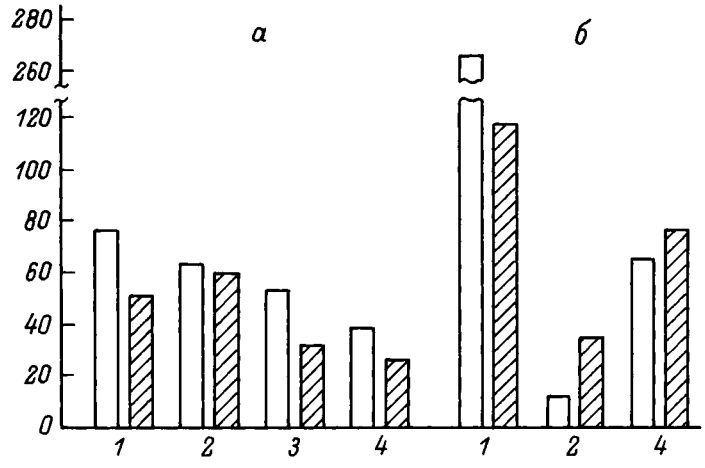


Fig. 20. Effect of boron deficiency on the content of free IAA in maize (1), wheat (2), sunflower (3), kidney bean seedlings (4); a, shoots; b, roots. Ordinate, concentration of IAA (ug per 10 g dry wt.); abscissa, unhatched blocks - with boron, hatched blocks - without boron (after Smirnov et al., 1977).

increase in free IAA in roots was observed in wheat, a plant of low sensitivity to a shortage of boron. Wheat roots, in contrast to those of dicotyledons, show good growth even under serious boron deficiency.

This conclusion is consistent with the data reported by Rajaratham et al. (1971) on the effect of boron deficiency on the level of IAA in the seedlings of the oil palm - a monocotyledonous plant. These workers found that the concentration of IAA in boron-deficient seedlings was 663±286 ppm, compared with less than 1 ppm in controls. Their experiments were performed on plants showing severe symptoms of boron deficiency. These huge difference in the auxin content of boron(+) and boron(-) plants are rather questionable; again, the limits of experimental error seem to be excessive. To demonstrate that a shortage of boron is equivalent in effect to IAA toxicity, these workers sprayed young palms with solutions of high concentrations of IAA. They succeeded in obtaining faint leaf symptoms closely resembling the symptoms of boron deficiency, but this might have been a result of the fact that IAA in high concentrations possesses a notable morphological effect (Smirnov et al., 1977). These arguments are further strengthened by observations

made in the author's laboratory. By treating bean and maize seeds with high concentrations of IAA, followed with and then supplying the compound in the nutrient solution, a marked suppression of growth and profound morphological alterations could be produced. The treatment produced browning of the tissues or decay of the growing points in bean plants, did not kill the maize plants. The above findings show that there is little supporting evidence for the view of Coke and Whittington that a relationship exists between the toxicity of high auxin concentrations and boron deficiency. A decrease in the IAA content in cases of boron deficiency is one of the causes of growth inhibition.

Ilyushchenko et al. (1978), having failed to find any decrease in proliferation after α-naphtylacetic acid treatment, considered the concept of the accumulation of auxins as a result of boron deficiency to be erroneous.

To summarize, it becomes clear that the poisoning of plants suffering boron deficiency cannot be accounted for by high concentrations of auxins. Which chemical compounds may, then, be responsible for the effects observed? One is left to suggest that this toxicity is attributable to phenols, the accumulation of which under conditions of boron deficiency has been well documented. The first to observe the accumulation of phenols in boron-deficient plants was Reed (1947). Later, other workers confirmed that levels of phenols are elevated by boron deficiency and the composition of phenol compounds becomes richer.

According to Perkins and Aronoff (1956), the blue fluorescence of the necrotic spots on the leaves of boron-deficient plants is due to the accumulation of caffeic and chlorogenic acids. Further elucidating this phenomenon, Dear and Aronoff (1965) found that the appearance of symptoms of boron deficiency coincides with a notable increase in the ratio of caffeic acid to chlorogenic acid (Fig. 21). The leaves of boron-deficient tobacco plants have been shown to contain elevated amounts of scopolin (Watanabe et al., 1961). In sunflower plants scopolin could be detected only in boron-deficient plants (Watanabe et al., 1964). In boron-deficient sunflower plants another compound has been found which is absent from boron-supplied plants - namely gentisic acid glycoside. Zane and Wender (1964) identify this compound as the 5-β-D-glycoside of gentisic acid. Troitskaya et al. (1971) found that the same compound occurred only in boron-deficient sunflower plants, appearing as early as two days after the removal of boron from the

medium. Moreover, another compound, preliminarily identified as glycosyl-2,3-dioxybenzoic acid, was detected in these plants.

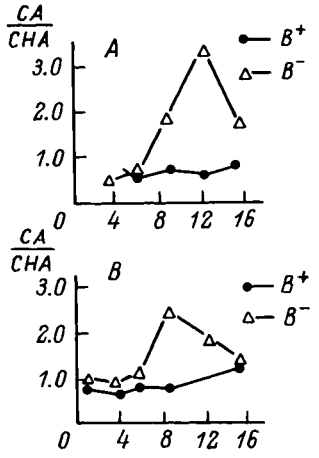


Fig. 21. Effects of boron on the relative contents of caffeic (CA) and chlorogenic (CHA) acids in the shoots (a) and leaves (b) of sunflower plants (after Dean and Aronoff, 1965). Abscissa, days; ordinate, caffeic/chlorogenic acid ratio.

In addition to phenol carbonic acid, flavonoid compounds including rutin and catechins with leucoantocyans accumulate in boron-deficient dicotyledonous plants (Shkolnik and Abysheva, 1975). Among these substances, not only inhibitors of auxin oxidase, but also growth inhibitors are found such as flavonol-3-glycoside, the concentration of which considerably increases in boron-deficient plants (Shkolnik and Abysheva, 1975; Fig. 22). As discussed above, the amounts of other growth inhibitors - caffeic acid and scopolin - also increase in boron-deficient plants.

Increases in the amounts of phenolic compounds in boron-deficient plants occur mostly in dicotyledons. Conversely in cereals, which exhibit a low sensitivity to boron deficiency during the formation of their vegetative shoots, the reverse situation may occur. In boron-deficient maize plants, the total content of flavonoid compounds was reduced (Krupnikova, 1970) and the concentrations of complex esters of oxycinnamic acids: 3-O- and 5-O-caffeylquinine (chlorogenic and neochlorogenic acids), 3-O- and 5-O-ferulylquinine and 3-O- and 5-O-cumarylquinine acids were lowered (Shkolnik et al., 1972; Fig. 23). A similar finding was reported

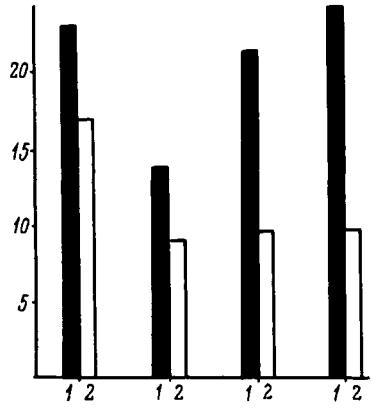


Fig. 22. Effect of boron deficiency on levels of the growth inhibitor flavonol-3-glycoside (mg per litre protein) in tomatoes (after Shkolnik and Abysheva, 1971). 1, without boron; 2, in the presence of boron. I, 1st and 2nd leaves; II, 3rd leaf; III, 4th leaf; IV, 5th leaf and apical meristem.

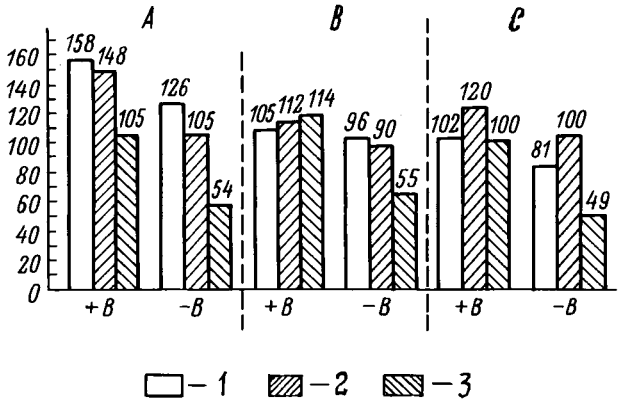


Fig. 23. Effect of boron deficiency on levels of oxycinnamic acids - p-cumaric (1), ferulic (2) and caffeic (3) - in maize leaves (after Shkolnik et al., 1972). (A) symptoms of boron deficiency absent, (B) signs of deficiency in the fruit, (C) advanced stages of deficiency. Ordinate, amounts of the acids (μg per g fresh wt.).

in a representative of the monocotyledons - a palm (Rajaratham et al., 1971). The concentration of caffeic and chlorobenzoic acids did not increase in the leaves of boron-deficient plants. An unknown compound with a blue fluorescence accumulated. At the same

time, in contrast to the leaves of boron-supplied plants, those of boron-deficient plants contained no leucoantocyanidines in the period before appearance of the signs of boron deficiency. A considerable increase in the accumulation of ferulic and vanillic acids, which are growth inhibitors, was reported in the boron-deficient palm (Rajaratham and Lowry, 1974).

In boron-deficient monocotyledons the total phenol content either is unchanged, is increased but to a less extent than in dicotyledons,, or is even decreased. Krupnikova and Smirnov (1981) found that in boron-deficient sunflowers, peas and maize the total phenol content is considerably increased; this is not found to occur in wheat, which is only moderately sensitive to boron shortage.

The above evidence indicates that phenols generally accumulate to a lesser extent in boron-deficient monocotyledons than in dicotyledons, however, some phenolic compounds may be found in monocotyledonous plants in considerably larger amounts. Again, it is not only in boron-deficient dicotyledons that individual phenolic compounds, which are absent from similar boron-supplied plants, may accumulate, but also in boron-deficient monocotyledons, as demonstrated in the helm palm.

Thus, it has become increasingly clear that inhibition of the growth of vegetative shoots as a result of boron deficiency is based on the accumulation of phenolic growth inhibitors, and that the more pronounced inhibition of growth in dicotyledons compared with some monocotyledons is associated with a more diverse and abundant complement of phenolic compounds, including growth inhibitors, in the former.

The more considerable accumulation of phenols in dicotyledons is consistent with the above-described decrease in the activity of auxin oxidase in boron-deficient dicotyledons (Shkolnik et al., 1964). This decrease is the result of an accumulation of phenolic inhibitors of auxin oxidase - i.e. caffeic and chlorogenic acids (Perkins and Aronoff, 1956).

The smaller inhibition of growth in boron-deficient cereals may be accounted for not only by a less extensive accumulation of phenolic growth inhibitors in these, but also by the higher resistance of cereals to high concentrations of these inhibitors, as is found in the case of germinating seeds of cereals treated with the growth inhibitor cumarin (Mayer and Poljakoff-Mayber, 1963). The treated seeds are more resistant to high concentrations of

the inhibitor than seeds of dicotyledons. Most of these inhibitors are flavonoids. In this context, it is of interest to note that Tauböck (1942a), discussing interactions between boric acid and flavonoids, noted that flavonoid-rich plants tend to suffer more from a shortage of boron.

The accumulation of phenols in boron-deficient plants follows from the stimulation of the pentose-phosphate pathway of the oxidation of sugars (Lee and Aronoff, 1967; Eichhorn and Augsten, 1974). Shkolnik and Ilyinskaya (1975) showed that this was the case only in dicotyledons and (to a lesser degree) in those monocotyledons (maize) that are more sensitive to boron deficiency. No stimulation of the pentose-phosphate pathway is observed in monocotyledons such as wheat which display low sensitivity to boron deficiency (Fig. 24).

The increased content of phenols in conditions of boron deficiency is also attributable to stimulation of the principal enzyme of phenol biosynthesis, that is phenylalanine ammonia lyase (Shkolnik et al., 1980; Fig. 25). Its activity increases in dicotyledons (bean and cucumber), as does the activity of glucose-6-phosphate dehydrogenase. This has never been observed in wheat and oats, which are monocotyledons of moderate sensitivity to boron shortage.

Studies on the involvement of boron in the metabolism of phenolic compounds have stimulated interest in the effects of boron deficiency on the activity of the enzymes concerned in this metabolism. β-glucosidase, located predominantly in the cell wall, is one such enzyme; it liberates aglycones, compounds which are considered as being more toxic than glycosides. β-glycosidase activity was found by Mayevskaya et al. (1974) to be higher in boron-deficient sunflower plants compared with boron-supplied plants, irrespective of the time lapse since boron was removed from the nutrient medium. Subsequent studies by these workers (Mayevskaya et al., 1975) revealed large differences in the activity of this enzyme in dicotyledons and monocotyledons. The activity of β-glycosidase was much higher in boron-deficient, than in boron-supplied dicotyledons. In contrast, this enzyme's activity was only slightly increased or not increased at all in the monocotyledons wheat and maize (Fig. 26). These findings indicate that the release of aglycones was increased in boron-deficient dicotyledons, whereas in monocotyledons aglycones were present either in very low concentrations or only in trace amounts. Again, these observations support the view that monocotyledons contain lower amounts and a smaller

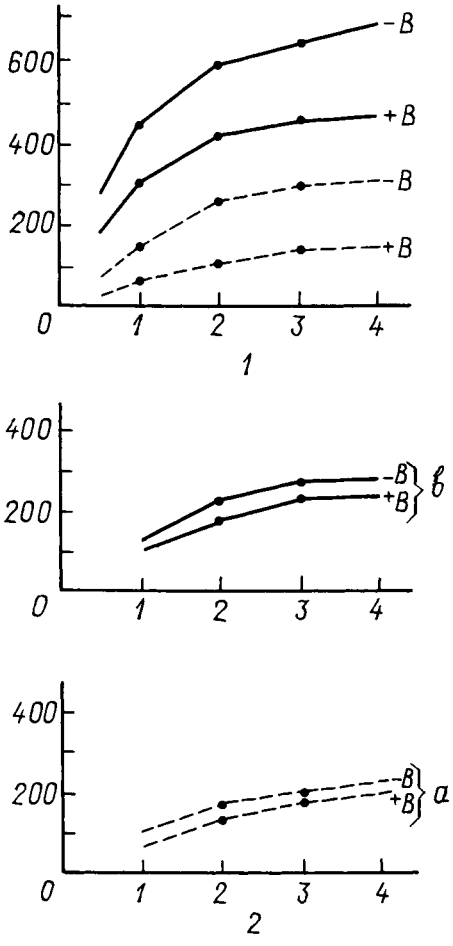


Fig. 24. Effect of boron deficiency on glucose-6-phosphate dehydrogenase activity in the roots of kidney bean plants (above), maize (centre), and wheat (below)(after Shkolnik et al., 1975). Unbroken curves, 9-day-old plants. Ordinate, μg NADH per mg protein; abscissa, time (minutes).

variety of phenolic compounds, compared with dicotyledons.

Interestingly, both the activity of β-glycosidase and the amounts of phenols decrease in seeds with the breaking of dormancy (Metlitsky et al., 1972). Boron has been found to stimulate the breaking of dormancy and germination of Themeda triandra seeds. Since gibberellins are also able to break dormancy, Creswell and Nelson (1972a,b) explain this effect of boron in terms of its influence on the synthesis of gibberellic acid.

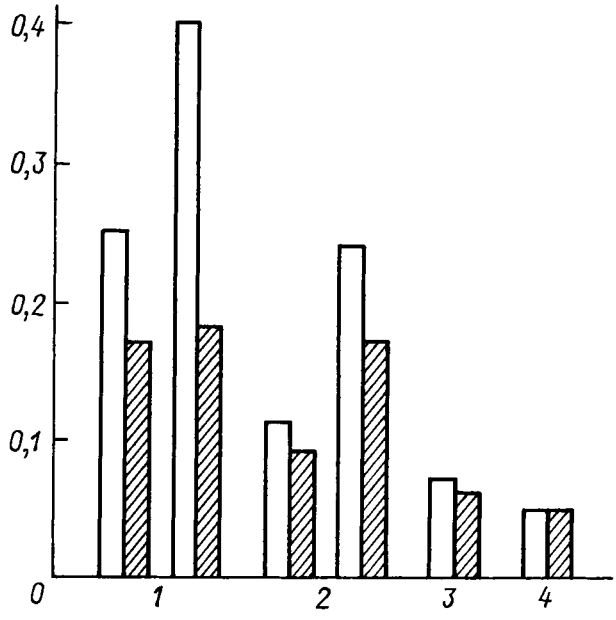


Fig. 25. Effect of boron deficiency on the activity of phenylalanine-ammoniumliase (after Shkolnik et al., 1981). Ordinate, enzyme activity (mg of coryc acid per g of raw tissue per hour). Abscissa, leaves of 1, phaseolus; 2, cucumbers; 3, wheat; 4, oats. First two blocks represent leaves showing initial symptoms of deficiency; second two blocks represent leaves showing strong symptoms of deficiency. Leaves of wheat and oats were taken with slight deficiency symptoms; unhatched blocks - with boron, hatched blocks - without boron.

These workers showed that the administration of boron, either alone or in combination with gibberellic acid, resulted in an increased RNA content and an increase in α-amylase activity in the germinating seeds (Creswell and Nelson, 1973). Gibberellic acid added alone produced a similar, but less pronounced effect. The positive effects of boron on the breaking of seed dormancy in T. triandra may not be entirely attributable to the element's influence on the biosynthesis of gibberellic acid, as suggested by Creswell and Nelson, but may also arise from its ability to decrease the high concentration of β-glucosidase in dormant seeds, and to lower the accumulation of phenolic growth inhibitors which preclude germination of the seeds. The increase in RNA content produced by boron and gibberellic acid is apparently related to

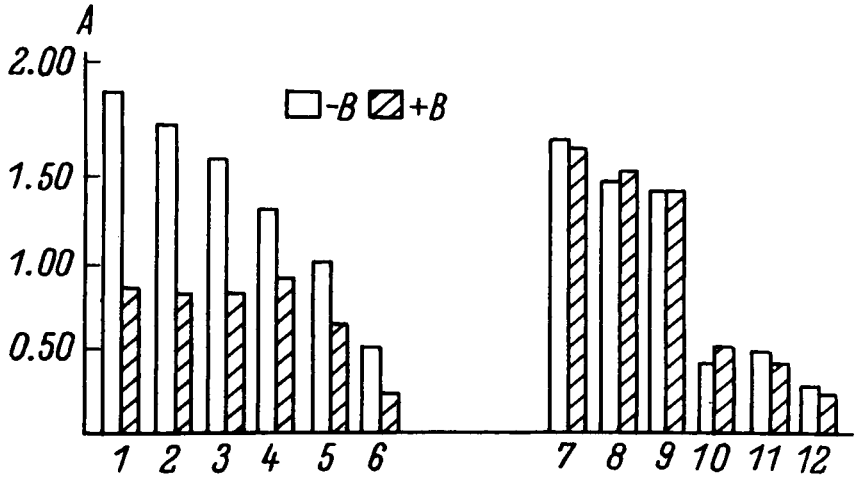


Fig. 26. Effect of boron deficiency on the activity of β-glucosidase in some mono- and dicotyledons (after Mayevskaya et al., 1975). A, enzyme activity (mg salicin per g fresh wt. per hour at 30°C). Left: 1, sunflower; 2, chick pea; 3, lupin; 4, broad bean; 5, kidney bean; 6, soybean. Right: 7, maize; 8, wheat; 9, sorghum; 10, oats; 11, sword-flag; 12, spiderwort.

the decreased content of phenols which in high concentrations are able to inhibit RNA synthesis (Korableva et al., 1971).

According to Lee and Aronoff (1967), the main symptoms of boron deficiency - browning of the tissues and degradation of the growing points in dicotyledons - are a manifestation of the accumulation of phenols occurring in an oxidized and more toxic state. Shiroya et al. (1955) has shown that browning of tobacco leaves is associated with the oxidation of caffeic and chlorogenic acids by polyphenol oxidase. These same acids accumulate in the tissues surrounding the necrotic spots characteristic of boron-deficient plants (Perkins and Aronoff, 1956). This assumption of Lee and Aronoff is certainly justified. In a number of studies the activities of tyrosinase, polyphenol oxidase, dehydrophenyl alanine oxidase were shown to increase in boron-deficient plants (McVicar and Burris, 1948; Klein, 1951; Nason, 1952).

Hewitt (1963) suggested that polyhydroxyboric compounds may control the rate of cyclic oxidation in phenolic oxidative systems. The inhibitory effect of phenols is attributed to the toxic action of their oxidation products.

Chlorogenic and caffeic acids are known to accumulate in infected organs, and in necrotic and paranecrotic tissues (Metlitsky et al., 1972). Judging from data on the toxicity of the enzymatic oxidation products of chlorogenic and caffeic acids, quinones may be among such oxidization products; sulfonic compounds of caffeic and chlorogenic quinones have been isolated (Metlitsky et al., 1972). Thus an accumulation of quinones may be responsible for the effects of boron deficiency. No accumulation of quinones occurs in boron-supplied plants, since in these plants the activity of polyphenol oxidase is much lower. Interestingly, in infected and necrotic tissues the scopolin content is sharply increased.

In this context mention should be made of the study by Reinert and White (1956), who observed browning of roots grown in culture. This effect was attributed to a diminution of growth, and the browning was thought to be caused by the activation of polyphenol oxidase since it could be eliminated by the addition of tyrosine or an inhibitor of polyphenol oxidase. Removal of the browning was accompanied by a simultaneous resumption of growth.

Electron microscopy of apple fruit tissues has revealed that the appearance of disease accompanied by the browning of tissues is associated with an increased permeability of the vacuole tonoplasts; this facilitates the penetration of polyphenols into the cytoplasm where they interact with polyphenol oxidase (Platonova and Metlitsky, 1972). Increased permeability of the tonoplast may be a consequence of the deposition of phenolic substances accumulating in the vacuoles. There are reasons to suppose that a similar enhancement of the permeability of tonoplasts occurs as a result of the accumulation of phenols in boron-deficient plants. Demyanets and Rybak (1974) found that in affected apple fruits the browning of the peel that occurred after prolonged storage of the fruits was accompanied by an increased accumulation of condensed catechins. This apparently occurs as a result of impairment of the structure of the vacuoles in which polyphenols are accumulating, and therefore the polyphenols become more accessible to the action of polyphenol oxidase present in the cytoplasm.

Shkolnik et al. (1981) compared levels of polyphenol oxidase activity in different boron-deficient plants showing various sensitivities to the absence of boron (Table 5).

The data in the table show that in boron-deficient dicotyledons of the first group (sunflowers and tomatoes), which under normal conditions show a high polyphenol oxidase (PPhO) activity in both

leaves and roots, a sharp rise in PPhO activity is observed (even where the symptoms of boron deficiency are slight). In plants of the second group, peas, which normally show a low PPhO activity, the same rise of PPhO activity is observed, although it is less pronounced.

TABLE 5 Influence of boron deficiency on the activity of polyphenol oxidase in dicotyledons and monocotyledons, ml 0.01 n.KJO_4 per g raw material (after Shkolnik et al., 1981)

Symptoms of boron deficiency	Leaves		Roots	
	+B	-B	+B	-B
	Controls	Untreated	Controls	Untreated
	Dicotyledons			
	Sunflower			
Absent	25.7	29.7	2.66	3.86
Slightly expressed	42.0	173.0	1.87	3.29
Expressed	39.0	192.0	2.28	3.69
	Tomato			
Slightly expressed	2.3	10.8	6.2	6.7
Expressed	3.6	12.7	6.5	6.7
	Pea			
Absent	0.14	0.43	0.83	0.88
Slightly expressed	0.30	1.04	0.58	1.06
Expressed	0.20	1.24	0.52	0.84
	Monocotyledons			
	Maize			
Slightly expressed	0.33	0.46	0.14	0.18
Expressed	0.66	1.64	0.16	0.25
Strongly expressed	1.12	3.46	0.14	0.20
	Sorghum			
Strongly expressed	0.10	0.11	0.18	0.22
	Wheat			
Small growth retardation	0.16	0.15	0.20	0.21

Changes in PPhO activity brought about by boron starvation in monocotyledons, which are normally characterized by low PPhO activity, are not quite the same as those observed in dicotyledons. The reactions of two plants of the first group of monocotyledons were different. Maize - the most boron deficiency sensitive monocotyledon, showed a rise in PPhO activity, whereas sorghum plant

showed the opposite reaction, although this plant, like maize, decays under conditions of boron starvation. Plants of the second group of monocotyledons, wheat plants, producing normal vegetative organs when starved of boron, show no rise of PPhO activity.

The author (Shkolnik et al., 1981) studied the formation of quinones from exogenous polyphenols in leaf homogenates prepared from boron-deficient sunflowers. In these experiments, stable derivatives of quinones with benzosulphonic acid were used instead of the less stable quinones. It was found that boron deficiency promoted quinone formation (Table 6). These data confirm the ability of boron to suppress the accumulation of the quinone-type products of phenol oxidation in dicotyledons.

The same phenomenon has been observed (Smirnov et al., 1981) in a maize variety that is sensitive to boron deficiency, but not in wheat, which is insensitive as far as the develoment of the vegetative organs is concerned. This result and the results presented in Table 6 are discussed from the point of view that the principal physiological role of boron is to protect dicotyledons and some monocotyledons (maize) from an increase in the quantity of quinoid products arising from phenol oxidation.

TABLE 6 Quantity of phenolsulphonic derivatives detected during incubation of leaf homogenates with various exogenous plants (after Shkolnik et al., 1981)

Experiment	Plant age (days)	Degree of boron deficiency	Mean daily air temp. (°C), 5 days before end of experiment	4-phenylsulphonylpyrocatequin		2-phenylsulphonylhydroquinone		6-phenylsulphonylcaffeic acid	
				-B	+B	-B	+B	-B	+B
1	16	Severe	17.8	20.5	10.0	17.0	11.0	3.3	1.3
2	21	Moderate	13.5	17.5	11.5	22.0	12.0	4.9	3.2
3	21	Mild	11.4	13.0	10.0	12.0	8.0	5.5	4.0
4	21	"	15.8	13.0	9.0	13.5	9.0	6.5	4.0
5	26	Severe	14.5	24.5	18.0	-	-	-	-

Experimental results showing that boron-deficient mono- and dicotyledons differ strikingly in their phenol metabolism, and that the boron requirements of mono- and dicotyledons are also strikingly different, have enabled the author not only to shed some light on the mechanism of these different reactions to boron

shortage, but also to conclude that the principal physiological role of boron is its involvement in the regulation of phenol metabolism.

These works confirmed the hypothesis of Shkolnik (1974a) concerning the physiological role of boron in dicotyledons. The keystone of this hypothesis is the role of boron in phenol metabolism. The concept is based essentially on the correlation found by the author and his collaborators between the greater sensitivity of dicotyledons, compared with monocotyledons, to a shortage of boron, and the greater accumulation of different phenols and increase in the content of oxidized phenols in the former. It is supposed that dicotyledons are poisoned by oxidized phenols of the quinone type as a result of boron deficiency. This poisoning is responsible for all or many of the observed metabolic disturbances in dicotyledonous plants (Fig. 27).

Data are available indicating an involvement of boron in membrane function. Thus, boron deficiency is accompanied by a decrease in the content of various phospholipids (Shkolnik and Kopmane, 1970; Alekseyeva, 1971; Timashov and Ilyushchenko, 1977).

According to Pollard et al. (1977), the specific activity of membrane components is regulated by boron, and boron plays a primary role in the control of membrane permeability. Robertson and Longham (1974) also report on the effect of boron on membrane permeability. Results show that boron restores the incorporation of phosphate into maize and bean roots (a process that is impaired in conditions of boron deficiency), and in the case of maize, restores the uptake of chlorine and lead. Boron also restores the activity of KCl-dependent ATPase. The authors believe that the observed membrane effect of boron deficiency is a direct one, and is not mediated by an accumulation of phenol compounds since the reversibly altered ATPase was isolated from boron-deficient plants under conditions in which all membrane-associated phenols would have been eliminated.

In line with these studies on the role of boron in membrane function, it is pertinent to take mention of the assumption of Tanada (1974), that boron affects the membrane structure and probably the membrane components that are involved in phytochrome-dependent shifts of membrane potentials. Glass (1973, 1974), Glass and Dunlop (1974) have revealed that exogenous phenols can considerably inhibit the assimilation of both anions and cations by the roots of barley and soy beans. Lewis (1980a) believes it will

be necessary to find out how in conditions of boron deficiency, elevation of the level of endogenous phenols affects membrane integrity and function.

Recently, Dave et al. (1980) noticed an increase in ribonuclease activity under conditions of boron deficiency, and they discuss this in the context of membrane permeability.

Some indirect information is available suggesting differences between monocotyledons and dicotyledons in their membrane apparatus. Cereals growing in areas known to be rich in boron reserves are resistant to increases in the level of boron and hence are less subject than dicotyledons to the toxic effect of high dosages of boron (Shakhova, 1960). This indicates that the membranes of monocotyledons may be more stable than those of dicotyledons.

Boron deficiency leads to an increase in the activity of enzymes which are in the membrane-bound and also in cytoplasmic form. These are: phenylalanineammonium liase, glucose-6-phosphate dehydrogenase, β-glucosidase, polyphenoloxidase, ATPase and RNAse. There are grounds to suggest that the disturbances in membranes noticed at boron deficiency lead to a release of membrane-bound form of named enzymes which results in an increase of their general activity. It is possible that this damage to membranes may be the primary cause of the injury observed in boron-deficient plants, since one of these enzymes is polyphenoloxidase, the great activity of which leads to the increase in the quinone level and the intoxication of plants (Fig. 27).

The advantage inherent in the author's hypothesis of the unique role of boron in the phenol metabolism of plants is the offer of an explanation for many of the above-mentioned problems which researchers working on the physiological role of boron in dicotyledons are facing.

Thus the following explanations can be advanced:

1. Animals do not need boron because: a) phenols are less numerous and less significant in animals than they are in plants; b) they lack the polyphenol-polyphenol oxidase system; c) they lack lignification processes.

2. Fungi survive without boron not only because they are vascular plants, but also because they have a potent hydrolytic enzyme complex for the degradation of phenolic compounds (Kunajeva and Auganbajeva, 1976).

3. Boron is non-essential for green algae because they are resistant even to high phenol concentrations (Kostyaev, 1973),

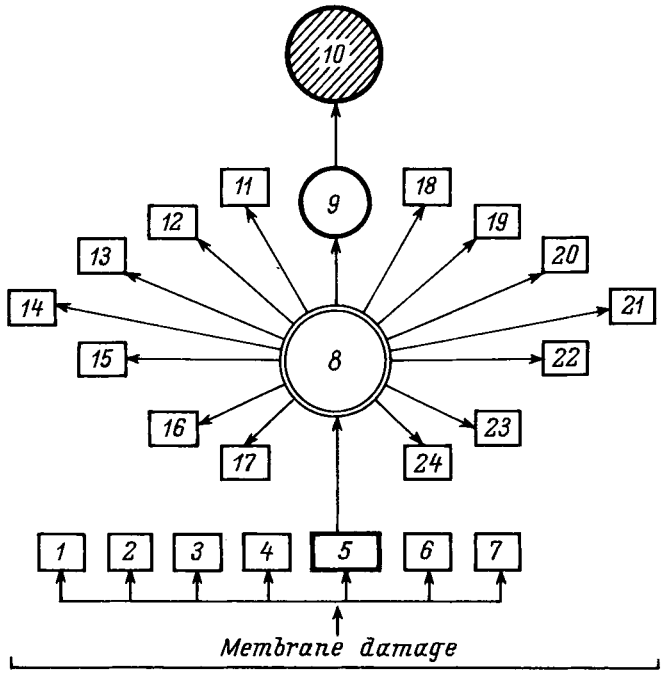


Fig. 27. A scheme of metabolic and structural disturbances under conditions of boron deficiency, caused by membrane damages, and the action of quinones: 1, boron deficiency; 2, activation of phenylalanineammonium liase; 3, activation of glucose-6-phosphate dehydrogenase; 4, activation of β-glucosidase; 5, activation of polyphenoloxidase; 6, activation of ATPase; 7, activation of RNAse; 8, increase in quinone content; 9, browning of tissues and degeneration of growing points; 10, decay of plants caused by toxicity of quinones; 11, decrease in content of free IAA; 12, changes in energy metabolism; 13, degradation of tRNA and rRNA; 14, disturbance of nucleotide composition of RNA; 15, changes in protein synthesis; 16, decrease in content of phospholipids and galactolipids; 17, disturbances of lignin synthesis; 18, disturbances of chloroplast structure; 19, disappearance of peroxysome; 20, disorganization of chromatin; 21, decrease in content of histone; 22, degradation of nucleoli; 23, disorganization of mitosis; 24, teratological alterations.

and because they lack polyphenol oxidase (Eynor and Kolesnikov, 1962). Diatomaceous algae need boron; Lewin and Ching-Hong Chen (1976) demonstrated a considerable increase in the level of phenolic compounds in boron-deficient Cylindrotheca fusiformis.

4. The elimination of boron deficiency symptoms by the introduction of hydrogen peroxide into the nutrient medium may result from the oxidation of phenolic growth inhibitors (Lewis, 1980a).

5. The elimination of boron deficiency symptoms by means of a nutrient medium supplemented with a mixture of nucleotide precursors of nucleic acids may be related to the formation of complexes involving nucleotides and quinones (Korchevaya et al., 1965). Probably the elimination of boron deficiency symptoms by the introduction of free RNA is also attributable to the complexing of quinones by RNA degradation products.

6. The alleviation of boron deficiency symptoms by the introduction of germanium into the nutrient medium may be explained by its ability to form complexes with phenols (Zerkovnitskaya and Epimakov, 1966).

7. An increased content of phenolic growth inhibitors and oxidized phenols may be responsible for the decay of all the organs of the flowers in boron-deficient dicotyledons.

8. The rise in the boron requirement at high temperatures is accounted for, as was mentioned earlier, by substitution of the polyphenol oxidase system for the oxidative system of peroxidase (Abayeva et al., 1967).

9. The cause of the appearance of teratogenic modifications in boron-deficient plants is revealed. In the author's review (Shkolnik, 1981) and in the chapter dealing with teratology, evidence is presented showing that teratogenic changes may be attributed to disturbances of hormonal regulation, i.e. the equilibrium of growth stimulants and growth inhibitors is shifted in favour of the latter. Such a phenomenon occurs in cases of boron deficiency, and contributes not only to a rise in the levels of growth inhibitors of the phenolic type, but also to a drop in the level of auxins.

An increase in the level of the toxin thiocyanate in Raphanus sativus plants suffering boron deficiency has been described recently by Bible et al. (1981). A sudden inhibition of the growth of the plants occurred as the concentration of CNS^- in the leaves increased. When boron was supplied at concentrations from 0.1 to 10.0 mg per 1, the concentration of CNS^- in the plants did not change. The authors suggested that boron regulates the synthesis, or accumulation of a CNS^- precursor. Yet these findings do not explain the toxic effect of boron deficiency on dicotyledonous plants, an effect which preceeded by browning of the tissues

caused by oxidized phenol accumulation.

The hypothesis on the physiological role of boron suggested by the author of this book refers mainly to the dicotyledonous plants. The physiological role of boron in the monocotyledonous plants is not yet thoroughly investigated and many facts remain unclear.

The decay of monocotyledons of the first group on sensitiveness to boron deficiency can be caused by different reasons. The intoxication and decay of some of them, such as maize, as well as dicotyledons, can occur probably as a result of increased quinone content. Other plants, such as sorghum, behave differently. The cause of the decay of the above-ground organs of boron-deficient sorghum plants in the absence of any increase in polyphenol oxidase activity is still not understood. This decay may possibly be connected with the accumulation of a growth inhibitor such as ferulic acid, which has been found to occur in monocotyledonous plants and to increase significantly in palms under conditions of boron deficiency (Rajaratham, 1974). Mayer and Poljakoff-Mayber (1963) showed that ferulic acid inhibited the germination of seeds to the same extent as p-cumaric acid, which is known to be a growth inhibitor.

It is possible that the decay of sorghum plants under conditions of boron deficiency is related with damage in the biosynthesis of lignin. The author of this book has pointed to the possibility of damage of this kind having a catastrophical result (Shkolnik,1956). It is possible that this damage is more expressed in monocotyledonous than in dicotyledonous plants.

Not understood facts have appeared: there is no adverse effect on the root system of sorghum as a result of boron-starvation (Fig. 28), although there is decay of the over-ground organs; there is poor development of the roots in the cucumber - a dicotyledonous plant (Fig. 68).

Thus boron, which is not a metal and unlike other metallic trace elements, is not an enzyme component or enzyme activator, plays a highly specific role in plant life processes on account of its unique significance in phenol and lignin metabolism and due to its influence on the state of membranes. It is of the greatest importance for further elucidation of the physiological role of this element that the study of its significance in phenol metabolism, in lignin biosynthesis, and in membrane function, should continue.

More than 40 years ago the author (Shkolnik, 1939a) suggested that a comparative physiological and biochemical investigation of monocotyledons and dicotyledons displaying different requirements for boron may provide a clue to the physiological role of this element. As can be seen from the above, this suggestion was fully vindicated by subsequent experimental findings.

Fig. 28. Sorghum grown with and without boron (after Shkolnik and Mayevskaya, 1977).

Chapter 2

MANGANESE

The typical symptom of manganese deficiency is spotted leaves (Fig. 29). The largest amount of manganese in plant cells is concentrated in the cytoplasm, and among cell organelles, the chloroplasts are the richest in manganese. Research on the physiological role of manganese was initiated by Bertrand (1897a,b).

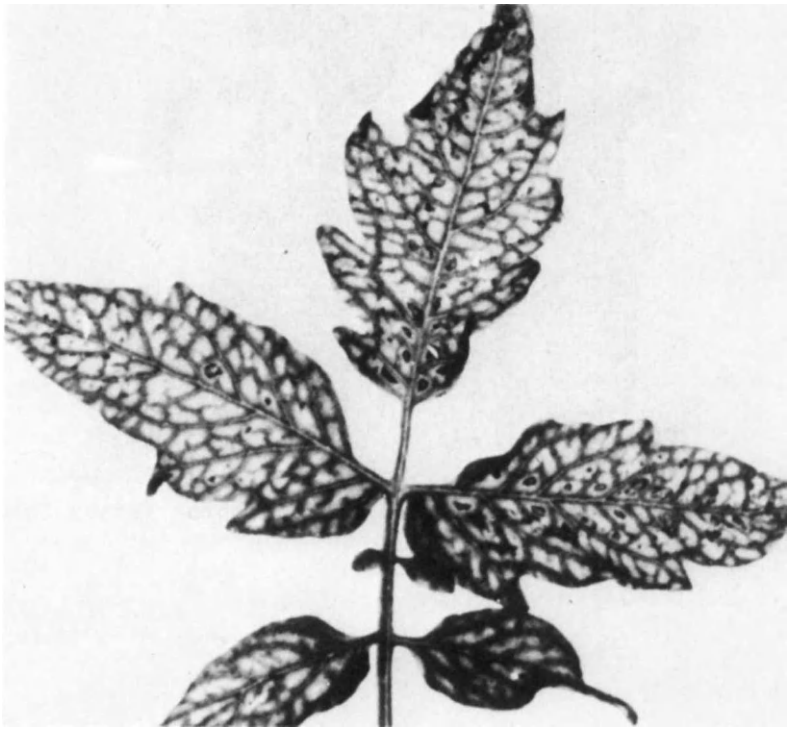


Fig. 29. Manganese deficiency in tomato (Lycopersicum esculentum). Orange-yellow chlorotic mottling with early development of profuse brown necrotic spots (after Hewitt, 1963).

Uspensky (1915) studied the effects of various concentrations of manganese on various groups of plants - algae, fungi, lichens and higher plants - and concluded that, by virtue of its oxidative properties, manganese may play a specific role in maintaining necessary oxidation-reduction conditions in the cells of plant organisms. This conclusion of Uspensky has found support in further research, and he should certainly be given credit for drawing

attention to this important aspect of the physiological role of manganese. He pointed to the relationship between the accumulation of manganese and the accumulation of tannides and organic acids in some lower and higher plants. Uspensky's views have also found strong support in the investigations of Levanidov (1957, 1961), who studied the role of manganese in manganese-rich plants, or so-called manganophiles. The manganophiles are particularly well represented among hydrophytes and woody plants. It has been established that the increased concentration of manganese in the tissues of these plants is related to their biochemical composition. Plants which take up large concentrations of manganese are usually also rich in tannides, and apparently often contain large amounts of alkaloids. According to the hypothesis of Levanidov (1961), the relationship between the accumulation of manganese and the accumulation of tannides is explained by the fact that the latter are potent reducing agents, while manganese in its highest oxidative state is one of the strongest oxidizing agents. The manganese as oxidizer counterbalances the large amount of reducing tannide.

The positive effect of manganese on alkaloid content has been confirmed by the studies of Jindra et al. (1959). Manganese, which belongs to those metals showing high oxidation-reduction potentials, can easily enter into biological oxidation reactions. Numerous direct and indirect data testify to the stimulation of oxidation processes by this metal (Hopkins, 1930).

For over 40 years the attention of investigators has been focused on the significance of the definite proportions of iron and manganese that are required by living organisms. Hopkins (1930) suggested that manganese, together with iron, maintains the activity of a particular oxidation-reduction system. Based on his studies on Chlorella, Hopkins concluded that manganese is able to regulate the ferrous-ferric interconversion ($Fe^{2+} \rightleftharpoons Fe^{3+}$).

Although the requirement of a definite ratio of iron and manganese cannot be denied, from evidence available to date it can be deduced that the relationships between the two metals are complex and hardly depend upon the control of the divalent/trivalent iron ratio by manganese.

Udelnova (1969) indicated that manganese may be found operative in a large number of catalytic reactions, and the structure of the manganese atom may to some extent explain its wide biological occurrence. Manganese belongs to metals of the transition group of the periodic system, a group featuring variable valence. Its

active deforming effect on the electron shells of the atoms of many molecules account for the tendency of the latter to form complexes. Various transitions can be observed in manganese:

$$Mn^{2+} \rightleftharpoons Mn^{3+} \rightleftharpoons Mn^{4+} \rightleftharpoons Mn^{5+} \rightleftharpoons Mn^{6+} \rightleftharpoons Mn^{7+},$$

and each of these transitions may underlie the involvement of manganese in a number of oxidation-reduction reactions. Thus, the $Mn^{2+} \rightleftharpoons Mn^{3+}$ system at neutral pH has an oxidation-reduction potential of +1.09 v. According to Boichenko (1968a), this oxidation-reduction potential is entirely sufficient for the release of a free oxygen atom from a molecule of water. No other system known to exist in plants can explain such a process, since no other has such a high oxidation-reduction potential. It was later found that manganese plays an important part in oxygen liberation as part of the process of photosynthesis. Manganese is an activator of a number of enzymes, including those which catalyze oxidation-reduction reactions, carboxylation, hydrolysis, and group transfer reactions.

The physiological role of manganese is largely determined by its ability to control the activity of enzymes (Table 7).

TABLE 7 Manganese enzymes (compiled from Dixon and Webb, 1958)

Enzyme	Source
Carbone-dioxide reductase	Higher plants
Hydroxylamino reductase (1.7.99.2)	Higher plants
Isocitrate dehydrogenase (1.1.1.41)	Animals, yeast, slime molds
Malate dehydrogenase (the malic enzyme) (1.1.1.38)	Animals, higher plants, bacteria
D-glutamyl transferase (2.3.2.1)	Bacteria
Prolidase (3.4.3.7)	Animals
Imidodipeptidase (3.4.3.7)	Animals
Assimilating enzyme	Higher plants
Imidazole glycerophosphate dehydrase (4.2.1.10)	Slime molds
Acetolactate decarboxylase (4.1.1.5)	Bacteria
Serine-oxymethyltransferase (2.1.2.1)	Animals (liver)
Oxaloacetate decarboxylase (4.1.1.3)	Bacteria
Serine-transoxymethylase (2.1.2.1)	Animals
Muconate-cycloisomerase (5.5.1.1)	Bacteria

There are indications in the literature that in addition to the enzymes listed in the table, glutamine synthetase from Escherichia coli (6.3.1.2), pyruvate carboxylase in animals (6.4.1.1), yeast apirase (3.6.1.5), and oxaloacetase (3.7.1.1) from slime molds are also manganese enzymes.

Kulayev et al. (1972) have found that polyphosphate phosphohydrolase is a metalloenzyme, requiring manganese or cobalt for its activity.

It has been found that for the activation of glutamatesynthetase in the chloroplasts of pea leaves, the presence of Mg^{2+} and (or) Mn^{2+} is essential; the activity in the presence of Mg^{2+} is 2.5 times greater than that conferred by Mn^{2+}. The greatest activity of glutamatesynthetase is observed in the presence of both Mg^{2+} and Mn^{2+} in the ratio of 10:1.

At the same time, manganese like other metals is an activator of a large number of nonspecific metalloenzyme complexes, especially those involved in glycolysis and the Krebs cycle (Fig. 30).

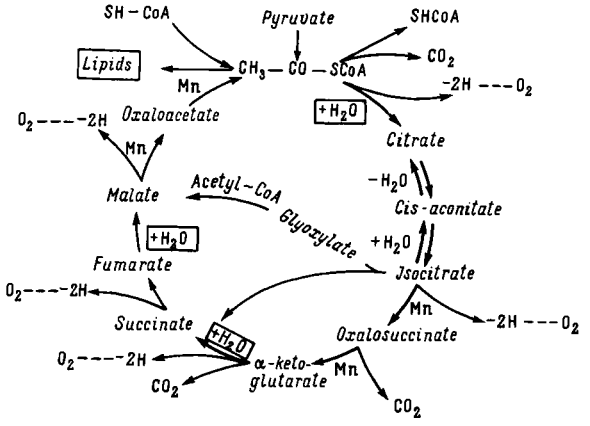


Fig. 30. A scheme of Mn participation in the enzyme reactions of Krebs cycle (after Chernavina, 1970).

As seen from the list of manganese-activated enzymes, this metal activates a large number of enzymes catalyzing oxidation-reduction processes, decarboxylation, hydrolysis, and group transfer reactions. It has also been found that manganese activates certain dipeptidases. Enolase is among these enzymes of the glycolytic cycle that are activated by manganese as are hexokinase and phosphoglucomutase (4.2.1.11).

Manganese plays a prominent role in the transformation of di- and tricarbonic acids. The significance of this is not difficult to appreciate, if one recalls that the Krebs cycle is a universal process in the plant kingdom.

Manganese is an activator of malic dehydrogenase, which oxidizes malic acid to form oxaloacetic acid. It is required for the conversion of isocitric acid to ocalosuccinic acid, and α-ketoglutaric acid to succinic acid. A shortage of manganese results in a considerable decrease in the activity of this enzyme (Nason, 1952). Manganese is also an activator of oxalosuccinic acid decarboxylase, which produces α-glutaric acid in the course of the decarboxylation. Manganese exerts a strong activating effect on malate dehydrogenase which catalyzes the decarboxylation of oxaloacetate (Tsai et al., 1971). Without manganese the malic acid enzyme which oxidizes malic acid to pyruvic acid does not function.

In collaboration with Ilyinskaya the author has found (Ilyinskaya and Shkolnik, 1970) that a distortion of the normal transformation pattern of organic acids occurred in plant leaves as a consequence of manganese shortage. This was manifested in an acceleration of the accumulation of citric acid, and retardation of the accumulation of malic acid. It is conceivable that manganese deficiency in plants serves to stimulate the conversion of malic acid to citric acid on the one hand, and retards further metabolism of citric acid on the other.

Brown et al. (1958) reported findings of a 12-fold decrease in $C^{14}O_2$ incorporation into glycolic acid, and a 10-12-fold decrease in the incorporation of the isotope into malic acid in Chlorella cells grown under manganese deficiency. The radioactivity of all the other intermediates of the Krebs cycle was found to be only two times lower than normal under conditions of reduced manganese supply.

Elstner and Heupel (1973) showed that a photoinduced decarboxylation of pyruvate, glyoxylate and glycolate occurs in isolated spinach chloroplasts. The rate of this decarboxylation is considerably increased by manganese ions or an addition of self-oxidized electron acceptors.

The processes of carboxylation and decarboxylation in which manganese plays an important part are basic to such cardinal life processes as the synthesis of proteins and fats, as well as to photosynthesis. Ochoa (1951) believes that one of the reasons for the important role of manganese in photosynthesis is its participation in the reversible decarboxylation of two and three carbon acids.

It is well-established that a close relationship exists between the manganese content of plants and their concentration of ascorbic

acid. Rudra (1944) found that manganese plays a specific role in the biosynthesis of ascorbic acid, and Devyatnin (1959) also confirmed that the biosynthesis of ascorbic acid requires manganese, which is apparently a constituent of the enzyme synthesizing ascorbic acid. This finding is supported by the results of model experiments on the synthesis of ascorbic acid in freshly prepared onion juice in the presence of glucose and 0.005 M manganese sulphate.

According to the above observations, it might be expected that manganese would be required for the processes of respiration. In fact, evidence for this was provided by Lundegardh (1939), who found that additions of manganese brought about an enhancement of oxygen uptake in roots by 155 to 470%. A decrease in oxygen uptake attributable to manganese deficiency was later reported by Kessler (1957), and Skvortsov (1950) indicated that manganese acts to stimulate respiration.

Brown et al. (1958) detected an increase in the intensity of respiration during three days' growth of autotrophic, myxotrophic and heterotrophic Chlorella cultures in the absence of manganese. These authors also reported variable effects of manganese on the endogenous respiration of algae, an increase or a decrease occurring according to the culture age.

Not only is manganese a component of, or activator for the enzymes catalyzing the various stages of respiration, but it is also associated with enzymes involved in nitrogen metabolism. Thus manganese was found to be a constituent of hydroxylamine reductase (Nason et al., 1954). These data may explain the earlier data reported by Burström (1939) on the involvement of manganese in the reduction of nitrates, as later confirmed by Jones (1949). According to Alberts (1941), plants supplied with nitrate nitrogen are more strongly affected by manganese deficiency than those grown on ammonium nitrogen. Kessler (1957) has shown that in green algae adapted to growth in hydrogen atmosphere the presence of manganese influences the reduction of nitrates by molecular hydrogen in the dark. Alberts (1941) found a strain of Chlorella which, if exposed to a limited manganese supply, preferred ammonium to nitrate nitrogen; Pirson (1958) however, was unable to reproduce accurately these experiments. Hewitt (1959) obtained indirect evidence that the rate of respiration in Chlorella is enhanced by manganese deficiency.

The importance of manganese for the reduction of nitrates is indicated by the fact that on soils of low exchangeable manganese

content and high nitrate nitrogen content, the so-called "marsh spot" disease of peas is wide-spread. By injecting nitrogen into pea plant tissues, Heintze (1946) produced symptoms of manganese deficiency in pea plants grown on manganese-rich soils. Friedrichsen (1944) showed that in leaves of spinach plants supplied with nitrate, the amount of manganese was greater than the amount in plants grown on ammonium nitrogen.

Manganese is not a constituent of nitrite reductase which in fact contains iron and copper, but manganese has been found to be an activator of this enzyme (Nicholas, 1961). Elliott (1953) succeeded in isolating an enzyme of nitrogen metabolism which requires manganese or magnesium for full activity. This enzyme catalyzes two reactions: the formation of glutamine from glutamic acid and ammonium, and the formation of glutamine or glutamine hydroxamic acid by transfer of the α-glutamic group from ammonium or hydroxylamine, respectively. In the first reaction magnesium proves to be a more effective actuator than manganese, whereas the opposite is true for the second reaction. It has been shown that manganese affects the activity of α-glutamine synthetase.

Like other metals, manganese is able to activate peptidases. The requirement for manganese is apparently highly specific in the case of prolidase. Heintze (1946) found high levels of free amino acids in pea plants suffering from "marsh spot" and these were further increased upon supplying zinc and molybdenum. A deficiency of zinc brings about an increase in the levels of a number of free amino acids together with a dramatic increase in amide concentration, whereas a shortage of manganese results only in an increased amino acid content (Possingham, 1956). The increase in free amino acids associated with manganese deficiency is apparently related to a suppression of protein synthesis.

Lipskaya and Godnev (1963) found that omitting manganese from a nutrient medium for sugar beet resulted in a decrease in the number of chloroplasts in the columnar and spongy parenchyma, a decrease in the chloroplast volume, and a reduced chlorophyll concentration. A comparison of results obtained by light and electron microscopy has revealed that depriving plants of manganese brings about a decrease in the chlorophyll content per chloroplast, corresponding with a decrease in the number of grana.

A few significant findings have been obtained concerning the effect of manganese on porphyrin biosynthesis; it was found that the addition of manganese relieved the inhibiting action of EDTA

on 5-aminolevulinate hydrolyase, which catalyzes the formation of porphobilinogen (Nandi and Waygood, 1967).

Only one study may be referred to regarding the problem of the involvement of trace elements in carotenoid biosynthesis. It has been established that the five-membered precursor of the carotenoids is isopentyl pyrophosphate. This compound is the end product of a complex chain of reactions involving several enzyme systems. The last stage - a decarboxylation yielding isopentyl pyrophosphate- is catalyzed by hydrocarboxylase of 5-pyrophosphomevalonic acid. Divalent manganese ions and ATP are cofactors in this reaction (Lynen et al., 1959).

Qureshi et al. (1974) found that $NADP^{+}$, FAD, Twin-80, as well as Mg^{2+} and Mn^{2+}, are cofactors in the enzyme system catalyzing the conversion of cis- and transphytoluin into higher saturated carotenoids. These findings confirm the earlier suggested sequence for the biosynthesis of acyclic, monocyclic, and dicyclic carotenoids from phytoin in tomato plastids. A considerable decrease in the photosynthetic activity of manganese-deficient plants has been noted (Gerretsen, 1949; Shkolnik and Saakov, 1964). In 1937 Pirson showed that manganese deficiency leads to a reduction of photosynthesis in Chlorella in spite of the absence of apparent chlorosis.

Positive effects of manganese have also been observed in higher plants grown in soil cultures, just as positive effects are obtained with boron and zinc. Thus the rate of photosynthesis at high ambient temperatures and during drought is enhanced (Tagi-Zade, 1957; Shkolnik and Davydova, 1959), and there is a diminution of the diurnal depression of photosynthesis (Tagi-Zade, 1957; Shkolnik and Greshishcheva, 1959; Ratskevich, 1970). The decrease in the midday depression of photosynthesis produced by some trace elements is related in the author's opinion to their ability to increase the heat tolerance of plants. This is expressed in a lesser degree of inactivation of plastids and enzymes at high temperatures on the one hand, and in the positive effects of trace elements on the rate of translocation of assimilates, on the other.

Information on the involvement of manganese in photosynthetic reactions is of considerable interest. The discoveries made in this field are of major significance and give grounds for believing that with the elucidation of the role of manganese in photosynthesis, some of the fundamental events in this process may become better understood (Brown et al., 1958).

As early as 1939 Emerson and Lewis found that by supplying manganese to culture media containing manganese-deficient Chlorella the normal value of the photosynthetic quantum yield can be restored in a short time. The effect of manganese, like that of nitrates, depends on the availability of potassium, so that the higher the concentration of the latter, the higher is the concentration of manganese that is required for attaining a high intensity of photosynthesis.

Strong evidence concerning the significance of manganese for photosynthesis has been obtained by Pirson and Bergmann (1955) in their studies on the growth of auxotrophic, heterotrophic and myxotrophic Chlorella strains. A shortage of manganese very rapidly affected the auxotrophic cultures, whereas the algae grown in darkness in the same medium with an addition of glucose (the heterotrophic cultures) showed no difference of response when placed in manganese-free or manganese-supplemented media (Fig. 31). It is of interest to note that in the myxotrophic cultures grown in the light and supplied with glucose, symptoms of manganese deficiency could be observed. The growth of these cultures ceased as a result of the manganese deficiency, but was immediately resumed upon the addition of this element to the medium. This testifies to the necessity of manganese for glucose uptake in the light, since glucose metabolism in the dark differs from that in the light. A reference should be made to the work of Eyster et al. (1958), who have shown that in Chlorella and other algae, as well as in duckweed, the requirement for manganese by the auxotrophic cultures is three orders of magnitude higher than the amount required by the heterotrophs. In algae containing hydrogenase the photochemical production of hydrogen, which is markedly increased upon the addition of glucose, is appreciably suppressed by manganese deficiency (Kessler, 1957).

The role of the photosynthetic reactions in isolated chloroplasts can be increased by manganese, as demonstrated by Arnon et al. (1956). Later, Arnon (1958) reported that vanadium and manganese can significantly intensify the role of photosynthesis in Scenedesmus obliquus. Moreover, manganese can increase the rate of photosynthesis both at high and low levels of illumination; vanadium, however, only produces this effect at high illumination intensities. From these findings, Arnon deduced that manganese is involved both in the dark reactions and the photochemical reactions, whereas vanadium is involved only in the dark process.

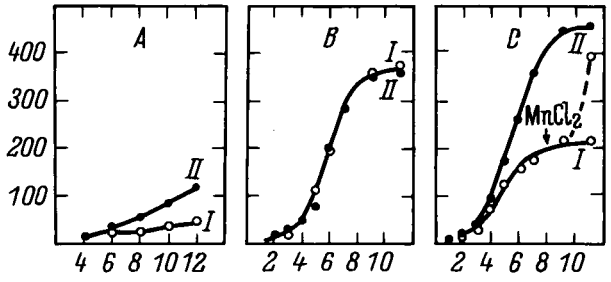


Fig. 31. Effects of manganese on the growth of Chlorella vulgaris (A) autotrophic (B) heterotrophic and (C) myxotrophic strains (after Pirson and Bergmann, 1955). I curves -Mn; II curves +Mn. Arrow indicates time of addition of $MnCl_2$. Ordinate, biomass (mg per 100 ml); abscissa, time (days).

A direct involvement of manganese in the photochemical reactions of photosynthesis is indicated also by the results of experiments in which the intensity of photosynthesis in cells grown in the absence of manganese could be restored within 20 minutes of supplying the element. In contrast, it took several hours for cells deprived of vanadium to return to their original intensity of photosynthesis upon addition of the metal to the growth medium, in spite of exposure to a high intensity of illumination.

Pirson (1958) noticed that the extent of the suppression of photosynthesis caused by an absence of manganese was independent of the intensity of illumination, and thus a similarity was observed between the effects of manganese deficiency and the action of hydroxylamine, - a compound that acts as an inhibitor of oxygen evolution in photosynthesis. This led the author to suggest that manganese plays an important role in the process of oxygen evolution during photosynthesis.

The work of Kessler (1957) has provided support for the hypothesis of Pirson. Kessler investigated the participation of manganese in the process of photoreduction (the reduction of carbon dioxide in the light in an atmosphere of hydrogen) in some species of algae. In this process the assimilation of carbon dioxide is not coupled to the production of molecular oxygen (Gaffron, 1940). According to the data reported by Kessler, photoreduction as opposed to photosynthesis, is absolutely independent of manganese, and an inadequate supply of manganese never brings about a decrease of the rate of photoreduction (Fig. 32). At the same time, an

increase in illumination intensity augments photoreduction. On the addition of manganese to the nutrient medium, and at high illumination intensities, the de-adaptation phenomenon can be observed accompanied by oxygen evolution, indicating the transition from photoreduction to photosynthesis.

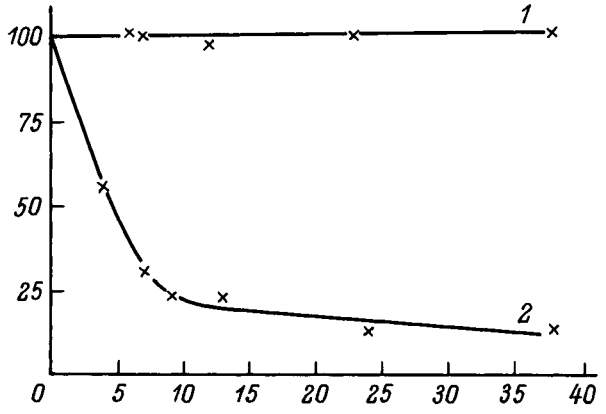


Fig. 32. Effects of increasing the manganese deficiency on the rate of photoreduction (1), and the rate of photosynthesis (2), in Ankistrodesmus sp. (after Kessler, 1957). Abscissa, time (days); ordinate, relative rate of process.

To summarize, these experiments demonstrate unequivocally that manganese is necessary for the photosynthetic reactions leading to oxygen evolution.

Investigations carried out on algae grown under conditions of phosphorus and iron deficiency have revealed the specificity of the action of manganese. A shortage of phosphorus slows down both photosynthesis and photoreduction, each to the same extent (Kessler, 1955), and the inhibition of photosynthesis in this case is observed at high light intensities. With an inadequate supply of iron, photoreduction is more strongly suppressed than photosynthesis (Kessler, 1957). According to Kessler, manganese undoubtedly acts as a link in the reactions which couple the photolysis of water to the release of oxygen. Kessler believes that manganese is specifically involved in the transformation of the first intermediate compound (designated $Y(OH)_2$) in the chain of reactions in which chemically combined oxygen is converted into a peroxide-like substance (designated $Z(OH)_2$). The latter eventually yields free

oxygen. $Z(OH)_2$ is assumed to be the component responsible for the re-adaptation and inactivation of dehydrogenase. In this respect, the hypothesized substance $Z(OH)_2$ is more active than free oxygen, since re-adaptation occurs more readily in the light than in the dark. Hence Kessler deduced that manganese influences this reaction which leads to the formation of a peroxide-like substance.

These concepts of the role of manganese gain support from experimental findings reported by other investigators - particularly from those which have demonstrated the involvement of manganese in the Hill reaction. Brown et al. (1958) emphasized that manganese plays a prominent role in the oxygen-producing system of photosynthesis. According to their observations, the Hill reaction is strongly suppressed in algae deprived of manganese, and this suppression can be relieved, increasing the rate of the reaction 15- to 20-fold by supplying adequate amounts of manganese (Fig. 33). The specificity of manganese in this process is clearly demonstrated; on addition of this metal, the Hill reaction is rapidly resumed and oxygen evolution begins.

Mechler (1951) also observed the influence of manganese ions on the Hill reaction. He discovered a special case of the Hill reaction in which oxygen acted as the Hill reagent, i.e. it replaced carbon dioxide as the electron acceptor in light reactions in chloroplasts. This type of Hill reaction (the Mechler reaction) was presented by Mechler in simplified form as follows:

$$2H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} 2(H) + 2(OH)$$

$$2(OH) \longrightarrow H_2O + 1/2O_2 ,$$

$$2(H) + O_2 \longrightarrow H_2O_2$$

In the experiments of Mechler, the production of hydrogen peroxide was not measured directly. The presence of hydrogen peroxide was, however, indicated indirectly by a stoichiometric reaction with ethanol, and by the formation of acetaldehyde. The author believes this reaction to be dependent on manganese. The results reported by Mechler offer further evidence for the involvement of manganese in oxygen evolution.

Later, confirmation of the involvement of manganese in photosynthetic oxygen evolution came from experiments employing the electron paramagnetic resonance technique (Treharne et al., 1960). Habermann (1960), using O^{18}, was able to detect catalytic stimulation by manganese of both oxygen evolution and oxygen uptake in

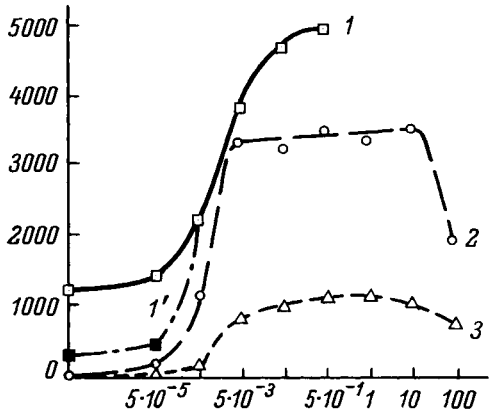


Fig. 33. Effect of manganese on the rate of photosynthesis, the rate of the Hill reaction, and growth in autotrophic Chlorella pyrenoidosa cells (after Brown et al., 1958). 1, rate of photosynthesis (rate of evolution of oxygen in ml per hour per mg of chlorophyll); 1', as 1, using purified Mn-free medium; 2, rate of Hill reaction, expressed in the same units; 3, growth of alga (μl biomass per 100 mg culture). For convenience the ordinates of the curves in 2 and 3 are magnified 5 times.

Phytolacca americana chloroplasts. In the process of oxygen release, manganese plays a part in the production of peroxide.

The investigations of Gerretsen (1949, 1950a, 1950b) also uphold the view that manganese is important for the process of oxygen evolution in photosynthesis. Gerretsen found that manganese considerably increased the rate of photosynthesis and the oxidation-reduction potentials in chloroplast suspensions exposed to light. From this finding, Gerretsen inferred that manganese plays a specific role in the process of the hydrogenation of oxygen to form hydrogen peroxide. Thus process, in turn, is the cause of the increase in oxidation-reduction potential. Manganese was thought to be involved in oxygen evolution occurring as a result of the photochemical cleavage of water:

$$HOH \longrightarrow H^+ + OH^- ,$$

the metal binding on to the hydroxyl group and thus preventing the reverse reactions from occurring:

$$H^+ + OH^- \longrightarrow H_2O .$$

Witt et al. (1963) suggested that there may be an unknown oxidation-reduction component that oxidizes water; this would be coupled to an enzyme system showing a strong requirement for the

manganese ion. According to Gaffron (1962), no oxygen evolution occurs during photosynthesis in the absence of the necessary manganese enzyme.

Kutyurin (1965) suggested that manganese is involved in the primary processes of the oxidation of water by an oxidized form of chlorophyll. Kutyurin (1965) believes it is still unclear which system is responsible for the oxidation of water - chlorophyll, cytochrome, or the manganese-containing complex. He concluded that the dissociation of water leading to oxygen evolution is a multistep process; in the first oxidative reaction an electron is liberated and the OH radical is produced, and in the second, the OH radical is oxidized, giving up an electron with the release of oxygen. The first reaction, according to the theory of Kutyurin, is associated with the activity of the chlorophyll-cytochrome system, the second with the complex that includes manganese.

Investigations by Sapozhnikov et al. (Sapozhnikov et al., 1957; Sapozhnikov et al., 1962; Sapozhnikov, 1967) have shown that an intimate relationship exists between the interconversions among xanthophils (violoxanthin and zeaxanthin) both in the light and in the dark, and oxygen evolution in photosynthesis. Sakharova (1966) has provided experimental evidence supporting the view that manganese is essential for the light-dependent conversion of violoxanthin into the lutein fraction (zeaxanthin). These findings also indicate that manganese is involved in reactions related to the release of oxygen in photosynthesis.

In 1955 Kenten and Mann for the first time studied the participation of manganese in photosynthesis with special reference to the properties of the metal intself. The possibility of catalysis of the oxidation of Mn^{2+} by the peroxidase system had been demonstrated earlier. Kenten and Mann suggested that if chloroplasts contain peroxidase, the hydrogen peroxide formed in the light might be used for the oxidation of Mn^{2+}. In fact, they were able to observe an oxidation of Mn^{2+} to Mn^{3+} in the light in a preparation derived from isolated chloroplasts; on the other hand, the chloroplast preparations showed only a very weak oxidative capacity in the dark.

These data of Kenten and Mann (1955) indicate the possible involvement of manganese in the oxidation-reduction reactions of photosynthesis. In considering this problem, it must be remembered that the oxidation of manganese may be effected by hydrogen peroxide produced earlier in the course of the Mechler reaction, and

if that is the case, then the oxidation should not be regarded as being a part of the photosynthesis process (Pirson, 1958). The probability of an oxidative manganese cycle has also been indicated by Andreae (1955) in his description of the sensitized photooxidation of manganese.

To summarize, it has been firmly established that manganese is a component of the oxygen-evolving system of photosynthesis. Later studies of the role of manganese of the oxidation-reduction systems of photosynthesis have been aimed at elucidation finding out the details of manganese involvement in the oxygen-evolving system.

According to current views, the photochemical reactions of photosynthesis require the participation of two pigment systems. The reactions responsible for oxygen evolution are coupled to pigment system II (Voskresenskaya, 1965). Anderson et al. (1964) have shown that manganese is predominantly associated with pigment system II. According to the results obtained by these investigators, the manganese content of the chloroplast fraction containing photosystem II is four to five times larger than that of the fraction containing system I. Photosystem II particles rich in manganese and chlorophyll have been shown to be active with respect to the Hill reaction in the presence of a stronger oxidizing agent.

Calvin (1965) believes that manganese oxidation takes place in the reaction between water molecules as the donors of electrons, and pigment system II. This proposal has been reinforced by theoretical calculations of oxidation-reduction potentials (Bachofen, 1966). Heath and Hind (1969) arrived at a similar conclusion. These investigators set out to find the site of manganese involvement in the process of photosynthesis in a series of studies of the behaviour of chloroplasts isolated from the leaves of plants grown in manganese deficiency. By supplying manganese to these chloroplasts the Hill reaction was restarted within 4 to 6 h, the chloroplasts reactivated in this way exhibiting a rate of electron transport 2.5 times as high as the rate in the absence of manganese. The site of action of manganese lies, according to these authors, between that part of the system involved in the oxidation of water, and that part of the system involved in the photochemical reactions of pigment system II.

More recent investigations have provided concluside evidence that manganese is an electron donor for system II. Cheniae and Martin (1968) have observed that a manganese shortage results in a decrease in the amount of oxygen released as a result of a light

flash, together with a decrease in both the quantum yield of photosynthesis and the reduction of quinones. The removal of manganese either specifically affects electron transport from water to the reducer of system I at an as yet undefined site of the electron transport chain, or it affects the reactions coupling the two pigment systems. The authors concluded that the decrease in quantum yield, the decrease in the reduction of quinones, and the lowering of the Hill reaction, all of which accompany a drop in the amount of bound manganese, together testify to photocatalyst E of system II being the first part of the system to suffer from a shortage of manganese. The effect of manganese deficiency resembles those of the action of dichlorophenyldimethylurea, or o-phenanthroline.

Walker and Ludwig (1970) studied light-dependent oxygen uptake and its subsequent dark-phase release, when excess catalase was added to chloroplast preparations showing no catalase activity. The addition of manganese chloride to the chloroplasts prior to illumination resulted in an increase both in the rate of oxygen uptake and in the rate of its noncatalytic and catalytic dark-stage evolution. The authors concluded that Mn^{2+} is the electron donor for system II and that it accelerates the electron transfer from water to oxygen in the light. The resulting Mn^{3+} oxidizes water and releases oxygen in the dark.

Ben-Hayyim and Avron (1970) studied the effect of divalent manganese ions on various types of Hill reaction in isolated spinach chloroplasts, and deduced that there was a possibility of direct interaction between manganese ions and the components of the electron-transport chain, with manganese acting as an electron donor at a site close to system II.

While studying the kinetics of photoinhibition in manganese-deficient Euglena gracilis cells inhibited with chlorophenylurea Gavalas and Clark (1971) found that treatment of the cells with chlorophenylurea increases the spread of photoinhibition. As suggested by these authors, the similarity between the effect of manganese deficiency and the effect of chlorophenylurea on photoinhibition shows that manganese functions at a site in the reducing part of system II.

Brown and Gasanov (1974) found that in the alga Dunaliella, exogenous divalent manganese competes with water as a donor for system II. Within 10 to 15 seconds of the start of illumination, the trivalent manganese produced as a result of the illumination acts as an oxidizer, while dichlorophenolindophenol yields

electrons via the supposed cyclic pathway. Diphenylcarbaside and Mn^{2+} act as donors at various sites of the electron transport chain. The amount of chlorophyll b occurring in various fractions correlates with the activity of system II in these fractions.

From a diatomaceous alga (Phalodactilum tricornutum) reared in a medium with a controlled concentration of trace elements, Holdsworth and Arshad (1977) isolated a metal-pigment-protein complex with a molecular weight of 850000, consisting of 40 protein units, 40 chlorophyll molecules, 20 molecules of chlorophyll C, 20 molecules of fucoxanthine, 8 g-atoms of Cu and 0.6-2.0 g-atoms of manganese. The authors believe this complex to be a part of system II.

Some researchers (Possingham and Spencer, 1962; Anderson et al., 1964) have suggested that the manganese participating in the oxygen-evolving system is not present in ionic form, but exists rather as a complex compound. These views stemmed from the difficulties encountered in attempts to isolate active manganese.

Hypotheses abound on the nature of complex manganese compounds. A likely candidate for the manganese compound which is involved in the process of oxygen evolution is manganese-porphyrin (Loach and Calvin, 1964; Calvin, 1965). In fact, Mn^{4+}-porphyrin is a potent oxidant and at pH 7 shows an oxidation-reduction potential sufficient to liberate free oxygen from water.

Peroxides might be involved in the steps of photosynthesis that lead up to the liberation of free oxygen; Kuzin and Shkolnik (1949) have presented evidence supporting this suggestion.

The nature of the manganese complex involved in photosynthesis has long remained unknown. Significant contributions towards a clarification of the situation have been made by Boichenko and associates (Boichenko, 1949, 1951; Boichenko and Zakharova, 1959; Boichenko and Sayenko, 1959, 1961a, 1961b, 1961c; Boichenko et al., 1965; Sayenko et al., 1965). From the chloroplasts of white clover and primula leaves they isolated an iron and manganese-containing enzyme which catalyzes the reduction of carbon dioxide in plants. Boichenko designated this enzyme the "assimilating enzyme". The isolation of this enzyme was an important step towards understanding the chemical aspects of the entire process of carbon assimilation.

The role of organic compounds of iron and manganese in the formation of the primary products of carbon dioxide reduction, as revealed by Boichenko and Sayenko, underlines the significance of organic catalysts containing metals, not only for respiration,

but also for photosynthesis.

According to current views, the process of photosynthesis is intimately associated with light-activated electron transport from excited chlorophyll to the terminal acceptor NADPH, and with ATP synthesis. Hence, the significance of the oxidation-reduction potentials of intracomplex systems, especially those of partially ionized metallocomplexes, becomes readily apparent. All the key enzymes involved in oxidation-reduction reactions are, according to recent findings, metalloenzymes. Therefore it is natural to suppose that they also play a role in one of the most important oxidation-reduction process - the assimilation of carbon dioxide by plants.

The so-called assimilating enzyme that has been isolated is a bimetallic metalloflavoprotein similar in structure to other hydrogenases. One of the metals is iron which cannot be replaced by any other metal, and the other, or supplementary one is manganese, which acts essentially as a donor of electrons. More than 1% iron, 0.13-0.14% manganese, as well as phosphorus have been detected in this enzyme preparation. Besides proteins, this enzyme complex also contains lipids, flavin adenine nucleotide, nonhaem iron and manganese.

In the light flavin reduces the iron in the enzyme, and in addition, participates in the oxidation of manganese. The reduced iron associated with coenzyme A is actively involved in the reduction of carbon dioxide. The oxidized metal, when it is bound to a lipid, produces a hydroperoxide which has a high oxidation-reduction potential (Boichenko and Sayenko, 1961a; Boichenko and Udelnova, 1964; Sayenko et al., 1965). This peroxide is a precursor of the oxygen that is released in photosynthesis. Boichenko (1963) noticed a similarity between the enzyme preparation and the electron transport particles studied by Green (1961).

From the above data and from the findings of numerous other investigators concerning the oxidation-reduction mechanisms operating in the reduction of carbon dioxide, Boichenko and Sayenko (1961a) arrived at the following scheme for the reduction of carbon dioxide (Fig. 34). In the light the flavin becomes a lipid peroxide, a process requiring the presence of manganese and following upon the photolysis of water. The peroxide is a precursor of the free oxygen that is released in the process of photosynthesis; the flavin also reduces the iron in the enzyme and the reduced iron participates in the photoreduction of carbon dioxide. During the latter process, a polyoxyacid is formed which is subsequently converted

into carbohydrates. It should be noted that Nezgovorova (1960) isolated an iron-containing complex from various plants, and found that this contained almost all of the carbon (C^{14}) assimilated following a brief (pulse) exposure to labelled carbon dioxide ($C^{14}O_2$) in strong light.

Studying an enzyme complex, which Boichenko and Sayenko (1961a) named "the assimilation enzyme", they confirmed the hypothesis suggested by K.A. Timiryazev and O. Warburg about the participation of iron in photosynthesis.

