RESEARCH PAPERS

THE RELATION BETWEEN CHEMICAL STRUCTURE AND UNCOUPLING ACTIVITY IN CONGENERS OF SALICYLATE

By D. V. Brostoff*, V. Moses† and M. J. H. Smith

From the Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London, S.E.5

Received November 11, 1960

The relation between chemical structure and uncoupling activity in congeners of salicylate has been studied by means of their effects on the oxygen consumption of a suspension of baker's yeast utilising a limited quantity of glucose. Salicylic acid, salicylaldehyde, 2-hydroxyacetophenone, salicylamide, 3-methylsalicylic and 1-hydroxy-2-naphthoic acids were found to show uncoupling activity.

Salicylate uncouples oxidative phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956) and many of its effects on the metabolism of isolated tissues and animals are explicable in terms of this action (Smith, 1959). However, the relation between chemical structure and uncoupling activity in the salicylate group of compounds has not been explored in any detail. In the present work, the uncoupling activity of a number of salicylate congeners has been studied by measuring the oxygen consumption of a suspension of starved baker's yeast cells incubated with a known amount of glucose. Uncoupling reagents decrease the proportion of the glucose which is assimilated by the yeast and increase the proportion oxidised, hence stimulating the oxygen consumption of the preparation (Simon, 1953). A preliminary account of the work has already been published (Brostoff, Moses and Smith, 1960).

Experimental

Materials

A 20 per cent (w/v) suspension of baker's yeast (Distillers Co. Ltd.) in 0·067 M KH_2PO_4 solution at pH 4·5 was starved for 16 to 20 hr. at 30°. After centrifugation for 20 min. at about 4500 *g*, the cells were resuspended at the same concentration in a further quantity of the phosphate solution. The salicylate congeners were obtained commercially and recrystallised from suitable solvents until their melting points remained constant. They were dissolved in 0·067 M KH_2PO_4 solution at pH 4·5 to give final concentrations, after admixture with the yeast suspension in the reaction mixtures, ranging from 0·1 to 20 mM.

Total Oxygen Consumption

Aliquots (0·8 ml.) of either the phosphate medium or congener solution were added to Warburg flasks, each of which contained 0·1 ml. of yeast suspension in the main compartment and 0·1 ml. of 0·08 M glucose in the side arm. The flasks were incubated at 32° and the oxygen consumption

* Smith, Kline and French Research Fellow.

† Wyeth Fellow in Pharmacology of the Empire Rheumatism Council. Present address: Lawrence Radiation Laboratory, University of California, Berkeley, California, U.S.A.

measured for 1 hr. to obtain the initial rate of endogenous respiration of the yeast. The contents of the flask and side arm were then mixed and measurements of the oxygen consumption continued until the phase of stimulated respiration due to the glucose present had ceased and a second rate of endogenous respiration had been established. Typical reaction

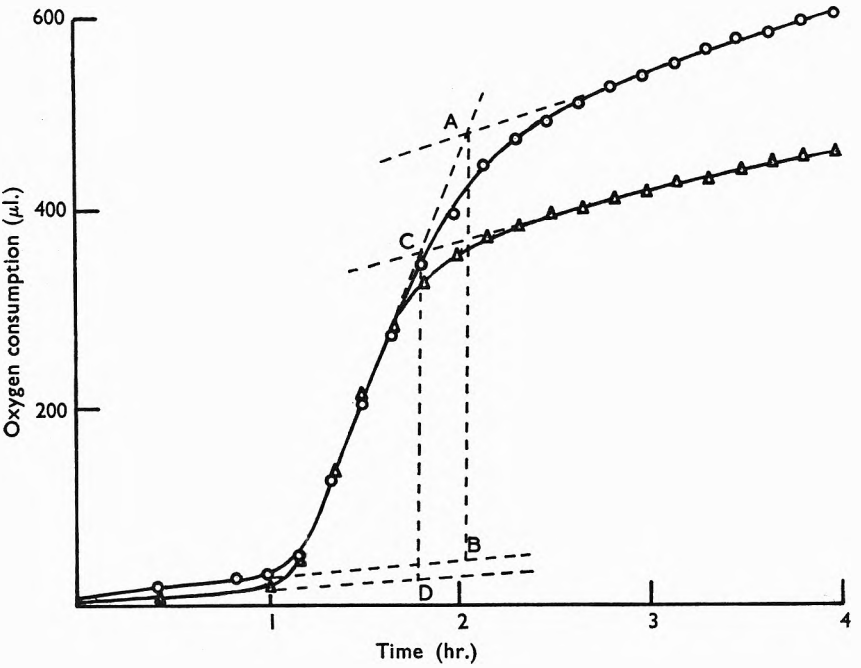

FIG. 1. Oxygen consumption of suspensions of starved baker's yeast cells, in the presence or absence of 10^{-2}M salicylate, before and after the addition of a limited quantity of glucose. Each flask contained 0·1 ml. of yeast suspension (20 per cent w/v of yeast) and 0·8 ml. of congener solution or phosphate buffer in the main compartment. The centre well contained 0·1 ml. of 10 per cent (w/v) KOH, and 0·1 ml. of glucose (8 μmoles) was added to the yeast suspension from the side arm at 1 hr. The total oxygen consumptions resulting from the addition of the glucose were calculated as follows; in the presence of salicylate the difference between the points A and B; control, in the absence of salicylate, the difference between the points C and D. ○, oxygen consumption in the presence of 10^{-2}M salicylate; △, oxygen consumption of the control.

curves are shown in Fig. 1 and the total oxygen consumption resulting from the addition of the glucose was calculated graphically from each experimental curve as the difference between the initial and final levels of endogenous respiration. The result for each concentration of each congener was calculated as the percentage of the corresponding control value obtained in the absence of the congener. A change of 15 per cent was considered to represent a significant effect.

Penetration Experiments

An important consideration in the present work was to determine if the congeners penetrated the cell membranes of the yeast. Although it

was not possible to determine if the active substances reached the enzyme sites concerned with oxidative phosphorylation reactions an attempt was made to assess if the congeners were excluded from the yeast cells. Fresh yeast cells (5·0 g.) were washed with three successive quantities of 200 ml. of tap water and finally resuspended in 50 ml. of 0·067 M KH_2PO_4 medium at pH 4·5. Aliquots (7·0 ml.) of this suspension were added to stoppered flasks containing 0·5 ml. of 0·01 M glucose and 0·1 to 0·5 ml. quantities of congener solution (0·0063 M in phosphate medium). When necessary the mixtures were made up to a total volume of 8·0 ml. with phosphate medium. The mixtures were shaken mechanically at 32° for 90 min. and centrifuged for 20 min. at about 4,500 *g*. The supernatant solutions were removed and successively frozen and thawed until they were optically clear after centrifugation. The optical densities of these solutions were measured at appropriate wavelengths in 1 cm. cells in a Hilger

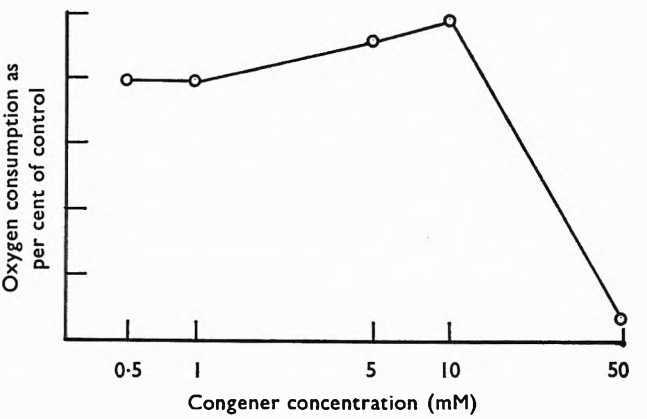

FIG. 2. Stimulation of total oxygen consumption of yeast preparation with increasing concentration of congener followed by depression at highest concentration.

Uvispek spectrophotometer against similar solutions prepared by replacing the congener solution in the reaction mixture with an equivalent quantity of phosphate medium. The salicylate congeners gave sharp absorption maxima in the range 225 to 350 mμ and individual calibration curves were constructed for each substance in phosphate medium. Hexahydrosalicylic acid did not give a suitable absorption maximum and its concentration could not be estimated. The proportion of the total suspension occupied by the yeast cells was found to be 42·5 per cent (v/v) when the suspension was centrifuged until no further packing of the cells occurred. Thus, when 0·5 ml. of 63 $\times$ 10^{-4} M congener solution was present in a total volume of 8·0 ml. of suspension, its concentration in the supernatant after removal of the cells by centrifugation should have been 4 $\times$ 10^{-4} M if it penetrated freely throughout the intracellular volume. A concentration of 7 $\times$ 10^{-4} M of the congener was interpreted as meaning that the congener was completely excluded from the yeast cells. Intermediate results, between 4 and 7 $\times$ 10^{-4} M, indicated partial penetration and values

below 4×10^{-4} M either a preferential binding of the congener by the cells or its chemical alteration.

RESULTS

When the change in total oxygen consumption after the addition of the glucose was plotted against concentration for each congener three types of response were distinguished. The first consisted of a stimulation of the total oxygen consumption with increasing concentration of congener followed by a marked depression at the highest concentration (Fig. 2).

TABLE I

CONCENTRATIONS OF CONGENERS PRESENT IN SUPERNATANT AFTER REMOVAL OF YEAST CELLS AFTER 90 MINUTES INCUBATION*

Congener	Concentration in supernatant (M × 10^{-4})
Phenol	0
Benzoic acid	1·0
Salicylic acid	0·6
3-Hydroxybenzoic acid	1·0
4-Hydroxybenzoic acid	2·3
2-Methoxybenzoic acid	1·0
Thiosalicylic acid	0·9
Salicylaldehyde	0
2-Hydroxyacetophenone	0
Salicylamide	0·7
Salicylic methyl ester	0
2-Hydroxyphenylacetic acid	6·4
3-Methylsalicylic acid	0
4-Methylsalicylic acid	0·1
3-Phenylsalicylic acid	1·0
1-Hydroxy-2-naphthoic acid	1·3
2,3-Dihydroxybenzoic acid	5·0
2,4-Dihydroxybenzoic acid	9·8
2,5-Dihydroxybenzoic acid	8·7
2,6-Dihydroxybenzoic acid	5·4
3,4-Dihydroxybenzoic acid	8·9
3,5-Dihydroxybenzoic acid	9·1
3-Nitrosalicylic acid	7·5
3,5-Dinitrosalicylic acid	7·4

* Values above 7×10^{-4} M show complete exclusion of congener from the cells, values between 4 and 7×10^{-4} M indicate partial penetration and values below 4×10^{-4} M suggest either preferential binding by the cells or chemical destruction of the congener.

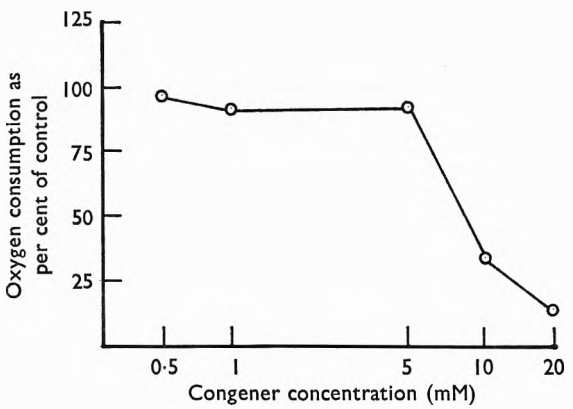

FIG. 3. Depression of total oxygen consumption of yeast preparation with increasing concentration of congener.

The substances which produced this response included salicylate, the classical uncoupling reagent 2,4-dinitrophenol, 3-methylsalicylic acid, 1-hydroxy-2-naphthoic acid, salicylaldehyde, 2-hydroxyacetophenone and salicylamide. All these compounds penetrated the yeast cells (Table I).

The second group of substances, consisting of benzoic acid, 2-methoxybenzoic acid and 3-phenylsalicylic acid, did not cause an initial stimulation of total oxygen consumption with increasing concentration, but produced an increasing depression at the higher concentrations (Fig. 3). All the members of this group were found to penetrate the yeast (Table I). The remaining substances did not cause either stimulation or depression of the total oxygen consumption with increasing concentration (Fig. 4). Some

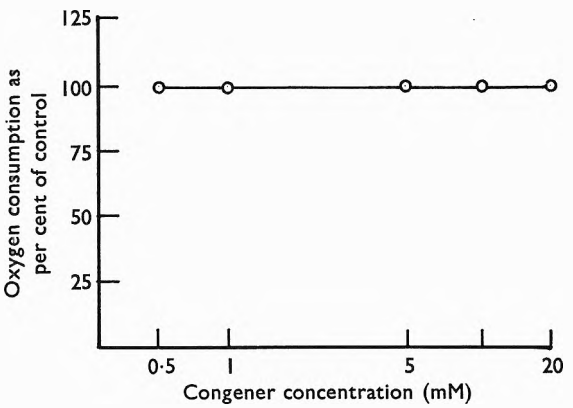

FIG. 4. Unchanged total oxygen consumption of yeast preparation with increasing concentration of congener.

of this last group (3- and 4-hydroxybenzoic acids, 2-hydroxyphenylacetic acid, 2,3- and 2,6-dihydroxybenzoic acids, thiosalicylic acid, 4-methylsalicylic acid, salicylic methyl ester and phenol) were found to penetrate the yeast but the remainder, including 2,4-, 2,5-, 3,4-, and 3,5-dihydroxybenzoic acids, and 3- and 3,5-dinitro-salicylic acids were completely excluded from the cells. *trans*Hexahydrosalicylic acid also gave this type of response but its degree of penetration could not be assessed.

DISCUSSION

When resting cells of baker's yeast are supplied with a limited amount of glucose they oxidise only about 30 per cent of the sugar and the energy produced is used to assimilate the remainder of the glucose into cellular components (Pickett and Clifton, 1943). Simon (1953) has shown that uncoupling reagents, such as the nitrophenols, when present in low concentrations, increase both the rate and total amount of oxygen consumption and reduce the proportion of glucose assimilated by the yeast cells. Thus, a stimulation of the total oxygen consumption of the yeast preparation is a criterion of uncoupling activity. However, this may not occur with all concentrations of the uncoupling reagent since high concentrations of the nitrophenols behave like respiratory poisons, such as

cyanide, in reducing both the oxygen consumption and glucose assimilation (Simon, 1953). In the present work only those substances which, over some portion of the concentration range tested, produced an increase greater than 15 per cent of the total oxygen consumption resulting from the addition of the glucose, were considered to possess uncoupling activity. Of the 25 congeners, only salicylic acid, salicylaldehyde, salicylamide, 3-methylsalicylic acid, 1-hydroxy-2-naphthoic acid and 2-hydroxyacetophenone fulfilled this requirement. The classical uncoupling reagent, 2,4-dinitrophenol, also behaved in the same way. 3-Methylsalicylic acid has been found to increase the oxygen consumption of the whole rat (Andrews, 1958) which presumably indicates an uncoupling activity. Salicylaldehyde (Packer, Austen and Knoblock, 1959) and salicylamide (Brody, 1956) have been reported to be devoid of uncoupling activity in respiring mitochondrial suspensions. The possibility that these two substances may have been almost quantitatively converted to salicylate by the yeast during the incubation was investigated by paper chromatographic analysis of both the incubation media and of extracts of the yeast cells. However, the substances were recovered unchanged, no free salicylate being detected. No information is available about the behaviour of 1-hydroxy-2-naphthoic acid or 2-hydroxyacetophenone in other test systems for uncoupling activity.