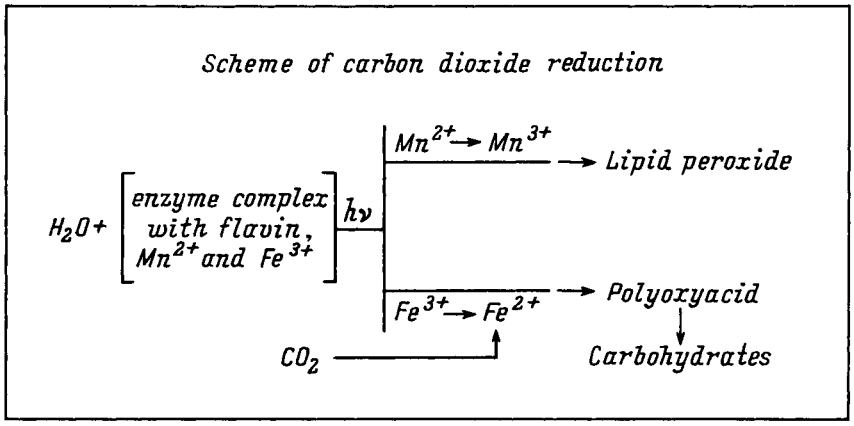


Fig. 34. The reduction of carbon dioxide in the photosynthetic process (after Boichenko and Sayenko, 1961a).

Zarin and Boichenko (1967) showed that the protein in the assimilating enzyme forms a complex with coenzyme A. This complex was studied by paper chromatography, and the authors suggested a scheme for the synthesis of the precursors of carbohydrates in which the iron-containing coenzyme A was also involved. According to the views they expressed, iron is closely involved in the formation of the first product of assimilated carbon dioxide functioning as an acceptor. The involvement of iron in the formation of further products becomes progressively less, since these do not contain iron.

In their later investigations, Zarin and Boichenko (1969) studied the participation of complexes containing non-haem iron in the assimilation of carbon dioxide and acetate in the light in the leaves and chloroplasts of various plants. These workers showed that in every case these complexes contained iron bound to coenzyme A and 4^1-phosphopantothein. A complex with such a composition is

endowed with the properties of both an acceptor and a reducing agent. These investigators deduced that the earliest products of carbon dioxide and acetate assimilation are formed not in a free state, but as acyls of coenzyme A and 4^1-phosphopantothein.

For the reduction of carbon dioxide in photosynthesis, the reducing agent must possess a low oxidation-reduction potential, near to $E_o' = -0.42$ V at pH 7. The iron complexes of the ferredoxin type and the $Fe(3\text{-KoA})_2$ complex isolated by Boichenko and Zarin display exactly this magnitude of potential.

In their studies of the assimilating enzyme, Boichenko et al. succeeded in solving a number of important problems, including that of the nature of the complex compound of manganese operating in photosynthesis. The work of these investigators deserves consideration in greater detail. Having discovered that manganese is involved in oxygen release from the lipid peroxide, Boichenko et al. (Boichenko and Udelnova, 1964; Udelnova and Boichenko, 1967, 1968) concentrated on finding a lipid compound containing manganese and possessing a high oxidation-reduction potential. Having isolated lipids to determine their manganese content, Boichenko and Udelnova (1964) found that over 80% of the cellular manganese is located in lipids. The results of deacylation treatment of the lipids indicated that manganese was associated with the unsaturated fatty acid fraction. Chromatography of the fatty acids indicated that manganese was bound to linoleic acid and linolenic acid. The role of linolenic acid in photosynthesis is well known; however, linoleic acid is found mainly in the galactolipids of leaves. The galactolipid fractions isolated from leaves were found to be 20 times as rich in manganese as other lipid fractions. The authors concluded that the association of manganese with these highly unsaturated fatty acids gives rise to the most potent of all the oxidation systems in plants, functioning to produce peroxides.

Boichenko (1975, 1976) has further purified, by crystallization, the "assimilating enzyme" assigning it another name - carbon dioxide reductase. A Fe-flavoprotein has been isolated from various plants (including algae and flowering plants) which is able to reduce carbon dioxide and dichlorophenol indophenol in vitro. The activity of the enzyme depends on the presence of phospholipids and galactolipids containing Mn^{2+}.

Udelnova and Boichenko (1967, 1968) found that a complex of manganese and monogalactosylglyceride isolated from clover leaves (and containing linolenic acid) displayed the highest oxidation-

reduction potential reported for plant cells. It has also been found that only the photosynthesizing organ of plants, the leaf, possesses a complex compound of manganese with a high oxidation-reduction potential. It could not be found in other parts of plants.

In the primrose, clover, and a number of other seed plants (Elodea, Tradescantia, potato and sunflower), Udelnova (1970) found another manganese compound in which the metal is bound to a galactolipid, namely, digalactosyldiglyceride. In mosses, ferns and in Elodea, this proved to be the main manganese compound occurring during periods favourable for photosynthesis. In both green algae (Chlorella vulgaris) and blue-green algae (Anacystis nidulans) a manganese compound has been found containing digalactosyldiglyceride, but no association of manganese with monogalactosylglyceride could be detected. In the photosynthesizing bacterium Chloropseudomonas ethylicum, no manganese compound of this type could be found, and this bacterium is known to be unable to liberate oxygen during photosynthesis (Udelnova et al., 1968). The authors did, however, find monogalactosylglyceride in green bacteria which had no polyunsaturated fatty acids. At the same time, a number of investigators believe linolenic acid to be an essential participant in photosynthesis, and it is also known that all organisms capable of releasing oxygen as a result of photosynthesis contain galactolipids, which in turn, contain linolenic acid.

Consequently at different evolutionary levels, plant organisms show not only different manganese contents, but also different types of association, characterized by high oxidation-reduction potentials, between manganese and lipids. In this context, the discovery of manganese-containing monogactosyldiglycerides and digalactosyldiglycerides in photosynthesizing organisms is of great interest. Udelnova believes that an analysis of the available data on the amounts and different forms of manganese-galactolipid compound in plants will be an important step towards understanding the evolution of the oxidation mechanism in plants. It can readily be seen that there is an evolutionary transition from the association of manganese with digalactosyldiglyceride in algae, in which this compound is the only one of its type, to the two types of association of manganese either with digalactosyldiglyceride, or with a more complex form - monogalactosyldiglyceride, both of which are found in the more advanced plants. Boichenko et al. (1972) offer many additional arguments in favour of the view that in the course of evolution, both the composition of complexes and their

roles in metabolism have undergone considerable modification.

Another site of action of manganese in the mechanism of photosynthesis must be mentioned. This has been established by the work of Allen (Allen et al., 1955b), in which it has been shown that manganese enhances the assimilation of carbon dioxide in isolated chloroplasts, only in the presence of ascorbic acid. Thus it cannot be ruled out that manganese may also act on another reaction in photosynthesis which requires the presence of ascorbic acid; this well known reaction is photophosphorylation (Arnon, 1958).

It may be of interest in this respect to discuss the work of Spencer and Possingham (1960), who studied the effects of manganese on photophosphorylation. These authors found that manganese deficiency decreased both the rate of the Hill reaction and the rate of noncyclic photophosphorylation without affecting cyclic photophosphorylation. Essentially the same observations had been reported earlier by other workers (Eyster et al., 1958). This might be considered as additional proof of the involvement of manganese in the reactions associated with oxygen evolution in photosynthesis. Vesk et al. (1966) believe that the decrease in photophosphorylation under conditions of manganese deficiency can be accounted for by alterations to the chloroplast structure resulting from a shortage of the trace element. In manganese-deficient material the authors were able to observe a decrease in the intergrana lamellae, the appearance of void spaces in the stroma, and a tendency to disruption of the discs of the grana.

Data have been obtained on the role of manganese ions in the stabilization of photosynthetic membrane structures. Structural alterations occur mainly in the granular thylakoids containing pigment system II, which is responsible for oxygen release in plants (Khmara, 1977).

Disorganization of the chloroplasts which occurs simultaneously with a decrease in the chlorophyll content and an increase in fluorescence, was observed by Homann (1967).

Accepting that manganese is principally involved in the oxygen link of photosynthesis, an involvement also in the reducing reaction of photosynthesis cannot be ruled out. This view is supported in the above-mentioned paper of Allen et al. (1955b), in which they showed that manganese enhances the fixation of carbon dioxide by isolated chloroplasts, but only in the presence of ascorbic acid. Other investigators (Tanner et al., 1960) also offered evidence for the role of manganese in the photosynthetic reduction

of NADP. Tanner et al. (1960) showed that Chlorella pyrenoidosa cells grown in the presence of manganese always exhibited the highest activity of NADP-reductase. The chloroplast grana isolated from Chlorella cells cultivated under conditions of manganese shortage were unable to reduce NADP, irrespective of whether they were supplemented with NADP-reductase from manganese-supplied or manganese-deficient alga. The authors deduced that manganese is required for the process of NADP reduction.

Interesting views regarding the significance of manganese in the evolution of aerobic organisms were expressed by Gaffron (1962), who believed that photosynthesizing organisms possess two exclusively characteristic features, the second Emmerson effect, and a relatively high manganese content. In photoreducing bacteria, which are known not to use water as the electron donor and consequently do not release oxygen, the second Emmerson effect is absent. It has also been established that the effect is absent from the alga Scenedesmus obliquus which is adapted to hydrogen, and also from a mutant of this alga incapable of liberating oxygen. Gaffron indicated that the requirements of obligate anaerobes (the purple bacteria) for manganese ions are at least 100 times lower than those of aerobic algae. Moreover, the requirement of the latter for manganese disappears during the transtion to photooxidation without the release of oxygen. Gaffron suggested that the manganese-containing enzyme catalyzes the dismutation of "photoperoxides" produced as a result of the photochemical reactions of pigment systems I and II. It was deduced by Gaffron that the incorporation of a manganese-containing enzyme, or of an unknown related catalyst, preceding the "free" chlorophyll, was the important mutation, which "put an end to the anaerobic era". Convincing evidence of the validity of Gaffron's arguments have been forthcoming from investigations employing the electron paramagnetic resonance technique.

The important part played by manganese in photosynthesis inevitably means that manganese deficiency affects other aspects of the carbohydrate metabolism in plants. However, the only evidence available on this problem is controversial. Eltinge (1941) found in his experiments on tomatoes that the most significant symptom of manganese deficiency was an abnormal diminution of the starch granules as seen by light microscopy. This could result directly or indirectly in an increase in sucrose concentration. Morita (1940) studied the effect of excluding manganese or iron from media supporting the growth of Alopecurus fulvus on the carbohydrate

content of various plant organs. A supply of manganese or iron resulted in an increase in the content of starch and sucrose in the stem, and of monosaccharides in the roots. Monosaccharides and sucrose were completely absent from the fruits when manganese and iron were deficient, while the starch content was changed only to a minor degree. This effect was apparently related to reduced translocation of carbohydrates associated with low manganese levels.

An essentially different situation is seen in Chlorella. Brown et al. (1958) found significant differences between normal and manganese-deficient Chlorella cells with respect to their ability to incorporate labelled carbon dioxide ($^{14}CO_2$) into sucrose. In the manganese-deficient algae, this ability was 18 times higher than that found in control plants. The authors concluded that a shortage of manganese either results in an enhanced production of starch, or interferes with its utilization in cells inadequately supplied with this element.

Hewitt (1959) found that the total carbohydrate chlorophyll ratio in heterotrophic and autotrophic cultures is drastically reduced by manganese deficiency, in agreement with the enhancement of glucose metabolism under conditions of manganese shortage.

Together with Saakov (Shkolnik and Saakov, 1964) the author has shown by using labelled carbon that manganese deficiency in plants is accompanied by an impaired translocation of assimilates. In the sugars upon restoration of the manganese supply has been shown in many plants grown in soil culture (Shkolnik et al., 1947; Tagi-Zade, 1957; Abutalybov and Aliyev, 1964).

Manganese is essential for the activation of phosphorylase (Fredrick, 1959). Magnesium, calcium, and iron do not affect the polysaccharide-synthesizing activity of the enzyme.

Manganese mitigates growth inhibition caused by organic acids (Shibaoka and Hurusawa, 1963).

The participation of manganese in the metabolism of auxins is of considerable interest. Bonner (1949) has demonstrated the stimulating action of the divalent manganese ion on the extension of oat coleoptile sections taking place under the influence of indoleacetic acid. These findings were later corroborated by a number of investigators (Cooil, 1952; Thimann, 1956). As indicated above, Sabbakh (1973) found that among the many cations studied in this respect, only manganese produces this stimulating effect on the response to indoleacetic acid. From his findings, Sabbakh concluded that Mn^{2+} plays a particularly important role in the mechanism of

action of indoleacetic acid during the extension phase of coleoptile cell growth. The author believes that this manganese effect lies in its influence on the synthesis of specific proteins essential for the continuous growth of coleoptile cells. Mn^{2+} and Ca^{2+} enhance the effect of IAA on coleoptile respiration (Ivanova and Salamatova, 1973). $MnCl_2$ added to maize coleoptile cuttings was found to be effective in restoring growth and respiration interrupted by applications of EDTA and o-phenanthrolin (Salamatova et al., 1975). Manganese ions may act as a cofactor in respiratory enzyme systems, or they may be involved in the ATPase system that is acted upon by IAA.

Manganese and calcium ions were shown by Ivanova and Salamatova (1973) to enhance the stimulating effect of IAA on respiration in coleoptiles. Later, treatment of maize coleoptile cuttings with EDTA and o-phenanthroline was shown to reduce the rate of growth and the rate of respiration, and to counteract the stimilating effect of IAA on respiration. The addition of $MnCl_2$ to the cuttings restored the influence of IAA on growth and respiration. Two possible mechanisms for the influence of Mn^{2+} on IAA-induced growth and respiration may be considered: (1) manganese may act as a cofactor in respiratory enzyme systems; (2) the element may act as a consituent of the ATPase system associated with the active transport of protons - a process which is influenced by IAA (Salamatova et al., 1975).

At the same time, manganese ions affect the non-enzymatic and enzymatic breakdown of indoleacetic acid. Its oxidation in the presence of manganese has been studied by Wagenknecht and Burris (1950). Currently, auxin oxidase is considered to be a system consisting of two components: an enzyme containing a heavy metal (a peroxidase), and a flavoprotein complex.

Siegel and Galston (1955) have shown that manganese is essential for both the enzymatic and non-enzymatic breakdown of indoleacetic acid in vitro. As shown by Masaki and Galston (1961), the activity of auxin oxidase is increased by prolonging the exposure to manganese in the incubation madium. Neither calcium, cobalt, copper, iron, magnesium, nickel, nor zinc has ever been observed to produce effects comparable to those of manganese.

Kenten and Mann (1950) and Kenten (1955) expressed interesting ideas on the role of manganese in the function of auxin oxidase, and have suggested a scheme for the role of auxin oxidase. The scheme was based on the assumption that this reaction is a chain

auto-oxidation reaction initiated and promoted by two enzymatic peroxidations: one leading to the formation of Mn^{3+}, the other resulting in an end-product of the oxidation of indole peroxide and a phenol cofactor. The authors have shown that the horseradish peroxidase, and the equivalent enzymes from other plants, are capable of catalyzing the oxidation of Mn^{2+} to Mn^{3+} in the presence of hydrogen peroxide and a thermostable cofactor. They proposed that manganese reduces the substrate oxidized by peroxidase by taking it through a cyclic oxidation-reduction process. The Mn^{3+} formed as a result of this reaction can interact with another oxidizing substrate at low peroxide concentrations, and can also be reversibly oxidized or reduced. In fact, indoleacetic acid is the substrate which is oxidized by Mn^{3+}.

Figure 35 shows the scheme postulated by Kenten and Mann (1950) to explain the oxidation of indoleacetic acid through a cyclic oxidation and reduction of phenolic and manganese cofactors.

Kenten (1955) offers convincing arguments in favour of the view that the Mn^{3+} formed in this cycle of reactions is involved in the oxidation of indoleacetic acid. Direct confirmatory evidence was given in the above-mentioned publication by Maclachlan and Waygood (1956), showing that Mn^{3+} (which can be chelated by versene) is able to oxidize indoleacetic acid, directly liberating carbon dioxide and possibly producing polymerizable free radicals of scatol.

Tomaszewski and Thimann (1966) have shown that polyphenols are synergists of indoleacetic acid which counteract its decarboxylation. Conversely, monophenols stimulate its decarboxylation, this effect being enhanced in the presence of divalent manganese ions. These ions stimulate the decarboxylation of indoleacetic acid, i.e., they stimulate auxin oxidase activity both in vivo and in vitro. Nonetheless, they inhibit growth elongation, and at relatively high concentrations may stimulate growth at both low and high concentrations of indoleacetic acid. Since manganese can stimulate growth induced by naphtylacetic acid and 2,4-D, the authors believe that the general effect of this element on growth is not connected with its direct role in auxin metabolism.

Bakardjieva and Jordanov (1967) studied the effect of manganese on the oxidation of ascorbic acid, using the electron paramagnetic resonance technique. The results indicated that free manganese ions can fulfil the function of a peroxidase and thereby promote the peroxidase-dependent breakdown of indoleacetic acid.

One of the important physiological roles of manganese is its participation in the biosynthesis of RNA and DNA. As has been shown, the activity of RNA- and DNA-dependent polymerases is sensitive to the presence of manganese or magnesium in the medium (Pegg and Korner, 1967), whereas the activity of tRNA-nucleotide transferase is dependent upon the presence of manganese (Klemperer and Haynes, 1967). Moore (1966) has found that poly-U and poly-C bind to the 50S ribosomal subunits from Escherichia coli, only if Mg^{2+} or Mn^{2+} are present in the incubation medium. Activation of the synthesis of non-ribosomal RNA occurs in vitro if divalent manganese ions and ammonium sulphate are added to the system.

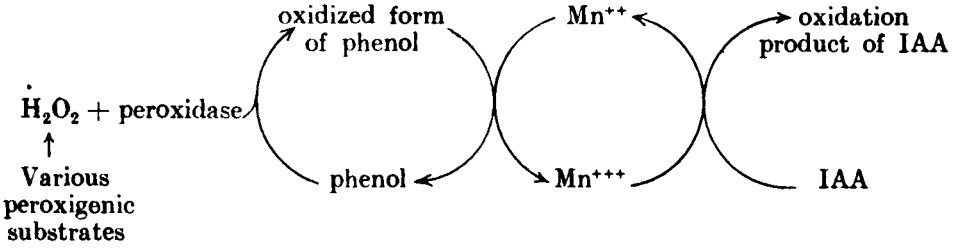


Fig. 35. A possible scheme for the oxidation of IAA by the cyclic oxidation and reduction of phenolic and manganous cofactors (after Kenten and Mann, 1950; Kenten, 1955).

Vlasyuk (1971) investigated the base composition of chloroplast nuclear fraction DNA and its dependence on manganese nutrition; also studied were the base compositions of DNAs of different degrees of heterogenity. It was found that manganese increased the content of thymine, and that the content of GC-pairs in DNA from manganese-deficient plants was higher than that from plants receiving manganese. Of all the DNA types differing from one another in heterogenity, the stable DNA possessed the molar fraction corresponding most closely to the Chargaff rule. This DNA from manganese-deficient plants contained less guanine and cytosine, while the contents of methylcytosine and thymine were increased.

An increase in the heterogeneity of chloroplast structural proteins has been found to be another effect of manganese. It is found (Zhidkov et al., 1978) that a manganese deficit caused structural disturbances at chloroplast DNA sites which are rich in basic guanine-cytosine pairs.

According to Klimovitskaya et al. (1975), manganese ions induce synthesis of various histone fractions. They also bring about a

shift in the absorption maximum of the DNA-histone 2 b complex toward shorter wavelengths. The presence of manganese ions apparently modifies the nature of the association between DNA and histone on account of conformational changes brought about in the DNA and the DNA-histone complex.

The presence of manganese leads to an increase in the content of chromatin histones, non-histone proteins, and RNA, and this might have important consequences for the regulation of gene activity. Manganese brings about modifications in the properties of the DNA-histone system. It may also exert a strong influence on the functional activity of chromatin by stabilizing or changing the complicated structure of this complex (Prokopivnyuk, 1979).

It has been established that the DNA of chloroplasts differs from nuclear DNA in that the former has no methylcytosine, and has a lower hyperchromism which increases with the supply of manganese to the plant. Under the influence of manganese, the proportions of free and membrane-associated ribosomes in the chloroplasts change in favour of the latter, an effect which might be attributable to changes in the binding properties of membranes (Klimovitskaya and Prokopivnyuk, 1981).

Interesting findings have been reported by Polevoy et al.(1973), who found that auxin exerts an inhibiting influence on degradative processes, namely decreases in RNA content, DNA content and protein content, and the breakdown of other substances. Such degradation processes were normally found to occur in mesocotyl cuttings of 4-day old etiolated maize seedlings during incubation for 24 h in distilled water. The efficiency of the inhibiting action of auxin on RNA degradation in the cuttings was increased in the presence of cations, especially manganese, and to a lesser degree magnesium. It was found that manganese chloride and magnesium chloride effectively prevent RNA breakdown when these are added to a homogenate.

Manganese is one of the elements which similarly to calcium, are involved in the control of the selective uptake of ions from the environment (Epstein, 1961). When manganese is not included in a nutrient medium this leads to an increase in the accumulation of basic mineral nutrients in plants (Lisnik, 1971). Ramamoorthy and Desais (1946) found that the deficiency disease of grasses attributable to manganese shortage is accompanied by a disbalance of trace elements.

Nitrogen-metabolism alterations caused by manganese deficiency were reported by Lerer and Bar-Akiva (1976). The leaves of 6 to 12-

month-old Mn^{2+}-deficient lemons contained 40% more total nitrogen than was found in the leaves of manganese-supplied plants; the nitric nitrogen content increased from 18.2 to 95.6 mg per g dry wt, the amino-acid nitrogen content was increased by 42%, and protein nitrogen rose by 22%. The dry weight of Mn-deficient leaves was less than that of normal ones (25.8% and 30.7%, respectively).

Lisnik (1971) reported that an inadequate manganese supply results in a decrease in the amounts of calcium and manganese in plants; this may bring about an alteration of membrane permeability and interference with the mechanism of ion uptake. He also found that the activity of magnesium and calcium-dependent ATPases is decreased by manganese deficiency. Furthermore, a decrease in the concentration of contractile actomyosin-like proteins could be observed; according to current views these proteins are involved in the transport of ions across membranes.

Evidence for the existence of manganese-activated ATPase is of interest. Thus, Lyubimova et al. (1966) reported that apirase from Mimosa pudica leaves could be separated into ADPase and ATPase, the latter requiring manganese for activation. These findings, as well as the discovery of a decrease in the total lipid content (Vlasyuk et al., 1971), testify to the effect of manganese deficiency in bringing about alterations of cell membrane structure. Vlasyuk et al. (1971) also found that the phospholipid ratio was changed by manganese deficiency.

Nagahashi et al. (1978) compared the stimulating effects of Mg^{2+} and Mn^{2+} on glycosyltransferases (contained in crude fractions of pea cotyledons), as indicated by the rate of transfer of carbohydrate entities from nucleoside diphosphates to lipids. They observed that Mn^{2+} preferentially stimulated the transfer of the label from ^{14}C-GDP-mannose to glycolipids, whereas Mg^{2+} stimulated the transfer to free lipids. In contrast, the transfer of the label from UDP-N-acetyl-^{14}C-glucosamine to glycolipids was Mg^{2+}-stimulated, the transfer to free lipids being exclusively Mn^{2+}-stimulated. This result is interpreted as suggesting that subcellular membrane-associated glycosyltransferases are stimulated by Mg^{2+} and Mn^{2+} in different ways. Scorpio and Masoro (1970) have found that in the presence of manganese the rate of fatty acid synthesis in the soluble fraction of liver cells was greater than that in the presence of magnesium. In experiments with partially purified acetyl-CoA-carboxylase, it was shown that manganese stimulates the activity of the promoter form of the enzyme. Moreover, manganese prevents

the decarboxylation of malonyl-CoA and elevates the activity of aconitase, the enzyme concerned in the metabolism of fats and carbohydrates.

Manganese apparently plays a prominent role in the control of the genetic activity in plants. Vlasyuk et al. (1973) found that a shortage of manganese affects the heterogeneity of the chromatin in plants; the content of histones and non-histone proteins, as well as the total protein content, RNA content and DNA content of condensed and diffuse chromatin all suffered a decrease. Manganese increases the concentration of polyanionic macromolecules in the diffuse chromatin, which is more active in RNA synthesis. The authors deduced that the absence of manganese from nutrient solutions adversely affects DNA-histone interactions. This causes some loci to be open, and the histones fail to control specific sites in the genetic apparatus that interfere with the normal biosynthesis of proteins. Manganese deficiency, according to their views, eventually leads to a loss of control over the differentiation processes as a consequence of the suppression of certain regions of the DNA. Manganese ions activate the synthesis of various histone fractions (Klimovitskaya et al., 1975).

To summarize, the evidence discussed in the above convincingly shows that manganese is principally involved in photosynthetic reactions. This element also plays a prominent part in respiration and the metabolism of nitrogen, auxin, and nucleic acids.

Chapter 3

ZINC

Different sensitivities to zinc can be observed not only among different plant species, but also among different varieties of the same species. Ambler and Brown (1939) found two Phaseolus varieties displaying different sensitivities to zinc. Fruit plants exhibit a considerable sensitivity to shortages of this element, and citrus plants show particularly severe symptoms of zinc deficiency (Chandler, 1937; Haas, 1937). A little-leaf-rosette disease attributable to zinc deficiency can be observed in Pinus radiata, and is similar to the symptoms of zinc deficiency in fruit trees (Smith and Rayliss, 1942).

The symptoms of zinc deficiency are shown in Fig. 36. A characteristic feature of all plants suffering from zinc deficiency is retardation of growth, with almost complete cessation of internodal growth. In the apple and other deciduous fruit plants the leaves may reach only 1/10-1/20 of their normal size when affected by little-leaf-rosette disease.

Zinc deficiency in fungi and bacteria is accompanied by impairment of the formation of pigments such as: melanin, chrisogenin, prodiglosin, subtyllin and others (Chernavina, 1970).

Plant species differ in their ability to take up zinc from the soil. In crop experiments, lucerne was shown to extract adequate amounts of zinc from soil on which grasses suffered a deficiency of this element (Hoagland, 1944). Sometimes it was noted that lucerne growing in gardens prevented the development of the little-leaf-rosette disease in fruit trees. Interestingly, wild plants exhibit a zinc content higher than that found in cultivated ones (Rogers et al., 1939). Lichens and conifers are conspicuous for their high zinc content, and the highest concentration of this element has been found in poisonous mushrooms (Vinogradov, 1965).

That zinc enters the plant passively has been indicated by findings obtained with ten-day-old Phaseolus seedlings. The pattern of zinc uptake, and the observation that inhibitors of respiration do not affect the process of ^{65}Zn uptake by the plant, both support this view (Cheryl et al., 1971).

The concentration and distribution of zinc in various plant organs have been studied by numerous investigators (Borovik and Borovik-Romanova, 1944; Wood and Sibly, 1950). They found that elevated zinc concentrations are typical of the leaves, generative

organs, and growth points. With the aid of radioautography it was possible to show that ^{65}Zn enters the young, growing parts of the plant in large amounts (Riceman and Jones, 1960).

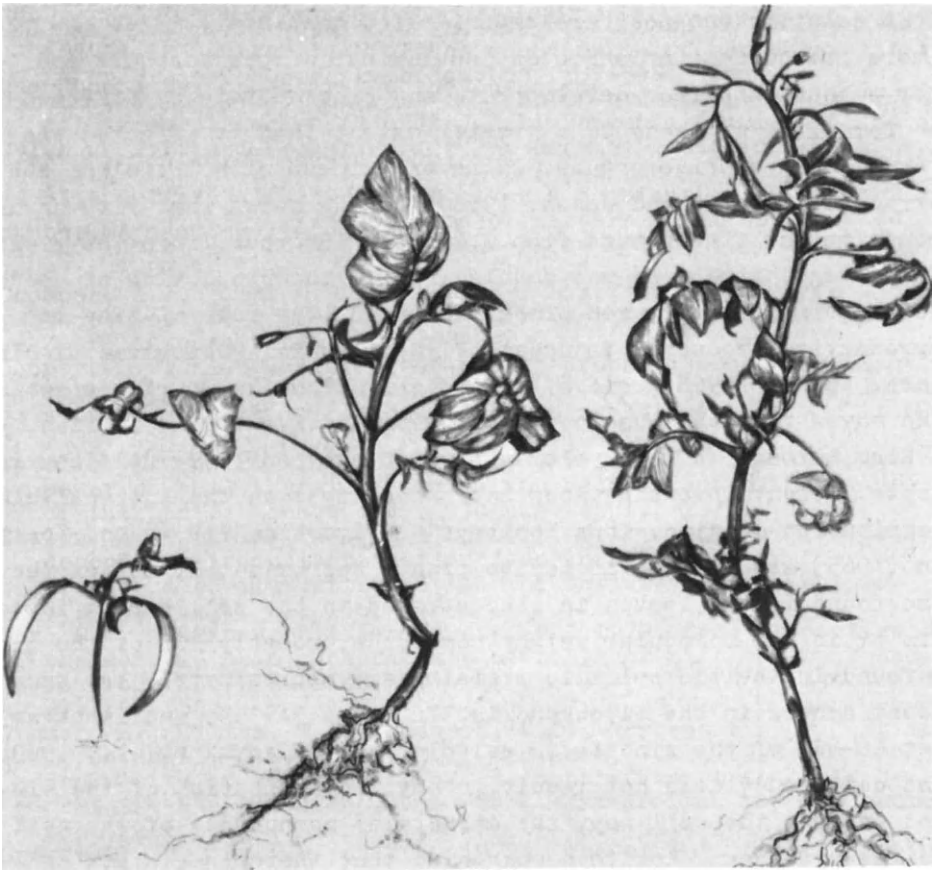


Fig. 36. Zinc deficiency in tomato (Lycopersicum esculentum) plants. Note shortening of the internodes, the diminutive size of the leaves with intercurling of the leaflets, and epinastic curvature (after Hewitt, 1963).

In contrast to manganese which accumulates in the leaves of higher plants, zinc, like copper, is stored predominantly in the seeds (Nikolaenko, 1971), where it is largely concentrated in the embryo (Riga and Bukovac, 1961). Fursov (1965) detected zinc in the female gametophyte cells, and the highest zinc concentration was found in the protoplasm of the oosphere and sinergids. These findings relate to the important role played by zinc in the formation of the generative organs and in fruit-bearing. Reed (1942)

found that pea plants grown under conditions of zinc deficiency produced no seeds, and in a later publication (Reed, 1944) he showed that zinc is required for normal development of the oosphere and the embryo. At zinc concentrations of 0.01 mg/dm^{-3} in the nutrient solution the oosphere deteriorated completely. Zinc exerted a more pronounced influence on development of the oosphere and embryo than on pollen development, and zinc deficiency affected the formation of seeds to a greater extent than it affected the growth of vegetative organs. In an experiment with Trifolium subterraneum (Riceman and Jones, 1958), it was shown that with an increase in the zinc dosage from 0.05 to 0.15mg per litre the yield of the vegetative mass was doubled, while the production of seeds and flowers was increased almost 100-fold. The role of zinc in reproductive processes is apparently of general biological significance. Voinar (1960) refers to an observation that during spawning, zinc moves from the muscles to the reproductive organs in fish.

Zinc belongs to those elements which are poorly re-utilized in plants. Recent investigations have shed light on the intracellular distribution of zinc. Thus Kositsyn and Igoshina (1964) and Kositsyn (1965) showed that in tomato plants approximately 80% of the zinc found in the leaves is concentrated in the cell sap in ionic form or in low molecular weight compounds; roughly 10% of the zinc is found in the protoplasmic proteins and approximately the same amount occurs in the mitochondria. In beans 34-35%, and in sugar beet 60-68% of the zinc is in soluble form (Vlasyuk et al., 1963). Zinc deficiency does not result in any redistribution of the element between the cell sap, the structural components of the cell and the cytoplasm. Kositsyn suggested that there is a state of equilibrium between the ionic zinc and the zinc combined in the physiologically important high molecular weight compounds. Considerable amounts of ionic zinc may thus play a protective role in maintaining the level of zinc-containing high molecular weight compounds. Fujii (1954) found zinc in nucleoli and stressed the important role of zinc in mitosis. This investigator reported that the mitotic apparatus contained a substance that was intensively stained by ditisone, and this was interpreted as indicating the presence of zinc. The substance shown up by ditisone accumulated in the spindle during the formation of the mitotic apparatus, and disappeared in anaphase. In subsequent publications, the author reported a specific affinity of the nucleolus for heavy metals (cobalt, zinc and silver). It has been observed that zinc inter-

feres with cell division in Ustilago sphaerogena, and in addition modifies their shape (Spoerl et al., 1957). Recent findings confirm the greater presence of zinc in the nuclei and mitochondria compared with other parts of the cell (Kathore et al., 1972). The role of zinc in mitosis was confirmed by Polikarpochkina et al. (1970) in their studies on suspension cultures of tobacco plant cells.

As shown by Kositsyn and Igoshina (1970), the bulk of the zinc in chloroplasts and mitochondria is associated with high molecular weight compounds.

In a later investigation employing disc electrophoresis and labelling with ^{65}Zn, Kositsyn and Igoshina (1971) found that zinc is associated with individual electrophoretic fractions (in the B and F zones) of the soluble protein of tomato chloroplasts. The Z zone exhibits a more diffuse pattern and cannot be associated with a specific protein fraction.

Considerable modifications of the palisade tissue and spongy parenchyma have been detected in the leaves of plants suffering from zinc deficiency (Reed, 1938). The palisade cells were 12-16 times larger, their number was reduced, and the intercellular spaces disappeared. Many of the cells of the mesophyll were strophied and the division and elongation of the cells, as well as the normal differentiation of the tissues, were impaired. The plastids were reduced in size and their differentiation was either retarded or it had ceased altogether. The mitochondria were elongated and twisted like a helix. Particularly serious damage was noted in the chloroplasts; their development was arrested and many were disintegrating; the palisade cell chloroplasts in the affected leaves were small and showed a tendency to accumulate in the lower parts of the cells. As a result of the degeneration of many plastids, their numbers became extremely low. Carlton (1954) found that zinc deficiency strongly affects the root meristem cells which become hypertrophied with abnormally elongated vacuoles and small nuclei.

Porokhnevich (1972) reported that a shortage of zinc in flax resulted in suppression of the extension of the columnar cells in the direction of their long axis, and brought about a reduction of the size of the chloroplasts. Molotkovsky and Dzyubenko (1969) found that zinc reduced the swelling of chloroplasts.

Zinc is apparently required for the development of mitochondria. The experiments of Price and Brown (1965) showed that more mitochondria were formed in Ustilago sphaerogena cells grown in the presence of zinc compared with the numbers formed in zinc-deficient counterparts.

In the cytochemical investigations of Reed (1938) it was shown that in apricots and walnuts suffering from zinc deficiency poly phenols and lecithin accumulate in the vacuoles as sphere-like formations (coacervates); the latter are also sites of accumulation of active polyphenol oxidase (Fig. 37).

The physiological role of zinc has been discussed in a number of reviews (e.g. Hewitt, 1963; Shkolnik et al., 1967), and follows largely from its incorporation in a multitude of metalloenzymes and also from its being a component of various metalloenzyme complexes. Numerous interesting findings have been obtained concerning zinc as a component in a large number of enzyme systems and its ability to activate various metalloenzymic complexes.

Zinc is the trace element which is most commonly found in metalloenzymes; it is found in 59 enzymes representing almost all enzyme groups (Riordan, 1976; Table 8). Accordingly, the metabolic functions of zinc are more various than those of any other trace element.

To illustrate the progress made in recent years in the discovery of further zinc-containing enzymes, it might be noted that Hoch and Vallee (1958) in their review referred to only seven zinc enzymes and proteins.

TABLE 8 Currently confirmed zinc metalloenzymes (after J.M. Riordan, 1976)

International Union of Biochemistry System		Number
E.C.1.	Oxidoreductases	7
E.C.2.	Transferases	8
E.C.3.	Hydrolases	23
E.C.4.	Lyases	19
E.C.5.	Isomerases	1
E.C.6.	Lipases	1

Bertrand and Wolf (1957) have shown that zinc is required for the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from Aspergillus niger. There are data indicating that 8-aminolevulinate dehydrogenase from Ustilago sphaerogena is a zinc enzyme (Komai and Neilands, 1968). The activity of this enzyme was shown to be strongly dependent upon the presence of zinc in the medium. It is inhibited by p-fluorophenylalanine

and its activity remains almost unaffected when Actinomycin D is administered to the cells.

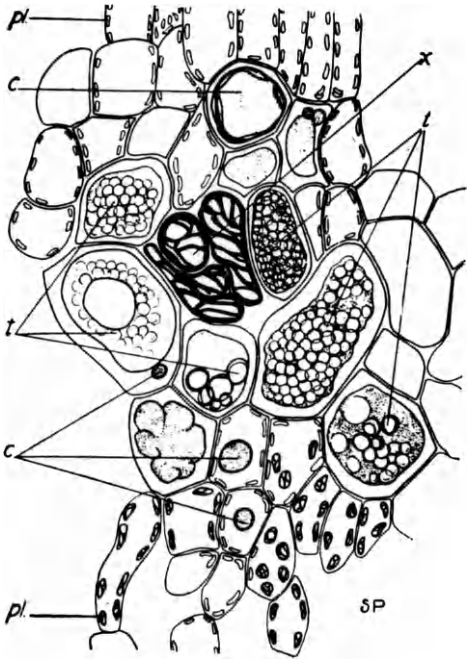


Fig. 37. Portion of transverse section of an apricot leaf affected by zinc deficiency, the perivascular cells having been filled with catechol. In the immediate vicinity of the bundle (x), the catechol masses have formed a continuous phase surrounding a number of vacuoles (t). Other cells contain spherical masses of catechol (c) enveloped by precipitation membranes; these masses may be considered to be auto-complex reserves. The plastids (pl) show abnormalities (after Reed and Dufrenoy, 1942).

Some of the metalloenzyme complexes activated by zinc include (after Parisi and Vallee, 1969): glycylglycine-dipeptidase (3.4.3.1; Zn), arginase (3.5.3.1; Zn, Mn, Fe^{2+}, Co, Ni, Cd), dehydropeptidase (3.5.1.14; Zn), glycylglycine-dipeptidase (3.4.3.1; Zn, Co), glycylglycine-dipeptidase (3.4.3.2; Zn, Mn), carnosinase (3.4.3.3; Zn, Mn), aminopeptidase (3.4.1.2; Zn, Co, Mn), histidine deaminase (4.3.1.3; Zn, Hg, Mg, Cd), lecithinase C (3.1.4.3; Zn, Cu, Mg, Co, Mn), dehydroorotase (3.5.2.3; Zn), l-mannosidase (3.2.1.24; Zn), oxaloacetate carboxylase (4.1.1.3; Zn, Mn, Co, Cd, Pb, Ni, Fe^{2+}), and enolase (4.2.1.11; Mg, Mn, Zn).

This list of metalloenzyme complexes involving zinc may be extended to include phosphopyruvate carboxylase (6.1.1.31), cysteine

amino oxidase (4.1.1.30) and leucylpeptidase (3.1.1.32). Mention should be made of β-mannosidase (1.4.3.4) which combines the properties of both metalloenzyme complexes and metalloenzymes, because zinc, which is specific for the enzyme, is only loosely bound to it and can easily be removed.

Among zinc enzymes are also methylmalonyl-CoA-carboxytransferase (2.1.3.1), thiosulphate-sulphide-transferase (rodanase; 2.8.1.1), and pyruvate carboxylase (6.4.1.1). Zinc metalloenzymes are represented among enzymes of all six classes (Table 8), although enzymes involved in the metabolism of carbohydrates, lipids and proteins constitute the majority of zinc metalloenzymes.

Not only is zinc a constituent of metalloenzymes and an activator of metalloenzyme complexes, but it is also involved in the allosteric regulation of enzymes by virtue of its ability to modify enzyme conformation. In beef liver glutamate dehydrogenase, zinc has been shown to bring about the dissociation of glutamate dehydrogenase into two subunits, resulting in a change in enzymic activity and substrate specificity of the enzyme. Thus, the glutamate dehydrogenase activity is lost and instead, the enzyme oxidizes alanine. Zinc thereby operates as a regulator of glutamate dehydrogenase activity by affecting the conformation of the protein molecule.

The first enzyme found to contain zinc was carbonic anhydrase which catalyzes the reversible decomposition of carbonic acid to carbon dioxide and water:

$$H_2CO_3 \rightleftharpoons H_2O + CO_2$$

Carbonic anhydrase from parsley, peas and beans is a zinc-containing enzyme (Tobin, 1970; Gerebtzoff and Ramaut, 1970; Risiel and Graf, 1972). However, no zinc could be detected in purified preparations of carbonic anhydrase isolated from leaves of Spinaceae oleracea (Rossi and Cortivo, 1968) and Petroselenium (Feliner, 1963).

Kositsyn and Khalidova (1974, 1976) reported that an extract of soluble protein from tomato chloroplasts displayed notable carbonic anhydrase activity. Electrophoresis of the preparations revealed the existence of two isozymes of carbonic anhydrase having R_f values of 0.31 and 0.40. The coincidence of these values with the R_f value of the zinc-containing chloroplast protein (Kositsyn and Igoshina, 1971) is evidence that the isozymes of carbonic anhydrase are zinc metalloenzymes.

Walk and Metzner (1975) found in the leaves of Lactuca sativa two iso-forms of carbonic anhydrase with molecular weights of 195000 and 25000.

Carbonic anhydrase activity was found in the root homogenates of 8 calcifuge species, but was not found in 6 others. Root homogenates of calcicoles and calcium-tolerant plants lacked carbonic anhydrase.

The activity of carboxypeptidase, the content and distribution of zinc have been studied in a number of plant species (Zuber and Matile, 1968).

Zinc is known to be a constituent of many of the dehydrogenases that require NAD for their activity (Hoch and Vallee, 1958). Of all zinc dehydrogenases, alcohol dehydrogenase has been studied in greatest detail.

Glyceraldehyde-3-phosphate dehydrogenase, like alcohol dehydrogenase, has been extensively studied with regard to its structure and function. Glyceraldehyde-3-phosphate dehydrogenase from pea seedlings has been found to be a zinc metalloenzyme. Malate dehydrogenase is also a NAD-dependent enzyme, and zinc is required for the maintenance of its quaternary structure (Vallee, 1960). Igoshina and Kositsyn (1975) obtained evidence that one of the isozymes of NADP-dependent glutamate dehydrogenase from tomato chloroplasts is a zinc metalloenzyme. Polikarpochkina (1975) also found that plant glutamate dehydrogenase contains zinc.

Under conditions of zinc deficiency, the cytochrome c content falls dramatically and the activity of cytochrome oxidase in Ustilago sphaerogena is considerably reduced (Grimm and Allen, 1954). High concentrations of cytochrome c, a and b are typical of cultures adequately supplied with zinc (Brown et al., 1966). Data have been obtained showing that zinc is involved in the biosynthesis of porphyrins and haemoproteins, including cytochromes.

Parallel with the suppression of the activity of some enzymes as a result of zinc deficiency, alterations in the activities of other enzymes are observed. Thus, Nason et al. (1951) noted that the activity of alcohol dehydrogenase in Neurospora crassa was entirely suppressed by zinc deficiency; that of tryptophane synthetase was strongly inhibited, while NAD-nucleosidase displayed a considerably higher than normal activity.

The above observations concerning zinc enzymes have provided for a better understanding of the physiological role of zinc. Thus, for instance, the fact that zinc is a constituent of many

of the enzymes of glycolysis and respiration, of many NAD- and some FAD-dependent enzymes, is a basis for investigating the involvment of zinc in the glycolytic and respiration cycles (Fig. 38).

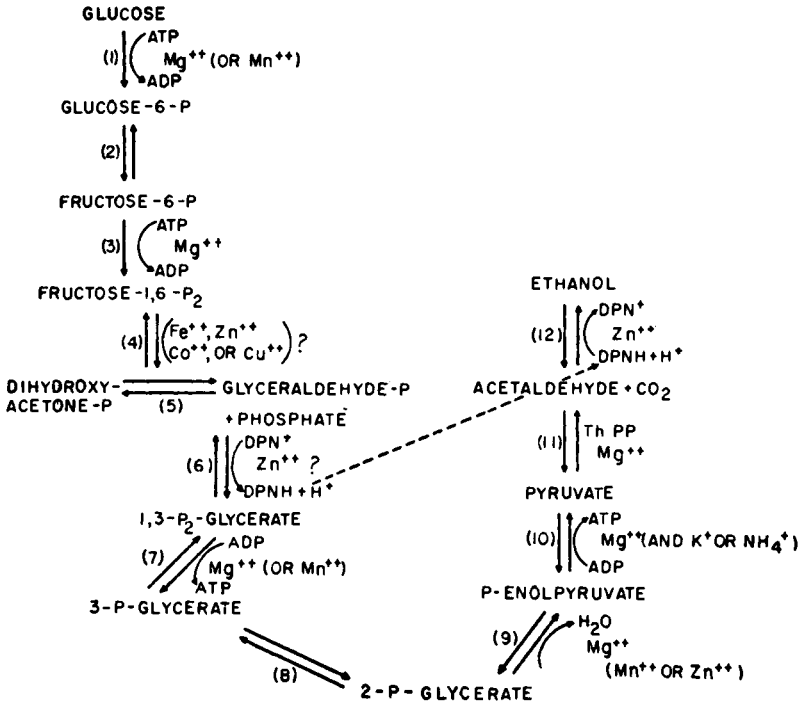


Fig. 38. Site of action of metal ions in the fermentation pathway (after Nason, 1958).

In animal tissues and lower plants zinc has been found to be incorporated in the following glycolytic enzymes: alcohol dehydrogenase, lactate dehydrogenase and glyceraldehyde phosphate dehydrogenase. Reports in the literature indicate that the activity of a number of glycolytic enzymes is decreased under conditions of zinc deficiency.

Zinc is also involved in that stage of glycolysis catalyzed by aldolase. The latter had been found to contain iron and copper (Warburg and Christian, 1943). The aldolase activity in oats is drastically reduced under conditions of zinc deficiency (although not when copper is in deficiency), which indicates that zinc is specifically involved in the activation of the enzyme (Quinland-Watson, 1951). A considerable increase in aldolase activity produced by a pre-sowing treatment of maize seeds with zinc has been observed by Shaltenene (1965).

The phosphorylation of phosphoglyceric aldehyde yielding diphosphoglyceric acid apparently requires the participation of zinc since zinc is a constituent of glyceraldehyde phosphate dehydrogenase, the enzyme involved in this conversion both in yeast and in some animal tissues. Zinc deficiency resulted in a suppression of this enzyme's activity in Aspergillus niger (Bertrand and Wolf, 1958).

Zinc activates enolase. It was found that the inhibition of potato enolase by fluoride could be prevented by introducing of zinc.

Zinc stimulates the activity of oxaloacetic dehydrogenase, which splits off carbon dioxide from oxaloacetic acid yielding pyruvic acid. Since pyruvic acid is an important intermediate in the metabolism of plant cells, the involvement of zinc in the production of pyruvic acid indicates the central role played by this element in the metabolic processes occurring in the plant cell.

Zinc is also involved in the conversion of pentoses. As found by Bertrand and Wolf (1958) in Aspergillus niger, an inadequate supply of zinc reduces the activity of two of the pentose phosphate cycle enzymes glucose-6-phosphate dehydrogenase and 6-phosphoglyconate dehydrogenase.

The importance of zinc for activity of the Krebs cycle enzymes has been demonstrated. The element has been shown to activate oxaloacetate decarboxylase in vitro, which catalyzes the splitting off of carbon dioxide from oxaloacetic acid to yield pyruvic acid (Nason and McElroy, 1963).

In their experiments with Rhizopus nigricans and Aspergillus niger, Foster and Denison (1950) showed that zinc plays a specific role in controlling the activity of pyruvate decarboxylase, which decreased almost to zero when these organisms were deprived of zinc.

Indications of the effect of zinc on the synthesis of organic acids were found by Foster (1939). Zinc enhances the synthesis of citric acid in some species, but in those organisms which produce gluconic acid, zinc inhibits citric acid synthesis. Zinc also promotes the synthesis of oxalic acid.

According to Paribok (1972), the rate of respiration in tomato leaves is decreased by zinc deficiency; the magnitude of the respiration quotient, however, remains the same. Experiments carried out by the author with inhibitors of cytochrome oxidase and the enzymes of glycolysis and the Krebs cycle indicated that the principal respiration pathway (glycolysis - the Krebs cycle - the

respiratory electron transport chain) is strongly inhibited under conditions of inadequate zinc supply. These observations are consistent with the already mentioned findings of a decrease in the activity of aldolase in higher plants, and a decrease in the activity of a number of glycolytic enzymes in lower plants and animals suffering zinc deficiency. In this context it should be noted that a decrease in NAD content and NADP content has been reported in zinc-deficient Mycobacterium smegmatis (Winder and O'Hara, 1962). The same holds true for the activity of NADP-diaphorase and NAD-diaphorase, the enzymes which oxidize the reduced forms of the respective coenzymes.

Petinov et al. (1963) reported that zinc has a positive influence on the heat resistance of plants. In their experiments with beet plants, additions of zinc decreased the sensitivity of the respiratory process to cyanide and other injurious effects such as that of overheating.

Chistyakov and Gendel (1968) reported a series of findings on the regulatory role of zinc in the functioning of the respiratory chain in animal mitochondria. They demonstrated that Zn^{2+} in concentrations of 10^{-6}-10^{-5}M inhibited electron transport in the region of the second site of energy coupling, i.e., between cytochromes b and c. In this case the inhibitory effect of zinc upon the respiratory chain may have been related to the high concentrations of the element employed in the experiments. It is important that these findings for plant mitochondria should be checked using low concentrations of zinc.

Using histochemical methods, Reed (1946) succeeded in demonstrating that leaves of zinc-deficient plants have high concentrations of inorganic phosphorus. As shown by Paribok and Alekseyeva-Popova (1965), zinc deficiency increases phosphorus uptake by pea and tomato plants, but normal utilization of phosphorus is disturbed. There is a several-fold increase in the inorganic phosphorus content and a decrease in the proportions of phosphorus in nucleotides (including nucleotides with high energy bonds), lipids and nucleic acids. Upon the addition of zinc to the nutrient medium, utilization of the phosphorus taken up by the plant returns to normal.

Kolev (1965) reported that external administration of zinc to the roots of Phaseolus plants grown on boggy soils increased the incorporation of ^{32}P into acid-insoluble phosphorus compounds (phospholipids, nucleic acids and nucleoproteins). An increase in

the amounts of organic phosphorus compounds in grapes was observed by Dobrolyubsky and Fedorenko (1969).

Stashauskaite et al. (1972) found that the activity of ATPase and acid β-glycerophosphatase in maize roots was suppressed by zinc, whereas the phosphorus content was lowered. The authors concluded that the zinc-related reduction of phosphorus accumulation by the roots, and the retarded transport of phosphorus to the aboveground plant organs were attributable to the observed suppression of the activity of these dephosphorylating enzymes.

Results have been obtained on the effects of zinc on oxidative phosphorylation. Paribok (1968) reported that zinc deficiency in tomato plants hardly affected the energy efficiency of mitochondrial respiration, as assessed by the P/O coefficient, in the early stages of development of zinc deficiency symptoms. In further experiments using 2,4-dinitrophenol to study respiration in intact tissues, Paribok has shown that in the initial stages of zinc deficiency respiration is efficient in terms of the energy produced. The observations reported by Zakharchishina and Klyuchko (1970) indicate that an inadequate supply of zinc results in an uncoupling of oxidation and phosphorylation in barley plants (the P/O coefficient decreased).

The role of zinc both in the biosynthesis of chlorophyll precursors and in the process of photosynthesis is of great interest. Shuvalov and Krasnovsky (1971) have detected zinc protoporphyrin in yeast cells and in both etiolated and green maize leaves. The authors are inclined, however, to regard zinc protoporphyrin as a precursor of iron porphyrins rather than a precursor of magnesium porphyrins.

In Neurospora crassa and Ustilago, zinc has been shown to be essential for the production and function of δ-amino levulinic acid dehydratase, which is responsible for the condensation of two molecules of δ-amino levulinic acid to yield a molecule of one of the precursors of the protoporphyrins, namely IX porphobilinogen; this is a precursor of chlorophyll (Komai and Neilands, 1968). Zaitseva et al. (1970), in their investigation into regulation of the synthesis of vitamin B_{12} and porphyrins in Propionibacterium shermani, confirmed that zinc is able to activate δ-amino levulinic acid dehydratase selectively. Komai and Neilands (1968) carried out experiments with amino acid analoques and inhibitors of protein synthesis, and deduced that zinc is involved in the biosynthesis of δ-amino levulinic acid dehydratase at the translation level.

The probability that zinc is a constituent of the enzyme itself cannot, however, be ruled out.

A decrease in the rate of photosynthesis has been observed under conditions of zinc deficiency (Fujiwara and Tsutsumi, 1962), but direct involvement of zinc in photosynthetic reactions has not yet been confirmed. Nonetheless, evidence is available suggesting that zinc may play a role in these reactions.

Zinc is not a variable valence metal and this may exclude its participation in the electron transport system. Nezgovorova (1963) suggested the existence of a lipoprotein complex which acts as an acceptor of carbon dioxide in the process of photosynthesis, and claimed that this complex contained 7% protein nitrogen, 1% phosphorus, as well as zinc and iron.

It should be kept in mind that zinc is a constituent of phosphoglycerol aldehyde dehydrogenase, and therefore plays a part in the reductive transformations of photosynthetic products. The possible involvement of zinc in the process of photosynthesis is indicated also by measurements of its concentration in chloroplasts and by its supportive role in maintaining chloroplast structure (Vesk et al., 1966).

Zinc may also be involved in photosynthesis by virtue of its association with carbonic anhydrase. The role of this enzyme in plants has not yet been elucidated. It produces a substrate for ribulose diphosphate carboxylase (carboxydismutase), and has been isolated from spinach chloroplasts (Waygood et al., 1969). In studies of the carbonic anhydrase content of maize leaves, Everson and Slack (1968) found that the amount was five to ten times lower than that occurring in spinach leaves, in which a typical Calvin cycle takes place. Moreover, the enzyme occurring in maize is not located in the chloroplasts, whereas in Calvin cycle plants carbonic anhydrase is found in the chloroplasts.

These findings have been confirmed by the studies of Waygood et al. (1969). They too noted that spinach chloroplasts displayed a high carbonic anhydrase activity, the enzyme possibly being associated with ribulose diphosphatase. Activity of carbonic anhydrase could not be detected in maize chloroplasts, in which the primary assimilation of carbon dioxide in photosynthesis is catalyzed by phosphoenol pyruvate carboxylase. This indicates that the substrate for carbonic anhydrase is carbon dioxide, and consequently the function of carbonic anhydrase in green plants is to fix carbon dioxide, which subsequently may be liberated by glycocol oxidase

in the process of photorespiration.

In the light of these findings of Waygood et al. (1969), a report concerning the different responses of two Phaseolus varieties to zinc as revealed by the electrophoresis of cell proteins is of interest (Jyung et al., 1972). Two Phaseolus varieties, genetically distinct in their response to zinc deficiency (resistant - Saginaw, sensitive - Salinac), were grown in a soil adequately supplied with zinc. At low external levels of zinc, fraction I featured prominantly in the electrophoregraphs of proteins from the zinc-resistant variety, but manifested only slightly in those from the sensitive variety. There was a corresponding decrease in the activity of ribulose diphosphate carboxylase by approximately 40% in the resistant variety and 95% in the sensitive one. With an adequate zinc supply, fraction I was more strongly represented in the sensitive variety.

The significance of phospholipids and various other lipid substances in the photochemical reactions of chloroplasts has long been recognized. The ability of zinc to produce complexes with the lipid compounds in chloroplasts (Igoshina, 1972), clearly demonstrates that zinc is involved in the photochemical reactions occurring in chloroplasts. There are reports of the Hill reaction being suppressed by zinc deficiency (Spencer and Possingham, 1960).

Suchdeo and Schultz (1974) showed that carbonic anhydrase facilitates the diffusion of CO_2 across artificial liquid membranes. It was assumed, therefore, that the enzyme also facilitates diffusion of the CO_2 assimilated in photosynthesis on its path through the liquid phase of the cell to the chloroplast (Hatch and Slack, 1970). Randall and Boumo (1973) measured the rate of photosynthesis and the level of carbonic anhydrase activity in spinach grown under conditions of zinc deficiency. Deficient plants contained approximately half the amount of total zinc per unit leaf area compared with normal plants, whereas carbonic anhydrase activity was drastically reduced to 1-13% of that in the controls. The rate of photosynthesis, however, was only slightly affected in deficient plants, and only in those leaves in which carbonic anhydrase activity was very low did the rate of photosynthesis decrease to 85% of that of the controls. Otherwise zinc-deficient leaves showed photosynthetic rates similar to those of control leaves at low CO_2 levels (0.02%), and higher photosynthetic rates at high CO_2 levels (0.03-0.05%). These results were interpreted by the authors as indicating that there was no close relationship between photo-

synthesis and carbonic anhydrase activity. Moreover, they believed that these findings testified against the hypothesis that carbonic anhydrase facilitates CO_2 transport into the chloroplasts, although they could not rule out the possibility that small amounts of carbonic anhydrase may play a minor role. Souginite (1978) obtained evidence in favour of carboanhydrase as a participant in the process of photosynthesis.

The role of zinc in carbohydrate metabolism is apparently related to its influence on photosynthesis and the processes of phosphorylation. A view has been expressed that a minimum zinc content of the cell is a prerequisite for the synthesis and utilization of carbohydrates. With a shortage of zinc in tomato, citrus and other plants, reducing sugars accumulate and the sucrose and starch contents of the plants go down. Chesters and Rolinson (1950) interpret the data relating to alterations in starch metabolism, as indicating a reduction in sugar utilization and an increase in the accumulation of organic acids under conditions of zinc deficiency. These effects arose presumably from an impairment of the enzyme activity responsible for the phosphorylation of sugars.

Shkolnik and Greshishcheva (1958) have shown that the influence of zinc on the synthesis and hydrolysis of carbohydrates is dependent on the form of nitrogen that is available to the plants. With a supply of ammonium nitrogen, the amount of sucrose in wheat and buckwheat is increased when zinc is added to the plants, whereas a supply of nitrate nitrogen results in the sucrose concentration decreasing; conversely, the concentration of monosaccharides decreases in the presence of ammonium nitrogen and increases with nitrate.

A typical symptom of zinc deficiency is retardation of growth and an almost complete cessation of internodal growth. A number of investigators (Skoog, 1940; Hoagland, 1944; Tsui Cheng, 1948) have found that the auxin concentration is considerably reduced in zinc-deficient tomatoes and sunflowers. In the experiments of Tsui Cheng (1948), the reduction in auxin concentration as a result of zinc deficiency was observed prior to any notable impairment of growth, and before any apparent symptoms of zinc deficiency developed. The concentrations of both free and bound auxins were reduced. Supplying zinc to the growth medium increased the concentration of auxins, and growth was resumed within 24 hours (Skoog, 1940).

The decrease in the auxin content caused by zinc deficiency is apparently associated, not with any enhancement of their oxidation, but rather with a disturbance of the biosynthesis of the precursor of indoleacetic acid, tryptophan, and probably other precursors as well. Tsui Cheng (1948) reported a decrease in the tryptophan content of zinc-deficient tomato plants. Subsequently, a dramatic reduction in the synthesis of tryptophan from indole and serine was detected in zinc-deficient plants (Tsui Cheng and Wu, 1960). Zinc shortage was thought to bring about the suppression of tryptophan synthesis by inhibiting the synthesis of serine. Serine and indole together have been found effectively to counteract the adverse effects of zinc deficiency, but not indole alone. Nason et al. (1951) discovered a relationship between the decrease in tryptophan content occurring under conditions of zinc deficiency and a concomitant reduction in the activity of the tryptophan synthesizing enzyme, tryptophan synthetase. Wood (1953) suggested that the diminished tryptophan content and reduced rate of protein synthesis brought about by zinc deficiency could be explained by heightened phosphatase activity and a breakdown of pyridoxal phosphate, since the latter functions as a coenzyme in the synthesis of tryptophan from serine and indole. In fact, zinc-deficient tomato plants have been found to exhibit a high phosphatase activity in the presence of glycerol phosphate, while additions of zinc reduced this activity (Sadasivan, 1950).

To digress from the main topic, it may be said that observations on the effects of zinc on tryptophan synthetase activity are supported by findings from other studies of the effects of zinc on the synthesis of alkaloids, of which tryptophan is a precursor. Thus, it was shown that the production of alkaloids in an Aspergillus fumigatus culture was dependent upon the concentration of zinc and potassium ions in the culture medium (Rao and Gupta, 1974). Both growth and the synthesis of alkaloids were suppressed in the presence of these two elements, the explanation given being that these ions stimulated tryptophan synthetase activity and this led to an accumulation of tryptophan, a precursor of alkaloids. When present in optimum concentrations, zinc and potassium caused the tryptophan synthetase activity to increase four-fold with a concurrent depression of the synthesis of alkaloids.

However, Mashev and Stanchev (1966) failed to observe any increase in phosphatase and peroxidase activity when the tryptophan content was depressed by high zinc concentrations. Thus they were

led to the conclusion that the increase in the synthesis of tryptophan from indole and serine effected by zinc was related to an elevation of tryptophan synthetase activity. The effects of zinc upon the biosynthesis of tryptophan are highly specific. Manganese, known for its stimulating influence on growth and its involvement in the metabolism of indoleacetic acid, was not found to affect tryptophan biosynthesis from serine and indole.

Another possible cause of the suppression of tryptophan synthesis under conditions of zinc deficiency is some change in vitamin B_1 and vitamin B_6 metabolism, since these compounds are known to play a major role in the synthetic processes concerned. Davydova (1966) reported that the removal of zinc resulted in a decrease in vitamin B_6 content of tomato roots, prior to the development of symptoms of zinc deficiency. With acute zinc deficiency, the concentration of both vitamins B_1 and B_6 was reduced in leaves and roots alike. It is known that in the reactions leading to the combination of ammonium with organic acids, the enzymes involved have an active group which is represented by vitamin B_6. It is conceivable that the decrease in vitamin B_6 content affects the biosynthesis of some amino acids, including tryptophan. The partial alleviation of the symptoms of zinc deficiency in tomato plants by adding vitamins B_1 and B_6 to the nutrient medium (Fig. 39) warrants the assumption that a shortage of vitamin B_6 is one of the probable causes of change in the rate of synthesis of tryptophan (Shkolnik and Davydova, 1962).

Vlasyuk et al. (1978) found that zinc regulates the activity of aromatic aminoacid transaminase in maize. As the symptoms of zinc deficiency become more clearly expressed, the capability of the enzyme to convert tryptophan into indolylpyruvic acid, which is the immediate precursor of indolylacetic acid, decreases, and the affinity of the enzyme for phenylalanine and tyrosine, which are the precursors of phenols, lignin and similar substances of secondary origin, increases.

Mashev (1972) found that zinc, and to some extent cobalt and copper also, have an effect on the synthesis of tryptophan from anthranilic acid. This process is accompanied by an increase in the content of indoleacetic acid and indole-3-acetonitrile.

Reports indicating that zinc affects the metabolism of indole compounds other than indoleacetic acid have appeared. Thus it has been shown that a pre-sowing treatment of barley and turnip seeds with zinc leads to an increase in the tryptophan contant of these,

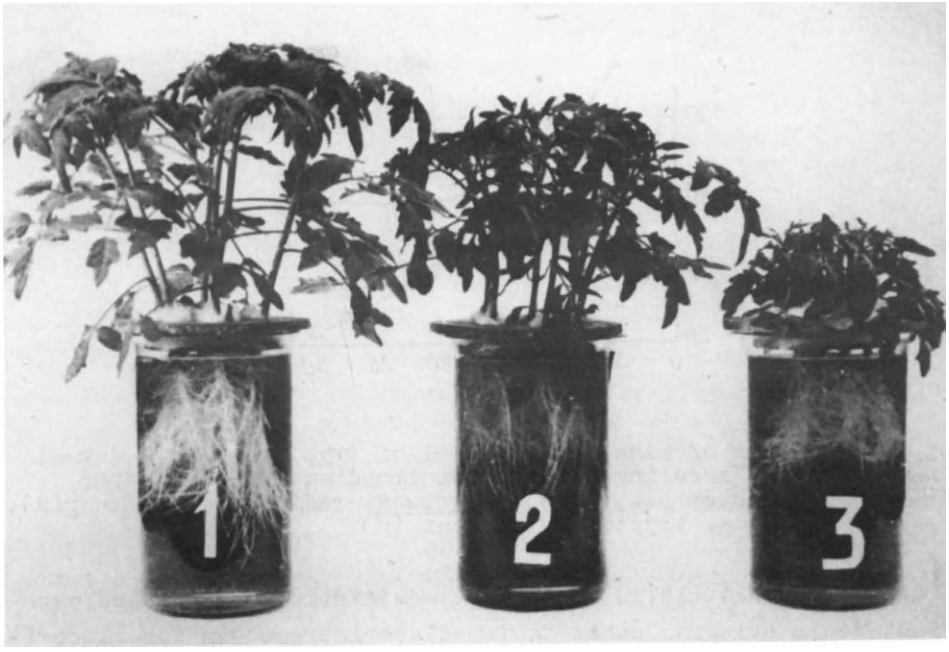


Fig. 39. Alleviation of zinc deficiency in tomatoes by supplying vitamin B_1 and B_6 (after Shkolnik and Davydova, 1962). 1, in the absence of both zinc and vitamins; 2, in the absence of zinc, but with a supply of vitamins; 3, with a supply of zinc.

as well as an increase in the levels of indole auxins, brassicin, glucobrassicin, indole-3-acetonitrile, indoleacetic acid and gibberellin-type substances (Mashev, 1967). The ability of zinc to eliminate or mitigate the inhibiting action of gamma irradiation on the production of tryptophan, indole auxins and gibberellin-type substances has been observed (Fig. 40; Kutacek et al., 1966). Purified preparations of tryptophan, indoleacetic and gibberellic acids exposed to very high doses of radiation in the presence of zinc showed no radiochemical degradation. The authors suggested that irradiation damages those plant enzyme systems that are involved in the turnover of growth substances. The injurious effect of irradiation on the auxins stems from its action at the level

of tryptophan synthesis, which is inhibited by this treatment. The authors are inclined to ascribe an increase in the synthesis of auxins and giggerellic acid in irradiated plants treated with zinc to activation of the remaining uninjured enzyme complement of the plants.

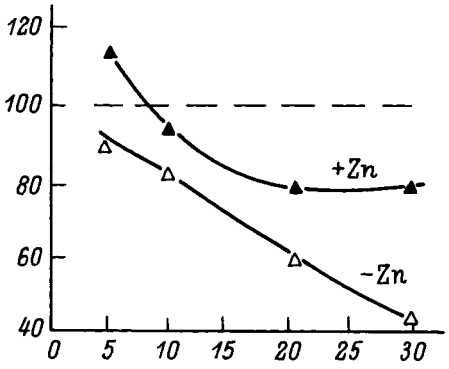


Fig. 40. Effects of zinc on the level of tryptophan in 7-day-old barley seedlings grown from irradiated seeds (after Kutacek et al., 1966). Abscissa, radiation dose (curie); ordinate, tryptophan content (%).

Jiracek et al. (1972) found that administrations of zinc were effective in bringing about an immediate increase in the glucosinalate content, especially the content of neoglucobrassicin.

In the initial stages of zinc deficiency in maize, decrease the concentrations of complex esters of oxycinnamic acids: 3-o- and 5-o-caffeyl-quinic acids (chlorogenic and neochlorogenic acids), 3-o- and 5-o-cumarylquinic acids, and 3-o- and 5-o-ferulylquinic acids (Fig. 41; Davydova et al., 1972). In this context mention should be made of the low levels of aromatic amino acids, in particular, tyrosine and phenylalanine - the precursors of oxycinnamic acids - that are typical of zinc-deficient plants (Bertrand and Wolf, 1961). Taking into account the role of oxycinnamic acids in the metabolism of tryptophan, a shortage of these may be considered as a possible cause of the observed decrease in the concentration of indoleacetic acid under conditions of zinc deficiency. The esters of oxycinnamic acids may influence the concentration of auxins via the indoleacetic acid-auxin oxidase system, since, according to available evidence (Gelinas and Postlethwait, 1969) ferulic

acid ester is the principal inhibiting agent acting on this enzyme in maize. Similar data have been reported for chlorogenic acid (Tomaszewski and Thimann, 1966).

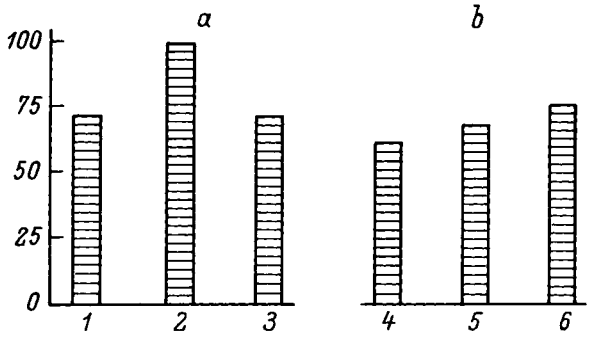


Fig. 41. Effects of zinc on the levels of oxycinnamic acids in maize leaves (after Davydova et al., 1972). Plants showing a, early signs of zinc deficiency, and b, very severe symptoms of zinc deficiency. Ordinate, amount of acids as % of their content in normal plants. Figures under columns, age of plants (days), (1) 17 days, (2) 12 days, (3) 15 days, (4) 25 days, (5) 14 days, (6) 17 days.

In view of the well-known role of zinc in the biosynthesis of auxins, a study has been made (Rudakova et al., 1981) of the role of zinc in regulating the activity of the aminotransferase for the aromatic aminoacids, this aminotransferase being a key enzyme in the biosynthesis of growth regulators of the indole and phenol types. It has been found that zinc deficiency in maize accelerates the degradation of high molecular enzyme forms and favours the accumulation of low molecular forms - a characteristic of ageing organisms. The addition of zinc ions to an enzyme preparation delays its degradation, especially where the preparation originates from a zinc-deficient plant. It has been assumed that the zinc ion is necessary for maintaining the protein structure of the aminotransferase enzyme of aromatic aminoacids.

Investigations of the influence of zinc on the metabolism of phenolic compounds are of interest in the light of the known involvment of some phenolic compounds in growth inhibition and the suppression of auxin oxidase activity. Krupnikova and Davydova (1972) have shown that the concentration of growth inhibitors increases with zinc deficiency.

In apple plants affected by little-leaf-rosette disease, Karakis (1968) observed seasonal variations in the accumulation of floridzin, which is the main growth inhibitor in apples, and which is important in controlling the rate of vegetative growth.

Evidence has been obtained indicating a relationship between zinc and gibberellins. Earlier we referred to the study of Mashev and Kutacek (1966) who observed an enhancement of gibberellin content in seeds treated with zinc preparations. In order to elucidate the mechanism of this influence of zinc on gibberellin content, it will be necessary to work with plants grown under conditions of artificial zinc deficiency.

Shkolnik et al. (1975) showed a significant increase in the content of gibberellin-like substances in leaves of Phaseolus plants supplied with zinc (Fig. 42). An increase in the content of gibberellin-like substances in stems (Davydova et al., 1977) was noticed only during the period of vegetative growth (A,B) and was weaker than that in the leaves. With the onset of reproductive development (C,D) in zinc-treated plants, the content of gibberellin-like substances was found even to decrease (Fig. 43). This may be connected with the fact that the zinc-treated plants started flowering whereas the zinc-deficient plants only reached the bud stage. Further study is needed on the effect of zinc on the content of gibberellin-like substances in stems of different plants. It is possible that a shortage of endogenous gibberellins during zinc deficiency is one of the factors which inhibit stem growth and cause shortening of the internodes - the symptoms of zinc starvation. These symptoms are seen particularly clearly in apple trees (Fig. 44) and other fruit trees and are named "little-leaf-rosette disease". The marked increase in the content of gibberellin-like substances in the leaves of zinc-treated plants noticed by the above-mentioned authors, suggests that a decrease in the content of gibberellin-like substances during zinc deficiency may be one of the causes of little-leaf-rosette disease.

An interesting observation of a synergic effect of zinc and gibberellin upon plant growth was communicated by Dancer (1959). Melisova (1970) reported a synergic effect of α-naphthylacetic acid, applied as a potassium salt, and zinc. Supplementing zinc with this compound as a treatment for apple trees enhanced the stimulating effects of zinc on rate of photosynthesis, the efflux of assimilates, the accumulation of total and organic phosphorus, drought-resistance, the observed activity of some enzymes, and the crop yield.

Studies by Davydova and Mochenyat (1980) did not reveal any dependence of the abscisic acid (ABA) content of bean leaves and stems on the zinc supply. However, the apical parts of zinc-deficient plants were found to contain higher quantities of ABA than those of zinc-supplied plants. Accumulations of ABA in the apical parts of the plants, including the uppermost internodes, may have a negative effect on growth and development. Thus it is quite possible that the shortening of the internodes observed in cases of zinc malnutrition is caused not only by a decrease in gibberellin content, but also by an increase in ABA content.

Zinc is intimately involved in the nitrogen metabolism of plants. A shortage of zinc eventually results in an accumulation of non-protein solyble nitrogen compounds - amides and amino acids. In tomatoes, for example, the glutamine concentration increases almost sevenfold, that of asparagine almost 50-fold (Possingham, 1956).

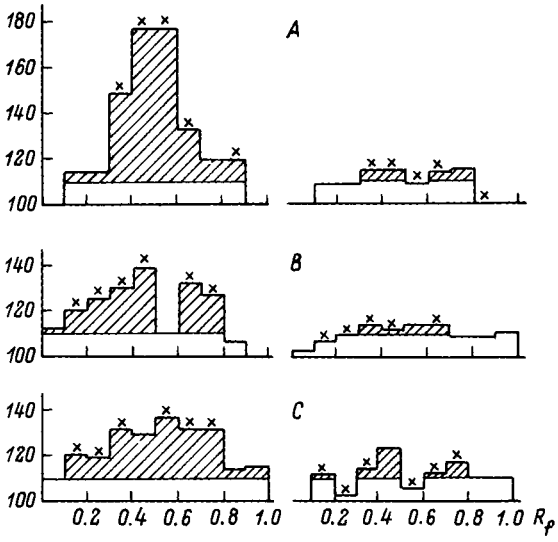


Fig. 42. Effect of zinc on the activity of gibberellin-like substances out of the 2-nd leaf of Phaseolus plants (in terms of growth reaction of Pisum seedlings). Age of plants: A, 19 days; B, 25 days; C, 28 days; left, plants provided with zinc; right, without zinc. Hatched blocks - reliable difference with control; x - reliable differences between analogous zones of comparative variants. Ordinate, gibberellin activity in terms of seedling length (% of control)

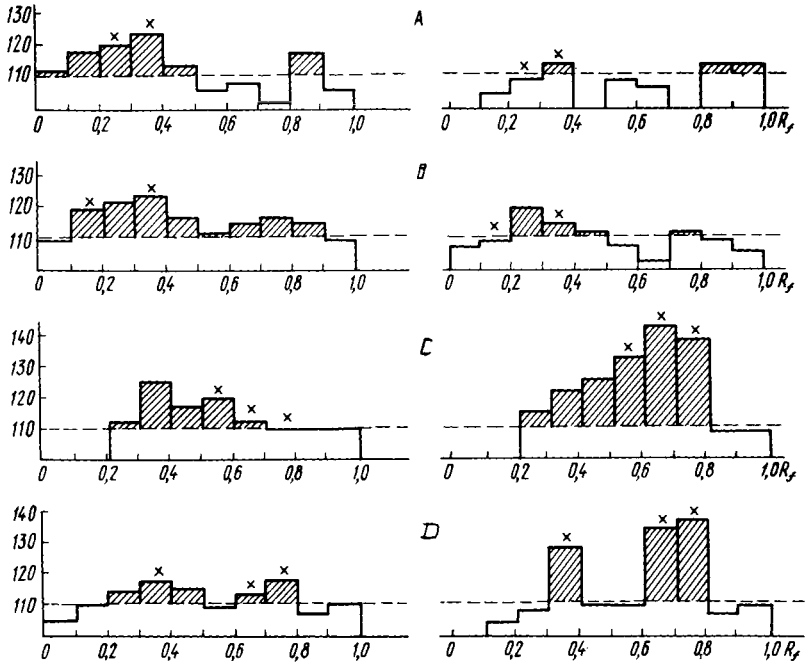


Fig. 43. Histogram showing the activity of gibberellin-like substances from Phaseolus stems, as a function of the supply of zinc (in terms of growth reaction of Pisum seedlings). Age of plants: A, 14 days; B, 16 days; C, 22 days; D, 25 days. Left, plants provided with zinc; right, zinc-deficient plants. Hatched blocks - reliable difference with control; x - reliable differences between analogous zones of comparative variants. Ordinate, gibberellin activity in terms of seedling length (% of control)(after Davydova et al., 1978).