Benzoic, 2-methoxybenzoic and 3-phenylsalicylic acids behaved as respiratory depressants in high concentrations but did not cause any stimulation of oxygen consumption over the wide concentration range tested. The remaining congeners possessed neither stimulating nor depressant properties but many of them were completely excluded from the yeast cells. However, 3- and 4-hydroxybenzoic acids, thiosalicylic acid, 4-methylsalicylic acid, salicylic methyl ester, 2,3- and 2,6-dihydroxybenzoic acids, 2-hydroxyphenylacetic acid and phenol penetrated the yeast. In this latter group, the lack of uncoupling ability was therefore due to molecular configuration rather than a failure to penetrate the cellular membranes of the yeast.

The present results show that modification of the hydroxyl group of salicylic acid caused a loss of uncoupling activity. Thus, its absence (benzoic), alteration of its position on the benzene ring (3- and 4-hydroxybenzoic acids), methylation (2-methoxybenzoic) or substitution of the phenolic oxygen by sulphur (thiosalicylic) all produced inactive substances. Alteration of the carboxyl group did not produce such drastic results. The corresponding aldehyde (salicylaldehyde), methyl ketone (2-hydroxyacetophenone) and amide (salicylamide) were active but the methyl ester was not. The absence of the carboxyl group (phenol) or the introduction of a methylene group between the carboxyl and the benzene ring (2-hydroxyphenylacetic acid) also removed activity. Substitution of the benzene ring by a 3-methyl group or by the introduction of a second benzene ring (1-hydroxy-2-naphthoic acid) retained activity but the presence of a 4-methyl, a 3-phenyl group or a second hydroxyl group at the 3 or 6 position produced inactive compounds. All the other ring substituted congeners tested failed to penetrate the yeast. It thus appears that the

essential requirement for uncoupling activity in this group of compounds is the presence of a phenolic hydroxyl group in the *ortho* position to a carboxyl group with the reservation that an aldehyde, ketone or amide group may substitute for the free carboxyl group.

Acknowledgements. We wish to thank Smith, Kline and French Research Institute for a grant towards the cost of the work.

References

Andrews, M. M. (1958). *Brit. J. Pharmacol.*, **13**, 419–423.
Brody, T. M. (1956). *J. Pharmacol.*, **117**, 39–51.
Brostoff, D. V., Moses, V. and Smith, M. J. H. (1960). *Biochem. J.*, **77**, 5P.
Packer, L., Austen, F. K. and Knoblock, E. C. (1959). *Proc. Soc. exp. Biol., N.Y.*, **100**, 239–244.
Pickett, M. J. and Clifton, C. E. (1943). *J. cell. comp. Physiol.*, **21**, 77–94.
Simon, E. W. (1953). *J. exp. Bot.*, **4**, 377–392.
Smith, M. J. H. (1959). *J. Pharm. Pharmacol.*, **11**, 705–720.

A NOTE ON THE APPLICATION OF THE FLASK COMBUSTION TECHNIQUE TO SULPHUR-CONTAINING SUBSTANCES

By C. Vickers and J. V. Wilkinson

From the Analytical Development Group, Standards Department, Boots Pure Drug Co. Ltd., Station Street, Nottingham

Received September 12, 1960

It has been shown that the flask combustion method is applicable to the assay of a number of sulphur-containing materials of pharmaceutical and horticultural interest and that results agree with those obtained by established methods.

The flask combustion method, which has been used for iodine-containing substances (Johnson and Vickers, 1959), has now been applied to pharmaceutical materials containing sulphur. Two methods have been investigated, one, that of Schöniger (1956), who used an alkalimetric titration and the other that of Wagner (1957) who applied the barium perchlorate titration procedure of Fritz and Yamamura (1955). The first of these methods is suitable only for samples which, on combustion, do not yield acidic or basic products other than sulphuric acid.

The alkalimetric determination has been applied to dibenzyl disulphide of micro-analytical reagent grade and to many pharmaceutical and

TABLE I

SULPHUR COMPOUNDS DETERMINED BY DIRECT TITRATION WITH ALKALI AFTER COMBUSTION

(Results expressed as per cent S unless otherwise stated)

Substance	Results by Fleck and Ward (1934) method	Results by flask method
Dibenzyl disulphide	26·03 (Theory)	25·9 (Mean of 10 determinations range 25·8 to 26·1.)
Sublimed Sulphur B.P.		99·7 (Mean of 6 determinations range 99·4 to 100·0.)
Precipitated Sulphur B.P.		99·5 (Mean of 4 determinations range 99·3 to 99·6.)
Sulphur Ointment B.P.*	10·3	10·3; 10·3; 10·2
Sulphur dust black-horticultural (containing charcoal)	90·7	91·3; 91·2; 91·2
Sulphur dust green-horticultural (containing charcoal and dyestuffs)	79·5	79·4; 79·6; 79·6
Tablets of sulphur and yeast (3 grains)	2·94 grains/tab.	2·99; 2·97; 2·98 grains/tab.
Tablets of guaiacum and sulphur (3 grains)	2·78 grains/tab.	2·97; 2·96; 2·98; 2·98 grains/tab.
Compound Tablets of Liquorice (containing Senna, Liquorice and a trace of Saccharin)	2·31 grains/tab.	2·36, 2·38, 2·37; 2·30 grains/tab.
Dimercaprol†	99·9 per cent $C_3H_8OS_2$ 100·3 " " " (by B.P. Method)	100·1 per cent $C_3H_8OS_2$ 100·3 " " " 100·2 " " "

* Ointments should be weighed onto grease-proof paper.
† Liquids should be absorbed on ashless filter paper floc contained in a small methylcellulose capsule.

horticultural preparations. Of the various methods for titration of sulphate which have been examined, that of Fritz and Yamamura (1955) was considered to be the most satisfactory. This has been successfully applied to dibenzyl disulphide and to many formulations.

METHODS

The recommended methods are as follows.

(i) Alkalimetric titration procedure. Burn a suitable quantity of the sample as described by Johnson and Vickers (1959) absorbing the combustion products in 15 ml. of distilled water containing an excess of hydrogen peroxide (about 1 ml. of solution of hydrogen peroxide B.P.) by

TABLE II

SULPHUR COMPOUNDS DETERMINED BY TITRATION WITH BARIUM PERCHLORATE AFTER COMBUSTION

(Results expressed as per cent S unless otherwise stated)

Substance	Results by Fleck and Ward (1934) method	Results by flask method
Dibenzyl disulphide	26·03 (Theory)	25·9 (Mean of 10 determinations range 25·6 to 26·0.)
Lozenges of Sulphur B.P.C. 1954	0·310 g./lozenge 0·318 g./lozenge	0·310; 0·310; 0·311; 0·310 g./lozenge
Proprietary tablets also containing guaiacum, mag. carbonate and potassium bicarbonate	1·86 grains/tab.	1·85; 1·85; 1·86 grains/tab.
Sulphur dust, horticultural, containing magnesium stearate	98·4	99·2; 98·7; 99·3
Ointment of Salicylic Acid and Sulphur B.P.C.*	3·11 2·98	3·15; 3·08; 3·08; 3·17
Proprietary ointment also containing charcoal and creosote*	2·40 2·39	2·36; 2·34; 2·47; 2·35
Proprietary ointment containing also resorcinol and hexachlorophene*	Fleck and Ward method inapplicable. Label declaration 8 per cent	7·92; 7·88; 7·82; 7·98
Sulphanilamide, B.P.C.	99·7 per cent $C_6H_8N_2O_2S$ (by B.P.C. method)	100·4; 100·9; 100·5 per cent $C_6H_8N_2O_2S$
Sulphacetamide, B.P.C.	99·7 per cent $C_8H_{10}N_2O_3S$ (by B.P.C. method)	101·6; 101·2; 101·8 per cent $C_8H_{10}N_2O_3S$
Saccharin, B.P.	99·7 per cent $C_7H_5NO_3S$ (by B.P. method)	100·7; 100·7; 101·0 per cent $C_7H_5NO_3S$

shaking for about 5 min. Wash the stopper and platinum gauze with water, boil the solution and washings for about 10 min. to destroy excess peroxide. Cool and titrate with standard sodium hydroxide solution (0·05 N or 0·02 N according to the material being assayed) using screened methyl red as indicator.

(ii) Barium perchlorate titration procedure. Burn a quantity of the sample containing about 8 mg. of sulphur by the method of Johnson and Vickers (1959), absorbing the combustion products in 15 ml. of distilled water containing an excess of hydrogen peroxide (about 1 ml. of solution of hydrogen peroxide B.P.) by shaking for about 5 min. Wash the stopper and platinum gauze with 60 ml. of industrial methylated spirit,

add 2 drops of a 0·2 per cent solution of Thoron in water and 2 drops of a 0·0125 per cent solution of methylene blue in water and titrate with 0·02 M or 0·01 M barium perchlorate solution until the yellow colour changes to pale pink. During the titration, which should be carried out in a good natural light, the solution should be stirred vigorously by means of a magnetic stirrer.

Results

Results from a selection of the substances to which the alkalimetric determination has been applied are given in Table I, and some results using the method of Fritz and Yamamura (1955) are listed in Table II. Although sulphur determinations are not used to assay sulphonamides, the method was applied to those listed in Table II. It will be noted that high results were obtained in every case and it was thought that this might be due to the presence of nitrate as suggested by Fritz and Yamamura. This suggestion was confirmed both by titrating sulphuric acid in the presence of varying quantities of nitrate and by burning mixtures of dibenzyl disulphide and urea. Soep and Demoen (1960) have also noted this effect and are investigating a different titration method for the determination of sulphur in sulphonamides.

Conclusions

The methods described may be applied to a wide range of pharmaceutical preparations and are in routine use for the determination of sulphur in complex ointments. They are not satisfactory for the determination of sulphur in organic compounds containing a high proportion of nitrogen.

References

Fleck, M. R. and Ward, A. M. (1934). *Quart. J. Pharmacol.*, **7**, 179–181.
Fritz, J. S. and Yamamura, S. S. (1955). *Analyt. Chem.*, **27**, 1461–1464.
Johnson, C. A. and Vickers, C. (1959). *J. Pharm. Pharmacol.*, **11**, Suppl. 218*T*–222*T*.
Schöniger, W. (1956). *Mikrochim. Acta.*, 869–876.
Soep, H. and Demoen, P. (1960). *Microchem. J.*, **4**, 77–87.
Wagner, H. (1957). *Mikrochim. Acta.*, 19–23.

THE FORMATION OF HISTAMINE IN THE RAT

By J. M. Telford and G. B. West

From the Department of Pharmacology, School of Pharmacy, University of London, Brunswick Square, London, W.C.1

Received October 19, 1960

Factors affecting the determination of histidine decarboxylase activity in adult rat tissues have been studied. The optimal conditions vary from tissue to tissue, and the most potent sources of the enzyme are the pyloric part of the stomach, the liver and the duodenum, with less in the kidney. There is no relationship between the histidine decarboxylase activity of a rat tissue and the amount of histamine it contains.

The existence of a mammalian enzyme capable of decarboxylating histidine to form histamine was first demonstrated by Werle (1936) and by Holtz and Heise (1937) more than 20 years ago. These authors and their colleagues subsequently made extensive studies of the distribution of the enzyme. Waton (1956) recently confirmed and extended their observations using in the incubation mixture a specific inhibitor of histaminase, the enzyme which inactivates histamine. Using a sensitive tracer technique, Schayer (1957) has found that the optimal pH value for incubation varies from tissue to tissue.

In the present work, the optimal conditions for determining the histidine decarboxylase activity of rat tissues have been re-examined. The enzyme activity of a tissue has then been compared with its histamine content so that further light might be shed on the mode of formation and the function of histamine in this species.

Methods

Female rats of Wistar strain weighing 120–150 g. were fed on a cube diet (No. 41B, Associated London Flour Millers Ltd.), allowed drinking water *ad lib.*, and housed at 70 $\pm$ 1° F.

Formation of Histamine from Histidine by Rat Tissues

The method of Waton was first used. Values of enzyme activity in some tissues were found to be lower than those previously reported, but when the amount of tissue homogenate was doubled, consistent results were obtained. The following experiments refer to the increased amount of tissue homogenate.

Pooled tissue from freshly killed rats was cleaned and weighed, cut into small pieces, and ground in a glass mortar with a little sand and Tyrode solution (5 ml./g. tissue). The resulting homogenate was allowed to stand and the supernatant fluid extract was removed for incubation. The composition of the incubation mixture was as follows.

Tissue homogenate (800 mg.)	4·0 ml.
Phosphate buffer (M/20-K_2HPO_4)	4·9 ml.
L-Histidine (15 mg./ml., neutralised)	1·0 ml.
Aminoguanidine (10 mg./ml., neutralised)	0·1 ml.
Benzene (1 drop)	20 mg.

The substrate (histidine) was always added last. The mixture was immediately shaken and incubated for 3 hr. at 37°. The reaction was then stopped by reducing the pH of the solution to 4·0 with N HCl and by cooling to 4°. After neutralising the mixture with N NaOH, its histamine content was determined. Mixtures containing boiled homogenate or no homogenate were similarly treated and assayed for histamine. In all experiments, mixtures without the substrate were also incubated and assayed for histamine. The final volume of the mixtures was maintained at 10 ml. The histamine content of the extract incubated in the

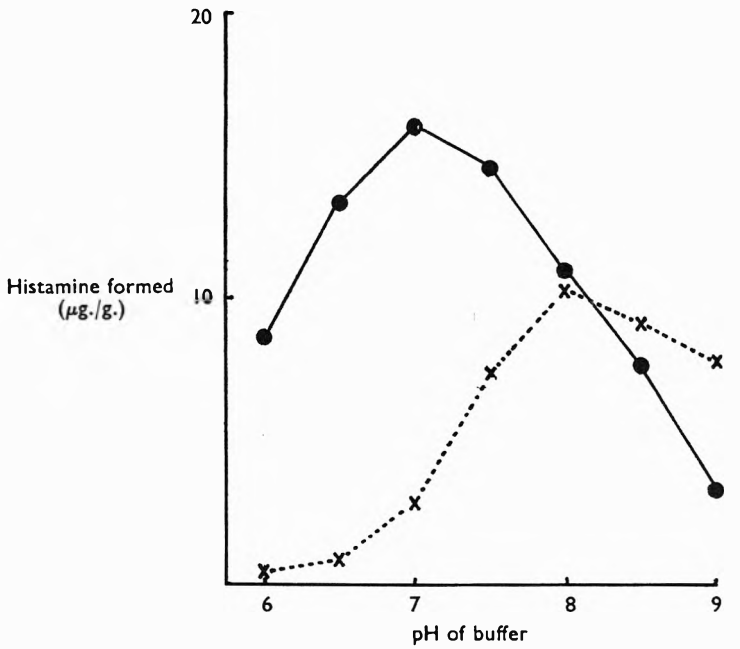

FIG. 1. The histidine decarboxylase activity of rat pyloric stomach (●——●) and liver (X - - - - X) expressed as μg. histamine formed/g. tissue/3 hr. Incubation at varying pH values.

presence of histidine less that of the extract incubated in the absence of histidine gives the amount of histamine formed from histidine during the incubation. This amount of formed histamine, when calculated per gram of tissue, was used as the index of histidine decarboxylase activity. Each result is the mean of at least three separate experiments. Each experiment uses tissue from at least 12 rats.

Extraction of Rat Tissues for Histamine

Pooled tissue from freshly killed rats was cleaned and weighed, cut into small pieces and extracted with 10 per cent (w/v) trichloracetic acid (5 ml./g. tissue) for 24 hr. Excess acid was removed by shaking the supernatant with 4 vol. of ether four times and discarding the ethereal layers. After gentle heating, the aqueous residue was assayed for its

histamine content. Each result is the mean of at least four separate experiments. Each experiment uses tissue from at least 4 rats.

Bioassay procedure. Bioassays were made on the isolated ileum of the guinea-pig. A 15 ml. bath of aerated atropinised Tyrode solution at 32° was used. On occasions, extracts were also assayed on the blood pressure of an anaesthetised cat. The specificity of the responses were checked using mepyramine maleate. All values of histamine refer to the base.

Results

Histidine decarboxylase and incubation pH. The histidine decarboxylase activity of various rat tissues was traced using phosphate buffers of various

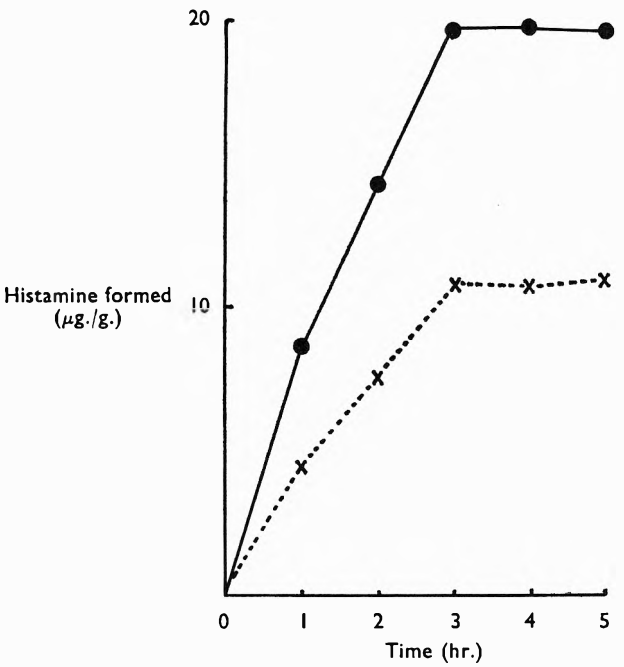

Fig. 2. The histidine decarboxylase activity of rat pyloric stomach (●——●) and liver (X - - - - X), expressed as μg. histamine/g. tissue/3 hr. Incubation for varying times at optimal pH values.

pH values. As may be seen from Fig. 1, the enzyme in the pyloric stomach showed maximal activity at pH 7·0, whereas that in the liver was highest at pH 8·0 with much less activity at pH 7·0. For kidney and duodenum, the maximal value was at pH 7·5. Only traces of enzyme activity were found over a pH range of 6·0–9·0 in jejunum, lung, abdominal skin, ileum and fundic stomach.

Histidine decarboxylase and incubation time. An incubation time of 3 hr. gave the maximal yield of histamine, no more being formed on longer incubation. The results for pyloric stomach and liver are shown in Fig. 2, and similar results were obtained for duodenum and kidney.

Histidine decarboxylase and tissue homogenate. For all tissues an optimal amount of tissue homogenate was required for enzyme activity (800 mg.). From some tissues, like liver, amounts in excess of this yielded less histamine, probably due to interfering substances. The results for pyloric stomach and liver are shown in Fig. 3.

Histidine decarboxylase and substrate. Alterations in the amount of histidine used in the incubation mixture also gave variations in the yield of histamine. Maximal enzyme activity was always obtained when

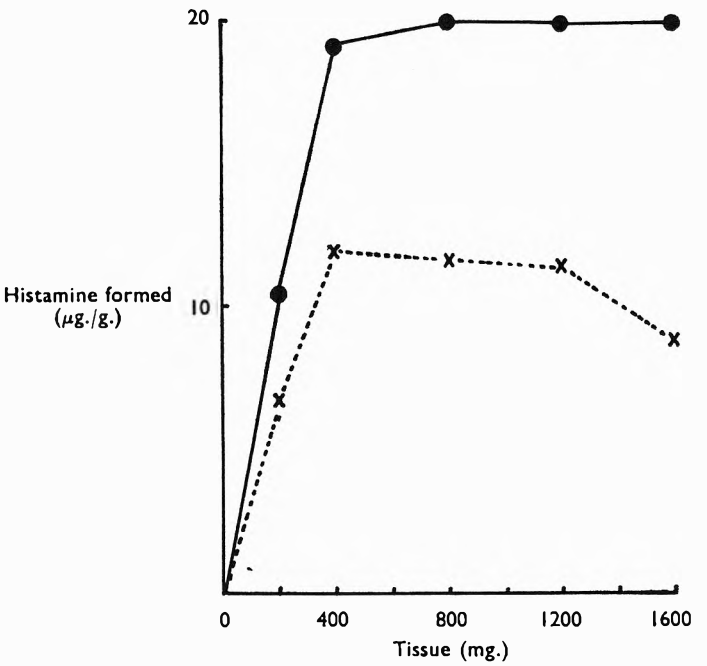

FIG. 3. The histidine decarboxylase activity of rat pyloric stomach (●——●) and liver (X - - - - X), expressed as μg. histamine formed/g. tissue/3 hr. Incubation with varying amounts of tissue.

15 mg. histidine was used, but in some experiments 5 mg. only was needed. The results for pyloric stomach and liver are shown in Fig. 4.

Histidine decarboxylase and benzene. A small quantity (20 mg.) of benzene was necessary to detect enzyme activity in rat liver, kidney and duodenum. When the amount was increased, the enzyme activity was reduced. Benzene was not necessary for, and did not potentiate, the histidine decarboxylase activity in pyloric stomach (Fig. 5).

Other Factors Affecting Histidine Decarboxylase

The presence of a small quantity (1–2 mg.) of aminoguanidine was necessary only when extracts of rat duodenum were incubated, since this tissue is one of the chief sources of histaminase. Pyridoxal, which has been reported to be a co-enzyme of histidine decarboxylase (Blaschko,

1957; Rothschild and Schayer, 1958), failed in all doses used (10–1,000 μg.) to increase the yield of histamine during the incubation of any of the four rat tissues.

Using the optimal conditions for determining the enzyme activity *in vitro*, the following values were obtained; pyloric stomach, 15·6 μg./g.; liver, 10·2 μg./g.; duodenum, 6·4 μg./g.; and kidney, 3·0 μg./g.

Histamine Content of Rat Tissues

These are shown in Table I. There is no relationship between the histamine content of a tissue and the amount of histamine-forming

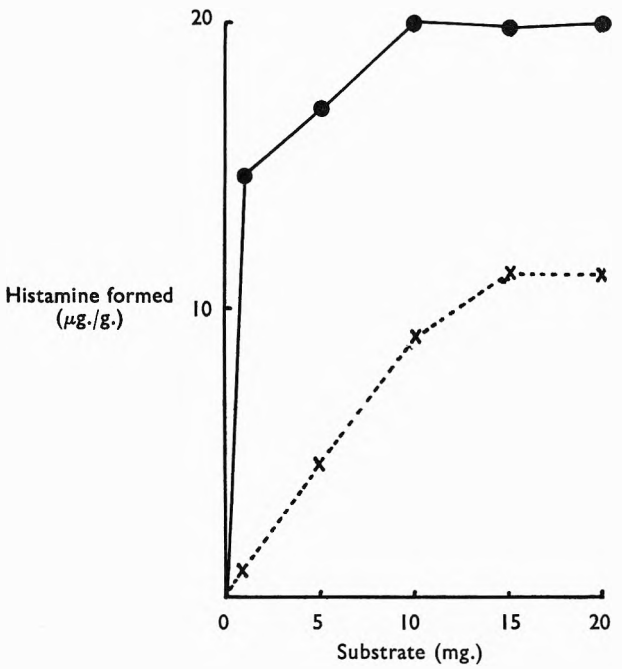

FIG. 4. The histidine decarboxylase activity of rat pyloric stomach (●——●) and liver (X - - - - X), expressed as μg. histamine formed/g. tissue/3 hr. Incubation with varying amounts of substrate.

enzyme it contains. The liver, for example, has a relatively strong histidine decarboxylase activity but very little histamine, whereas the duodenum contains much histamine and possesses less enzyme activity. It is of interest that the fundic part of the stomach, unlike the pyloric part, possesses no histidine decarboxylase activity yet it contains much histamine.

DISCUSSION

The finding that the optimal conditions for determining histidine decarboxylase activity vary from tissue to tissue agrees with the results of previous workers. Schayer, for example, found that the enzyme in rat pyloric stomach and rabbit platelets was most active at pH 7·2 whereas

for rabbit kidney it was most active at pH 8·0. The present results also confirm that, in terms of histamine formed per gram of tissue, the pyloric stomach is the most potent source of the enzyme in the rat. However, the high activity of adult rat liver has not been noted before (Kahlson,

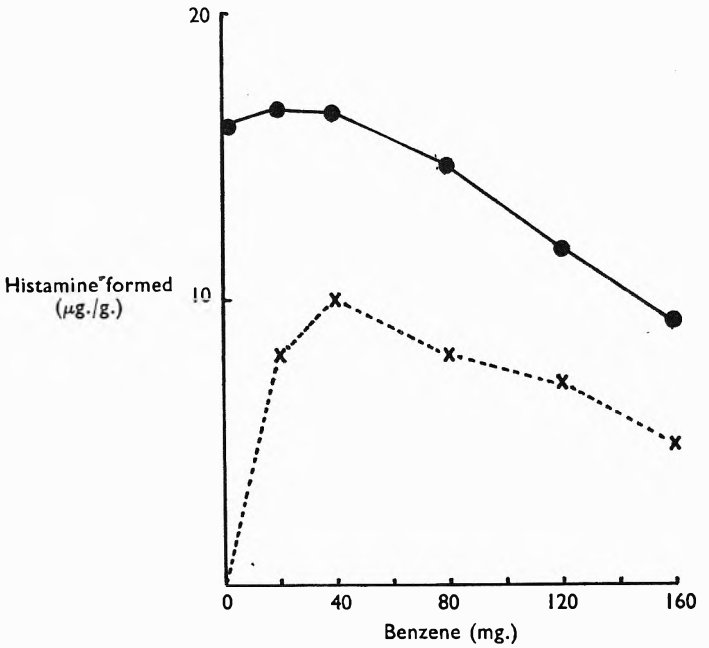

FIG. 5. The histidine decarboxylase activity of rat pyloric stomach (●——●) and liver (X - - - - X), expressed as μg. histamine formed/g. tissue/3 hr. Incubation with varying amounts of benzene.

Rosengren and White, 1960; Mackay, Marshall and Riley, 1960). This activity, unlike that of the pyloric stomach, is detectable only in the presence of a small quantity of an organic solvent such as benzene.

The weight of the liver of an adult rat is 6–10 g., and with an enzyme activity equivalent to 10·2 μg./g. this organ is capable of forming at least 60 μg. histamine in 3 hr. or 480 μg./day. Although the enzyme activity

TABLE I

COMPARISON OF THE HISTIDINE DECARBOXYLASE ACTIVITY (AS INDICATED BY THE AMOUNT OF HISTAMINE FORMED IN μg./g. TISSUE) AND THE HISTAMINE CONTENT (μg./g.) OF RAT TISSUES

Rat tissue	Histidine decarboxylase	Histamine content
Pyloric stomach	15·6	29·9
Liver	10·2	2·0
Duodenum	6·4	25·2
Kidney	3·0	1·0
Jejunum	1·2	25·0
Lung	1·2	8·0
Abdominal skin	1·0	31·3
Ileum	0·9	23·8
Fundic stomach	0·4	12·6

in the pyloric stomach is equivalent to 15·6 μg./g., this tissue weighing 0·25–0·5 g. can form only 18 μg. histamine in 3 hr., or 64 μg./day. Likewise, the duodenum and the kidney are capable of forming daily only 40 and 32 μg. histamine respectively. Thus, in terms of absolute amounts of histamine formed, the liver is by far the most potent source of histidine decarboxylase in the rat.

Kahlson (1960) has recently reported that histidine decarboxylase activity may play an important role in processes of tissue growth and repair, since a high histamine-forming capacity is found in the rat foetus, in the adult rat after partial hepatectomy, and in healing skin wounds. Waton, on the other hand, suggests that the intracellular decarboxylation of histidine is not the major pathway in the formation of histamine, and that decarboxylation occurs in the lumen of the gut and histamine is absorbed as such. Sufficient histamine may be absorbed from the alimentary tract to meet all physiological needs and local histidine decarboxylase may not be necessary. He also states that, although histidine decarboxylase is identified in a tissue by an *in vitro* method, this is not proof that such a decarboxylation occurs *in vivo*. Nevertheless, as the present work shows, the rat is capable of forming very large quantities of histamine. The pyloric stomach, liver, duodenum and kidney, for example, can form between them over 1 mg. of histamine in a day.

The presence of histidine decarboxylase in the pyloric stomach and its absence in the fundic stomach suggests that the amount of histamine available to stimulate the oxyntic and peptic cells in the fundus is controlled by the rate of formation of histamine in the pyloric portion. It is in the pyloric portion of the stomach where gastric ulcers most commonly occur. The presence of the enzyme in rat duodenum but not in jejunum or ileum is surprising, and this finding too may be linked with the fact that intestinal ulcers are found most frequently in the duodenum.

Much of the histamine in the body is held in tissue mast cells (Riley and West, 1953). In the rat, the high histamine content of the skin is reflected in its high mast cell population, and the same is true for lung. But the histamine content of the alimentary tract is not related to tissue mast cells. The fundic stomach, for example, contains a high number of mast cells per unit area yet its histamine content is relatively small and its histadine decarboxylase activity is low. The pyloric stomach contains very few mast cells but much histamine and histidine decarboxylase activity, and a similar situation is found in the duodenum. In the jejunum and ileum, there is a high histamine content, very few mast cells and very little histidine decarboxylase activity but these two tissues are the chief sources of histaminase activity in the rat. The liver and kidney possess the power to form histamine but not the power to store it. Thus, the histidine decarboxylase activity of rat tissues is not related to the amount of histamine they contain nor to the mast cell population.