The accumulation of these amides when zinc is in deficiency has been reported by other investigators for various plants (Steinberg, 1956). An increase in the total content of free amino acids can also be noted (Naik and Asana, 1961; and others). Some of these (lysine, histidine, serine, threonine, aspartic acid, leucine, valine, proline and arginine) are accumulated at high concentrations under conditions of zinc deficiency, while the concentration of other metabolites (citrulline, ethanolamine, glycocholate, phenylalanine, tyrosine) decreases (Possingham, 1956).

It should be pointed out that the amine nitrogen content of plants shows an increase before the outward symptoms of zinc deficiency appear (Naik and Asana, 1961). Some visible signs of an inadequate zinc supply are obviously attributable to accumulations of specific compounds, in particular amine nitrogen compounds. In

Fig. 44. Little-leaf-rosette illness caused by zinc deficiency (after Childers, 1954).

experiments in which various amino acids were added to sterile cultures of tobacco seedlings, blotchy chlorosis developed and morphological changes occurred in the leaves, closely resembling the symptoms of zinc deficiency (Steinberg, 1949). L(+)-isoleucine proved to have the greatest effect of the amino acids studied. Amides (glutamine and asparagine) which usually accumulate in larger amounts than amino acids, were less effective in producing visible changes (Possingham, 1956). This does not support the assumption that a relationship exists between the accumulation of specific nitrogen metabolites and some of the symptoms of zinc deficiency. Other data have been obtained relating some of the manifestations of zinc deficiency to nitrogen nutrition. Thus, Haas (1937) reported that the different stages of chlorosis typical of zinc deficiency in citrus plants could be reproduced by introducing compounds containing an amino group (urea, a mixture of

urea and calcium nitrate cyanamide).

An abundant nitrogen supply aggravates the symptoms of zinc deficiency in plants (Ozanne, 1955). Thus in clover, zinc accumulates in the roots proportionately with the protein nitrogen content. Ozanne believes that complexes of zinc and protein are formed in the roots, but that these are not translocated to the above-ground plant organs.

Some investigators have reported that nitrates accumulate in zinc-deficient plants (Possingham, 1956). This accumulation, however, is not associated with changes in nitrate reductase activity, since it has beeb shown that the concentration of nitrate compounds in tomato plants increases well before the visible symptoms of zinc deficiency become obvious, while the activity of nitrate reductase remains unchanged (Davydova, 1966). The accumulation of soluble nitrogen compounds when zinc is in deficiency apparently points to disturbances in protein synthesis (Steinberg, 1956). As shown by many workers (Schneider and Price, 1962; Shkolnik and Davydova, 1965), the protein content in both higher and lower plants decreases when there is a zinc shortage. According to Shkolnik and Davydova (1965), zinc promotes the incorporation of ^{14}C-tyrosine into the proteins of tomato plant tissues, indicating an enhancement of protein synthesis. Disturbances in the synthesis of some amino acids (for example, tryptophan) have been implicated as being responsible for the reduced protein synthesis (Hoagland, 1944; Tsui Cheng, 1948). Although it is known that the synthesis of tryptophan is impaired by zinc deficiency, a more likely probability is that a shortage of zinc interferes with the steps of assembly of amino acids in the course of protein synthesis (Nason et al., 1951). The view has been expressed that zinc is involved in the synthesis of peptides (Steinberg, 1956).

The effect of zinc on protein synthesis apparently may operate through glutamate dehydrogenase, which in animal tissues has been shown to be a zinc enzyme.

Praske and Plocke (1971) also discussed the effects of zinc on protein synthesis. These workers found that the ribosomes in Euglena gracilis were completely degraded in cells grown in the abcence of zinc. The breakdown of the ribosomes proceeded hand-in-hand with the retardation of growth in the culture. These findings indicate that zinc is involved in the supramolecular organization of intracellular components. Not only does zinc promote the association of ribosomes from the products of their dissociation, but

it also stabilizes polysomes isolated from imbibed pea seeds (Backer and Rieber, 1967).

Sherstnev et al. (1970) have discovered in zinc-deficient Euglena gracilis cells a decrease in the number of protein stripes in the course of electrophoretic analysis of ribosomal proteins and a different way of their distribution. This gives evidence of the destruction of ribosomes due to the lack of zinc and gives ground to the supposition, that this metal participates directly in the structural organization of ribosomes.

Evidence on the involvement of zinc in nucleic acid metabolism is available supporting the general view that this is the most important aspect of the physiological role of zinc. The author is here referring to evidence pointing to the importance of zinc in transcription. Many nucleotidyltransferases have proved to be zinc-containing enzymes (Valenzuella et al., 1973). The identification of zinc as an integral part of RNA-dependent DNA-polymerase (reverse transcriptase) (Springate et al., 1973) opens up a challenging area of study in the field of genetic engineering.

Zinc ions inhibit the replication of adenoviruses (Korrant et al., 1974). Taking this into consideration together with the available information on the involvement of zinc in polyribosomal protein biosynthesis, one can see that further investigation into the physiological role of zinc will probably contribute much to the development of molecular biology.

In zinc-deficient cells of the green alga Euglena gracilis, Falchuk and Hardy (1977) found a duplication of the mRNA content accompanied by a twofold increase in the $\frac{G+C}{A+U}$ ratio. An increase in the manganese content of the nutrient medium from 2 mM to 5 mM, or even to 10 mM can considerably modify the function of RNA-polymerase in Zn-deficient Euglena cells.

Some investigators believe that zinc regulates protein synthesis through its influence upon the metabolism of nucleic acids. The first to observe a decrease in RNA content caused by zinc deficiency were Kessler and Monselise (1959), working with orange tree leaves. The decrease in RNA content was paralleled by a diminution of the protein content. Decreases in RNA content caused by a shortage of zinc have also been observed in other fruit trees, as well as in tomatoes and barley. Zinc deficiency in Nocordia opaca was accompanied by decreases in RNA content and DNA content (Webly et al., 1962). In Euglena gracilis, RNA content and DNA content were shown to depend on the zinc concentration in the medium. At the

same time, the essential nature of zinc for the synthesis of nucleic acids was observed in Mycobacterium smegmatis (Winder and Denneny, 1959), and its necessary presence for the synthesis of ribose and deoxyribose in Aspergillus niger was demonstrated (Bertrand and Wolf, 1961); in particular, a notable suppression was reported for ribosomal RNA, which is consistent with the slowdown of protein synthesis (Price, 1962; Schneider and Price, 1962). In the same organism, Wacker (1962) observed that zinc deficiency led to a decrease in the RNA content, but not the DNA content; indeed the latter markedly increased. In barley, severe symptoms of zinc deficiency were accompanied by a decrease in the RNA content of the leaves (Tsutsumi and Fujiwara, 1964). It has been suggested that zinc influences growth primarily because of its role in RNA synthesis (Price, 1962; Schneider and Price, 1962; Wegener and Romano, 1963). Price refers to evidence that the earliest metabolic change to occur when zinc is in deficiency is a decrease in the RNA content accompanied by a decrease in the protein and DNA contents. This has also been found to be the case in zinc-deficient Rhizopus nigricans and Mycobacterium smegmatis. A decrease in the RNA content accompanied by a decrease in protein content has been observed by Davydova (1967) in zinc-deficient tomato plants. A decrease in protein content occurring simultaneously with a decrease in nucleic acid concentration as a result of zinc deficiency has been reported for maize by Vlasyuk et al. (1969).

Reference was made above to the suppression of ribosomal RNA synthesis when zinc is in deficiency. In this respect the finding of Altman et al. (1968) that zinc concentrations in cells of Chlorella pyrenoidosa (strain 211-86) are proportional to the rate of mRNA synthesis is of great interest. These workers found a strong correlation between variations in zinc concentration throughout the mitotic cycle and the rate of guanine incorporation into mRNA, and inferred that zinc ions influence the synthesis of mRNA. This study points to the possibility of zinc being involved in the expression of genetic information. Pilipenko (1975) found that zinc deficiency is accompanied by considerable changes in the high-polymer RNA content, which decreases considerably compared with the low molecular weight RNA content. A decrease in the molar proportions of adenylic and uridylic acids in the high polymer RNA fraction could be observed.

It may be that zinc is concerned in the synthesis of nucleotides. Such a possibility is indicated by the increase in the concentra-

tion of free nucleotides occurring in cultures of Rhizopus nigricans following the addition of zinc (Wegener and Romano, 1963). In Mycobacterium smegmatis, an ultraviolet-absorbing fraction of acid-soluble substances was reduced by zinc deficiency; this fraction contains nucleotides as well as other substances (Winder and Denneny, 1959).

The observed decrease in the RNA contant accompanying zinc deficiency has been attributed to an increase in RNAse activity. Evidence for this has been obtained by Kessler (1957) in his experiments with citruc plants, and RNase activity has been reported to be increased when zinc is in deficiency (Vlasyuk et al., 1969). Zinc inhibits this enzyme.

A suggestion has been advanced that zinc interferes with protein synthesis through its effect on the activity of RNase (Kessler and Engelberg, 1962). In the leaves of citrus plants, apple trees, and vines inadequately supplied with zinc, the activity of RNase increased and simultaneously the protein content and RNA content decreased (Kessler and Monselise, 1959).Similar conclusions were arrived at by Sarin and Saxema (1965). In cotton, an increase in RNase activity accompanied by a decrease in protein content were detected before any visible symptoms of zinc deficiency developed (Naik and Asena, 1961).

Interesting findings have been reported by Sodek et al. (1970), who isolated two ribonucleases from wheat leaf extracts: WL-RNases I and II. Ethylenediaminetetracetic acid was found to inhibit their activity strongly. Of the four metals studies - magnesium, manganese, zinc and copper - only zinc ions were effective in counteracting the inhibiting action of EDTA on ribonuclease activity.

The involvement of zinc in the metabolism of cell structural components should certainly be kept in mind. A correlation has been observed between the amount of zinc available and the phospholipid content of the cell (Wachter and Spoerl, 1961). A shortage of zinc has been shown to result in a decrease in the phospholipid contents of barley, tomato and pea seedlings (Tsutsumi and Fujiwara, 1964). Reference has already been made to an investigation by Ispolatovskaya (1967), who reported that zinc was a constituent of Clostridium perfringens lecithinase. There are grounds, therefore, for believing that zinc plays a part in the assembly of intracellular membranes. This view also finds support in those observations which suggest that zinc deficiency is responsible for

structural changes in chloroplasts and mitochondria.

Zinc seems to be involved in the formation and maintenance of polymer structures and supramolecular complexes. According to available information (Ernst and Weinert, 1972), zinc has a stabizing effect on macromolecules and biomembranes. About 10% of the total zinc in Silene cucubales leaves is combined in stable complexes in the cell membranes. Considerable quantities of zinc occur in nucleoli, in the mitotic apparatus, and in ribosomes (Tal,1968).

Prask and Plocke (1971) observed a disappearance of ribosomes in zinc-deficient cells of the alga Euglena gracilis and suggested a special role for zinc in the formation of the tertiary and quaternary structures of cytoplasmic ribosomes.

Zinc apparently plays a role in the function of membranes. It is known that divalent cations exert a considerable influence on cell membranes, and as shown by Epstein (1961) in experiments with isolated barley roots, zinc ions and calcium ions may be concerned in regulating ion transport across cell membranes.

Unicellular algae are very sensitive to zinc deficiency, and normally zinc is accumulated in the cell membranes of these organisms. Zvereva and Sherstnev (1981) have detected 5-6 substances giving a lipid stain reaction, 3 of these being phospholipids. Of the latter phosphatidylcholine is predominant, and the two others are identified as phosphatyl ethanolamine and phosphalylserine. Two stain reactions are apparently attributable to galactolipids. Zinc has no effect on the qualitative composition of the lipids detected, but significantly increases their quantity.

Of great importance is the work of Falchuk et al. (1975). These authors examined the part played by zinc in the premitotic stages of cell division in Euglena (Table 9). The results are summarized in table 9; normally G_1 is the stage preceding DNA replication; then (phase S) follows the duplication of DNA. G_2 is the phase of assembly of the apparatus needed for accomplishing mitosis. In a zinc-deficient medium the DNA duplicates, but the cells do not divide, and DNA accumulates. If, however, the cells are placed in a zinc-deficient medium after the premitotic phase, no DNA duplication occurs. The authors concluded that zinc is essential for the biochemical processes of interphase and the transition into mitosis. The duplication of DNA in a zinc-deficient medium occurs by virtue of the minute amount of zinc still present in the medium- an amount sufficient to enable the zinc-containing enzymes to function normally.

From these findings and other information on the involvement of zinc in cell division, one may conclude that this particular role of zinc is the most important of its biological effects. The process of cell division is obviously governed by a large number of cooperating enzymes, many of which contain zinc.

TABLE 9 The role of zinc in biochemical and morphological processes of the cell cycle (after K.Falchuk, 1975)

Stage of cell cycle	Biochemical and morphological processes involved	Processes or cellular components with known Zn requirement
G_1	RNA synthesis Protein synthesis Increase in size	Uridine incorporation into RNA DNA-dependent RNA polymerase RNA stabilization
S	DNA synthesis	Thymidine incorporation into DNA DNA-dependent DNA polymerase DNA stabilization
G_2	Assembly of apparatus for nuclear and cell division	Unknown
Mitosis	Nuclear division	Dithizone staining of nucleoli Spindle apparatus Chromosomes

This view is also supported by the demonstration of increases in the concentrations of several trace elements: copper, iron, manganese, boron, molybdenum, in various zinc-deficient plants (Millikan, 1953; Steinberg et al., 1955; Naik and Asana, 1961). In experiments with tomato plants it has been shown that the uptake of iron, copper, manganese, molybdenum and phosphorus is enhanced under conditions of zinc deficiency (Paribok and Alekseyeva-Popova, 1965). According to Wacker (1962), zinc-deficient Euglena gracilis cells accumulate 3-60 times as much magnesium, calcium, iron and manganese as that taken up by cells adequately supplied with zinc. Rudakova et al. (1970) reported that in plant tissues showing the little leaf symptom a decrease in zinc content was always accompaneid by a heavy accumulation of iron. The increase in iron content is more typical characteristic of the disease than the decrease in zinc concentration, since the disease may be encountered when large amounts of zinc are present in plants and soils. As revealed in experiments with mitochondria, zinc modifies the energy-dependent permeability of mitochondrial membranes to potassium and magnesium (Brierley and Knight, 1967; Brierley and Settlemire, 1967).

Of paramount interest, however, is the problem of phosphorus-zinc antagonism. A review of all pertinent findings has been given by Paribok (1970a). Investigations dealing with the phosphorus-zinc relationship have revealed a typical case of ion antagonism, there being a reciprocal dependence of phosphorus concentration upon that of zinc in plant tissues. The same reciprocal relationship has been found with respect to the effluxes of these elements from plants. The essence of this antagonism has not so far been explained. According to the evidence presented in the review cited, this relationship between phosphorus and zinc can be observed in the surface cells of roots and in the root cells involved in the translocation of these elements to the aboveground plant organs. This, however, does not rule out the possibility that zinc and phosphorus interact in the soil, a phenomenon that is currently being intensively studied.

A decrease in the zinc content of plants resulting from high doses of phosphorus may eventually produce adverse effects. Laganovsky (1952) recommended that zinc fertilizers should be supplied to flax and other plants whenever high doses of phosphorus are present in, or applied to the soil.

It has been shown by Tarasov and Zhuravleva (1981) that the little-leaf-rosette disease of apple trees and other fruit trees, which is common in hot regions, is caused mainly by a lack of zinc arising primarily from the lack of balance between macro- and micronutrients, particularly between copper and zinc. With the introduction of 500 mg of a potassium salt per 1 kg soil, the mass of the aboveground system of three-year-old plants fell 20% below the control mass, and the condition of the rosette disease remained unchanged. The combined effect of potassium and phosphorus brought about an increase both in growth and in the severity of the disease as a result of the increase in mobile phosphorus. Nitrogen fertilizers decreased the severity of the disease, but when introduced together with phosphorus, enhanced its deleterious effects.

It has been demonstrated (Dogar and Van Hai Tung, 1980) that an increase in the soluble nitrogen level, contrary to the effect observed when the level of phosphorus increases, leads to an increase in the zinc content of plants. The major part of the nitrogen remained in the roots, and this increased the sensitivity of the plants to low zinc concentrations (0.05 μM).

Recent data concerned with the role of zinc in the sexual fertilization of plants are reported by Polar (1975). Tobacco and

fodder beans were grown in a nutrient solution containing 22 curie per litre ^{65}Zn in a total of 0.3 mg per litre of zinc. In the flowers, the label became concentrated mainly in the pollen, and during pollen germination the zinc migrated into the pollen tube. After pollination of the flowers of unlabelled plants with labelled pollen, seeds were obtained which contained 68% of the label of the pollen. It was concluded that zinc is involved in the process of fertilization.

The zinc requirement of plants is largely dependent on temperature, being greater at higher temperatures. Palamar-Mordvintseva and Stupina (1968), while studying the effects of zinc on the growth and development of Ankistrodesmus braunii, observed an enhancement of growth at lower temperatures accompanied by a decrease in zinc content. The opposite was true at elevated temperatures. The little-leaf-rosette disease of fruit plants attributable to shortage of zinc is mostly encountered in southern regions.

Summarizing the evidence available on the physiological role of zinc, the polyfunctional nature of this element must be emphasized. A shortage of this metal brings about changes in a number of the fundamental physiological processes of plants.

Thus among the most important physiological functions of zinc are its involvement in growth regulation, nucleic acid metabolism and protein biosynthesis, cell division, the maintenance of membrane structure and function, and sexual fertilization. Zinc is a cofactor of many enzymes, and it is essential for the formation and maintenance of the macromolecular structure of a number of the most important cell components (e.g. the ribosomes).

Chapter 4

COPPER

The omnipresence of copper in both the plant and animal kingdoms was recongnized as early as 1816 (Buchholtz, 1816). When a copper requirement had been established for a large number of plants (Fig.45), copper was introduced into practical plant cultivation, especially on peat-bog soils deficient in readily utilizable forms of copper (Lashkevich, 1937).

Fig. 45. Variations in tomato leaves caused by copper deficit (after Stiles, 1961). Below, leaf of a normal plant. Above, leaf of copper-deficient plant.

A characteristic of such soils is the appearance of a disease of cereals - "treatment illness", the main symptom being wilting of the leaf tips (Fig.46). Adding copper to the soil eliminates this problem (Fig.47).

Copper deficiency results in malformations of the tissues and influorescences, and changes in the fertilization process.

Over a relatively short period of time, outstanding new discoveries and concepts have contributed much to our understanding of

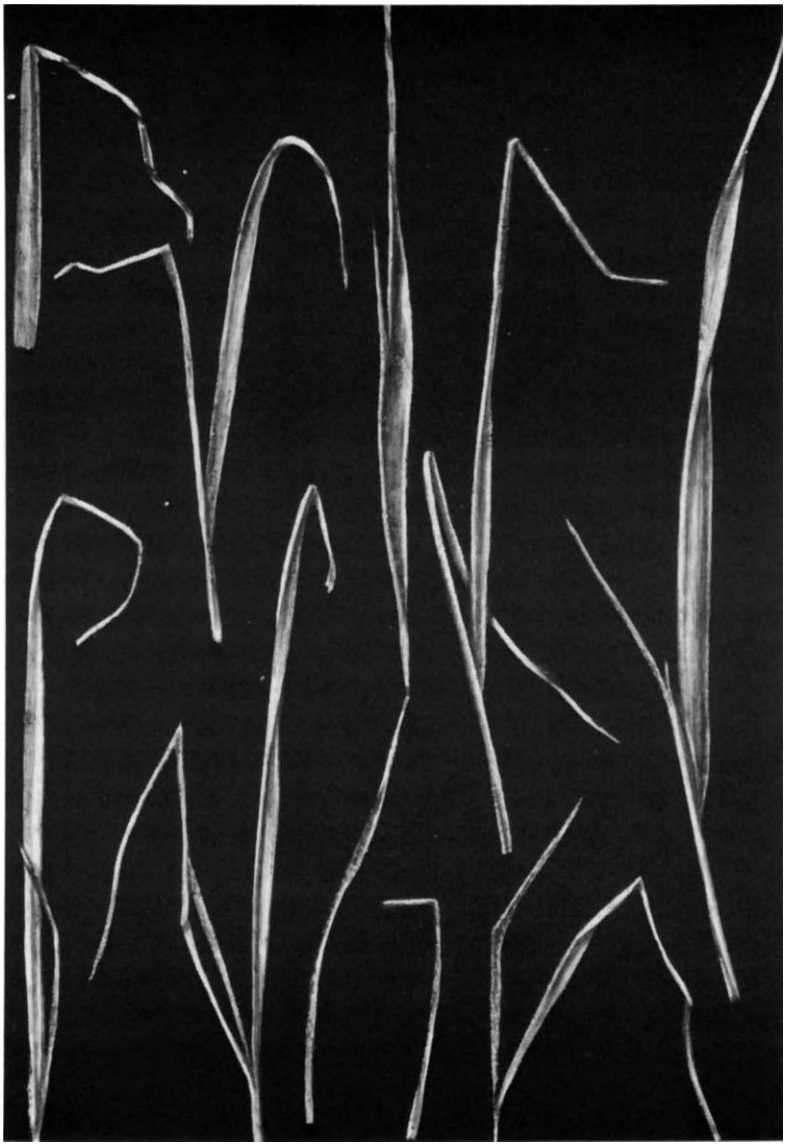


Fig. 46. Wilting of oat leaf tips as a result of copper deficit (after Piper, 1942).

copper as a trace nutrient.

Copper is usually found in the mitochondria, and in animal cells, copper has been shown to be present as a constituent of all isolated mitochondrial fractions (Balevska, 1972), It has been detected in the proteins of the intermembrane space, in the matrix, in the structural proteins, and in the detergent-soluble proteins.

Fig. 47. Alleviation of copper deficiency symptoms in wheat plants growing on boggy peat soils by means of copper (after Lazarev, 1939).
1, without NPK and without Cu; 2, with NPK, but without Cu; 3 and 4, with both NPK and Cu.

These findings point to the important role played by copper in the processes taking place in the mitochondria; the most active copper proteins are those of the soluble mitochondrial fraction. In this respect copper is not limited to being a constituent of enzymes, since the total amount of copper in mitochondria is considerably higher than what would be required for the monoamino oxidase, cytochrome oxidase, ascorbinate oxidase, urate oxidase and other enzymes of the mitochondria.

The nature of the involvement of copper in metabolic processes is determined by the specific physico-chemical properties of the metal. Firstly, copper ions react with amino acids, proteins and other biopolymers, producing stable complexes. In this regard

copper is more active than other metals. Secondly, copper ions possess catalytic properties which are enhanced upon binding of the ion to a protein molecule. Thirdly, copper ions readily release or accept one electron, which accounts for the behaviour of copper either as a donor, or as an acceptor of electrons (Frieden, 1968).

The physiological significance of copper lies predominantly in its being a constituent of a number of copper proteins and enzymes (Table 10) mostly oxidation-reduction enzymes. Among copper enzymes are those which catalyze the oxidation of diphenols and the hydroxylation of monophenols, namely, orthodiphenol oxidase (polyphenol oxidase and tyrosinase) and dophamine hydro-oxidase, respectively. These enzymes are omitted from our discussion, because detailed information on phenolases is given in a number of reviews (Rubin and Loginova, 1968; Soboleva and Bokuchava, 1969; Peive, 1970).

Cytochrome oxidase is the most thoroughly studied of the copper proteins. It has been suggested that copper and iron are found at a single active site in cytochrome oxidase (Williams et al., 1968).

Cytochrome oxidase attracts considerable attention because of its important role in respiration and in photosynthesis. Green et al. (1956) have found cytochrome oxidase to be abundant in bovine mitochondria, and offered evidence indicating that copper is concentrated in components of the respiratory chain.

Pirie (1962) believes plant amino oxidase is a copper enzyme. The element is found in a di- and monovalent state in monoamine oxidase, the latter being located in the outer mitochondrial membranes. Amino oxidases carry out the oxidative deamination of mono- and diamines.

Among copper enzymes concerned with the oxidation of substrates by atmospheric oxygen is galactose oxidase isolated from Polyporus circinatus. Tryptophan pyrrolase from Pseudomonas has been found to be a copper enzyme. Along with copper, this enzyme contains a dissociating haemin co-enzyme. It is believed that the copper of the enzyme is bound by oxygen molecules, and tryptophan is bound to the haematin component (Maeno and Feigelson, 1965).

Besides copper-haematin enzymes such as cytochrome oxidase and tryptophan pyrrolase, copper-pyridoxal enzymes are also known. Thus copper, together with pyridoxal phosphate, has been found in benzylamine oxidase. In contrast to the copper in polyphenol oxi-

dase, laccase, ascorbate oxidase, and ceruloplasmin, the copper in benzylamine oxidase does not change its valency during catalysis.

TABLE 10 Copper enzymes
(after Kovalsky and Rish, 1970)

Enzyme	Copper content		Mol. wt.	Source
	w/w protein	atom per molecule		
O-diphenol oxidase Polyphenoloxidase	0.21%	4	119,000	higher fungi
O-diphenol oxidase	0.27%	1	23,000	potato
P-diphenol oxidase (laccase)	0.22%	4	120,000	lac tree
Cytochrome oxidase	0.09%	2	230,000	heart, liver
Ascorbate oxidase	0.25%	6	146,000	pumpkin
Galactose oxidase	0.085%	1	75,000	fungi
Xanthine oxidase (iron+FAD)	17 µg/g	4	236,000	small intestinal wall (cattle)
Monoamino oxidase	0.09%	4	255,000	blood, plasma
Diamino oxidase	0.07--0.12%	3	130,000	kidney peas
Urate oxidase	0.06%	1	120,000	liver
Benzylamino oxidase	0.08--0.09%	4	195,000	blood plasma
Tryptophan pyrrolase	-	-	-	liver
Ribulose diphosphate carboxylase	-	-	-	spinach
Dophamine-hydroxylase	1.6 µg/g	2	-	liver
Sulphide transferase	-	-	-	liver
Glutamyl transferase	-	-	-	bacteria
Hyponitrite reductase	-	-	-	slime molds
Nitrogen oxide reductase (probably identical to nitrite reductase)	-	-	-	bacteria
Haemocyanin	0.18%	26	950,000	haemolymph in the Pacific crawfish

The polyfunctional role of copper has been demonstrated in a number of oxidases. Thus four copper atoms have been detected in Polyporuc versicolor laccase: one copper atom of type I, one of type II, and two diamagnetic copper atoms. Such an arrangement

provides for a cooperative effect between the one-electron transfer occurring in the oxidation of substrates, and the four-electron transfer taking place during the reduction of oxygen.

Zamarayev (1967) analyzed the electron paramagnetic resonance spectra of copper proteins and enzymes (laccase, ceruloplasmin, cytochrome oxidase, benzylamine oxidase, diamine oxidase, Cu^{2+}--carboxypeptidase, Cu^{2+}-carbonic anhydrase, and erythrocuprein). He also studied the copper protein from the enzyme system of Nitrosomonas europea which oxidizes hydroxyl amine. The spectra obtained indicated that in all of the cases studied, Cu^{2+} was found as part of a complex comprising protein functional groups. Copper and iron are activators of nitroreductases and reductases acting upon nitrogen oxides (Nicholas, 1961).

Apart from copper enzymes in which the metal is firmly bound to the protein, there is a number of copper enzyme complexes in which the copper is loosely bound to the protein and can be replaced by other metals. Among such complexes one finds tyrosine iodinase, which is involved in the synthesis of di-iodotyrosine, and sulphide oxidase, which oxidizes hydrogen sulphide to thiosulphate.

A shortage of copper results in decreased activity of the copper enzymes, as shown in a number of studies (Okanenko and Ostrovskaya, 1950; Ostrovskaya, 1961; Kovalsky and Maslyanaya, 1964) involving polyphenol oxidase and ascorbate oxidase. A decrease in copper enzyme activity occasioned by copper deficiency is accompanied by increasing activity among iron enzymes (Kohan, 1955).

An interesting observation has been made regarding polyphenol oxidase, which is responsible for the oxidation of polyphenols and tanning agents in the fermentation of tea. Recently investigators in Ceylon have made a discovery which has very important practical implications namely that no fermentation of black tea occurs if the leaves contain less than 15 mg per kilogram of copper. With less than this amount the activity of the fermenting polyphenol oxidase is markedly reduced. At the same time, tea plants containing less than 10 mg copper per kilogram of leaf tissue show normal growth.

Endowed with the ability to change valency, iron, manganese, copper and molybdenum occupy central positions in the mechanisms of biological oxidation-reduction reactions, including those of respiration, photosynthesis, and the assimilation of molecular nitrogen. The involvement of iron and manganese in photosynthetic

processes has long been recognized, and recently a similar function has been discovered for copper. Various early observations had pointed indirectly to the possible involvement of copper in photosynthesis, for example, there was the observed accumulation of copper in chloroplasts (these structures are known to contain 70% of all the copper found in the leaf), as well as the observation of the essential nature of copper for the synthesis of iron porphyrin complexes. Jamada-Ankei et al. (1977a) showed that coproporphyrin III, produced by Bacillus cerens in a glucose-rich medium, is a copper-containing compound. It was also shown that copper is essential for the synthesis of both coproporphyrin III and coproporphyrin, and cannot be substituted by any other metal.

Sorokina (1967) has demonstrated that copper may be involved in the biosynthesis of chlorophyll at the level of protochlorophyll conversion.

According to current views, polyphenol oxidase and peroxidase acting upon phenolic substrates are involved in the intermediate stages of electron transport, whereas cytochrome oxidase operates in the terminal stages of the respiratory chain. Since it has been established that copper is a constituent of cytochrome oxidase in animals, it seems very probable that the same holds true as regards the involvement of copper in plant mitochondria functions. Gamayunova and Ostrovskaya (1964) found an increased activity of cytochrome oxidase when the supply of copper salts had been augmented.

Of considerable interest is the metal-enzyme superoxide-dismutase, with its two different forms in the tissues of higher plants containing either copper and zinc (McCord et al., 1971; Fridovich, 1975), or manganese (Sevilla et al., 1980). There also exists an iron-containing superoxide dismutase, regarded as an enzyme belonging to prokaryons and algae only. The main bulk of the superoxide dismutase of chlorophyll-containing cells is concentrated in the chloroplasts.

The enzyme protects the chloroplast from the destructive effect of the superoxide radical which is formed during the reduction of oxygen by the reduced products of the chlorophyll light reaction. According to existing evidence superoxide-dismutase is confined to the fraction of sub-chloroplast particles containing photosystem I, and the superoxide radicals are formed on the reducing arm of photosystem I.

Low levels of zink, copper, manganese and iron nutrition result in the inhibition of the corresponding forms of superoxide-dismutase and reduce the protection afforded against ultraviolet light, the oxidation of lipids and chlorophyll, and the disintegration of membranes. Data obtained by Klyavinya and Ozolinya (1978) show that superoxide-dismutase activity in copper-deficient plants is decreased. The authors suggest that this enzyme is a highly sensitive indicator in the early diagnosis of copper deficiency.

In another publication by Ozolinya et al. (1979) it was repeated that in spinach, a plant with a low sensitivity to copper deficienty, a high superoxide-dismutase enzyme-ascorbatoxidase - was maintained in the leaves of plants showing a low copper content; these high activities were not observed in barley, a plant with high demand for copper. According to data obtained by Klyavinya (1980), cabbage also belongs among plants with a low sensitivity to copper deficiency.

Cells with a low copper content retain their total Cu/Zn superoxide-dismutase. This is attributable to stimulation of the Mn--dependent form of this enzyme, which is located in the mitochondria associated and is cyanide-resistant (Shatzman and Kosman, 1978).

The appearance of lime chlorosis in the grape-vine is caused by a low availability of iron, zinc, copper and manganese. Superoxide-dismutase in higher plants is usually a bimetallic enzyme containing zinc and copper, as well as iron or manganese proteins, and plays an important role in protecting cellular structures from the effect of oxygen radicals. It has been found (Ostrovskaya et al., 1981) in determinations of superoxide dismutase activity in preparations of chloroplasts and supernatants from vine leaves, that the activity of the enzyme increases at the mid-way stage of development of lime chlorosis. The assumption has been made that the increase in enzyme activity is a response to the greater appearance of oxygen radicals during lime chlorosis.

Data are available which testify to the effect of a number of trace elements, copper in particular, in promoting greening, the destruction of chlorophyll in the dark and in the senescence of leaves, and the binding of protein to chlorophyll (Okuntsov, 1946; Makarova and Solovyeva, 1959).

Rubin and Chernavina (1959) found that inhibitors of oxidative enzymesystems consisting of copper and iron proteins suppress the

synthesis of green pigments. Chernavina and Kartashova (1967) suggested that the resumption of chlorophyll synthesis effected by copper in tissues which had lost this capacity, was attributable mostly to activation of that part of the respiratory chain involving copper-containing oxidases. Rubin and Chernavina (1970) showed that iron and the enzyme systems activated by this metal form the energy source and substrate material for chlorophyll biosynthesis. With suppression of the biosynthesis of green pigments because of a shortage of iron, the role of the principal metal functioning as activator of the catalytic systems is transferred to copper (copper proteins and related enzyme systems). Under these conditions, however, respiration is not very effective.

A series of interesting studies have appeared presenting direct evidence for the involvement of copper in photosynthetic reactions (Arnon, 1950; Trebst and Eck, 1963; Bishop, 1964,1966). These investigators found that both photosynthesis and the Hill reaction were sensitive to copper chelating agents. Spencer and Possingham (1960) reported that the Hill reaction in chloroplasts was considerably slowed down in copper-deficient plants. Bishop (1964,1966) found that copper, in contrast to manganese, markedly affects photoreduction and only slightly influences the Hill reaction (Fig. 48).

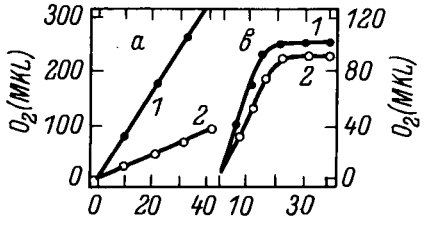


Fig. 48. Effects of copper on the rate of photosynthesis (a), and the rate of the Hill reaction (b), in Scenedesmus obliquus (after Bishop, 1964), 1, normal plants; 2, copper-deficient plants. Abscissa, time (minutes); ordinate, µl of O_2 evolved.

When copper is in deficiency, and in the presence of the herbicide DXMM, photoreduction is lowered by 78%, the rate of photosynthesis is reduced by 65%, while the Hill reaction is only 25% inhibited. Bishop suggests that copper is an essential part of the electron transport mechanism of pigment system I.

Interesting studies of the electron transport chain in photosynthesis have recently been carried out particularly with regard to those components concerned with the photoreduction of NADP in chloroplasts. The important role of ferredoxin has been widely recognized; this is a protein containing nonheam iron which has a low oxidation-reduction potential, and is responsible for the reduction of NADP in intact chloroplast preparations. It is now known that in addition to ferredoxin, a protein of the flavin type is required for the light-induced production of $NADP \cdot H_2$. This protein is ferredoxin-NADP-reductase which catalyzes the transfer of electrons from reduced ferredoxin to NADP. Not long ago a new component of the NADP-reducing system in higher plants and algae, plastocyanin, was isolated from spinach. This is an acidic copper protein of molecular weight (about 21,000 with 2 Cu atoms per molecule), and is bright blue when oxidized, showing a main absorption band at 597 nm and a typical EPR signal at low temperatures (Blumberg et al., 1967). The redox potential of this protein (+0.37 v at pH 7) is close to that of cytochrome f and b-559. The molecular weight of plastocyanin of other plants is 10,500 with 1 atom Cu per molecule with one copper atom per molecule of the protein (Ramshaw et al., 1974).

Plastocyanin was discovered by Katoh and Takamiya (1961) in chloroplasts isolated from spinach and Chlorella. The spectral characteristics of the protein indicate that plastocyanin is rich in tyrosine and phenylalanine, whereas tryptophan is present, if at all, in extremely low amounts (Mutuskin and Pshenova, 1970). In the leaves of some plants, almost one half of the total copper is found associated with this copper protein. The blue colour of plastocyanin is attributable to the Cu^{2+} component which is linked to the protein moiety by four coordinate bonds. Plastocyanin is a participant in oxidation-reduction reactions, but in contrast to true oxyreductases it is incapable of autoreduction, and when it is in the reduced state it does not react with molecular oxygen.

Since the purple bacteria do not contain plastocyanin, it was suggested initially that plastocyanin is involved in the process of oxygen evolution, which does not occur during photosynthesis in purple bacteria. Subsequently, however, it became evident that plastocyanin participates in the release of oxygen not directly, but rather in the reaction yielding a strong photo-reducing agent. Thus plastocyanin (as has been shown in chloroplasts of Scenedesmus obliquus) is an essential component of the electron transport

chain of pigment system I (Bishop, 1964; Fork and Urbach, 1965; Trebst and Elstner, 1965).

Trebst and Elstner (1965) have demonstrated the dependence of NADP reduction on plastocyanin, and their data may be interpreted as indicating that plastocyanin is an obligate electron carrier in photosynthesis. Wessels (1965) found that plastocyanin could restore the photo-reducing activity of $NADP^+$ in digitonin-disrupted spinach chloroplasts. Akulova and Mukhin (1968) also studied the role of plastocyanin in the reduction of NADP by chloroplast fragments from digitonin-treated pea leaves. The results indicated that plastocyanin is involved in the NADP-reducing system, and the introduction of a sufficient amount of plastocyanin as a substrate may affect the position of the saturation point of the light-dependence curve. This finding warrants the suggestion that plastocyanin is involved in the adaptation of the NADP-reducing system of the chloroplast to the illumination conditions.

Plastocyanin has been found to possess the same redox potential as cytochrome f, and to be active in the electron transport chain between pigment systems I and II. Being a component of the electron transport mechanism of system I, it is involved in the synthesis of a strong oxidizing agent. Cytochrome f is an immediate donor for system I(Fork and Urbach, 1965; Trebst and Elstner, 1965).

Kok and Rurainski (1965) have suggested that since plastocyanin and cytochrome f have almost the same redox potential, they may function in the electron transport chain in parallel. Experiments on Chlamydomonas reinhardii mutants, which have neither plastocyanin nor cytochrome 553, have revealed that plastocyanin is located in the electron transport chain after cytochrome 553, the latter being similar to cytochrome f isolated from parsley (Gorman and Levine, 1965).

Elstner et al. (1968) suggested two possible electron donors for pigment system I, plastocyanin and cytochrome f. They visualize the existence of two pigment systems, presumably spatially uncoupled; one is concerned with the cyclic electron transport and contains cytochrome f, while the other is concerned with noncyclic electron transport and contains plastocyanin. The latter has been found to be a better electron acceptor than cytochrome f, as demonstrated in the photo-oxidation of water by pigment system II of sonicated chloroplasts. It may be regarded as an established fact that plastocyanin is a mediator of the light-induced oxidation of

cytochrome, and thereby serves as an electron carrier between cytochromes and system I (Avron and Schneyour, 1971). These findings indicate that copper (in plastocyanin) and iron (in cytochrome f) interact in the process of photosynthesis.

Measurements of light-induced absorption at 597 nm have failed to yield unequivocal evidence of the photo-oxidation of plastocyanin. However, comparatively recent EPR investigations carried out on oxidized plastocyanin at low temperature (25 K) have revealed that the protein is oxidized by system I and reduced by system II (Malkin and Bearden, 1973). Photo-reduction of plastocyanin system II is inhibited by 1-dimethylurea (DCMU). Various treatments which inactivate plastocyanin by complexing with the Cu^{2+} (e.g. KCN or $HgCl_2$), which separate plastocyanin from membranes (e.g. sonication, detergent or polyene antibiotics), or which form complexes with the protein (e.g. polylysine, histones), generally inhibit the photo-oxidation of cytochrome f in the same manner as they inhibit electron flow from $NADP^+$ from water and from artificial electron donors, which feed system I. A Chlamydomonas mutant devoid of plastocyanin has been found which behaves like treated chloroplasts (Gorman and Levine, 1965).

Treatment of chloroplasts with KCN was shown to inhibit system I (Berg and Krogmann, 1975). After this treatment together with sonication, both control and KCN-treated chloroplasts could be stimulated by purified plastocyanin, with the initial activity of system I restored. This finding suggests that plastocyanin may be a site of action of KCN, which was shown to remove copper from chloroplasts.

The amount of copper removed from chloroplasts by KCN was almost equal to that usually found in plastocyanin. In experiments with purified plastocyanin, KCN was shown to remove the copper completely, leaving an apoprotein. This apoplastocyanin has an N-ethylmaleimide (NEM) group, a reactive sulfhydryl group not present in holoplastocyanin. Since plastocyanin has only a single cysteine residue and since the cysteine sulfhydryl is only NEM reactive in the apoplastocyanin, it is tempting to speculate that either the copper blocks access to the free sulfhydryl or that the sulfhydryl is one of the ligands of the copper. Of the three proteins mentioned, plastocyanin is the most firmly bound within the chloroplasts: even hypotonic disruption of intact plastids releases only a minor portion of the plastocyanin content.

Polyene antibiotics, which interact with membrane steroids, have been shown to damage the electron transport chain in chloroplasts (Mutushin et al., 1977; Makovkina et al., 1978). This injury can be relieved by the addition of plastocyanin, which is supposed to be incorporated into the chloroplast membrane where it is surrounded by steroids.

Although these observations point to the role played by plastocyanin in photosynthesis, the details of the involvement of copper in this process have yet to be explained. Its participation can hardly be limited to the functions of plastocyanin. Evidence has been offered showing that in Anabaena variabilis, the copper in plastocyanin is not involved in the inhibition of the Hill reaction by copper chelating agents, and it may be that some other link is sensitive to the effect of the copper chelating agents. This again brings us to the positive involvement of copper in the mechanism of oxygen evolution in photosynthesis (Lightbody and Krogmann, 1967).

The electron transport pathway in chloroplasts is an extensively studied subject. The sequence of individual electron carriers, and their location in the photosynthesizing membrane are as yet unknown.

The involvement of plastocyanin and cytochrome f in the transport of electrons from system II (where they arise from the oxidation of water) to the reaction centre of system I, is known. The problem of their relative disposition, of their proximity to the reaction centre of system I, remains to be solved.

A scheme of photosynthetic electron transport and photophosphorylation in chloroplasts (Fig.49; after Boardman, 1975) is offered, where the place of plastocyanin action is shown; the place of action of other trace elements (Mn, Fe, Cl) is also given.

Interesting observations have been communicated by Yuferova et al., (1969) concerning the copper content of the polar lipid fraction of chloroplasts. The involvement of these compounds in the oxidation-reduction reactions of photosynthesis is beyond doubt, especially since the role of lipids in the electron transport system of photosynthesis is well established. The participation of copper in photosynthetic reactions may also have some connection with its being a constituent of cytochrome oxidase.

It is known that spinach leaf ribulose-diphospate-carboxylase incorporates firmly bound copper (Wishnick et al., 1970), and it

has been suggested that the copper is involved in maintaining the conformation of this enzyme (Wishnick and Mildvan, 1969). Thus it seems that the role of copper in photosynthesis is not only that of a plastocyanin component, but also that of a component of ribulose-diphosphate carboxylase.

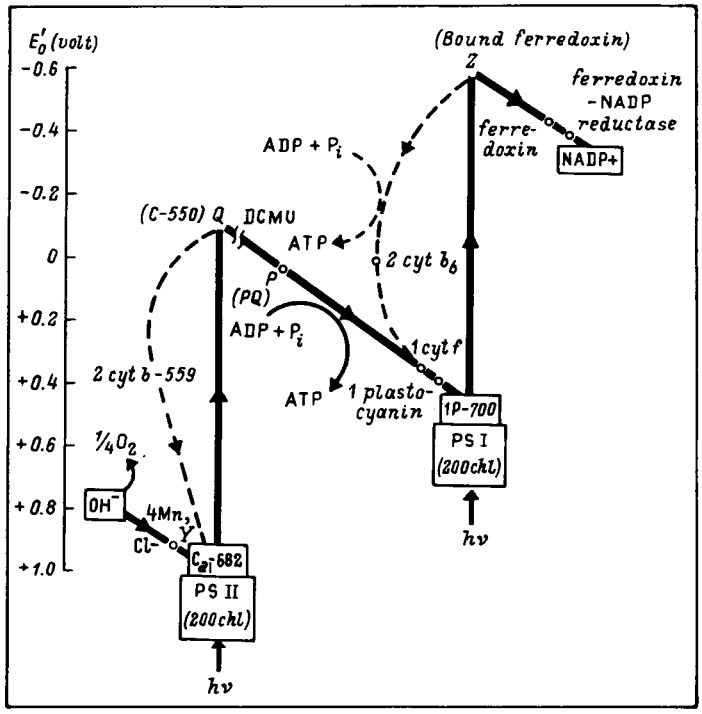


Fig. 49. Scheme for photosynthetic electron transport and phosphorylation in chloroplasts (after Boardman, 1975). Note the position of plastocyanin in the electron transport chain.

Subchloroplast particles, largely composed of components of system II, have been isolated and shown to be able to release O_2 in the light. Pherulanid and silicium tungstate served as electron acceptors; tris-treatment of chloroplasts also inhibited the Hill reaction. After incubation with a Cu^{2+}-d-albumin complex, electron transfer from water to silicium tungstate was restored. It may be concluded from this that copper is an integral component of the photo-decomposition system of potato chloroplasts.

Under conditions of copper shortage, a striking decrease in chlorophyll content and in the rate of synthesis of all phenyl

lipids of the thylakoids has been observed. Plastoquinone formation was much more strongly inhibited than the formation of phytol derivatives and carotenoids. The rate of photosynthesis is depressed by copper deficiency, system I and the process of cyclic phosphorylation being markedly inhibited. The inhibition of system II is less pronounced. The plastocyanin content and the total copper content of chloroplasts decreased to less than a half of the corresponding control values (Baszynski et al., 1978).

Ozolinya et al. (1975) found that a decrease in the total copper content of the leaves of plants sensitive to copper deficiency (wheat, barley) strongly affects the concentration of this trace element in the soluble low molecular weight proteins. In less sensitive plants such as clover and spinach, it is the high molecular weight protein fraction that is sensitive to the copper level in the plant. The dark reactions of photosynthesis are inhibited in the latter type of plant by copper deficiency, whereas in sensitive plants the light-dependent photosynthetic reactions are also inhibited by a copper shortage. It is suggested that the reason for differing sensitivities of plants to copper deficiency lies in the different stabilities of the complexes of copper and low molecular weight proteins involved in photosynthetic electron transport.

The above observations have provided some explanation for the earlier observed increase in the intensity of photosynthesis in some plants produced by copper (Ostrovskaya et al., 1959; Shkolnik and Greshishcheva, 1959).

Evidence is also available regarding the positive effect of copper on the translocation of assimilates (Shkolnik and Abdurashitov, 1958; Shkolnik and Greshishcheva, 1959), and its positive influence on the activity of some enzymes of phosphocarbohydrate metabolism (Shaltenene, 1965).

Some reports suggest that the rate of respiration is increased by the administration of copper (Maslyanaya, 1954; Shkolnik and Abdurashitov, 1958); however, contradictory findings have also been communicated (Nason et al., 1952). Ostrovskaya (1961) found that the influence of copper deficiency on respiration varied among different plants. The respiration rate was generally found to be reduced in copper-deficient sugar beet and oats, whereas in kok-sagyz it was increased. In experiments with maize, we detected an increase in the respiration rate as a result of adding copper (Shkolnik and Abdurashitov, 1958).

There are no indications so far that copper influences energy metabolism in plants, although there is evidence that it influences membrane-bound ($Na^+ + K^+$)-dependent ATPases and the swelling of mitochondria (Peters et al., 1965).

The copper stimulation of ATP-ase was demonstrated by Pilet et al. (1978). Simultaneous additions of manganese considerably enhanced the effect of copper.

Copper plays an important role in nitrogen metabolism, being involved in the reduction of nitrates. Medina and Nicholas (1957) found that copper increased the activity of nitrate reductase.

Peive et al. (1964, 1966, 1967) have published a number of papers in which they seek to demonstrate that copper (along with molybdenum and cobalt) is responsible for increasing nitrate reductase activity in nodules. This enzyme mediates the process of nitrate reduction, and probably is involved in the assimilation of molecular nitrogen. These investigators also noticed that copper raised the activity of dehydrogenase in the nodules of legumes. It is this type of enzyme that provides for a continuous flux of protons and electrons in the reduction of molecular nitrogen. Moreover, copper influences the content of ferredoxin, a protein occupying an important position in the electron transport system of nitrogen fixation.

The positive influence of copper on the fixation of molecular nitrogen had been established earlier by Gribanov (1954).

The introduction of copper to copper-deficient peat soils resulted in an increase in the activity of malate- and succinate triphenyltetrazolium reductases in lupin nodule homogenates (Zhiznevskaya et al., 1969). With aging of the nodules, the activity of these enzymes decreased although the presence of copper helped to maintain a generally higher activity level. The fact that the malate- and succinate tetrazolium reductase activity in the nodules is attributable mainly to the bacteroids and not to the plant tissues, suggested that the supply of copper activated the respiratory chain of the bacteroids at the position of haemoprotein P-450, which has copper at its active site.

Zhiznevskaya et al. (1967) showed that copper exerts a stabilizing effect on the level of haemoglobin in the nodules, and Zhiznevskaya and Borodenko (1969) believe that copper plays a part in the biosynthesis of haemoglobin in the nodules by regulating iron uptake and increasing the proportion of haem iron.

Zhiznevskaya (1972) studied the effects of copper and molybdenum on the amino acid pools in legume nodules, with special reference to nitrate nutrition. She was the first to investigate the effects of these trace elements on the amino acid content of nodules, and to observe a maximum concentration of amino acids during flower bud formation in the plants, i.e., at the moment of the most intensive nitrogen fixation. Zhiznevskaya concluded that copper and molybdenum significantly affect the amino acid content, especially the content of the essential amino acids. This pointed to the possibility that these trace elements were operating in the initial stages of the synthesis of amino acids from ammonium nitrogen. The latter and the other possible intermediate, hydroxyl amine, are products of molecular nitrogen fixation, obtained via 1-ketodicarbonic acid.

Evidence has been found that amides accumulate under copper deficiency, indicating some disturbance in protein synthesis. Using the stable isotope ^{15}N, Ostrovskaya and Geller (1955) found that copper is an essential factor for the normal assimilation of mineral nitrogen, and for protein synthesis in plants. These investigators detected differences in the assimilation rates of nitrogen of different degrees of oxidation - ammonium and nitrates. Copper deficiency in plants provided with ammonium nitrogen resulted in a notable suppression of protein synthesis.

The investigations carried out by Ozolinya and Lapinya (1965) on the role of copper in the nitrogen nutrition of plants showed that the copper requirement of plants is strongly dependent upon the level and type of nitrogen nutrition available. Having noted that the nucleic acid content decreases under copper deficiency, the authors deduced that the effect of copper on the assimilation of nitrogen is connected with its involvement in nucleic acid metabolism. In a later investigation, Ozolinya and Livdane (1970) found that a change in the composition of the soluble proteins in barley seed endosperm tissue could be produced by copper, which led them to conclude that copper plays a role in the control of protein biosynthesis in maturating seeds.

The role of copper in nucleic acid metabolism is of considerable interest, especially because of the influence it has on the structure and function of nucleic acids; this influence can apparently be accounted for by the strong complexing capacity of copper (Frieden and Alles, 1958). These authors studied the ability

of copper to associate with nucleic acids and their precursors, by estimating the decrease in the rate of ascorbate oxidation catalyzed by Cu^{2+}. They detected a surprisingly high affinity of nucleic acids and some nucleic acid precursors for copper ions. The complexing strength of nucleotides exceeded that of the majority of amino acids. Such interactions, according to these investigators, are related to the biological role of RNA and DNA.

In experiments with wheat seedlings, Okuntsov et al. (1966) showed that the biosynthesis of adenine, adenosine and adenosine monophosphate was in each case enhanced by copper ions. The rate of synthesis of adenine was increased most, and was 2.2 times higher than the rate in control plants. The contents of ATP and ADP was not affected by copper.

Copper plays a role in auxin metabolism. Erkama (1950), Virtanen (1953) and Ostrovskaya (1956) reported a direct dependence of germination vigour in cereal and legume seeds on copper content. Ostrovskaya (1956) found that a copper deficiency could be alleviated by treating the seeds with β-indoleacetic acid, which significantly enhanced germination of the copper-deficient seeds. No such effect could be observed in seeds showing normal copper levels on treatment with copper salt solutions. A comparison of these findings with reports of the acceleration of germination in seeds having a high auxin content led Gamayunova (1965) to infer that copper is concerned in the metabolism of growth substances. Other investigators (Vanyugina, 1959) reported a positive correlation between the indoleacetic acid content of plant tissues and the activity of a copper enzyme, ascorbinate oxidase.

Gamayunova (1965) found that the tryptophan content was greatest in copper-enriched seeds during their germination. From this, she deduced that one of the functions of copper in seed germination was to play a part in processes leading to an enhancement of tryptophan synthesis, tryptophan being a precursor of indoleacetic acid. Vonsavichene et al. (1978) found that copper deficiency is associated with a marked depression in levels of free IAA in all the tissues of the axial organs, and subsequently substantiated the role of copper in IAA biosynthesis.

At the same time, iron and copper have been shown to promote the oxidation of indoleacetic acid by hydrogen peroxide (Turian, 1957). Copper ions and copper complexes alike may also catalyze the oxidation of this acid by atmospheric oxygen. Since indolea-

cetic acid and products of its degradation can also bind copper, Turian inferred that there is an autocatalytic mode of oxidation in the case of copper.

The indoleacetic acid oxidase of barley roots was stimulated when the copper concentration reached 1000 µg per g of dry residue, while higher concentrations of copper had an inhibitory effect (Coombs et al., 1976).

For the biosynthesis of ethylene from methionine, a copper-containing enzyme and O_2 are essential. Ethylene rapidly inhibits lateral auxin transport, the inhibition being reversible. Ethylene also inhibits tropic reactions in plants (Burg, 1973).

Briggs and Ray (1956) found that a copper enzyme, polyphenol oxidase, behaves in a similar way to the iron enzymes peroxidase and catalase, in that in inactivates indoleacetic acid oxidatively with the addition of catechin or pyrogallol. Fujiwara and Tsutsumi (1954) reported a considerable decrease in the activity of auxin oxidase in copper- and molybdenum-deficient barley. The activity could be restored by supplying copper, but not molybdenum. Shortages of zinc, manganese and boron did not affect the activity of this enzyme, a finding which led the investigators to suggest that auxin oxidase is a copper enzyme.

Plants of the genus Brassica are known to have a diversity of indole compounds. Glucobrassicin which has been detected in these plants is a precursor of the growth stimulators indolescetonitrile and indoleacetic acid. Mikhalovsky and Sedlak (1972) found that copper ions influence the conversion of glucobrassicin into indoleacetonitrile. This effect of copper is a very specific one. Ions of other metals, namely, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Hg^{2+}, Cd^{2+} and Zn^{2+} have been found to increase the level of auxin only slightly.

In wheat coleoptiles and dwarf pea seedlings, it has been demonstrated that indoleacetic and gibberellic acids applied in growth--stimulating concentrations show antagonism to metal ions the strongest antagonism being observed between gibberellic acid and copper (Ivanova and Bakardjieva, 1968).

Growth-promoting substances have been shown to produce intra--complex compounds with metals. Intra-complex compounds of 2,4--dimethyl, 2-methyl-4-chloro- and 2,4-dichlorophenol oxyacetic acide together with copper have already been studied (Armarego et al., 1959).

It is important to be aware of the role of copper in phenol metabolism, and to understand the effect of copper on the concentrations of various polyphenols which include both inhibitors and stimulators of plant growth. Copper is known to form complexes with various phenolic substances. Yuferova and Udelnova (1971) found in water-soluble leaf extracts a compound of copper and phenol containing 18.1% copper.

Copper is frequently found complexed with antocyans; in fact this metal plays an important part in the biosynthesis of antocyan (Edmonson and Thimann, 1950). Thimann and Radner (1955) succeeded in inhibiting the synthesis of antocyan by supplying 2-thioracil. Interestingly, this inhibition could be reversed not only by thymine, uracil and hypoxanthine, but also by copper. It cannot be ruled out that this phenomenon is, to some degree, related to the known positive effect of copper on the biosynthesis of adenine (Okuntsov et al., 1966). Grinkevich et al. (1970) reported that copper raised the concentration of various flavonoid compounds, especially antocyans, in buckwheat plants. These workers also found a close correlation between the accumulation of copper and the total flavonoid content.

Fraig (1972-1973), discussing in his review the biosynthetic pathways and metabolism of the principal mono- and polyphenols and the enzymatic systems involved in their methylation, glycosylation and oxidative decarboxylation, emphasizes that copper and iron are constituents of those enzymes that regulate amounts of phenols on account of being involved in phenol synthesis.

Several investigators have reported a positive influence of copper on resistance to wilting; this effect is apparently related to the effects of copper on the phenolic inhibitor content. A decrease in these inhibitors results in excessive elongation of the stem and greater susceptibility to wilting (Prusakova, 1966). It is known that under conditions of copper deficiency, for example on peat-bog soils, disturbances in plant development, particularly in the formation of reproductive organs, can frequently be observed.

Polukhina and Maslyanaya (1962) studied the effect of copper on the development of ovaries and karyopsis in cultured gasses. They showed that copper deficiency impairs development of grain, and as a result weak seeds are produced. Anatomical studies of grain, performed at the time of formation, have revealed that copper deficiency affects ovaries with mature embryo sacs, bringing about

degeneration of the mature embryo sac; this in turn results in the formation of a weak seed. Livdane et al. (1970) observed morphological alterations in the male generative organs and extensive degeneration of the female generative organs during the flowering of plants severely affected by copper deficiency.

Copper has been shown to enhance the synthesis of β-indoleacetic acid from tryptophan (Vonsavichene and Shaltenene, 1975). Rooted seedlings growing in copper solutions or containing copper in very small amounts showed chlorosis and then necrosis of the leaves. Initiation and development of the flower buds in such plants was retarded or repressed entirely. The critical level of copper content in the culture solution was 0.0005 mg per litre and at lower concentrations the development of the buds was considerably delayed. Upitis (1975) showed that a shortage of copper is responsible for depression of the formation of spores in fungi and bacteria.

Hillman (1962) reported that the short-day Lemna perpusilla 6746 and long-day Lemna gibba G_3 grown on Hoagland solution showed normal photoperiods. With an addition of 2 mg per litre copper, the short-day Lemna perpusilla becomes neutral, whereas the long-day Lemna gibba ceases to flower. A similar, but less pronounced effect has been reported for mercury. Cobalt, chromium, manganese, nickel, lead and zinc failed to produce similar effects, but some of them tended to modify slightly the effect of copper. This influence of copper was interpreted as being caused by a reduction in photoperiodic sensitivity, involving phytochrome.

Clarification of the role of copper in the structural organization of the cell, in particular the effects of copper on intracellular membrane structure would be of considerable interest. Gallacher et al. (1956) showed in animal material that phospholipid synthesis was notably depressed by copper deficiency. It would be interesting to see whether the same holds true for plants. It is noteworthy that acetyl-coenzyme A, like copper, plays an important role in the biosynthesis of antocyan and serves as a common link relating antocyan biosynthesis to the metabolism of lipids.

Sharafutdinova et al. (1975) studied the dependence of the fatty acid composition of chloroplast and cotton seed lipids on copper content. Cotton leaf chloroplasts were shown to contain high levels of unsaturated fatty acids (oleic, linolenic and linoleic). Copper was found to increase the total amount of unsaturated fatty acids in chloroplasts.

In the author's investigations (Shkolnik, 1939b, 1955; Shkolnik and Makarova, 1958), as well as in others (Okuntsov and Levtsova, 1952; Sviderskaya, 1959), the positive effects of copper on drought-, frost-, and heat resistance of plants and on the underlying processes have been demonstrated. A more detailed discussion of relevant findings will be presented in the final chapters of this book.

The various physiological functions of copper in plants are intimately associated with interactions that take place between copper and other mineral nutrients. We have found an antagonism between iron and copper (Shkolnik and Makarova, 1950), and in our experiments with flax, we were able to eliminate the toxic effects of copper by supplying iron to the plants. These findings were later confirmed by other investigators who have shown that the uptake of iron by plants decreases when high doses of copper are given (Lingle et al., 1963). Consequently, shortages and excesses of copper alike interfere with the uptake of iron.

The pattern of interaction between copper and iron is governed by their molar proportions and the pH of the medium (Gamayunova and Ostrovskaya, 1964). As shown by Ostrovskaya et al. (1966), antagonism between iron and copper appears in an alkaline environment, and may be absent in a slightly acidic medium. Ovcharenko (1965) has shown the importance of maintaining the appropriate iron and copper ratio in the tissues for maximising the activity of some oxidative enzymes. She also demonstrated that copper and iron enzymes, as well as related systems, may partially substitute for one another when either element is in short supply. Copper is presumed to play a prominent role in the oxidation of iron in plants. Erkama (1950) arrived at the conclusion that copper, by oxidizing iron in plant tissues, is converted into an insoluble form.

Interesting findings regarding the interaction between copper and iron in the metabolism of root nodules were obtained by Zhiznevskaya (1972). She arrived at this conclusion from observation (reported in the literature) of a relationship between copper and haematin coenzymes at the active sites of a number of oxidative enzymes, (cytochrome oxidase, tryptophan pyrrolase, hemoprotein R-450), and from her own findings on the effects of copper on the activity of the terminal copper- and iron-containing portion of the respiratory chain in nodules. One aspect of the activation of nitrogen metabolism in legume plants in the presence of copper is

the effect this element has on the metabolism of iron, and on the functioning of iron proteins and iron-copper proteins. Special investigations have shown that uptake of iron is increased with increasing copper uptake by the plant. Supplying copper to the soil tends to bring about an increase in the concentration of both haemin and nonhaem iron in the nodules of broad beans during the formation phase of the green beans. Copper also precludes a decrease in the iron content of the nodules when increased doses of lime are supplied to the soil.

Zhiznevskaya (1972) revealed an interaction between iron and calcium in the nutrition of leguminous plants. In her experiments, supplying copper resulted in an increase in the amount of calcium in lupin plants when these were grown in acidic soils having practically no labile copper. In liming, an addition of copper somewhat diminished the calcium content of lupins, thus preventing any excessive accumulation of calcium. According to Zhiznevskaya, copper acts in this way as a regulator of the calcium level in leguminous plants. There is also evidence for an interaction between copper and molybdenum.

The problem of the interaction between copper and phosphorus is also of interest. High doses of phosphorus may produce both zinc and copper deficiencies, as shown in experiments with lemons (Bingham and Martin, 1955). Taking into account all the above data, Schütte (1964) suggested that there are grounds for speaking of an antagonism between phosphorus and zinc and between phosphorus and copper.

To recapitulate, it may be said that the principal function of copper in plants is its participation in oxidation-reduction processes. Together with manganese, copper, as a constituent of antocyan, plays an important part in photosynthesis, it also plays a prominent role in the metabolism of phenols, nitrogen, nucleic acids and auxin. Copper also plays a part in the fixation of molecular nitrogen.

Chapter 5

MOLYBDENUM

The requirement of plants for molybdenum is considerably lower than that for other trace elements. This becomes fully evident from calculations which show that for the normal growth and development of one cell of, for example, Scenedesmus obliquus, as few as 10 atoms of molybdenum are sufficient (Arnon, 1954).

The essentiality of molybdenum has been demonstrated in both higher and lower plants, as well as in microorganisms. The highest requirements for molybdenum have been shown in plants of the Leguminosae family (Fig.50) and the genus Brassica (Anderson, 1942; Hewith and Jones, 1947) (Fig.51).

Shortages of molybdenum are most frequently observed in plants growing on acidic soils, where this element is found in a largely immobile state (Yakovleva and Skvortsov, 1956; Burkin, 1968).

Molybdenum fertilizers are successfully used on acid soils on which legumes crops - peas, lupins, soybeans, clover and alfalfa - (Fig.50) are cultivated. The molybdenum fertilizers are administered either by spraying the seeds or by applying it to the aboveground plant organs (Yakovleva and Skvortsov, 1956; Burkin , 1964). These fertilizers are also used on pastures to increase the protein content and yield of the crop, particularly of the grass species (Skripchenko, 1950; Koryakina, 1963). In Florida, molybdenum is used for treating citrus groves to combat yellow spot disease.

The highest molybdenum concentration are found in leguminous plants (Vinogradova, 1943). In these the lowest level of molybdenum is found in the root, the nodules showing large concentrations. (Vinogradova, 1943). Most of the molybdenum is located between the leaf veins, the conductive tissues containing less molybdenum. Molybdenum accumulates in the reproductive organs (Dautova, 1971), and this is probably a reflection of the specific role of molybdenum in the fertilization and development of the embryo.

With administration of molybdenum to the roots, its concentration in the aboveground plant organs is found to increase by an order of magnitude, as has been shown in our experiments with clover (Shkolnik and Bozhenko, 1956).

Potatuyeva (1968) has reported that molybdenum occurs in the

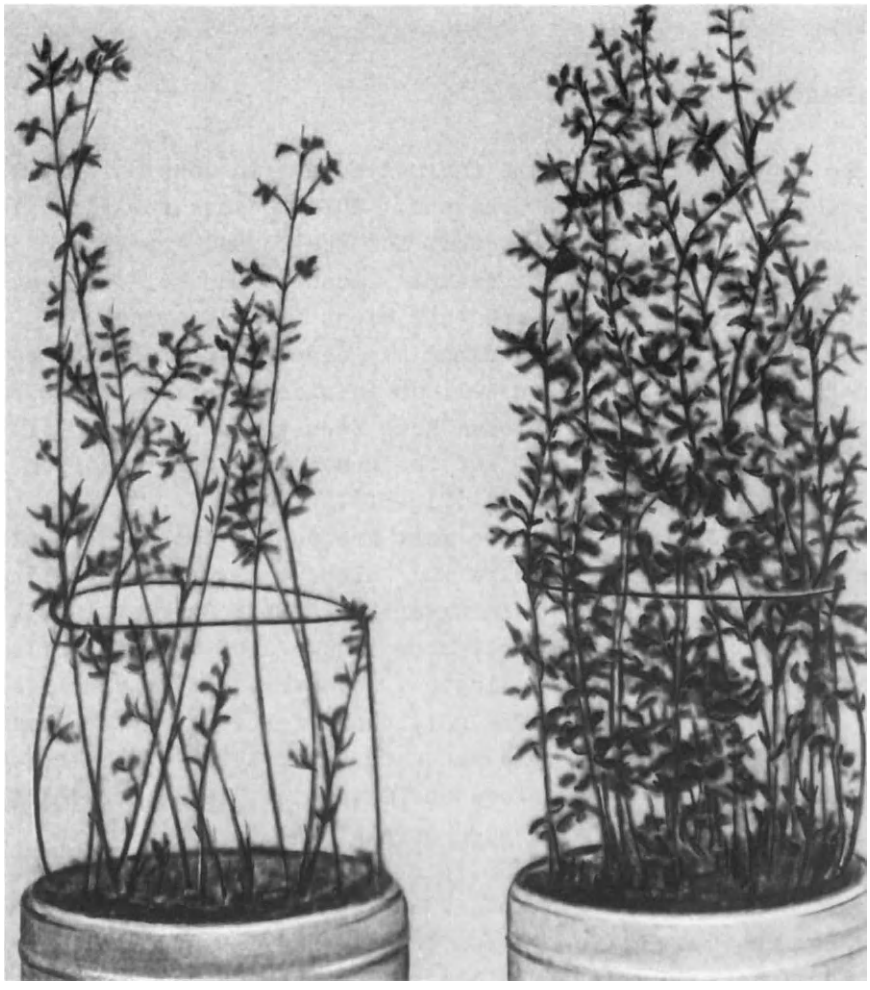


Fig. 50. Effect of molybdenum on the development of Medicago sativa (after Borys and Childers, 1964). 1, without Mo; 2, with Mo.

chloroplasts. By omitting iron from the nutrient solution, the molybdenum content of the nucleus increased and that of the chloroplasts decreased.

In many plants the initial symptoms of molybdenum deficiency appear as yellow-green or pale-orange interveinal spots.

A molybdenum deficiency eventually results in a diminished number of flowers on the plant. In deficient tomatoes, the flowers are very small and lose their capacity for opening.

Severe disturbances in the formation of the reproductive or-

gans, and especially in the development of pollen, have been found when molybdenum is deficient (Agarwala et al., 1979).

In plants of the genus Brassica, there is another symptom of molybdenum deficiency, namely, the whiptail leaf symptom (Fig.51).

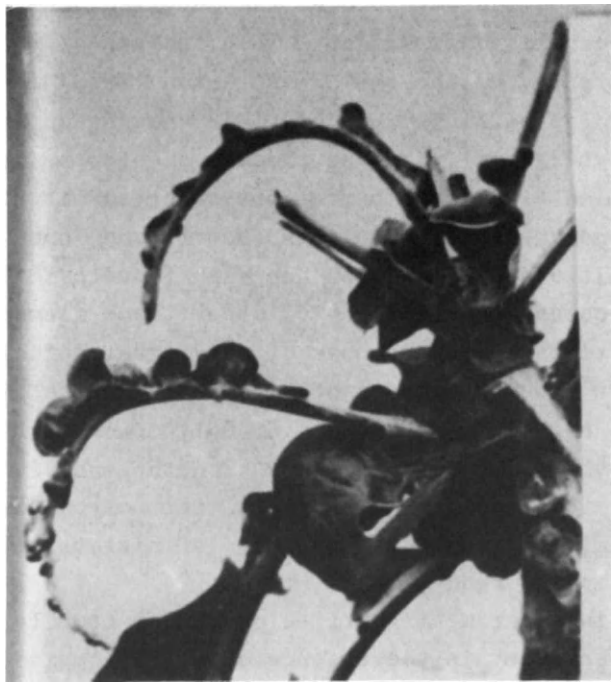


Fig. 51. Teratological alterations (whiptail) in cauliflower leaves produced by molybdenum deficiency (after Hewitt and Jones, cit. by Shütte, 1954).

In molybdenum-deficient cauliflower plants, the inflorescences become deformed and diffuse. The leaf laminae either do not develop or grow as distorted thickened structures.

The most important aspect of the physiological role of molybdenum is its involvement in nitrogen metabolism, particularly in the reduction of nitrates and the fixation of molecular nitrogen.

Molybdenum-containing enzymes are numerous. Commonly found in living organisms are nitrogenase, nitrate reductase, xanthinoxidase, xanthindehydrogenase, aldehyde oxidase, sulphite oxidase, and formic dehydrogenase, which belong to the important group of redox enzymes catalizing the reduction of free nitrogen, nitrates and formate, and the oxidation of purines, aldehydes, sulphites and other metabolites.

Steinberg (1937) was the first to recognize the significance of molybdenum in the reduction of nitrates, In a study on the effect of molybdenum on the growth of Aspergillus niger, he observed an enhancement of this organism's growth in the presence of molybdenum, provided that the medium contained nitrates as the sole nitrogen source. If ammonium nitrogen was supplied, the effect of molybdenum was usually only slight. The findings of Steinberg were later corroborated by Mulder (1948) and Arnon (1958) in tomatoes, Azotobacter and Aspergillus. Mulder (1948) suggested that molybdenum may act catalytically on the process of nitrate reduction. This view may also find support from the observed nitrate accumulation in molybdenum-deficient plants (Hewitt and Jones, 1947). Although such nitrate accumulation has been found by Steinberg et al., (1955) in cases of deficiency of other trace elements, it is particularly noticeable in the case of molybdenum deficiency.

Numerous data have since been obtained showing that in higher plants there is a positive influence of molybdenum in the presence of ammonium as the source of nitrogen. Nonetheless, it should be recognized that the requirement of plants for molybdenum is usually much more strongly manifested in the case of nitrate nitrogen nutrition (Hewitt and McCready, 1956).

Much later, in experiments carried out under sterile conditions, it became apparent that in the absence of nitrification the positive influence of molybdenum could only be observed against a nitrate nitrogen background (Hewitt and Gundry, 1970).

The physiological role of molybdenum largely centres on its being a constituent of a number of enzymes. This element is found in at least five enzymes: nitrogenase, nitrate reductase, xanthine oxidase, aldehyde oxidase and sulphite oxidase, which catalyze different unrelated reactions.

Nicholas (1975) indicates that these are complex enzymes, each containing additional non-protein groups acting as redox-carriers. Thus nitrogenase contains Fe-S-proteins in addition to Mo-Fe-S-proteins. Nitrogenase is a molybdoflavoprotein; xanthine and aldehyde oxidases contain flavin and Fe-S-proteins, whereas sulphite oxidase has a haem component in addition to Mo. The enzymes which have two substrates, an oxidant and a reductant, facilitate low-energy electron flow from the reducing agents to the oxidants. In each case molybdenum interacts directly with the oxidant.

The function of molybdenum in the reduction of nitrates was fully recognized when the element was shown to be a constituent of nitrate reductase (1.6.62) isolated from Neurospora crassa and soybean. The credit for this discovery goes to Nason, Evans and Nicholas (Nason and Evans, 1953; Nicholas et al., 1953).

Nitrate reductase contains flavine adenine dinucleotide (FAD) or flavine mononucleotide (FMN) as its prosthetic group, and molybdenum is involved in the reduction of nitrates to nitrites. Reduced nicotinamide-adenine dinucleotide (NAD·H) or reduced nicotinamide adenine dinucleotide phosphate (NADP·H) serve as the donors of electrons and protons.

Nicholas and Stevens (1955) have found that in the nitrate reductase system, the reduction of nitrates is executed on account of the presence of Mo^{5+}; the pentavalent molybdenum serves as an electron acceptor, and is converted into the hexavalent ion. The transfer of electrons occurs, presumably, according to the widely accepted scheme depicted in Fig.52. It was shown that Mo^{4+} may be converted into Mo^{6+} (Stiefeld, 1973).

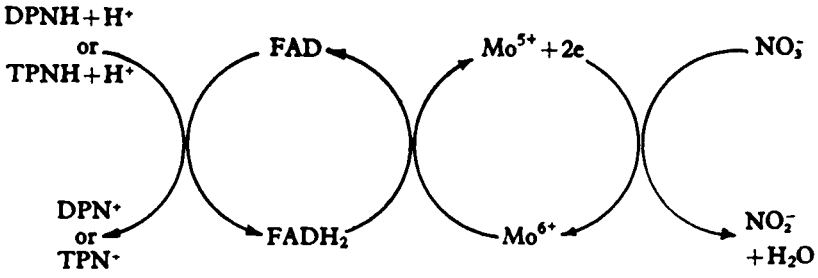


Fig. 52. Electron transport chain of the nitrate reductase system (after Nicholas and Stevens, 1955).

Nitrate reductase was considered to be an enzyme that was adaptable to nitrates (Candela et al., 1957), until it was conclusively shown that nitrate induces the synthesis of the enzyme de novo (Zielke and Filner, 1971).

Recently Maldonado et al. (1980) discovered that partly purified nitrate reductase isolated from spinach leaves and inactivated by incubation with NAD-H_2 and cyanide, was reactivated by Mn^{3} pyrophosphate or Mn^{3+} ions, formed by the oxidation of Mn^{2+} by illuminated chloroplasts.

Interesting findings have been obtained concerning the nitrate--reducing system in algae. It has been shown that nitrate reductase in Chlorella (1.6.6.1-2) is an intricate enzyme complex having a molecular weight of 500,000. The enzyme consists of the FAD-dependent diaphorase (Zumft et al., 1970) and the terminal nitrate reductase itself, which contains molybdenum (Aparicio et al., 1971; Vega et al., 1971).

Consequently, two enzymes are involved in the transport of electrons from NAD·H to nitrates, operating consecutively. These enzymes may be studied independently without physically separating them; the first is NAD·H disphorase and the second is the nitrate reductase itself, which is capable of utilizing reduced flavin nucleotides as donors of electrons. Using ^{99}Mo and ^{59}Fe, Aparicio et al. (1971) have shown that , in addition to molybdenum, iron is also a constituent of nitrate reductase.