Recently, whilst investigating the mechanism of action of corticosteroids in allergic diseases and asthma, we have found (Telford and West, 1960) that prolonged treatment of rats with glucocorticoids causes a marked depletion of histamine in many tissues, although the histamine

content of the pyloric stomach is increased. It is possible that these changes are brought about by an alteration in the rate of formation of histamine. The effect of glucocorticoids on the histidine decarboxylase activity of tissues is now being studied.

References

Blaschko, H. (1957). *Experientia*, **13**, 9–12.
Holtz, P., and Heise, R. (1937). *Arch. exp. Path. Pharmak.*, **186**, 269–273.
Kahlson, G. (**1960**). *Lancet*, **1**, 67–71.
Kahlson, G., Rosengren, E., and White, T. (1960). *J. Physiol.*, **151**, 131–138.
Mackay, D., Marshall, P. B., and Riley, J. F. (1960). *Ibid.*, **153**, 31P.
Riley, J. F., and West, G. B. (1953). *Ibid.*, **120**, 258–537.
Rothschild, A. M., and Schayer, R. W. (1958). *Fed. Proc.*, **17**, 136.
Schayer, R. W. (1957). *Amer. J. Physiol.*, **189**, 533–536.
Telford, J. M., and West, G. B. (1960). *Brit. J. Pharmacol.*, **15**, 532–539.
Waton, N. G. (1956). *Ibid.*, **11**, 119–127.
Werle, E. (1936). *Biochem. Z.*, **288**, 292–293.

ANATOMICAL STUDIES IN THE GENUS *RUBUS*

PART IV. ANATOMICAL VARIATIONS IN THE LEAVES OF CULTIVATED VARIETIES OF *R. idaeus* L. AND *R. loganobaccus* L. H. BAILEY, AND OF CERTAIN SPECIES OF BRAMBLE

BY K. R. FELL AND J. M. ROWSON

From the Pharmacognosy Research Laboratory, Bradford Institute of Technology

Received October 10, 1960

Differential anatomical characters of the leaves of cultivated varieties of *R. idaeus* L. and *R. loganobaccus* L. H. Bailey, and of certain species of bramble, are described and illustrated. The comparative anatomical characters of raspberry, blackberry and loganberry leaves, including these varieties and species, is discussed.

THE anatomy of the leaves of wild plants of *R. idaeus* L., cultivated plants of *R. loganobaccus* L. H. Bailey and a wild bramble has been described in earlier communications (Fell and Rowson, 1956; 1957; 1960). There are many well-established cultivated varieties of *R. idaeus* and commercial samples of raspberry leaves are invariably derived from cultivated crops, the thornless variety of *R. loganobaccus* occurs in cultivation, moreover 386 species of indigenous brambles have now been recognised by Watson (1958). To determine if the leaves of certain of these varieties and species could be distinguished amongst themselves and from the materials already described, an investigation was carried out on leaves of eight well-established commercial varieties of *R. idaeus*, on the thornless variety of *R. loganobaccus*, and on seventeen species of bramble, representative of the seven Sections of the Sub-genera *RUBUS* (*MORIFERI* Focke), *GLAUCOBATUS x RUBUS* and *IDAEOBATUS x RUBUS* (Thoms, 1931).

MATERIALS

Authenticated leaf materials of *R. loganobaccus* L. H. Bailey, thornless variety and of *R. idaeus* L., commercial varieties *Lloyd George*, *Malling Enterprise*, *Malling Exploit*, *Malling Jewel*, *Malling Promise*, *Newburgh* and *Norfolk Giant* were supplied from the Gardens of the Royal Horticultural Society, Wisley, Surrey. Leaves of *R. idaeus*, variety *St. Walfried* were obtained from the East Malling Research Station, Kent. Leaves of the following British brambles were provided by a taxonomist specialising in this genus and selected to represent all seven Sections recognised by Watson (1958) in the three Sub-genera comprising this group (shown on following page.)

EXPERIMENTAL

The techniques employed for the examination of all materials were those previously described (Fell and Rowson, 1956; 1957; 1960).

Leaflets, rachis and stipules of the eight commercial varieties of *R. idaeus* were examined by means of surface preparations and by sections,

The subject matter of this communication forms part of a thesis by one of us (K.R.F.) accepted by the University of London for the Degree of Doctor of Philosophy.

Sub-genus	Section	Species
IDAEOBATUS x RUBUS	SUBERECTI	*R. fissus*, Lindl.
GLAUCOBATUS x RUBUS	TRIVIALES	*R. corylifolius* Sm., *R. bucknallii* White, *R. balfourianus*, Blox ex Bab.
RUBUS (MORIFERI Focke)	SYLVATICI	*R. schlechtendalii* Whe., *R. pyramidalis* Kalt, *R. hirtifolius* Muell. & Wirtg., *R. danicus* Focke.,
	DISCOLORES	*R. pubescens*, var. *subinermis* Rogers, *R. cornubiensis* Rils.
	SPRENGELIANI	*R. lentiginosus* Lees
	APPENDICULATI	*R. thurstonii* Rilst., *R. fuscus*, var. *macrostachys*, *R. nuticeps*, Barton & Ridd.
	GLANDULOSI	*R. koehleri* Weihe, *R. hystrix* Weihe, *R. cognatus* N.E. Br.

the rachis being cut above and below the point of insertion of the lateral leaflets. Particular attention was directed to the characters of the epidermal cells, cuticle, stomata, hydathodes and trichomes, and also the arrangement of vascular and fibrovascular tissue and meristeles. The majority of the structural characters of wild *R. idaeus*, already described

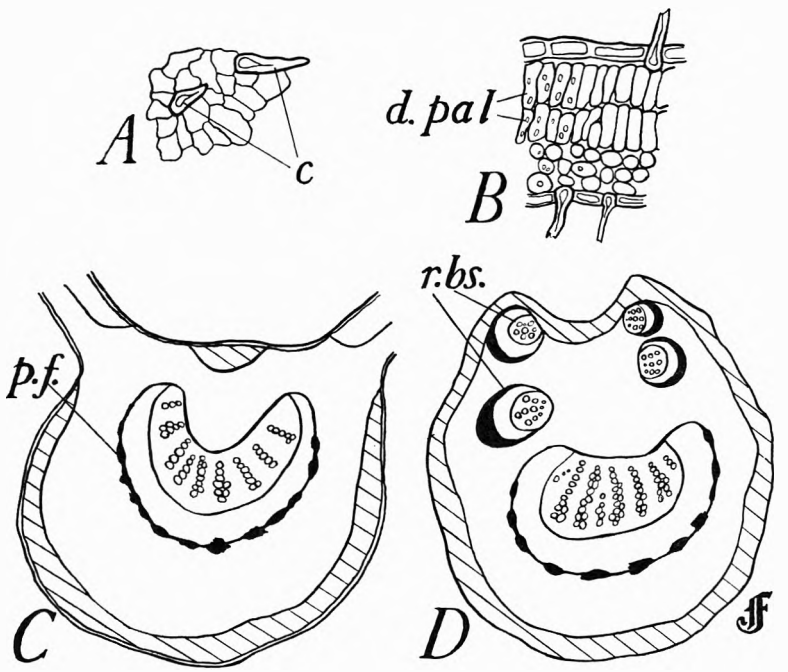

FIG. 1. Leaves of cultivated varieties of *R. idaeus* L. *A*, var. *Lloyd George*. Upper epidermis of interneural lamina, showing short, covering trichomes. *B*, var. *Malling Enterprise*. Transverse section of interneural lamina, showing well-formed double palisade. *C*, var. *Malling Jewel*. Transverse section of midrib of leaflet cut near base, showing pericyclic fibres. *D*, var. *Newburgh*. Transverse section of upper rachis, cut near the top, showing four fibro-vascular ridge bundles. *A* and *B* x 200; *C* and *D* x 25. *c*, covering trichomes; *d. pal*, double palisade; *p.f.*, pericyclic fibres; *r.bs.*, ridge bundles.

and illustrated (Fell and Rowson, 1956), were found in the eight commercial varieties. Several differences from the wild material were also noted, these are given in Table I and are illustrated in Fig. 1.

Preliminary examination of the 17 bramble species showed a uniform anatomical pattern, similar to that already described and illustrated in this series of papers for a *Radula* sp. of bramble (Fell and Rowson, 1957). Some variations in armature have been reported by Thoms (1931) and Moeller-Griebel (1928a) and our bramble materials also showed such variations in trichome structures, thus detailed investigations were

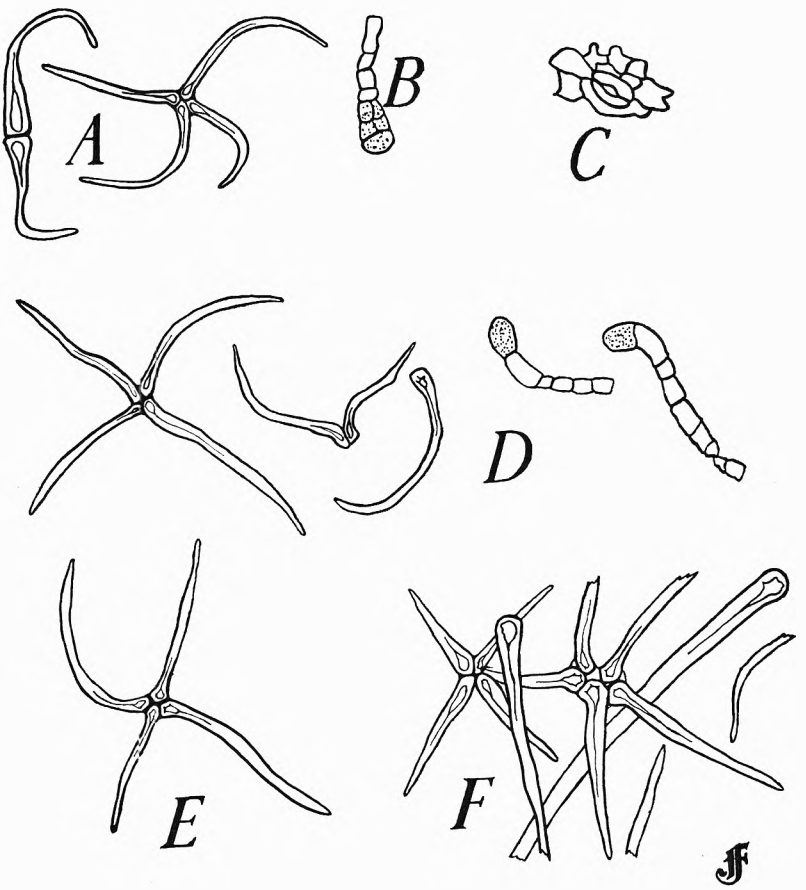

FIG. 2. Hydathode and trichomes from the leaves of certain bramble species. *A*, 2- and 4-celled covering trichomes from the lower epidermis of the lamina of leaflet of *R. corylifolius*. *B*, Glandular trichome from the lower epidermis of leaflet of *R. bucknallii*. *C*, Hydathode from upper epidermis of leaflet of *R. pyramidalis*, seen some distance in from the edge of the lamina. *D*, 1-, 2- and 4-celled, unlignified, covering trichomes, and glandular trichomes from the lower epidermis of lamina and midrib, respectively, of leaflet of *R. hirtifolius*. *E*, 4-celled, lignified, stellate, covering trichome from the lower epidermis of leaflet of *R. danicus*. *F*, Fragment of the tomentum of lignified, covering trichomes from lower epidermis of leaflet of *R. thurstonii*. All x 200.

directed to the upper and lower epidermises of lamina and midrib of each species. The characteristic unicellular, lignified covering trichomes with well-marked crosslines and the different glandular trichomes of bramble (Fell and Rowson, 1957) were found in the 17 species examined. In addition, certain differential characters were noted and these are summarised in Table II and illustrated in Fig. 2.

TABLE I

ANATOMICAL CHARACTERS DISTINGUISHING CERTAIN CULTIVATED VARIETIES FROM WILD LEAVES OF *Rubus idaeus*

Variety	Covering trichomes of			Transverse section	
	Leaflet upper surface	Rachis	Stipules	Leaflets	Rachis
Lloyd George	30–50μ long (Fig. 1, *A*)	Lignin ±	Lignin very slight	Double palisade	
Malling Enterprise			lignin ±	Very well-marked double palisade (Fig. 1, *B*)	
Malling Exploit		Lignin ±	Lignin ±	Well marked double palisade.	
Malling Jewel	Lignin ±			Lignified pericyclic fibres also pitted vascular elements in some leaflets (Fig. 1, *C*)	
Malling Promise	Sparsely scattered		Lignin ±	Well marked double palisade	At top of rachis two ridge bundles in each of two ridges commonly found
Newburgh			Lignin ±	Very well marked double palisade	As for *Malling Promise* (Fig. 1, *D*)
Norfolk Giant				Double palisade	
St. Walfried				Double palisade	

Note: lignin ± means that the covering trichomes gave only a slight reaction for lignin.

The structure of the epidermises of the leaflet and rachis of the thornless variety of *R. loganobaccus* was investigated, the results are summarised in Table III.

DISCUSSION AND CONCLUSIONS

A. GENERAL

In the work described above, and in the earlier work, it has been shown that the morphological and anatomical characters of the leaves of *R. idaeus* L. (wild form and eight cultivated varieties), *R. fruticosus* L. (18 wild British species according to Watson's classification (1958)) and *R. loganobaccus* L. H. Bailey (two cultivated varieties) are similar in most general respects, but that there are differences in detailed structures, some of which are of diagnostic value.

B. MORPHOLOGY

The *leaves* of all varieties and species examined are imparipinnately compound, usually with five leaflets, but sometimes three or, occasionally,

TABLE II

EPIDERMAL CHARACTERS DISTINGUISHING CERTAIN SPECIES OF BRAMBLE

Species	Upper epidermis	Lower epidermis	
		Stellate trichomes number of arms	Glandular trichomes
Rubus fissus ..		2	
R. corylifolius ..		2–6	
R. bucknallii ..		2–5	Multicellular head on multicellular uniseriate stalk, occasional collapsed cell in stalk (Fig. 2)
R. balfourianus ..		2	
R. schlechtendalii ..	Trichomes rare except on midrib and veins	2–8	
R. pyramidalis	Few trichomes on margins only Hydathodes prominent (Fig. 2)	Abundant unicellular trichomes varying much in length and in amount of lignification	Abundant with unicellular head on multicellular uniseriate stalk
R. hirtifolius ..	Trichomes rare except on midrib and veins	2–4 (on midrib lignin ±)	Unicellular head on multicellular uniseriate stalk, frequently with collapsed cell in stalk (Fig. 2)
R. danicus		2–6 (Fig. 2)	As *R. pyramidalis*
R. pubescens var. *subinemis* ..	Glandular trichomes rare	2–5	
R. cornubiensis ..	Trichomes rare	2–4	
R. lentiginosus ..	Few trichomes near the margin only	2	
R. thurstonii	Covering trichomes mainly confined to the veins	2–8 Covering trichomes forming a dense tomentum	Abundant on interneural surface and on veins
R. fuscus var. *macrostachys* ..		2–6 as *R. thurstonii*	Few
R. nuticeps		2–6	
R. koehleri		2–4	
R. hystrix ..		2–4	
R. cognatus		2–4	

TABLE III

ANATOMICAL CHARACTERS OF *R. loganobaccus* AND OF A THORNLESS VARIETY

	R. loganobaccus	*R. loganobaccus* (thornless)
1. *Sclereids*	Present, as described*	Absent
2. Upper epidermis of lamina	Cells beaded	Cells not beaded
Lower epidermis of lamina	Tomentum: hairs only give a positive reaction for lignin with phloroglucin and hydrochloric acid after preliminary treatment with ether	Very hairy tomentum; hairs give a positive reaction for lignin with phloroglucin and hydrochloric acid without difficulty and without preliminary treatment with ether
4. Lower epidermis of midrib	Cells beaded. Covering hairs give a positive reaction for lignin only after preliminary treatment with ether	Cells very slightly beaded, covering hairs plentiful, lignified and tending to be tomentose
5. Epidermis of rachis ..		Cells particularly well beaded; covering hairs lignified and *very* numerous

* Fell and Rowson, 1960.

seven. Individual *leaflets* have sharply pointed apices, the margin is coarsely serrate-dentate with mucronate teeth and the base cordate. The venation is pinnate; each vein terminates at the apex of one of the marginal teeth. The lamina is hairy in all species, but much more so on the lower surface; since the dense tomentum on the lower surface of the

lamina of leaflets of *R. idaeus* imparts a distinctly *white* colour to this surface, this latter is a valuable macroscopical diagnostic character, even when the leaves are in a coarsely chopped and dried condition, as is commonly the case with commercial material. This character is not seen in the leaves of *R. loganobaccus*; those of the bramble species vary in appearance (due to the greatly differing degrees of hairiness encountered in different species) but in no species does the underside of leaflets appear

TABLE IV

KEY FOR MACROSCOPICAL IDENTIFICATION OF SPECIES

1.	Colour	Leaves, or fragments of lamina showing white lower surface	2
		Not as above	3
2.	*R. idaeus:*	odour pleasantly aromatic; confirmed by histological examination (see Table V)	
3.	Prickles	Present	4
		Absent	5
4.	*R. loganobaccus*	or a species of bramble	6
5.	*R. loganobaccus*	thornless variety: confirm by histological examination (see Table V)	
6.	Leaves green;	leaflets (if leaves whole) large, and usually five in number *R. loganobaccus* —confirm by histological examination (see Table V)	
		Leaves varying in colour from green to greyish-green, brownish-green, light brown or greyish-white; leaflets not large .. *Sp. of bramble*—confirm histologically (see Table V)	

white (see Table IV). Terminal leaflets are, in general, larger than the lateral ones and are symmetrical; lateral leaflets are slightly asymmetrical, and are sessile on the rachis in the cases of *R. idaeus* and *R. loganobaccus*, but this detail is very variable in the brambles, where (depending on the species) either or both lateral and basal leaflets may be stalked. Leaflets of *R. loganobaccus* were the largest of any species examined, but several

TABLE V

KEY FOR HISTOLOGICAL IDENTIFICATION OF SPECIES

1.	All of the trichomes of the lower interneural epidermis unlignified (even after treatment with ether or chloral hydrate solution) and forming a tomentum	2
	Some, or all, lignified (after treatment with ether)	3
2.	*R. idaeus* or variety.	
3.	Trichomes of the lower interneural lamina lignified (after treatment with ether) and forming a loose tomentum. No stellate trichomes	4
	Covering and glandular trichomes present, former frequently stellate; some covering trichomes, at least, lignified	7
4.	Sclereids present	5
	Sclereids absent	6
5.	*R. loganobaccus.*	
6.	*R. loganobaccus*, thornless variety.	
7.	Species of bramble. Refer to Table II.	

leaflets of cultivated varieties of *R. idaeus* were almost as large, and mere size cannot be considered as a useful differential character between these two species.

The *rachis* does not exhibit any significant variation in external structure; in specimens examined, its dimensions always lay between limits of 4 to 15 cm. in length, and 1 to 5 mm. in width, the latter increasing from top to base. It is terete except for a single groove running down the middle of the upper surface.

The elongated-conical, laterally compressed *prickles* found on the midrib and rachis are common to all species and varieties examined with

the single exception of the thornless variety of *R. loganobaccus* (see Table IV). The precise shape, size and frequency of the prickles vary with the species or variety, but such variations have not been used to distinguish between the 28 species or varieties possessing prickles and described in this series of papers.

It is not considered that the *stipules* have any differential diagnostic significance in the species and varieties so far examined.

The *sensory characters* are of greatest value in distinguishing commercial samples of leaves of *R. idaeus*, fragments of which show a green upper surface and a white lower surface. The colour of bramble leaves varies greatly with the species and age of the specimen—from green to greyish-green, brownish-green, pale brown, and, in the case of the lower surface, occasionally greyish-white (but not white); leaves of *R. loganobaccus* are green. The taste, for all species and varieties, is astringent, due to the high tannin content, the odour is distinctive in the case of *R. idaeus*, dried samples of which are quite pleasantly aromatic; the texture of all leaves in the dried condition is fragile. These differential morphological and sensory characters are summarised in Table IV as a key for the identification of the species here considered.

C. Anatomy

A number of anatomical characters are common to the 29 species and varieties examined, these are the general distribution of tissues, similarity in sizes of corresponding cells (except trichomes, see below), stomatal type and distribution, hydathodes, also the cell inclusions of calcium oxalate and starch granules. Characters which may be employed for the distinction of the species and varieties from each other are principally those of the epidermis (trichomes, epidermal cells and prickles) but also include certain characters of the mesophyll, cortex and stele. They will be discussed under the following headings, and they are employed in Tables II and V.

(*a*) *Epidermal tissues*

(1) *The trichomes.* The present work and other records (Thoms, 1931; Moeller-Griebel, 1928b) show that, in general, the *types* of trichome found on the leaves of the brambles serve adequately to distinguish them from the leaves of *R. idaeus* and *R. loganobaccus* but, as shown previously (Fell and Rowson, 1957) and in Table II, there is a considerable variation in character (e.g., whether single or stellate), location, frequency and distribution of both covering and glandular trichomes between leaves of different species of the brambles. It is possible that still further variation in the epidermal characters exists in leaves of other indigenous species of bramble recognised by Watson (1958). The considerable infra-specific variation in detailed anatomical characters, especially of the trichomes, which we have found within *R. fruticosus* L. does lend support to the work of Watson in splitting up this Linnaean species.

Covering trichomes only were found on the *upper epidermis* in the *interneural* region of leaflets of all leaflets examined; they were abundant

except in *R. idaeus* var. *Malling Promise*, in *R. schlechtendalii* and in *R. lentiginosus* where they were rare and in *R. cornubiensis* where they were confined to the margin of the leaflet. The trichomes are unicellular, lignified (except in *R. idaeus* var. *Malling Jewel* which only gave a slight reaction for lignin), thick walled, the cross-line effect being well marked in the brambles and in *R. loganobaccus*, less well-marked in cultivated varieties of *R. idaeus* and absent in the wild form of this species. The trichome apex is often solid, the lumen extending only about half-way up the arm of the hair; the base is thickened and pitted, adjacent epidermal cells being arranged in a radiate manner around the base, this being well-marked in *R. idaeus* and *R. loganobaccus*. The lengths of the hairs varied between species and varieties, in *R. idaeus* they were 200 to 500 μ (except var. *Lloyd George* which were 50–60 μ), 300 to 1,000 μ in the brambles and 180 to 1,400 μ in *R. loganobaccus*.

Both covering and glandular trichomes were found on the *upper epidermis of the midrib* in all materials except *R. pyramidalis*. The covering trichomes are similar to those of the interneural lamina; the glandular trichomes consist of a subspherical, multicellular glandular head about 60 μ in diameter, on a uni-or bi-seriate stalk, 3 to 7 cells long.

The trichomes of the lower epidermises of the laminae and midribs give the clearest demarcation between leaves of *R. idaeus* and its cultivated varieties, the brambles and *R. loganobaccus*. Thus *R. idaeus* is characterised by the presence of abundant *unlignified* covering trichomes forming a tomentum. They are unicellular, about 150 to 500 μ long, usually with the lumen obliterated at the upper end only. *The brambles* show great variation within the 18 species examined; the covering trichomes differ much in size and are either unicellular or 2 to 8 stellate, the former being the more common on interneural laminae whilst the latter are usually found over the veins. The glandular trichomes also vary in these species. Table II indicates the range of variation and by comparing it with the tabulation of materials examined it is seen that no relationship exists between trichome structure and the taxonomic divisions of species, Sections and Sub-genera. As might be expected from its hybrid nature, the trichomes of the lower lamina of *R. loganobaccus* are somewhat intermediate in character between those of *R. idaeus* and the brambles (Fell and Rowson, 1960). They are mainly of the covering type, about 300 to 700 μ long, and form a tomentum which is less dense than that found in *R. idaeus*. They give a positive reaction for lignin with phloroglucinol and hydrochloric acid, only after preliminary treatment with ether or chloral hydrate solution, whereas the trichomes of the lower surface of the leaflet of *R. idaeus* will not stain, even after this treatment, and the corresponding trichomes of the brambles give a positive reaction without any such preliminary treatment (see Table V).

The trichomes on the *rachis* are of the covering type in *R. idaeus* and *R. loganobaccus*, where they resemble those on the midrib. Both covering and glandular types were seen in the *Radula* sp. of bramble examined earlier (Fell and Rowson, 1957), where it was shown that the glandular trichomes were of larger size and different character from those present

on the midrib of the leaflet, thus providing a useful method for detecting rachis in powdered bramble leaflet.

The trichomes of the *stipules* are mainly of the covering type in *R. idaeus* and *R. loganobaccus* but large numbers of both types occurred in the *Radula* sp. of bramble where the glandular ones were large, resembling those of the rachis. Covering trichomes are lignified and show the "cross-line" effect even in the case of the wild specimen of *R. idaeus*.

(2) *Epidermal cells*. In all species examined, the cells of both upper and lower epidermises of the lamina exhibit wavy anticlinal walls, the waviness being more pronounced on the cells of the lower epidermis. Slight beading was observed on cells of the *Radula* sp. of bramble; beading is well-marked on cells of both upper and lower lamina of *R. loganobaccus*, where it forms a distinctive character.

(3) *Prickles*. These are very common throughout the genus. In the species examined, they consist of elongated or fibre-like sclereids, lignified, and with pitted walls. The presence of these sclereids, or their fragments, is therefore to be expected in powders of all species examined, with the single exception of *R. loganobaccus*, thornless variety (see Table V).

(*b*) *Mesophyll*

In all cases, the mesophyll is well differentiated; in *R. loganobaccus* and all cultivated varieties of *R. idaeus*, there is a well-marked double palisade; in the wild plant of the latter, there is a tendency towards this condition, but the double palisade appears to be a feature associated with cultivation. Calcium oxalate is very common.

(*c*) *Cortical tissues*

The cortical tissues of both rachis and midrib follow a similar pattern in all species examined. The *endodermis* is rather ill-defined, and its cells contain minute starch grains; in the *Radula* sp. of bramble it is further characterised by the presence of lignified reticulate parenchyma which we have not observed in specimens of *R. idaeus* or *R. loganobaccus*.

(*d*) *Stelar tissues*

The *xylem* and *phloem* elements are similar in all species examined. *Pericyclic fibres* have also been found in every specimen of rachis examined. In the midrib, pericyclic fibres were found in some, but not all leaflets of *R. idaeus* var. *Malling Jewel*, they were absent from the other seven cultivated varieties and the wild form of this species; they were also present in some, but not all leaflets of *R. loganobaccus* (Fell and Rowson, 1960) and were always present in the *Radula* sp. of bramble (Fell and Rowson, 1957). Since the amount of fibre present in a leaf can vary with age, the diagnostic significance of presence or absence of pericyclic fibres in the leaf midrib is not stressed.

References

Fell K. R. and Rowson, J. M. (1956). *J. Pharm. Pharmacol.*, **8**, 334–345.
Fell, K. R. and Rowson, J. M. (1957). *Ibid.*, **9**, 293–311.

Fell, K. R. and Rowson, J. M. (1960). *Ibid.*, **12**, 473–487.
Moeller-Griebel, (1928a). *Mikroscopie der Nahrungs—und Genussmittel aus dem Pflanzenreiche*, 3rd ed., p. 365. Berlin: Springer.
Moeller-Griebel, (1928b). *Ibid.*, p. 366.
Thoms, (1931). *Handbuch der Pharmazie*, Vol. V, p. 1024. Urban & Schwarzenberg,
Watson, (1958). *Handbook of the Rubi of Great Britain and Ireland*, Cambridge University Press.

CURARE-LIKE DRUGS AND CARDIOVAGAL SYNAPSES: COMPARATIVF STUDY *IN VITRO* ON ISOLATED GUINEA-PIG VAGUS-HEART PREPARATION

By D. Della Bella, F. Rognoni and K. Gopal*

From the Research Laboratories of the Laboratorio Bioterapico Milanese SELVI & C., Milan, Italy

Received November 9, 1960

* Indian Government Scholar in Italy.

Isolated vagus-heart preparations from guinea-pigs have been used for a comparative study of the ability of curare-like drugs to block the effects of preganglionic stimulation of the vagi on the heart, *in vitro*. The following drugs all had this property and have been arranged in order of decreasing potency in this respect: hexafluorenium, gallamine, laudexium, decamethonium, tubocurarine, suxamethonium and succinyldisulphocholine.

Though observed for a long time and considered by some workers to be a general property, the ganglion blocking effect of drugs which interrupt the transmission at the level of neuromuscular junctions of striated muscle has not received much study (Bovet, 1959; Paton, 1954; Paton and Perry, 1953; Rosenblueth, 1950).

It was felt that a direct demonstration of this side-effect of curare-like drugs could prove of indubitable interest in adding to our knowledge of the cardiovascular troubles sometimes occuring in a curarised patient (Foldes, 1957).

Using the isolated guinea-pig vagus-heart preparations, we assessed the anti-vagal action of the following curare-like drugs: tubocurarine, decamethonium, suxamethonium, succinyldisulphocholine, gallamine, hexafluorenium and laudexium.