Interesting observations have been reported by Zumft et al. (1971) indicating that the enzyme contains iron and that there may be electron-donor specificity in the enzyme from Ankistrodesmus. In experiments with ^{55}Fe, evidence has been obtained to indicate that iron is present in highly purified nitrate reductase preparations from this alga. No definitive conclusions can as yet be drawn as to the nature and functional role of iron in this enzyme. Nitrate reductase responds to a multitude of artificial donors and acceptors of electrons, the mechanisms of action of which still remain unclear. The end product of the first step of reduction, during which two electrons are transferred, is nitrite. The latter is reduced by nitrite reductase, which contains two atoms of iron, at the stage of the transfer of six electrons to ammonium. The electron donor at this stage of the reduction is either ferredoxin or flavodoxin, recently detected in Chlorella and Ankistrodesmus. Suggested is a scheme for the enzymatic reduction of nitrates in green algae (Fig.53).

Non-haem iron or cytochromes act as additional electron carriers in the chain of electron transfer to nitrate (Garrett and Nason, 1969). Ivanova (1975) found that a haem-containing enzyme (peroxidase), under physiological conditions and in the presence of diethylthiocarbamate and sodium sulphite, is also capable of catalyzing the reduction of nitrates to nitrites with high efficiency (up to 700 mM of nitrate per 1 mg protein per minute).

Ivanova (1959) has shown that when nitrate reductase

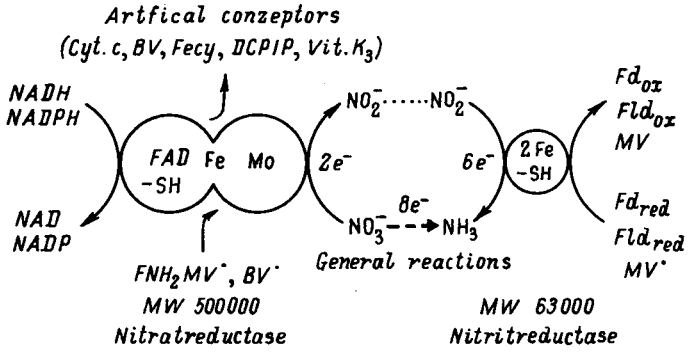


Fig. 53. Scheme for nitrate reduction in green algae (after Zumft et al., 1971). Abbreviations: Cyt.c - cytochrome c; BV,MV - benzylviologen, methylviologen and their reduced forms; Fecy - ferricyanide, DCPIP - dichlorophenolindophenol; Vit.K3 - vitamin K3; FNH_2 - reduced flavinnucleotide; Fd - ferredoxin; Fld - flavodoxin; SH - sulfhydryl groups involved in the reaction.

activity is suppressed by introducing tundsten into the nutrient medium, the nitrate-reducing peroxidase system is activated.

As well as the assimilating nitrate-reducing system which is typical of higher plants and some fungi (Neurospora crassa, Aspergillus niger, etc.), there is another, dissimilating nitrate-reducing system found only in bacteria and a few fungi. This system is referred to as the nitrate reductase of nitrate respiration (Nason and Takahashi, 1958), in which nitrates are used under anaerobic or partially aerobic conditions as terminal oxidants in the energy-dependent respiration processes by which microorganisms obtain energy.

Nason and Takahashi (1958) suggested the following scheme to explain the then available evidence concerning nitrate respiration:

$$\text{NAD·H (NADP·H)} \longrightarrow \text{FMN (FAD} \longrightarrow Fe^{3+} \longrightarrow \text{bacterial cytochrome} \longrightarrow \begin{cases} \rightarrow O_2 \\ \rightarrow \text{nitrate reductase} \longrightarrow NO_2 \end{cases}$$

Thus it is seen that at one point in nitrate respiration, the process is coupled to the cytochrome system and to compounds having non-haem iron. During respiration, nitrate reductase carries electrons from reduced cytochrome to nitrite.

A series of studies has shown that molybdenum appreciably enhances the activity of nitrate reductase in plant leaves. The assimilating nitrate reductase in Neurospora crassa is amino acid

stimulated. These phenomena were observed by Ketchem et al. (1977), with NADP-H-dependent nitrate reductase isolated from the mycelium of Neurospora crassa wild type FGS 354. Histidine, tryptophan, glycine, arginine, glutamate, glutamine and alanine stimulated nitrate reductase 25- to 46-fold, EDTA being absent. Citrate also stimulated nitrate reductase. EDTA in low concentrations is also a potent stimulator, so that the stimulating effect of amino acids and citrate was always masked in its presence. Heiner et al. (1969) demonstrated that the activity of nitrate reductase decreases tenfold, and approaches zero as the tungsten concentration in the medium increases from 0 to 20 μM. This is accompanied by an increase in the concentration of nitrates. Molybdenum added 24h after the tungsten is introduced, opposes the inhibiting action of the latter. The finding that another molybdenum enzyme, xanthine dehydrogenase from Pseudomonas aeroginosa, is also inhibited by tungsten in vivo, and that its activity can be restored by molybdenum, testifies to the specificity of the effect of tungsten on molybdenum enzymes.

Molybdenum has not been detected on purified nitrate reductase from some higher plants (wheat, tobacco and spinach). Nitrate reductase has been found in the root nodules of leguminous plants (Evans, 1954).

It is widely accepted that the highest nitrate reductase activity in leaves is found in the chloroplasts and the cytoplasmic fraction. Nitrate reduction occurs in the chloroplasts in the light, but occurs in the cytoplasmic fraction in the dark. Nitrate reductase is found not only in leaves, but also in roots.

The enzyme apparently plays an important physiological role in the nodules. Thus a positive correlation has been established between the nitrogen-fixing capacity of various races of bacteria in root nodules and the activity of nitrate reductase in suspensions of the bacteria (Cheniae and Evans, 1960). A view has been expressed that in actively growing nodules, this enzyme may serve to transfer electrons and protons from reduced compounds (NAD·H and succinate) to molecular nitrogen (Peive et al., 1965). Ivanova (1973) has reviewed the numerous observations made on nitrate reductase.

In the experiments of Daniel and Gray (1976), the nitrate reductase activity of free living Rhizobium japonicum cells depended on the parcial pressure of O_2, but not on the nitrate concentration.

In aerobically and anaerobically grown cells and in bacteroids, nitrate reductase occurred in both the soluble and the particulate fractions.

From a cell free extract prepared from a suspension culture of rice cells, a proteinaceous factor of molecular weight 20,0000 was isolated, which inactivated nitrate reductase, although the NAD-H-diaphorase function was not affected. The activity was reduced by methylviologen. Some authors have demonstrated that this proteinaceous inhibitor is located in the cytoplasm (Venkatramana et al., 1978).

Recently some progress has been made in the identification of the chromosomes carrying the genes which are responsible for controlling the nitrate reductase level.

Sherar et al. (1976) compared the nitrate reductase, nitrite reductase, and acid proteinase activities in the first leaves of wheat seedlings and 21 disomic hybrid-substituted strains. Two chromosomes - 7B and 7D were found to control the level of nitrate reductase. Nitrite reductase is controlled by two major genes residing in chromosomes 4D and 7D, and by two minor genes residing in chromosomes 3D and 5A.

Garret and Cove (1976) studied the synthesis of NADP-H-nitrate reductase in nia D and cnx mutants of Aspergillus nidulans, and revealed that NADP-H-nitrate reductase consists of a polypeptide component, the synthesis of which is controlled by the gene nia D, and a molybdenum-containing component produced cooperative action of the genes cnx.

As well as nitrate reductase, nitrite reductase and hydroxylamine reductase are involved in the process of the reduction of nitrates. Nitrite reductase contains iron and copper (Walker and Nicholas, 1961), and is also stimulated by molybdenum and manganese; hydroxylamine reductase contains manganese.

The nitrite reductase in many microorganisms is a metalloflavoprotein, which uses NAD as a donor of electrons to reduce nitrite. In Escherichia coli, a specific nitrite reductase of an assimilative type has been detected, differing from other assimilative nitrite reductases in that it contains no flavins or metal ions (Lazzarini and Atkinson, 1961).

In summary, it may be concluded that all the enzymes involved in the reduction of nitrates to ammonium are metalloenzymes. The entire pathway of the reduction of nitrates may be depicted as

follows (Medina and Nicholas, 1957):

$$NO_3^- \xrightarrow{Mo} NO_2^- \xrightarrow{Fe,Cu} NO \qquad N_2O_2^- \xrightarrow{Fe,Cu} NH_2OH \xrightarrow{Mn} NH_3$$

Reductases acting on nitric and nitrous oxides have also been found. These are metalloflavoproteins, the activity of which depends on copper and iron.

Inhibition of the primary process of nitrogen assimilation as a result of molybdenum deficiency, should lead to a decrease in the concentration of amino acids. In fact, in contrast to shortages of other trace elements - boron, copper, zinc, manganese - molybdenum deficiency usually brings about a decrease in the content of free amino acids (Hewitt et al., 1949; Possingham, 1957). Hewitt et al. (1949) reported a diminution in the concentrations of alanine, aspartic acid, glutamic acid, proline, arginine, and the amides asparagine and glutamine, in molybdenum-deficient plants. Zhiznevskaya (1972) observed increased contents of asparagine, aspartic acid and glutamic acid in barley, lupin leaves and the nodules of lupins and broad beans when molybdenum was supplied. It should be emphasized that the amino acid levels in molybdenum-deficient plants are strictly dependent on the nitrogen source and the degree of molybdenum deficiency. Steinberg et al. (1955) observed a decrease in the content of amino acids only in those molybdenum-deficient plants which were supplied with nitrate nitrogen. Conversely, when the plants were supplied with nitrites, ammonium salts, urea or glutamic acid, the reverse situation arose, the levels of some amino acids showing a steep increase. Peterburgsky and Mikolov (1970) observed that the tryptophan content of legume seeds tended to increase when molybdenum was supplied.

As early as 1930, Bortels discovered the importance of molybdenum for the fixation of molecular nitrogen in the aerobic bacterium Azotobacter chroococcum. He found that Azotobacter failed to survive and to assimilate molecular nitrogen in a pure culture without molybdenum. These findings were soon confirmed by Birch-Hirschfeld (1932).

The stimulating action of molybdenum on nitrogen fixation was later confirmed by Burk and Horner (1935), who regarded molybdenum as a specific catalyst in this process. Van Niel (1935) reported that the bacteroids in the nodules completely failed to fix nitro-

gen in the absence of molybdenum. In 1936, Bortels proved the essentiality of molybdenum for nitrogen fixation in the anaerobic bacterium Clostridium pasteurianum.

Evidence has also been obtained on the important role of molybdenum in nitrogen fixation in other organisms, including the blue-green algae. The essentiality of molybdenum for the nitrogen fixation has also been proved in the following microorganisms: Azotobacter chroococcum, Azotobacter agile, Azotobacter vinelandii, Azotobacter serogenes, Mycobacterium rocoalbum, Mycobacterium flavum, Rhizobium japonicum found in symbiosis with soy, lupin, broad bean and clover.

Several reviews have been written dealing with the details of biological nitrogen fixation (Virtanen and Miettinnen, 1963; Mishustin and Shilnikova, 1968; Shilov and Lichtenstein, 1971; Bergersen, 1971), and the role of molybdenum in this process (Peive, 1971).

The biochemical mechanism of the fixation of molecular nitrogen are somewhat complicated. The process of biological nitrogen fixation is unique, in that no other example exists of similar activation of the nitrogen molecule at normal temperatures and pressures. The exceptional non-reactivity of the nitrogen molecule is presumably a consequence of the extraordinarily high energy of its triple bond. In biological nitrogen fixation, this high energy barrier is overcome. The most difficult task has been to discover which enzymes carry out the activation of molecular nitrogen, and it has taken a long time to isolate these enzymic catalysts.

Investigations of nitrogen fixation in cell-free systems have shown that the fixation of atmospheric nitrogen is accomplished by the cooperative action of two enzyme systems - the nitrogen-activating and the nitrogen-evolving systems. Mortenson et al. (1962) isolated two fractions from cell-free extracts of Clostridium pasteurianum, one of which contained the hydrogen-evolving system, the other showing nitrogen-activating properties. The former was shown to possess a hydrogenase and a phosphoroclastic action, while the latter exhibited nitrogenase activity. The hydrogen-evolving system contains hydrogenases and components (as well as co-factors) of the phosphoroclastic system, whereas the nitrogen-activating system comprises enzymes (or a group of enzymes) which are specifically involved in the process of nitrogen fixation. The latter process only takes place when the two systems

operate simultaneously.

The hydrogen-evolving system consists of dehydrogenases and hydrogenases which are responsible for the reduction reactions. They create the large reducing pool typical of nitrogen-fixing organisms (Zhiznevskaya, 1972). Evidence is available indicating that molybdenum activates the dehydrogenases both in legume nodules (Peive et al., 1964) and in free-living nitrogen-fixing organisms (Krylova, 1963; Ilyina, 1966). Peive et al. (1967) observed a positive effect of molybdenum on the activity of hydrogenase in legume nodules. Active, nitrogen-fixing nodules display a high level of hydrogenase activity. Several hydrogenases and several isozymes of hydrogenase have been found in nitrogen-fixing microorganisms (Yakovlev and Levchenko, 1964).

In addition to non-haem iron, Mo^{5+} has been detected in the hydrogenase from the nitrogen-fixing cell-free system of Azotobacter vinelandii (Nicholas et al., 1962). This finding suggests that molybdenum might be associated with those systems which are responsible for the transfer of protons to molecules of atmospheric nitrogen.

Elucidation of the mechanism of the primary activation of gaseous nitrogen molecules presents a more complicated problem. In the mid-1960s a major discovery was made which contributed much to our understanding of the mechanism of nitrogen fixation and the functions of molybdenum and iron in this process. Molybdenum and iron were shown to be constituents of nitrogenase, which is nitrogen-activating in function and catalyzes the fixation of molecular nitrogen.

Investigations involving separation of the nitrogenase complex into individual components were undertaken after Carnahan et al. (1960) had succeeded in obtaining active nitrogen-fixing preparations from extracts of dessicated Clostridium pasteurianum cells.

Bulen and Le Compte (1966) were the first to isolate and purify nitrogenase from the aerobic microorganism Azotobacter vinelandii. They found two nitrogen-fixing fractions in the nitrogen-fixing complex obtained from Azotobacter. The first of these had a molecular weight of 120,000-135,000 and contained 16 atoms of non-haem iron and 1-2 molybdenum atoms per molecule of the enzyme. The second fraction (mol. stet wt 40.000) contained 3 atoms of non-haem iron per molecule of the enzyme. The ATP-dependent reduction of molecular nitrogen, the evolution of hydrogen and corresponding release of inorganic phosphate, required the simultaneous

presence of both fractions.

Mortenson (1966) described some of the properties of nitrogenase obtained from the aerobe Clostridium pasteurianum. Mortenson et al. (1967) isolated two components participating in the fixation of molecular nitrogen in Clostridium pasteurianum, and designated these molybdenum ferredoxin and nitrogen ferredoxin. These two components were absent from extracts of cells grown on media containing ammonium. Both enzymes are required for nitrogen fixation.

When the first fractionation of nitrogenase from cell-free extracts of bacteriods was carried out (Klucas et al., 1968), the enzyme was separated into two components, analogous to those isolated from free-living nitrogen-fixing organisms. In one of the components iron and molybdenum were detected, while in the other, only non-haem iron was found. Only upon combining both components does nitrogen fixation proceed. Nitrogenase is capable of reducing not only N_2 and C_2H_2, but also KCN, $NaNO_3$ and NO_2.

In 1970 Bergensen and Turner (1970) fractionated nitrogenase from soy bean nodules and also found two components. Similar findings have been obtained with regard to nitrogenase isolated from bacteroids from lupin root nodules (Peive et al., 1971).

Separation of the nitrogenase complex into two components was also achieved by Shilov and Lichtenstein (1971).

Molybdenum and non-haem iron have been reported in nitrogenases from Azotobacter vinelandii and Clostridium pasteurianum by a number of workers (Lyubimov et al., 1968; Yakovlev and Gvozdev, 1968; Manorik, 1971).

Nitrogenase is a Mo-Fe tetrameric protein with a molecular weight of 220,000-250,000. Its monomers are of two types, differing both in molecular weight and in amino acid content, particularly the content of arginine containing peptides. 1 molecule of nitrogenase contains 2 Mo atoms and 18-36 Fe atoms the latter being associated with labile sulphur in Fe-S-clusters. The Fe-protein is a dimer of two identical sub-units; its molecular weight is 50000-60000 (Kennedy et al., 1976; Swissher et al., 1977).

Mo-Fe-protein from the nitrogenase of Azotobacter vinelandii has been found to inhibit nitrogenase from Klebsiella pasteurianum with respect to all its characteristic reactions. However, the Fe-protein does not affect the Klebsiella pasteurianum nitrogenase. In contrast to this, the Fe-protein, but not the Mo-Fe-protein, of

Klebsiella pasteurianum nitrogenase inhibits the nitrogenase of Azotobacter vinelandii. It was demonstrated that in both cases the inhibition resulted from the appearance of an inactive complex derived from the Mo-Fe-protein of the Azotobacter vinelandii nitrogenase and the Klebsiella pasterianum nitrogenase Fe-protein. The Fe-protein from Klebsiella pasterianum binds with Mo-Fe-proteins from 6 various organisms, forming active or inactive complexes. The heterologous Fe-protein is believed to compete with the homologous Fe-protein for the binding sites of the Mo-Fe-protein (Emerich and Burris, 1976).

According to the currently held view, nitrogenase is a protein complex with a sophisticated quaternary structure (Gvozdev et al., 1971). In 1971 Hardy and his collaborators succeeded for the first time in isolating and describing a crystalline nitrogenase preparation containing molybdenum and iron (Hardy et al., 1971a).

Bahadur and Tripathi (1975) found that in the presence of lanthanum sulphate the rate of fixation of nitrogen by bacteria was doubled. Thus in three different strains of microorganisms the specific rate of fixation (expressed in mg N_2 per g of organic carbon) was increased. The rates of uptake of nitrogen and oxygen was simultaneously raised.

The macromolecular structures of nitrogenase and its components obtained from various sources are strikingly similar. A combination of the Mo-Fe-protein from one strain of microorganism with the Fe-protein from other cells yields an active nitrogenase complex (Kelly, 1969). Each of the nitrogenase components consists of subunits, there being eight subunits in the Fe-Mo-protein and two subunits in the Fe-protein (Gvozdev et al., 1971).

Spirillum lipoferum nitrogenase has been fractionated into 3 components (Mo-Fe-protein, Fe-protein, and an Fe-protein activation factor). All of these are needed for enzyme activity. A combination of 0.5 M Mn^{2+} with 10-20 M Mo^{2+} and ATP stimulates nitrogenase activity. If pre-incubation of the nitrogenase components with ATP, $Na_2S_2O_4$ and Mo^{2+} is omitted, the time-curves of nitrogenase activity are not linear. Pre-incubation of the Fe-protein and its activation factor with these three reaction components eliminates the non-linearity of the curves, indicating interaction between the Fe-protein activation factor and the Fe-protein, but not between the activation factor and the Mo-Fe-protein (Ludden et al., 1978).

What are the functions of the molybdenum and the iron in nitrogenase? Molybdenum is assumed to be the only element that effectively weakens the N—N bond. Moreover, both metals are thought to be involved in the reduction of nitrogen. According to the views put forward by Hardy et al. (1971b), nitrogenase binds the nitrogen to the iron moiety of the enzyme, and molybdenum becomes bound to the free end of the nitrogen ligand thus promoting reduction of the nitrogen. These workers suggested a step-by-step scheme for the nitrogen reduction mechanism, with diimide and hydrazine as intermediates. The active site of nitrogenase is thought to contain molybdenum and iron linked to a sulphur bridge. The reaction is initiated by the formation of a linear complex of nitrogen with the iron in the nitrogenase. The subsequent action of molybdenum yields binuclear diimide, and then the addition of two electrons and protons gives rise to hydrazine, while binding of the terminal electrons disrupts the N—N bond. Splitting of the ammonium from the iron, and the hydrolysis of Mo-NH_2, yields two NH_3 molecules.

Bacteria (Klebsiella pneumoniae strain M5) were shown to be able to fix nitrogen under anaerobic or microaerophillic conditions (Yoch, 1974). Electron carriers, capable of transferring electrons from spinach chloroplasts to nitrogenases isolated from Klabsiella pneumoniae and Azotobacter vinelandii were isolated from extracts of Klebsiella pneumoniae. One carrier was identified as a flavoprotein with a molecular weight of approximately 21,000, and was designated Klebsiella flavodoxin. The second carrier was not identified but was assumed to be ferredoxin.

Opposing views are held by Shilov and Lichtenstein (1971) and by Syrtsova et al. (1971), who maintain that the iron in both the Mo-Fe-protein of nitrogenase does not interact directly with the nitrogen molecule, but rather is involved in the activation and transport of electrons.

Shilov and Lichtenstein (1971) report interesting observations regarding the structure of the active sites in nitrogenase, and the function and structure of the Fe-Mo- and Fe-components; the latter are incorporated within the interpretation of the nitrogenase active site (Fig.54).

Lichtenstein et al. (1978) have shown that all the 32 iron atoms in nitrogenase are gathered together in clusters, and have discovered the relative disposition of these clusters. The ATP-ase centre of the enzyme has been found to be linked directly to the

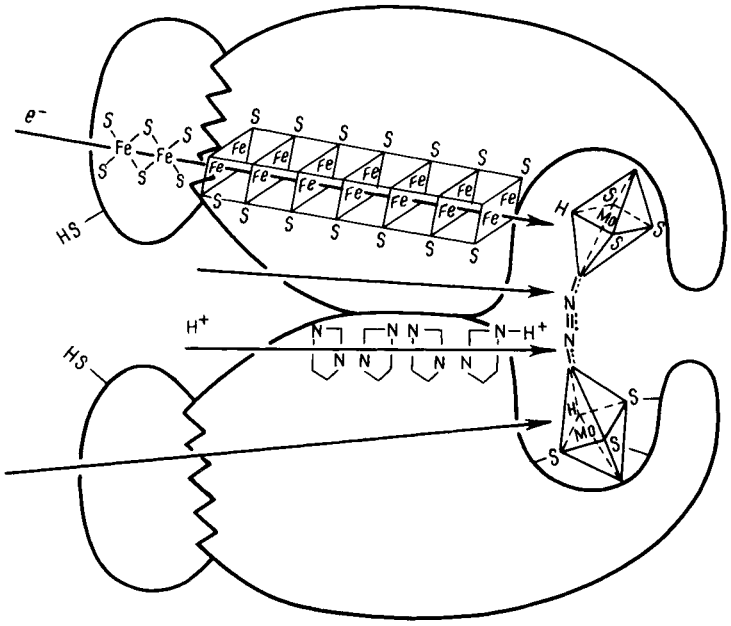


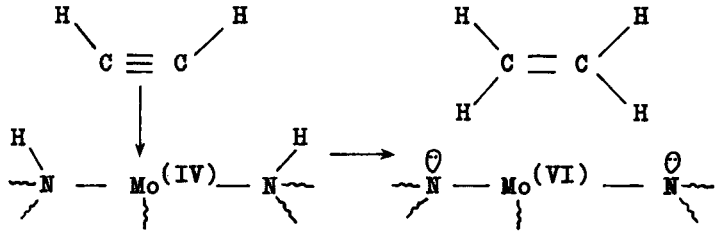
Fig. 54. A model of the nitrogenase active centre (after Shilov and Lichtenstein, 1971).

electron-transport system of the clusters, and to be set apart from the nitrogen-binding centre. Using specific inhibitors, Pisarskaya et al. (1977) have established that the substrate binding site of nitrogenase is localized on the Mo-Fe-protein, whereas the sulphhydryl groups of the ATP-ase centre are situated both on the Mo-Fe-protein and on the Fe-protein.

The nitrogen-fixing site is located inside the protein complex, and is in the immediate neighbourhood of the reducing site. Two molybdenum atoms are involved in the activation of the nitrogen, a binucleate complex of two metal ions and a nitrogen molecule being formed in the process.

This scheme accounts for the specificity of both molybdenum and iron. An asymmetric reduction of molecular nitrogen is assumed to take place without such intermediates as diimide and hydrazine.

Stiefel (1973) believes that after the substrate (whether this be acetylene or molecular nitrogen) binds to enzyme I, enzyme II (Fe-S-protein) yields up electrons to the Mo^{4+} in enzyme I reducing it to Mo^{6+}, which in turn transfers two electrons to the substrate. Moreover, protons are carried from the coordinated nitrogen of enzyme I to the substrate. Thus, acetylene is reduced to ethylene according to the following scheme:



Schrauzer (1974) published a review of model nitrogenase systems involving complexes of molybdate with thiol ligands, in particular, with L(+)-cysteine. Such systems, in the presence of a reducing agent ($NaBH_4$ or $Na_2S_2O_4$) and substrate amounts of ATP, catalyzed the reduction of all the known substrates of nitrogenase. The active complex capable of the reducing function contains Mo^{4+}. A model analogue of ferredoxin, Fe_4, $S_4(SR_4)^{2-}$, takes the part in model nitrogenase systems of a catalyst, promoting electron transport in the conversion of molybdate-thiol complexes into an active reduced form. The function of ATP in the model systems is not specific. The mechanisms of reduction in model systems of a number of nitrogenase substrates, including molecular nitrogen, were discussed by this worker. In the case of molecular nitrogen the reaction yields a precursor of ammonium - diamide.

That the biological fixation of nitrogen proceeds as a reduction reaction has been conclusively demonstrated. A molecule of nitrogen coming into contact with the surface of the nitrogenase enzyme complex is reduced to ammonium, the reaction requiring ATP. This reduction occurs by virtue of the action of hydrogen atoms brought to the nitrogenase via a chain of ferredoxins.

This process may be summarized as follows:

$$N_2 + AH_2 + ATP \longrightarrow NH_3 + A + ADP + P,$$

where AH_2 is a donor of electrons, and A is the oxidized form of the donor. In fact the enzyme catalyzes three coupled reactions: the reducing agent-dependent breakdown of the ATP high energy bond, the ATP-dependent evolution of hydrogen, and the reduction of nitrogen to ammonium. The hydrolysis of ATP in nitrogen-fixing systems has been shown to be an essential part of enzymatic nitrogen fixation. The details of the hydrolysis of ATP in the mechanism of nitrogenase action during biological nitrogen fixation are poorly understood.

Promising research is continuing using chemical models of nitrogenase. Schrauzer et al. (1975) studied the effect of ATP and

some acids of similar ionic strength (H_3PO_4, $HClO_3$, H_2SO_4, CH_3COOH, HCl) on the reduction of 4 substrates (NC, CN^-, Ch_2=CH-CN, C_2H_2) in a molybdenum-thiolic form of the nitrogenase model. The activation of ATP in this system occurs via the formation of intermediate protonic complexes of ATP with the oxidyzed molybdate ion; these are subsequently hydrolysed, producing ADP and phospate. The effect of this is obviously acceleration of the release of molybdenum-bound hydroxy groups, which in turn accelerates the transition of the catalyst from the oxidized form to the active reduced state. These studies are believed to contribute to the understanding of the role of ATP in nitrogenase reactions.

An important recent achievement is the finding that each e--transfer, coupled with ATP-hydrolysis, is followed by dissociation of the nitrogenase into the Mo-Fe- and Fe-protein components (Hageman and Burris, 1978).

Banerjea and Samarjit (1975) reported that Professor Chata of Sussex University had succeeded in camping out the fixation of molecular nitrogen under relatively mild experimental conditions. Following this fixation, nitrogen in the form of NH_3 could be separated from molybdenum by ethanol. This process of fixation corresponded to the enzymatic process, mediated by nitrogenase, in Azotobacter cells.

The involvement of ATP in the process is largely dependent upon magnesium. In experiments with Clostridium, the activity of nitrogenase with respect to nitrogen fixation and hydrogen evolution has been shown to be dependent upon the Mg^{2+} :ATP ratio, but not on the absolute concentration of magnesium (Kennedy et al., 1968). Mn^{2+}, Co^{2+}, Fe^{2+} or Ni^{2+} may replace Mg^{2+} in the hydrolysis of ATP by Azotobacter nitrogenase, whereas Cu^{2+} and Li^{2+} inhibit the hydrolysis of ATP (Burris, 1969).

On the basis of available experimental results, the following sequence of reactions in the nitrogenase-mediated reduction of free molecular nitrogen is suggested: reduction of the Fe-protein, activation of the Fe-protein by Mg-ATP, binding of the substrate to the Mo-Fe protein, association of the Fe and Mo-Fe proteins to form an active complex, electron transfer to the substrate, release of the reduced product as well as ADP and Pi, and dissociation of the complex (Postgate et al., 1978).

The nitrogenase from various sources requires ATP for its activity. No details of the involvement of ATP in the reduction of

nitrogen (or acetylene) have yet been disclosed. Some pertinent evidence has been provided by Aseyeva et al. (1973) in their studies of some of the properties of nitrogenase. Thus while investigating the specificity of nitrogenase with regard to a number of nucleoside triphosphates, these workers found that nitrogenase from lupin bacteroids is most active in the presence of ATP, and that activity of the enzyme in the presence of UTP and GTP is low. An estimation of the acetylene reduction rate as a function of ATP concentration revealed that the pattern of saturation of the enzyme with ATP shows some complexity. It can readily be seen from that plot shown in Fig.55 that the shape of the curve varies from hyperbolic to S-shaped, which indicates the presence of several ATP binding sites. From this finding, the authors concluded not only that ATP is bound to the active site, but that it also acts allosterically as a controller, the nitrogenase from lupin bacteroids being a regulated enzyme. Consequently, ATP functions not only as an energy source for the nitrogenase reaction, but also as an important controller.

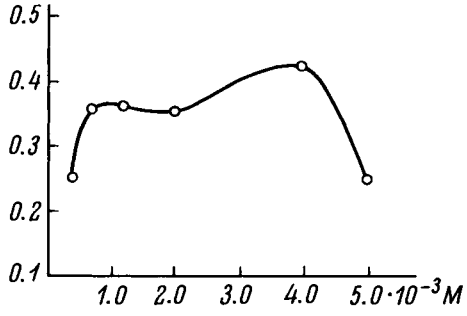


Fig. 55. Effect of ATP concentration on the reduction of acetylene by nitrogenase from lupin bacteroids (after Aseyeva et.al., 1973). Ordinate, enzyme activity in terms of the quantity of ethylene umoles reduced per minute per mg protein; abscissa, ATP quantity M.

In the process of nitrogen fixation in Clostridium pasteurianum a prominent role is played by electron carriers with low oxidation--reduction potentials. Of outstanding importance in this respect is the iron protein ferredoxin which was discovered in, and initially isolated from cell-free extracts of Clostridium pasteurianum

(Mortenson et al., 1962). D'Eustachio and Hardy (1964) demonstrated the involvement of ferredoxin as an electron carrier in the process of nitrogen fixation. The electron flow from the reducing agent to nitrogen has been found to pass via ferredoxin. It can reduce nitrogenase, which, by interacting with ATP, is able to act upon molecular nitrogen.

Zhiznevskaya (1972) found another non-haem iron protein which could be isolated from the cytoplasmic fraction of root nodules under the same conditions as those favourable for the isolation of ferredoxin from leaves. Maximal amounts of this protein were present during periods of active nitrogen fixation. It is worth emphasizing that the non-haem iron protein fraction displays characteristics similar to those of the ferredoxin isolated by Syrtsova et al. (1968) from Azotobacter.

The requirement for ferredoxin and ATP to sustain nitrogen-fixing activity in Clostridium pasteurianum extracts was confirmed by Mortenson et al. (1963).

In the course of the reduction process, ferredoxin operates between pyruvate dehydrogenase and hydrogenase. The former carries electrons from pyruvate to ferredoxin. It should be stressed that ferredoxin is also involved in the conversion of nicotinamide adenin dinucleotides and in the reduction of flavin co-enzymes according to the following scheme:

$$2F_D(\text{reduced}) + NADP \rightleftharpoons NADP{\cdot}H + H^+ + F_D(\text{oxidized})$$

Following from this, Hardy offers a scheme for that part of the nitrogen fixation process which is responsible for the electron flow leading to the reduction of nitrogen (Hardy et al., 1965 Fig.56).

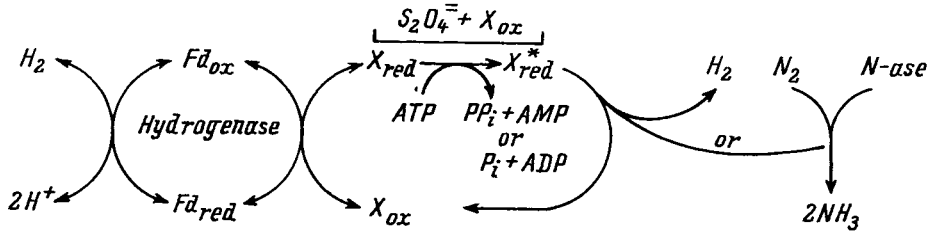


Fig. 56. Supply of electrons for the reduction of nitrogen (after Hardy et al., 1965). Asterisk indicates an activated form of X.

According to this cheme, hydrogen reduces an electron carrier X (to become $X_{reduced}$) via hydrogenase and ferredoxin. The reduction of the carrier X may also be induced by $Na_2S_2O_4$ without hydrogenase and ferredoxin. It is further supposed that $X_{reduced}$ is converted into an activated form of $X_{reduced}$ by the intervention of ATP. Subsequent oxidation of the reduced intermediate is accompanied either by oxygen evolution or by reduction of the nitrogen chemically bound to the nitrogenase. The hypothetical electron carrier (X) is an iron protein displaying paramagnetic properties. The immediate electron donor for the nitrogenase might be flavodoxin.

Yoch et al. (1969, 170) identified a flavoprotein (nitrogen flavine) and ferredoxin in an Azotobacter vinelandii extract. These proteins carry electrons to nitrogenase when they are provided with an artificial electron donor system, such as illuminated chloroplasts and reduced 1.6-dichlorophenolindophenol. Using an essentially similar method, a fraction from soybean bacteroid extracts has been identified as containing ferredoxin-like, non-haem iron proteins which function as electron carriers. Later they were purified, partially characterized, and shown to transport electrons from illuminated chloroplast fragments to the bacteroid nitrogenase; they were not involved in the photochemical reduction of NADP by chloroplasts.

This bacteroid non-haem iron protein contains an acid-soluble sulphide with a molecular weight around 9400. Azoflavin and a non-haem iron protein were isolated from soybean bacteroids by Wong You Cheong and Chan (1973). The authors set up a reduction pathway of five successive, including various electron carriers associated with nitrogenase. In each of the systems Wong You Cheong and Chan (1973) used nitrogenese preparations isolated from soybean nodule bacteroids. In system I, they used hydrogenase and ferredoxin from Clostridium pasteurianum; in system V, azoflavin could be replaced by FAD or FMN. According to these workers, system V of the electron transport pathway from glucose-6-phosphate to the bacteroid nitrogenase was a very close approximation of the conditions encountered in vivo. This system also contained glucose-6-phosphate hydrogenase, NADP, NADP.H-ferredoxin reductase, azoflavin (the bacteroid flavoprotein) and the bacteroid iron protein $Fe_{non-haem}$-protein.

Much progress has been made in the elucidation of the principal properties of nitrogenase. At present, efforts are mostly con-

centrated on investigating its active site and the cellular milieu in which nitrogenase functions. In studies of the active site much attention is given to the molybdenic factor (molybdofactor). Molybdenum is a component of the active site, but its role in enzymatic nitrogen fixation is still obscure. For studying the role of molybdenum in the enzyme and the role of the molybdenum-protein linkages, the cofactor is a more convenient object to work with than the high molecular weight enzyme complex.

The existence of a common cofactor in different molybdenum-containing enzymes was first shown by Nason et al. (1971).

Of considerable interest is the publication by Nason et al. (1971), discussing the appearance in vitro of a new, assimilating NADP.H nitrate reductase, in the course of mixing cell-free preparations of Neurospora crassa mutants with components of molybdenum enzymes. The results of these investigations allow for the presence of a molybdenum component, as the supposed molybdenum co-factor which is of wide occurrence in microorganisms, plants and animals in molybdenum enzymes.

The well-known, highly specific phenomena of the recognition, interaction and association of protein subunits yielding multimeric enzymes, led the authors to suggest that in the course of phylogenesis the molybdenum-containing component has remained a single non-mutant polypeptide chain. The authors concluded that the molybdenum part of the enzyme exists as part of a comparatively small organic molecule or co-factor, probably as a small polypeptide. According to this hypothesis, the Mo-cofactor serves as a link between the enzyme subunits thus forming the total enzyme complex, and also acts as an electron carrier. It is supposed that both of these functions are universal in all molybdenum enzymes.

Recently it has become clear that the molybdenum co-factor isolated from other molybdenum-containing enzymes. Thus two types of co-factor appear to exist, one belonging to nitrogenase, and one occurring in all other molybdenum-containing enzymes.

In the pioneering work carried out in the laboratory of Brill at the University of Wisconsin, the research was aimed at obtaining Azotobacter vinelandii mutants, deficient in different components of nitrogenase. A mutant was found, UW 45 (Bishop and Brill, 1977), with an inactive Mo-Fe-protein; activity was restored by adding an acid-treated wild type Mo-Fe-protein isolated from various nitrogen-fixing organisms (Azotobacter vinelandii, Klebsiella

pneumoniae, Rhizobium rubrum, Clostridium pasteurianum) (Nagatani et al., 1974).

Making use of this co-factor test the authors isolated, from a homogenate of Azotobacter vinelandii, the nitrogenase-specific Fe--Mo-co-factor (FeMo-co), which restores activity to the defective nitrogenase from UW 45, but failed to reactivate the defective nitrogenase from Neurospora crassa nit-I mutant (Shah and Brill, 1977). In the experiments of Nason et al. (1971), crystalline Mo--Fe-protein from different nitrogenases were successful in reactivating the nit-I mutant nitrogenase. Shah and Brill attributed this variation in effect to the fact that inadequately purified Mo-Fe-protein contains trace amounts of nitrate reductase, which provides the nit-I mutant restoring co-factor.

FeMo-co is under thorough study, but its molecular weight and its nature - whether it is a protein or a peptide - remain unknown. It dissociates from the Mo-Fe-protein at pH 2,5 and contains all of the molybdenum and a half of the non-haem iron of the protein, Fe and S being arranged in a cluster which contains 8 atoms Fe and 6 atoms S per 1 atom of Mo. This cluster differs from the "ferredoxin" type (4, Fe, 4S), which contains the other half of the non--haem iron of the nitrogenase Mo-Fe-protein (Rawlings et al., 1978).

Fe-Mo-co displays a nitrogenase-like activity of its own (it reduces acetylene to ethylene), amounting to 8% of the original nitrogenase activity (Shah et al., 1978). Like nitrogenase, it is inhibited by carbon monoxide. These facts indicate that Fe-Mo-co is indeed a part of the active site of nitrogenase.

An important result of a comparative study of the Mo-Fe-protein and Mo-Fe-co by X-ray absorbtion spectroscopy was the recognition that in the enzyme, Mo is bound to sulphur and to iron (Cramer et al., 1978).

The assumption that the molybdenum in nitrogenase is sulphur- and iron-linked is confirmed by the data of Zumft (1978), showing that sulphur is one of the possible ligands. Molybdenum was isolated from the nitrogenase Mo-Fe-protein in thiolmolybdate complexes (Zumft , 1978).

Lvov et al. (1975) studied a low molecular weight factor common to molybdenum enzymes (nitrogenase, nitrite reductase, xanthine oxidase). This was isolated from bacteria, fungi and other sources, and was capable of restoring the activity of nitrate reductase in a mutant of the fungus Neurospora crassa deficient in

this enzyme. The factor is obtained by treatment of the molybdenum enzyme and its highest activity is observed when it is associated with protein. When dissociated from the protein as a low molecular weight compound it is rapidly inactivated.

Lvov et al. (1978) have shown that nitrogenase and nitrate reductase from the bacteroids of lupin nodules contain two forms of molybdenum co-factor, both of which restore activity to the deficient nitrate reductase of the Neurospora crassa mutant. One is a low molecular weight fluorescent peptide, the other is a low molecular weight (24000) protein. Both co-factors were active only if they were supplemented with sodium molybdate, as their own molybdenum was lost during isolation and purification.

Alikulov (1979) has found a similarity between the properties of the molybdenum cofactors (Mo-co) isolated from nitrate reductase and xianthine oxidase. The Mo-co in enzymes carries out at least two functions: a catalytic one taking place at the substrate binding site, and a structural one involving the linking of the enzyme subunits.

Alikulov et al. (1980) developed a quantitative method for the anaerobic isolation of the molybdo-cofactor from two molybdenum enzymes, nitrate reductase from lupin bacteroids, and xanthinoxidase from milk. It was established that the cofactor is a complex consisting of an aromatic component and amino acid residues. The structural and catalytic functions of the Mo-cofactor in enzymes were demonstrated as well.

One of the interesting findings arising from investigation of the molybdenum cofactor is that there is a strong likeness between different molybdenum-containing enzymes, including the very important nitrogenase and nitrate reductase. Up to now there has been little agreement about the likeness between these enzymes. Lvov et al. (1978) suggested genetical, regulatory, and structural likenesses between these enzymes, as a possible explanation for the involvement of nitrate reductase in nitrogen fixation.

The detection of a cofactor common not only to nitrogenase and nitrate reductase but also to a number of Mo-enzymes, suggests strong similarity among these enzymes. Indeed, the investigations of recent years have established considerable structural and functional likenesses between various Mo-enzymes (Ganelin, Lvov, 1975). All the Mo-enzyme-catalyzed reactions yield products which differ from the corresponding substrate by two electrons and two protons. Besides two molybdenum atoms, the Mo-enzymes contain the same redox

cofactors, and the same iron-sulphur components, flavins, and haem. The molybdenum atoms of these enzymes reside at the substrate binding sites and are involved in substrate activation. Under appropriate conditions all Mo-enzymes, with the exception of nitrogenase, produce a Mo-type electron paramagnetic resonance (EPR) signal (V). It is valid to assume that Mo-enzymes, except nitrogenase, contain similar polypeptide blocks, with the same sets of cofactors. This gains support from the fact that some substrates are common to several Mo-enzymes, e.g., aldehydes are substrates for xanthinoxidase, xanthindehydrogenase, and aldehydoxidase.

In the review of Lvov et al. (1981) results are presented which show that the Mo-co's imolated from various enzymes (xanthinoxidase, nitrate reductase, sulphitoxidase) have similar properties. All contain an aromatic pterin-related compound. The other component of Mo-co obviously consists of amino acid residues.

Molybdocofactors perform both catalytic and structural functions. The catalytic function concerns the role of the cofactor at the active site of the enzyme, where association with the substrate and its reduction take place. This function ceases in air-oxidated molybdocofactors. The stabilization of Mo-co-activity by ascorbate is evidently attributable to the anti-oxidative protection afforded to the molybdenum coordinating region of the cofactor.

The structural role of molybdocofactors is to link the enzyme subunits (at present, this has only been proven for the Mo-co).

Many new and interesting facts about the molybdenum cofactors of molybdenum-containing enzymes are presented in the review of Lvov et al. (1981). In this review the basic approaches to the isolation and investigation of molybdenic factors are discussed, as well as details of their structure, function, and biosynthesis.

Of considerable interest are studies of the conditions influencing the intracellular functioning of nitrogenase. Scherings (1978) found that one of the two ferredoxins of Azotobacter can protect nitrogenase from the effects of oxygen. In general, protection from oxygen depends on interactions between three proteins, including molybdo-azo-ferredoxin and ferredoxin. According to Hill and Kennedy (1978), the oxygen-sensitive regulatory proteins are responsible for the oxygen sensitivity of nitrogenase biosynthesis.

Wilcockson and Werner (1978) have shown that the arabinose content of the medium strongly affects the nitrogenase in Rhizobium.

Considerable progress has been made both in understanding the genetics of nitrogen-fixing microorganisms, and in the study of nitrogenase with respect to the genetics of nitrogen fixation. Research into the genetics of nitrogen fixation follow two main directions. One involves thorough study of, and experimentation on the regulatory sequences of the nif Genes (genes of nitrogen fixation) and Klebsiella pneumoniae, with the purpose of obtaining an "ideal" bundle of nif genes and transfering this by the techniques of genetical engineering to suitable and promising genetical systems, e.g. gramineous cells. The other direction of research is concerned with improving the symbiosis between root nodule bacteria and bean plants.

Only the genetical problems relating to nitrogenase and nitrogen fixation will be discussed here.

Wall et al. (1975) studied the genetical transmission of nitrogenase-hydrogenase genes in Rhodopseudomonas capsulata. The transmission of nitrogen fixation genes (nif) in photosynthesising Rhodopseudomonas capsulata was carried out successfully. Nif^- mutants were obtained by N-methyl N'-nitro-N-nitrosouridine treatment of the B_{10} strain of the wild type.

Ammonium sulphate was used as the source of nitrogen, and 5 types of nif^- mutants were obtained (W11-W16). These retained all the characteristic features of the B10 strain, the mutants W12, W15 and W16 lacking nitrogenase, while W11 and W13 were phenotypically nif^- and appeared to lack glutamate synthesis or glutamate metabolism. A filtrable factor synthesized by B10 cells transferred to the mutant W11-W16 cells the lost nif^- marker. Recombination analysis of W12, W15 and W16 mutant lines revealed that their nif^- mutations were not identical. It is suggested that in photosynthesizing bacteria the nitrogenase-hydrogenase dimer forms a single enzyme.

In a research paper on nitrogenase and the genetics of nitrogen fixation (Dixon, 1977), the most desirable achievements in the genetical modification of existing nitrogen-fixing organisms were pointed out. Thus the following are recommended as research goals: reduction of the release of H_2 during nitrogen fixation; enhancement of the nitrogenase content by increasing the quantity of nif (nitrogen fixation) genes; isolation of constitutive nitrogen--fixing, ammonia-producing mutants. Such mutants of blue-green algae for example, may be employed to utilize solar energy for the

production of N-fertilizers. For the improvement of symbiotic nitrogen fixation, the author recommends that the genetics of the host plant and the ecology of the symbiont be taken into account. As regards nif gene transfer, the following factors which may be expected to limit the expression of nif genes should be taken into account: the extremely nigh O_2-sensitivity of nitrogenase, the necessity of an appropriate reducing agent; the accessibility of the Mo-containing cofactor in the host cells; the high energy requirement and accessibility of suitable regulatory proteins in the host organism. Thus it is probable that the introduction of additional genes (besides the nif genes) will be necessary for establishing new nitrogen-fixing strains.

The review published by Shanmugam and Valentine (1975) deals with the molecular biology of nitrogen fixation; the problem of the transfer of nitrogenase complex coding genes (nif genes) into organisms that are unable to fix N_2 is discussed. Other subjects discussed include progress in the investigation of nif genes, localization of the sites of these genes in the genomes of Klebsiella pneumaniae, their transfer into Escherichia coli cells, and their transfer from Rhizobium trigolii into Klebsiella aerogenes. The role of plasmids in the transfer of nif genes is taken into account. Attention is given to the involvement of glutamate dehydrogenase in the regulation of nif gene expression in N_2-fixing bacteria. Research prospects in the field of the molecular biology of N_2 fixation are considered together with the possibilities for agricultural application.

Interesting evidence points to the possible involvement of glutamate synthetase in molecular nitrogen fixation. The presence of glutamine synthetase has been induced in the cytoplasm of nodules during their development.

It was shown that in Spirillum lipoferum, as in Klebsiella pneumoniae, glutamine synthetase plays an essential part in the regulation of nitrogenase biosynthesis (Gauthier et al., 1977).

The problem of how glutamine synthetase regulates nitrogenase biosynthesis has not been finally solved. Kleiner (1978) found that glutamine synthetase was not involved in the regulation of nitrogenase biosynthesis in Clostridium pasterianum, but suppression and anti-suppression of nitrogenase did correlate with levels of glutamine and glutamate synthetase.

Recently two forms of glutamate synthetase were found in Rhizobium japonicum cells (Darrow and Knots, 1978).

Chemical models simulating the process of biological nitrogen fixation are of considerable interest. The study discussed above has elucidated a number of important details of the chemical mechanism of the enzymatic fixation of nitrogen, and has disclosed a series of reactions involving nitrogen which challenge former ideas about the non-reactivity of nitrogen. Among new reactions discovered were some displaying a close resemblance to biological nitrogen fixation.

The first step in this direction was taken by Volpin and Shur (1964,1967), who demonstrated the ability of molecular nitrogen to react with a number of systems based on transition metal compounds. Complexes of such transition metals as titanium, chromium, molybdenum, vanadium and iron, upon interaction with organic compounds of magnesium, lithium and aluminum, were shown to be able to react with molecular nitrogen at normal temperatures and pressure. As a result, compounds of nitrogen and transition metals are produced which yield ammonium upon hydrolysis. In this way the reduction of nitrogen in the presence of transition metal compounds has been achieved.

Following this, Allen and Senoff (1965) succeeded in synthesizing the first complex of molecular nitrogen and a compound of a transition metal (ruthenium). Hydrazine served as the source of nitrogen, but molecular nitrogen did not undergo reaction under the conditions employed. The synthesis of this complex, however, suggested the possibility of obtaining the same result directly from molecular nitrogen. In fact, Shilov (1972) observed the formation of complexes of nitrogen and transition metals in solution, by reducing zinc amalgama, chloride and ruthenium hydrochloride ($RuCl_3$ and $RuOHCl_3$) in tetrahydrofurane.

Analysis of this reaction revealed that nitrogen can take part in reactions in the presence of water, oxygen and relatively mild reducing agents, i.e., the reactions can occur under typically biological conditions. However, all attempts to reduce nitrogen within a complex were unsuccessful.

It was only later that success came in the detection, isolation and investigation of compounds of a metal (titanium, iron) and molecular nitrogen, with the nitrogen being capable of further reduction. The formation of hydrazine during the subsequent reduction of nitrogen in these complexes could be observed. In the search for systems operating in water and approximating closely to

enzymatic nitrogen fixation, compounds of trivalent titanium, divalent chromium and divalent vanadium were chosen as reducing agents. These compounds had negative reducing potentials, i.e., they were capable of liberating hydrogen from water, in the same way as biological systems. The compounds of molybdenum which activate nitrogen in nitrogenase were successfully used for the activation of nitrogen. Denisov et al. (1970) found systems able to reduce nitrogen to hydrazine and ammonium in water and water-alcohol alkaline solutions (Denisov et al., 1970). Molybdenum salts proved to be effective catalysts which, however, functioned only in the presence of trivalent iron. Many of the characteristics of this system were found to resemble those of nitrogenase. It was also possible to demonstrate that vanadium compounds reduced nitrogen to hydrazine and ammonium very efficiently in alkaline media, even at room temperature and atmospheric pressure.

Further details of these investigations may be found in the reviews by Shilov and Likhtenstein (1971) and Shilov (1972).

Silverthorn and Green (1971) synthesized a molybdenum complex capable of direct reaction with molecular nitrogen. Results obtained in the chemical modeling of biological nitrogen fixation have contributed much to the understanding of this vital process.

Thus, the goal of creating systems operating under conditions similar to those encountered in enzyme reactions has been obtained. Although complicated enzyme systems are fundamentally different from the relatively simple model systems, investigations along these lines are of prime importance for elucidation of the mechanism of nitrogen fixation. The results obtained to date support the idea that multi-metal protein complexes are involved in nitrogen fixation.

The above data demonstrate the extent of the involvement of molybdenum and iron in the biochemical processes of nitrogen fixation. Although the process of nitrogen fixation in root nodules resembles in many respects that occurring in free-living nitrogen--fixing organisms, symbiotic nitrogen fixation is by far the more complex process. The nodule bacteria are incapable of nitrogen fixation in pure culture.

Of considerable interest are experiments involving nitrogenase induction. Nitrogenase was induced in Petunia seedlings with Rhizobium strain 32 HI, which is specific for the common cow pea. Both the seedlings and the bacteria lack nitrate reductase (Hess

and Gölz, 1977). The Petunia seedlings were grown for 16 h at 24°C in the light, in an agar nutrient medium containing yeast extract and mannitol.

The Rhizobium 32 H I was grown in the same medium, and was used for inoculation after 10 days. 4 days after inoculation, a thin layer of bacterial cells surrounded the roots. In spite of thickening of the root hairs, penetration of the bacteria into the root tissue was never verified. The presence of nitrogenase was recorded only in 23-day-old seedlings, inoculated at the age of 13 days. In earlier inoculated (10-day-old) and later inoculated (14-23--day-old) seedlings no nitrogenase induction was found (Hess and Gölz, 1977).

That nitrogen fixation occurs inside the nodule in the bacteroids is now a widely acknowledged fact (Mortenson, 1966; Kretovich et al., 1969). The higher plant host supplies the bacteroids with an appropriate energy source and various substrates and co-factors. Having demonstrated the possibility of studying the process of nitrogen fixation in squashed nodules, Bergersen (1966) found ways of overcoming the difficulties that arose in studies of the mechanism of symbiotic nitrogen fixation. Among difficulties encountered was that of the extreme lability of nitrogenase isolated from cell-free extracts of the bacteroids of legume nodules. Discoveries made with the aid of this technique have been discussed above.

Molybdenum is involved in symbiotic nitrogen fixation not only directly in the biochemical processes of nitrogen fixation itself, but also by virtue of its role in the metabolism of leguminous plants. Molybdenum participates in those aspects of metabolism which are associated with the biosynthesis of carbohydrates in the leaves and the translocation of these metabolites to the nodules where they are required for nitrogen fixation. Stimulation of nitrogen fixation by copper, manganese and boron may be also explained in terms of their role in the biosynthesis of carbohydrates (Gerretsen and Hoop, 1954; Gribanov, 1954). Gribanov (1954) found that copper and manganese (each at a concentration of 5 mg per litre) stimulated the process of nitrogen fixation in Azotobacter chroococcum. Gerretsen and Hoop (1954) concluded that this species of Azotobacter required boron for nitrogen fixation. Copper, moreover, plays an important part in the development of the nodules (Zhiznevskaya and Borodenko, 1969). Some trace elements, for

example tungsten, compete with, or are inhibitors of molybdenum in nitrogen fixation (Keeler and Varner, 1957). Tungsten and copper in free-living nitrogen-fixing organisms competitively suppress the functioning of molybdenum in nitrogen fixation, and increase the dose of molybdenum that is required for maximal nitrogen fixation (Gradova-Krylova, 1967).

An inseparable constituent of the nitrogen fixation system is the haemoglobin of the nodules, leghaemoglobin. It is found in nodules that develop with active strains of Rhizobium and is absent from ineffective nodules (Kretovich et al., 1972). Bergersen (1962) visualizes leghaemoglobin as being involved in the uptake of oxygen, which is essential for the respiration of the bacteria in the nodules and for ATP generation. Without leghaemoglobin, oxygen diffusion through the nodule tissue would be insufficient. Bergersen (1970) expressed the view that leghaemoglobin is a system specific to bacteroids, protecting the nodule nitrogenase from an excessive oxygen supply.

Peive et al. (1967) observed a species specificity, and heterogeneity of the leghaemoglobins in the nodules of soybeans, broad beans and lupins. Leghaemoglobin from different lupin varieties has been crystallized, the shape of the crystals varying according to the variety and the amino acid composition of the leghaemoglobin (Peive et al., 1970). Interesting observations on the relationships between leghaemoglobin and the other iron proteins occurring in nodules may be found in a monograph of Zhiznevskaya (1972).

Zhiznevskaya (1974) summarized her investigations on iron proteins in the nodules of leguminous plants. She concluded that the haem of leghaemoglobin is synthesized by the bacterial partner in the symbiosis between legumes and nodule bacteria, while the globin is produced by the plant. From her findings she suggested that copper and cobalt preclude premature degradation of the haemoglobin. An assessment of the oxygen capacity of leghaemoglobin has revealed that the maximum capacity occurs during flower bud formation and flowering. In the soluble cytoplasmic fraction of the nodules (after removal of the bacteroids) haemoprotein fractions could be found possessing catalase and peroxidase activity. The observed positive correlation between the contents of haemin and non-haemin iron proteins in the soluble cytoplasmic fraction led Zhiznevskaya to suggest that these proteins interacted in the metabolism of leguminous plants with nitrogen-fixing root nodules.

Zhiznevskaya (1976) summarizes the history of investigation on the iron-containing protein leghaemoglobin, and contemporary conceptions of its structure and function are presented. Leghaemoglobin is formed in the vegetal part of the nodule as the product of symbiosis of the nodule and the leguminous plant. It is located outside the bacteroids. The conformation of the globin part of the molecule is similar to that of myoglobin and the monomeric haemoglobins of invertebrates, although there is a considerable difference in the amino acid sequence. Oxyleghaemoglobin is an important component of the symbiotic nitrogen fixation system, being responsible for forming optimal oxygen regime in the nodule, facilitating oxygen diffusion towards the bacteroids, and bringing about an increase in the amount of ATPH in the oxidative phosphorylation system of the bacteroids.

Peive and Zhiznevskaya (1976) discuss current views on the structure and function of the leghaemoglobin present in bacteroids. The structure of a part of the leghaemoglobin molecule resembles that of myoglobin and the monomeric haemoglobins of invertebrates, in spite of considerable differences in the amino acid sequences. Oxyleghaemoglobin does the job in symbiotic nitrogen fixation of producing an optimum oxygen regime inside the nodules, facilitating oxygen diffusion and stimulating ATP synthesis.

On the citozole of the root nodules of leguminous plants, a non-haemin ferroflavoproteid has been detected which is capable of reducing 2,6-dichlorphenolindophenol, cytochrome-c and leghaemoglobin in the presence of NAD(P)H. It has been assumed that the bringing of root nodule leghaemoglobin to an active reduced state is a physiological function of the non-haemin ferroflavoproteid (Fe_{H2}·FP) (Zhiznevskaya and Borodenko, 1981).

Melik-Sarkisyan and Bashirova (1978) have found ascorbate oxidase activity in the soluble fraction of root nodules and in the roots of yellow lupine. The ascorbate oxidase activity is noticeably reduced when the symbiosis is ineffective. Nitrogenase, the chief enzyme of nitrogen fixation localized in the bacterioids at the centres of the nodules, is known to be inhibited by oxygen. The authors suggest that the oxidation of ascorbic acid in the nodule cortex and in the roots of lupine is probably associated with the regulation of the oxygen flow towards the centre of the nodule. Thus, together with the iron-containing protein leghaemoglobin, Cu-proteid ascorbate oxidase may participate in a complex mechanism for protecting the nitrogenase from an excess of oxygen.

Evidence is available that trace elements (Mo,Fe) may be involved in the biosynthesis of leghaemoglobin (Cheniae and Evans, 1960; Zhiznevskaya, 1972).

Bergersen (1971) presented a novel scheme which included his ideas about the fundamental processes of nitrogen fixation in legumes (Fig.57). Although the author believes that the scheme

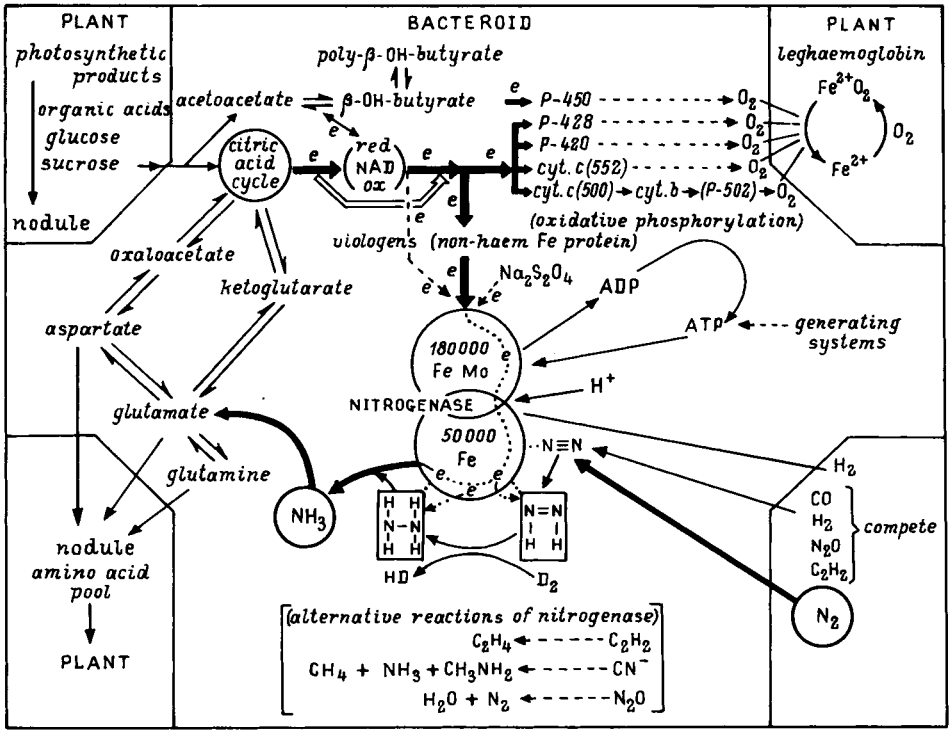


Fig. 57. Scheme for nitrogen fixation in legumes (after Bergersen, 1971). P - haemoproteins.

Bergersen proposed will inevitably have to be modified on the strength of new evidence, some of its points may now be considered as firmly proved. Bergersen (1971) believes that further research will find out more about the metabolism of bacteroids; in particular, further investigation is required into the pathways of electron transport, the terminal utilization of oxygen, and the details of the metabolism of carbon compounds.

A review has been published (Lvov et al., 1980) which surveys the literature on the relationship between nitrogenase and nitrate

reductase, and the pathways of nitrogen assimilation by nitrogen--fixing microorganisms and legume root nodules. The article discusses the factors responsible for the change from bound nitrogen assimilation to nitrogen fixation and vice versa, and the possibility of obtaining the simultaneous functioning of both processes.

Is the physiological role of molybdenum limited to its involvement in the reduction of nitrates and the fixation of molecular nitrogen? There is evidence indicating that molybdenum is involved not only in the initial stages of nitrogen metabolism, but also in subsequent processes, including protein biosynthesis. The protein content of various plant organ, including that of nodules, is enhanced by supplying molybdenum (Zhiznevskaya, 1972), and the concentration of glutamic acid increases when molybdenum is supplied (Hewitt et al., 1949; Zhiznevskaya, 1972). The involvement of molybdenum in the reduction of nitrates cannot alone account for this effect of the metal on protein content.

Vlasyuk and Ivchenko (1975) presented additional evidence for enhancement of reductive amination and transamination by molybdenum. They also reported findings testifying to the possible involvement of molybdenum in protein synthesis; there was an increase in the content of glutamic acid and a decrease in the levels of ketoacids and ammonium. These results were explained in terms of a positive effect of molybdenum upon the association of ribosomes with messenger RNA, thereby enhancing protein biosynthesis.

Lyukova (1972) studied the effect of molybdenum on the content of protein components and the amino acid composition of soluble proteins. She found that qualitatively, the composition of protein components in leaves and roots was not changed by varying the molybdenum supply, but the proportions of individual protein components were changed. The amino acid composition of hydrolysates of the total soluble proteins from pea leaves and roots was not modified by changes in the molybdenum supply. At the same time, the contents of individual amino acids (leucine, phenylalanine, valine, methionine and others) were increased. The relative proportions of some amino acids were modified under conditions of molybdenum deficiency. Lyukova (1972) found that there was suppression of the incorporation of ^{14}C-leucine and ^{14}C-glycine into proteins in the leaves and roots of molybdenum-deficient plants.

Although molybdenum is principally involved in nitrogen metabolism, clear observations of its involvement in other processes - nucleic acid and phosphorus metabolism, the metabolism of vitamins and the biosynthesis of porphyrins - have been made. These observations may explain the positive effect of molybdenum on plants growing on substrates with reduced nitrogen.

Other findings indicate that molybdenum is involved in other important metabolic processes apart from nucleis acid and nitrogen metabolism. A molybdenum shortage interferes with the metabolism of phosphorus compounds; the inorganic phosphorus content increases, while that of organic phosphorus compounds, including nucleoside di- and triphosphates with their energy-rich bonds,decreases (Chernavina, 1952; Possingham, 1954; Hewitt, 1959).

Spencer (1954) found that the inorganic phosphorus content in molybdenum-deficient tomato leaves was 4-10 tumes higher than that in normal plants. Possingham (1954) reported that the total organic phosphorus content of molybdenum-deficient tomato plants began to increased as early as 2-4 days after re-supplying molybdenum.

Some workers relate changes in phosphorus metabolism caused by molybdenum deficiency to the activation of phosphatases (Spencer and Wood, 1954; Hewitt, 1957), while others believe that these changes are associated with disturbances in phosphorylation (Rubin and Chernavina, 1970). In fact, Spencer and Wood (1954) observed an increase in the activity of acid phosphatase in the roots of tomato plants growing in the absence of molybdenum. A similar increase was also noted in homogenates of the plant tissues. These investigators suggested that one of the chief physiological roles of molybdenum is to protect phosphorylated compounds from hydrolysis, by inhibiting the activity of acid phosphatase.

Ternavsky (1971) reported that molybdenum inhibits non-specific acid phosphatases, namely α-glycerol phosphatase and p-nitrophenyl phosphatase, which are found in the lysosomes and spherosomes that carry out the lytic functions in the cell.

The ability of molybdenum to affect the activity of acid phosphatase has been assumed to be the result of formation of a molybdenum phosphate complex. Details of the relevant studies may be found in a paper by Chernavina (1970).

Data are available to support the view expressed by Wolfe (1954), that molybdenum is important in energy metabolism. Hewitt (1958) found that molybdenum stimulated the coupling of respiration

and phosphorylation, thereby increasing the resistance of plants to 2,4-dinitrophenol.

Chernavina and Voronina (1971) reported that the rate of cyclic photophosphorylation was lowered in isolated chloroplasts of molybdenum-deficient lettuce supplied with nitrate nitrogen. In plants grown on ammonium nitrogen, no changes in cyclic photophosphorylation occurred. On the other hand, the intensity of non-cyclic photophosphorylation in molybdenum-deficient plants was maintained, under some conditions ($NADP^+$ taking part in the Hill reaction and nitrate nitrogen nutrition), at the same level as that occurring in molybdenum-supplied plants.

Study of the interaction between molybdate ions and RNA isolated from pea leaves has shown that the formation of complexes of this metal with RNA occurs through the phosphate groups and involves a transfer of charge, especially in an acid (pH 5) environment. This is indicated by the appearance of an additional absorption band in the area of 220 nM. Results obtained provide evidence of the incorporation of the molybdate ion into the RNA structure, apparently more rigidly than the incorporation of molybdenum into DNA, as indicated by the shift of absorption bands in the ultra-short spectrum of RNA (Ivchenko, 1981).

Molybdenum influences the metabolism of vitamins in plants. A number of studies show that a dramatic fall in ascorbic acid content occurs in molybdenum-deficient plants, this now being taken as a specific sign of molybdenum deficiency (Hewitt et al., 1950; Burkin, 1968). Such a decrease is not observed when other essential trace elements are in deficiency. Suffice it to say that in the studies of Hewitt et al. (1950) the ascorbic acid content decreased 3–4 times in molybdenum-deficient Brussels sprouts, borecole, cauliflower and sugar beet. These workers did not rule out the possibility that molybdenum is involved in the biosynthesis of ascorbic acid.

A rather characteristic sign of molybdenum deficiency is a considerable decrease in chlorophyll content (Hewitt and McCready, 1956). Burkin (1968) advanced the hypothesis that the positive action of molybdenum on the synthesis of chlorophyll is connected with an increase in the ascorbic acid content; ascorbic acid plays a prominent role in the regeneration of chlorophyll and bacteriochlorophyll from their photooxidation products.

Agarwala (1952) found that in preparations of cauliflower leaves (in which the main symptom of molybdenum deficiency was the thread-like appearance of the leaves), the leaf cells appeared as if they were separated from each other, and the middle lamella could not be seen, indicating a possible effect on pectin metabolism.

A possible influence of molybdenum on cell membrane structure is indicated by the studies of Ivchenko (1981), who showed that molybdenum stimulates phospholipid synthesis and increases the concentration of inosine phosphatides in the roots of peas, and increases the levels of choline phospholipids in the leaves of the same plants.

Numerous observations have been made with respect to antagonisms and synergisms occurring between molybdenum and other trace elements. Thus the antagonism between molybdenum and aluminium is well known. Molybdenum gives protection from the toxic influence of mobile aluminium, an excess of which is typical of acidic soils. Millikan (1948) has reported that molybdenum is able to counteract the toxic effect of copper and other heavy metals in flax. Barrocio (1962) found that a synergism exists between molybdenum and potassium. Zhiznevskaya (1972) observed synergism between molybdenum and copper, and found that a definite mutual proportion of these elements is required for the normal development of plants. Conversely, supplying high doses of molybdenum alone may bring out the effects of antagonism between copper and molybdenum. Candela et al. (1957) have shown that the relationship between molybdenum and manganese in their effects on plant growth is such that an excess of manganese adversely affects the growth of plants suffering from molybdenum deficiency, although the molybdenum content of these plants is increased.

In summary, it may be said that despite the considerable progress that has been made in the study of the physiological role of molybdenum in nitrogen metabolism, many aspects of its activity remain obscure. Further investigations of the involvement of molybdenum in physiological processes will have to determine the degree of specificity of its activity in the individual biochemical reactions.

Chapter 6

COBALT

This trace element has been found in all higher and lower plants. High cobalt contents are found in legumes, but less cobalt is found in grasses (Kovalsky and Chebaevskaya, 1951). In the legumes, the cobalt is concentrated in the nodules where this metal performs a specific function in the process of nitrogen fixation.

High concentrations of cobalt are also found in the generative organs (Vergnano, 1958). Yamada (1958, 1960) found that cobalt accumulated in the pollen, stile and stigma. Cobalt accelerates the germination of pollen, and thus it has some influence on the processes of fertilization. This is fairly consistent with the findings of Kedrov-Zikhman et al. (1956), who demonstrated that the action of cobalt is most strongly manifested in the generative organs.

Various cobalt compounds occur in plants: ionic cobalt, cobalt porphyrin structures (in particular vitamin B_{12}; Fig.58), and cobamide co-enzymes (Wilson and Hallsworth, 1965). Cobalt is also present in some unidentified stable organic complexes.

Barker et al. (1958, 1960) isolated a new form of vitamin B_{12}, the so-called cobamide co-enzymes. A series of vitamin B_{12} derivatives has been isolated which are active as co-enzymes in an enzyme system from Clostridium tetanomorphum acting upon glutamate-β-methyl aspartate. The vitamin itself was inactive in this reaction. The component isolated represented an activated or co-enzymic form of the vitamin and proved to be 5,6-dimethyl-benzimidazole cobamide. It should be emphasized that cobamide co-enzymes and other corrinoid-co-enzymes are the most common form of vitamin B_{12} in nature. Detailed information on these compounds may be found in a monograph by Yagodin (1970).

A number of interesting investigations have appeared more recently, showing that cobalt-methylcorrinoid may act as a donor of methyl groups in the methylation of t-RNA (Walerych et al., 1966). Even before these studies were undertaken, transfer RNAs had been found to contain methylated bases (Littlefield and Dunn, 1958).

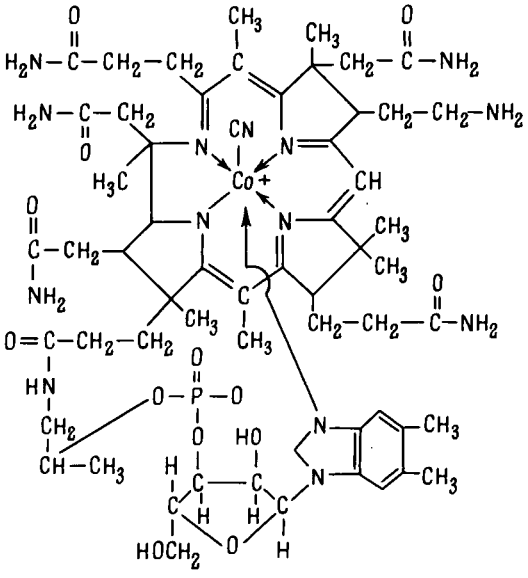


Fig. 58. Structure of vitamin B_{12}.

Vitamin B_{12} and cobamide co-enzymes are involved in nucleic acid metabolism. Thus RNA isolated from vitamin B_{12}-deficient tissues has been found to be less active in binding to amino acids than that isolated from tissues of normal animals (Wagle, 1958, cited after Walerych et al., 1966). There is a possibility that cobamide co-enzymes are involved in nucleic acid metabolism at the point of the reduction of nucleoside triphosphates (Abrams, 1965).

Vitamin B_{12} and cobamide co-enzymes have been detected in root nodules (Burton and Lockhead, 1951 ; Kliewer and Evans, 1962,1963), and Burton and Lockhead (1951) established the presence of vitamin B_{12} in the nodule bacteria. Kliewer and Evans (1962,1963) reported that 5,6-dimethylbenzimidazole co-enzyme resides in the nodules of soybeans, lucerne, peas, clover and the alder. These workers have also shown that the ineffective and parasitic races of nodule bacteria, Rhizobium japonicum and Rhizobium meliloti, in both pure culture and in the nodules of soybeans contain appreciably less cobamide co-enzyme than the amounts occurring in effective races. Observations concerning the synthesis of cobamide co-enzymes by active strains of nodule bacteria have also been made by Shemakhanova and Bunko (1969). The amounts of vitamin B_{12} and cobamide co-enzyme in nodule bacteria notably increases with an increasing

concentration of cobalt in the culture solution (Ahmed and Evans, 1961). In the experiments of Kliewer and Evans (1963), the nodule bacteria were completely devoid of the cobamide co-enzyme when they were placed in a cobalt-deficient or cobalt-free medium.

Yagodin and Ovcharenko (1969) found that in lupin and soybean nodules there were small amounts of another cobamide (besides 5,6--dimethylbenzimidazole cobamide), which had no nucleotide moiety. This was an analogue of vitamin B_{12} - the factor B_1 being more abundant in the soybean nodules than in the lupin nodules.

Nicholas et al. (1962) found 5,6-dimethylbenzimidazole cobamide co-enzyme also in cultures of the free-living nitrogen-fixing organism Azotobacter vinelandii.

In algae of the order Chrysomonadales, a growth response to vitamin B_{12} can be observed, but there is no responce to similar compounds. In the algae Rhodymeria palmata, vitamin B_{12} has been discovered, but no cobamide co-enzymes have ever been reported in this organism (Scott and Ericson, 1955). There are indications also that vitamin B_{12} occurs in the fungus Aspergillus oryzae.

Vitamin B_{12} has only rarely been detected in higher plants. Fries (1962) succeeded in demonstrating the presence of vitamin B_{12} in pea plants grown under sterile conditions.

It is important to intensify the search for other cobalt proteins and complex compounds containing cobalt. In this context, Wilson and Nicholas (1967) found low molecular weight cobalt-containing compounds, other than the known cobamide compounds, in two clover species and soybeans (Glycine max) no cobamide compounds having been detected in these hitherto. The authors suggested that low molecule weight cobalt compounds are probably present in all higher plants, and that these are similar to the cobamides occurring in microorganisms.

Cobalt has long been regarded as an essential element for animals and microorganisms, but has not been recognized as such for plants.

Buddhari (1960) reported that cobalt is required for the growth of the blue-green algae Nostoc muscorum, Calothrix garietina, Coccochloris peniecystis, Diplocystis aeroginosa. In the experiments carried out by Holm Hansen et al. (1954) on Nostoc muscorum, additions of cobalt eliminated chlorosis and resulted in a resumption of normal culture growth. Wilson and Nicholas (1967) showed that under steril conditions cobalt is essential for the growth of clover and wheat; control plants which received no cobalt showed

growth retardation and chlorosis.

What specific role does cobalt play in plant metabolism?

Cobalt is a variable valency metal, which accounts for the high value of its oxidation-reduction potential in the system $Co^{3+} \longrightarrow$ $\longrightarrow Co^{2+}$ in an acid medium. This allows cobalt ions to participate efficiently in oxidation-reduction reactions (Boichenko, 1966). Cobalt, however, has never been found to be a constituent of the active sites of the respiratory chain enzymes, nor is it involved in photosynthesis. In the 1950s metal enzymes were discovered in which cobalt was a specific metallic component. Among these enzymes are lecithinase from the alga Clostridium perfringens, acetylornithinase from bacteria, D-β-oxybutyryl-CoA dehydrogenase from animal tissues, and glycylglycine dipeptidase from animals and plants (Wakii, 1955). Cobalt was found to enhance the stability of the tobacco mosaic virus RNA, with an effect similar to that produced by nickel, manganese and zinc (Schofield and Zemecnik, 1968).

Cobalt ions were found to promote the incorporation of amino acids into isolated ribosomes of peas (Abdul-Nour and Webster, 1960), and like divalent ions of manganese, calcium and magnesium, cobalt positively influenced the interaction between aminoacyl--tRNA and ribosomes (Hershko et al., 1961), 1961).

There are indications that vitamin B_{12} is involved in nucleic acid metabolism. Thus, Lascelles and Cross (1955) reported that vitamin B_{12} takes part in the biosynthesis of deoxyribosides and DNA. Deoxyribosides of thymine, cytosine, adenine or guanine were found to substitute adequately for vitamin B_{12} in supporting the growth of Lactobacillus lactis and Lactobacillus leichmanii, but the concentrations of these compounds required to produce the same effect were 1000 times higher that those of vitamin B_{12}; pure pyrimidine and purine bases failed to produce any effect.

Studies on the role of vitamin B_{12} in protein biosynthesis have been carried out in animals and microorganisms. Areshkina et al. (1961) showed that vitamin B_{12} was required for protein biosynthesis in a B_{12}-deficient strain of Escherichia coli; the incorporation of amino acids into the structure of the proteins was significantly reduced in the absence of vitamin B_{12}. Supplying vitamin B_{12} appreciably raised amount of ^{14}C glycine incorporated into Escherichia coli proteins. These workers explained the stimulating effect being the result not of any direct influence on protein synthesis, but of the vitamin's role in methionine synthesis.

The involvement of vitamin B_{12} in the biosynthesis of the methyl groups of methionine and thymine has been demonstrated in experiments with microorganisms.

The administration of cobalt tends to elevate the protein content of legumes and other plants (Danilova and Demkina, 1967; Yagodin, 1970). Adding cobalt ions to a nutrient solution supporting the growth of oats increased the protein nitrogen 1.5 times, and the amine nitrogen 4 times (Kostir and Iracek, 1959).

One of the important physiological functions of cobalt is its participation in respiration and energy metabolism (Danilova, 1961; Yagodin, 1970). Yagodin (1970) observed an increase in the respiration rate of the nodules of broad beans after carrying out a presowing treatment of the seeds, and providing an extraroot supply of cobalt to the plants. In the latter case, the rate of respiration increased as early as 2 h after supplying cobalt. Cobalt stimulates the Krebs cycle enzymes, does not affect the glucuronic pathway, and suppresses the pentose phosphate pathway. Cobalt also stimulates the activity of phosphoglucomutase, which catalyzes the mutual conversions of glucose-1-phosphate and glucose-6-phosphate. Thus the process of glycolysis can be enhanced by cobalt (Niebroj and Kozubska, 1964).

Cobalt can elevate the activities of catalase and peroxidase (Timashov, 1958), in accord with reports on the influence of cobalt on the synthesis of haem (Laforet and Thomas, 1956). The latter is the prosthetic group of the iron-porphyrin enzymes (catalase, peroxidase and some cytochromes).

Available evidence points to the possible involvement of cobalt in one of the initial stages of porphyrin biosynthesis, that is, cobalt takes part in the synthesis of coproporphyrin and influences the activity of succinyl-CoA-synthetase. The latter enzyme catalyzes the synthesis of an early precursor of porphyrins - succinyl--CoA - from succinate and co-enzyme A (Palmer and Wedding, 1966). This is a further indication of the involvement of cobalt in the respiratory process.

That cobalt plays a part in energy metabolism has been demonstrated by Loercher and Liverman (1964), who discovered that cobalt positively affects oxidative phosphorylation. At the same time, this element plays an important part in the structural and functional organization of leaves. It contributes towards maximum occupation of the leaf surface by chloroplasts and pigments, at

at the same time increasing the leaf surface area.

In experiments in vitro, cobalt was shown to exert a positive influence on the Hill reaction. The increased data of this reaction under the influence of cobalt is accompanied by a parallel increase in the labile chlorophyll percentage (Lipskaya, 1980).

In the light of the known role of ascorbic acid in oxidation--reduction processes, data reported by Ramakrishna (1956) on the effect of cobalt on the synthesis of ascorbic acid are of interest. The experiments were carried out on Aspergillus tamanii which is normally unable to synthesize ascorbic acid, but proceeded to do so in the presence of cobalt and manganese salts.

Ting Chin-Lu and Tsung Le-Loo (1950) found that the growth of oat coleoptiles could be stimulated by cobalt. Thimann (1956) conducted a detailed study comparing the effect of cobalt on the elongation of stem cuttings with that of other metals. He observed that the elongation of pea stems was enhanced by cobalt in the presence of indoleacetic acid, the effect of cobalt being more pronounced at the elongation stage than during the twisting of the epicotyl. Thimann found that oat coleoptiles responded both to cobalt and to manganese, but the concentrations of these elements required to elicit comparable responses were different. Pea plants were almost insensitive to manganese. Thimann concluded that cobalt stimulates a stage of oxidative metabolism, probably the synthesis of ATP, which provides energy for growth. This suggestion of Thimann has recently gained support from studies of the effects of cobalt on oxidative phosphorylation (Loercher and Liverman, 1964).

Scott and Liverman (1956), Liverman (1959) carried out a series of studies on the role of cobalt in controlling growth processes. These workers showed that cobalt, like other substances such as purine analogues - (kinins) - influences the growth of etiolated leaves either in the presence or in absence of photomorphologically active red and far red light.

Liverman (1959) reported that by supplying cobalt to short-day plants during photoperiodic induction, flower-formation in these plants was inhibited. Peroxidase substrates such as catechol and pyrogallol also inhibit flowering. Liverman offers the explanation that these substrates, like auxin which also inhibits the flowering of short-day plants, cause the activity of auxin oxidase to be reduced.

Referring to a study by Galston and Siegel (1954), who observed a reduction in the rate of peroxidase synthesis in etiolated tissues upon the addition of cobalt, Miller (1954) deduced that the reason for cobalt-mediated stimulation of stem elongation lies in a decrease in the breakdown of indoleacetic acid by peroxidase.

However, Tomaszewski and Thimann (1966), who also observed a cobalt-dependent decrease of indoleacetic acid degradation by peroxidase, did not believe that this could account for the metal's growth-stimulating activity. They argued that Co^{2+}, like Mn^{2+}, stimulates elongation of the stem even in the presence of optimum doses of indoleacetic acid, and interacts with naphthylacetic acid as well as with indoleacetic acid (Fig.59). Busse (1959) offered evidence that cobalt inhibits the deposition of the secondary thickening of the cell wall because of its inhibiting effect on those reactions which restrict extension. In this way, cobalt prolongs the activity of the auxin.

Evidence has been obtained of the inhibition by cobalt of ethylene synthesis at the point of the conversion of methionine into ethylene (Abelles, 1973).

Evidence is available which indicates that cobalt is involved in other physiological processes. Thus cobalt can activate the synthesis of fatty acids, and can affect the oxidation of keto-acids, unsaturated fatty acids (linoleic and linolenic), and organic acids.

An important aspect of the physiological role of cobalt is its involvement in the fixation of molecular nitrogen. This discovery was made almost simultaneously by several investigators in 1960 (Ahmed and Evans, 1960a, 1960b, 1961; Hallsworth et al., 1960; Reisenauer, 1960).

In 1960 Ahmed and Evans, in their experiments on soybean plants, showed that when the plants received bound nitrogen no cobalt was required, whereas in the absence of bound nitrogen the plant's growth was strictly dependent upon the size of the cobalt supply (Fig.60). Vitamin B_{12} had a considerable effect, although not as great as that of pure cobalt. It has been found that a number of trace elements fail to substitute for cobalt with respect to its influence on symbiotic nitrogen fixation. Thus, Delwiche et al. (1961) reported that nodules grown in the presence of cobalt fix nitrogen much more actively than those grown in the absence of cobalt.

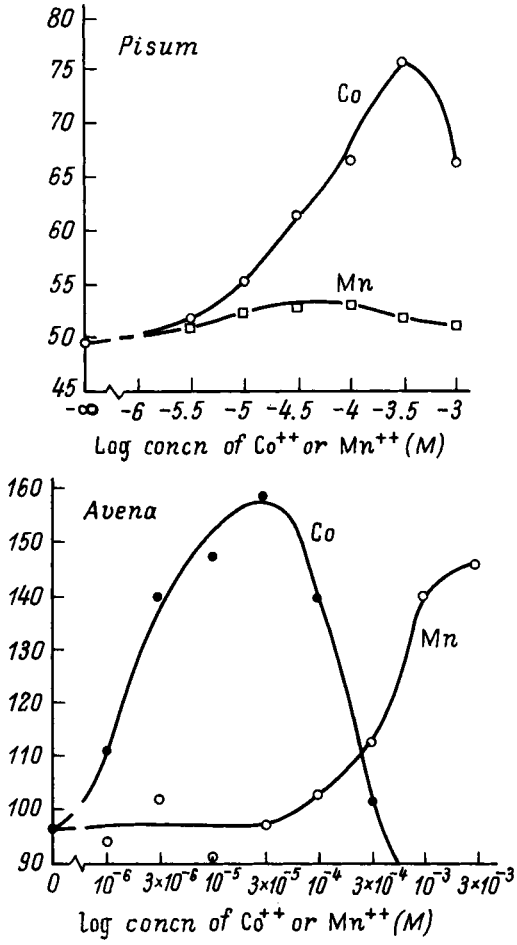


Fig. 59. Effects of cobalt and manganese on the rate of growth of pea stems (a), and oat coleoptiles (b), treated with optimum doses of indoleacetic acid (after Tomaszewski and Thimann, 1966). Abscissa, by concentration (M) of Co, Mn; ordinate, length (mm).

Bond and Hewitt (1962) observed that cobalt exerted a positive effect on nitrogen fixation in some non-leguminous plants (Alnus and Causarina trees). Johnson et al. (1966) demonstrated that cobalt is essential for the symbiotic growth of the fern Azolla filiculoides in the absence of bound nitrogen.

Cultivating Trifolium plants under sterile conditions with added Co, and infecting them with an effective Rhizobium strain produced a significant increase in vegetative mass, which was

accounted for by an enhanced rate of symbiotic nitrogen fixation (Fig. 61).

Fig. 60. Effects of cobalt on the development of soybean plants in the absence of bound nitrogen (after Ahmed and Evans, 1960a). 1, in the absence of cobalt; 2, in the presence of cobalt.

Cobalt produces considerable alterations in the ultrastructure of the nitrogen fixing apparatus. Generozova and Yagodin (1969) found that lupin nodules supplied with cobalt showed a more rapid development of the bacteroid tissue, with accelerated entry of the bacteroids into the nodule cells, and a rapid formation of the capsules surrounding the bacteroids. Cobalt increases the number of ribosomes in the cytoplasm, both in the plant cells and in the bacterial cells. A general stimulation of metabolic activity by cobalt is manifested in the enhanced mobility of the bacteroids.

Simultaneously with the discovery of the significance of cobalt in the processes of symbiotic nitrogen fixation, the important role of vitamin B_{12}, and the lesser role of cobalt in the process of nitrogen fixation in the free-living microorganism Azotobacter chroococcum were established in 1960. Credit for this discovery should be given to Iswaran et al. (1960). An increase in nitrogen fixation brought about by vitamin B_{12} could be observed even in the

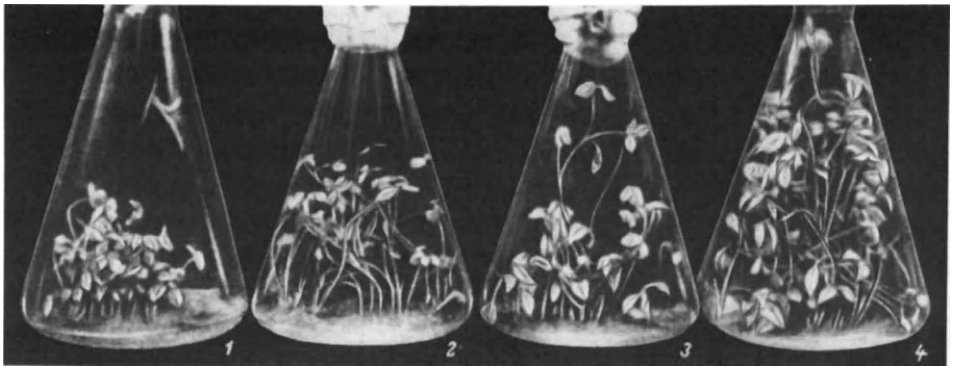


Fig. 61. Effect of cobalt and infection by nodule bacteria on the growth of Trifolium pratense in sterile culture (after Yagodin et al., 1970). 1, without inoculation; 2, without inoculation, Co added; 3, with inoculation; 4, with inoculation, Co added.

absence of cobalt. That the effect of vitamin B_{12} on nitrogen fixation cannot be attributed solely to cobalt is evidenced by the fact that under the action of cobalt itself, the effect produced is considerably weaker. Later, the essential nature of cobalt, or of vitamin B_{12} in the process of nitrogen fixation in Azotobacter vinelandii was demonstrated (Nicholas et al., 1962).

The cobalt-containing vitamin B_{12} and cobamide co-enzymes have been detected only in root nodules in which the process of symbiotic nitrogen fixation takes place and no compounds of this type have been found (except for two cases of the occurrence of vitamine B_{12}) in the other organs of higher plants. Evans and Kliewer (1964) found that cobamide compounds participate in certain enzyme reactions including group transfer reactions.

To a certain degree, the role of cobalt in nitrogen fixation may be associated with the positive effects of this element on the activities of the individual enzymes involved in nitrogen fixation. This holds true primarily for the effects of cobalt on the hydrogen donor system. There is evidence available that cobalt participates in the metabolism of hydrogen in the cell. Winfield (1955) holds that cobalt is the only metal capable of promoting the initial reaction step of hydrogen in the process mediated by hydrogenase. Peive et al. (1967) have found that the bulk of this

enzyme's activity is concentrated in the bacteroids.

In the light of the pronounced dependence of the nitrogen fixing activity of nodule bacteria on their dehydrogenase activity, of considerable interest too, is evidence concerning the activation of isocitric acid dehydrogenase by cobalt, and the positive effects of cobalt (supplied in ionic form or as chelate compounds) on the activity of melate- and succinate dehydrogenases in the nodules of legumes. The highest dehydrogenase activity is observed in the bacteroids, the activity being considerably lower in the vegetative tissues of the nodule (Peive et al., 1968). It has, moreover, been shown that the peak of dehydrogenase activity in root nodules coincides with the phase of flower bud formation, i.e., during the period of the most intense nitrogen fixation (Peive et al., 1964; Peive et al., 1965).

Zhiznevskaya (1972) suggested that the role of cobalt (in the form of cobamide compounds) in nitrogen fixation amounts to activation of the processes of protein biosynthesis in the nodules, and therefore cobalt may also activate biosynthesis of the nitrogenase protein as well as biosynthesis of the nodule haemoglobin.

A paper by Troitskaya (1976) presents a survey of publications on the distribution of compounds related to the vitamin B_{12} group or corrinoids in root nodules and nodule bacteria. The correlation between the content of corrinoids and the nitrogen-fixing activity of the nodules is shown. The author suggests that since they participate in the synthesis of principal metabolites such as succinate, methionine, and desoxyribonucleoproteids, and thus control some of the chief metabolic pathways corrinoids have an indirect effect on the formation and function of the nodule nitrogen-fixing apparatus.

Yagodin (1970) succeeded in demonstrating that cobalt exerts a considerable influence on the metabolism of the leguminous host plant itself, including those aspects of metabolism associated with biosynthesis in the leaves and the supply of metabolites which are important for the process of nitrogen fixation to the nodules.

The possibility of the involvement of cobalt at certain stages of chlorophyll biosynthesis have been reported in the literature. Relevant evidence is summarized in a book by Yagodin (1970), who observed an increase in the contents of chlorophyll, carotenoids, total haematin and vitamin E, all of which are related to chlorophyll. He also obtained indirect evidence that cobalt controls

the utilization of phytol in seedlings, and the afflux of haematin compounds to the nodules. The amount of cobalt available to the nitrogen-fixing apparatus is related to the degree of formation of cobamide compounds in Rhizobium. Brownstein and Abeles (1961) suggested that in many reactions, hydrogen transfer is performed by a cobalt cobamide.

Cowles and Evans (1968) have found that the B_{12}-coenzyme in bacteroids and nodule bacteria is a constituent of ribonucleotide reductase. The synthesis of the apoenzyme of ribonucleotide reductase proceeds under conditions of cobalt deficiency, but the synthesis of the B_{12}-coenzyme is disturbed, which results in a considerable reduction in the activity of this enzyme. The reduced activity of ribonucleotide reductase when there is cobalt deficiency interferes with the synthesis of deoxyribonucleotides and DNA, and thus interferes with cell division (Cowles et al., 1969).

The specific influence of cobalt on nitrogen fixation is accounted for by the part played by vitamin B_{12} and cobamide coenzymes in this process, is corroborated by the following observations. Rhizobium strains which are ineffective in nitrogen fixation, as well as nodules formed by these strains, contain considerably less vitamin B_{12} than active strains and nodules formed by active bacteria (Shemakhanova and Sidorenko, 1970; Kliewer and Evans, 1963). Moreover, bacteroids of the nodules formed by ineffective Rhizobium strains have notably reduced amounts of the catalytically active, protein-bound form of the B_{12}-coenzyme, whereas in the bacteroids of nodules formed by effective strains, most of the B_{12}-coenzyme is represented by its proteinbound form.

Yagodin et al. (1973), taking into account the above findings, agree that there is a close relationship between compounds of the vitamin B_{12} group and the nitrogen fixation system in legume nodules.

Chapter 7

NICKEL AND VANADIUM

Nickel

Nickel is widely distributed in the plant kingdom, some plants being concentrators of nickel (Malyuga, 1946). An outstanding representative among such plants is Alyssum bactolonii. The ash of this plant's leaves contains 10% NiO, and the ash of its seeds contains 9.24%. As a rule, the amounts of nickel present in plants is larger than the amounts of cobalt.

Knypl (1980) provided data on the content of nickel in plants, its distribution in different plant organs, and the different forms of nickel occurring in plants. In a survey by Welch (1981) the biological importance of nickel is elucidated in detail. This element is necessary for the growth of some bacteria and blue-green algae. It is needed for the normal growth of some Pinus species. Being a component of the enzyme urease which hydrolyses carbamide to give NH_3 and CO_2, it is required for the culture of tissues from soybean, Lemna, Spirodella and Wolfia, where the cultures are grown on media containing carbamide as the only source of nitrogen.

Nickel and cobalt are intimately associated geochemically. Their atomic weights are very close (58.94 and 58.69), which is reason to expect they have similar physiological roles. In fact, animal physiology has accumulated much evidence to support this view. Nickel is involved non-specifically in a number of metto- - enzyme complexes. Arginase from Canavalia ensiformis L., converting arginine into ornithine and urea, may be activated by nickel (Agarwala, 1952). Nickel was found to stimulate α - amylase from barley (Corello and Boisio, 1955). The elevated activity of inorganic acid pyrophosphatase in ageing detached rice leaves decreases when the leaves are kept in a solution containing 237.7 ppm nickel (Mishra et al., 1973). Mishra and Kar (1974) reported that an excess of nickel depresses catalase activity in mature plants and stimulates its synthesis in rice seedlings. This is attributable to the fact that in the first case excess nickel prevents the incorporation of iron into protoporphyrin, thus causing catalase activity to decrease, whereas in the second case excess nickel

enhances the synthesis of the protein part of catalase, or at least increases the accessibility of iron to the porphyrin of the enzyme.

Rubin and Chernavina (1970) presented evidence to show that nickel acts as a stabilizer for antocyan pigments. This action of nickel is associated with a dramatic increase in the activities of ascorbate and phenol oxidases.

Fujiwara and Kikuchi (1950) reported that nickel is essential for soybeans. Venkata (1961) succeeded in eliminating the symptoms of blister blight in Camelina sinensis by supplying nickel. Probably the requirement of some legums for nickel is related to the role of this metal in symbiotic nitrogen fixation. As has been noted, the role of cobalt in nitrogen fixation has been firmly established, but evidence for any part played by nickel in this process is lacking. From experiments on models of the process of nitrogen fixation it has become clear that nitrogen forms linear complexes on metal surfaces (Me... N = N) analogous to carbonyl complexes and organic diazo-compounds. Such complexes have been observed on various metals, including nickel. Although there is a large difference between the complexing of nitrogen on a metal surface and enzymatic nitrogen fixation, further reactions of the nitrogen on metal surfaces being obscure, these model experiments support the view that elucidation of the positive involvement of nickel in the process of nitrogen fixation may be a promising line of research.

Observations have been made which indicate that nickel positively influences the activity of nitrate reductase, an important enzyme both in the reduction of nitrates and in nitrogen fixation (Naranville, 1970). These findings, however, have not been made in leguminous plants, although in the crop plant sorghum, a six fold increase in activity was found on account of the ability of nickel to counteract the inhibitory effect of cyanide (Mishra and Kar, 1974).

Interesting observations have been communicated by Bushnell (1966), who studied the effects of kinetin and benzimidazole on the ageing of leaves. The application of a drop of these compounds to detached yellowing wheat leaves resulted in the appearance of a green spot at the site of application. The administration of nickel salts ($1 \cdot 10^{-3}$M) also prevented the leaf from yellowing. All those substances which delay yellowing of the leaves

have been shown to produce a localized accumulation of nitrogen and starch. In non-detached leaves on the other hand, nickel salts, in contrast to kinetin, failed to delay yellowing but were efficient in preventing any decrease in nitrogen.

Crooke and Knight (1955) reported on the relationship between nickel toxicity and the uptake of both nickel and iron. In oat plants growing in sand cultures with a high nickel concentration, it could be shown by autoradiography that the extent of leaf necrosis caused by nickel was related to the nickel content, whereas chlorosis was attributable to changes in the nickel-iron ratio.

The uptake of Ni^{2+} and its transport into stems was inhibited by Cu^{2+}, Zn^{2+}, Fe^{2+} and Co^{2+} (Cataldo et al., 1978).

It is apparent from the material presented here that the problems of nickel as an essential nutrient for plants and of the specificity of its activity in plant metabolism still await solution.

In studies reported by animal physiologists, nickel is regarded as a bioelement.

Vanadium

The involvement of vanadium in biological processes was first recognised in 1911 by Henze, when he discovered an organic compound containing vanadium in the blood of some marine animals.

In a number of studies, Bortels (1936) showed that vanadium is able to stimulate the fixation of molecular nitrogen and the growth of Azotobacter in cultures devoid of bound nitrogen. Similar results have also been reported by other investigators (Burk and Horner, 1935). Subsequently Bortels (1937) found that vanadium, like molybdenum, is essential for the growth of a number of Azotobacter species, but the maximum stimulation obtained with vanadium is only 50%-80% of that obtainable with molybdenum (Horner et al., 1942). Bortels (1940) also reported that vanadium can replace molybdenum as a catalyst in the process of molecular nitrogen fixation in a number of soil microorganisms. This conclusion was later extended to all nitrogen-fixing blue-green algae, and in recent model studies of biological nitrogen fixation, it was found that only vanadium was capable of replacing molybdenum. In alkaline media, vanadium compounds have been shown to be able to reduce nitrogen to hydrazine and ammonium at a rapid rate, even at room temperature and in an atmosphere of nitrogen at 760 mm Hg.

It should be emphasized that Bortels (1940) reported data on the substitution of molybdenum by vanadium that were less convincing than data obtained previously. Arnon (1938) referred to these latter findings to support the view that the function of molybdenum cannot be taken over by vanadium in Anabaena.

McKenna et al. (1970) carried out detailed studies to find out whether vanadium can replace molybdenum in the process of nitrogen fixation in various Azotobacter races. By substituting molybdenum by vanadium in a nutrient medium supporting the growth of nitrogen--fixing Azotobacter vinelandii OP cells, he was able eventually to obtain cell-free extracts which contained considerable amounts of non-dialysable vanadium. These extracts showed considerably reduced nitrogenase-specific activity, a reduced content of molybdenum and a lower ability to reduce acetylene, in comparison with other extracts derived from the cells grown in media supplied with molybdenum. The authors conclude that vanadium can replace molybdenum in supporting nitrogenase activity.

In a later publication (Benemann et al., 1972) it was demonstrated that in Azotobacter vinelandii OP and Azotobacter chroococcum, vanadium is able to meet efficiently the requirements for molybdenum in nitrogen-fixing cell cultures. Measurements of nitrogenase activity in growing cultures have shown, however, that the efficiency of vanadium is 10 times as lower than that of molybdenum. With respect to specific activity, resistance to oxidation in air and resistance to destruction by heating, the enzyme isolated from cells grown on vanadium displays characteristics that are intermediate between these of nitrogenase from cells cultivated with and without molybdenum. Purification of nitrogenase from cells grown on vanadium has revealed that vanadium is incorporated into the enzyme as a constituent of the protein, in the same way as are the molybdenum and the iron-containing protein moiety of nitrogenase. The effects of vanadium observed in cells cultivated in molybdenum deficient media are determined, according to these workers, by the nature of the incorporation of vanadium into the nitrogenase complex, and by the degree of stabilization afforded to the enzyme's structure; the metal is not responsible for maintaining the catalytic activity of the enzyme.

Peterburgsky and Tormasova (1969) believe that vanadium complements and enhances the functioning of molybdenum, and stimulates, moreover, nitrogen fixation in legumes. On strongly acidic soils

vanadium is as efficient as molybdenum, and although it is already clear that some sort of relationship exists between vanadium and molybdenum in the process of nitrogen fixation, this issue is far from being resolved unequivocally. Bortels (1937) found only a mild effect of vanadium in legumes. Dmitriyev (1938) failed to observe any effect of vanadium on clover grown on sub-limy soils. According to Peive (1971), no effect of vanadium can be seen in the presence of molybdenum.

Velours et al. (1973) found that vanadium at a concentration of 2 mM completely inhibits the succinate-oxidation-dependent mitochondrial swelling induced by phosphate or acetone, but that does not influence swelling that is dependent upon ATP. Vanadium was shown to suppress succinate dehydrogenase activity by 83%, although it did not affect malate and NADH dehydrogenases. This element does not interfere with the transport of phosphates into mitochondria, but it considerably increases the release of dicarbonic acids (malate and succinate) from mitochondria into the ambient medium. It also inhibits the transport of dicarbonic acids into mitochondria.

Bertrand (1941) showed that vanadium is essential for the growth of Aspergillus niger. Vanadium is also essential for the growth of Scenedesmus obliquus, in which it is involved in photosynthesis (Arnon, 1954). A shortage of vanadium, in contrast to a shortage of manganese, resulted in a considerable decrease in the chlorophyll content of this alga. The rate of photosynthesis could be elevated only at a high illumination intensity, (Fig.62). Arnon (1958) reported that vanadium increased the maximum rate of the Hill reaction in isolated chloroplasts. Warburg et al. (1955) have shown that vanadium plays a role in photosynthesis in Chlorella.

Meish and Bielig (1975) studied the effect of vanadium on the growth of Chlorella pyrenoidosa, Scenedesmus obliquus and Chlorella vulgaris. Together with iron in the form of $FeCl_3$, vanadium accelerated the growth rates of these algae five to six times, but the iron when present as Fe(III)EDTA or Fe(III) citrate, vanadium had little effect. The rate of formation of protoporphyrin IX was 83% higher. It was suggested that vanadium stimulated the biosynthesis of chlorophyll precursors at the stage of protoporphyrin IX.

The problem of the requirements of higher plants for vanadium has received considerable attention. Positive evidence has been

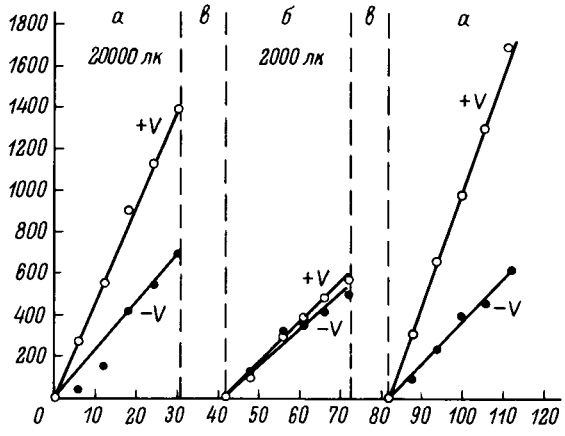


Fig. 62. Effect of vanadium on the rate of photosynthesis in Scenedesmus obliquus at different intensities of illumination (after Arnon, 1962). Ordinate, light intensity (mcal per hour per mg); abscissa, time (minutes).

obtained that peas, asparagus, lettuce, maize, and flax grown in both water and sand culture do require vanadium (Maze and Maze, 1939; Peterburgsky and Tormasova, 1969; Kevorkov and Kudryashov, 1970). However, no symptoms of vanadium deficiency have ever been recognized in higher plants.

Interesting observations have already been discussed, indicating that vanadium and molybdenum inhibit ribonuclease (Vlasyuk and Kuznetsova, 1972). In the chapter dealing with molybdenum, it was pointed out that vanadium plays a role in the initial stages of seed germination, by virtue of its activating effect on genom(e) - total genetic apparatus of the cell. All these somewhat inconclusive findings necessitate that further efforts be made to establish the significance of vanadium in the life of higher plants.

Chapter 8

LITHIUM, SODIUM, RUBIDIUM AND STRONTIUM

Lithium. Numerous studies are available on the accumulation of lithium in brown algae; the relevant information can be found in a review by Vinogradov (1953) and Borovik-Romanova (1969). This latter author reported comparatively high concentrations of lithium in Anodimenia palmato and Chara hispida. High lithium levels (66--100 mg per kilogram raw weight) have been observed in marine flowering plants (Zostera nana, Zostera marina and Ruppia spirales). In the brown algae the concentration of lithium is 18 times lower than that of rubidium.

Although marine organisms feature the highest levels of lithium, one can also find lithium-concentrator species among terrestrial plants. Thus a special lithium flora is known (Vinogradov, 1957) comprising, among others, several species of the Ranunculaceae and Solanaceae.

Lithium concentrations in plants vary within wide limits; for example, plants growing in the Zarafshan valley which features sulphate salinity in the soils and high lithium concentrations, show strikingly large variations in lithium content from 0.01 to 9000 mg per kg. By supplying lithium to the soil, the concentration of the metal in tobacco leaves of the lower layer may reach as much as 20.3 g per kg ash weight (equivalent to 6.1 g per kg dry weight) (Ezdakova, 1973).

Among concentrators of lithium, Ezdakova (1973) believes that Nicotiana tabacum, Datura stramonium and Hyoscyamus niger are notable lithiumphiles. She maintains that lithium is essential for lithiumphiles, and has shown this to be the case in the three latterly named species.

An important problem is that of the relationships between lithium and sodium and between lithium and potassium. In animal cells these relationships have been studied in detail by Skulsky (1969), and Ezdakova et al. (1970) reported similar relationships in plants. The sodium and potassium content of plants is a function of the lithium concentration and is subject to considerable variation, so that the concentration of potassium is less sensitive to lithium variations than is the amount of sodium. Ezdakova et al. believe that this effect of lithium should be regarded not

only as a consequence of direct ionic interactions, but also as a catalytic effect on the active transport of potassium and sodium across membrances. There is evidence of inhibition by lithium of cellular uptake and release of potassium and sodium, as well as inhibition of calcium uptake, The effect of lithium on the permeability of membranes is associated with the activation of acetylphosphatase (Meis, 1969), which is involved in the supply of energy for active ion transport. Epstein and Hagen (1952) believed that the role of lithium in the control of permeability amounts to a modification of a carrier protein.

Antagonism between lithium and calcium has long been known (Frerking, 1915). Calcium prevents the uptake of lithium and potassium by the roots of plants, whereas low doses of lithium significantly lower the calcium content of plants (Epstein, 1960). Frerking (1915) made the interesting observation that lithium is poisonous only to those organisms which require calcium, but is harmless to those which do not take up calcium, such as algae and fungi. The data referred to show that the physiological role of lithium may be assessed largely in terms of its interactions with other mineral nutrients.

Observations have been made indicating similarities between the effects of lithium, sodium and potassium on some metabolic processes.

In studies of the effect of the ionic environment and the effect of the substitution of one alkaline metal cation for another on the secondary structure of DNA, Kuznetsov et al. (1967) showed that the magnitudes of the intrinsic viscosity of DNA in lithium, sodium and potassium salts in 0.14 M solutions of the respective chlorides were very nearly the same (12 de g^{-1}). Both lithium and sodium activate poly-β-hydroxybutyric acid depolymerase in halophilic plants (Evans and Sorger, 1966). Holmes and Halvorson (1965) concluded that halophilic enzymes are completely inactivated in the absence of monovalent cations as a result of denaturation of their proteins. All this evidence indicates that lithium is probably essential for halophilic plants, which are concentrators of this element.

Of considerable interest is the role of lithium in the metabolism of nitrogen compounds. AMP-aminohydrolase, or adenyldeaminase, which hydrolyses adenosine monophosphate to yield inosine monophosphate and ammonium, is activated by ATP. Setlow and

Loewenstein (1967), and later Severin et al. (1970) have shown that adenyldeaminase can also be activated by alkaline metal ions. According to Setlow and Loewenstein, the addition of chlorides of alkaline metals (up to a concentration of 0.2 M) to adenyldeaminase from calf brain results in a considerable enhancement of the rate of hydrolysis, even in the absence of ATP. Of the alkaline metals studied, lithium proved to be the most potent stimulator of adenyldeaminase. Setlow and Loewenstein explained this strong effect of lithium ions in terms of its greater ability, compared with that of other ions, to penetrate into the negatively charged regions of the enzyme. This points to one of the important aspects of the specific role of lithium in metabolic processes.

Majer (1930) reported that the activity of chlorophyllase, inactivated by the removal of electrolytes from the enzyme preparation, could be restored by supplying various salts. Of the salts tested, the most effective in this respect proved to be the chlorides of alkaline metals, especially lithium chloride. Also, a considerable increase in chlorophyllase activity was reported by Ezdakova (1973) in tobacco and cotton.

Golovina (1964) also reported findings indicating that lithium has an influences on enzyme processes. In her experiments, lithium considerably increased the activity of invertase in sugar beet leaves, especially when sugar was being accumulated. It also affected the transport of sugars from the leaves to the roots.

Lithium has been found to exert a positive influence on photosynthesis in tobacco leaves (Ezdakova, 1973). Vlasyuk et al. (1968) observed that lithium increases the photochemical activity of chloroplasts, and increases the chlorophyll content of potato and pepper plant leaves.

One of the specific features of the physiological role of lithium is its ability to control the biosynthesis and accumulation of alkaloids and alkaloid precursors. Thus, Ezdakova and Osmolovskaya (1964) found that the application of lithium to the soil resulted in an increased nicotine content in the leaves and roots of Nicotiana tabacum. Moreover, they demonstrated the positive effects of particular doses of lithium on the synthesis of tropane alkaloids (atropine and hyoscyamine) in Datura leaves. They also revealed a close dependence between the concentrations of these alkaloids and their precursors (the amino acids phenylalanine and ornithine).

The fact that many lithiumphiles belonging to the Solanaceae and Ranunculaceae are alkaloid-bearing plants also testifies to the involvement of lithium in the metabolism of alkaloids. A high alkaloid content is typical also of saltworts (Sokolov, 1952), many representatives of which belong to the lithium flora. Ezdakova (1973) believes that the general characteristic of a high alkaloid content among lithiumphiles is confirmed by the fact that in the absence of lithium the amount of alkaloids in the leaves of Datura stramonium, for example, is very low in both young and mature leaves.

The lithium ion has the smallest radius of the alkaline metals, but in water it acquires the largest radius of the water of hydration. According to Grinchenko and Golovina (1962), the appearance in cellular colloids of hydrated lithium ions should increase the amounts of free and bound water in cells. In fact, their observations indicate that lithium increases the water retention capacity of tissues. Ezdakova (1961) found that the amount of colloid-bound water was increased by lithium.

A teratological effect of lithium has been reported in animal physiology. Thus it has been shown that lithium interferes with the course of histogenesis in amphibia.

The problem of morphological alterations in plants caused by lithium salts has received little attention. Interesting findings were obtained by Haccius (1956) who observed joining of the seed lobes of Eranthis hiemalis treated with $LiCO_3$. Prolonged watering of pink plants with lithium salts over several years resulted in fusing of the segments of the calyx (Puccini, 1957). Ezdakova (1973) reported the emergence of petals of various forms, as well as changes in the colouring of the corolla in Dianthus uzbekistanicus, which is notable for its high lithium content. She also observed morphological changes in the vegetative and reproductive organs of Eranthis longistipitata plants which were rich in lithium. She found morphological alterations attributable to high lithium concentrations also in representatives of the Solanaceae.

Teratological alterations appear as a result of disturbances in the process of mitosis. In this context, reference should be made to the studies of Korovina and Dampel (1945), who noted an inhibiting action of lithium on mitotic division in onion root meristem cells. In fact, evidence is available which shows that DNA synthesis is inhibited by lithium ions (Turkington, 1968; Volm et al.,

1970). With respect to their stabilizing action on viral RNA, ions of alkaline metals may be arranged in the following order: Li^+ K^+ Rb^+ Cs^+; with respect to the strength with which they bind to DNA, the order is: Li^+ Na^+ K^+ (Soroka, 1969).

Berg (1968) reported that lithium in high doses produced an effect similar to that of Actinomycin D, considerably reducing DNA--dependent RNA synthesis in sea urchin eggs. Belitsina et al. (1971) reported that lithium was a potent reducing agent acting within ribosomes.

Sodium. The first indications of the essential nature of sodium for plants were reported in a paper by Hellriagel and Willfarth (1898).

Later, investigations were focused on the positive results obtained with sodium in potassium-deficient plants (Lehr, 1941; Dorph-Petersen and Steenbjerg, 1950; Harmer et al., 1953). The response to sodium under conditions of potassium deficiency varies among different plants and also depends upon the composition of the nutrient medium. In spinach, for example, sodium can replace up to 7/8 of potassium.

Harmer et al. (1953) divided plants into four groups: (1) plants requiring sodium in the absence of potassium (lucerne, barley, oats, tomatoes, Brussels sprouts, carrots); (2) plants showing a low requirement for sodium under potassium deficiency conditions (maize, red clover, lettuce, onions, potatoes); (3) plants displaying moderate requirements for sodium when adequately supplied with potassium (some members of the Cruciferae, wheat, peas); (4) plants showing a strong dependence on sodium when adequately supplied with potassium (celery, beet, turnips, and particularly sugar beet).

Joanson and Joman (1971) reported on the effects of calcium on the uptake of sodium by isolated cotton roots, and the effects of sodium on the uptake of calcium. At low concentrations (0.45--0.82 mM), sodium ions competitively inhibited the uptake of calcium ions; an increase in sodium ion concentration (0.82-1.64 mM) enhanced the uptake of calcium ions. Conversely, calcium ions at low concentrations stimulated sodium uptake, whereas at high concentrations of calcium (above 0.2 mM), sodium uptake was inhibited.

The first decisive evidence that sodium is essential for plants was offered by Allen and Arnon (1955). These workers showed that small amounts of this element are essential for the growth of Anabaena cylindrica. They also found that sodium was essential for

growth of other blue-green algae. Long before these discoveries were made, it had been established that sodium is required for growth in sugar beet (Lehr, 1941).

Brownell and Wood (1957) found that sodium is an essential element in Atriplex vesicaria; in the absence of sodium the growth rate of Atriplex vesicaria was one tenth of the normal rate. The essential nature of sodium was later demonstrated for other species as well. The addition of as little as 0.1 mgEq of sodium per litre of medium restored normal growth. Williams (1960) reported his finding that sodium is indispensible for growth of the halophyte Halogenton glomeratus, which accumulates oxalic acid. The concentration of the latter is directly proportional to the sodium content of the plants. Moreover, sodium increases the resistance of the plant to wilting, and increases its yield as a crop fivefold. By supplying even minor quantities of sodium, the crop yield of tomato plants was considerably increased (Woolley, 1957).

In the survey conducted by Brownell (1979), evidence is presented that sodium is an indispensable element for plants, and the metabolic and physiological effects of low sodium concentrations in the higher plants and algae of Australia are considered.

A requirement for sodium has been found in species that depend on the C_4-dicarbonic photosynthetic pathway. In six such species grown on nutrient media containing 0.08 mgEq of sodium per litre, the crop yield is depressed and the leaves become chlorotic and necrotic. In Portulaca grandiflora, no flowering can be observed under such conditions. Additions of sodium chloride at a concentration of 0.1 mgEq per litre make for normal development of the plants, and increase crop yields two to three times. No symptoms of sodium deficiency appear in plants that feature the C_3-dicarbonic route.

The physiological role of sodium has been little studied, but its significance in plant respiration has been demonstrated. Thus it has been found that in sodium-deficient Atriplex nummularia, respiration is considerably reduced. It takes several hours following the addition of sodium to the nutrient medium for respiration to be restored, while the symptoms of deficiency in the leaves take 5 days to disappear (Brownell and Jackman, 1966).

Gordon and Bichurina (1973) observed a considerable increase in the respiration rate of 6–8-day-old wheat seedling roots immersed in 0.05 M sodium chloride. The authors carried out inhibitor

studies which led them to suggest that this stimulatory effect of sodium chloride was a result of activation of the transport system of the cells, in particular the ATPase system of the plasmalemma, in addition to the immediate influence of sodium ions on the respiratory chain. Activation of Na-K-dependent ATPase may also account for the observed stimulation by sodium ions of oxygen uptake (Stroganov et al., 1970). (Na^+-K^+)-dependent ATPases have frequently been discovered in plant tissues (Vyskrebentseva and Krasavina, 1971; Bouling et al., 1972. A sodium pump is also thought to operate in root cells.

Judging from the available evidence, sodium is not a physiological analogus of potassium (El-Sheikh and Albert, 1970).

Mazel and Zhitneva (1973), in studies of the kinetics of potassium, rubidium and sodium uptake by maize roots, found that sodium is taken up by plant cells at a rate considerably lower than the rate at which potassium (or rubidium) is taken up. There are also considerable differences in the behaviour of these metal cations. Thus it has been found that as soon as a particular concentration of sodium is reached in the cell, the element begins to be excreted from the cell. That the release of sodium occurs in the cells of all root tissues, including the meristemic cells which have no vacuoles, led the authors to suggest that the excretion occurs through the plasmalemma. This excretion of sodium, according to their view, is not connected with the sodium pump since the latter operates continuously in the cell, while the excretion takes place only when a threshold concentration of the element is reached.

Bowen (1966) drew up a classification of all elements essential for plant growth, grouping them according to their function electrochemical, catalytic, structural, or other. He considers sodium as belonging to the electrochemical group of elements comprising the cations Na^+, K^+, Ca^{2+} and Mg^{2+}, and anions Cl^-, NO_3^-, HCO_3^-, SO_4^{2-} and PO_4^{3-}. According to Bowen, Valonia cells growing in sea water as well as human erythrocytes are richer in potassium and magnesium, but contain lower concentrations of sodium and calcium than are present in the respective ambient media. The situation in the cell, according to Bowen, represents that of a storage battery charged with free energy. In fact, the cell is able to maintain a potential of 50 to 100 mV across its membranes. Metabolic energy is required for operation of the sodium-potassium pump, and it has been calculated that some 18-28 sodium ions traverse the

cell membrane per oxygen molecule used. Differences in the ability to transport alkaline metals in different cells may be accounted for by variations in the distribution of ATPases on both sides of the mebrane. The stored energy may be readily accessible in some types of cells; the stimulation of nerve cells, for example, causes potassium and magnesium to leak out of the cell, while sodium and calcium are taken up. Referring to these observations, Bowen points out that both calcium and magnesium apparently control the permeability of cell membranes to sodium and potassium. However, it remains unclear how the differential flux of ions is linked to energy utilization.

Gordon and Bichurina (1973) used antimycin A to study possible energy sources for sodium transport in root cells. The antibiotic, which inhibits respiration, was found to diminish the stimulating effect of sodium - a result attributed by the authors to disturbances in the intramitochondrial electron transport and strong inhibition of ATP synthesis. Gordon and Bichurina (1973) refer to the available evidence indicating that monovalent metals are essential for the respiratory electron transport system to function properly (Kudzina, 1970; Tashmukhamedov, 1971). These cations act at a site in the respiratory chain located between cytochrome b and c-1 (the antimycin-sensitive site).

In the conductive bundles of Beta vulgaris and Heracleum sosnowskyi leaf stalks, Bouling et al. (1972) detected $(Na^+ + K^+)$-dependent ATPase activity. The enzyme can be activated by sodium ions over a wide range of concentrations. This activation by sodium and potassium ions is pronounced in the ATPase and only weakly in the ATPase reactions. These workers discuss the involvement of $(Na^+ + K^+)$-ATPase in the mechanism of transport of organic compounds in the phloem. It is suggested that the presence of ion concentration gradients in the sieve tubes and ATPase in the conductive tissues may create conditions favourable for a mass flow of liquid, which probably serves to translocate assimilates over long distances.

Brownell and Nicholas (1967) reported on the effects of sodium on nitrogen metabolism. Anabaena cylindrica growing on a medium containing nitrates required high doses of sodium (0.4 mgEq of NaCl per litre). On media deficient in sodium and containing the nitrate form of nitrogen, nitrates accumulated in the medium. At low concentrations (0.1 mM), various nitrogen compounds including

ammonium chloride, amides and amino acids notably suppressed the accumulation of nitrite in sodium-deficient algal cultures. Upon supplying the same amount of nitrite as that usually accumulated in sodium-deficient cultures to normal cultures of the alga, and normal plants developed chlorosis. The loss of chlorophyll in this case was caused by the toxicity of the nitrite.

Brownell and Nicholas (1967) have also showed that a shortage of sodium resulted in enhanced rates of incorporation of $^{15}NO_3$, $^{15}NO_2$, $^{15}NH_3$ and ^{14}C-glutamate into algal proteins. The activity of nitrate reductase increased considerably with the omission of sodium from the medium. Inhibitor studies performed with chloramphenicol indicated that sodium controls the activity of nitrate reductase via protein synthesis. A chortage of sodium brought about a reduction in the rate of nitrogen fixation in Anabaena cylindrica cells. The problem of the possible involvement of sodium in molecular nitrogen fixation in blue-green algae is of major interest, and research along these lines seems to be promising.

The effects of high doses of sodium chloride on plants are omitted from our discussion, since a vast body of literature on this subject has now been accumulated. The interested reader is referred to a detailed review by Stroganov et al. (1970).

Rubidium. This element is widely distributed in plants. Some groups of algae are concentrators of rubidium, the concentration in some Macrocystis plants being as high as 130 mg per kg dry matter; in the brown algae rubidium concentrations are 19-120 mg per kg ash.

Attempts have been made to replace potassium with rubidium in media supporting the growth of various plants. In the experiments of Richards (1941,1944) rubidium showed the best results against a low potassium background, and a partial substitution of potassium by rubidium could be observed. This effect was not observed in the presence of high levels of phosphorus, suggesting an antagonism between phosphorus and rubidium. Thus in plants growing on very low levels of potassium or phosphorus, rubidium augments sterility, whereas with a moderate or high potassium concentration, and in the presence of a plentiful supply of phosphorus or ammonium nitrogen, rubidium enhances the yield of fertile seeds.

El-Sheikh and Albert (1970) studied the interactions between rubidium, sodium, and potassium in the nutrition of sugar beet. These workers evaluated the growth of beet plants at various con-

centrations of three elements in the medium, and found that if rubidium was supplemented with a ranged potassium levels in the strict absence of sodium, it increased both the dry weight of the plants and their sugar content. When sodium was present in the nutrient solution, no such positive effect of rubidium could be observed. Rubidium and sodium accelerated the translocation of potassium from the stalks to the leaf blades.

At this point we may consider some of the findings that give some explanation, of the ability of rubidium and sodium to replace potassium. Thus, for example, it has been shown that in higher plants rubidium or sodium can replace the potassium that is required (together with magnesium or manganese) for the activity of pyruvate kinase (Miller and Evans, 1957). Latsko and Claus (1958) reported that potassium could be replaced by rubidium as far as photosynthetic phosphorylation was concerned, but that the efficiency of the process was then reduced. Richards and Berner (1954) have established that the positive effects of rubidium and sodium in cases of potassium shortage in barley are attributable, at least in part, to the ability of these elements to prevent the synthesis or accumulation of putrescine. The abnormal level of amino acids produced by potassium deficiency can also largely be normalized by rubidium or sodium, whereas excess rubidium can reproduce some of the symptoms of potassium deficiency. The effects of rubidium and potassium on the transformation of ammonium nitrogen into non-toxic products may be explained by the influence of these elements on amino acid metabolism; ATP is required for the activation of the amino acids used in protein synthesis and for the synthesis of amides from ammonium, and the synthesis of the necessary ATP may be disturbed as a consequence of the reduced activity of the pyruvate system (Hewitt, 1963).

Interesting findings concerning the relationships between potassium, rubidium and sodium have been communicated by Hiatt and Evans (1960) from their studies of the acetate thiokinase system in higher plants. Acetate thiokinase, which catalyzes reversible pyrophosphorylation (acetate+ATP+CoA $\rightarrow$ acetyl-CoA+AMP+pyrophosphate), resembles pyruvate kinase in that it has a requirement for a divalent ion (magnesium or manganese) in addition to a monovalent ion (potassium, rubidium or ammonium). Both enzymes are equally active at similar concentrations.

In contrast to rubidium and potassium, sodium does not support acetate thiokinase activity, which accounts for its inability to substitute completely for potassium. At the same time, sodium and replace potassium in the activation of ketohexokinase (Dixon and Webb, 1958). The greater ability of rubidium, compared with that of sodium, to replace potassium may be a result of the greater difference that exists between the properties of sodium and potassium, than between those of rubidium and potassium. For example, the difference in the radii of potassium and rubidium ions is only 11%, whereas the corresponding difference between potassium and sodium is 33%.

However, only in sugar beet has it been found that there is a definite requirement for rubidium (El-Sheikh and Albert, 1970).

Summarizing, it may be said that the role of rubidium in plants is not limited to its ability to replace potassium. It may be speculated that in algae, and very probably in the brown algae that are capable of concentrating rubidium, this element performs yet unknown, but specific role.

Strontium. Mevius (1927) demonstrated that strontium is able partially to replace calcium in those plants which exhibit an enhanced requirement for calcium (e.g. sainfoin). Walsh (1945) summarizes the various observations that have been made concerning the positive effects of strontium on calcium-deficient plants. Thus oats growing on neutral soils and showing no response to applied calcium carbonate, respond positively to applications of strontium carbonate; in the absence of strontium the crop yield is drastically reduced. Applications of barium carbonate or sodium carbonate have no effect. A similar ability of strontium to replace calcium partially has been observed in maize plants. In experiments with cereal plants on the other hand, strontium could only replace calcium with respect to the effect on seed crop yield. These observations were made in oats grown in sand culture, as well as in wheat and barley. In oats and wheat, strontium was found to raise the concentration of calcium, but this effect was not observed in barley. One cannot, therefore, explain the observed effects of supplying strontium to plants as being entirely the result of stimulation of calcium uptake.

Walsh (1945) believes that strontium carbonate is able to replace calcium carbonate only with respect to its neutralizing function under conditions of low environmental pH, but that it cannot substitute for calcium in those plant functions associated with

metabolic processes. However, the close similarity in the dimensions of the calcium and strontium ions allows one to speculate that strontium may be able to replace calcium in some metabolic functions.

The algae may be divided into three groups with respect to their response to strontium (Walker, 1953, 1956): (1) those that absorb strontium instead of calcium, (e.g. Chlorella), (2) those which do not use strontium, but which are not injured by this element (e.g. Scenedesmus obliquus), and (3) those algae which are injured by strontium (e.g. Coccomyxa pringsheimii).

Calcium can be replaced by strontium in algae (Moss et al., 1971). Some green algae grow without ever producing zoospores when strontium is substituted for calcium in the growth medium. Interestingly, spore formation in Penicillium occurs less readily under partial borium substitution for calcium than under strontium substitution for calcium (Basu, 1951). Partial replacement of calcium by strontium has been noted also in microorganisms, which, however, show retarded growth and start to secrete amino acids into the medium when the replacement is made (Sactel, 1956).

It may be inferred that strontium is an essential element for some plants.

Wolf and Cesare (1952) found that peach trees suffering from chlorosis recovered when sprayed with a weak solution of strontium chloride. Other elements, such as iron, were ineffective. An analysis of the leaves revealed that the strontium content of ash from the chlorotic trees was 0.0002%, while that of ash from healthy trees was 10 times greater.

Further investigation is needed to find out whether strontium is indispensible for plants.

Chapter 9

ALUMINIUM

Not all plants show a requirement for aluminium. The essential nature of this trace element for plants has interested researchers for a long time. Growing various hydrophytes in water cultures and in silica gel cultures with and without aluminium led Stoklasa (1922) to conclude that aluminium does play an unknown, yet important role in the physiological functions of hydrophytes. Some plants were unable to grow normally without aluminium and eventually died. Thus Glyceria aquatica grown in silica gel culture died on the 22nd day of aluminium deficiency in the medium, whereas Juncus effusus survived 56 to 69 days in water culture. Aluminium was found to improve significantly the growth of wheat, barley and oats.

Znamensky (1927) carried out an interesting study with closely related plants which differed in their requirements for water. Two varieties of wheat - a mesophyte (Triticum vulgare var. pseudohostianum 330/16) and a xerophyte (Triticum vulgare var. ferrugineum 81/4) - were used. Aluminium given to the plants in the form of a sulphate was found to be poisonous to both varieties at high concentrations. Lowering of the aluminium concentration gave rise to different phenomena in the two varieties; the growth of the xerophyte was inhibited, whereas that of the mesophyte was stimulated. The same concentration of aluminium stimulated respiration in the mesophyte (330/16) and suppressed this function in the xerophyte (81/4).

To check whether aluminium was essential for plants, Sommer (1926) set up a series of experiments with pea and millet plants, taking rigorous precautions to purify the salts used in making up the nutrient media. She could not observe any effect of aluminium deficiency on the pea plants, but a supply of aluminium was found to produce a considerable increase in the crop yield of millet seeds (from 0.2 g in control plants to 4.98 g in aluminium-supplied plants). Tauböck (1942b) studied the aluminium requirements of various flowering plants. He grew 124 species of plants on nutrient media containing less than 25 ug of aluminium. No symptoms of aluminium deficiency could be noted. Nevertheless, Tauböck reported that some plants which accumulated aluminium did show a

requirement for it. He provided clear evidence that three ferns (Alsophila australis, Aspidium filis-mas and Polypodium proliferum) that are accumulators of aluminium cannot develop normally when aluminium is in short supply. The requirement of this metal by ferns had been shown earlier by Kratzmann (1913), who found that the spores of one fern species were unable to produce normal gametophytes when aluminium was absent from the neutrient solution.

Another aluminium accumulator species is tea, the growth of which depends upon a supply of aluminium. Chenery (1948) found that the pronounced chlorosis of tea plants growing on an alkaline soil could be eliminated by applying aluminium, whereas iron salts were ineffective.

The absorbed Al is localized in the epidermis and the root tips, and within the cells it is mainly confined to the nucleus and cell walls. Al readily becomes bound to nucleic acids, especially DNA. The concentration of Al in old leaves is significantly (about 10 times) higher than the concentration in young leaves. A stimulating effect of Al on the growth of tea seedlings has been observed (Matsumoto et al., 1976).

It is assumed that the physiological role of the element is connected with the promoting of root growth. Aluminium is not a constituent of true metalloenzymes, but it acts non-specifically as an activator of some enzymes (Dixon and Webb, 1958). At the time aluminium has been found to be a specific activator of ascorbate oxidase (McElroy and Nason, 1954). Moreover, the transamination reaction involving pyridoxal and the majority of amino acids is catalyzed by copper, iron and aluminium salts (Metzler and Shell, 1952).

Phosphorus uptake in sugar cane has been observed to be enhanced by aluminium (Randal and Vose, 1963; Wong You Cheong and Chan, 1973); in contrast to silicon, however, aluminium does not have any influence on phosphorylation, and the amounts of sugar esters and nucleotides remain the same when aluminium is provided.

Aluminium has been detected together with other trace elements in highly purified preparations of DNA and RNA (Wacker and Vallee, 1959a), suggesting that aluminium may possibly play a role in nucleic acid metabolism. In this context, the observation of McLean and Gilbert (1927) that aluminium accumulates in the nucleus may be significant.

Iron has also been reported to occur in the nucleus, and later it was inferred that iron played a specific role in the functioning of DNA (Goldstein and Gerasimova, 1963; Ivanov and Minchenkova, 1965).

Shestakov (1940) reported interesting effects of aluminium on the development of root nodules in peas (Fig.63). In plants abundantly supplied with aluminium the nodules developed more rapidly

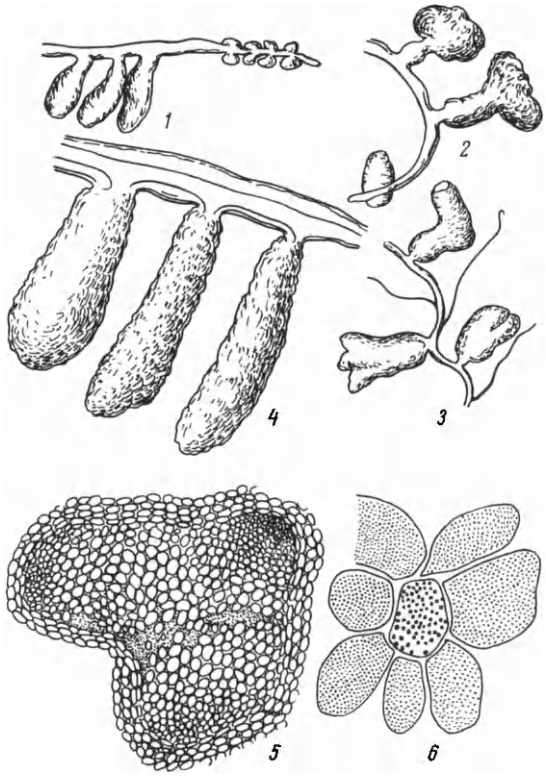


Fig. 63. Morphology of nodules on Pisum roots receiving aluminium (after Shestakov et al., 1940). 1–4, nodules observed under a binocular microscope; 5, nodule section under light microscope (low magnification); 6, cells in bacteroid tissue under light microscope (high magnification).

and were more numerous than those in control plants. Many nodules were abnormally elongated and had markedly dilated cells. In contrast to the starch distribution in the untreated controls, the starch in the aluminium-affected nodules was not concentrated in

the starch sheath, but was distributed throughout other tissues. Shestakov believed that this was evidence of the ability of aluminium to influence carbohydrate metabolism in the nodules.

Schnabl (1970) reported a stimulating effect of Al on starch synthesis, which was more intense in the lower epidermis when there are large numbers of stomata. This author thinks that Al is able by this means to regulate the movement of the stomata.

Interestingly, aluminium has been detectected in phytochrome (Mumford and Jenner, 1966).

The amount of mobile aluminium in acidic soils is usually relatively high. Klimashevsky (1966) has shown that, not only different species, but also different varieties of plants give essentially contrasting responses to aluminium. Calcium uptake into root cuttings and wheat seedlings was considerably reduced at pH 4 when aluminium was present at a concentration of 10^{-4}M or lower.

Aluminium precipitates most of the phosphorus in the cell, thereby making much of the phosphorus pool unavailable for metabolism, and eventually leading to phosphorus deficiency. For example, the amount of phosphorus in the sap of aluminium-poisoned plants is five to seven times less than the amount in normal plants. Treatment of excised roots with aluminium more than doubled the rate of incorporation of ^{32}P into nucleotides and hexosephosphates (Rorison, 1965). In barley grown in water culture, Sampson et al. (1965) observed large disturbances in nucleic acid metabolism and cell division when high doses of aluminium were given to the plants. Histochemical techniques revealed that cell division is blocked by aluminium sulphate at a concentration of 10^{-3} M. DNA isolated from the treated plants was found to have an unusual nucleotide composition; the proportions of G+C in the DNA of the control plants were 52% and 42% respectively, whereas the corresponding proportions in the DNA of plants treated with high doses of aluminium sulphate were 51% and 53% respectively.

Clarkson (1968), working with onion root tips, found that the inhibition of root growth, which was associated with a considerable reduction in the numbers of mitotic cells, could be observed not only in cells treated with high doses of aluminium, but also in cells exposed to other trivalent metals (gallium, indium and lanthanum). The authors concluded that some part of the mechanism of cell division is extremely sensitive to high concentrations of aluminium, as well as other trivalent and divalent metals, as will be shown later.

Klimashevsky et al. (1970) demonstrated that the more rapid uptake of ^{32}P by roots in water cultures supplied with aluminium is typical of the more unstable varieties which are sensitive to aluminium. The synthesis of nucleic acids and free nucleotides in the root tissues of these varieties is more strongly inhibited than the synthesis of nucleotides in aluminium-resistant varieties. ATPase activity in the roots of sensitive varieties is markedly reduced. The amino acid composition of proteins from the roots and above-ground plant organs of sensitive and resistant varieties was found to be modified by the administration of high doses of aluminium, and these changes were different in aluminium-sensitive and resistant plants. The modified amino acid composition of the proteins in aluminium-resistant varieties, according to the authors, endows the plants with a notable tolerance to high doses of the metal.

Aluminium absorbed by cell walls may strongly inhibit the active sites of enzymes situated in the cell walls, thereby significantly impairing the uptake of nutrients and active transport (Salyayev, 1969).

Chapter 10

SILICON

It is known that silicon is present in all plants, and in many it accumulates in large amounts in the cell walls. That silicon is an important structural element and that the exoskeletons of many unicellular plants and animals are made of silicon is a notable phenomenon, since sea water contains as little as 3 mg of silicon per litre. The highest silicon contents have been detected in the stems of grasses; rounded amorphous concretions containing 98.8-99.7% of SiO_2 have been found in the internodal regions of some bamboo varieties. The ash of the horse-tail sometimes contains up to 70% silicon.

Silicon has been shown to be essential for higher plants (rice, millet, beet, maize, tomatoes, horse-tails) (Fig. 64; Sommer and Lipman, 1926; Raleich, 1939; Hewitt, 1963; Potatuyeva, 1968; Chen Ching-Hong and Lewin, 1969).

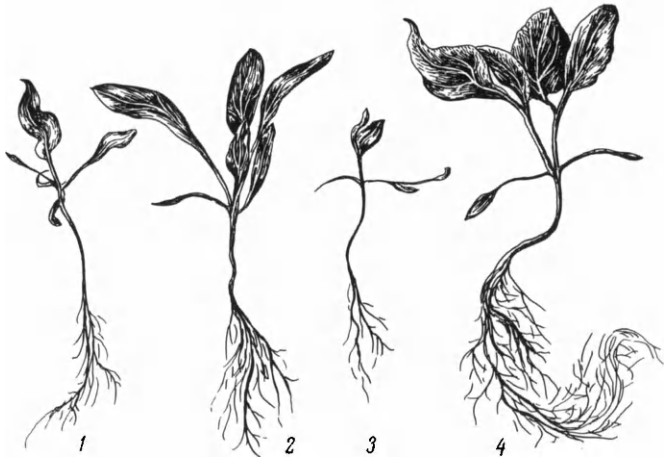


Fig. 64. Effects of silicon on the growth of beet (after Raleich, 1939). 1, 3, in the absence of silicon; 2, 4, in the presence of silicon.

Of considerable interest are results showing that silicon is essential for diatoms, and that disturbances in the metabolism of these algae are caused by a shortage of this element. The essential nature of silicon for diatoms has been demonstrated by Krause (1958), and as shown by Lewin (1962), cell division is partially or completely inhibited in diatoms when silicon is absent from the nutrient medium. The cells, however, remain viable if they are kept in the dark or in weak light, but the silicon of their cell walls undergoes considerable modification; in some cells the entire silicon skeleton changes.

Lewin (1969) observed dramatic alterations in the structure of cell organelles in Cylindrotheca fusiformis and attributed this to silicon deficiency.

Growth, tillering, and the chlorophyll content in rice plants treated with a solution of Si salts (concentration $400 \cdot 10^{-6}$) were significantly increased. The rate of $^{14}CO_2$ incorporation, which began to increase 5 seconds after Si was introduced, doubled after one hour of exposure to Si (Pawar and Hedge, 1978).

The phosphorylation of sugars in sugar cane has been shown to be impaired by silicon deficiency (Wong You Cheong and Chan, 1973). Removal of silicon from the medium blocks the synthesis of proteins, chlorophyll, DNA, RNA, xanthophyll and lipids, and photosynthesis is inhibited completely (Werner, 1966). Werner (1968) found that the respiration quotient was lowered in silicon-deficient Cyclotella cryptica.

The transpiration rate is higher in silicon-deficient plants (Yoshida et al., 1959), and silicon increases the salt tolerance of plants.

Morani and Fortini (1963), working with oat plants, found that sodium and potassium markedly reduce the silicon content of plants. An increased accumulation of manganese and iron in leaves has been observed in silicon-deficient rice plants. Furthermore, omitting silicon from the nutrient medium results in the appearance of necrotic spots on barley leaves, the spots becoming larger as the manganese supply is increased. The addition of silicon effectively eliminates the necrotic symptoms (Williams and Vlamis, 1957).

Silicon exerts a considerable influence on the manganese nutrition of plants. As reported by Kluthcouski and Nelson (1980), when the manganese level was 0.5 mg per litre faint symptoms of manganese deficiency were observed; they did not appear in the presence of silicon. Signs of manganese toxicity developed when its

concentration reached 5 mg per litre, but disappeared upon the addition of silicon.

A relationship between silicon and boron has been found. Thus, a similarity in the requirements for these elements by plants of various taxonomic groups can be noted. Diatoms, for example, require both silicon and boron for their growth and resemble higher plants in this respect (Lewin, 1966a). Many fungi and some species of Chlorella do not exhibit a requirement for silicon or boron (Bowen, 1966).

Considering the similarity between these two elements, one might expect them to compete with one another. A high Si/B ratio in the nutrient medium causes the growth rate of marine brittleworts to be reduced, indicating that silicon influences either the uptake or the utilization of boron (Lewin, 1966a).

It is interesting that germanic acid is a specific inhibitor of silicon metabolism in diatoms, and that only those organisms are sensitive to germanium which have a strict requirement for silicon (Lewin, 1966b). Thus, germanium in low concentrations is extremely toxic to Diatomaceae and higher plants; however, the toxic effect of germanium can be eliminated by supplying silicon (Lewin, 1966b; Werner, 1966, 1967). Germanic acid inhibits the growth of Sinapis alba, Lemna minor, Wolffia arrhiza, Nicotiana tabacum, Tradescantia fluminensis, Zinnia elegans and Secale cereale, and Werner (1967) believes that this points to the necessary presence of silicon for the normal development of these plants. At the same time, various algae, bacteria and fungi are not inhibited by comparatively high doses of germanium.

To recapitulate, the physiological role of silicon has not been thoroughly studied, least of all in higher plants. Nevertheless, it has been established that silicon is an essential element for Diatomaceae and some higher plants - the concentrators of silicon. There is no evidence so far that silicon is essential for all plants.

Chapter 11

SELENIUM

Selenium attracted the attention of investigators when it became apparent that the widely occurring disease "alkalosis" or "alkaline disease" encountered in cattle in some parts of the USA may be attributed to the toxic effects of selenium in forage. Toxic amounts of selenium in the soil have since been found not only in the USA, but also in South America, Australia, New Zealand, South Africa, Algeria, Marocco, Spain, France, Bulgaria, Germany and the USSR. A province geochemically rich in selenium has been recognized in Tuva (Ermakov, 1967), detailed information about this area having been given by Ermakov and Kovalsky (1974).

The flora of the selenium province is much more limited than that of other Tuva regions, the total number of species not exceeding 30. On the stony chestnut soils of the selenium area no more than 10-15 species are found, typically Nanophyton erinaceum, Caragana bungei, Caragana spinosa, Alyssum biovulvatum, Alyssum lenense, Artemisia glauca, and Thymus bituminosis. Among the higher plants the notable accumulators of selenium are members of the Cruciferae, Leguminosae and Compositae, as corroborated by the observations of Trelease and Beath (1949). Mushrooms, especially inedible types, accumulate selenium in high concentrations. The selenium contents of higher plants may be correlated with the selenium concentration in the soil, but this is not the case with fungi such as Lycoperdon gemmatum.

Plants growing on selenium-rich soils, usually accumulate high concentrations of selenium. Those plant species which possess this ability are known as concentrators of selenium; they are the primary selenium indicators, since their growth is restricted to specific regions. Detailed information on the primary selenium indicators, among which species of Astragalus are by far the most important, may be found in a number of publications (Rosenfeld and Beath, 1964). Experiments with water and sand cultures, as well as field observations, have shown that selenium stimulates the growth of those plants that are concentrators of selenium (Trelease and Trelease, 1937; Rosenfeld and Beath, 1964; Broyer et al., 1972). Interestingly, selenium concentrators such as Astragalus

pectinatus and Aplopappus fromontii do not grow on selenium-free soils. Selenium apparently is involved in some vital function in these plants, which show abundant growth on selenium soils.

On selenium soils one can also be found species which are not accumulators of selenium. These contain only traces of selenium. Not all Astragalus species are selenium concentrators, there being some which accumulate only insignificant amounts of selenium. Miller and Byers (1937) have shown that while Astragalus racemosus and Astragalus bisulcatus accumulate over 1000 mg of selenium per kg dry weight, the selenium content of, for example, Astragalus nissouriensis growing in the same soil is as low as 1-5 mg per kg.

The range of variation of selenium content in plants is strikingly wide: from 0.1 to 14,900 mg per kg dry weight. Plants of different species growing on the same soil may accumulate very different amounts of selenium.

Karelina (1971), having studied the accumulation of selenium in wild plants, reported that mushrooms concentrate selenium to levels 10-1000 greater than the levels in higher plants; particularly high concentrations of selenium have been found in Amanita muscaria and Boletus edulis. Cereals belong to plants which have a low capacity for accumulating selenium.

Plants accumulate selenium more from organic sources than from mineral forms (Trelease and Disoma, 1944). Painter (1941) believed that the selenium accumulated in seeds may replace the sulphur in proteins. Small amounts of a crystalline amino acid complex containing selenium and sulphur have been isolated from Astragalus pectinatum. Selenium is able to replace the sulphur in sulphur amino acids (e.g. in methionine) as has been shown in wheat. Similar findings are summarized by Rosenfeld and Beath (1964). The replacement of sulphur by selenium is obviously a widely occurring phenomenon in nature. Van Niel (1931) has shown that the sulphur purple bacteria may use not only hydrogen sulphide, but also molecular sulphur for photoreduction. Later Sapozhnikov (1937) revealed that sulphur may be replaced by selenium for the photoreduction of carbonic acid in the sulphur purple bacteria. A number of enzyme systems that act upon sulphur compounds in various metabolic processes may also use selenium analogues.

Furthermore, it has been found that as a result of the replacement of the methionine-bound (and probably also cysteine-bound) sulphur in enzymes, changes occur in the conformational stability of synthetic seleniumoxytocin and selenium-β-galactosidase

isolated from Escherichia coli. In spite of changes in the conformational stability of selenium-β-galactosidase in which 50%-70% of the methionine-bound sulphur is replaced by selenium, the activity of the enzyme remains unaltered. On the other hand, the transformation of the cysteine in oxytocin into selenocysteine results in minor modifications in the hormonal activity of the protein, depending on the extent of the replacement.

Toxic concentrations of selenium primarily affect those enzyme systems involving enzymes with sulphhydryl groups. This inhibition can be relieved by methionine. Interestingly, cytochrome oxidase and arginase which have no sulph-hydryl groups in their molecules are resistant to inhibition by selenium (Wright, 1938).

A reduction in tissue respiration produced by applications of selenites was found to be partially or completely restored by administering glutathione. If, however, glutathione was applied some time (30-60 minutes) after a dose of selenium was given, then the inhibitory effect could not be blocked (Wright, 1938). These findings provide some insight into the mechanisms underlying the antagonism between selenium and sulphur.

Hurd-Karrer (1938) reported another effect of antagonism between sulphur and selenium, namely the ability of sulphur to counteract th the inhibitory action of selenium on the growth of wheat. It was later found that the inhibitory action of selenium on the yeast Saccharomyces cerevisiae could be partially blocked by supplying methionine. The ability of sulphur compounds to counteract inhibition caused by selenium has now been demonstrated in many organisms. In experiments with Escherichia coli, this effect of sulphur has been obtained with cysteine and glutathione, whereas methionine proved to be ineffective (Fels and Chelderin, 1948).

Antagonism between selenate and sulphate with respect to uptake has been investigated by Leggett and Epstein (1956), who studied the kinetics of sulphate uptake by barley roots. These authors believed that in this case there was competition between selenate and sulphate ions for a carrier in the cell membrane. Other ions (PO_4^{3-}, NO_3^- and Cl^-) do not exhibit any notable affinity for the SO_4^- - SeO_4^{2-} binding site.

Data have been obtained on the detoxification of selenium-affected plants by using phosphorus (Singh and Singh, 1978). Singh and Singh (1979) showed that of all the selenium forms present in soils, the most harmful to plants is SeO_4^{2-}.

What metabolic changes are produced by toxic concentrations of selenium, and what are the physiological mechanisms underlying the resistance of selenium concentrators to high levels of this element?

The reason for the toxicity of selenium is thought to lie in its interference with the metabolism of sulphur. This point of view is based on the similarity between sulphur and selenium compounds. Both elements are in the VIth group of the periodic system, and most biologically important sulphur compounds have a selenium analogue, which displays physical and chemical properties similar to those of the sulphur counterport, (e.g. selenium cysteine, selenium methionine, selenium cystathionine). The following compounds have been detected in plants which accumulate selenium: selenomethylcysteine, selenohomocystine, γ-L-glutamine-selenomethylselenium-L-cysteine, selenocystathionine, and dimethyldiselenide. In non-accumulator species occur selenocystine, selenomethionine, selenomethionine-selenoxide, selenomethylselenium--methionine, selenocysteine-selenic acid, selenopropenyl-selenium--cysteine-selenoxyl. Selenium analogues function as substrates for enzymes involved in the assimilation of sulphur. This has been demonstrated in Escherichia coli and animals.

Very little is known of the physiological mechanisms underlying the resistance of selenium concentrator species to high concentrations of this element.

Interesting proposals concerning the defensive reactions of plants to high concentrations of selenium are presented in a paper by Peterson and Butler (1967). In experiments with the selenium concentrator Neptunia amplexicaulis, these workers found that much of the selenium makes its appearance in free cysteine and methionine. The authors suggest that the presence of selenium in such amino acids is typical of selenium-accumulating plant species, in which selenocysteine and selenomethionine are not incorporated into proteins. They believe this may be the mechanism by which plants deal with high doses of selenium. It is possible that the differences in resistance to selenium observed in concentrator and non-concentrator species may find explanation in the results reported by Shrift (1972). These results indicate that there is a difference between selenium-resistant and selenium-sensitive plants with respect to the composition of those compounds in which sulphur is replaced by selenium.

Kovalsky et al. (1972) reported on the genetic transformation of Bacillus megaterium which occurs in the Tuva selenium-rich province and is adapted to high concentrations of selenium. DNA from these cells, when introduced into a culture medium supporting a growth of recipient Bacillus megaterium cells maintained at low selenium concentrations, brought about the transformation of the sensitive form which then developed normally under conditions of high selenium concentration. An important discovery accounting for the resistance of the Tuva strain of Bacillus megaterium to selenium was that of the presence in the cells of selenium reductase, which reduces selenium to insoluble, non-assimilable forms. No such enzyme could be found in a Moscow strain of the organism, but by means of genetic transformation, the latter cells were enabled to synthesize this enzyme (Letunova et al., 1968). This ability of the transformants to synthesize selenium reductase persists for several years under laboratory conditions.

At present it is not possible to explain why some Astragalus plants - (concentrators of selenium) - cannot develop normally in the absence of sufficient amounts of selenium in the soil. It must be borne in mind that the replacement of sulphur by selenium does not always result in changes in metabolism, although in some metabolic processes the selenium analogues may react more readily than the corresponding sulphur compounds. Thus DL-selenomethionine is a better substrate than DL-methionine for the methionine-metabolizing enzymes in yeasts (Peterson and Butler, 1967). It is also possible that in higher plants which are selenium concentrators, some selenium analogues of sulphur metabolites may participate more efficiently than the sulphur compounds in metabolic processes.

For a long time the study of Se was focused on its toxicity to organisms. Evidence that low concentrations of this element averted liver necrosis in rats and exudative diatesis in chickens, marking the beginning of its use in the therapy of white-muscle disease and toxic liver distrophy in cattle and swine, has confirmed that Se is essential for animals.