Methods

The guinea-pigs weighed 300–350 g. The isolated vagus-heart preparations were made from them as previously described (Della Bella, Rognoni and Villani, 1959). The apex of the perfused heart was connected to an isotonic lever which recorded contractions on a kymograph. The preganglionic fibres of the intact vagus were placed on a pair of platinum electrodes, near the heart, to avoid any stretching. Rectangular pulses of 1 millisec. duration, 15 to 20/sec. were applied for 5 sec. at 10 min. intervals of these fibres from an electronic stimulator. Drugs were dissolved in Ringer solution and were injected into the perfusion fluid close to its entry into the heart, 2 min. before a period of vagal stimulation.

Results

Table I shows the minimum doses of the curare-like drugs required to modify the effects on the heart of preganglionic stimulation of the vagus. Hexamethonium has been included as an example of a drug which has nearly pure ganglionic blocking effect. The doses of these drugs required

to produce "head drop" in rabbits have been collected from the literature (Bovet, 1957; Foldes, 1957; Della Bella and others, 1959; Della Bella, Villani and Zuanazzi, 1956) and are included for comparison.

It was found that the most active compound was hexafluorenium, which proved even more active than hexamethonium in blocking the effects of preganglionic vagal stimulation on the heart *in vitro*. This is

TABLE I

GANGLION BLOCKING AND CURARE-LIKE ACTIVITIES OF DRUGS TESTED

Drugs	Minimum active dose on the cardiovagal synapses (*in vitro*) mg.	"Head-drop" dose in rabbits, mg./kg.
Hexamethonium iodide	0·075	—
Hexafluorenium bromide	0·025	0·08
Gallamine iodide	0·050	0·50
Laudexium methylsulphate	0·075	0·03
Decamethonium iodide	0·250	0·15
Tubocurarine chloride	0·300	0·15
Suxamethonium chloride	0·500	0·20
Succinyldisulphocholine iodide.. ..	2·500	2·00

of interest because hexafluorenium produces a marked fall of blood pressure when injected intravenously in rabbits (Fig. 1) and in dogs (Macri, 1954) resembling that caused by the intravenous injection of hexamethonium. Hexafluorenium does not, however, block the effects of preganglionic stimulation of the vagi on the heart either in the rabbit (Fig. 1) or in the dog (Macri, 1954). Fig. 1 shows that hexafluorenium potentiates both the vagal responses the action of acetylcholine: this may be attributed to inhibition of cholinesterases by the drug (Rizzi, 1957; Foldes, Molloy, Zsigmond and Zwartz, 1958). Gallamine and laudexium also have high *in vitro* action in blocking the effects on the heart of

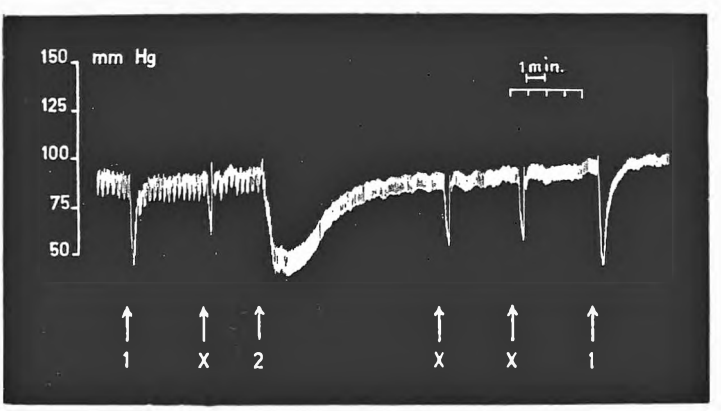

FIG. 1. Rabbit, 2·7 kg. (chloralose: 80 mg./kg. i/v.). Registration of pressure at the carotid. It can be seen that after the administration of hexafluorenium (0·5 mg./kg., i.v. at 2), the response to electrical stimulation of the vagus appears to be potentiated (at X: electrical stimulation of the peripheral trunk of the right vagal nerve for 5 sec.; frequency, 15 pulses/sec.; duration of each pulse: 1 millisec.). Also, the response to the administration of acetylcholine (at 1) is more potentiated (0·5 μg./kg., i.v.).

preganglionic vagal stimulation (Fig. 2) and are almost as active as hexamethonium (Table I). This observation in the case of gallamine (Fig. 3) is in agreement with the clinical and experimental findings that the drug has an inhibitory action on the cardiac effects of vagal stimulation (Bovet, Depierre, Courvoisier and Lestrange, 1949; Jacob and

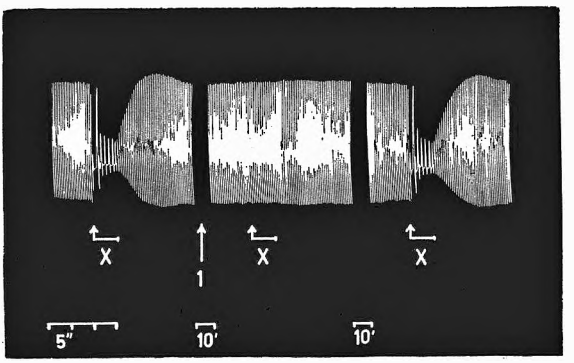

FIG. 2. Isolated guinea-pig vagus-heart preparation. Registration of the responses to electrical stimulation (at X) of the cardiovagal fibres for 5 sec. (frequency, 20 pulses/sec.; duration of each pulse, 1 millisec.). The blocking effect due to hexamethonium treatment (75 μg. at 1) carried out 2 min. before stimulation, is evident.

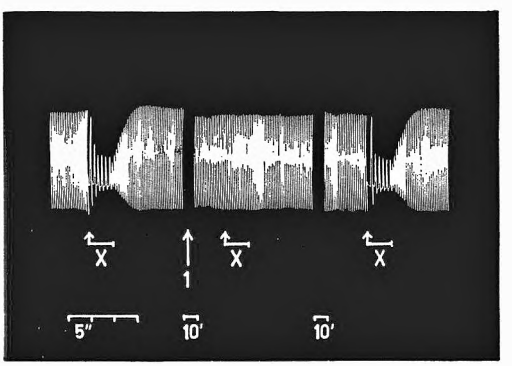

FIG. 3. Isolated guinea-pig vagus-heart preparation. Registration of the responses to electrical stimulation (at X) of the cardiovagal fibres for 5 sec. (frequency, 20 pulses/sec.; duration of the single pulse, 1 millisec.). Treatment with gallamine (50 μg. at 1) causes a vagal block practically identical to that obtained with hexamethonium in the experiment described in Fig. 2.

Depierre, 1950; Riker and Wescoe, 1951). The vagal blocking action of laudexium appears to be approximately three times that of tubocurarine (Table I). But when their activities were compared using isolated guinea-pig intestine stimulated with nicotine, tubocurarine was found the more potent (Collier and Macauley, 1952). Finally, suxamethonium had only slight *in vitro* vagal blocking action (Table I). This has already been noted *in vivo* by workers using electrocardiographic methods of recording

in cats; its neuromuscular blocking activity is also of short duration (Purpura and Grundfest, 1956). The disulphonium analogue—succinyldisulphocholine proved even less active (Fig. 4).

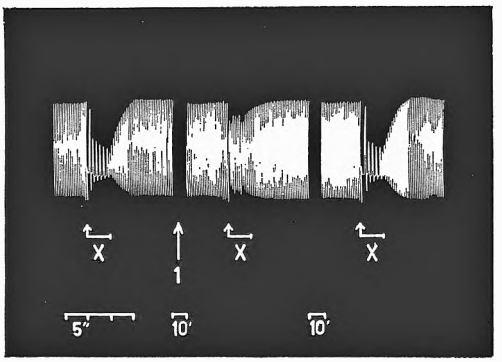

FIG. 4. Isolated guinea-pig vagus-heart preparation. Recording of the responses to electrical stimulation (at X) of the cardiovagal fibres for 5 sec. (frequency, 20 pulses/sec., duration of each pulse, 1 millisec.). Partial disappearance of the vagal response, following succinyldisulphocholine treatment (2 mg. at 1), is evident.

DISCUSSION

It is not possible to determine the nature of the anti-vagal effect shown by the drugs tested with the isolated vagus-heart preparations used. However, since it is well known that these drugs do not possess atropine-like properties and do not interfere with the liberation of the cholinergic mediator, it may well be assumed that the anti-vagal effect recorded is a result of the inhibitory action exerted on the cardiovagal synapses.

Table I shows that the ganglion blocking effect of curare-like drugs *in vitro* has a very close relationship to their curare-like action *in vivo* except for gallamine. On the contrary, the same relationship does not persist when the *in vivo* ganglion blocking activity is considered. For example, hexafluorenium which is found to have a very high ganglion blocking activity *in vitro*, does not have any such activity *in vivo*. Instead, the suxamethonium activity *in vitro* is still retained *in vivo*. It is difficult at the present moment to give a satisfactory explanation for these differences.

REFERENCES

Bovet, D., Bovet-Nitti, F. and Marini Bettolo, G. B. (1959). *Curare and Curare-like Agents*, 1st ed., pp. 252–287, Amsterdam: Elsevier Publ. Co.

Bovet, D., Depierre, F., Courvoisier, S. and De Lestrange, Y. (1949). *Arch. intern. physiol. biochem.*, **80**, 172–188.

Collier, H. O. J. and Macauley, B. (1952). *Brit. J. Pharmacol.*, **7**, 398–408.

Della Bella, D., Villani, R. and Zuanazzi, G. F. (1956). *Boll. Soc. Ital. Biol. Sper.*, **32**, 483–487.

Della Bella, D., Rognoni, F. and Villani, R. (1959). *Arch. ital. sci. farmacol.*, **9**, 544–549.

Foldes, F. F., Molloy, R. E., Zsigmond, E. K. and Zwartz, J. A. (1958). *Fed. Proc.*, **17**, 367.

Foldes, F. F. (1957). *Muscle Relaxants in Anaesthesiology*, 1st ed., Springfield: Thomas.
Jacob, J. and Depierre, F. (1950). *Arch. int. Pharmacodyn.*, **83**, 1–14.
Macri, F. J. (1954). *Proc. Soc. Exp. Biol. N.Y.*, **85**, 603–606.
Paton, W. D. M. (1954). *Pharmacol. Rev.*, **6**, 59–67.
Paton, W. D. M. and Perry, W. L. M. (1953). *J. Physiol.*, **119**, 43–57.
Purpura, D. P. and Grundfest, H. (1956). *Science*, **124**, 319–320.
Riker, W. F. Jr. and Wescoe, W. C. (1951). *Ann. N.Y. Acad. Sci.*, **54**, 373–392.
Rizzi, R. (1957). *Curaro-curarosimili-curarizzanti*, 1st ed., Venezia, I.T.E.
Rosenblueth, A. (1950). *The Transmission of Nerve Impulses at Neuroeffector Junctions and Peripheral Synapses*. 1st ed. New York, Wiley and Sons, Inc.

A PHYTOCHEMICAL SURVEY OF MALAYA

Part II. Alkaloids

By A. K. Kiang, Bryce Douglas* and Francis Morsingh†

From the Chemistry Department, University of Malaya, Cluny Road, Singapore, 10

Received October 31, 1960

A phytochemical survey has been made of the plants of Malaya. The results of screening for alkaloids in 708 species of plants belonging to 104 families and 408 genera is reported.

In Part I (Douglas and Kiang, 1957) and in a communication to the Third Congress of the Pan Indian Ocean Science Association (Kiang and Douglas, 1957), the results were reported of the screening of the first 200 plants of the Malayan flora for the presence of alkaloids. These marked the beginnings of a systematic survey which has received much stimulation from, and is closely associated with, the phytochemical surveys conducted in the neighbouring countries especially in Australia (Webb, 1949), Papua-New Guinea (Webb, 1955), Borneo (Arthur, 1954) and South Moluccas (Bisset, 1957).

The collaboration between the University of Malaya in Singapore and various other institutions in Malaya in the Phytochemical Survey has been described in Part I. This report contains the screening results of 708 species of plants belonging to 104 families and 408 genera. Many of the plants screened are reputed by the aborigines, or recorded in the literature (Burkill, 1935), to possess medicinal or poisonous properties.

Amongst the plants screened, 144 species marked with * were collected in Singapore in the Botanic Gardens and around the University campus and 18 species were collected in Selama, Perak, by Miss B. E. Copeland, who was attached to the St. John's Ambulance in Perak in 1957. The rest of the plants were collected by an officer of the Department of Chemistry, Federation of Malaya, often with the aid of one or two aborigines, in the following districts: Pulau Langkawi, Kedah; Fort Kemer, Perak; National Park, Pahang; and Ulu Langat Forest Reserve, Selangor.

The plants were identified by Professor H. B. Gilliland, Mr. Wong Phui Weng and Mr. K. M. Kochuman and herbarium specimens are kept in the herbaria of the Botany Department of the University of Malaya in Singapore, the Botanic Gardens, Singapore, and the Forest Research Institute, Kepong, Selangor.

Procedure

The procedure for alkaloid screening is as follows. 15 g. of finely-ground, well-dried plant material is mixed thoroughly with 15 ml. 2 N ammonium hydroxide and packed into a Soxhlet extractor using a 150 ml.

* Visiting Research Associate, Chemistry Department, University of Malaya in Singapore; present address, Smith, Kline and French Laboratories, Philadelphia 1, Pa., U.S.A.

† Present address, Chemistry Department, University of Malaya in Kuala Lumpur.

capacity flask and extracted continuously with chloroform for about 6 hr. The chloroform extract is concentrated under reduced pressure at 50 to 70°. The solvent-free residue is rubbed thoroughly with 6 ml. 2 N hydrochloric acid. The acid extract, filtered through paper, if necessary, is divided into three equal quantities and placed in three test-tubes (capacity 4 ml. approx.). Each test solution is treated with 4 to 8 drops of one of the alkaloid reagents, Bouchardat, Dragendorff and Mayer. The production of a precipitate is indicative of the presence of alkaloidal material. A visual assessment of the quantity is made, for each of the reagents, on the following basis: heavy precipitate ++++, strong precipitate +++, weak precipitate ++, negligible precipitate +. The Bouchardat's reagent is prepared by dissolving 4 g. potassium iodide and 2 g. iodine in 100 ml. water. The Dragendorff's reagent is prepared by adding 8 g. bismuth nitrate, 20 ml. concentrated nitric acid, 27·2 g. potassium iodide to 50 ml. water, decanting off the supernatant from the deposited potassium nitrate and diluting the solution in 100 ml. with water.

Results

The list of plants screened is given in Table I.

We have found that in the isolation of crude alkaloids from 2 to 5 kg. quantities of materials, generally plant parts giving strong precipitates with all three reagents would yield favourable amounts of alkaloids. Sixty-six species (9·3 per cent of total species tested) which have given strong precipitates are listed in Table II. From most plants we had selected from this table and from the similar table in Part I, we have been able to isolate crude alkaloids in various quantities. These plants and their approximate alkaloidal contents are listed in Table III. It is noteworthy that we have found that with *Trivalvaria pumila* (Anonaceae), *Lindera pipericarpa* (Lauraceae), *Coptosapelta flavescens* (Rubiaceae) and *Uncaria pteropoda* (Rubiaceae), the crude alkaloids could not be extracted by dilute acids from the residues of alcoholic extracts of the plant materials. The alkaloids were best isolated by treating the residues with ammonia and extracting with chloroform.