In many countries throughout the world, provinces and regions deficient in Se have been recognized (Ermakov and Kovalsky, 1974). From his observations, Guylakhmedov et al. (1974) concluded that 60% of the territory of the Soviet Azerbaijan Republic has soils with low or intermediate Se concentrations. A very low Se content is characteristic of mountain-forest brown steppe soils, and it is

on these soils that the white-muscle disease of buffaloes occurs. The Se content of bog and marsh soils is particularly low, contrasting with the levels in dry soils. The highest Se contents of fodder plants have been found in Artemisia hay and Medicago hay, whereas Carex hay and Phragmitas hay have very low Se contents.

The application of Se salts to wheat plants growing on Se-deficient soils has a pronounced positive effect; it increases considerably the numbers of organisms in the soil and creates more favourable conditions for mineralization and humification (Shakuri, 1974). An addition of 1.5 kg ha Mo and 1 kg ha Se fertilizer was found to increase considerably the yield of Indigofera tinctoria crop on the grey soils of Apsheron which are poor in Mo and Se (Gyulakhmedov and Teimurova, 1978).

Chapter 12

IODINE AND CHLORINE

Iodine. Iodine is a widely occurring element in the plant kingdom. Many algae concentrate iodine (Vinogradov, 1965); for example Laminaria digitata concentrates it so effectively that the concentration in the plant exceeds 30,000 times the concentration in sea water. The important functions of iodine in the physiology of animals are well recognized. Lewis and Powers (1941), using highly purified nutrient solutions containing only one part of iodine per 10^{18} parts solution, failed to observe any sign of a requirement for iodine in maize, barley, tomatoes and Azotobacter agile.

Although iodine cannot be regarded as an essential element for higher plants, a supply of this element is desirable for stimulating the growth of isolated roots (White, 1938). Iodine has been shown to stimulate the growth of plants on iodine-deficient soils. Thus on light (in terms of mechanical properties), low-humus soils in Buryatia, largely underlain by acidic mountain-volcanic soils conspicuous for their low iodine content, iodine applied to the soil or sprayed on to seeds prior to sowing has been shown to stimulate the growth of various agricultural plants and promote development of the microflora (Kashin, 1972). Similarly, significant increases in crop yields of barley have been obtained by supplying iodine to peat-bog soils. The latter contain iodine mostly in the form of low solubility compounds that can be utilized by plants only to a very small extent (Golovneva, 1970).

Referring to the ability of some algae to concentrate iodine, it may be said that a correlation has been found between the iodine contents of these algae and their taxonomic positions (Vinogradov, 1965) (Fig.65). Among the Laminariaceae - one of the most widely distributed families of the brown algae - genera and species are known, particularly species of Laminaria, which are strikingly rich in iodine. All the species of another common family, the Fucaceae, are less rich in iodine than the representatives of the Laminariaceae growing in the same marine locality. The red algae comprise genera and species which vary greatly in iodine content. Representatives of the three main genera of the green algae - Ulva, Enteromorpha and Cladophora - contain very little iodine.

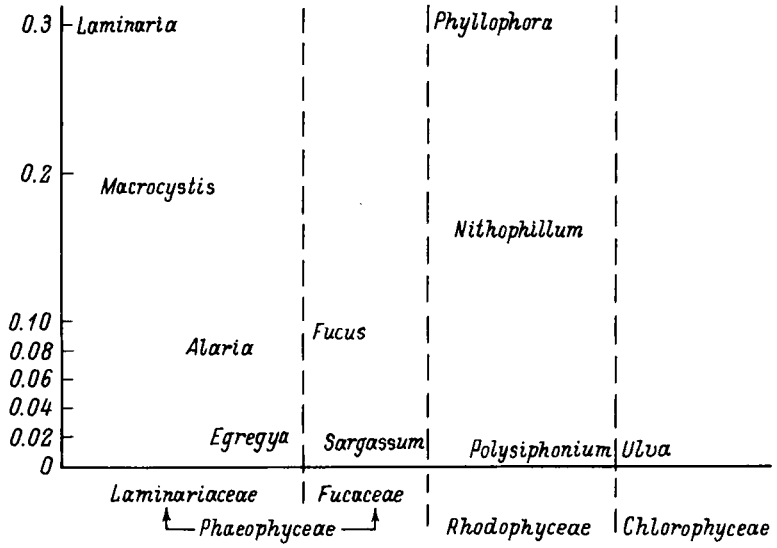


Fig. 65. Iodine levels in algae of different systematic groups (after Vinogradov, 1965). Ordinate, quantity of iodine (% dry matter).

By analogy with what is known about essential requirements for aluminium, it might be speculated that among concentrators of iodine species must exist for which iodine is an essential element. These expectations have been fulfilled. In 1962, Stosh was able to observe the cessation of growth and the appearance of specific morphological changes in gametophytes of the red alga Asparagopsis armata subjected to iodine deficiency. A similar retardation of growth was observed in another two species of red algae, and in three species of brown algae. However the green algae Diatomaceae and Coccolitoforidae, which do not concentrate iodine, showed no reaction to the removal of iodine from the nutrient medium.

Fries (1966) has shown that iodine at a concentration of 1 to 8 μM is absolutely indispensible for the normal growth of Polysiphonia urceolata. Nemalion multifidum was unresponsive to the addition of iodine to the medium, whereas the growth of Coniotrichum elegans was inhibited at iodine concentrations above 0.4 uM. Bromine is an inhibitor at a concentration equal to that in sea water, but can stimulate growth slightly when present at concentrations of less than 50 mg per kg.

Nothing is known of the role of iodine in the metabolism of those algae for which iodine is an essential element. Reference may be made only to one paper (Kowallik, 1968) regarding the effect of potassium iodide upon light-stimulated endogenous respiration in algae.

It may be speculated that some iodo-organic compounds have acquired importance in the metabolic processes of some of the algae for which iodine is an essential element. It is known that most of the iodine is bound to protein molecules through the aromatic amino acids (tyrosine and to a lesser extent tyronine), which are readily iodized. In algae, iodo-organic derivatives of tyrosine have also been detected (Vinogradov, 1965). Fowden (1959), by carrying out a chromatographic separation of protein hydrolysates and some free amino acids from both halophytes and mesophytes, discovered the presence of 3,5-di-iodotyrosine and 3,5,3-tri-iodotyrosine, which are precursors of thyroxine. Evidence is available concerning the synthesis of organic compounds of iodine in wheat roots (Böszörményi et al., 1959) and the incorporation of radioactive iodine into organic compounds in various angiosperms (Fowden, 1959). Most of the iodine in the barley seed is located in the protein fraction; and little of the iodine is in a soluble form (Golovneva, 1970).

The type of iodo-organic compounds present largely determines the degree of uptake and the distribution of ^{131}I in the different organs of the plant (Darkanbayev and Niretina, 1965). The considerable accumulations of iodine in the above-ground plant parts comprise iodides, and iodate predominates in the root system. Dependence of the degree of iodine accumulation upon the biological characteristics of the plant has been reported. Iodine is concentrated in the young leaves and stems of flowering plants, especially in the cambium zone. During the formation of the reproductive organs iodine is located predominantly in the ovary, stigma and stamens.

Only fragmentary evidence is available regarding the effect of iodine on physiological processes in higher plants. Barker and Mapson (1964) reported on the influence of high doses of iodoacetate on respiration and carbohydrate metabolism. Golovneva (1970), in a study of the effect of iodine on physiological processes in barley, found a dependence of the accumulation and metabolism of mobile carbohydrates upon the iodine content. Iodine accelerated

the process of the transformation of translocated forms of carbohydrate into complex polymers, as indicated by an increase in the amount of polysaccharides in the stems. Iodine stimulates the synthesis of cellulose and the lignification of the stem tissues, which results in a mechanical strengthening of the stem. Niretina (1969) also reported effects of iodine on carbohydrate metabolism.

Iodine has been shown to increase the concentration of ascorbic acid in plants (Hageman et al., 1942). Portyanko et al. (1969) found that iodine produced a dramatic increase in the total content of free amino acids in plants grown in water culture.

Seitz (1970) reported that potassium iodate, in contrast to potassium chloride and potassium nitrate, inhibited the rapid movements of chloroplasts in response to illumination by ultraviolet and blue light. No inhibition was observed when chloroplast movement was elicited by red light illumination.

Portyanko and Kudrya (1966) observed an enhancement of the germination of pollen under the influence of iodine and other halogens.

An antagonism takes place between iodine and chlorine, the latter reducing the uptake of iodine (Katalymov, 1965). Conversely, a decrease in chlorine content attributable to iodine has been reported (Nazarova, 1972). An increase in salt tolerance produced by iodine, which lowered chlorine uptake, has also been observed (Nazarova, 1972). The antagonistic activity of iodine ions towards chlorine is several orders of magnitude greater than that of chlorine ions towards iodine (Nazarova, 1972). Manganese increases the uptake of iodine by plants (Niretina, 1969).

Finding out why iodine is essential for some algae, and the quantification of the requirements of some higher plants for iodine are important issues yet to be resolved. In the light of the significant role played by 3,5-di-iodotyrosine and 3,5,3-tri-iodotyrosine in the synthesis of the thyroid hormone thyroxine, it would seem appropriate to study the function of these compounds in the halophytes and mesophytes in which they occur.

Chlorine. The stimulatory effect of sodium chloride on the halophyte Salicornia herbacea was thought to be an effect not only of the sodium ions but also of the chloride ions (Lyubimenko, 1921). A favourable influence of this element on the growth and crop yield of members of the family Chenopodiacea (e.g. beet) was subsequently reported (Lipman, 1938). Celery, spinach and cotton

also respond positively to chlorine. Broyer et al. (1954), working with water cultures of tomatoes, showed that chlorine is an essential element for these plants. One could observe chlorosis, browning and twisting of the leaves, reduced fruit bearing, and a cessation of root growth in chlorine-deficient plants (Johnson et al., 1957; Fig.66). Supplying chlorine at a concentration of 100 mM

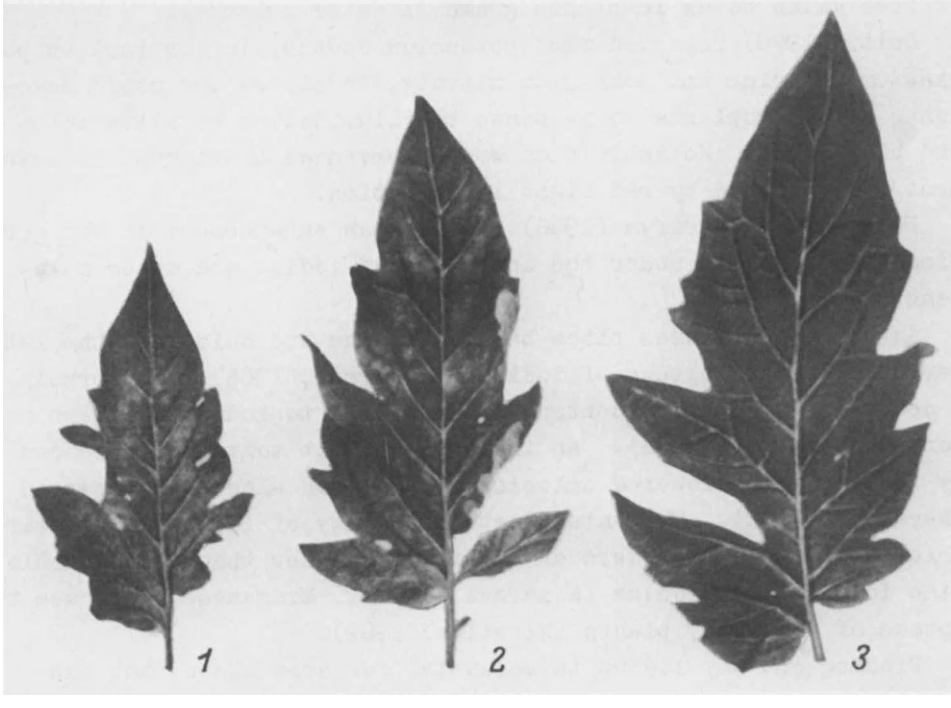


Fig. 66. Effect of chloride supply on growth of tomato (Lycopersicum esculentum) plants in water culture. 1, without chloride; 2-3, with chloride (after Johnson et al., 1957).

relieved the deficiency symptoms. Bromine introduced at a concentration ten times higher that that of iodine was found to be effective in substituting for chlorine, but after two years the symptoms of chlorine deficiency reappeared in sugar beet plants (Ulrich and Ohki, 1956). The symptoms of this deficiency appear primarily in the middle leaves, and initially resemble those of magnesium deficiency in that the inter-vein areas of the leaf blade show a patchy chlorosis which can be seen only in reflected light. Moreover, thickening of the secondary roots can be observed in affected

plants. These symptoms disappear upon adding 6.7 to 20 mgEq of chlorine per litre to the nutrient medium. The place of chlorine may be taken only by bromine at a concentration of 20 mgEq per litre; manganese, sodium, silicon and other elements are ineffective in counteracting the symptoms of chlorine deficiency. Johnson et al. (1957) described the symptoms of chlorine deficiency in 11 plant species. The requirements for chlorine have proved to be different in different plants, high concentrations of the element being necessary for tomatoes, lettuce, spinach and cabbage, and low levels being sufficient for the growth of pumpkins, beans and maize.

Bromine cannot take the place of chlorine on a 1:1 basis, being only 75-95% as efficient as chlorine in fulfilling the nutritional requirements of plants. Nonetheless, bromine elevates the level of chlorine in various plants, an effect which may be accounted for by stimulation of the transport of chlorine from the roots to the above-ground plant organs. A severe deficiency of chlorine cannot be ameliorated by supplying bromine.

Chlorine deficiency in the field has been observed in Australia and in California (Ozanne, 1958). The essential nature of chlorine has been demonstrated in Lemna minor plants cultivated on nitrate soils supplied with various amounts of iodine. After 12 days of growth in the absence of chlorine, the plants showed signs of poor development and had short, thin, poorly pigmented roots. Iodine proved to be toxic, and in contrast to bromine failed to function as a substitute for chlorine.

Both heterotrophic and autotrophic strains of the brittlewort Spirodela polyrhiza require chlorides for growth (Eyster et al., 1958).

Experiments on the uptake of ^{36}Cl by higher plants have revealed that chlorine ions enter the plant not only via roots, but also through the leaves. Like sulphur, chlorine can enter the leaves through the stomata as a vapour. From the older leaves, chlorine is translocated to the roots (Mengel, 1960). In contrast to sulphates, for example, chlorine accumulates to a greater extent in the stalks than in the leaves (Ulrich and Ohki, 1956).

The plasmodesmata of higher plants contain structural components with a close affinity for Cl^-, which is absent from the plasmodesmata of algae. The main part of the intracellular Cl^- is located on membranes between the tonoplast and the plasmalemma, and

much is found in the plasmodesmata. A considerable presence of Cl^- was noticed in chloroplasts, the Cl^- being evenly distributed throughout the organelle (Van Stevenick, 1976).

Although the physiological functions of chlorine in plants are not fully understood, some pertinent information is available. We may refer to the role of chlorine in the functioning of chloroplasts, and to its role in photosynthesis. Warburg and Lutgen (1946) demonstrated that chlorine was essential for oxygen evolution in chloroplasts isolated from spinach and beet leaves. These findings have been corroborated by Arnon and Whatley (1949). In the experiments described by these authors, chloroplasts were exhaustively washed until they lost the ability to produce oxygen, and then a cytoplasmic fraction was added which restored this capacity. Chlorine or bromine ions at a concentration of 3.10^{-3} M also aided recovery. If, illuminated in the absence of ferricyanide (an oxidant in the Hill reaction), then they irreversibly lost their capacity to evolve oxygen; chlorine had to be added during the preliminary illumination to prevent irreversible inhibition. Simultaneous additions of chlorine and ferricyanide were ineffective. The authors suggested that chlorine ions might protect isolated chloroplasts, although this protection might not be indispensible for intact cells. So far as the author is aware, this proposal has never been verified.

Bove (1959) observed that chlorine at a concentration of 3.10^{-4}M affected the photosynthetic activity of chloroplasts, oxygen evolution, and the reduction of NADP. Non-cyclic photophosphorylation was stimulated by the addition of chlorine ions, and cyclic photophosphorylation catalyzed by riboflavin-phosphate was also stimulated by chlorine, although to a considerably lower extent. Cyclic photophosphorylation catalyzed by vitamin K or phenazine-metasulphate was insensitive to chlorine and continued actively in its absence. From these results, Bove concluded that chlorine ions function as electron carriers between the two cytochromes, and that vitamin K is able to bypass this chlorine-dependent stage. To our knowledge, no direct evidence supporting this view has ever been found.

The discovery by Eyster et al. (1958) that chlorine is essential for the growth of autotrophic strains, but almost completely unnecessary for heterotrophic strains of Chlorella, also testifies to the involvement of chlorine in photosynthesis. A similar pheno-

menon has been reported for manganese, an element playing a prominent part in photosynthesis.

Chlorine positively influences the Hill reaction (Yamashita et al., 1972), and Arnon (1958) has found that chloride ions stimulate the process of electron transport between components of the cytochrome system in the process of phosphorylation. Nieman (1956) reported that chlorine increases the uptake of oxygen by roots, and thus promotes the respiratory oxidation of sugars. Nieman also found that chlorine activates cytochrome oxidase, being superior in this respect to the sulphate ion.

Ulrich and Ohki (1956) found that in beet leaves chlorine lowers the sucrose content in the presence of high potassium levels, and elevates it when the potassium concentration is low.

Chlorine apparently plays an unknown, yet important role in nitrogen metabolism. In experiments with beet, Schmalfuss and Reinike (1960) observed that adding chloride in combination with nitrate resulted in the disappearance of the latter; the same effect was observed under conditions of ammonium nutrition. This finding points to the important role of chlorine in the biosynthesis of amino acids, and probably also protein synthesis. Lyzhenko and Portyanko (1970) found that the halogens - chlorine, iodine and bromine - increased the amounts of both free and bound amino acids.

Summarizing, it may be said that chlorine is essential for plants probably on account of its specific role in photosynthetic reactions and, apparently, in nitrogen and energy metabolism.

Chapter 13

TITANIUM AND SILVER

Titanium. This element is one of the most widely distributed in nature. Because titanium is unable to produce readily soluble compounds in the biosphere, its concentration in the soil solution is very low (7.10^{-6}%) in spite of the large amounts present in the soil.

Titanium is found in abundance in the plant kingdom, and concentrators of titanium have been reported. Among these is the alga Lithothamnia sp., which concentrates titanium to levels thousands of times greater than the levels in sea water. The highest concentration of titanium found in a plant is 2.10^{-3}%. Vernadsky (1937, p.255) wrote: "Organisms, so far as one can judge without clear corroboration from experimental findings, seem to pump in titanium atoms from aqueous media and channel these into the metabolic processes of the living substance".

Titanium is a potent reducing agent. It may be speculated that this element plays a role in photosynthesis, and probably also in the fixation of molecular nitrogen. Bertrand and Voronka-Spirt (1930) suggested that titanium takes part in the process of photosynthesis. More recently it has been established that titanium, like iron, manganese and copper, plays a major role in photosynthesis and accumulates in the leaf (Udelnova et al., 1971). According to current views, various metalloproteins associated with lipid groups are involved in electron transport, and indeed high levels of the polyvalent metals titanium, nickel, iron, manganese and copper have been detected in plant cell lipids. The latter three elements are important in the process of photosynthesis. It may be speculated, therefore, that titanium and nickel also play an important part in this process, but further investigation is required to find out whether such an assumption is valid.

Data are available indicating the possible effect of titanium on the rate of nitrogen fixation Baum (1939). Reference has already been made to the discovery by Volpin and Shur (1964) of the reduction of nitrogen in solutions containing metal compounds, including compounds of titanium. Again, complexes of titanium or iron with molecular nitrogen have been discovered in which the

nitrogen is capable of being further reduced. Furthermore, the reduction of nitrogen to hydrazine and ammonium by Ti^{3+} and Cr^{2+} in the presence of molybdenum compounds has been discovered. Although there is a considerable gulf between the complexity of enzyme systems and the comparative simplicity of model systems, the evidence obtained from the latter testifies to the high reducing power of titanium, which is probably used in the processes of the reduction of nitrogen and carbon compounds.

Gryzhankova et al. (1975) isolated a titanium compound derivative of iron phosphopanthotein from four organisms - the red alga Anufelia plicata, the brown alga Laminaria japonica, the green alga Ulva fenestrata, and the flowering plant Zostera marina. This compound proved to be a potent reducing agent. Thus it may be assumed that titanium compounds are involved in the reductive processes in living cells, particularly under conditions of illumination as may be found, for example, at great depth in the sea or during diurnal or seasonal cydes of illumination intensity. Being strong reducing agents, titanium compounds may play a role in the protection of plant cells from excessive oxidation, especially in the absence of photosynthesis. Such compounds occur as constituents of phosphoproteins, and form a reserve of reducing potential in plant cells.

It is reported that when tomatoes are grown in aquatic culture, their chlorophyll content increases under the influence of titanium. In the field, tomato plants receiving extra-radical titanium nutrition, show an increase in yield, more rapid ripening of the fruit, and a greater chlorophyll content of the leaves and sugar content of the fruit. Similar effects are observable in grapes and apples. Spraying the leaves of apple trees and vines with titanium in solution was found to increase markedly their contents of Zn, Mn, Cu, Ni, Co and Cr. When maize was provided extra-radically with a titanium solution, the yield increased by 25-30 per cent; the weights of the spadices of various maize cultivars, and the concentration of sugars in them increased as well (Pais et al., 1979).

In a study by Sagi et al. (1980) the introduction of 15-30 mg per kg titanium in the form of extra-radical feeding was found to contribute to increased tillering in wheat. Under the influence of titanium the protein content of the grain of the wheat increased, an effect which was supposed to have been connected with titanium-induced stimulation of nitrate reductase activity. In

tissue culture titanium suppresses the formation of callus in maize embryos and prevents abnormal differentiation of maize calluses.

It is quite possible that titanium, being a strong reducing agent, is an element that is essential for plants. Confirmation of this will require that experiment are carried out with all traces of titanium removed from the nutrien media. It is worth while pointing out, however, that although the literature abounds in data on the stimulating effects of various microelements on the growth of plants, the absolute indispensability of these microelements for plant growth is unlikely ever to be proven. Similarly, non-specific effects of these microelements on a number of physiological processes are commonly observed. This problem is discussed in detail in our publication of 1963 (Shkolnik, 1963).

Silver. Silver is not an element that is essential for plants growth. However, existing studies on the comparative effects of gibberellins and silver ions on the induction of male flowers on female hemp plants and female cucumber lines have attracted a great deal of attention to the influence of silver on the physiology of plants.

In an investigation by Sarath and Moham Ram (1979) it was shown that after the application to the top part of female hemp plant shoots of either 10 mg per litre $AgNO_3$, or gibberellin acid for a period of 10 days during which several female flowers were formed on the first nodes, the formation of male and hermaphrodite flowers took place. It has been discovered that the effect of silver ions on the development of masculine tendencies in hemp plants is connected with the capacity of silver to block the activity of ethylene.

Tolla and Peterson (1979) established in field experiments that spraying plants with $AgNO_3$ solution at concentrations of 100, 200, and 400 mg per litre resulted in the formation of a greater number of staminal flowers than that formed on plants treated with gibberellin A 4/7 solution. The usage of $AgNO_3$ for stimulating the formation staminal flowers suggests the possibility of obtaining commercially profitable quantities of hybrid seeds from crossings of female lines.

Atsman and Tabbak (1979) discovered that two successive sprayings of female cucumber plants (var. Delila) with silver nitro--oxide, gibberellin, or aminoethoxyvinyl glycine, in each case resulted in the development of staminal plants. $AgNO_3$ and aminoetho-

xyvinyl glycine contributed to the development of normal pistillate flowers and buds at each node as well. Whereas silver nitrate and aminoethoxyvinyl glycine blocked ethylene activity, this effect was not produced by gibberellin A 4/7, indicating that gibberallin possesses some different and as yet unknown mechanism of influencing sex.

Nijis et al. (1980) also succeeded in obtaining male flower formation on female cucumber plants by means of silver treatment. Silver ions induced the development of male flowers even in very stable female lines. Moham Ram and Sett (1980) found that silver nitrate and cobalt chloride, which is known also to suppress the synthesis of ethylene, induced the formation of male flowers with viable pollen in normal, strictly pistillate terminal inflorescences. Female flowers pollinated with the pollen of induced male flowers formed fruit with viable seeds.

PART III. TRACE ELEMENTS IN BIOSPHERE

Chapter 1

THE ROLE OF TRACE ELEMENTS IN THE EVOLUTION OF PLANT METABOLISM IN THE BIOSPHERE

Research on trace elements is pursued not only by plant physiologists, biochemists and ecologists, but also by various other specialists in botanical and non-botanical disciplines.

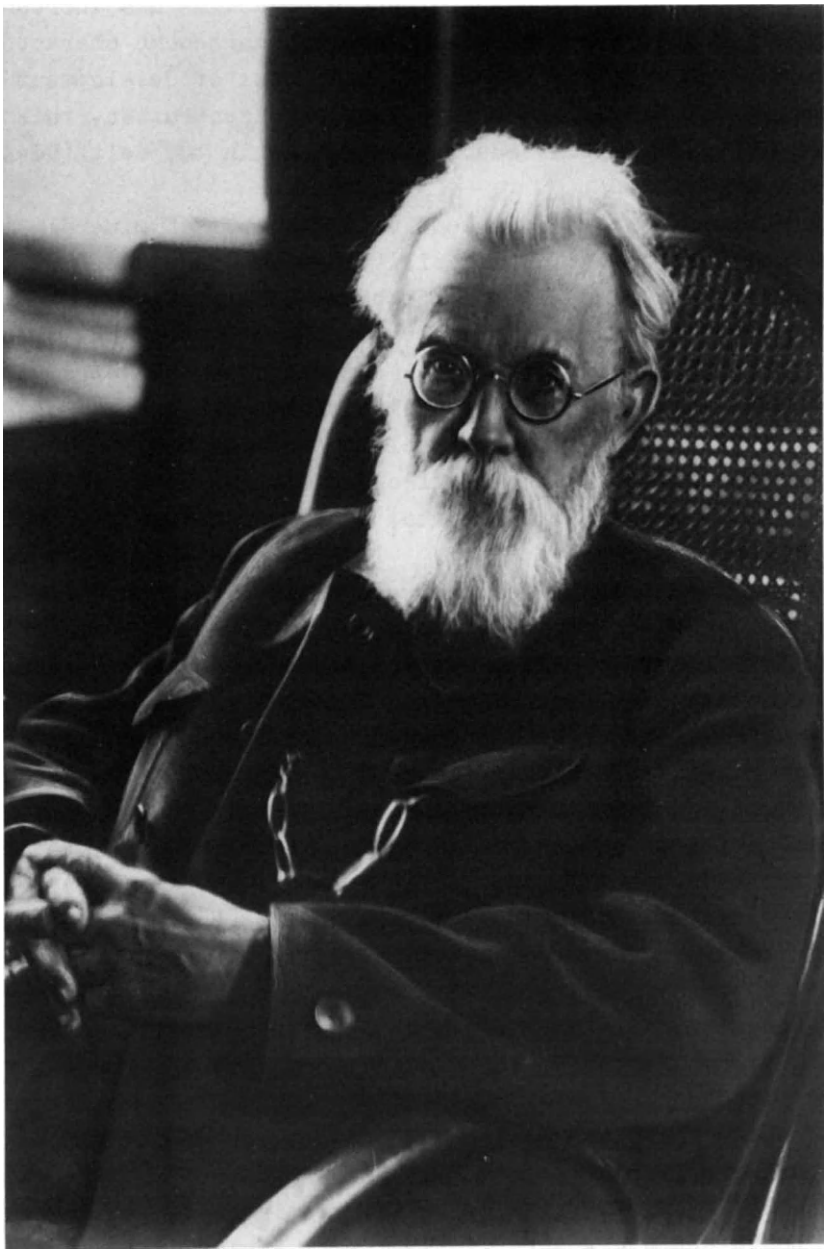
V.I.Vernadsky believed that the mysteries of living processes could not be solved purely by the study of living organisms. Solutions must also be sought through investigation of the sources of life, one of which is the Earth's crust; thus the properties of the chemical elements comprising the Earth's crust must be thoroughly understood. The scientific progress that has been made in subsequent years has validated these views, which embrace the general problems of the origin of life and the evolution of the plant kingdom.

Bakardjieva (1980) assigns a significant role to micronutrients in the primary syntheses of simple organic substances. She points out the following possible functions of the micronutrients in biopoiesis - the evolution of the first living organisms, the chemical history:

- a catalytic function - catalysis of the initial abiogenic processes;
- a structural function;
- an electron transport function;
- contribution towards the formation of the inorganic matrix;
- participation in establishing the conformation of macromolecules;
- the promotion of structural and functional diversity, which was acted upon by natural selection at the molecular level;
- participation as photosensibilizators in primary reactions;
- an integrating function in macromolecule self-assembly;
- the promotion of molecular asymmetry.

It is also pointed out that pairs of elements acting together established the basis of conjugate processes. These listed functions of microelements can be recognized in contemporary living organisms as well. N.Bakerdzhieva considers microelements to be factors influencing biological evolution at all levels including

that of the macromolecule, the organism, the species and the biosphere.



V.I. Vernadsky, founder of the science of the biosphere.

The study of the polyvalent metal (Fe, Mn, Cu, Ti, Ni, Co, V, Cr) composition during photoheterotrophic and photoautotrophic development in Chromatium vonosum, has indicated that after the phase of autotrophism, comparatively high contents of manganese and chromium (metals whose presence in phototrophic organisms has increased in the course of the evolution of these organisms) become characteristic of bacteria. The photoheterotrophic phase of development was characterized by an increase in titanium concentration, this metal being a participant in reducing reactions in the cell (Udelnova et al., 1977).

Plants are known to take up selectively a number of mineral elements from the environment. This ability has changed according to the changes that took place in the biosphere, and changes that occurred in plants themselves as they evolved, so that various types of plant organism with different metal compositions developed at different times. In the course of evolution the greatest changes occurred in the contents of those metals the compounds of which were most closely involved in overall changes of metabolic pattern (Bowen, 1966; Bogorov, 1967; Boichenko, 1968a; Gryzhankova et al., 1973).

Referring to the ideas of Vernadsky on the evolution of organisms and their involvement in the turnover of elements in the biosphere, Boichenko (1976) indicates that, because they are continuously interacting with their environment, living organisms absorb metals, create modified metal compounds in the course of their metabolism, and excrete these modified products into the biosphere. Consequently, both modification of the organisms themselves under environmental influences, and an increasing influence of these organisms on the biogeochemical processes of the biosphere have taken place in the course of evolution. Plants are the most important organisms in this respect. Boichenko (1976) presents data on the changes that have occurred in the concentrations of metals in plants throughout evolution (Table 11). It is apparent that various types of plant, having appeared at different times in evolution, are able to concentrate metals to various degrees. Table 12 shows the changes that have occurred in the ratios of metal compounds in the evolution of plants. As seen from the Table, percents of Mn, Cu, V, Ni, Mo has in plants have increased throughout evolution; these trace elements together with lipids from complexes that are important in oxidation-reduction processes.

TABLE 11 Changes in the concentrating capacities of plants in the course of evolution (after Boichenko, 1976)

Geological Era (% O_2)	Dominant plants	Dominant processes	Elements concentrated in plants
Archeozoic (0.02-0.2)	Cyanophyta	Anaerobic reduction	Fe
		Oxidation with fermentation	Zn
Pterozoic (0.2-2.0	Rhodophyta	Rise to predominance of oxidative processes	Co Ni Cr
	Chlorophyta	Respiration	
Paleozoic (2-20	Phaeophyta	Differentiation of organisms	Cu Ti V
Mesozoic (20-23)	Angiosperms	Further elaboration of photosynthetic organs	Mn Mo
Cenozoic (23.01)	Cultivated plants	Refinement of various processes	Many metals

TABLE 12 Changes in the proportions of metals in plants in the course of evolution (after Boichenko, 1976)

Dominant plants	Ratios of metal concentrations in plants				
	Fe:Mn	Zn:Cu	Ti:V	Ni:Co	Cr:Mo
Cyanophyta	109.7	22.5	6.5	0.8	7.1
Rhodophyta	11.9	5.3	5.3	0.8	14.0
Chlorophyta	7.9	4.8	5.7	2.7	32.8
Phaeophyta	8.3	6.7	4.5	1.7	5.8
Angiosperms	1.7	7.5	1.6	20.0	2.0

Numerous findings throwing light on the place of polyvalent metals in the evolution of plants, in particular their involvement in the evolution of autotrophic plant nutrition, have been reviewed by Boichenko (1966, 1968a,b). Boichenko based her hypothesis on the assumption that metals and their complexes within the cell are located at the active sites of enzymes which catalyze important oxidation and reduction reactions, and that each of these reactions is characterized by a specific oxidation-reduction potential (E_0). Important changes must have occurred in plants during the evolutionary transition from the anaerobic to the aerobic biosphere.

In agreement with Uspensky (1915), Boichenko concluded that during this process, plant metabolism changed to include new oxidation--reduction reactions taking place at higher redox potentials. This was accompanied by the emergence of enzymes operating at higher potentials. Boichenko recognizes several main groups among the numerous metalloenzymes catalyzing various types of oxidation reaction. These are flavoproteins containing non-haem iron, zinc proteins, haemoproteins and copper proteins. She suggests a role for each of these groups of metalloenzymes in the evolution of plant metabolism (Fig.67).

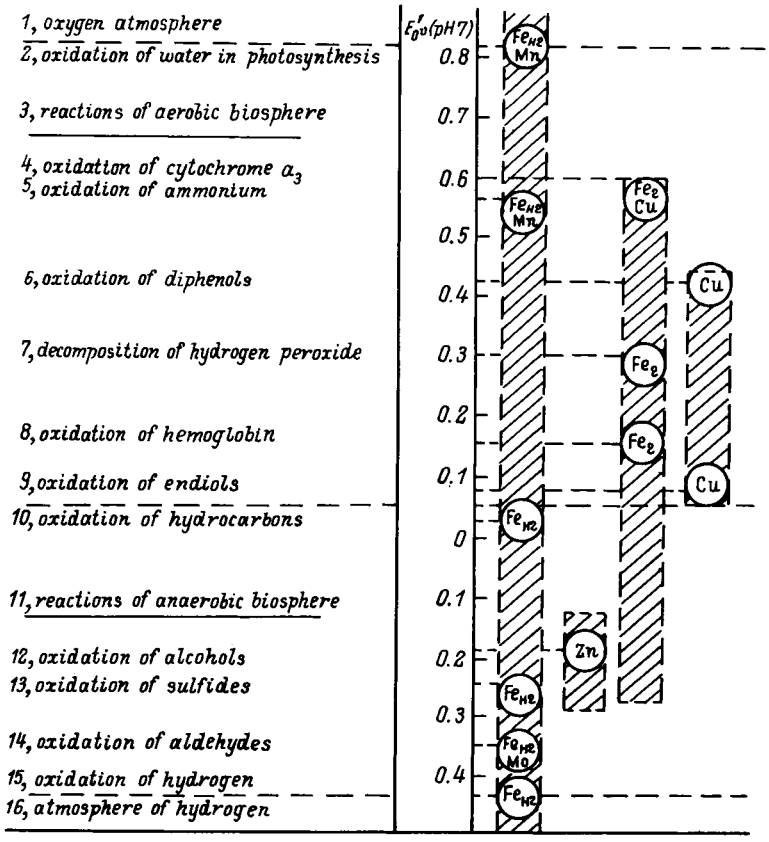


Fig. 67. Metal compounds in the evolution of the biosphere. E_0', oxidation-reduction potential (after Boichenko, 1968a).

In anaerobic oxidation various substances may act as electron acceptors, whereas in aerobic oxidation processes the terminal acceptor of electrons generally tends to be oxygen, which is thus reduced to water.

Boichenko supposed that the most ancient enzymes are those displaying a maximum activity at low oxidation-reduction potentials, their substrates being substances such as might have been found in the primeval biosphere. Among the oxyreductases, hydrogenases (flavoproteins containing non-haem iron) are enzymes of this type. They may catalyze the oxidation of molecular hydrogen by reducing carbon dioxide either to an organic compound with one carbon atom, or to an inorganic compound containing sulphur or nitrogen. All hydrogenases feature extremely low oxidation-reduction potentials (E_0' = -0.421), which accounts for the fact that they can carry out the reduction of a wide range of poorly reducible substances.

Compounds containing non-haem iron were utilized over a very wide range of oxidation-reduction potentials by organisms at different stages of their transition from anaerobiosis to aerobiosis. In the anaerobic bacteria, iron is represented by such non-haem compounds as the iron-containing protein-ferredoxins, which reduce various substances at low redox potentials. For reduction to occur in the presence of ferredoxins, co-enzyme A must also be present, since this is the acceptor leading to carbon dioxide and various acyl compounds in many heterotrophic and autotrophic organisms (Mortenson et al., 1963). Consequently, in the early stages of the evolution of cells, a link was established between acylation and electron transport mediated by iron compounds.

In addition to that of non-haem iron, a lesser roles were played by molybdenum, manganese and zinc in the evolution of anaerobic reactions. The oxidation-reduction potential of the reactions in which these metals are involved never exceeds the value of the so-called Pasteur point (E_0' = 0.05) around which a transition occurs from oxidation to aerobic respiration.

In the course of the subsequent evolution leading to aerobiosis, organisms could have utilized not only compounds containing non-haem iron, but also metals such as manganese, copper and zinc. With the adoption by plants of aerobic oxidation, which is more effective than anaerobic oxidation in terms of providing energy, a prominent role was assumed by haem iron compounds which both reduce hydrogen peroxide and transfer electrons to oxygen. Elevation of

the oxidation-reduction potential above the Pasteur point, according to Boichenko, allowed the algae to bring into use oxyreductases such as those comprising copper proteins and bimetallic flavoproteins.

When in the course of evolution aerobic photosynthesis began to occur, the reduction of carbon dioxide was mediated by oxyreductases containing non-haem iron - i.e. by hydrogenases. Further modifications of these enzyme reactions have involved the formation of stable iron-flavoprotein complexes with co-enzyme A, these retaining their activity under the increasingly oxidizing conditions of the environment. As shown by Zarin and Boichenko (1967), a complex of non-haem iron and co-enzyme A, which reduces carbon dioxide, is associated with the phospholipid moiety of chloroplasts. The incorporation of manganese into this system led to the formation of a complex involving the galactolipids of chloroplasts, - a complex which was capable of oxidizing water and releasing oxygen (Boichenko and Udelnova, 1964).

The incorporation of manganese into flavoproteins was an important step in the creation of the aerobic biosphere. This metal associated with chloroplast lipids established the oxidation-reduction potential required for oxygen evolution in the process of photosynthesis. An increase in the intensity of illumination together with the addition of manganese to the incubation medium, have been shown to promote the transformation of photoreduction into photosynthesis (Gaffron, 1944; Kessler, 1955). According to Boichenko (1966,1968a,b,1976) a similar transition could have occurred from anaerobic to aerobic photosynthesis in the primeval biosphere.

Metalloflavoprotein molecules which take part in aerobic reactions contain a second metal in addition to non-haem iron. Examples of second metals are molybdenum in nitrate reductase, copper in nitrite reductase (Medina and Nicholas, 1957), and manganese in hydroxylamine reductase (Nicholas, 1961). These enzymes can oxidize pyridine nucleotides. Also belonging to this group of flavoproteins is a manganese-containing enzyme, carbon dioxide reductase, which oxidizes water and thus liberates oxygen in photosynthesis (Boichenko, 1966). The existence of such bimetallic enzymes widens the scope of possible oxidation and reduction reactions, in many cases providing for reactions at considerably elevated oxidation-reduction potentials.

Boichenko (1963) regards bimetallic enzymes as belonging to a transitional form leading to still more complex enzyme systems,

the so-called electron transport particles. Each such particle represents a single enzyme system comprising several individual proteins each with its own active site and some sort of association with lipids. As a result, an electron transport chain is established which functions as an energy source in all living cells. Such particles have been isolated from the mitochondria of various organisms, and it has been found that the principal enzymes in these particles are represented by flavoproteins and haemoproteins (Green, 1961). Large amounts of non-haem iron and copper, as well as phospholipids, are found in these structures. Further investigations carried out on electron transport particles have revealed that they contain four lipoprotein complexes (I-IV), which transfer electrons from oxidized substrates to oxygen.

In actively photosynthesizing chloroplasts, the transport of electrons from water to carbon dioxide is mediated by a complex which is also a lipoprotein system. This contains almost all of the non-haem iron, and some manganese as well (Boichenko and Sayenko, 1961a); in many respects it resembles the electron transport system of mitochondria.

Mention should be made of the problem of the role of metals in the evolution of intracellular particles, primarily those of mitochondria and chloroplasts (Boichenko, 1968b, 1969). These particles are the sites of the principal oxidation-reduction processes; furthermore one finds that the concentrations of the polyvalent metals iron, copper and manganese in these particles are higher than the concentrations in other parts of cells. Boichenko emphasizes that in plant organs, plant cells and plant cell organelles, quantitative relationships have arisen in the course of evolution between metals which act as complex-forming agents in various plant species.

During evolution there has occurred an increasing concentration of individual polyvalent metals in intracellular structures, as more complexes involving substances of a lipid nature have come into being. This process can still be widely recognized in bacterial, plant and animal cells. The association of individual metal complexes with various lipids controls the direction of electron transport in cells. Such associations have emerged together with the appearance of the lipid-rich cell organelles-mitochondria and chloroplasts. The concentrating of polyvalent metals has played an important role in the evolution of these organelles themselves and the processes of respiration and photosynthesis that take place within them.

In the course of the evolution of chloroplasts from photosynthesizing bacterial chromatophores containing various non-haem iron compounds, there emerged in these organelles an association between manganese and galactosyldiglycerides. On account of the high oxidation-reduction potential of this new compound, there came about a transition from anaerobic bacterial photosynthesis, proceeding from the oxidation of various electron donors at low oxidation-reduction potentials, to the oxidation of water and release of oxygen. In the evolution of mitochondria, a transition occurred from the uncoupled functioning of individual dehydrogenases in the oxidation of substrates by non-haem iron, to the orerly transfer of electrons to oxygen. The latter process employs cytochrome oxidase as the terminal link in the chain. This enzyme contains iron solely in the haemin form, and copper. Such a transition was made possible by the development of the high lipid-content complexes which constitute electron transport particles.

To recapitulate, it is the view of Boichenko that at different stages of the evolutionary transition of chloroplasts and mitochondria from anaerobiosis to aerobiosis, changes have occurred in the associations between metals and individual lipid groups, and new complexes have been produced. These changes have resulted primarily in the advancement of the oxidation process in cell organelles. It is known that many of the enzymes found in organelles are involved in specific reaction cycles taking place in these organelles. As indicated above, the control of metabolism in organelles, and the emergence of their biochemical specificity have became possible by virtue of the important contributions made by complex metal compounds, many of which were non-enzymatic, although the greatest influence was exerted by metalloenzymes.

Boichenko (1975) summarizes views on the role of polyvalent metal compounds in the evolution of the metabolism of plants. Table 12 taken from her later paper (Boichenko, 1976), illustrates the changes in the relative proportions of metal compounds in the evolution of plants.

One of the principal processes taking place in the biosphere is the assimilation of carbon dioxide (Vernadsky, 1926). Therefore, the pattern and rate of assimilation are among the chief features characterizing different stages of evolution, together with the oxidation-reduction potentials at which the process proceeds (Boichenko, 1966, 1968a,b; Table 13).

TABLE 13 Role of metal compounds in the evolution of the biosphere (after Boichenko, 1976)

Evolutionary stages and characteristics of biosphere	Assimilation of CO_2	Involvement of metal compounds	E'_o at pH 7
Anaerobic biosphere	$n \cdot 10^{-3}$	Fe in reductases acting on C, S, N compounds	+0.05
Assimilation of CO_2 during the oxidation of organic substances		Zn in carboxylases and dehydrogenases	
Transitory biosphere	$n \cdot 10^{-2}$	Fe, Ti in reductases and dehydrogenases	+0.30
Chemosyntheses associated with the oxidation of inorganic substances		Co, Ni, Cr in dehydrogenases and other enzymes	
Aerobic biosphere	$n \cdot 10^{-1}$	Mo, V in reductases	+0.55
Photoreduction with production of peroxides		Cu in oxireductases	
The "oxysphere"	$n \cdot 10^{0}$	Mn in oxidases	+0.82
Photosynthesis with liberation of O_2 from water			
Transition to noosphere			
Photosynthesis in cultivated plants	$n \cdot 10^{1}$	Fertilization with compounds of various metals	+0.82

With the transition from the heterotrophic pattern of carbon dioxide assimilation to chemosynthesis, and further to photoreduction and photosynthesis, the rate of reduction becomes thousands of times greater. This is accompanied by a simultaneous increase in redox potential, effected by various metals (Boichenko, 1974). Increased rates of oxidation resulted in the formation of an anaerobic biosphere somewhere in the middle of the Protozoic era, and a transition back to an aerobic biosphere at the beginning of the Paleozoic era.

Further release of oxygen into the atmosphere converted the biosphere into its modern form known as the oxysphere. Compounds of metals such as copper and manganese, active at high oxidation-

-reduction potentials, acquired greater significance, and oxidation came to depend upon the oxidation of water in the light with the liberation of free oxygen ($\underline{E}_0$ = +0.82). This oxidation is mediated by manganese compounds and galactosylglycerides present in chloroplasts. In contrast to the amounts of substances that were oxidized in the earlier oxidative processes, the amount of water available in the biosphere is practically unlimited. Thus photosynthesizing algae, and later higher plants, were able to spread over the entire planet. The progressive perfection of the carbon dioxide assimilation process significantly influenced all the other biochemical processes occurring in plants.

The iron, titanium, manganese and copper compounds isolated by Boichenko (1976) are of particular interest with respect to the evolution of photoautotrophism in plants. The iron compounds are found in carbon dioxide reductase, which mediates the reduction of carbon dioxide at a very low $\underline{E}_0$ value - close to that of a hydrogen atmosphere. This enzyme is ferro-flavoproteid with a high content of lipids (Table 14). Titanium compounds, being strong reducing agents, protect plant cells from peroxidation (Gryzhankova et al., 1975). The copper and manganese compounds associated with chloroplast lipid perform the important function of raising the potential at which oxidative reactions take place in these organelles. Manganese has been found to play a particularly important role in oxygen evolution.

Boichenko indicates that the functioning inside the cell of these metals with their diverse $\underline{E}_0$ values is only feasible by virtue of compartmentalization. This is achieved primarily by the association of metals with various lipid groups such as phospholipids (Fe, Ti), neutral lipids (Cu), and galactolipids (Mn). The increasing number of such interactions has been very important in the evolution of catalytic activity among metal compounds. To illustrate this point, Boichenko refers to changes in the composition and activity of carbon dioxide reductase (Table 15). This enzyme is a metalloflavoprotein associated with large amounts of lipid (Boichenko and Gryzhankova, 1974). The latter workers found that there was a range of lipid contents in different forms of the enzyme isolated from plants differing in their evolutionary advancement.

TABLE 14 Metal compounds in autotrophic development (after Boichenko, 1976)

Metal compounds	Properties of the compounds	Changes in evolution	Involvement in photosynthesis
Fe(II) associated with sulphide groups and flavin in carbon dioxide reductase	Lipoflavoprotein 0.3% Fe, E_o = -0.42	Increase in lipid content of flavins	Reduction of carbon dioxide
Ti(IV) associated with 4 phosphopanthetein	A component of phosphoprotein 0.03% Ti, E'_o -0.22	Increase in percentage of P	Protection against peroxidation
Cu(II) associated with amino sugars or phenols	A component of proteins 0.2-1.1% Cu, E'_o up to +38	Increase in percentage of Cu-phenol compounds	Elevation of the E'_o value
Mn(III) associated with galactolipids in chloroplasts	Galactosylglycerides 0.2-0.4%, Mn E'_o +0.55	Increase in percentage of Mn-monogalactosylglycerides	Oxygen evolution from lipid peroxides

TABLE 15 The reducing capacity of carbon dioxide reductase (after Boichenko, 1976)

Sources	Composition % lipids	Fe:flavin	Reduction (μmoles per μg Fe)
Cyanophyta and Rhodophyta	1.1-2.4	61.0-74.1	0.22-0.25
Chlorophyta and Phaeophyta	2.7-10.4	31.4-60.4	0.65-0.77
Angiosperms (in autumn)	9.4-25.0	17.9-36.9	0.68-4.76
Angiosperms (in spring)	26.5-32.5	17.7-17.8	4.55-11.99

Referring to the vast growth of biogenic processes as a consequence of biological evolution on Earth (Tugarinov and Voitkevich, 1970; Ronov, 1972), present data on the annual migration of some elements attributable to the living processes of plants in various biogeochemical situations (Table 16).

TABLE 16 Participation of plants in migration of elements in the biosphere (after Boichenko, 1976)

Element	Annual migration with plants		
	Total m	Lower plants %	Higher plants %
O	$3.60 \cdot 10^{11}$	21.5	78.5
C	$1.35 \cdot 10^{11}$	21.5	78.5
N	$8.77 \cdot 10^{9}$	22.0	78.0
S	$2.32 \cdot 10^{9}$	66.8	33.2
P	$9.76 \cdot 10^{8}$	46.3	53.7
Fe	$1.22 \cdot 10^{8}$	74.0	26.0
Zn	$4.21 \cdot 10^{7}$	45.8	54.2
Mn	$2.12 \cdot 10^{7}$	32.1	67.9
Cu	$4.61 \cdot 10^{6}$	30.8	69.2
Ti	$3.37 \cdot 10^{6}$	46.0	54.0
Ni	$1.07 \cdot 10^{6}$	36.2	63.8
V	$6.23 \cdot 10^{5}$	41.4	58.6
Mo	$2.63 \cdot 10^{5}$	22.0	78.0
Co	$2.04 \cdot 10^{5}$	44.3	55.7
Cr	$1.81 \cdot 10^{5}$	77.3	22.7

Comparison of the amounts of polyvalent metals concentrated annually in plant cells with the world deposits of ores, reveals the major biogeochemical significance of the uptake of metals by plants (Vernadsky, 1931; Boichenko, 1968a,b). The ore deposits of many of these metals laid down in all the known geological eras are smaller than the amounts contained in the plant kingdom. Moreover, polyvalent metal compounds are involved in the assimilation by plants of many other elements (carbon, hydrogen, nitrogen and sulphur) on a large scale. This assimilation is accompanied by changes in valency as a result of reduction in the process of photosynthesis or oxidation in the process of respiration (Boichenko, 1974). Conse-

quently, metal compounds augment the influence of plants on the biosphere by promoting the concentration, oxidation and reduction of various elements (Vernadsky, 1965).

In the survey carried out by Zakharova and Udelnova (1977) the content of polyvalent metals in algae cells is reported to be tens or hundreds of thousands times higher than the content in sea water. It has been established that the concentration of these elements is to a large extent achieved by the formation of complexes. The accumulation of metals by the formation of metallo-complexes means that metals exert an influence on the various metabolic reactions that take place in living cells.

The paper considers a number of complex compounds of polyvalent metals occurring in algae, the part played by these in the evolution of metabolism, and their role in the biogeochemical functions of organisms in the biosphere.

Lewis (1980) has expressed interesting views on the role of boron in the colonization of dry land by vascular plants. According to his hypothesis, boron became an indispensable element only after sucrose had been selected as the basic carbohydrate in phloem transport. Because of the absence of cis- hydroxyls in the phloem the latter seems to be largely incapable of forming boron complexes, whereas sugar-alcohols (polyols), which possess cis-hydroxyl pairs, are formed readily.

Lewis supposes that in a majority of the fungi and algae, with the exception the green algae, all the borate absorbed or the main bulk of it, becomes bound in the form of boron-polyol complexes (Raven, 1980). Thus boron is incapable of exerting any regulatory effect in the metabolism of these organisms, and consequently they do not have any specific requirement for boron. It was only when sucrose, with its low capacity for complexing with borate, became a metabolically important carbohydrate in the green algae - the ancestors of higher plants - that boron acquired a significant role in plant metabolism, creating the possibility for this element eventually to achieve a regulatory role in the metabolism of vascular plants. We refer here to the views of Lewis regarding the role of boron in lignin biosynthesis and in cell differentiation in the development of the xylem.

Lewis draws attention to the fact that the use of sucrose as a transport carbohydrate is almost entirely restricted to algae and green plants.

Raven (1977) believes that green algae were the ancestors of the terrestrial flora. He points out that although the green algae do not synthesize lignin, they possess the key enzyme (phenylalanineammonium-lyase) for the formation of the initial phenolic acid; green algae also contain the peroxidase which is known to stimulate lignin synthesis, and which is very similar to an enzyme found in higher plants. Thus the green algae are preadapted to the synthesis of lignin-like polymers, and this points to them as the most probable ancestors of vascular plants.

According to Lewis, boron has gradually become involved in two of the major activities of plant life on Earth. One of these is the exploitation of soils for water, minerals, and in particular boron itself, since boron is necessary for the development of lateral roots (Fig.68) (Shkolnik, 1935). The other major activity requiring boron is the germination of pollen and growth of the pollen tubes in angiosperms (Fig.69) (Bobko and Tserling, 1938; Visser, 1955 and others). Lewis is of the opinion that, irrespective of the exact mechanism of boron participation in the germination of pollen, the role of boron in this process is another example of the indispensibility of this element for vascular plant life.

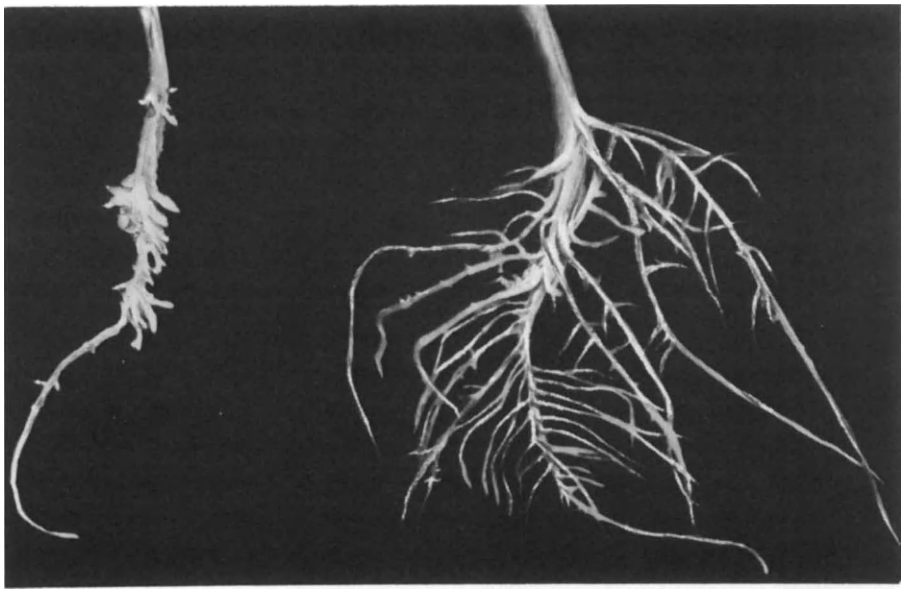


Fig. 68. The effect of boron deficiency on the development of the root system in Cannabis: left, without boron; right, with boron.

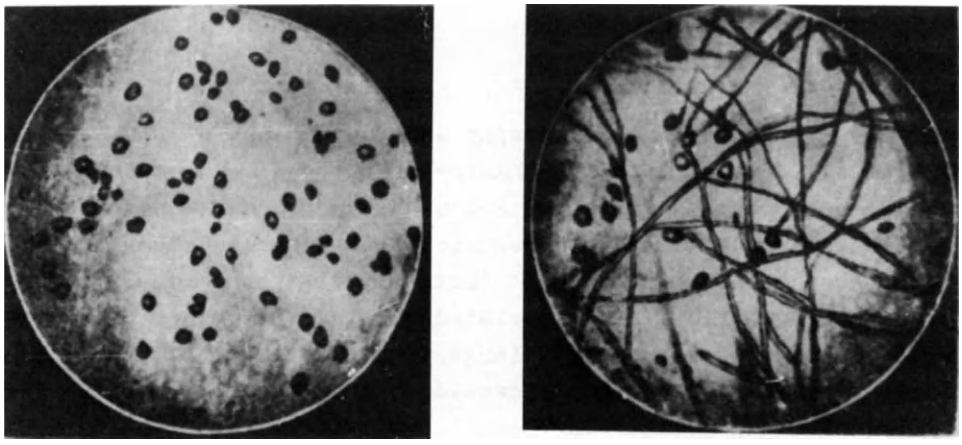


Fig. 69. Germination of pear pollen (after Vasilyev, 1937).
Left, without boron; right, with boron.

Chapter 2

GEOCHEMICAL ECOLOGY

Progress in botanical geography and the ecology of plants, has enabled the effects of the chemistry of the environment on vegetation to be better understood. Botanical geographers and taxonomists have long noticed the specificity of flora in areas of chalk deposits and other lime rocks. Another well-known example is the peculiar halophilic flora associated with salinated soils. Halophilic plants are represented largely by the families Chenopodiaceae, Frankeniaceae and Tamaricaceae, which apparently evolved on highly salinated soils. The peculiarities of vegetation in territories enriched with individual trace elements are well known. Various floras have been described such as halmeine (occurring on zinc-enriched soils), selenium, copper, nickel, chromium, cobalt, barium, berillium, and other floras, according to the predominent element in the soil.

Among plants permanently associated with halmeine floras one finds varieties and new species which have emerged under the influence of the specific soil conditions of the locality. These plants include Viola calaminaria, Thlapsi alpestre spp. calaminare, Minuartia verna spp. hercynica, Armenia calaminaria, Armenia halleri (Baumeister, 1954).

Particular attention has been given to the study of the serpentine flora which develops on ultrabasic rocks - periodotites, olivinites, and the products of their metamorphosis, serpentinites. These soils are rich in magnesium, nickel, chromium and cobalt, but are poor in calcium (Paribok and Alekseyeva-Popova, 1966). All the serpentinite regions of the worled show a number of common features: (1) low fertility of the soil; (2) a vegetation that is more sparse and xerophytic than the vegetation occurring on other mountain rocks of the same region; (3) a flora that is rich in indigenous species, showing a sharp boundary with vegetation of neighboring territories. Serpentine floras are relatively poor both in terms of species composition and numbers of plants. Some species are represented by a number of distinct races which differ in their ecology and sometimes also in their morphology from the original type. Cerastium alpinum var. serpentinicola and Visicaria alpina var. serpentinicola are well-known serpentinite races (Rune, 1953).

To illustrate the extent of the influence of serpentinites on the pattern of vegetation an aerial photograph from a book by Schütte (1964) is shown (Fig.70). According to Schütte, the prevailing

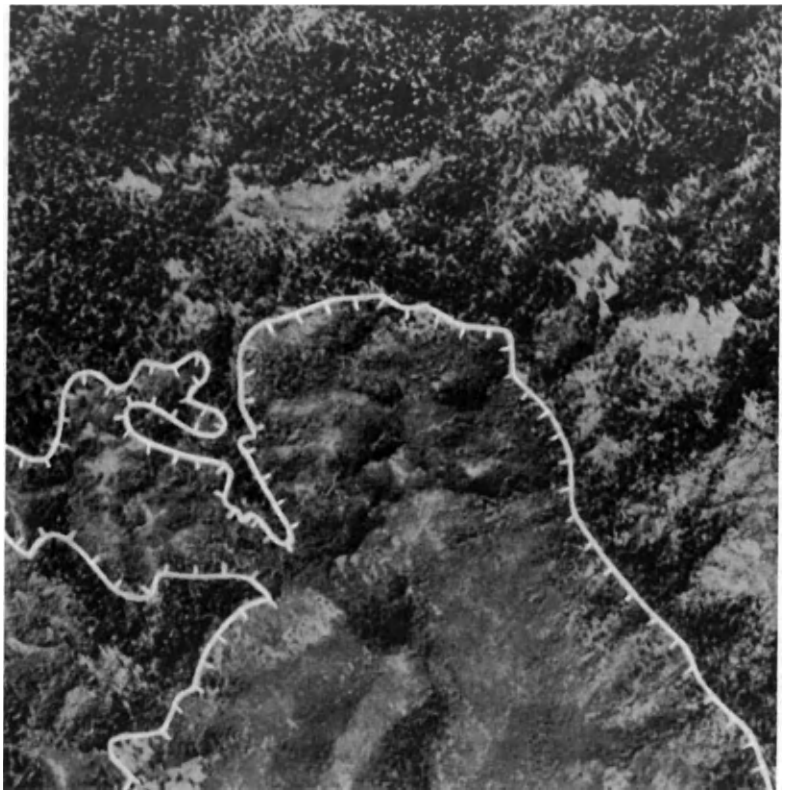


Fig. 70. Differences in the pattern of vegetation growing on serpentines (aerial photograph, after Schutte, 1974).

vegetation in the area of California depicted in the photograph is forest growing on sandy soil, and featuring such species as Pseudotsuga menziesii, Pinus ponderosa and Quercus kellogii. These species, however, cannot grow on serpentinite soils on which is found a specific flora consisting of Cupressus sargenti, Arctostaphylos viscida, Adenostoma fasciculatum, Quercus aurata, Pinus attenuata. The boundary between the two communities is very distinct, changes in the vegetation coinciding directly with changes in soil composition and underlying rock type.

Igoshina (1966) showed that the flora of the ultrabasic massif of Rai-iz in the Polar Urals comprises half as many species as that of the neighboring slanetz mountains. Some plant species grow on

both types of mountain rock, but most of the species occurring on the slanetz soils are entirely absent from the flora of the ultrabasic massif. On the other hand, another group of species is typical of the ultrabasic massif, and of rare occurrence on slanetz soils. High concentrations of magnesium, nickel and chromium have been found to be toxic to those plant species which are widely distributed on slanetz soils and absent from the ultrabasic massif (Paribok and Alekseyeva-Popova, 1966). It is conceivable that species growing on both types of mountain soil are represented by two ecotypes which have evolved under the influence of different edaphic conditions (Alekseyeva-Popova, 1972).

Nemec (1957) reported that forest species died on serpentinite soils in southern Czechoslovakia because they were unable to the levels of nickel, cobalt and chromium in the soil.

Zakharov and Zakharova (1970) found bare areas on rock fields and rock deposits of cobalt in Tuva, where copper predominates over cobalt and nickel. In these areas Artemisia frigida and species of genus Actogeron are sometimes found.

By the early nineteen-twenties, a vast body of information had been accumulated relating to the floristic and chemical properties of plants, and indicating a relationship between plants and their geochemical environment. A thorough understanding of this relationship did not come until the science of the relationship between living organisms and their geochemical environment, biogeochemistry - became established. The founder of this science was Vernadsky (1922, 1926, 1931, 1965). The ideas expressed by Vernadsky concerning the biogenic turnover of chemical elements gave impetus to the development of new trends in science, including indicator geobotany and biogeochemical approaches to the surveying of natural deposits. These disciplines yielded much material relevant to the geochemical ecology of plants.

The foundation laid by V.I.Vernadsky was developed by A.P.Vinogradov, who suggested a definition for a biogeochemical province (Vinogradov, 1938, 1963). Thus regions or areas within which organisms show similar responses to excesses or deficiencies of macro- and micronutrients in the environment, are designated biogeochemical provinces. Underlying this definition of the term biogeochemical province is the idea of the turnover of the micro- and macronutrients within the system: soil - plant organism - animal organism. Because of a sharp deficiency or excess of one or more

elements within a particular biogeochemical province, an endemic disease of the plants and animals may exist.

Vinogradov (1938, 1963) recognizes two types of biogeochemical province. Those of the first type occur as small localities or large areas and are always found within specific soil-climate zones; they are the so-called zonal biogeochemical provinces. Thus, a number of biogeochemical provinces and associated endemic conditions can be discerned within the podzol and turf-podzol forest soils of the northern hemisphere. These span from the USA throughout the whole of Europe, the Netherlands, Denmark, Poland, the Baltic Republics of the USSR, across the USSR to Moscow and further to the Urals, and then across Siberia to the east as far as the rivers Zea and Burea. These biogeochemical provinces are characterized by shortages of calcium, phosphorus, potassium, cobalt, copper, iodine, boron, molybdenum and some other trace elements. Such provinces and their associated endemic deficiencies are zonal and negative in type, emerging as a result of shortages of individual elements.

The second type of biogeochemical province, the distribution of which does not relate to specific soil-climate zones, is the so--called azonal type of province (interzonal, by Vinogradov). Biogeochemical provinces of this type are found in regions characterized by salt deposits, volcanic emanations, rock beds and natural deposits, and are of the positive type, i.e., they are associated with excesses of chemical elements in the environment and native organisms. Examples of such provinces are the boron province situated in the boron-deposit region of Lake Inder, fluorine provinces that occur around active volcanos, and the molybdenum provinces of the Caucasus, etc. (Kovalsky, 1974).

As far back as 30 years ago, biogeochemical provinces were known which had excesses or deficiencies encompassing more than 30 chemical elements, including cobalt, iodine, zinc, molybdenum, copper, selenium, manganese, beryllium etc. (Vinogradov, 1946; Rademacher, 1940; Malyuga, 1946; Shkolnik, 1960, and others). Information on these can be found in the books by Schütte (1954) and Kovalsky (1974), and in many other publications. The book by Kovalsky gives data on the biogeochemical provinces of the Soviet Union, with deficits or excesses of, elements covering vast territories. The description of the biogeochemical provinces is taken mainly out of this book.

Biogeochemical provinces with a deficiency of cobalt. The taiga-forest non-chernozem zone is rich in provinces featuring cobalt deficiency of the soil, and various forms of acobaltosis among the plants.

Animal diseases such as bush sickness have been recognized as being attributable to cobalt deficiency for over a hundred years.

Underwood (1956, 1971) described the history of the early investigations on the role of cobalt in animal physiology. After the publication of his studies, the essential nature of cobalt for animals was acknowledged in many countries.

In Latvia and some regions of Lithuania and Estonia, a severe and widespread form of acobaltosis once took a high toll among cattle. The disease was eradicated by supplying cobalt in tablet form to the animals (Berzin , 1952) (Fig.71). In the campaign to combat acobaltosis, the observations made by Davis (1958) on the efficiency of supplying this element in extremely small amounts have been fully validated. Acobaltosis is common in many districts of the non-chernozem zone because of a deficiency of cobalt in the forage. B_{12} hypovitaminosis associated with cobalt deficiency is progressively aggravated by an increasing degree of copper deficiency. In such cases, only a combined administration of cobalt and copper is effective.

Kovalsky and Chebayevskaya (1951) studied cobalt levels in many meadow plants and found that legumes contained comparatively high levels of cobalt.

Biogeochemical provinces with a deficiency of copper in the soil and plants are found on the peat and peat-bog soils. Approximately 30% of the plants growing in these provinces contain less than $3 \cdot 10^{-4}$% copper (the lower limit of the normal range), and some contain even less than $7{:}2 \cdot 10^{-5}$% in some places. This accounts for the common occurrence of anaemia and haemosiderosis in some areas (especially the less wet peat bogs), and the absence of these conditions in other localities.

On peat bog soils and in lesser peat bogs that are poor in utilizable copper, endemic deficiency diseases of grasses-wilting and the so-called "treatment disease" - are common. Sugar beet, hemp and hemp-mallow suffer from copper deficiency in these localities (Lashkevich, 1937), as well as wild and cultivated grasses (Bakhulin, 1952). Applying copper sulphate, or industrial waste containing copper, restores normal plant growth.

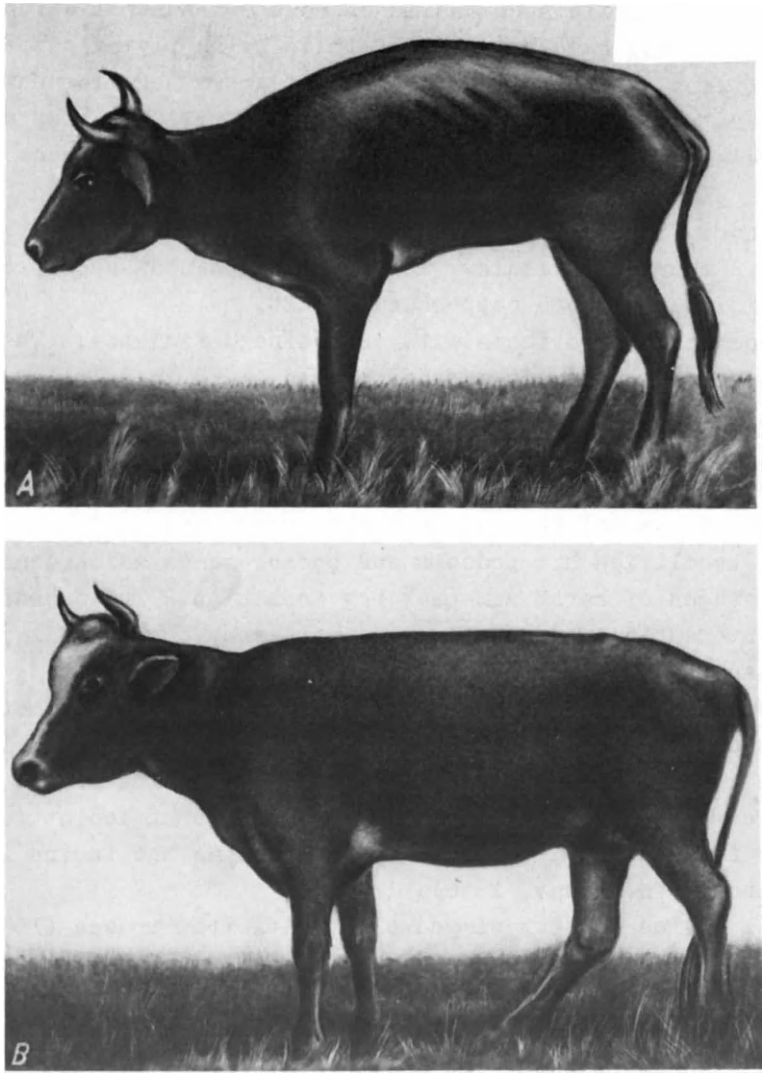


Fig. 71. Effect of application of cobalt on eradication of acobaltose sickness among cattle (after Berzin, 1952). Above - a sick cow before cobalt supply; below - the same cow after cobalt supply during 35 days.

Biogeochemical provinces with a deficiency of copper in the soil are found in various countries including Germany (the North West German Valley), the Netherlands and Denmark (Sjollema, 1933; Rademacher, 1940), Australia (Bennets et al., 1942), South Africa (Schütte, 1964), the USSR (Lashkevich, 1955) and other countries with extensive areas of peat bog and sandy soil.

The non-fertile sandy soils of Southern Australia (ca. 2.5 million hectares) have been made fertile by supplying copper and zinc fertilizers. Research on the mode of action of micronutrient fertilizers was initiated in South-West Australia in response to the progressive deterioration of tea plantations, and yucca and eucalyptus plants. Sheep breeding was severely restricted by the occurrence of enzootic ataxy, which was responsible for the death of large numbers of animals. The cause of this disease proved to be a deficiency of both copper and cobalt.

Biochemical provinces with an iodine deficiency. The incidence of endemic enlargement of the thyroid gland in animals and humans arising from a deficiency of iodine in the soil and vegetation is high in many regions of the USSR. Biogeochemical provinces with a shortage of iodine occur more commonly in mountain areas and the plains of the taiga-forest non-chernozem zones. Typical soils in these localities are podzols and podzol-sands which commonly display stages of marsh and peat bog formation. The disease is also encountered in arid desert zones with grey, brown soils, and along the banks of riverbeds.

Atmospheric iodine plays only a minor role in the metobolism of organisms in continental and mountain regions, whereas in coastal areas the atmosphere may be the principal source of this element. Furthermore, atmospheric iodine as a source of iodine for the soil is of fundamental importance in maintaining the iodine level of a province (Vinogradov, 1946).

The iodine content of podzols varies from traces (10^{-7}%) to $7.5 \cdot 10^{-4}$%. Eighty per cent of soil samples contain less than $1 \cdot 10^{-4}$% and a quarter of these contain less than $1 \cdot 10^{-5}$%.

Iodine determinations carried out on forage plants growing in non-chernozem regions have shown that 75% of all the species studied contained less than $8 \cdot 10^{-6}$% of iodine (minimum $2 \cdot 10^{-7}$%). Problems encountered in cattle and livestock on account of iodine deficiency include low stature, infertility, reduced milk and wool productivity, loss of hair, miscarriage (in pigs), etc.

Endemic enlargement of the thyroid gland tends to affect both humans and livestock, however the reaction to iodine deficiency in man is more pronounced and of more frequent occurrence.

Particularly severe manifestations of endemic goitre occur in provinces poor in both iodine and cobalt (Kovalsky, 1957). A correlation between the occurrence of goitre and deficiencies of

iodine, copper and cobalt has been reported for the Altai region (Kolomijtseva and Neimark, 1963). Both excesses and shortages of manganese inhibit the synthesis of iodine compounds in the thyroid gland (Kovalsky, 1974).

There is an interesting mercury biogeochemical province in the Mountain Altai Autonomous district, which differs notably from other localities in the Mountain Altai in that there is a considerably larger amount of iodine in the environment. In spite of this goitre of medium severity is endemic there (Kolomijtseva and Neimark, 1963). Iodine prophylaxis carried out over a period of three years has reduced the incidence of goitre in the Altai by 24.2%, but no response to prophylaxis has ever been recorded in the mercury province. An excessive intake of mercury from the air (Hg concentrations are: 3.2 mg m^{-3} in the air, 0.04 mg per litre in milk, and 0.012 mg per litre in the water) has been implicated as the cause of the failure to combat goitre there.

Biogeochemical provinces with an iodine deficiency are encountered in many countries.

Little is known of the levels of iodine in higher plants. Only a few studies may be cited, among which is that of Darkanbayev and Niretina (1965) who succeeded in elevating the iodine content of cultivated plants by supplying the element as a fertilizer.

Biogeochemical provinces with an excess of molybdenum and sulphates and deficiency of copper. In many countries - Peru, Great Britain, Canada, South Africa, Morocco, Australia, New Zealand, Kenya, the United States, the Netherlands, West Germany, Greece, Norway and the Soviet Union - there are biogeochemical provinces characterized by a shortage of copper and an excess of molybdenum and sulphates. Such provinces in the Soviet Union are found in the arid steppe, semi-desert and desert biogeochemical zones described in detail by Kovalsky and Rish (1970); Aliverdiyev et al. (1970) and Kovalsky (1974). The incidence of endemic ataxy in sheep, goats and cattle is high in these provinces. In other countries this form of ataxy also affects llamas, pigs and horses, but always ruminants tend to suffer the most.

As has been found in the biogeochemical provinces of the USSR and other countries, endemic ataxy can arise in the presence or absence of an adequate supply of copper. Even with an adequate level of copper in the soil, this disease can be instigated by an elevated concentration of molybdenum, or more particularly, of

sulphate. As was shown by Mills (1960), an excess of sulphate or molybdenum in the diet of ruminants interferes with the absorption of copper in the gastro-intestinal tract.

Kovalsky (1974) recommended the investigation of other factors of the geochemical environment that might affect copper metabolism and lead to ataxy. He indicated that a shortage of copper is insufficient by itself to account for the spread of endemic ataxy, and pointed to the deficiency of cobalt and phosphorus in provinces in which endemic ataxy is common. In arid steppe, semi-desert and desert zones there tends to be a large amount of boron which is antagonistic to copper (Shkolnik and Makarova, 1949). It is conceivable that excess copper in forage reduces the retention of this element in the organism, and this may aggravate any copper deficiency associated with ataxy. Kovalsky (1974) also refers to the fact that in copper-deficient provinces in Dagestan (North of the Caucasus) the lead/copper ratio is abnormally high (Aliverdiyev et al., 1970); a similar situation is observed in Derbishire in England. A high level of lead is thought to render copper unutilizable by the organism on account of impeded intestinal absorption.

As in the case of iodine deficiency, we now have findings of major significance relating to the emergence of endemic nutritional disease.

Biogeochemical provinces with an excess of boron in the soil. In the chapter on boron, mention was made of biogeochemical provinces in the Aralo-Caspian depression notable for their high levels of boron. The author considers it appropriate to discuss these here in detail, since they are unique indeed, comparable in detail investigations of such provinces have made such a large contribution to the geochemical ecology of plants.

Investigations have been carried out over a vast territory of boron enrichment stretching in an east-west direction (Kovalsky (1974). Mosaic patches of extremely high boron concentrations (up to 0.356% in brown desert steppe soils) occur in high boron regions. Compared with tuft-podzol soils, brown desert soils, solonetz soils and solonchak soils are 688, 73 and 63 times richer in boron, respectively. Meadow soils losing boron during spring floods are an exception.

A high level of boron specifically affects plant growth. With a moderately high boron content of the soil, plants are larger than normal (sometimes 2-3 times larger) and have more succulent leaves

which remain large and green even during the driest part of the season. This latter effect is apparently related to the positive influence of boron upon drought resistance (Shkolnik, 1939b). Populations of Kochia prostrata Schrat feature strong multi-stemmed bushes reaching 80-90 cm in height and 60-70 cm in diameter. Artemisia lercheana Web grows to 50 cm in height and 30-40 cm in diameter. Individual plants have as many as 60-80 stems each developing a large number of shoots with large inflorescences and many flowers. Anabasis aphyla L., Eurotia ceratoides C.A.M., Limonium suffruticosum Ktze as well as many saltworts also grow larger. Plants usually develop at a faster rate under these conditions.

In localities in which there are high boron concentrations or boron is present in a readily accessible form, the less tolerant plants die while others show teratological modifications, retarded growth, or a spreading, cushion-like habit (as in Anabasis salsa) resulting from inhibited development of the growing points (Shvyryayeva and Malashkina, 1960) (Fig.72).

Plant cover is almost entirely absent where borates have come up to the surface. Isolated Salsola lenuta Pall. and Limonium suffruticosum Ktze plants represent the only vegetation in such areas, but even these eventually die. Only Salsola nitraria Pall. is able to grow on pure ulexite. Different species show different responses to boron excess according to the soil type. Anabasis lercheana Web , Limonium suffruticosum Ktze and Salicornia herbaceae L. are also boron concentrators.

Such observations have shown that there is considerable intrapopulational variability with respect to the ability to concentrate boron under different soil conditions. The grass Agropyron repens L. contains only minor amounts of boron irrespective of the concentration in the soil of the biogeochemical province. Other grasses behave likewise. This is conceivably related to specific properties of the cell membranes in grasses, including a higher degree of stability under extreme environmental conditions. It has also been found that grasses, in contrast to dicotyledons, take up only minor amounts of selenium when growing on soils enriched with this element.

A high boron content of the forage crops grown in boron-rich biogeochemical provinces results in an endemic disease of the cattle and other livestock (mostly sheep), Fig.73 affecting the lungs and gastro-intestinal tract. This disease has been

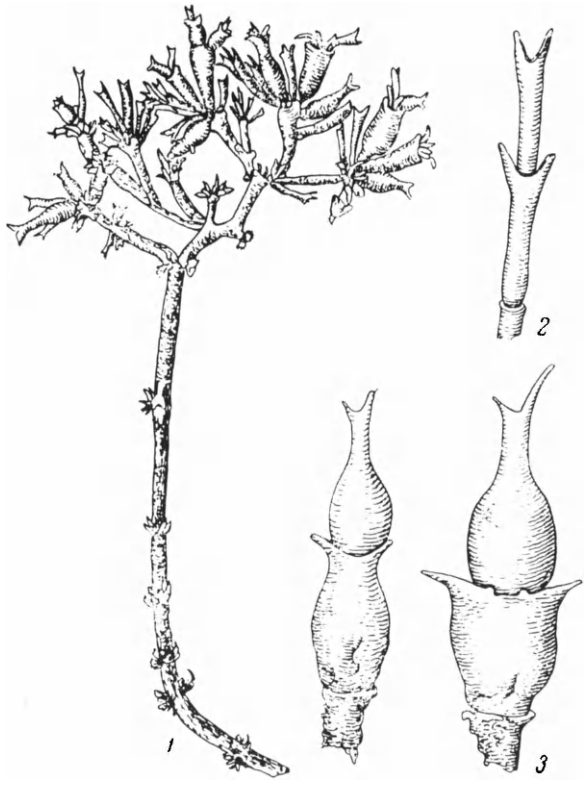


Fig. 72. Teratological modifications of Anabasis salsa var. depressa in the biogeochemical province with boron excess (after Shvyryaeva and Malashkina, 1960). 1, whole plant; 2, normal internodes; 3, bottle-like abnormal internodes.

designated boron enteritis; it occurs in approximately 16% of animals, the lethality being 41% of the affected stock.

Kovalsky (1974) indicates that endemic disease caused by an excess or deficiency of a trace element in the environment generally occurs in 10-20% of the animals of a population, the majority of the animals remaining healthy. Particularly severe symptoms have developed in sheep brought to Kazakhstan from other regions in order to improve the local breeds. Thus according to Kovalsky, in any population of animals exposed to high concentrations of a trace element the number of adapted individuals exceeds the number of non-adapted animals; the reverse holds for plants, the number of adapted plants generally being lower than the number of non-adapted ones. He believes that this can be ascribed to the more immediate dependence of plants upon soil conditions, and thus to the greater

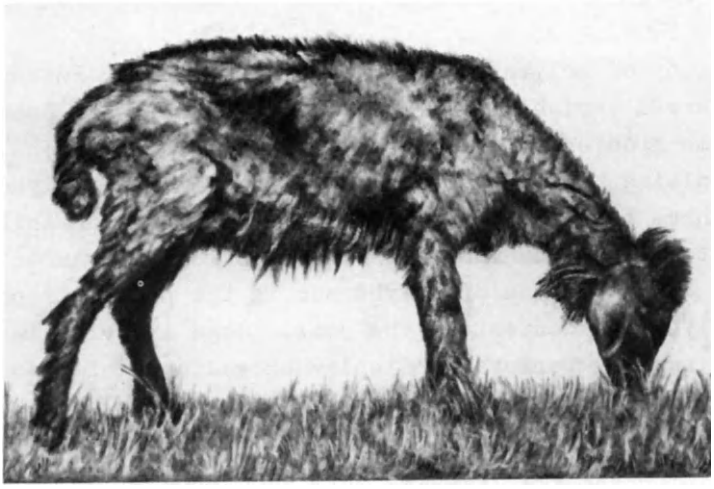


Fig. 73. A sheep sick in boron enterit in biogeochemical province with boron excess (after Kovalsky, 1974).

influence on plants of the overall geochemical environment.

The occurrence of biogeochemical provinces with high levels of boron in the United States has already been mentioned. There can be little doubt that similar provinces occur in arid steppe, desert and semi-desert zones in other countries, although they may not yet have been recognized as such.

The antagonism between boron and copper in plants observed by the author (Shkolnik and Makarova, 1949) has found practical application in attempts to eliminate boron enteritis. The administration of extra copper to afflicted sheep has been suggested (Kovalsky, 1974) as a means of stimulating the excretion of boron accumulated in organs and tissues. This approach has indeed proved to be effective. Affected sheep given 252 mg of copper sulphate daily showed a rapid excretion of boron, the rate of excretion from the kidneys being enhanced 79-fold; at the same time over half of the boron taken with the food was retained by the animals.

A biogeochemical province with an excess of molybdenum. In sheep and cattle feeding on plants accumulating high concentrations of molybdenum (above 10^{-3}% per unit dry weight), a number of investigators have reported molybdenosis manifesting in severe diarrhoea, loss of weight and hardening of the hair with eventual hair loss. This disease occurs in sheep and cattle in England, the USA, Canada, New Zealand and Sweden. The disease develops very rapidly (within

2–4 days) in animals grazing in a molybdenum-rich environment (Kovalsky, 1974).

In a study of molybdenum-rich soils in Armenia, Petrunina (1970, 1974) observed variability of concentrating capacity among plants of the same species under the same ecological conditions. Populations displaing positive and negative gradients of molybdenum accumulation have been found. In Thymus kotschyanus the ability to concentrate molybdenum manifests as an ecological characteristic, i.e., the concentration of molybdenum in the plants is proportional to the molybdenum content of the soil. Some individuals of the species Hypericum perforatum display exceedingly high concentrations of molybdenum. Thus according to Petrunina, the adaptation of an organism to high levels of this element is a taxonomic character. Physiological variants have also been found in lower organisms - fungi and bacteria. Kovalsky and Letunova (1966) described a number of genetically determined physiological variants of microorganisms adapted to high concentrations of molybdenum, vanadium, copper, cobalt and selenium.

Kovalsky et al. (Kovalsky et al., 1961; Kovalsky, 1974), taking the results of similar biochemical examinations of humans and animals in the molybdenum biogeochemical province, deduced that an increase in the activity of xanthine oxidase and disturbances of purine metabolism eventually result in a condition which in terms of its clinical presentation, resembles gout.

A biogeochemical province rich in copper has been discovered in Bashkiria. The mean copper content of the soils in this province is $3.9 \cdot 10^{-2}$%, or about 40 times higher than that of the soils in neighbouring regions with normal copper levels. Plants growing on pasture land rich in copper accumulate the metal; some Artemisia species, for example, concentrate copper up to $5.4 \cdot 10^{-2}$%. Gololobov (1960) found that the organs and tissues of cattle and sheep in the biogeochemical province rich in copper contain notably higher amounts of this element. He detected a specific form of anaemia affecting the livestock of the eastern regions of Bashkiria where the soils contain high concentrations of copper. This disease is strictly endemic, occurring mostly in lambs. It affects up to 50% of the animals with a lethality rate of 20–30%, the most severe symptoms developing at the time of weaning. The transition to feeding on forage accounts for the increasing incidence of the disease, since the copper content of plant fodder is some tens to

hundreds of times higher that that of the animals' milk. Anaemia in lambs caused by high levels of copper in the fodder is associated with a progressive loss of weight and malfunction of the liver (jaundice).

An antagonism between iron and copper in plants has been confirmed in the author's laboratory (Shkolnik and Makarova, 1950). It is the author's belief that these findings, if used as a basis for deeper investigation into the effects of iron on the excretion of copper, may lead to the development of a therapy for copper-induced anaemia. The practical application of research findings on the antagonism between boron and copper has proved to be effective in eliminating endemic disease caused by excess boron in animals.

Teratogenic alterations occur in plants growing in provinces with high levels of copper, as exemplified by Potentilla bifurca on the mineral soils of the Southern Urals where copper predominates over nickel. The plants develop an extreme dwarfness, with the formation of dense ball-like cushions consisting of many juvenile shoots with underdeveloped leaves.

Biogeochemical provinces with high levels of copper also occur in other countries. In Norway Viscaria alpina and Melandrum dioicum grow in abundance on copper-rich soils. Other plants able to adapt to these conditions include (Vogt, 1942): Equisetum arvense L., Equisetum palustre L., Equisetum limosum L., Juncus trifidus L., Agrostis canina, Agrostis borealis, Betula odorata, Betula nana L., Eriophorum vaginatum L., Eriophorum polystachian L., Carex vastrara Stokes, Salix reticulata L., Salix herbaceae L., Salix glauca L., Salix arbascua L., Oligotrichum hercinicum (Hedw) Lav.

Biogeochemical provinces with high levels of nickel have been discovered in the Southern Urals (Malyuga, 1950), the plants in these areas being 20 times as rich in nickel as those exposed to normal concentrations of this element. Gololobov (1960) reported that sheep grazing on the pastures of such provinces receive 6 times as much nickel as those kept elsewhere. Nickel becomes concentrated in the hypophysis ($3 \cdot 10^{-4}\%$), lungs ($2.25 \cdot 10^{-4}\%$) and medulla oblongata ($1.58 \cdot 10^{-4}\%$) of the sheep in nickel-rich provinces. Particularly large amounts of the element accumulate in the skin, hair, and cornea. Local veterinarians report atypical symptoms of dermatological diseases in these regions. In the Southern Urals, Storozheva (1954) found teratogenic alterations in Pulsatilla patens resulting in changes of flower colour and considerably

reduced lobes of the perianth or even complete absence of the perianth (Fig.74).

Fig. 74. A complete reduction of coronilla in Pulsatilla patens flowers grown in the biogeochemical province with nickel excess (after Shvyryaeva and Malashkina, 1960).

A biogeochemical province with a high level of nickel and a deficiency of copper has been discovered by Egenbayev et al. (1975) in the Aktyubinsk region of Kazakhstan. A high level of nickel in the soil is responsible for blindencess in 10-15% of the sheep and 10-12% of the cattle. The content of copper, which is antagonistic to nickel, is small, the average amounts in hay and grass being 4.45 and 2.94 mg per kg dry weight, respectively, compared with normal levels of 6-12 mg per kg. Supplying 44-84 mg of copper sulphate per day to each calf between the ages of 2 and 6 months was found to increase the body weight by 50% and completely eliminate nickel blindness. The optimum dose was found to be 9 mg per kg dry weight of the fodder, lower or higher doses failing to produce any effect.

Biogeochemical provinces with high levels of lead in northern Armenia have been described by Kovalsky (1974). In these areas the lead content of the soil reaches $9.2 \cdot 10^{-3}$%, and the levels in foodstuffs are 2-5 times higher than normal levels. Since lead tends to accumulate in tissues, various effects of lead poisoning (e.g. gingivitis) occur there. Biogeochemical investigations in regions of lead-zinc enrichment in the Armenian Republic have been carried out by Malyuga et al. (1959).

Malashkina (1960) reported teratogenic alterations in Papaver macrostomum, involving dissection of the flower petals.

Strontium-calcium biogeochemical provinces. The Urov provinces of the USSR are renowned for being relatively poor in calcium and rich in strontium (Vinogradov, 1949; Kovalsky, 1974). Similar biogeochemical provinces occur in northern China, North Korea, Japan, Sweden, the Netherlands and Mongolia (Kravchenko, 1961). The "Urov" disease affects the whole organism, the most striking defects occurring in the growth of the epiphyseal and articular cartilages. These defects are attributable to disturbances of nerve trophic function. The most commonly presented symptoms of the disease are brachydactylia and deformation of the interphalangeal joints, muscle atrophy, and restricted mobility in affected humans and animals. The disease takes a heavy toll on the local economy.

Vinogradov (1946) put forward a biogeochemical theory of the "Urov" disease taking the cause to be a shortage of calcium. According to Vinogradov (1949), the calcium oxide content of the hay varies considerably, the animals developing osteoporosis at levels below 0.52% and "Urov" disease at 0.56% (in the Trans-Baikal Regions); only at a calcium oxide content of 1.20% are the animals healthy.

The deficiency of calcium in the soils of the Urov biogeochemical province is accompanied by high levels of strontium and barium (Kovalsky, 1957; Samarina, 1960), the content of strontium being 7 times, and that of barium 3 times higher than the respective levels in wet podzolic soils. The relative proportions of these and other trace elements are also distorted in the Urov province, the soils there containing small amounts of sodium, cobalt and iodine and featuring elevated levels of titanium and gallium.

Kovalsky (1974) described a new strontium-calcium province in Tadzhikistan. The calcium content of forage in this province is relatively high (9.0-14.0%), but the strontium content is also high

so that the Ca/Sr ratio for forage in the province (52) is lower than that found outside the province, and is significantly lower than that of the non-chernozem zone.

Phosphatase activity in preparations of the epiphyseal parts of bones from animals of the strontium-calcium province increases as the concentration of strontium in the homogenate increases from 0.001 to 5 mg% (pH 9.2); phospatase activity in similar material originating from control regions decreases in response to the same increase in strontium concentration. Kovalsky believes that this large difference in the behaviour of phosphatase from the epiphyseal cartilage is indicative of the adaptability of enzymes to the strontium content of the geochemical environment. Endemic chondriodystrophy manifesting in a shortening of the terminal and middle phalanx occurs, as well as endemic fracturing of the extremities in sheep (3-4%, sometimes up to 10%).

Plant concentrators of strontium (Echium italicum, Alhagi kirghisorum, Ampelopsis vitiflia, Glycyrrhiza glabra, Artemisia sp. and Risa sp.) have been found in the Tadzhikistan strontium-calcium province. Thus, Echium talicum normally contains 0.008% strontium in terms of dry matter, whereas in the regions of primary and secondary concentration of strontium the level in this species is 0.9%, i.e. strontium is concentrated 450-fold within the province, and only 4-fold in regions outside the province. Anabolic alterations occur in non-adapted grass and tree concentrators of strontium, and Roemeria refracta D.C. additionally develops a number of teratogenic abnormalities, including sectioning of the petals. A similar modification of the petals has been reported by Malyuga et al. (1959) in Papaver macrostomum in zinc-lead provinces in Armenia. Concentrations of lead and zinc in Roemerii refracta D.C. are the same in normal and modified variants. Members of the Papaveraceae have been reported to undergo teratogenic modifications such as segmentation of the petals in response to changes in environmental conditions (Fedorov, 1958).

A biogeochemical province with a high level of uranium has been found in the Issyk-Kul plain extending along the bank of a lake. The area is 5-12 km wide in some places and only a few hundreds of meters wide in others. The lake itself is included as part of the province, since the waters and wildlife of Issyk-Kul contain elevated concentrations of uranium. Biogeochemically, this province has been studied in detail by Kovalsky et al. (Kovalsky, 1974).

Earlier reports by other investigators give information on the uranium content of the mountain rocks and soils in this province (ibid).

The highest uranium contents were found in sedimentary rocks of various ages - carbon-silicon schists ($1.07 \cdot 10^{-3}$%), clays and organogenic limestones ($3.0 \cdot 10^{-4}$%). Uranium levels in the soils of the province vary from $1.2 \cdot 10^{-4}$% to $6.4 \cdot 10^{-4}$%.

Kovalsky (1974) presents detailed information on the uranium content of plant concentrators of uranium and the morphological variability of these plants.

Measurements of the uranium levels in plants of the Issyk-Kul plain and plants of the Central Non-Chernozem Zone (the Kursk preserve) show that even with the relatively low level of uranium enrichment of the Issyk-Kul soils (2.0-8.5 times the normal amount), amounts of the element taken up by the plants may be very considerable. The uranium contents of plants in various regions of the Issyk-Kul province range from $3.7 \cdot 10^{-6}$% to $5.1 \cdot 10^{-4}$% in terms of dry matter, and are 1.5-240 times greater than uranium levels in the plants of the virgin chernozem steppes ($2.1 \cdot 10^{-6}$%).

Teratogenic alterations in plants growing in regions of elevated uranium content have been described in Ferula gigantea and Eremurus stenophylus. Mis-shapen forms appear in plants with uranium concentrations of 0.01-0.1%: stems become crooked and leaves twisted, the flowers are deformed, and the normally pinnate leaf plate acquires a palmate form. Modification of the flowers has been described in Opilobium angistifolium growing on territory rich in uranium in Canada.

Morphological variability among the plants of the Issyk-Kul plain is observable only in two regions, and the feature that shows the greatest variability in this respect is the pigmentation of the petals in Geranium collinum and Oxytropis nutans. There is a close relationship between the distribution of Dracocephalum bipinnatum Rupr. and the uranium content of the soil, this plant being most abundant on soils containing high uranium concentrations of between 1.5 and $3.3 \cdot 10^{-4}$%. Dracocephalum bipinnatum concentrates uranium and constitutes a separate group of morphologically modified variants. The leaves of this species have become simplified, changing from the original compound double pinnate form to a simple pinnate form or even to a simple leaf with a narrow, undivided leaf lamina. Astragalus borodinii plants contain from $2 \cdot 10^{-4}$ to $7.1 \cdot 10^{-4}$% uranium

and also display marked teratogenic alterations. Similarly, Lagochilus dicantophyllus growing on soils of high uranium content develops only as a dwarf plant and shows a persistent albinoism of the flowers. On moist sandy meadow soils with a somewhat lower uranium content some plants show no reduction in size but display other teratogenic modifications. Kovalsky (1974) believes that teratogenic disturbances occurring on soils of low uranium content arise on account of a modified threshold sensitivity of the plants to this element.

Although some species show a depression of growth, others such as members of the genus Caragana and the species Scutelaria prszewalskii. Perovskia obratonoides and Peganum ermala flourish producing many flowers with 6-9 petals instead of the usual 5.

Very considerable intrapopulational variability has been observed with regard to uranium accumulation and the manifestation of teratogenic alterations, the latter appearing in 1-20% of the plants growing in uranium provinces.

The uranium content of forage plants and food given to sheep has been studied in detail. An interesting fact emerged from the evaluation of the uranium balance, namely that sheep of the Issyk-Kul plain display an exceptional ability to excrete uranium via the gastrointestinal tract. Thus sheep fed on forage containing large amounts of uranium excreted 155.5 ug in one region and 617 µg in another, whereas sheep in the non-chernozem zone excreted 18.3 µg.

Sheep adapted to elevated uranium levels in the environment accumulate the element mostly in the hair, skin and bones. They get rid of some of this uranium in the process of shedding the hair. In the fish of the Issyk-Kul lake the element is distributed as follows: 92.7% in the skeleton and muscles, 3.3% in the gonads, 0.48% in the kidneys and 0.19% in the blood.

In Orostachus thyrsiflora plants containing high levels of uranium, one frequently observes teratogenic modifications which include dwarfing with branched inflorescences instead of straight lanceolate inflorescences. Morphological alterations also occur in Caragana lacta on uranium-rich soils. Caragana leucophloea, however, concentrates uranium without developing teratogenic alterations, while Caragana leucophloea Pojark undergoes strong modification on soils rich in uranium.

No pathological changes have ever been detected in humans and animals inhabiting uranium biogeochemical provinces, although

the metabolism of animals has received very little study under these conditions. Concentrations of uranium at which biological effects occur in animals are possibly not attained in these provinces. In those plants that accumulate uranium to a greater degree than animals, such effects have been very evident.

Interesting data have been obtained from the Issyk-Kul lake, which is a part of the Issyk-Kul province. Uranium levels in the water, the calcareous concretions in the silt, and the plankton, fish, and various components of the fish's food are high. The highest concentrating capacity for uranium was reported for Chara, which was found to concentrate the element 100–1000 times over the concentration in the lake to a level one and a half times that of the underlying silt. The uranium content of Chara was proportional to the amount in the silt. The primary accumulation of uranium in the silt was shown to be biogenic. The uranium contents of other algae (Cladophora glomerata and Enteromorpha salina) and water plants (Potamogeton dophora glomerata and Enteromorpha salina) were considerably lower than that of Chara.

The tenthic animals were found to accumulate much less uranium than the plants. Fish were intermediate in terms of uranium content, and generally, fish caught for food contained 3–5 times less uranium than other fish species. Information on selenium-rich biogeochemical provinces in the USA is given in the chapter on selenium in Part II.

An excess or deficiency of certain chemical elements in a biogeochemical province affects the pattern of synthesis and function of many of the biologically active substances including enzymes, vitamines and hormones, in the living organisms of the province (Yarovaya, 1960, and others). This leads to changes in the pattern of metabolism of animals and plants, often giving rise to a pathological condition. In the course of adjustment to a new geochemical environment, organisms develop the regulatory physiological mechanisms that are necessary to deal with reduced or elevated concentration of an element in the environment. However, there are always concentration limits, or threshold concentrations of chemical elements which are beyond the ability of any regulatory system to cope with, and thus endemic disease occurs.

The study of biogeochemical provinces constitutes the basis of geochemical ecology, a field of science concerned with investigating the effects of environmental geochemical factors on living

organisms. It sets out to study the effects of deficiencies and excesses of micronutrient elements on metabolism, morphology, the adaptation of organisms to the geochemical environment, and the origin of endemic disease in biogeochemical areas.

Even when Vinogradov first pointed out the need of developing geochemical ecology, he pointed out that the knowledge gained might lead towards solving the major theoretical problems of evolution. This is a very important statement.

Azonal biogeochemical provinces with soils rich in micronutrient elements attract the greatest interest, for it is in these regions, that the pressure of natural selection is most intense. Metabolic variability and adaptive metabolical change enable species to survive. Increased variability and divergence of physiological and morphological characters provide a basis for the origin of new forms and varieties.

Thus, the enrichment of soils with heavy metals is known to promote clearly manifested, intraspecific differentiation. Plant populations growing on soils rich in heavy metals possess a genetically determined resistance to heavy metals, whereas populations growing on normal soils do not show such a property. Plant populations on heavy metal soils are composed of edaphic ecotypes capable of morphological and physiological differentiation.

Thus, for example, there are populations of Festuca ovins, which are very sensitive to concentrations of lead and lead-resistant populations growing on soils with lead contents as high as 4 per cent (Wilkins, 1960). Numerous examples of such phenomens exist. Differences in resistance are not necessarily accompanied by morphological variations. Here we are concerned principally with physiological forms, as described by many authors (Bradshow, 1952). Species capable of growing on serpentinites are also known to be represented by two ecotypes, or physiological races. In 12 out of 21 species belonging to a number of families, segregation into serpentinite and non-serpentinite races has been found (Kruckenberg, 1951).

Vinogradov (1938) wrote: "Various floras and faunas have in the course of time spread throughout existing and newly formed biogeochemical provinces. A biogeochemical province constitutes a selective force in their evolution, and therefore the investigation of the effect of these provinces on the evolution of organisms presents a very interesting challenge".

In some cases, the geochemical differentiation of populations apparently gives rise to indigenous races, or even species. Such races and species have been described for soils rich in copper, nickel, chromium, zinc, aluminium, selenium, and other elements. One may speculate that the evolution of the entire genus Astragalus occurred under conditions of high selenium content in the soil, and similarly such families as the Chenopodiaceae, Frankeniceae and Tamaricaceae, evolved in associated with salinity. The results of research on selenium are summarized by Rosenfeld and Beath (1964).

Similar examples may be cited from among the concentrator species of aluminium, including many species of the families Theaceae, Melastomaceae, Euphorbiaceae (Baccaurea and Aporusa), all species of the Diapensiaceae and Symplocaceae. It has been found that Vaccinium varingifollum var. lucidum, Rhododendron retusum and Fious diversifolia, growing on very acidic Javanese soils with high levels of mobile aluminium, require this element for their growth although the accumulation of aluminium occurs only in the roots. Three fern species - Alsophila australis, Aspidium filixmas and Polypodium proliferum - which accumulate aluminium, cannot grow in its absence (Tauböck, 1942b). These findings indicate that the specificity of concentrator activity in some plants is intimately associated with the metabolism of the plant. When the element that is concentrated is absent, the entire organism suffers.

Of the 1324 species of the Australian flora, some 80 aluminium concentrator species have been found. The accumulation of this element is most evident in 69 dicotyledonous and 11 fern species. The fact that the aluminium concentrator species are, as a rule, the most primitive among dicotyledons and ferns suggests that this physiological characteristic should be regarded as having been acquired relatively early in evolution.

In accord with Vinogradov's view that the chemical composition of organisms reflects their evolutionary history, Rish and Ezdakova (1960) attempted to reconstruct the conditions under which species of the genus Lycium arose by looking at lithium concentrations in Lycium ruthenicum and Lycium turkomenicum in relation to the distribution of these plants in various localities. It was concluded that both species arose on the lithium-enriched soils of flooded plains, and became adapted to high salinity. Lycium ruthenicum is an indicator species for lithium in soils. The red-flowered areas in Soviet Central Asia (The Turkestan Ridge, Karatau) have

a flora which is peculiar to those areas (Popov, 1923).

A higher plant that accumulates titanium has also been discovered. This is Alternanthera sessilis, which accumulates Ti, Al and V to a degree that is an order of magnitude greater than average levels of accumulation, and Si to levels that are 10^3 times average. The first three elements are localized mainly in the roots, while Si is equally distributed in the roots and shoots. Alternanthera sessilis is the only plant known thus far to accumulate Ti and Si (Nadkarni and Chaphekar, 1977).

Particular emphasis has always been placed by investigators on the concentrator plants. Unexpected degrees of adaptation plants to high trace element concentrations have been reported. Penicillium glaucum can live on 21% copper sulphate solution, whereas Spirogyra dies when as little as $8 \cdot 10^{-7}$ M copper is present in the culture medium. Viscaria labine and Melandrium dioicum grow on deposits of copper silphide in Norway. Some other species have also been found growing on these deposits (Vogt, 1942). More than 13% zinc has been detected in the ash of Thlaspi calaminari, a halmeine plant. Such a concentration of zinc causes the death of all other plants. The ash of Silene inflata contains 2.1% zinc (Baumeister, 1954). There are many plants which are concentrators of nickel, the ash of Alyssum biovulatum containing about 1% nickel (Petrunina, 1965), and that of Alyssum murale (growing on serpentinites in Georgia) containing up to 15% of nickel (Doksopulo, 1961). Not only individual species, but entire genera and even families may be concentrators of trace elements.

Kovalsky and Petrunina (1964) divided concentrator plants into obligate and facultative types. Obligate concentrators, as a rule, absorb considerable amounts of a chemical element from the soil, even when the concentration in the soil does not exceed normal levels. Facultative concentrators show a variable capacity to concentrate elements, accumulating then only on soils that are rich in the element. Among obligate accumulators the ability to absorb some elements selectively is a taxonomic characteristic.

Kovalsky and Petrunina (1964) concluded that physiological forms constitute important evolutionary steps in the adaptation of plants to the geochemical environment. These forms may be found where both obligate or facultative concentrators provide a basis for the divergence of characters and development of new physiological varieties (by analogy with morphological varieties). The terminal stage of selection in the case of non-adapting forms is death,

whereas in adapting forms it is the development of new species, i.e., species formation (Fig.75).

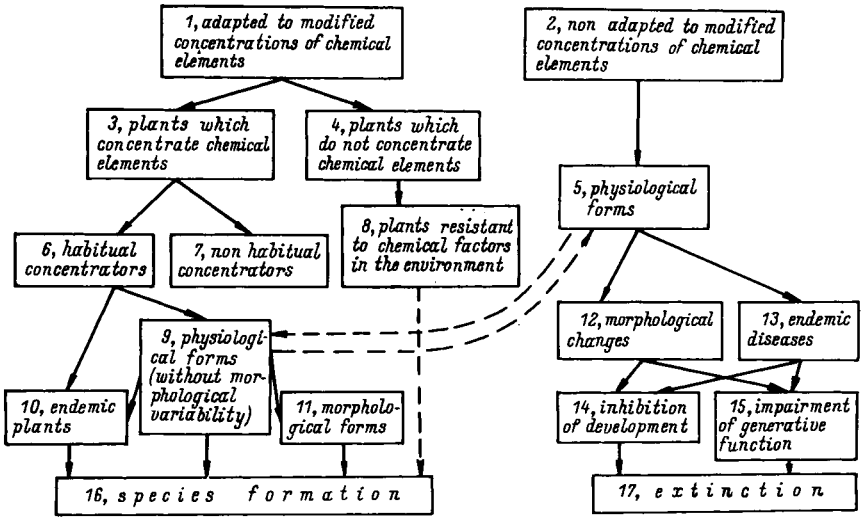


Fig. 75. Scheme illustrating the mode of response of plants to high concentrations of chemical elements in the environment (after Kovalsky and Petrunina, 1964). 0, plants; 1, plants adapted to extreme concentrations of chemical elements; 2, plants, that are not adapted to extreme concentrations of chemical elements; 3, plants which concentrate chemical elements; 4, plants which do not concentrate chemical elements; 5, physiological variants; 6, unconditional concentrators; 7, conditional concentrators; 8, plants showing tolerance to chemical factors in the environment; 9, physiological variants (not showing morphological variability); 10, plants characteristic of locality; 11, morphological variants; 12, morphological changes; 13, endemic diseases; 14, inhibition of development; 15, impairment of regenerative function; 16, creation of new species; 17, extinction.

A vast body of information has accumulated on the adaptation of plants to various levels of trace elements, this being of profound interest in the context of the general theory of adaptation in plants.

During recent years a number of publications dealing with the mechanism of such adaptation has appeared.

The mechanisms of population adaptation to an excess of a heavy metal are diverse and have not as yet been clarified. Some importance might be attached to the accumulation in roots of excessive amounts of metal in the form of complexes of one kind of another,

(Wu et al., 1975), or to build up within the structures of leaf coats (Ernst, 1969). Wu et al. concluded from the results of their studies that the tolerance of Agrostis stolonifera to the copper is attributable to a Cu-complexing mechanism in the roots.

Ernst (1976) points out that within some plant species growing on soils with high heavy metal concentrations, ecotypes arise possessing an increased tolerance to large quantities of heavy metals. The author is of the opinion that resistance is achieved by excuding heavy metals from active metabolism and accumulating them in the cell walls.

Ernst reports that in Great Britain a genofund of plants resistant to heavy metals is being created with the purpose of obtaining suitable material for soils that are rich in these metals. Sowing such metal-resistant plants may provide a means of rehabitating soils containing heavy metals.

In Great Britain and Central Africa the successful removal of Zn, Cu and Pb from soils has been achieved by growing Agrostis tenuis, which is resistant to these metals.

Winwright and Woolhouse (1977) investigated some of the physiological aspects of the resistance of Agrostis tenuis to copper and zinc, including the effects of these on the elongation of cells and membrane function. The extension of root cells, in a non-resistant pasture clone of Agrostis was suppressed to a greater degree than extension in the roots of clones resistant to Cu and Zn. Incubation of root segments in copper-containing solutions resulted in greater K^+ losses from the cells compared with losses during incubation in water. Roots of the clone not resistant to Cu^{2+} lost K^+ more readily than those of the resistant clone. Incubation in zinc solutions did not influence losses of K^+ from the cells.

In two Agrostis gigantes clones differing in their resistance to excess copper in the environment, it has been found that in the roots of the more resistant clone the content of exchangeable copper is markedly higher than that in the roots of the non-resistant clone. After the removal of the exchangeable copper fraction, the copper contents of the roots of both clones was found to be equal. In the more resistant clone more copper entered the above-ground parts of the plants than was translocated upwards in the less resistant clone. Possible mechanisms of plant resistance to excess concentrations of metals, are discussed, in particular the role of organic acids and proteins in detoxification by means of metal

complex formation, by Hogan and Ranser (1981).

Lee et al. (1977), in experiments with 176 Allysum species from New Caledonia, isolated and identified a citrate complex from plants accumulating nickel. The authors concluded that the major part of the nickel in plants that accumulate this metal exists in the form of a negatively charged citrate-nickel complex containing $Ni(H_2O)_6^{2+}$.

A clear correlation between levels of nickel and citric acid has been found. Three plants (Allysum bertolonii, Allysum serpyllifolium and Pearsoma metallimera) collected from areas outside New Caledonia did not contain unusually high quantities of citric acid. In these plants nickel is complexed with other organic acids.

In the leaves of Allysum bertolonii, as well as the leaves of some other Allysum species capable of accumulating large amounts of nickel, amounts of malic acid and malonic acid ten times greater than those in non-accumulating controls have been found. In Allysum, malic and malonic acids are thought to take part in the process of nickel complex formation (Pancaro et al., 1978). In fact in the leaves of this plant, nickel has been discovered in the form of complexes with malic and malonic acids (Pelosi and Fiorentini, 1976).

Publications on the resistance of enzymes to the effects of heavy metals have appeared. Mathys (1975) studied the influence of Cu, Zn, Cd, Ni, Co and Mn on the activities of nitrate reductase, malate dehydrogenase, glucoso-6-phosphate dehydrogenase and isocitrate dehydrogenase in zinc-copper-resistant and normal populations of Silene cucubalus. The nitrate dehydrogenase and isocitrate dehydrogenase of zinc-resistant populations were activated when zinc was added to the nutrient solution. Nitrate reductase showed a particularly high activity at concentrations which caused an almost total loss of nitrate reductase activity in populations not resistant to zinc.

The results of an investigation by Cox et al. (1976) show that in a tolerant population of Anthoxanthum odoratum, an acid phosphatase is produced which is more zinc-resistant than that found in normal sensitive population.

The major physiobiochemical mechanisms underlying the resistance of plants to metals are now considered on the basis of metal-resistant plant populations that have arisen in the course of evolution. The mechanism of resistance is seldom associated with a

delay in absorption, more often involving internal neutralization of excess amounts of metal. Metals can be immobolized in physiologically inactive forms, or molecular forms of physiologically active compounds can be produced, particularly enzymes, that are more resistant to the toxic effects of heavy metals (Alekseeva--Popova and Kositsyn, 1981).

The natural selection of plants showing resistance to heavy metals can proceed very rapidly. Kakes (1977) described a situation in which a stable zinc-tolerant population of Viola calaminaria was found inhabiting a very small area rich in zinc; the area was surrounded by the species Viola aroensis, with which Viola calaminaria is easily intercrossed. Genoecological studies of this are of considerable interest and are held to be highly fruitful.

It is interesting that the competitive ability of populations tolerant to heavy metals is sharply reduced under normal conditions. This was observed when normal and metal-resistant populations of Agrostis tenuis, Anthoxanthum odoratum, Plantago lanceolata and Rumex acetosa were grown together. Under normal conditions the competitiveness of the metal-resistant population of Anthoxanthum odoratum proved to be 1000 times lower than that of the normal population. The reason for this phenomenon is not yet clear (Hikey and Neilly, 1975).

Lichens are known to be capable of accumulating enormous quantities of heavy metals. Reasons for the high tolerances of lichenes to high concentrations of metal ions were proposed by Lange and Zilgler (1963). They suggested three mechanisms of resistance: 1) internal cytoplasmatic resistance; 2) cytoplasmatic immobilization and inactivation by means of chemical combination; 3) expulsion of cations to regions external to the plasmalemma, or even beyond the cell wall.

Of no lesser interest are some recently published data on the possible appearance of higher plant races, both resistant and non--resistant to lead and copper, in comparatively recent times. Genetically determined tolerance to lead has been discovered; a clone found under conditions of strong lead contamination showed a high tolerance to lead, whereas a clone from a less lead-polluted region possessed a lower resistance to this metal (Briggs, 1972). In the same author's experiments (Briggs, 1976) data have been obtained which lead him to suppose that the high tolerance to lead in roadside topodemes is of recent origin.

Japanese workers (Minagawa et al., 1952) found that the resistance to high doses of copper in Saccharomyces may be transferred to sensitive yeast cells via RNA isolated from resistant yeast cells. Brenes-Pomeles et al. (1955) found in genetic experiments with Saccharomyces cells that sensitivity to copper is determined by a single pair of genes.

Kovalsky et al. (1972) carried out a genetic transfer from Actinomyces indigocolor (strain 1100) adapted to excess boron. DNA was isolated and introduced into the culture medium of a recipient (strain 1029) which was sensitive to high boron concentrations. As a result of the genetic transfer, a new Actinomyces indigocolor strain was obtained, which showed a higher resistance to boron than the original recipient (Fig.76). Kovalsky (1974) deduced that the

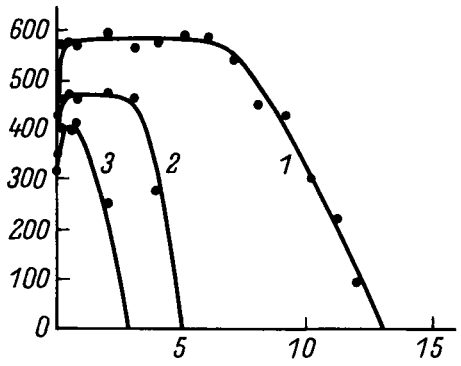


Fig. 76. Effects of borax on the rate of growth of Actinomyces indigocolor (after Kovalsky et al., 1972). 1, donor (strain 1100); 2, transformant (strain T-17); 3, recipient (strain 1029). Abscissa, sodium borate, g per litre; ordinate, biomass (mg dry wt.).

resistance of microorganisms to high concentrations of chemical elements under extreme geochemical conditions may be genetically determined, and suggested that under such conditions mutants may well be formed. New forms adapted to the extreme factor then rise to prederminance by natural selection.

Klimashevsky et al. (1970) concluded that DNA transfer endowed the recipients with the ability to synthesize a specific protein which was the basis of resistance to high concentrations of chemical elements, such as aluminium. This conclusion was supported by analyses of the amino acid composition of proteins from aluminium-sensitive and resistant plants.

Working with wheat and pea plants, Karabach (1973) was able to give one of the reasons for the variety of trace element concentrations in plants. She studied the ability of grasses to take up considerably lower amounts of boron than those taken up by dicotyledons in the Northern Kazakhstan boron-rich biogeochemical province. The difference was accounted for by the quantitatively and qualitatively different composition of the root secretions of these species. The secretions may either enhance or reduce the uptake of boron by the plants. Thus in the root secretions of wheat there are more components tending to reduce the uptake of boron, whereas in pea plants the components responsible for enhanced uptake of boron prevail.

Mention should also be made of adaptations taking place in biogeochemical provinces characterized by trace-element shortages. An excellent example of a biogeochemical province with a shortage of copper is the North German plain (Fig.77), a region of humus--rich sandy soils in which the organic matter readily binds copper and makes it unavailable to most plants. Under these conditions, the ability to survive is a function of the capacity of plants to absorb copper, and therefore this province has a specifically adapted flora including, for example, Calluna vulgaria, Erica tetralix, Betula pubescens, Triticum vulgare, and Fagopyrum esculentum, which contain large amounts of copper. Rademacher (1940) was able to explain why some varieties of oats grow well in this regions, while other, more economically profitable varieties fail to grow there. The successful adaptation of those varieties that grew well was found to be a consequence not of a lower requirement for copper, but rather of a greater capacity to take up copper in the early stages of growth. The more valuable varieties absorbed copper from the soil very slowly in the early stages of development, and therefore suffered from deficiency later in their development as copper became less available with drying of the soil in the summer months.

The study of biogeochemical provinces has yielded valuable evidence on concentrators of trace elements, morphological variability, the adaptation of organisms to the geochemical environment, biogeochemical endemic plants and diseases, physiologically separate races, and intraspecies differentiation arising from adaptation to high concentrations of trace elements.

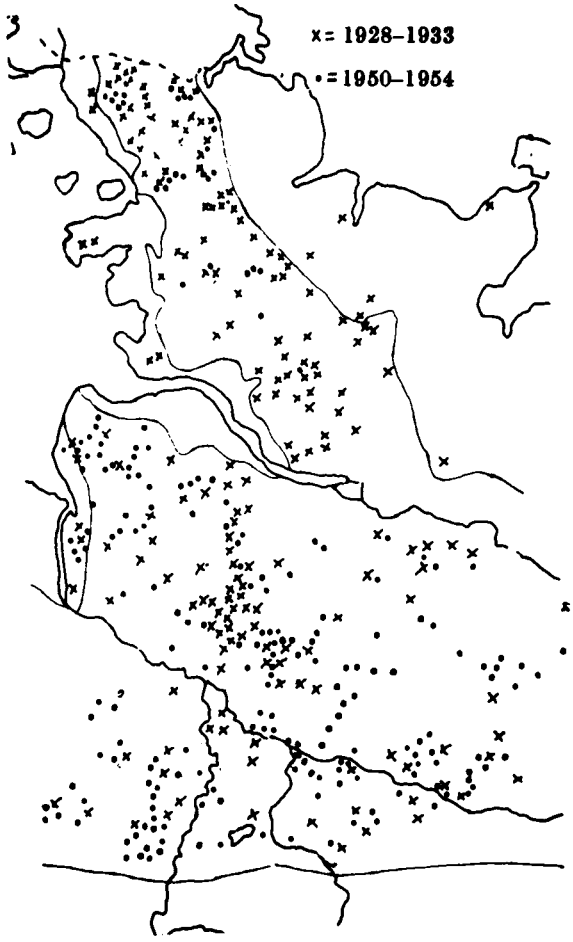


Fig. 77. Copper deficiency in North-West Germany (after Schlichting, 1962 cit. after Shutte, 1954). The map shows the wide distribution of the deficiency. It is of particular importance to note that this condition appears to be spreading, as the distribution occurring during the 1950–1954 survey shows.

Chapter 3

TRACE ELEMENTS AND TERATOLOGY

Shortages or excesses of trace elements frequently result in morphological anomalities in plants - the so-called "teratological alterations" (Figs.78,79) which are particularly common in biogeochemical provinces with excess and deficiencies of trace elements (Petrunina, 1970; Malashkina, 1960).

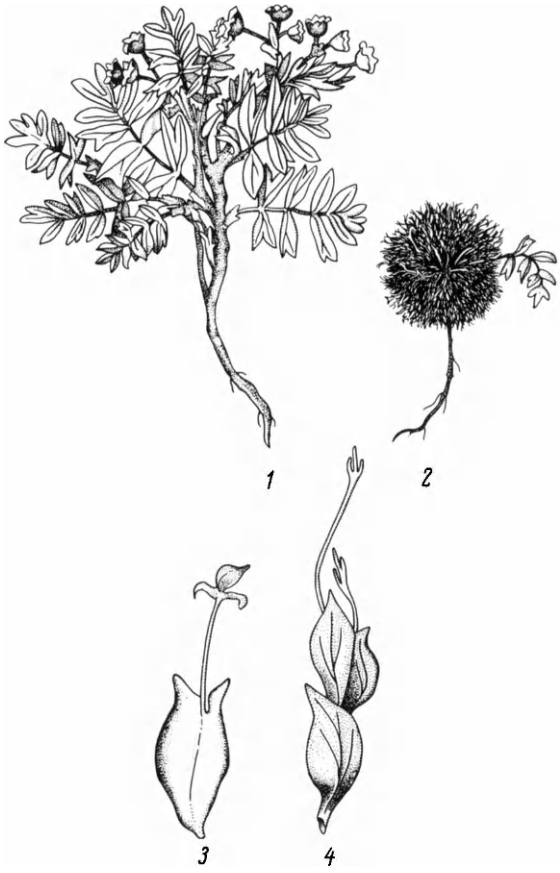


Fig. 78. Teratological modification of Potentilla bifurca, produced by excess copper (after Petrunina, 1950). 1, normal form; 2-4, modified form; 2, general view; 3, underdeveloped leaf, 4, shortened shoot (increase in 3.5 times).

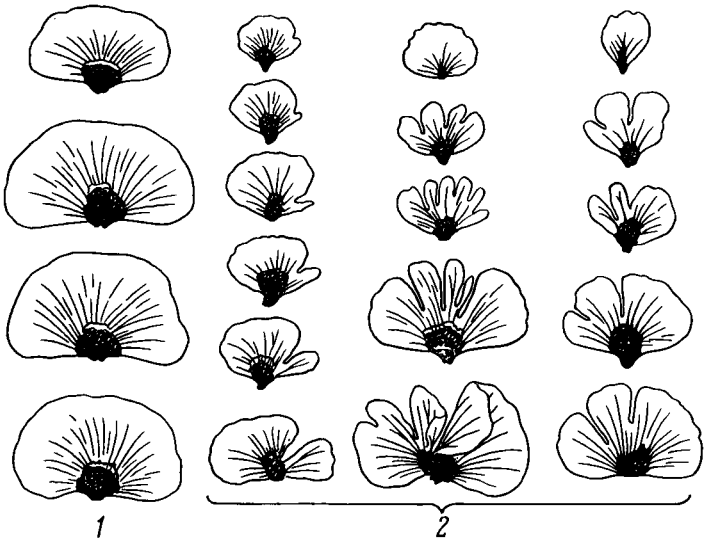


Fig. 79. Corolla petals of Papaver macrostomum flowers (after Malashkina, 1960). 1, normal corolla petals; 2, different degrees of variation in the corolla petals of Papaver plants growing on ore deposits.

High concentrations of heavy metals result in anomalies, not only of the morphology, but also of the anatomy of plants (Fig.80). Deficits of some trace elements may impair the formation of some organs, for example under B-deficient conditions vines do not form any flowers, and wheat flowers may lack pistils and anthers (Fig.12) (Shkolnik and Solovyeva-Troitskaya, 1962). Under the influence of phenylboric acid, 2- and 3-pistillate flowers appear in Pisum sativum (Haccins, 1976) (Fig.81).

Severe anomalies are observed also in the ultrastructure of cell organelles. Deficits of Mn and Mg lead to destruction of the ribosomes, and a shortage of B affects polysomes. Shortages of Fe, Mn, B, Zn and Cu induce disturbances in the lamella-grana structure of chloroplasts (Fig.89) (Possingham et al., 1964); Alekseyeva, 1974). In cases of Mn deficit, the structure of the mitochondria is damaged. B starvation leads to disappearance of the peroxysomes and disturbances in the structure of both cell walls and nucleoli (Alekseyeva, 1971) (Fig.14).

Nothing was known about the physiological causes of teratological alterations until about 1960.

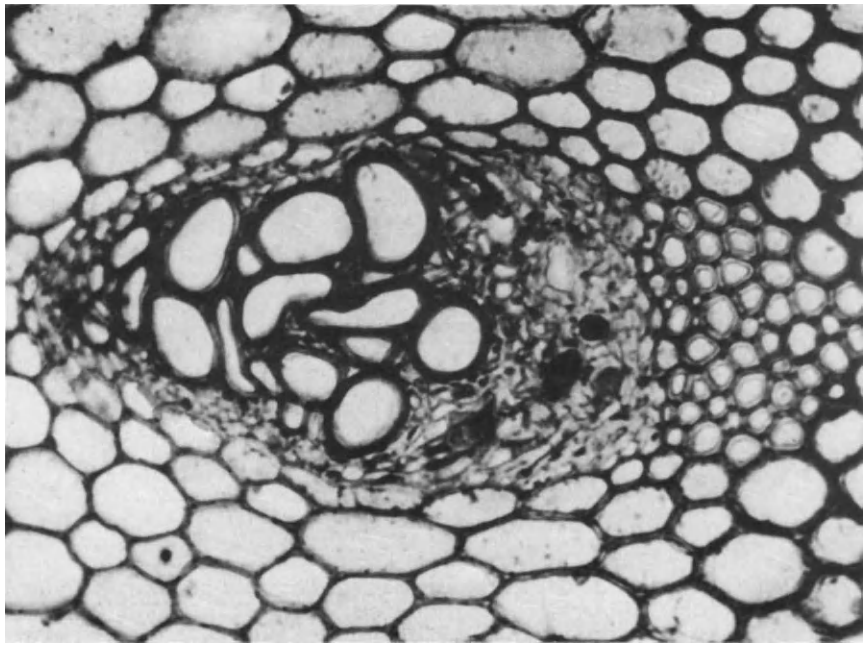


Fig. 80. Anatomical modifications in Papaver macrostomum produced by excesses of Pb and Zn in the soil (after Malashkina, 1960).

The author of this work (Shkolnik and Maevskaya, 1961; Shkolnik et al., 1964) initiated a systematic study of the physiological causes of teratological alterations, observing various teratological changes in the vegetative organs of sunflowers and tomato plants deprived of boron deficiency for a short time (Fig.11).

Results obtained from morphological, cytological and biochemical studies of plants have contributed to the formation of a hypothesis as the starting point in the search for the physiological mechanism of teratological changes in plants. Evidences is available in the literature describing various teratological alterations resulting from disturbances of normal mitoses in apical meristems (Danilova, 1961). Several investigators have reported disturbances in cell division induced by teratogens such as maleic acid hydrasid, 2-4-D (Darlington and McLeish, 1951), excesses of Ni and Cr (Smirnov and Shkolnik, 1978), IAA (Araratyan, 1970), analogues of the nitrogenous bases of nucleic acids (Hotta and Osawa, 1958; Heslop-Harrison, 1962), colchicine, deficiencies of Zn and B (Smirnov and Shkolnik, 1978).

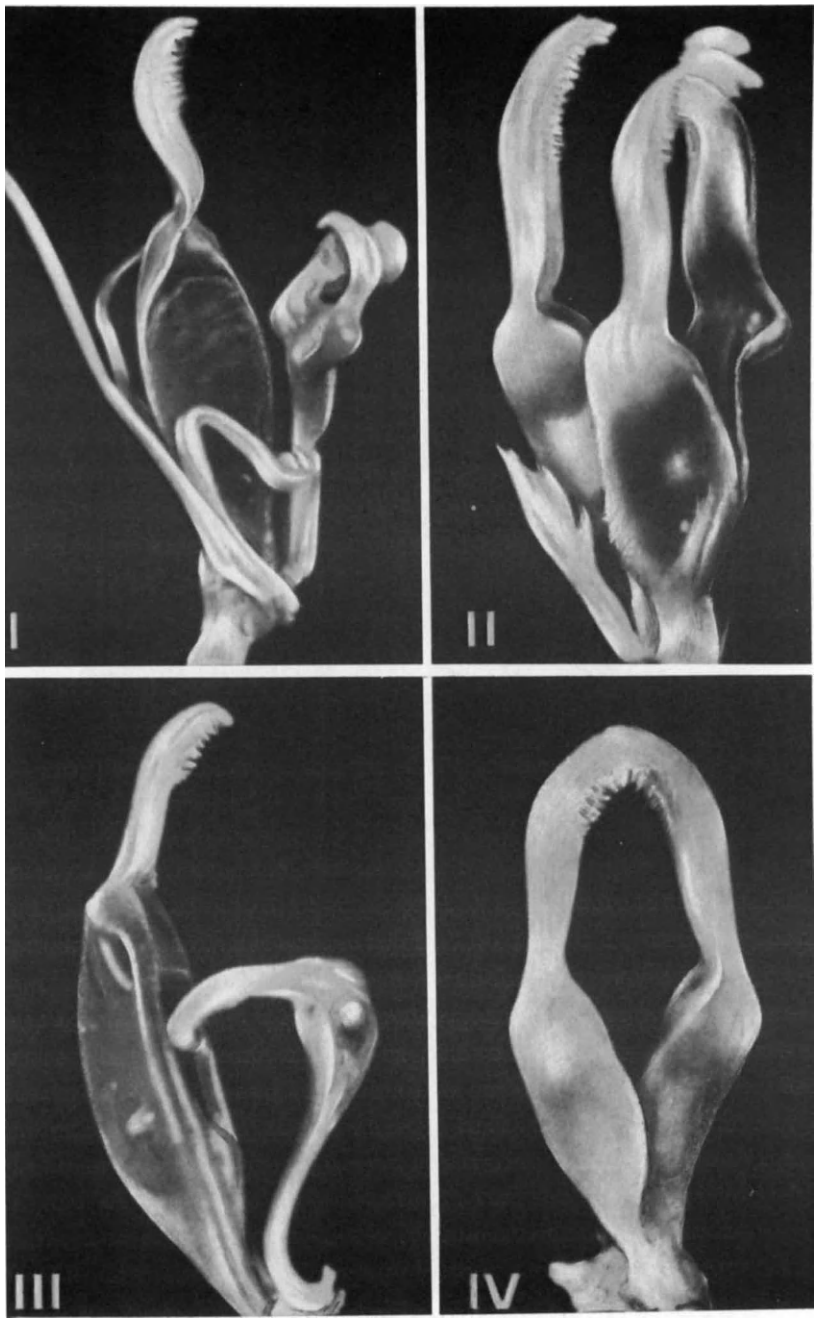


Fig. 81. Distorted Pisum sativum flowers after treating with phenyl-boric acid (after Haccius, 1976). I, transition form to tripistillie form; II, tripistillate form, with all other organs of the flower, except the sepals, aborted III, transitional form to dipistillate form; IV, dipistillate form

Teratogens affect the chromosome apparatus of the cell. Chromosome aberrations arise as a result of the action of high B concentrations (Matter and Turian, 1961) (Fig.82), high temperatures, auxins, maleic acid hydrasid, B deficiency, and excesses of nickel and chromium.

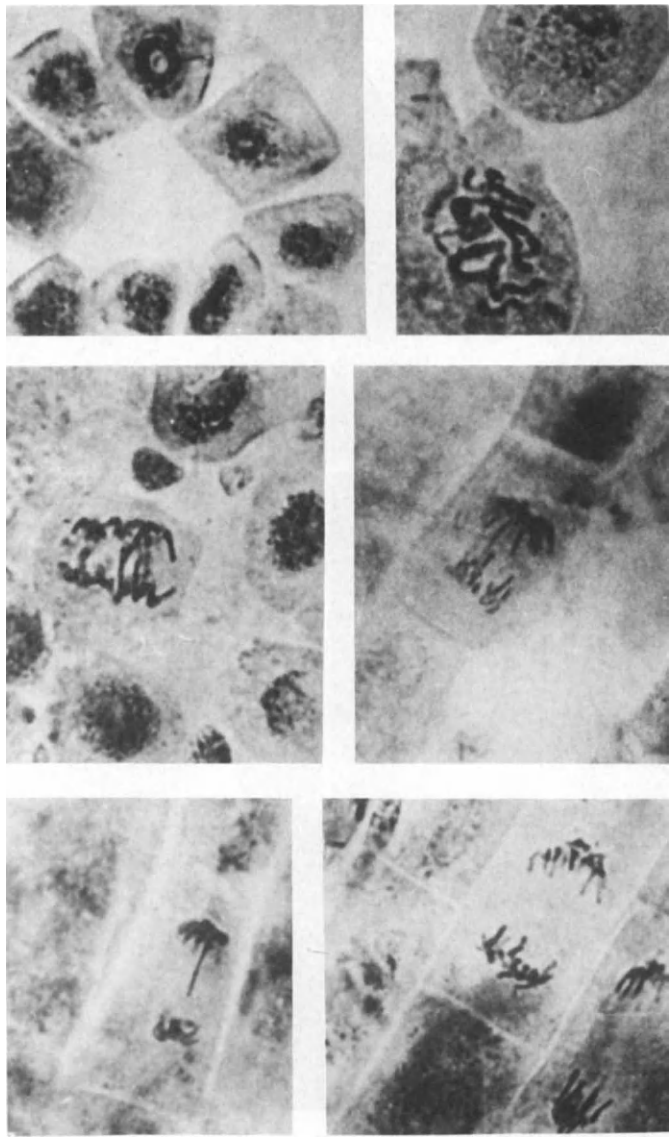


Fig. 82. Effect of excess boron on chromosome apparatus in actively functioning cells. Note chromosome aberrations (after Matter and Turian, 1961).

The publication of three papers on the effects of amino acid analogues and analogues of the nitrogenous bases of nucleic acids on the appearance of teratological modifications (Hotta and Osawa, 1958; Hotta et al., 1959; Heslop-Harrison, 1962) has advanced the search for the physiological mechanisms underlying teratological alterations.

The first of the above-mentioned authors studied the development of Dryopteris erythrosa gametophytes under the influence of chemicals known to interfere with the synthesis of protein and nucleic acids. Under normal conditions the spores of this fern begin to germinate five days after dusting into the culture medium. The primary linear growth of the protonema proceeds up to the seven--cell stage, which is followed by planar growth beginning approximately 25 days after germination; it takes about 100 days for the mature prothallium to develop. Being aware that cell and tissue differentiation involves the synthesis of new kinds of protein, the authors decided to follow the changes in protein content that occurred during the formation of the young gametophyte. The representative results shown in Fig.83 indicate a gradual decline in protein concentration during linear growth, whereas planar growth is accompanied by a rapid increase in protein concentration. Adding analogue of amino acids such as ethionine and 5-methyltryptophan inhibits the planar growth and converts it into "artificial" linear growth. The same interference of planar growth is also brought about by 8-azaguanine, an effective inhibitor of nucleic acid synthesis. Substitution of the amino acid analogue by methionine and tryptophan, and of 8-azaguanine by guanine alleviates the inhibition of protein synthesis and restores planar growth (Fig.83). Regardless of the nature of the inhibitors used, the inhibition is always accompanied by an immediate cessation of the increase in protein concentration, followed by gradual decrease. The authors suggest that planar growth is casually connected with rapid protein synthesis, which probably occurs in response to increased RNA formation. The protein synthesized is specifically inhibited by amino acid analogues and 8-azaguanine.

This interpretation is supported by the evidence obtained by Botta et al. (1959), concerning changes in the nucleotide composition of RNA at different stages of growth of Dryopteris erythrosora gametophytes, and in response to doses of 8-azaguanine. Upon

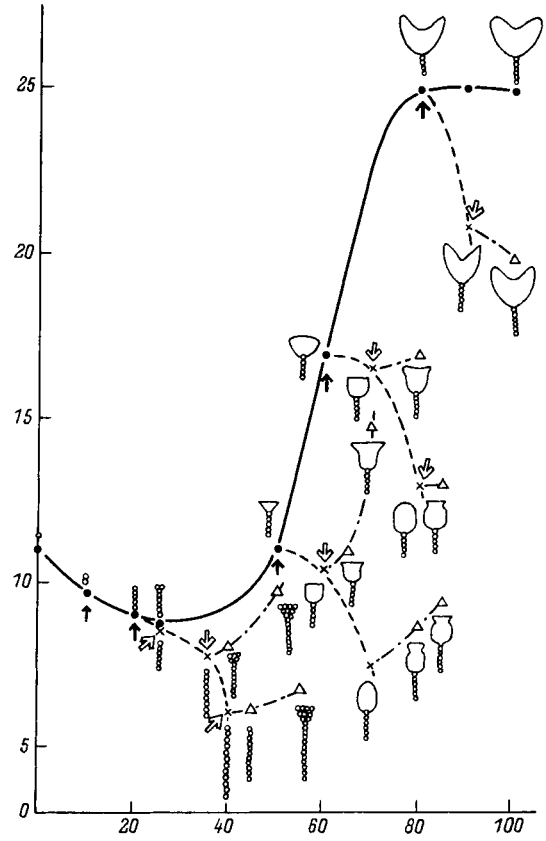


Fig. 83. Effect of 8-azaguanine on the differentiation of protein content of the young prothallium of Dryopteris erythrosorum, and the reversal of 8-azaguanine effect by the addition of guanine. o --- o protein-N per mg dry weight in control culture; x --- x protein-N per mg dry weight in cultures to which M/12500 8-azaguanine had been added at the time indicated by the closed arrow; --- protein-N per mg dry weight in cultures to which 8-azaguanine was added, followed by an addition of the same concentration of guanine at the time indicated by the open arrow. The appearance of the prothallium is schematically presented at each point (after Hotta and Osawa, 1958).

treating Cannabis sativus leaves with an analogue of a nucleic acid base (2-thiouracyl), Heslop-Harrison (1962) observed several morphological alterations.

When Morsilea vestita and Morsilea drummondi were grown in sterile cultures with additions of 2-thioracyl and 5-fluorineuracyl, White (1966) found that there was retardation of leaf formation in the young plants, followed by the development of leaves with a

structure more characteristic of land plants (Embryophyta). Generally adult acquatic forms acquired some of the characteristics of land forms when they were exposed to these compounds. The authors believe that these alterations are connected with the inhibition of protein synthesis.

Somewhat later the author of this volume succeeded, by adding 8-azaguanine, to reproduce in the presence of boron morphological alterations (Fig.84) that are characteristic of B-deficient plants

Fig. 84. Teratological alterations in the leaves of sunflower plants grown from seeds treated with 8-azaguanine solution (after Shkolnik et al., 1965).

(Shkolnik et al., 1965). In the light of these findings Shkolnik (1966) suggested that the physiological cause of teratological alterations in plants was the synthesis of modified proteins, leading to disturbances in cell division. Ladonin and Switser (1967) explained fatal disturbances in cell division in plants as being the

result of the transfer of deleterious information to the sites of protein synthesis.

Observations which support the general hypothesis come from the already mentioned studies of Hotta and Osawa (1958), Hotta et al. (1959), Heslop-Harrison (1962) and Shkolnik et al. (1965). Also worthy of mention are the studies of Bodansky (1950) on the action of viruses in blocking important links in nucleic acid synthesis and switching these processes over to the synthesis of vital nucleic acids and proteins with concomitant teratological modifications.

Evidence has been obtained of the formation of new proteins under the influence of teratogenic GMA (Rakitin et al., 1978). Stroganov et al. (1970) found changes in the composition of the histone fraction occurring as a result of the accumulation of a soil salt considered to be a teratogenic agent. It is suggested that in this case a disturbance in the link DNA-histone was observed, that is, a dissociation of the histone complexes occurred necessarily leading to the loss by the cell of its capacity to control the genetic apparatus.

Since the analogues of the nitrogenous bases of nucleic acids (8-azaguanine, 2-thiouracyl and others) are artificially synthesized compounds which do not occur naturally in plants, it was essential to find the biochemical mechanism which, under particular conditions in plants, leads to the same effects as those produced by nitrogen base and amino acid analogues, namely, the synthesis of modified proteins and consequently the appearance of teratological modifications.

In the search for this mechanism, the author's attention was drawn to the fact that many teratogenices influences, including boron deficiency (Perkins and Aronoff, 1956; Shkolnik and Abysheva, 1971), zinc deficiency (Reed and Dufrency, 1942) and others, bring about phenol accumulation. Phenols are able to induce distrubance in the biosynthesis of nucleic acids (Dzhokhadze and Papelishvili, 1976) and changes in the macromolecular structure of DNA (Kazakov and Chebotar, 1969), which must result in the synthesis of modified proteins and disturbance of the process of cell division (Frankfurt et al., 1963).

It may be concluded, therefore, that phenol accumulation is part of the mechanism underlying teratological modifications.

This suggestion also gains support from the fact that phenols and quinones induce disturbances in the structural integrity of chromosomes and are responsible for the appearance of chromosome aberrations (Krogulevich and Stom, 1969) typical of those produced by teratogenic agents. As observed by Kulikov et al. (1970), chromosome aberrations arising under the influence of 2-4-D are directly linked with a disturbance of macromolecular structure.

Recently the author (Shkolnik et al., 1978) obtained experimental evidence in support of the suggestion that the accumulation of phenols is the primary cause of many of the teratogenic modifications produced by different teratogens. In these experiments sunflower seeds were treated with phenols and quinones (hydroquinone, pyrokatechin, rezorcinol and p-benzoquinone) at concentrations of 10^{-2}M. The proportion of plants showing morphological alterations when treated with the first three of these substances was 30%, the proportion similarly affected by p-benzoquinon being 44.5%. The observed modifications were the same as those arising from B deficiency, the presence of high concentrations of Ni, Cr, Co and B, and treatment with IAA and the herbicids 2-4-D and GMA. They were; asymmetry, narrowness and deformation of the leaf blades, doubling of the leaves on one stem (Fig.85), and disturbances of phyllotaxis (Fig.86). Plants treated with hydroquinone two fused cotyledons and a mature leaf in place of one cotyledon. The cotyledons of plants treated with p-benzoquinone showed severe teratogenic alterations; their cotyledons resembled raspberries (Fig.87).

These facts led to the hypothesis that the accumulation of phenols in plants was a factor which, like the presence of analogues of the nitrogenous bases of nucleic acids, induces both synthesis of modified proteins and disturbances in cell division leading to teratogenic alterations. Many of the known facts about fungal and animal teratology stand in favour of this hypothesis. Kazakova and Chebotar (1969) describe teratogenic modifications in embryos arising under the influence of Na-salycil acid. Verticillium daliae Kleb. growing on Chapek-Dok media with different concentrations of phenolic compounds extracted from the leaves of Juglans regia and Churma orientalis showed morphological and karyological alterations. The cell membranes of Verticillium daliae were particularly sensitive to the action of these phenols, which finally resulted in the reduction of mitochondria crist, loss of endoplasmatic reticulum integrity, and destruction of the mitochondrial and

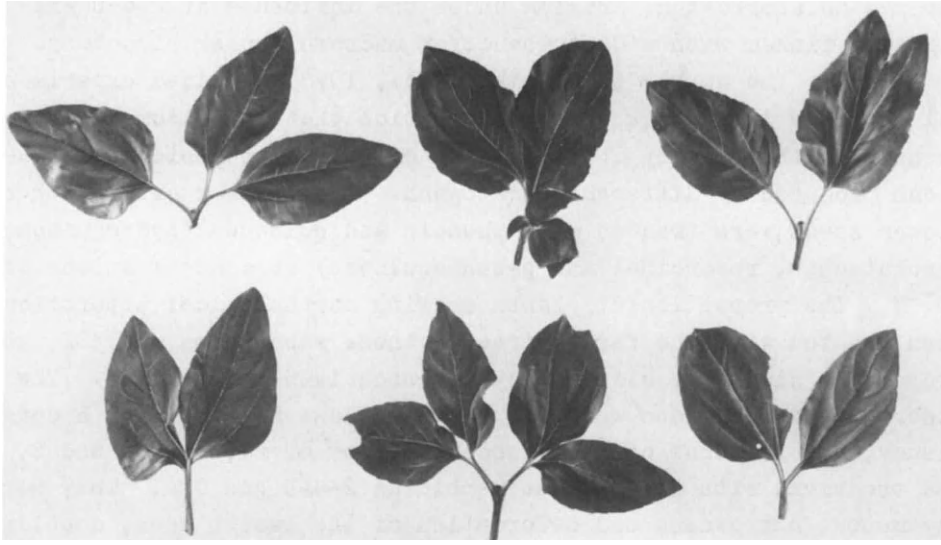


Fig. 85. Phenol-induced morphological alterations of the leaf blade in Helianthus annuus. Appearance of two leaf blades on the same petiole (after Shkolnik et al., 1978).

nuclear membrances (Shulman and Safyazov, 1980).

Studying the effect of high (teratogenic) concentrations of Ni, Cr and B on polyphenoloxidase activity in the young leaves, hypocotyls, cotyledons and roots of sunflower plants, Smirnov (1978) detected a significant increase in polyphenoloxidase activity in response to teratogenic concentrations of these elements. This suggests that there may have been an increase in the oxidised phenol--quinone content. Under the influence of high concentrations of the same three elements there occurred a significant accumulation of flavonoids and of a phenol growth inhibitor - flavonoid-3-glucoside (Shkolnik and Abysheva, 1982). Phenol accumulation and increased quinone levels are induced by other teratogenic factors such as B deficiency, 2-4-D, etc.

It is well known that many structural anomalies have a genetic origin, these being referred to as "mutations" (Takhtadjan, 1954). Haccius (Haccius, 1976; Haccius and Garrecht, 1963) succeeded in

Fig. 86. Disturbances of leaf disposition in Helianthus annuus induced by polyphenol compounds (after Shkolnik et al., 1978).

inducing such mutations with organic B compounds. Under the influence of phenylboric acid lanceolate leaves appeared in Lycopersicum plants, and other teratogenic alterations were induced in other dicotyledonous species.

Mathan (1965) and Mathan and Jenkins (1962) described a mutant lanceolate variety of Lycopersicum (Fig.88) with leaves similar to those observed by Haccius in plants exposed to phenylboric acid. The suggestion was made (Haccius, 1976) that the lanceolate forms in plants induced by phenylboric acid may be phenocopies imitating the developmental disturbances occurring in mutant varieties under natural conditions. The term "phenocopy" proposed by Goldschmidt (1935) refers to a phenotypic aberration induced by exogenous factors, imitating some natural manifestation of a modified genotype.

A spontaneous mutant of tomatoes, the lanceolate mutant, has one mutant gene and shows a morphological modification of the leaf--shape from feather-lanceolate, characteristic of normal tomato plants, to a simpler smaller leaf of lanceolate shape (Mathan, 1965). The mutant allele of the lanceolate gene induces not only a change in leaf shape but also increases in the activity of four oxidizing enzymes: tyrosinase, lackase, peroxidase and catalase. Treating normal tomato seedlings with phenylboric acid promotes changes like those associated with the mutant allele of the

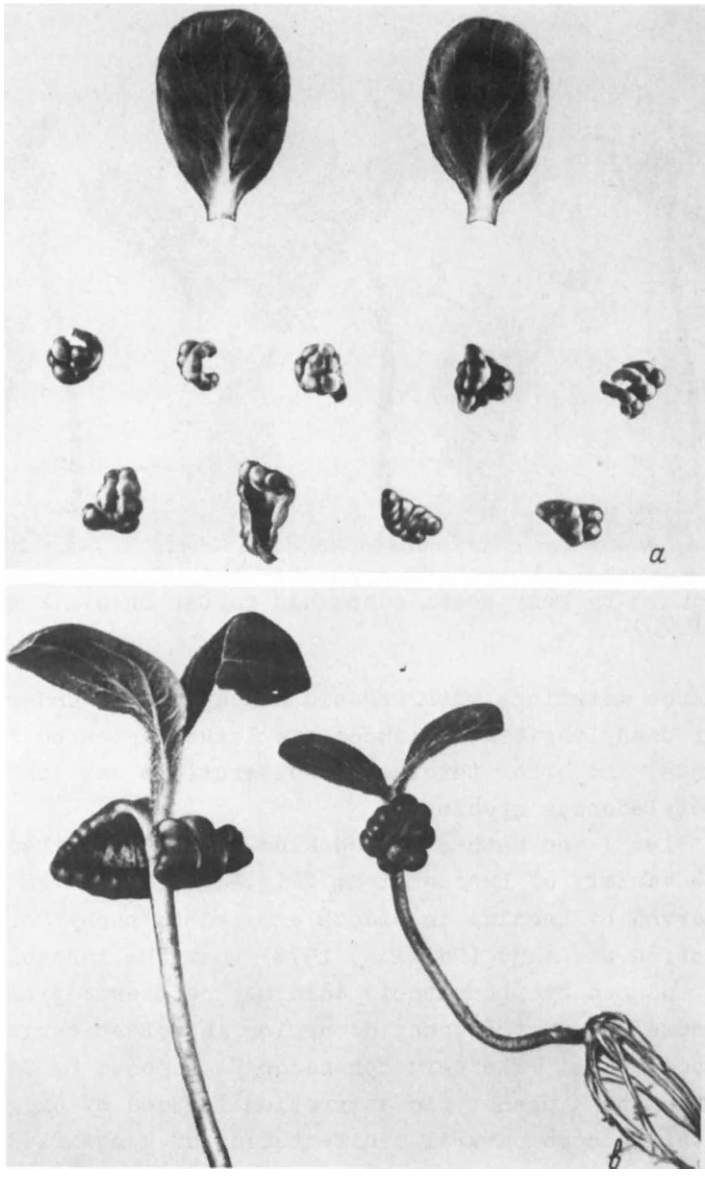


Fig. 87. Modifications of the cotyledons of Helianthus annuus under the influence of para-benzoquinone. Above, cotyledons of control plants; upper middle and lower middle, cotyledons of experimental plants; below, effect of soaking seeds in p-benzoquinone solution (after Shkolnik et al., 1978).

Fig. 88. Effect of treatment with phenylboric acid on the shapes and dimensions of the first and second leaves of tomato seedlings. Left, the first true leaves of control and treated plants.

lanceolate gene; it induces the appearance of lanceolate leaves and increases the activity of the four oxidizing enzymes. This author considers that both the mutant allele of the lanceolate gene and phenylboric acid bring about the same chain of events. In both cases there is an increase in oxidizing enzyme activity bringing about oxidation and inactivation of proteins (mainly of those of the cell walls), and this results in premature elongation growth and maturing of the cells, with consequent morphological changes in the normal leaf shape. The tyrosinase and peroxidase oxidation of proteins occurs through oxidation of the tyrosyl groups, and this is manifested in irreversible inactivation of the proteins (Sizer, 1953).

The disease of tomatoes "stem fragility" caused by B deficiency is controlled by a single recessive gene, btl, responsible for the transport of B from the roots to the overground organs (Wall and Andrus, 1962). Its expression, as symptoms of B deficiency, is governed by several environmental factors, including daylength, illumination intensity, temperature, and N, P and Ca levels.

Dubinin and Pashin (1977) think the cause of teratogenic modifications to be the influence of specific factors (including environmental mutagens on the functioning of genes). Excesses or

shortages of trace elements may be regarded as being among these factors. It would be interesting to know whether high concentrations of phenols could affect the functioning of genes. We already know that disturbances of the nucleolus and other cell structures are induced by B deficiency and high temperatures, both of which are strong teratogenic agents, and that these disturbances are probably connected with poisoning of the cell by accumulated phenols. The damage done to the nucleolus must be cause of the depression of histone synthesis that takes place in cases of B deficiency (Khudzhanazarov et al., 1973) together with consequent disturbance of the genetic activity of affected cells. The lowest, most significant decrease observed by these authors occurred in the histone fraction rich in lizin, which may be responsible for the organization of the highest-order supermolecular structures in the chromatin.

The question arises whether the accumulation of phenols and quinones is the sole cause of teratogenic alterations. On the strength of existing evidence, teratogenic alterations in plants may be supposed to have more than one cause. It is known that phenols bring about a decrease in the content of the growth stimulator, IAA (Runcova et al., 1972; Le Tsung, 1977), and thus it may be that teratogens which induce phenol accumulation have the effect of decreasing auxin levels to the extent that hormonal regulation is disturbed (the balance of auxins and phenolic growth inhibitors being shifted in favour of the latter). The fact that treating plants with auxin may significantly decrease the appearance of teratogenic forms (Dancy et al., 1975), and the fact that teratogenic factors such as B deficiency (Shkolnik et al., 1964; Smirnov et al., 1977), Zn deficiency (Skoog, 1940, and others) and Ni excess (Smirnov and Krupnikova, 1978) simultaneously increase the phenol content and decrease the auxin content, together confirm this suggestion.

Teratogens, it seems, lead to decreases in auxin content not only through the accumulation of phenols, but also through the inhibition of auxin synthesis.