TABLE I

List of plants screened for alkaloids

(Plant parts are in parenthesis; l = leaves, s = stems, b = bark, r = root, p = pericarp, wp = whole plant, flr = flower, frt = fruit, sd = seed.)

Acanthaceae—*Acanthus ilicifolius* (l, s); *Andrographis paniculata* (l and berries, s, r); *Asystasia coromandeliana** (l); *Gendarussa vulgaris* (l, s, r); *Ptyssiglottis obovata* (l, s, r); *Rhaphidophora korthalsia* (wp); *Sanchezia nobilis** (l); *Staurogyne longifolia* (l, s, r); *Thunbergia laurifolia** (l, s).

Alangiaceae—*Alangium javanicum* (l, s, b, frt).

Amaryllidaceae—*Curculigo villosa** (wp).

Anacardiaceae—*Mangifera sp.* (l, frt); *Spondias pinnata* (r, frt).

Anisophylleaceae—*Anisophyllea sp.* (l, s, r).

Annonaceae—*Anaxagorea javanica* (l, s); *Cyathostemma hookeri* (l, s, r); *Desmos chinensis* (r); *Enicosanthum macaranthum* (s, r); *E. membranifolium* (l, s, r, b, frt); *Fissistigma sp.* (l, s, r); *Goniothalamus curtisii* (l, b); *G. giganteus* (l, s, r, b, flr, frt); *G. ovariodes* (l, s, r, b, frt); *G. ridleyi* (l, s, r, b, frt); *G. tenuifolius* (l, s); *Miliusa longipes* (l, s, b, r, frt); *Mitrephora maingayi* (l, s, r, b, frt); *Monocarpia marginalis* (l, r, frt); *Orophea polycarpa* (r); *Oxymitra affinis* (b, r, frt); *O. biglandulosa* (l, s, r); *O. kingii* (l, s, r); *O. latifolia* (l, s, r, frt); *O. sp.* (l, s, r, b); *Phaeanthus nutans* (l, s, b); *P. ophthalmicus* (l, s, r, b); *Polyalthia bullata* (l, s, r); *P. cauliflora* (l, s); *P. cauliflora var. desmantha* (l, s, r, b, p); *P. cinnamomea* (l, s, r, b, frt); *P. clavigera* (r, b); *P. laterifolia* (l, s, r, b); *P. rumphii* (l, s, r, b, frt); *P. stenopetala* (l, b, frt); *P. sumatrana* (l, s, r, b, frt); *P. sp.* (l and flr, r, b); *P. sp.* (l, r, b); *Popowia pisocarpa* (l, s, r, b, frt); *P. ramosissima* (l, s, r, b); *P. tomentosa var. crinita* (l, r, s); *P. sp.* (l, s, r, b); *Pseuduvaria setosa* (l, s, r); *Sageraea elliptica* (l, b, frt); *Trivalvaria macrophylla* (l, s, r); *T. pumila* (l, s, r); *Uvaria grandiflora* (l, s, frt); *U. sp.* (l, s, b, r).

TABLE I—*continued.*

APOCYNACEAE—*Aganosina marginata* (l, s, r); *Alstonia spathulata* (l, s, b, r); *Cerbera manghas* (l, s); *Chilocarpus costatus* (l, s, r, p, sd); *Chonemorpha penangensis* (l, s); *Epigynum maingayi* (r); *Hunteria corymbosa* = *H. zeylanica* (l); *Leuconotis griffithii* (l, s, r); *Lochnera rosea** (l, s, r); *Strophanthus caudatus var. marckii* (l, s); *Tabernaemontana coronaria* (l, s); *T. curtisii* (l, s, r); *T. malaccensis* (l, s, r); *T. peduncularis* (l, s, r, b); *Thevetia neriifolia* (sd); *Willughbia coriacea* (r, b, frt).

ARACEAE—*Aglaonema oblongifolium* (wp); *Amorphophallus campanulatus* (l, s. r, frt); *A. variabilis* (wp); *A. sp.* (s, tuber); *Anadendrum marginatum* (wp); *Colocasia antiquorum* (r); *Epipremnum giganteum* (l, s, r); *Homalomena propinqua* (l, s, r); *Scindapsus picta* (l, s); *Syngonium phodophyllum* (l, s).

ARALIACEAE—*Aralidium pinnatifidum* (l, s, r, b, frt, sd); *Arthrophyllum borneense* (l, s. r); *A. diversifolium* (s, r, b, flr); *Scheffera biternata* (l, s, r); *S. subulata* (s, r, flr); *S. venulosa var. curtisii* (l, s, r); *Trevesia cheirantha* (l, s, b, r, flr).

ARISTOLOCHIACEAE—*Aristolochia curtisii* (r); *Thottea parviflora* (s, r).

ASCLEPIADACEAE—*Hoya diversifolia** (l, s); *H. ridleyi* (l, s).

BIGNONIACEAE—*Arrabidaea corymbosa** (l, s); *A. magnifica* (l, s); *Bignonia magnifica** (l, s); *Oroxylum indicum* (l, s, r); *Stenolobium stans** (l, s, frt).

BIXACEAE—*Bixa orellana** (l, s, r, flr, frt); *Hydnocarpus kurzii** (l, s).

BOMBACACEAE—*Durio griffithii* (l, s, r, b, frt); *D. lowianus* (r, b, p, sd).

BORAGINACEAE—*Cordia cylindristachya** (l, s, r); *Heliotropium indicum* (l, s).

BURSERACEAE—*Canarium littorale* (l, s, r, frt); *C. maluense* (l, s, r, b, frt); *C. sp.* (l, s, r, b, frt); *Dacryodes floribunda* (l, s, r, b, frt); *D. rostrata* (l, s, r, b, frt); *D. aff. rugosa* (r, b); *Pachylobus edulis** (l, s); *Santiria laevigata* (r, b, frt); *S. rubiginosa* (l, s, r); *S. sp.* (l, s, b, frt); *Triomma malaccensis* (l, s, b, frt).

CAMPANULACEAE—*Isotoma longiflora* (l, s, r, flr); *Pentaphragma begoniifolium** (l, s, r); *P. horsfieldii* (l, s, r).

CAPPARIDACEAE—*Capparis sp.* (l, s, r).

CASUARINACEAE—*Casuarina equisetifolia** (l, s).

CELASTRACEAE—*Euonymus javanicus* (l, s, r, b, frt); *Glyptopetalum quadrangulare* (l, s, r); *Kurrimia paniculata* (r, frt).

CHLORANTHACEAE—*Chloranthus officinalis* (l, s, r, wp).

COMBRETACEAE—*Quisqualis indica** (l, s); *Terminalia belerica* (l, r, b, frt); *T. innelleri** (l, s).

COMMELINACEAE—*Floscopa scandens* (wp); *Forrestia cf. griffithii* (l, s, r); *Pollia sorzogonensis* (wp).

COMPOSITAE—*Ageratum conyzoides* (l, s, r, flr); *Same sp.** (l, r, flr); *Blumea myriocephalia* (l, r, s); *Cosmos bipinnatus* (s); *Elephantopus scaber* (l, s, r); *Erechtites valerianifolia** (l, r, flr); *Leuzea speciosa** (l, s); *Mikania scandens* (wp); *Synedrella nodiflora** (l, s).

CONNARACEAE—*Cnestis palala* (l, s, r, sd); *Connarus sp.* (l, s, r, b, flr); *Rourea fulgens** (l, s).

CONVOLVULACEAE—*Argyreia capitata* (l, s, r); *A. kunstleri* (l, s, r, b, flr); *Maripa nicaranguensis** (l, s).

CORNACEAE—*Aralidium pinnatifidum* (l, s, r).

CUCURBITACEAE—*Gymnopetalum integrifolium* (frt); *Hodgsonia capniocarpa* (l, s, r, b, sd, p); *Melothria affinis* (wp); *Trichosanthes celebica* (wp).

CYPERACEAE—*Hypolytrum latifolium** (l); *Scleria sp.** (wp).

DILLENIACEAE—*Dillenia eximia* (l, s, r, b, frt); *D. ovata* (r, b, frt); *Tetracera akara* (l, s, r, frt); *T. fagifolia var. fagifolia* (l, s, r); *T. scandens* (l, s, sd, flr); *T. sylvestris** (l, s); *Wormia suffruticosa* (l, s, r, flr, frt); *Same sp.** (l, s).

DIOSCOREACEAE—*Dioscorea glabra var. pahangensis* (l, s, r); *D. hispida* (l, s, r); *D. piscatorium* (s, tuber); *D. scortechinii* (l, s, r, frt); *D. sp.* (s, tuber); *D. sp.** (wp).

DIPTEROCARPACEAE—*Shorea ovalis* (l, s, r).

EBENACEAE—*Diospyros argentea* (l, s, r, b); *D. malabarica* (l, b, r, frt); *D. malayana* (l, s, r, b, frt); *D. notans* (b, frt); *D. oblonga* (l, s, b, frt); *Same sp.** (l, s); *D. pendula* (l, s, r); *D. subrhomboidea* (l, s, r, b, frt); *D. sumatrana var. decipieus** (l and twigs, s, r); *D. sumatrana var. flavicans* (l, s, r, b); *D. wallichii* (l, s, r, b, frt).

ELAEOCARPACEAE—*Elaeocarpus petiolatus* (l, s, r, b, flr, frt); *E. sphaericus* (l, r, b, frt); *E. stipularis* (l, s, r); *E. sp.* (l, s, r, frt).

EUPHORBIACEAE—*Acalypha macrophylla** (l, s); *A. siamensis* (l, s); *Same sp.** (l, s); *Antidesma bunius** (l); *A stipularis* (l, s, r, frt); *A. velutinosum* (l, r,, s, b, frt); *Aporosa arborea* (l, s, r); *A. aurea* (l, s, r, b, frt); *A. benthamiana* (l, s, r, b, flr, frt); *A. confusa* (l, s, r, b, frt); *A. nervosa* (l, r, b); *A. selangorica* (l, s, r, b); *A. symplocoides* (l, s, r, b, frt); *Baccaurea brevipes* (l, s, r, b, frt); *B. griffithii* (l, s, b, frt); *B. lanceolata* (l, s, r, b, frt); *B. macrophylla* (l, s, frt); *B. motleyana* (l, s, p, frt); *Same sp.** (l, s); *B. parviflora* (l, s, r, b, frt); *Same sp.** (l, s); *B. sapida* (l, s, r, b, frt); *B. sp. aff. lanceolata* (l, r, frt); *B. sp.** (l, s, frt); *Blumeodendron tokbrai* (b, frt); *Bridelia tomentosa* (l, s, r, flr); *Same sp.** (l, s); *Claoxylon longifolium* (b, frt); *Cleistanthus hirsutulus* (l, s, r, b, frt); *Coccoceras muticum* (l, s, r, b, frt); *Croton argyratum* (r); *C. caudatus* (l, s, r, b); *C. griffithii* (l, s, r, b, flr); *C. sp.** (l, s); *C. sp.* (l, s, r, b, frt); *C. sp.* (l, s, r, b, frt); *Elateriospermum tapos* (l, s, frt); *Endospermum malaccense* (l, s, r); *Epiprinus malayana* (l, s, r, b, frt); *Euphorbia heterophylla* (wp); *E. neriifolia** (l, s); *Excoecaria agallocha* (l, b, s); *Galearia fulva* (l, s, r); *G. phlebocarpa* (l, s, r, b); *Glochidion superbum* (l, s, r, b, frt); *Hevea brasiliensis** (l, s, r, sd); *Jatropha curcas* (l, s); *J. gossypiifolia* (l, s, r); *Macaranga hypoleuca* (b); *M. lowii* (l, r, b); *M. robiginosa* (l, s, b); *M. tanaria* (l, s, r); *M. triloba* (l, s, r, b, frt); *Mallotus paniculatus* (l, s, r, b, flr, frt); *M. porterianus* (l, s, b); *Neoscortechina paniculata* (l, s, r, frt); *Phyllanthus frondosus** (l, s, r); *Pimeleodendron griffithianum* (l, s, r, b, frt); *Trigonostemon longifolius* (l, s, b); *T. villosus* (l, s, r),

FAGACEAE—*Castanopsis curtisii* (r, frt); *Pasania encleisocarpa* (r, b, frt); *P. semiserrata* (l, r, b, frt); *Quercus spicata* (l, r, b, frt).

FLACOURTIACEAE—*Barleria cristata** (l, s); *Casearia clarkeii* (l, s, r, b); *C. sp.* (l, s, r, b, frt); *Flacourtia rukam** (l, s); *Hydnocarpus sp.* (l, s, frt); *Osmelia maingayi* (l, s, r, b, frt); *Pangium edule* (l, r, b); *Ryparosa fasciculata* (l, s); *R. scortechinii* (l, s and frt, r).

FLAGELLARIACEAE—*Susum malayanum* (wp, frt).

GESNERIACEAE—*Cyrtandra cupulata* (l, s, r); *C. lanceolata* (l, s and R); *C. pilosa* (l, s, r); *Cyrtandromoea acuminata** (l, s, r).

GNETACEAE—*Gnetum gnemon** (l, s); *G. gnemon var. domesticum* (l, s, r); *G. gnemon var. tenerum* (l, s, r); *G. macrostachyum* (l, s, r, frt); *G. tenuifolium* (l, s, r); *G. aff. tenuifolium* (s, r, b, frt).

GRAMINEAE—*Brachiaria mutica** (wp); *Centotheca lappacea** (wp); *Lophatherum gracile* (r).

GUTTIFERAE—*Calophyllum inophylloide* (r, b, frt); *Garcinia atroviridis* (l, s, r); *G. bancana* (l, s, r, frt); *G. dulcis** (l, s); *G. eugeniaefolia* (l, s, r, b); *G. prainiana* (l, s, r, b, frt); *G. sp.* (l, s, r, b); *G. sp.* (l, s, r, b, frt); *G. sp.* (l, s, r, b, frt); *G. sp.* (l, s, r, b); *G. sp.** (l, s); *Kayea racemosa* (l, r, b, frt).

HYPERICACEAE—*Cratoxylon sp.** (l, s).

ICACINACEAE—*Gomphandra affinis* (l, s, r); *Phytocrene bracteata* (s, r, b, frt); *P. oblonga* (l, s, b, frt); *Sarcostigma aff. kleinii* (s, r, frt).

LABIATEAE—*Gomphostemma scortechnii* (l, s, r); *Hyptis capitata* (wp); *Orthosiphon grandiflorus** (l).