Some data have been obtained indicating that the cause of the appearance of teratogenic alterations is a disturbance of the gibberellin-phenol balance. It is a well established fact that different teratogens (high concentrations of Zn, B and Ni, in particular) being about a significant shortening of the internodes

leading to dwarfism in plants. This effect is in large measure induced by a decrease in gibberellin synthesis. It was shown that in cases of Zn shortage, the content of gibberellin-like substances in leaves and stems does in fact decrease (Shkolnik et al., 1975; Davydova et al., 1978), while that of phenols increases (Reed and Dufrenoy, 1942).

As a result of these findings much more attention is now being given to studying disturbances of hormonal regulation caused by teratogenic agents. It is likely that the study of disturbances of the auxin-ethylene balance will be fruitful since it is disturbance of this balance that affects DNA synthesis and finally results in teratogenic alterations. In fact, shifting of the auxin-ethylene balance was observed in studies of the causes of thickening in the transitional zone or "hypocotyl-root", produced by a N--nitrosocompound which is an atropogenic environmental pollutant and teratogenic agent (Becker, 1979).

Thus we may conclude that teratogens induce disturbances of hormonal regulation, resulting in the synthesis of modified proteins that are unnatural to the affected organism. These proteins disturb the processes of mitosis and bring about the appearance of teratogenic alterations.

The discoveries presented in this chapter show that teratology is no longer simply a descriptive discipline. The information obtained on the physiological causes of teratogenic alterations in plants may serve not only to further our understanding of the physiological causes of the appearance of anomalous plant forms under the influence of shortages and excesses of trace elements and other teratogenic factors, but also to increase understanding of the effects on plant metabolism of spontaneous and atropogenic environmental pollution.

PART IV. TRACE ELEMENTS AND BOTANICAL PROBLEMS

Chapter 1

TRACE ELEMENTS AND TAXONOMY

Vernadsky (1926,1931) and Vinogradov (1937,1952,1965) suggested that the elementary chemical compositions of plants may be taken as species-related characteristics. Vinogradov (1937,1952) points out that the concentrations of many trace elements in living organisms obey laws which are specific to a given taxonomic unit (genus or family), and which may even differ from species to species. Naturally, wide variations can sometimes be observed in the elementary composition of representatives of a single species, especially where these derive from different habitats; nevertheless the principal specificity of the elementary composition of each species remains unaltered.

An impressive example of a correlation between the elementary composition and the taxonomic position of the species may be seen among the marine algae, for which iodine is often a species characteristic (Vinogradov, 1965; Fig.55). Another example is given by the distribution of molybdenum in plants (Vinogradova, 1954). The highest molybdenum contents (especially in seeds) are found in species of the families Fabaceae and Caesalpiniaceae, and species of the subfamily Mimosoideae. Among these one finds species the molybdenum content of which is a species-related characteristic. The molybdenum contents of the seeds of woody representatives of these families are as follows, expressed as a percentage of the dry weight (Vinogradova, 1954):

Fam. Fabaceae (Papilionaceae)	
Tribe Genisteae	
Cytisus sessilifolius	2.10^{-4}
Cytisus hillebrandtii	7.10^{-4}
Spartium junceum	$3.4.10^{-4}$
Tribe Galegeae	
Robinia hispida	2.10^{-4}
Robinia pseudoacacia	5.10^{-4}
Fam. Caesalpiniaceae	
Gleditsia caspica	7.10^{-5}
Gleditsia triacanthos	8.10^{-5}
Gleditsia japonica	1.10^{-5}

A good illustration of the ideas expressed by Vinogradov on the correlation between elementary composition and the taxonomic position of the species is given by the results obtained by Teshabayev and Rish (1974) regarding the taxonomic significance of the trace element composition of a sand desert on the genus Calligonum. These authors demonstrated that Calligonum alatiforme and Calligonum aphyllum, although they show no visible differences, are notably different with respect to levels of elements belonging to the iron group. Thus, Calligonum aphyllum has 3.4 times as much iron, 3.2 times as much cobalt and 1.8 times as much nickel as Calligonum alatiforme. They showed also that changes in the contents of the majority of elements studied within sections of the genus Calligonum obey a definite law. Thus species of the more ancient section Pterococcus, evolving under the conditions of a gypsum desert, are relatively rich in iron, manganese, cobalt and copper. Species of the youngest section Eucalligonum, which have become adapted in the course of evolution to low levels of trace elements in the soil, feature a poor trace-element composition. The concentrations of trace elements in representatives of the section Pterigobasis, as well as the structure of the fruits of these species, are intermediate between those of species in the sections Ptericoccus and Eucalligonum. The former exhibit a high concentration of calcium that is associated with their adaptation to clay soil conditions. This may be regarded as a taxonomic characteristic of Pterigobasis species. The only representative of the section Calliphysa, Calligonum junceum, contains high levels of magnesium, calcium and sodium. Its phylogenetic development has followed a specific pattern determined by its adaptation to gypsum rocks of the Tertiary and Cretaceous eras, and to salinated clay substrates. Elevated contents of sodium and magnesium and a low content of manganese may be considered taxonomic features of this species.

In a study of the accumulation of boron in the plants of Tadjikistan, Amanova (1980) found that the boron contents of the plants could to some extent be regarded as taxonomic characteristics. Low boron levels were characteristic of the studies species of gymnospers (Juniperus, Ephedra). Average levels of accumulation were found in the pteridophytes (Equisetaseae, Polypodiaseae). High, and extremely high accumulations were characteristic of the angiosperms only, being a more common property of dicotyledons (45% species) than of monocotyledons (13% species). The majority of

grasses studied accumulated very little boron. High boron contents were found in all the investigated species of the Zygophyllaceae, Chenopodiaceae, Cappariaceae and others.

The author's findings on the occurrence of borophiles among grasses are of considerable interest, since previously, all grasses have been considered as being plants of low accumulating potential (borophiles constituted 13% of the species studied).

Chemical analyses of the elementary composition of plants, in conjunction with serological investigations, may contribute considerably towards solving problems of taxonomic separation especially in cases of poorly distinguishable species. Research in this area is certaily promising.

Chapter 2

TRACE ELEMENTS AND PHYTOCENOLOGY

Geochemical factors of the environment influence not only the evolution of species, but also the evolution of vegetative types such as phytocenoses.

Reference should be made to studies carried out in the Latvian SSR on the structural formation of phytocenoses in natural meadows and how this is affected by the trace elements molybdenum, copper and boron (Klyavinya, 1967; Sabardina et al., 1970). In addition to a number of plant species belonging to the meadow flora, groups of phytocenoses and some types of meadows have been recognized which to some degree serve as indicators of the trace element content of the soils. Trace elements have been found to stabilize existing relationships between the components of phytocenoses; they strengthen the position of dominants, and interfere only with the processes of change in the vegetation. Gorokhova (1968) described the interesting dynamics of marsh vegetation in the Yaroslavsk-Kostroma regions along the Volga, and related the vegetation pattern to the trace element content of the upper layers of the turf.

Interesting information is available concerning the balance of trace elements in high mountain biogeocenoses, in plants of the taiga-permafrost landscapes, and in plants growing on accumulations of the rarer elements in the Far North. It has been shown that different plant groups and organisms differ in their ability to concentrate trace elements.

Lalayan and Vladimirov (1970) studied the trace element content of plants growing on mountain pastures and hay meadows in northern Osetia. They found that alpine low grass meadows and matgrass communities showed elevated levels of trace elements. Optimum levels were recorded in subalpine meadows with a prevalence of grasses (brome-grass, wood-reed, timothy) and in mid-mountain herb and grass meadows. The poorest trace element composition was reported for the steppe vegetation on dry southern slopes and on alluvial soils. A study of the trace element balance in high mountain desert biogeocenoses of the Eastern Pamirs revealed that the trace element content of plant ash was largely determined by the composition of the phytocenosis from which the plant was taken

(Tyuryukanov and Saboyev, 1970). For example, in the phytocenoses of association of spear-grass, the manganese content was high. Strontium was found in higher amounts in the phytocenoses of associations in which Eurotia was predominant, whereas increased vanadium and lead levels were reported in phytocenoses with a predominance of sedge-grass. In the permafrost-taiga landscapes of Buryatia, Veratrum lobelianum and Aconitum arcuatum were predominant in meadow grass stands growing on soils with an excess of manganese (Zotova and Kovalevsky, 1970).

Studies of iron and manganese accumulation by plants in the permafrost-taiga landscapes revealed that the highest iron content was to be found in Sphenopsidae, progressively decreasing in the Compositae, Scrophulariaceae, Rosaceae, Gramineae, Cyperaceae, Geraniaceae, Onagraceae, Leguminosae, Labiatae, Umbelliferae and Ranunculaceae. In order of decreasing manganese content the families studied could be arranged as follows: Cyperaceae, Sphenopsidae, Polygonaceae, Scrophulariaceae, Compositae, Labiatae, Rosaceae, Gramineae, Rubiaceae, Onagraceae (Sazonov and Reshetnikov, 1970).

Paribok (1970b) showed that in all plants of the tundra and forest tundra of the Urals, and in all those of the arctic tundra of the Polar Urals, manganese, copper, strontium, vanadium and nickel were the elements most intensively accumulated. Molybdenum, cobalt, titanium, iron and chromium were accumulated least. Levels of elements accumulated proved to be different in various biological plant groups. Shrubs of the genera Betula and Salix, and especially shrublets of the genus Vaccinium, are notable for their vigorous accumulation of manganese, copper and barium. Herbs usually display low levels of trace elements, except for strontium. Lichens and mosses show exceedingly high accumulations of iron, titanium, lead, strontium and chromium, the levels being one or two orders of magnitude greater than those reported for other plants.

Amanova (1980) succeeded in showing that different species of one and the same phytocoenosis are characterized by different levels of boron accumulation. The range of the boron content is especially wide in components of mesothermal, xerophilic and halophilic phytocoenoses. The increased boron content is more often encountered with in the predominant plants of the chernolesie (deciduous forests), shibliak (Mediterranean decidous shrub popula-

tions), savannoids, ancient Mediterranean-type deserts, and halophytones. A much lower level of boron accumulation is characteristic of the predominant plants of steppes, heathlands, tragacanth populations and semi-savannas. There are more plants with a high boron-accumulating capacity among species whose habitats are connected with the territory of the Ancient Mediterranean region, than among species of essentially Boreal origin (39 and 26 per cent, respectively).

Ecological factors play an important part in the regulation of boron levels in plants. This is indicated by the wide range of boron levels in plants of the same species growing under different conditions, as well as by the clear differentiation of boron levels in different ecological groups of the same species. Plant glycophytes concentrating boron may be considered as being true borophiles because they are distinguished for their high levels of boron accumulation.

Interesting observations have been reported on the levels in meadow and pasture grasses of those trace elements (cobalt, iodine, iron, copper, manganese and others) which play important roles in animal physiology. However, only limited information is available concerning the trace element content of forage grasses. Reference may be made to a study carried out by Kazaryan (1970) on the copper content of forage plants in mountain regions of Armenia, and also to his study of the contents of copper and molybdenum in the plants and grass stands of the Agdaksk copper - molybdenum province (Kazaryan and Della-Rossa, 1970). Kozyreva (1970) reported on interesting findings with respect to the copper content of pasture plants growing in the biogeochemical province of the Zaamin district of the Syr-Darya region, notable for its low copper levels. Legumes have been shown in a number of investigations to contain more cobalt than most grasses (Kovalsky and Chebayevskaya, 1951; Hill et al., 1953; Katalymov and Shirshov, 1955; Matveyeva and Znamenskaya, 1959). Kovalsky and Chebayevskaya (1951) found that among herbs and grasses, some species are no less rich in cobalt than legumes.

The botanical composition of a grass stand may be improved by supplying trace elements to the soils, This leads to an increase in the total mass of leguminous plants (Fig.89), and the yield of protein per unit area increases, therebeing more protein both in the legumes and the grasses (Koryakina, 1963). Important information on the trace element content of plants and forage crops is

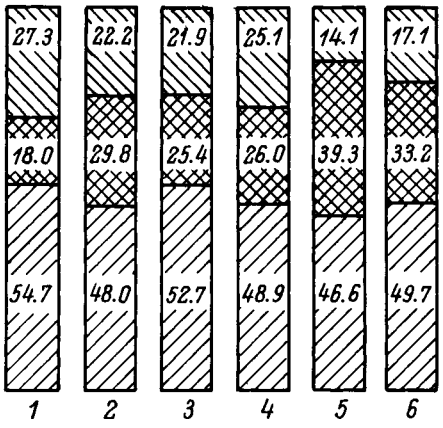


Fig. 89. Effects of root external supply of trace elements on the composition of a grass stand in a meadow (after Koryakina, 1963). Ordinate, weight of component (% total air-dry weight of grass stand). Below - grasses; middle - legumes; above - herbs. Abscissa: 1, control; 2, Mo; 3, Zn; 4, B; 5, Mo+Zn; 6, B+Zn.

presented in a book by Kovalsky et al. (1971).

Biocenological studies of the turnover of trace elements in biogenocenoses of various types, as well as in the biosphere as a whole, are of great value. Two investigations deserve discussion in greater detail. In the first of these (Snytko and Dubynina, 1970) results are given of a study of the turnover of trace elements in facies of the Onon-Argun steppe; the results show that there are varying degrees of concentration of trace elements among the principal cenosis-forming plants of different facies. Thus barium accumulates in plants of the sheep's fescue facies, whereas its content is low in plants of the tansy and herbaceous-woody reed facies. Manganese concentrates largely in the vegetation of the herbaceous-woody reed facies, whereas the sheep's fescue facies features lower quantities. The facies studied vary considerably in terms of the rate of trace element turnover. The highest rates were reported for the herbaceous-woody reed and sheep's fescue facies. The results obtained on test sites may be regarded as being representative of most of the Onon-Argun steppe.

The second paper (Titlyanova, 1970) presents a general scheme for the turnover of carbon, iron and manganese in four different biogenocenoses: mixed spruce forest, birch and grass forest, high pine peat moss bog, and herb grass steppe. Material is given for the estimation of specific parameters of the turnover of carbon,

iron and manganese. It was found that the rates of the soil-plant fluxes of iron and manganese were not correlated. The rate of the iron flux was greatest in the steppe areas and least in the spruce forest areas; in the case of manganese the flux rate was high in birch forest and low in the steppe. Estimates of the period of turnover for each element in the plant "block" were: 40 years for carbon, 13 years for iron and 5 years for manganese. Birch is a component of biocenoses which specifically accelerates the turnover of manganese. Very high, and almost equal values for the turnover rates of the three elements have been reported for the steppe, where the elements largely return to the soil upon the death of the roots, and for the forest biogeocenoses where the elements return with the leaf fall. Rough estimates have revealed that in birch forest the rate of the litter-soil iron flux is 30 times lower than the corresponding carbon flux rate, and 3 times lower than the corresponding manganese flux rate. This difference is less pronounced in steppe biogeocenoses.

Investigation of the turnover of trace elements in biogeocenoses of different types has begun only recently. Multidisciplinary studies may be expected to contribute significantly to our understanding of the dependence of biocenoses' structure and dynamics upon the geochemical environment, and should enrich our knowledge by providing important information on the biogenic movements of chemical elements. Such investigations would play a prominent role in developing the ideas expressed by Vernadsky on the biosphere.

Chapter 3

TRACE ELEMENTS, ANATOMY AND THE STRUCTURAL ORGANIZATION OF THE CELL

Extensive anatomical abnormalities may develop in plants growing in soils with excesses or shortages of trace elements. Thus, striking anatomical modifications have been reported in plants suffering from deficiencies of boron (Fig.90), manganese, zinc and copper (Warington, 1926; Eltinge, 1936; Reed and Dufrenoy, 1942).

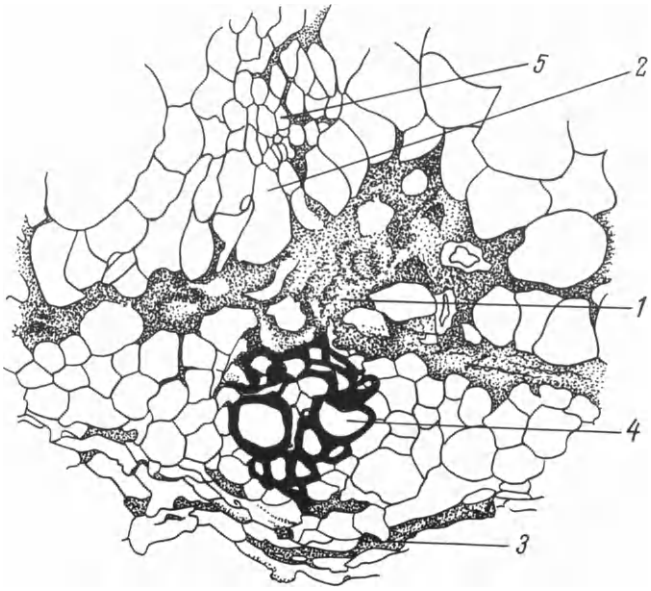


Fig. 90. Effects of boron deficiency in the advanced stages on the anatomy of the shoot tips of broad beans (after Warington, 1926). 1, disintegration of originally elongated cambium cells; 2 and 3, degradation of the main parenchyma; 4, woody xylem; 5, phloem.

Eltinge (1941) studies the effects of manganese deficiency on the anatomy of Lycopersicum esculentum. Only a few normal plastids could be found in the palisade cells of manganese-deficient plants, the majority of the plastids becoming vacuolized or showing a granular appearance, with blurring of the contours. At later stages of the deficiency disease the plastids dissolved, producing a yellow-green solution in the cytoplasm. Products of abnormal

metabolism steadily accumulate in the cells of plants exposed to prolonged manganese deficiency. Frequently, areas in which every palisade cell is at least occupied by calcium oxalate crystals may be seen. Stems of manganese-deficient plants also undergo modification in that they become thinner. Only the newly formed xylem is viable in such plants, while the old xylem shows numerous non-viable degraded cells. The xylem and parenchyma cells are completely filled with calcium oxalate crystals. Starch grains are fewer in number in the tissues of the stem, and are smaller and simpler in structure in comparison with those of normal plants.

Reed (1939) reported large cavities between the palisade cells in copper-deficient tomato plants (Fig.91).

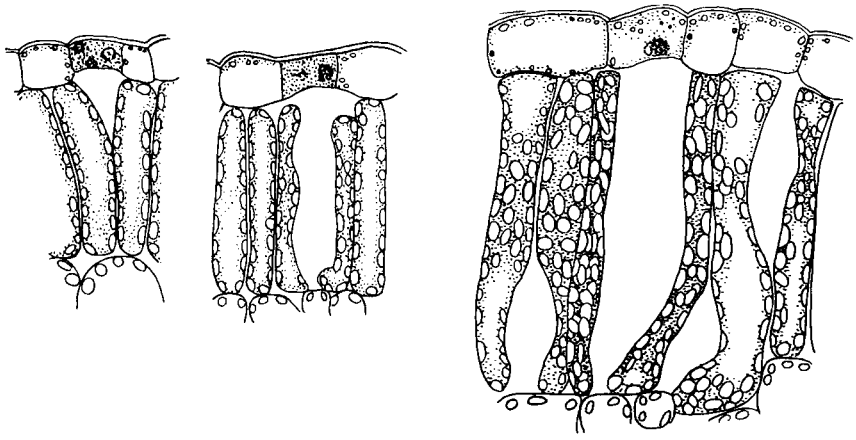


Fig. 91. Effects of copper deficiency on the anatomy of the palisade tissue of tomato leaves. Large cavities are present between the cells (after Reed, 1939).

Molybdenum deficiency is also accompanied by considerable anatomical changes. For example, the epidermal cells of cauliflower leaves disintegrate, even before destruction of the chloroplasts has occurred. This is followed by extension and deterioration of the spongy parenchyma cells. Chloroplasts shrink and disintegrate in affected areas. Similar alterations occur in plants exposed to excessive amounts of trace elements in the environment. High boron concentrations in the soil cause specific abnormal cells to appear which are absent from normally developing plants of the same species. In Anabasis aphylla, for example, numerous stony cells appear in the central axial region of the stem, and in Limonium suffriticosum this effect is also accompanied by the appearance of thick-walled cells in the cambium, the latter cells being modified

filaments performing a mechanical function. In Salicornia herbacea, elongated cells develop with helical thickenings of the walls. These cells resemble water-conducting tracheids which provide a pathway for the efflux of excess salt solution from the plant (Petrunina, 1965).

Shortages of trace elements result in serious alterations in cell ultrastructure. With the progressive chlorosis that occurs in iron-deficient plants, the structure of the chloroplasts undergoes changes from the early stages of the disease. Ostrovskaya et al. (1968) reported alterations in chloroplast structure in field-grown apple trees suffering iron chlorosis, and in pea plants cultivated under conditions of artificially induced iron deficiency. They found that the grana contained fewer discs, and that the intergrana structure progressively deteriorated. Large osmiophilic globules have been detected in chlorotic grape leaves.

Chloroplast degeneration has been observed by Kibalenko (1966a) and Lee and Aronoff (1966) in boron-deficient plants. Alekseyeva (1971) found that boron deficiency in flax and maize resulted in ultrastructural modifications of the cell walls, chloroplasts, hyaloplasm, Golgi apparatus, microsomes, ribosomes and nuclei of the mesophull cells. In addition, she found that mesophyll cells degenerated very early in their development in boron-deficient flax plants. In chloroplasts, these disturbances involve specific alterations, including underdevelopment of the lamellar system and the appearance of symptoms of structural disorganization, primarily the disappearance of the plastid wall. These ultractructural modifications extended to the cytoplasmic of the cell and the membrances of the chloroplast stroma. In the chlorotic areas of maize leaves, swelling of the grana discs and intergrana lamella, a reduction in the number of discs in the grana, and extrusions of the chloroplast wall were observed. In some cells the chloroplasts were abnormally small, showing a considerable reduction in the volume of the lamellar substance and markedly swollen thylakoids (Fig.92(1), 92(2)).

Alterations of the lamellar-granular structure of chloroplasts and mitochondria also occur under conditions of manganese and zinc deficiency (Vesk et al., 1966) (Fig.93). The number of grana is reduced, the lamellac inside these structures appear to be fused together, the number of discs is dramatically reduced, and the intergrana links consist of only a few frets or are replaced by

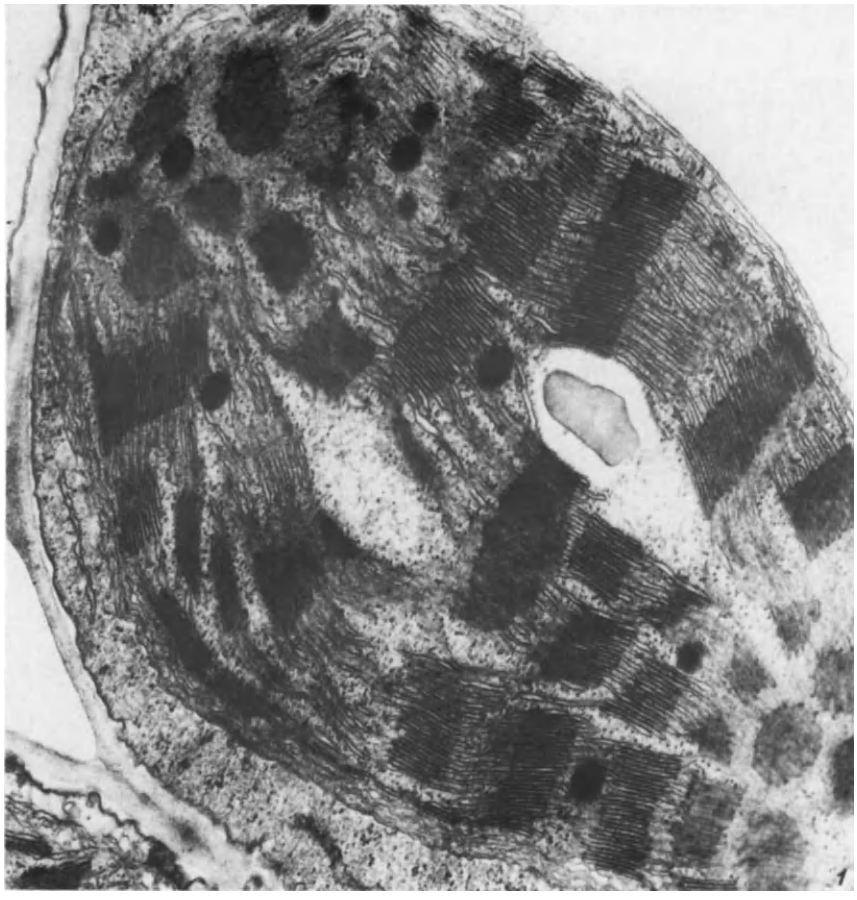


Fig. 92(1). Effect of boron starvation on the structure of mesophyll chloroplasts in maize plants (after Alekseyeva, 1971). 1, chloroplasts in normal plants.

vesicles. In zinc-deficient tomato plants the mesophyll chloroplasts contain starch "vacuoles" and show a separation of the grana, a reduction in the volume of the frets, an increase in the overall stroma area and a depression of the plastid membrane which separates a portion of the stroma from the remainder of the chloroplast interior. As shortage of zinc or a shortage of magnesium either distorts the grana or completely inhibits their development.

Among factors that are responsible for ultrastructural damage to complex cell organelles, in particular the chloroplast, we may include a diminished content of phospho- and galactolipids, as observed in association with boron deficiency of boron and deficiencies of some essential divalent metal trace elements. It has been

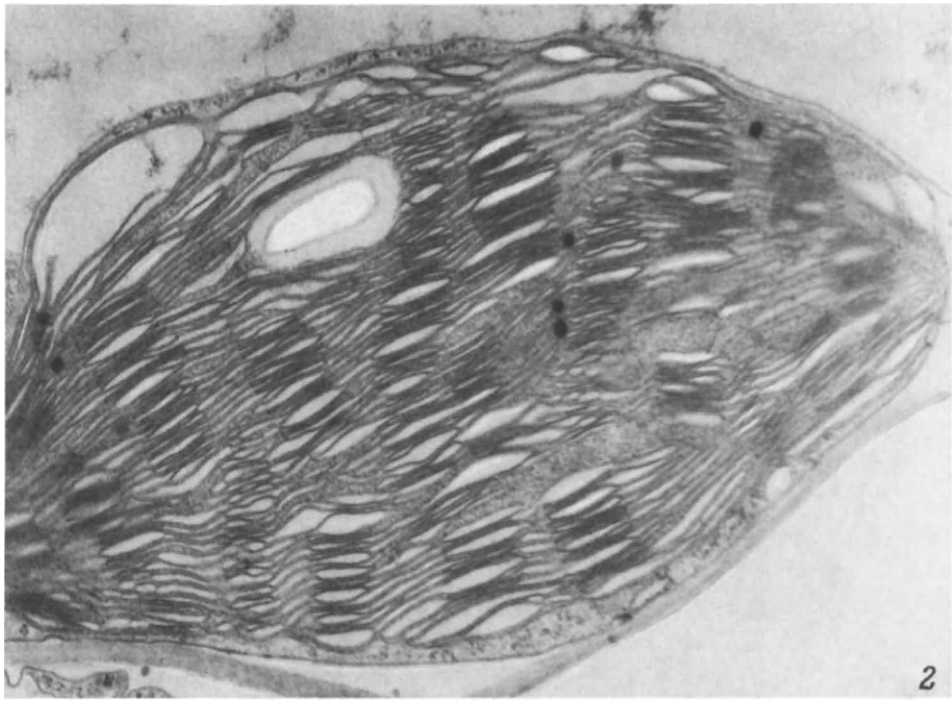


Fig. 92(2)(continuation). 2, chloroplasts in boron-deficient plants. Note swelling of grana and intergrana lamellae.

shown that 8 enzyme-mediated steps in lipid metabolism require the presence of metals as constituents of co-factors (Stumpf and Bradbeer, 1959).

Trace elements play a prominent role in maintaining the integrity of the intracellular membrane structure. Kavanan (1962) suggested that divalent metals form bridges between neighbouring phosphoryl groups in lipids, and covalently link carboxyl groups in both lipids and proteins. At low concentrations of divalent cations associations between membrane components become weaker, accounting for the loss of selective permeability and other important properties of membranes.

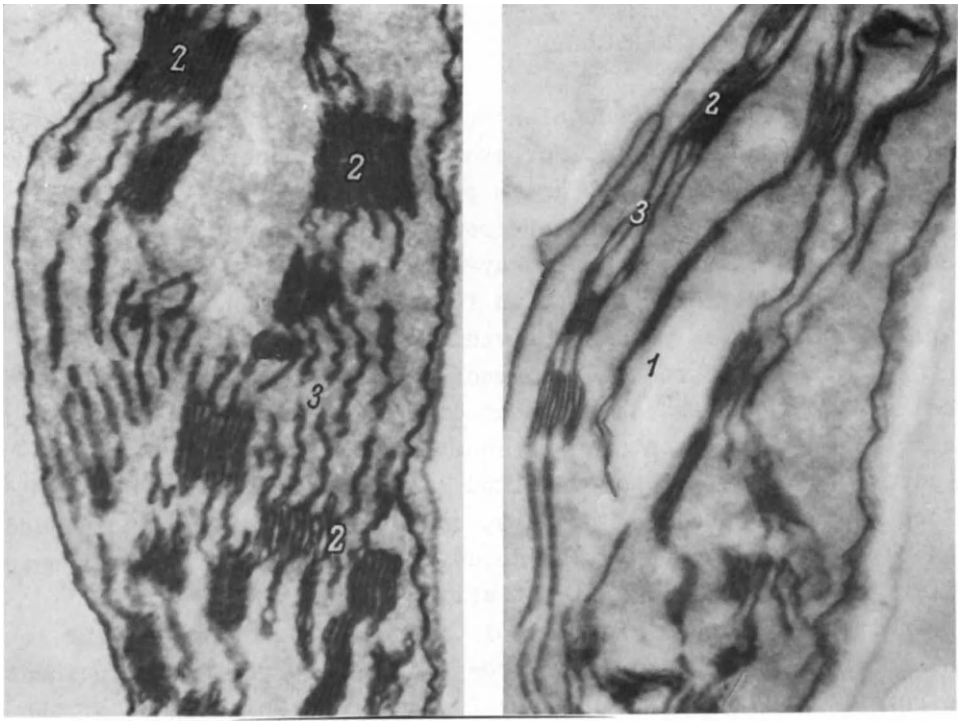


Fig. 93. Chloroplasts from the mesophyll cells of normal and zinc-deficient tomato plants (after Vesk et al., 1966). Left, control (+Zn) detail of chloroplast of normal tomato. Right, detail of chloroplast of zinc-deficient tomato leaves. 1, starch "vacuoles"; 2, grana; 3, frets.

Chapter 4

TRACE ELEMENTS AND EMBRYOLOGY

Shortages of some trace elements may be responsible for the absence of organs in plants, for example an absence of inflorescences occurs in boron-deficient, grape plants (Woodbridge, 1955), and stamens and pistils are absent from boron-deficient wheat plants (Shkolnik and Solovyeva-Troitskaya, 1962).

The important role of zinc in fertilization was recognized as early as the 1940s; plants thrived vegetatively when deprived of this element, but produced no seeds. Reed (1944) showed that zinc was essential for the development of the cosphere and embryo in peas. When the element was in short supply, the embryo was degraded. Boron deficiency resulted in an extensive loss of the flower buds in cabbages, peanuts, and various fruit-producing and other plants. In cereals, boron deficiency produces complete sterility of the ears (Shkolnik et al., 1956).

Troitskaya and Batygina (1970) offered evidence testifying to the importance of boron for macro- and microsporogenesis in wheat, as revealed by major alterations in the sporogenic tissue of the anthers. Anomalies could be seen primarily in the process of reduction division in the parent cells of the spores; the separation of the chromosomes was asynchronous and never reached completion. As a result of disturbances in the later stages of meiosis considerably enlarged microsporocytes with large nuclei and extrusions of the chromatin were observed. The nuclei of such microsporocytes are usually polyploid (Fig.94). The anther lobes are either destroyed or are empty, no pollen being produced.

In boron-deficient wheat the embryo sacs degenerated; similar phenomena have been reported for soy and pomegranate plants (Povolochko, 1940; Shestakov et al., 1956).

Modilevsky (1953) used boron treatment for studying the biology of heterostyle plants. Heterostyly in these plants is a peculiar adaptive device for achieving cross pollination. Spraying such plants (e.g. buckweat) with boron solutions was found to increase the frequency of fertilization by illegitimate pollen, and significantly affect the ratio of long-column and short-column individuals among the progeny.

Son (1973) found that all stages of embryogenesis, beginning from the first division of the zygote and that of the primary nucleus of the endosperm, were accompanied by the synthesis of nucleoproteins and carbohydrates, and by influxes of boron, calcium and magnesium into the embryo, endosperm and aleurone layer cells.

Development of the ovary is affected by copper deficiency (Polukhina and Maslyanaya, 1962). Livdane et al. (1970) described morphological changes in the anthers, and extensive degradation of the ovaries in copper-deficient plants. Interestingly, cobalt has been shown to enhance pollen germination (Yamada, 1969).

Elevated levels of trace elements may result in serious interference with the formation of the generative organs. Thus excess iron has been suspected as a cause of sterility in rice flowers (Ponnamperuma et al., 1955). Petrunina (1965) reported interesting findings on the sterility of flowers in biogeochemical provinces with excesses of trace elements. Thus, she was able to observe underdevelopment of the flowers and abnormal development of the inflorescences in Lynosyris villosa and Lynosyris tatarica.

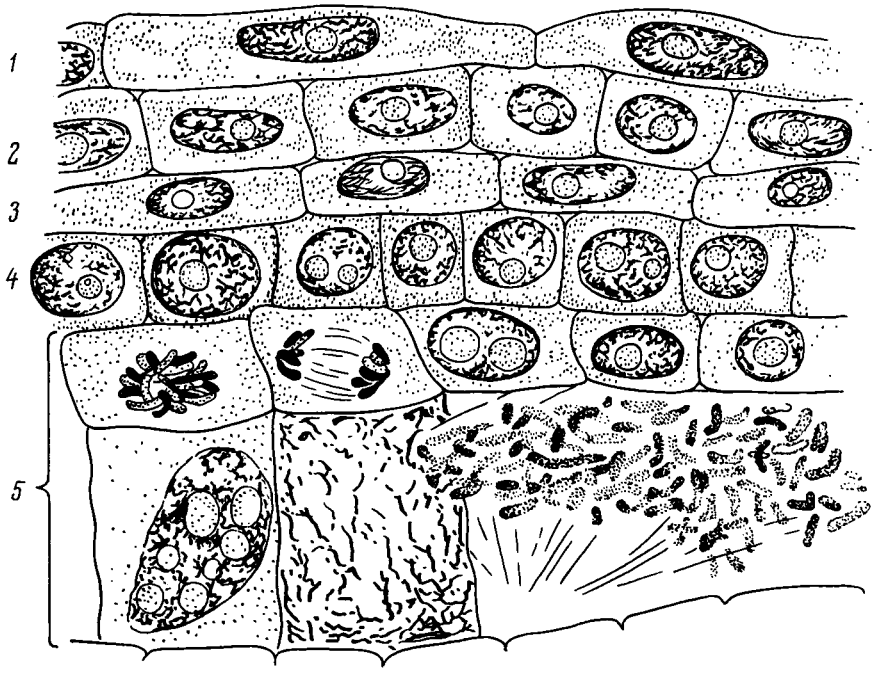


Fig. 94. The effect of boron starvation on the structure of microsporangia in wheat plants. Longitudinal section of microsporangium (after Troitskaya and Batygina, 1970).
1, epidermis; 2, endotecium; 3, middle layer; 4, tapetum; 5, sporogenous tissue (notice an increase in chromosome number).

Chapter 5

TRACE ELEMENTS AND GENETICS

One series of reports has dealt with the importance of a balance of trace elements in the cell for the structural integrity of the chromosomes (Mazia, 1961). In a study of isolated polytene chromosomes from nuclei of the salivary glands of Chironomus dorsalis larvae, divalent metals were found to participate in chromosome formation (Gruzdev and Belaya, 1973). Intrachromosomal associations have been suggested to depend upon the existence of a complex of histone fI and divalent metal cations. Since variations in the dimensions of chromosomes are produced primarily by changes in the electrostatic forces acting between the phosphate groups of the DNA in the elementary chromosomal filament, it has been assumed that divalent cations and molecules of lysine-rich histone serve to stabilize the structure of the elementary chromosomal filament.

Moutschen-Dahmen and Moutschen-Dahmen (1963) provided evidence indicating that metals increase the probability of chromosomal aberration. These workers studied the effect of ethylmethanesulphonate on the appearance of chromosomal aberrations and on seed germination and plant growth as a function of temperature, duration of exposure to this agent, and the presence or absence of metal cations. Mutations produced by ethylmethanesulphonate are essentially similar to those produced by uv irradiation. The activity of the mutagen was found to be enhanced in the presence of copper and zinc.

Robert (1975) gave brief account of the influence of changes in the ionic composition of the medium upon the structural and functional state of chromosomes. Results were given of the biochemical analysis of giant chromosomes and chromatin during consecutive stages of condensation and decondensation produced by changes in the ionic environment. A correlation between the extent of the template activity of chromosomal preparations and their degree of condensation was observed. Ionic mechanisms are presumed to be involved in the regulation of the structural and functional activity of chromosomes.

The effects of metals on chromosomes are of major interest. Mahler (1961) believed that the genetic material itself existed as a complex with metals or metal chelates. Kihlman (1957)

suggested that iron and other metals are probably constituents of chromosomes, and that chelate radicals are able to induce changes in chromosomal structure by establishing firm metal-containing complexes.

In the 1930s, when progress was being made in the study of chemical mutagenesis in Drosophila, mutations were also reported as having been produced by various trace elements, including iodine (Zamyatina and Popova, 1934; Sakharov, 1938), manganese (Naumenko, 1936) and copper (Magrzhikovskaya, 1936). Roberts and Aldous (1951) reported that manganese and copper evoked mutations by affecting the structure of DNA. Lawrentz (1963) cultivated a tryptophan-deficient Bacillus subtilis strain on media supplemented with copper. Within 4 days, new morpological variants appeared which by the 13th day had completely replaced the original cells. The DNA nucleotide composition of the newly developed variants was essentially different from that of the original strain.

By carrying out numerous transfers together with selection, Bershova (1967) obtained variants of Azotobacter in media containing boron and molybdenum. The new variants differed from the original strains with respect to their morphology and rate of nitrogen fixation. Subsequent cultivation of the modified bacteria for several months in media without boron and molybdenum did not eliminate the properties acquired.

Bowen (1966) found that the mineral composition of tomato seeds notably affected the number of mutations induced by irradiation. The proportion of mutant pale-green seed lobes developing from manganese-deficient seeds after irradiation was considerably higher than that produced by zinc-deficient seeds. Deficiencies of boron, calcium and phosphorus also resulted in notable, albeit less dramatic, alterations. The frequency of lethal mutations increases under conditions of nitrogen or copper deficiency, and is less with a shortage of magnesium.

Wall and Andrus (1962) found that the boron deficiency fragile stem disease of tomato plants is controlled by a single recessive gene, btl, responsible for the transport of boron from the roots to the above-ground plant organs. Activation of the gene is effected by a number of environmental factors including daylength, illumination intensity, temperature, and the levels of nitrogen, phosphorus and calcium, i.e., those factors which are known to modify the effects of boron deficiency.

It has been found that the behaviour towards boron differs in different maize variaties. For example, boron take in hybrids is a maternally inherited characteristic. Hybrids accumulate boron in the leaves, whereas in the parental lines boron is mostly held in the roots. Boron is more mobile in hybrids than in their parental lines, possibly on account of the greater activity in the accumulating organs (in this case the leaves). It may be concluded that boron exerts its influence on the plant through participation in the processes of gene regulations. This might be of relevance in the context of studies on heterosis and the breeding of new plant lines (Aseyeva, 1981).

Extensive research into the mutagenic activity of metal trace elements was carried out by Rosen (1964). He studied the effects of 65 elements on the nucleus by taking a cytological approach, and revealed that chromosomal alterations were produced by elements of atomic weight 65 or less which readily enter into complex formation. This finding might suggest that metals are effective mutagens. By treating plant seeds with various complex-forming trace elements - copper, iron, cobalt, zinc, manganese and others - Rosen obtained evidence indicating that gene mutations are induced not only by shortages, but particularly by excesses of essential trace elements. The frequency of mutations, albeit lower than that resulting from ionizing radiation of many of the chemical mutagens, was shown to be moderately high. The spectrum of mutations produced by metals proved to be much like that produced by ionizing radiation.

Simultaneous treatment with several trace elements was found to have a particularly strong mutagenic effect. Also strongly mutagenic was combined exposure to ionizing radiation and metal cations; some metals in this case did not affect the spectrum of specific chlorophyll mutations, but were able to induce mutations of other types.

According to Rosen, the ability of metal ions to form complexes with proteins accounts for their mutagenic activity. It is suggested that metals which form complexes play a limited, but nevertheless significant role in the evolution of plants. This is the result partly of their direct influence, and partly of an indirect influence on active radicals produced by natural radiation. Some gene mutations are known to be produced by the action of various kinds of ionizing radiation. Others are produced by any of a large group of chemical mutagens, including: sulphonates, nitric acid,

mustard gases, and peroxides. Investigations carried out by Rosen have demonstrated that another important group of substances - the salts of trace elements - are mutagenic. Rosen believed that treatment with metal ions, either alone or in conjunction with other mutagenic agents, will offer plant breeders a new approach to breeding research.

Ponomarev (1967) grew plants from seed irradiated with high velocity neutrons on media supplemented with zinc and copper salts, and succeeded in obtaining variations in some of the characteristics of the plants. Among these he reported a considerable displacement of the period of spike emergence, a change which persisted up to the fourth generation.

Treating seeds with Fe and Mn salts under strong irradiation was found to increase the yield of vegetative tillers, with no effect on seed yield (Ilyina and Kuznetsova, 1975).

The ability of the salts of some metals to mitigate irradiation injury in plants may be related to the fact that metal ions can neutralize the negative charges on phosphate groups, so that in spite of the disruption of hydrogen bonds brought about in DNA by irradiation, individual DNA strands are maintained in their original position. This may facilitate restoration of the secondary structure.

Kovalsky (1974), in a discussion of intrapopulational physiological and biochemical variability in the organisms of biogeochemical provinces, expressed the belief that considerable reserves of unmanifested variability attributable to minor mutations and gene recombination may accumulate in populations of organisms. Such reserves of variability have been detected in soil microorganisms exposed to high concentrations of molybdenum, vanadium, boron, selenium and cobalt. The intrapopulational variability of microorganisms was evaluated in terms of the adaptability of individual strains isolated from single colonies to the full range of natural plant concentrators of chemical elements. For example, Bacillus megaterium strains isolated from uranium-rich soils normally develop in the presence of high concentrations of this element, and suffer when it is present in low amounts. Conversely, bacteria of this species isolated from soils poor in uranium show the most rapid growth at low concentrations of the element. Similar behaviour has been observed in other microorganisms in response to chemical elements. However, mutant strains have also been found which show

a different type of response; such strains isolated from boron rich soils were able to develop normally when exposed to wide range of boron concentrations.

A reference was made above to the demonstration, by genetic transformation, of the hereditary nature of adaptation to extreme geochemical environments in microorganisms. Kovalsky (1974), citing these studies, expressed the belief that in biogeochemical provinces rich or poor in chemical elements, mutations may be induced by the prevailing extreme conditions. Enrichment of the gene pools of populations takes place, making for an intensification of the processes of natural selection and species formation, and eventually resulting in genetic and ecological changes within populations in response to extreme variations of the geochemical environment.

An interesting topic is that of the effect of genetic factors on the metabolic role of trace elements. A large amount of evidence has accumulated in animal physiology concerning alterations in trace element metabolism that can be related to genetic disturbances of the synthesis of the protein moieties of metalloproteins (which occur in abundance in man and animals). Thus Rish (1976) referred to defects in the synthesis of gastroferrin responsible for haemochromatosis in man and animals, and the defective synthesis of ceruloplasmin resulting in Wilson and Konovalov's disease. There is a sex-linked recessive genetic defect in man ("kinky hair") involving interference with the absorption of copper from the gastro-intestinal tract; this leads to the expression of typical symptoms of copper deficiency, such as disturbances of the nervous and cardiovascular systems and an effect on hair growth (Evans, 1972). Among the many other metabolic disturbances of this type is the inherited inability, arising as the result of a genetic defect of specific peroxidase to use iodine in the synthesis of thyroid hormones. Hunt (1974), who studied abnormalities of metabolism in mottled mutants of mice, showed that the mutant mice were defective in the metabolism of dophamine and noradrenalin as a result of reduced activity of dophamine-β-hydroxylase in the brain tissues. The activity of enzyme extracts from the brain tissues of the mutant was restored in media supplemented with copper. An abnormally low level of the copper protein ceruloplasmin was detected in the blood serum of the mutant.

Rish (1970) pointed to another group of genetic modifications which alter the pattern of trace element metabolism without giving rise to pathological effects. These modifications include:

(1) hereditary differences in the rate of biosynthesis of those metalloproteins that occupy central positions in the metabolism of trace elements in animals (enzymes involved in transcuprination, iodination, etc); (2) hereditary physiological characteristics that mitigate or aggrevate trace element deficiency symptoms (bitter taste in the mouth resulting from phenylthiocarbamide-iodine deficiency; depression of succinic dehydrogenase activity resulting from a reduced uptake of selenium, etc); (3) the synthesis of enzymes containing a less than normal amount of metal per unit functional activity (e.g., isozymes of zinc alkaline phosphatase), and the synthesis of isozymes metal of which is replaced by another metal (e.g. the copper and molybdenum forms of xanthine oxidase).

Rish maintained that these modifications made it possible for a population to avoid the adverse consequences of excesses or shortages of trace elements in the environment, that is, to use scarce trace element reserves with greater economy or tolerate high concentrations of trace elements. The metabolic functions of trace elements are determined essentially by the protein components of metalloproteins; this accounts for the hereditary modifications and specific characteristics of trace-element metabolism.

The effects of trace elements on the radiosensitivity of organisms and the frequency of mutations induced by ionizing radiation should be mentioned. Bowen and Cawse (1959) found that shortages of some polyvalent cations, in particular manganese and zinc, somewhat increased the sensitivity of tomato seeds to gamma irradiation. Boron deficiency did not affect the radiosensitivity of the seeds. Conversely, other workers (Skok, 1958) have reported that boron enhanced increases the sensitivity of plants to X-rays and thermal neutrons. Hazama et al. (1963) showed that pretreatment of barley seeds with salt solutions of polyvalent metals (cobalt, nickel, iron, and more especially manganese) considerably elevated the resistance of the seeds to gamma irradiation. By pre-soaking the seeds in magnesium salt solutions the opposite effect was produced, whereas zinc failed to modify the radiosensitivity. These workers concluded that paramagnetic ions offer some protection against ionizing radiation. Other investigators have obtained corroborative evidence that heavy metal ions display radioprotective properties, as revealed by their ability to mitigate the injurious effects of radiation on plants (Vlasyuk et al., 1966; Guseynov, 1966; Ponomarev, 1967; Abdullayev et al., 1974).

Abdullayev et al. (1974) reported on the radioprotective properties of selenium. Experiments carried out in albino rats, selenite administered at a concentration of 4.5 mg per kg prior to irradiation had a radioprotective effect, considerably enhancing the survival (90%; $P > 0.1$) and average longevity (28 ± 3.6 days; $P > 0.1$) of the rats.

Irradiation is known to produce free radicals in the cell which trigger a chain of radiochemical reactions involving the peroxidation of lipids, and leading eventually to the death of the cell. The process of repair commences only when the chain reactions are interrupted by the intervention of naturally occurring anti-oxidants. The authors explain mechanism of radioprotective effect of selenium by referring to findings indicating that selenium compounds are strong anti-oxidants.

Ermakov and Kovalsky (1968) studied the radioprotective effect of sodium selenite in combination with vitamins A and E on rats irradiated with a dose of 490 rads. A notable degree of radioprotection was afforded, increasing the survival of the animals by 50% ($P > 0.02$) over that of the controls. The percentage survival was even higher (83.3% versus 33.3% for the controls ($P > 0.05$)) in another experiment in which rats were exposed to 570 rads.

Reference has been already made to the significant role played by magnesium, zinc and manganese in the activation of DNA-polymerase. In addition, zinc is a co-factor for a number of endonucleases.

No less significant than the repair of DNA molecules in the cell is the repair of intracellular membranes, DNA-membranes and DNA-protein complexes. In other important repair processes, damage done to non-genetic structures - cellular and nuclear envelopes, mitochondria, centrioles, etc., is repaired (Korogodin and Korogodina, 1976). In these processes, the long-recognized contribution of trace elements to the stabilization of cell structures plays an important part. This topic has been dealt with in Chapter 3 (Part IV) on the role of trace elements in the structural organization of the cell.

Kutacek et al. (1966) found that zinc was able to eliminate the inhibitory effects of gamma irradiation on synthesis of the auxin precursor tryptophan, indole auxins and gibberellin-like compounds. This property of the element may be associated with its role as a stabilizer of macromolecules, especially those that form the components of various membranes (Chvapil, 1973).

What might be the mechanism of the radioprotective effects of metals? Jones (1960) believed that the answer lay in the formation metals and chelate ligands of strong metallochelate complexes in the cell, and more particularly in the chromosomes. Beatty and Beatty (1967) found that the administration of various components of nucleic acids reduced the frequency of radiation-induced chromosomal aberrations by 25 to 50%. All of the substances tested were found to facilitate the processes of repair rather than diminish radiation injury. Thus supplementation with nucleosidetriphosphates, in particular ATP, was thought to provide an additional energy source for the repair of radiation-induced breaks in DNA strands. Since trace elements have been shown to elevate contents and improve energy metabolism, it seems probable that this is one of the mechanisms by which trace elements exert a radioprotective ei effect. However, is just one on the many functions of trace elements, which take part in a large number of cell processes, including the influence on the oxidative processes.

Chapter 6

TRACE ELEMENTS AND THE ADAPTATION OF PLANTS TO UNFAVOURABLE ENVIRONMENTS

Aluminium, cobalt and copper have been shown by a number of workers to increase considerably the resistance of plants to drought (Fig.95), both under experimental conditions and in the

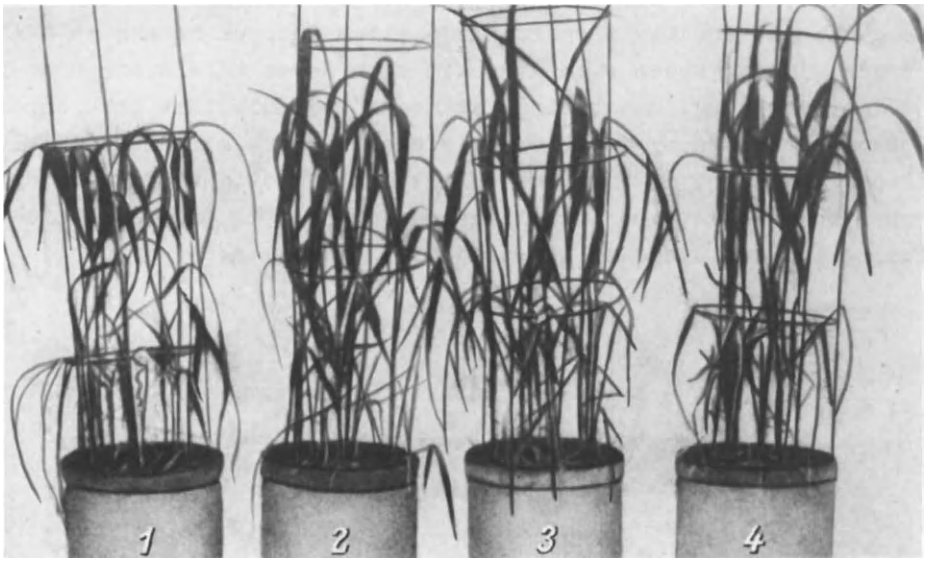


Fig. 95. Effects of cobalt, molybdenum and aluminium on drought resistance of oat; irrigation stopped for 10 days (after Bozhenko and Shkolnik, 1965).

wild in arid regions (Shkolnik, 1939a, b; Okuntsov and Levtsova, 1952; Shkolnik and Makarova, 1958; Novitskaya, 1957; Shkolnik and Bozhenko, 1959, 1974; Shkolnik et al., 1968; Shkolnik et al., 1970). The heat resistance of plants has been shown to be raised by the trace elements zinc (Petinov and Molotkovsky, 1956), boron and copper (Shkolnik and Makarova, 1958), and manganese (Barabalchuk, 1970).

Skazkin et al. (Skazkin and Rozhkova, 1956) observed the most pronounced effects of trace elements on drought resistance in those cases in which the drought coincided with the most critical period (as regards water supply) in the development of the plant, namely, the period of pollen tetrad formation. The ability of

trace elements to increase the drought resistance of plants has been put to practical use. In the author's works (Shkolnik et al., 1968,1970; Shkolnik and Smirnov, 1975) a method is given for increasing the drought resistance of wheat and barley by spraying the seeds with solutions of trace elements. Aluminium, cobalt and copper have mainly been used for this purpose (2-3 g metal salt per litre; 2 litres solution per 100 kg seeds).

Plants are known to adapt to drought in a number of ways, and therefore it has been a matter of interest to find out which of these adaptive mechanisms are affected by trace elements. Thus, trace elements have been discovered to increase the content of bound water and the water-retaining capacity of plant tissues (Shkolnik, 1950; Okuntsov and Levtsova, 1952; Startseva and Vasilyeva, 1958; Shkolnik and Makarova, 1958), as well as facilitating other processes involved in drought resistance. The content of bound water increases at the expense of colloid-held water, and the concentration of water in free solution decreases.

Shortages of the trace elements zinc, boron, copper and manganese reduce the transpiration rate of plants (Schütte, 1959). Trace elements augment transpiration in the morning and lower it later in the day during the period of greater water demand (Fig.96) (Dorfmüller, 1941; Tagi-Zade, 1957; Shkolnik and Makarova, 1958). This decrease in transpiration rate brought about by trace elements is related to changes in the proportions of bound water (Shkolnik, 1950; Startseva and Vasilyeva, 1958; Guseynov and Guseynov, 1963). In this case the danger of overheating becomes less likely, owing to the ability of trace elements to increase cytoplasmic viscosity (Natanson, 1952). Changes in the state of the water in plants have been shown to be related to metabolic changes brought about by trace elements, as revealed by the increases in the contents of hydrophilic proteins and nucleoproteins which occur in response to the presence of trace elements (Shkolnik, 1939b; Okuntsov and Levtsova, 1952; Startseva and Vasilyeva, 1958), and the greater degree of hydration of these molecules (Vasilyeva, 1966).

Veen (1979) discovered that silver in the form of silver thiosulphate completely blocked the increase in ethylene content that preceedes the withering of petals. As a result the life of cut flowers could be increased by 100 per cent, and flowers pretreated with thiosulphate became insensitive to treatment with ethylene.

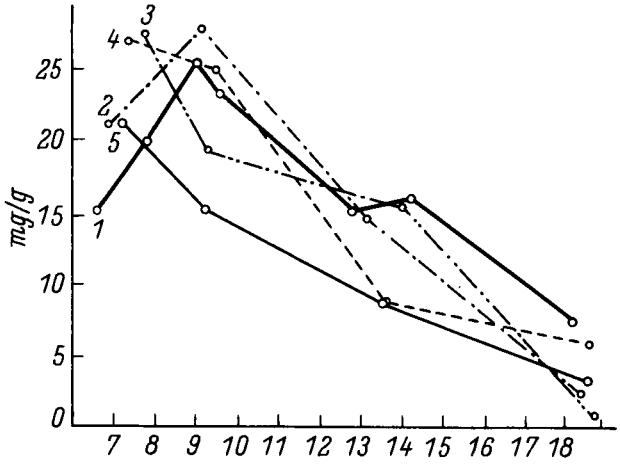


Fig. 96. Effects of boron and molybdenum on the rate of transpiration in sainfoin at the rosette stage (after Ipekdjiyan; cited from Shkolnik and Makarova, 1958). 1, control (seeds not soaked); 2, single soaking and drying of the seed (to harden against drought, according to Henkel); 3, double soaking and drying of the seed; 4, treatment 3 + boron; 5, treatment 3 + boron + molybdenum.

Drought affects nitrogen metabolism by increasing the nitrate levels in plants (Bozhenko and Shkolnik, 1963), there being a failure of the nitrate reduction mechanism. Drought is accompanied by a considerable (three to five fold) increase in the amino nitrogen levels, especially in old leaves, and this is attributed to an enhanced rate of hydrolysis of proteins. The greatest increase usually occurs in the stem, which serves as a reserve of amino acids during drought (Shkolnik and Bozhenko, 1974). In contrast to what happens in the leaves, the protein nitrogen content of the stem is not reduced, which indicates that the increase in amino acid nitrogen is a consequence of enhanced transport of amino acids from the leaves to the stems, and is not a result of greater hydrolysis of proteins. This is one of the most interesting reactions of plants to drought, since it is of great adaptive significance. Such a response allows plants to preserve their amino acids, which can then be re-utilized for protein synthesis upon restoration of

the water supply. The function of storage is performed by the stems. Only when the protein content is reduced considerably below the critical level does an extensive and irreparable loss of hydrophilic colloids occur, together with a loss of the water-retaining and water-absorbing capacity of the affected plant (Mothes, 1928).

Chromatography of the amino acids of drought-affected leaves has revealed that the proline content of such leaves is significantly elevated (Shkolnik and Bozhenko, 1959), a finding which led the author to suggest a defensive role for proline, since it is the most hydrophilic of the amino acids.

Aluminium, molybdenum and cobalt have been found to reduce the content of ammonium and increase that of amide nitrogen in drought conditions (Shkolnik and Bozhenko, 1974). An increase in amide nitrogen suggests that ammonium may be neutralized by the extra amide nitrogen. It has been discovered (Bozhenko, 1981) that an intensive accumulation of free proline occurs in plant tissues during drought as a kind of adjustment response to the drought. Under conditions of water deficiency a variety of wheat that was not drought-resistant accumulated 4 times as much free proline as that accumulated by a resistant variety. Treatment of the seeds with aluminium and cobalt prior to sowing resulted in a significant increase in the proline levels in both varieties studied. The stimulating effect of microelements was manifested more strongly in the non-resistant variety.

Drought and high temperatures cause the rates of photosynthesis and translocation of carbohydrates to decrease, whereas boron, manganese, zinc, copper and molybdenum stimulate photosynthesis, especially under conditions of low water supply (Tagi-Zade, 1957) and high temperature (Shkolnik and Davydova, 1959). These elements are also able to counteract the midday photosynthetic depression (Shkolnik and Greshishcheva, 1959), and stimulate the translocation of carbohydrates from the leaf to the spike in wheat and in barley (Novitskaya, 1957; Shkolnik et al., 1960).

Respiration in plants suffering from drought is known to further deplete the already low levels of those phosphorus compounds that are important in energy metabolism (Zholkevich, 1958). Shkolnik et al. (1963) showed that copper, boron, aluminium and cobalt were able to elevate the ATP content of the growing points of sunflower plants (Fig.97). According to these workers, the adverse effect

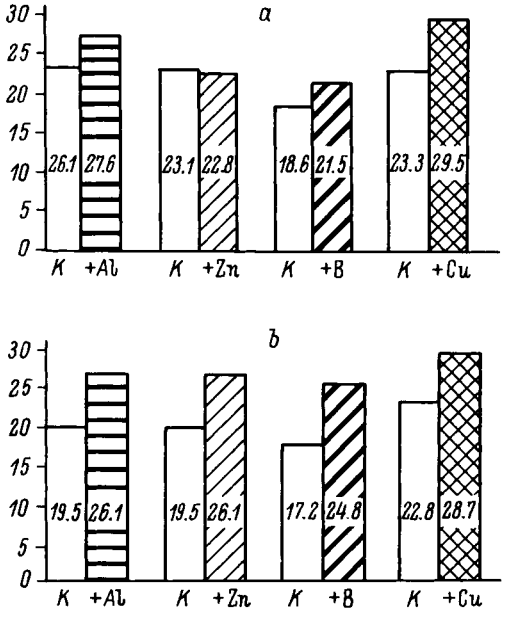


Fig. 97. Effect of trace elements Al, Zn, B and Cu on the ATP content of sunflower leaves (after Shkolnik et al., 1963). a, at 20°C; b, at 50°C; k, control without trace elements. Ordinate, ATP content (in terms of ukr phosphorus per g fresh weight).

of drought on the oxidation-reduction processes of plants is reflected in the decrease in ascorbic acid content caused by water stress. Again, the trace elements referred to above notably increased the level of ascorbic acid. Ascorbic acid is regarded as possessing drought protection properties as suggested by the high content of this substance in drought-resistant varieties (Shkolnik and Makarova, 1958). Water stress has been shown to result in a considerable increase in RNAse activity, and a concomittant decrease in the amount of cell RNA (Kessler and Monselise, 1959; Kessler and Engelberg, 1962). RNAase activity is confined within the boundaries of subcellular structures under optimal water-supply conditions, but spreads throughout the cell under conditions of water stress. Bozhenko (1968) showed that the pretreatment of seeds with aluminium or cobalt increased the RNA and DNA contents of the growing points during water stress, and reduced the RNAse activity (Fig.98). It is thus conceivable that the effects of these trace elements on nucleic acid metabolism form the basis of

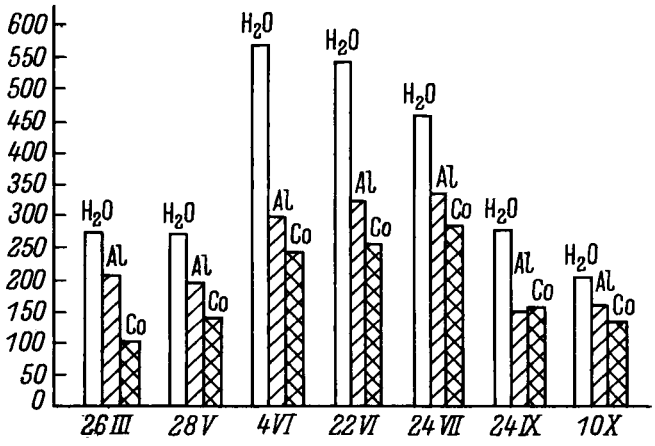


Fig. 98. Effect of treating seed with aluminium and cobalt on ribonuclease activity in sunflower leaves during drought (after Bozhenko, 1968). Ordinate, enzyme activity in conventional units per mg protein.

the increase in plant drought resistance that is attributed to these elements.

Bozhenko (1978) showed that water deficit leads to a decrease in the degree of methylation of the primary DNA structure; the content of 5-methylcytosine was reduced. Treatment of sunflower seeds with solutions of aluminium and cobalt salts was found to increase the drought resistance of the plants and increase the 5-methylcytosine content. The treatment thus had a protective effect, since 5-methylcytosine promotes the stability of DNA.

The favourable influence of a number of microelements (manganese, zinc, boron, molybdenum, and particularly aluminium, cobalt and copper) on plant drought-resistance has now been recognized. Microelements show a multiple array of effects upon the physiological processes that determine drought-resistance in plants (Shkolnik and Bozhenko, 1974).

Drought is usually accompanied by high temperatures. Petinov and Molotkovsky (1956) reported an increase in heat resistance produced by zinc salts, which they attributed to an increase in the activity of dehydrogenase in the presence of added zinc, leading to a greater production of organic acids. The latter are acceptors of ammonium, which accumulates in plants exposed to high temperatures. This effect of zinc upon heat resistance may be related, as shown by Vasilyeva et al. (1974), to the fact that zinc

increases the conformational flexibility or lability of protein macromolecules in leaves affected by high temperatures. This observation is consistent with the views of Alexandrov (1977), who believes that the most important characteristic of proteins, their conformational flexibility, determines to some extent the flexibility of protein macromolecules, which in turn determines the degree of conformational rearrangement that can occur as a necessary aspect of the biological activity of proteins. According to Vasilyeva (1966) zinc increases the flexibility of protein macromolecules, and this may be one of the reasons for the increased heat resistance produced by zinc.

Positive effects of trace elements on the frost resistance of plants were observed as early as the 1930s. Freckmann (1934) demonstrated that copper sulphate could prevent the premature freezing of a number of plants growing on marshy soils in Germany. At the same time, Mowry and Camp (1934) showed that tunga trees suffering from browning of the leaves as a result of zinc deficiency recovered when they were supplied with this element; plants receiving zinc were found to over-winter better. Dickey et al. (1948) observed that tunga trees showing symptoms of copper deficiency were also less cold-resistant. An increase in the frost resistance of ananas plants, achieved by supplying them with copper and zinc, was reported by Lawless and Camp (1940). Similar effects on the frost resistance of plants were later reported for manganese (Lawless, 1941). Manganese was found to elevate cold resistance of grape vines (Mininberg, 1960). Shear (1953) demonstrated that the higher the dose of zinc applied to tunga plants, the higher was its concentration in the leaves and the greater was the cold resistance of the plants. The cold tolerance was at a peak when comparatively high doses of zinc and potassium were administered. In the author's investigations, zinc, copper and manganese were found to increase the cold resistance of a number of citrus plants including orange (Fig.99), lemon and shaddock (Shkolnik, 1955).

Increased cold resistance has been achieved by supplying copper to herbaceous plants (Okuntsov and Sileva, 1950; Ocheretenko, 1951; Storozheva, 1954). In experiments carried out by Storozheva (1954) on the cultivation of clover in the northern Urals, only those plants which had been supplied with copper, or copper in combination with boron, survived.

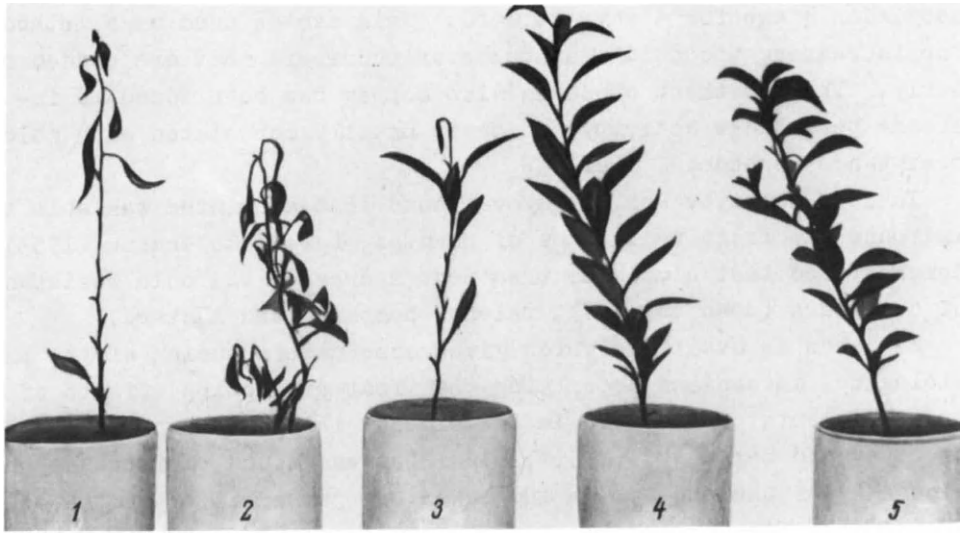


Fig. 99. Effect of boron, zinc and manganese on frost resistance in orange trees (at -7°C for 2 hours). 1, without trace elements; 2, with boron; 3, with zinc; 4, with a double dose of zinc; 5, with manganese.

Several investigators found that molybdenum increases the cold resistance of clover plants (Drobkov, 1952; Bozhenko, 1958; Burkin, 1968). Furthermore, Mikhalovsky and Sopilnyak (1953) found that an increase in the cold resistance of clover could be obtained by soaking the seeds in solutions of trace elements, and similar observations were reported for winter wheat (Mikhailovsky and Sopilnyak, 1954). Vlasyuk (1955) and Fisher (1955) obtained an increase in the cold resistance of ornamental plants supplied with zinc and boron. Zinc was also effective in improving the cold resistance of winter wheat (Vasilyeva and Lebedeva, 1963). Lebedeva and Palm (1970) reported that fruiting cucumbers were more resistant to ground frosts if they were supplied with zinc, copper and boron. Rafikova and Smolyaninov (1970) observed an increase in the cold resistance of winter wheat in responce to treatment with cadmium, iodine, or vanadium. Vasilyeva and Safonova (1974) demonstrated

the feasibility of producing frost-resistant apple hybrids by treating the plants with boron during the embryonic phase of their development.

It has been observed that soaking the cucumber seeds in a 0.02% solution of copper sulphoxide has a favourable effect on the cold resistance of cucumbers placed in refrigerators at the unfolded cotyledon stage for 4 days at +4°C. This can be used as a method for increasing the cold-resistance of cucumbers that are bedded out early. The treatment of seeds with copper has been found to increase peroxidase activity, which is usually correlated with cold resistance (Lebedeva, 1981).

In 1939 Sergeyev and Sergeyeva found that aluminium was able to increase the frost resistance of plants. Later, Cojeneanu (1953) demonstrated that aluminium treatment increased the cold resistance of cucumbers (down to -4°C), melons, pumpkins and mustard.

Evidence is available which gives some understanding of the physiological mechanisms underlying the frost-protecting effects of trace elements on plants. In the already mentioned experiments of Sergeyev and Sergeyeva (1939), aluminium was found to increase the viscosity of the protoplasm and lower the permeability of the cell membranes. Zinc, which is effective in increasing the frost resistance of winter wheat was found to increase the amount of bound water and degree of hydration of high molecular weight polymers, and decrease the rate of ice formation (Vasilyeva and Lebedeva, 1963). One of the reasons for the increased frost resistance conferred on plants by trace elements is the ability of the latter to stimulate photosynthesis and raise levels of translocated and accumulated carbohydrates. Evidence for this was obtained by the author in experiments carried out as long ago as 1936-1938 on orange oranges, lemons and shaddocks (Shkolnik, 1955). Manganese, copper, zinc and, to a lesser extent, boron were found to elevate the cold resistance of the plants studied. Similar results have been obtained by other investigators (Rafikova and Smolyaninov, 1970; Vasilyeva and Safonova, 1974). Bozhenko (1958) suggested that one of the reasons for the increased frost resistance bestowed on clover by zinc and molybdenum might be the ability of these elements to improve the translocation of sugars from the leaves to the roots throughout the autumn.

Interesting results pertaining to the mechanisms underlying zinc-induced increases in frost resistance have been obtained by Vasilyeva and Estrina (1970). They showed that in winter wheat, zinc enhances the accumulation of mono- and oligosugars in preparation for the coldest period of the winter, modifies the pattern of energy metabolism, decreases the mobility of water, enhances the hydration of molecules, and brings about changes in the proportions of different protein fractions and the conformation of protein molecules. The latter has a direct bearing on the degree of orderliness of the water in the cell.

Evidence has also been obtained showing that zinc affects the structural orderliness of water during the 1st and 2nd stages of hardening of the seedlings. Zinc is able to modify the secondary and tertiary structures of protein macromolecules by substituting for hydrogen in the imidazole ring of histidine and in the side chains of lysine and aspartic acid. In a later investigation (Vasilyeva et al., 1974) evidence was found pertaining to the effect of zinc on the activity of actomyosin-like proteins in the vegetative organs of winter wheat at different stages of hardening. Thus zinc was shown to affect the ATPase properties of these proteins, and to enhance the structural orderliness of water associated with actomyosin-like proteins. These changes brought about by zinc in the properties of the actomyosin-like proteins of the vegetative organs of winter wheat are accounted for by conformational changes that may have a role in increasing frost resistance.

The positive effects of zinc and copper on the orderliness of water have also been observed in experiments with cucumber plants (Lebedeva and Palm, 1970), leading these workers to suggest that this is linked with the low sensitivity of cucumber plants to ground frosts. Lebedeva (1966) found that copper raised both the frost resistance and the ATP and RNA contents of winter wheat. Khisamutdinova (1970) observed a zinc-induced enhancement of sugar metabolism by the pentose-phosphate pathway in winter wheat during the pre-winter period. This was considered to be an adaptive reaction to adverse environmental conditions. Boron and manganese, which increase the frost resistance of grape vines, also increase the levels of ascorbic acid and gluthatione.

Experiments with ornamental plants and rye grass have established that zinc increases more readily than copper plant tolerance to spring and autumn frosts, while copper more effectively increases

plant winter hardiness. The effect of injurious low temperatures considerably increases β-glucosidase activity in ryegrass leaves. Copper, zinc and aluminium decrease the activity of this enzyme in plants which are not hardened-off, i.e., they have a positive effect on frost hardiness, if it can be assumed that there is a correlation between frost hardiness and β-glucosidase activity (Volodko and Shkolnik, 1981).

Trace elements are effective in boosting the cold resistance of many warmth-loving plants. Abdurashitov (1957) obtained an increase in the cold resistance of young maize seedlings by treating the seeds with solutions of boric acid, ammonium molybdate, sulphates of manganese, zinc and copper. Plants receiving trace elements prior to, and after exposure to low temperatures showed increased levels of chlorophyll, carbohydrates (sucrose, starch, hemicellulose) and ascorbic acid, indicative of greater cold resistance of the tissues. Abayeva and Khodzhayev (1970), who observed an increase in the cold resistance of cucumber plants treated with trace elements, also found that the content of ascorbic acid increased. In the author's experiments (Shkolnik et al., 1960), the treatments of maize seeds which were effective in raising the cold resistance of the plants (i.e. treatment with solutions of copper, aluminium and cobalt salts), were also effective in elevating the levels of free amino acids, especially that of glutamic acid. It is conceivable that glutamic acid possesses a protective property against low temperatures similar to that of proline, since its content increases during exposure to low temperatures.

Experiments with ornamental plants and ryegrass have established that zinc is more efficient than copper in increasing plant tolerance to spring and autumn frosts, while copper more effectively increases plant winter hardiness. It has been found that one effect of injurious low temperatures is to increase considerably -glucosidase activity in ryegrass leaves. The trace elements copper, zinc and aluminium were shown to decrease the activity of this enzyme in cold-treated plants which were not properly hardened-off, thus indicating that they have a positive effect on frost hardiness (if it can be assumed that there is a correlation between frost hardiness and β-glucosidase activity), (Volodko and Shkolnik, 1981). Volodko and Bozhenko (1980) observed a significant increase in the proline content of plants treated with Cu, Mn, and more especially Zn. This was thought to be directly connected with the promotion of cold resistance in plants.

One of the reasons for the increased frost resistance conferred on plants by trace elements is the ability of the latter to modify the growth rhythms and bring toward the completion of important stages of growth and maturation of tissues (Mininberg and Shatkovska, 1957; Pudova, 1972).

As early as 1939, the author (Shkolnik, 1939b) detected an ability of boron, manganese and aluminium to raise the salt tolerance of plants. The effect of aluminium in this respect was considerable, especially where salinity was combined with drought. Similar results on the positive effect of boron on salt tolerance were obtained simultaneously for cotton (Novikov and Sadovskaya, 1939), and in wheat (Bobko and Aginyan, 1939). Subsequently it was demonstrated that the salt tolerance of some vegetables was also increased by boron, manganese and copper (Matukhin et al., 1961). Gyulakhmedov (1961) found that trace elements increased the salt tolerance only of plants that grew on weakly salinated soils, and exerted a negative influence in situations of strong salinity.

Evidence concerning the physiological mechanisms of the enhancement of salt tolerance by trace elements is very limited. One of the main factors underlying this effect of trace elements is a decrease in the permeability of the plasma to Cl^- and SO_4^-. This has been established with respect to the action of boron, manganese, zinc, copper and molybdenum (Novikov and Sadovskaya, 1939; Matukhin et al., 1961; Gyulakhmedov, 1961). Other possible causes include an increase in the content of hydrophilic colloids under the influence of trace elements (Matukhin et al., 1961), an increase in the content of sugars which would thus tend to normalize a water balance disturbed by elevated salinity, and an increase in the stability of plasma colloids (Shkolnik et al., 1949).

Considering the above evidence, it is apparent that trace elements play a prominent role in the metabolic processes of plants growing under extreme environmental conditions, and are therefore important factors in the adaptation and acclimatization of plants to such conditions. In fact, recent years have witnessed the experimental use of trace elements to facilitate the introduction of Picea excelsa seedlings and other plants to the Northern Caucasus (Smirnova, 1970). Stepanov (1968) reported the successful use of trace elements in agricultural and forest breeding practice in the arid climates of the South-Eastern regions of the European part of the Soviet Union. Boron has been used in the development of apple hybrids that are frost-resistant during the embryonic phase of development (Vasilyeva and Safonova, 1970). Trace elements have been found to influence positively the water balance and drought resistance of tree species in the arid climate of Kazakhstan (Pudova, 1970).

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