TABLE I—*continued.*

LAURACEAE—*Actinodaphne glomerata* (s, r); *Actinodaphne sesquipedalis* (l, s, r, b, frt); *Alseodaphne pedunculars* (l, s, r); *A. petiolaris* (l, s, r, b, frt); *Beilschmiedia sp.* (r, b, frt); *Cinnamomum iners* (l, s, r, b, flr, frt); *C. iners*• (l, s); *Cryptocarya griffithiana* (l, s, r, flr); *Lindera pipericarpa* (l, r, b); *Litsea cubeba* (l, s); *L. firma* (l, s); *L. robusta* (l, s, r, b, frt); *L. spathacea* (l, s, r, b, flr, frt); *L. spathacea var. tomentosa* (l, s, r); *L. sp.* (l, s, r); *L. sp.* (l, s, b, frt); *Nothophoebe panduriformis* (l, s, r, b); *Phoebe opaca* (l, s, r, b); *P. taroyana* (l, s, r); *Stemmatodaphne perakensis* (l, s, r, b, frt).

LECYTHIDACEAE—*Barringtonia cylindristachya* (l, s, r, b, frt); *B. fusiformis* (l, s, r, b); *B. macrostachya* (l, s, r, b, frt); *Couroupita guianensis*• (l).

LEGUMINOSAE—*Adenanthera pavonina*• (l, s, sd); *Alysicarpus nummularifolius* (wp); *Andira surinamensis*• (l, s); *Bauhinia calycina* (s, r, b, frt); *B. integrifolia* (l, s, r, b); *B. purpurea* (frt); *B. sp.*• (l, s); *Caesalpinia pulcherima*• (l, s, sd); *Calapogonium mucunoides* (l and s); *Calliandra surinamensis*• (l, s); *Camoensia maxima*• (l); *Cassia leschenaultiana* (wp); *C. nodosa* (l, s, flr); *C. occidentalis*• (l, s, r, sd); *C. surattensis*• (l, s, flr, frt); *Centrosema pubescens* (l and s); *Clitorea sp.*• (l, s, r, sd); *Crotalaria striata*• (l, s, r, sd); *C. verrucosa*• (l, s, r, sd); *Cynometra inaequifolia* (l, s, r, b, frt); *Derris malaccensis var. aptera* (l, s, frt); *D. sinuata*• (l); *D. sp.* (l, s, r, flr); *Desmanthus virgatus cf. also D. depressus* (s, r); *Desmodium ovalifolium*• (l, r, frt); *Entada scandans* (s, r, b, frt); *E. spiralis* (l, r, b); *Enterolobium saman*• (s, l); *Flemingia congesta*• (l, s, r, sd); *F. strobilifera* (l, s, r); *Glyricidia maculata* = *G. sepium* (l, s, frt); *Same sp.*• (l, s); *Indigofera teysmanii* (l, s); *Koompassia malaccensis* (l, s, r, b, frt); *Leucaena glauca*• (l, s, sd); *Milletia albiflora* (l, s, r, b, frt); *M. atropurpurea*• (l, s); *Mundulea sericea* (l, s, r); *Padbruggea maingayi* (s, b, sd); *Parkia singularis* (l, r, p); *P. speciosa*• (l, s, frt); *Peltophorum pterocarpum*• (s); *Phyllocarpus septentrionalis*• (l, s); *Pithecellobium jiringa*• (l, s); *Poinciana regia*• (l, s, flr); *Pterocarpus indicus* (l and s, b); *Pueraria phaseoloides* (l and s); *Saraca sp.*• (l, s); *Sesbania punctata* (l, s, r); *Swartzia pinnata*• (l, s); *Tephrosia maxima*• (l, s, r, sd); *Xylia kerrii*• (l, s).

LILIACEAE—*Dianella ensifolia* (wp); *Dracaena conferta* (l, s, r); *D. congesta* (wp); *D. finlaysoni* = *D. graminifolia* (l, s, r); *D. graminifolia*• (l, s); *D. umbratica* (l, s, r, b, frt); *Peliosanthes lurida* (wp); *Smilax barbata* (l, s); *Same sp.*• (l); *S. calophylla* (l, s, r); *S. myosotiflora* (l and s, r and tuber).

LINACEAE—*Ixonanthes icosandra*• (l, s); *R. griffithiana* (l, s, r, b); *Same sp.*• (l, s); *R. griffithii* (l, s, r).

LOGANIACEAE—*Fagraea sp.* (l, s, r); *Strychnos ovalifolia* (s, r).

LORANTHACEAE—*Dendrophthoe grandifrons* (l, s, r).

LYCOPODIACEAE—*Lycopodium cernuum*• (wp).

LYTHRACEAE—*Duabanga sonneratioides* (l, r, b, frt); *Lafoensia punicarfolia*• (l, s).

MALPHIGHIACEAE—*Brysonima crassifolia*• (l, s).

MALVACEAE—*Hibiscus macrophyllus*• (s, r); *Sida rhombifolia* (l, s, r); *Urena lobata*• (l, s).

MARATTIACEAE—*Angiopteris evecta* (wp).

MELASTOMACEAE—*Allomorphia malaccensis* (l, s, r); *Anplectrum pallens* (l, s, r, frt); *Blastus caudatus* (l, s,, r, flr, frt); *Clidemia hirta* (l, s, r); *Dissochaeta hirsuta* (l, s, r, flr); *Marumia rufolanata* (l, s, r); *Medinilla speciosa* (l, s, frt); *Melastome sp.* (l, s, r, frt); *Memecylon coeruleum*• (l, s); *M. dichotomum* (l, s, r, b, frt); *M. minutiflorum* (l, s, r, b); *M. myrsinoides* (l, s, b); *Phyllagathis rotundifolia* (l and s, r, flr) *Pogonathera pulverulenta* (l, s, r, frt); *Pternandra echinata* (l, s, r, b); *Same sp.*• (l, s); *Sonerilla begoniaefolia* (wp); *S. heterostemon* (wp).

MELIACEAE—*Aglaia argentea* (l, s, b, flr, frt); *Amoora ridleyi* (p, sd); *Carapa granatum*• (l); *C. guianensis*• (l, s); *Chikrasia tabularis* (r, b, p); *Chisocheton macrothyrsus* (s, r, b, rb); *C. sp.* (r, b, p, sd); *C. sp.* (l, s, r, b, frt); *C. sp.* (l, s, r, b); *C. sp.* (l, s, b, flr); *Dysoxylum arborescens* (l, s, r, frt); *D. cauliflorum* (l, s, r); *D. costulatum* (b, frt); *D. thyrsoideum* (r, b, p, sd); *D. sp.* (r, b, p, sd); *D. sp.* (l, s, r, b, frt); *D. sp.* (l, s, r, b, frt); *D. sp.* (l, s, r, b); *D. sp.* (l, s, r, b, frt); *Lansium domesticum* (l, s, r, b, frt); *Melia indica* (l, s, flr).

MENISPERMACEAE—*Arcangelisia loureiri* (l and s, r); *Coscinium wallichianum* (l, s); *Cyclea laxiflora* (wp); *Fibraurea chloroleuca* (l, s, r, b, frt); *Limacia oblonga* (l, s, r); *L. velutina* (l, s, r); *L. sp.* (l, s, b, r); *Tinomiscium petiolare* (l, s, r, frt); *Tinospora tuberculata* = *T. crispa* (s).

MORACEAE—*Artocarpus gomezianus* (r, frt); *Same sp.*• (l, s); *A. griffithii* (l, s, r, b, frt); *A. integer* (l, s); *Same sp.*• (l, s); *A. lanceifolius* (l, b, frt); *A. lowii* (s); *A. kemando* (l, s, p, sd); *Conocephalus sp.* (l, s, r); *Ficus alba* (l, s, r, b, frt); *F. annulata* (l, b, r, frt); *F. aurantiaca* (, s, r, frt); *F. aurata* (l, s, r, frt); *F. deltoidea var. lutescens* (l, s, r, b); *F. depressa* (s, b, frt); *F. fistulosa* (l, s, r, b, frt); *Same sp.*• (l, r, sd); *F. fulva* (l, s, b, frt); *F. geocarpoides* (l, s, b, frt); *F. kurzii*• (l, s); *F. lepicarpa* (l, s, r, frt); *F. montana var. arbuscula* (l, s, frt); *F. villosa*• (l, s); *F. sp.* (l, s, r, frt); *F. sp.*• (l, s); *Hulletia dumosa* (l, s, r); *Sloetia elongata* (l, s, r, b); *Treculia africana*• (l, s).

MYRISTICACEAE—*Gymnacranthera forbesii* (l, s, r, b, flr, frt); *Horsfieldia superba* (l, s, r, b, frt, sd); *Knema curtisii* (l, s, r, b, frt); *K. glaucescens var. patentinervia* (l, s, r, b); *K. laurina* (l, s, r, b, frt); *K. oblongifolia var. monticola* (l, s, r, b, frt); *K. cf. laurina* (r, b, sd, p); *Myristica hollnugii* (l, s, r, b, frt).

MYRSINACEAE—*Ardisia andamanica* (l, s, r); *A. colorata var. complanata* (l, s, r); *A. fulva* (l, s, r); *A. goodenoughii forma* (l, s, r); *A. lanceolata* (l, s, r, b, flr); *A. lurida* (l, r, b); *A. solanacea* (l, s, frt); *A. villosa* (wp); *A. sp.* (l, s, r, b); *Embelia garciniafolia* (s, r); *E. ribes*• (l, s, r, flr); *E. rugosa* (b, r, frt); *Labisia pothoina* (l, r): *L. pothoina var. alata* (l, s, r); *Maesa macrothyrsa* (l, s, r, b, flr); *M. ramentacea* (l, s, r, b, frt).

MYRTACEAE—*Eugenia grandis* (l, s, b, flr); *E. macrocalyx*• (l); *E. nigricans* (b, r, frt); *E. pachyphylla* (l, s, r, flr); *E. papillosa* (l, s, r, flr); *E. polyantha* (l, s, r); *Same sp.*• (l, s); *E. scortechinii* (l, r, frt); *E. tumida* (l, r); *E. variolosa* (l, s, b, flr); *Lecythis ollaria*• (l, s); *Psidium guajava*• (l, s); *Rhodamnia trinervia*• (l).

OCHNACEAE—*Gomphia lanceolata* (l, s, r, b).

OLACACEAE—*Erythropalum scandens* (l, s, r); *Ochanostachys amentacea* (l, s, r, b, frt); *Phlebocalymna pyriforme*• (l, s); *Phytocrene bracteata* (s).

OLEACEAE—*Jasminum bifarium* (l, s); *Linociera insignis* (l, s, r, b, frt); *Myoxopyrum nervosum* (l, s, r, frt).

ORCHIDACEAE—*Calanthe ceciliae* (wp); *Ceratostylis subulata* (wp); *Corymborchis veratrifolia* (l and s, r); *Oberonia anceps* (wp).

OXALIDACEAE—*Oxalis corniculatus*• (wp).

PALMAE—*Calamus orantus* (l, s, flr, p, sd); *C. sp.* (l, s); *C. sp.* (l, s, frt); *C. sp.* (l, s, frt); *C. sp.* (l, s, r, frt); *C. sp.* (l, s, r); *Caryota mitis* (l, r, s); *Orania macrocladus* (l, frt, Palm Cabbage); *Pinanga cf. disticha* (l, s, r); *Plectocomiopsis geminiflorus* (l, s, frt).

PASSIFLORACEAE—*Adenia sp.* (l, s, r, frt); *Passiflora foetida*• (l, r, frt); *P. laurifolia* (l and s).

PIPERACEAE—*Peperomia pellucida*• (wp); *Piper caninum* (l, s, r); *P. kurzii* (l, s, r); *P. muricatum* (l, s, r); *P. porphyrophyllum* (l, s, r); *P. ribesioides* (l, s, r); *P. ridleyi* (l, s, r); *P. sarmentosum*• (wp); *P. semangkoanum* (l, s, r); *P. stylosum* (l, s, r); *P. triandrum* (l, s, r); *P. sp.* (wp).

PLUMBAGINACEAE—*Plumbago zeylanica* (l, s, r).

POLYGALACEAE—*Polygala venenosa* (l, s, r); *Xanthophyllum affine* (l, s, r, b, frt); *X. palembanicum* (l, s, r, b).

POLYPODIACEAE—*Antrophyrum callifolium* (wp).

PROTEACEAE—*Buckinghamia celsissima*• (l, s); *Helicea attenuata* (l, s, r, b, frt).

RHAMNACEAE—*Ventilago malaccensis* (l, s, r, b).

TABLE I—*continued.*

RHIZOPHORACEAE—*Anisophyllea corneri* (s, r, frt); *A. griffithii* (r, b, frt); *Bruguiera cylindrica** (l); *Carallia suffruticosa* (l, s, r); *Pellacolyx axillaris* (s, r, b).

ROSACEAE—*Eriobotrya japonica** (l, s); *Parastemon urophyllum** (l, s); *Pyrus gramelosa* (l, s, r, b, frt); *Raphiolepis indica** (l, s).

RUBIACEAE—*Argostemma elatostemma* (wp); *Aulacodiscus premnoides* (l, s, r, b); *Canthium horridum* (l, s, r); *Chasalia pubescens* (l, s, r); *Coptosapelta flavescens* (l, s, r); *Greenia jackii* ((l, s, r); *Hedyotis cangesta* (l, s, r); *H. capitellata* (l, s, r); *Ixora congesta* (l, s, r); *I. javanica* (s, r); *I. kingstonii* (l, s, r, b, flr); *I. pendula* (l, s, r); *I. sp.** (l, s); *Lasianthus attenuatus* (l, s, r); *L. maingayi* (l, s, r); *L. sp.* (l, s, r); *Morinda citrifolia* (l, s, frt); *M. elliptica** (l, r, b, frt); *Mussaenda glabra* (l, s); *M. mutabilis* (l, b); *M. oblonga* (l, s, r); *M. villosa* (l, s, r, b, frt); *Mycetia javanica* (wp); *Nauclea junghuhnii* (l, r, b); *N. maingayi* (s, r); *N. subdita* (l, s, r, b, frt); *Ophiorrhiza communis* (l, s, r); *O. discolor* (l, s, r, flr); *O. hispidula* (s, r); *Pavetta graciliflora* (l, s, r, frt); *P. indica* (l, s, r, b); *Psychotria calocarpa* (l, s, r); *P. griffithii* (l, s, r); *P. malayana* (l, s, r, b, frt); *P. montana* (l, s, r); *P. sarmentosa* (l, s, r); *P. viridiflora* (l, s, r, b, flr); *P. viridifolia** (l, s, r, frt); *Randia anisophylla* (l, r, b, flr); *R. densiflora* (l, s, r, b, frt); *R. exaltata* (l, s, r, b, frt); *R. scortechinii* (l, s, r, b, frt); *R. stenopetala* (s, r); *Rennellia speciosa* (l, s, r); *Stylocoryne mollis* (l, s, r, b, frt); *Tarenna salicina* (l, s, r); *T. wallichii* (s, r); *Timonius wallichianus* (l, s); *Uncaria gambir* (s, r); *U. glabrata** (l, s, r); *U. parviflora* (l, s, r); *U. pteropoda* (l, s, r); *U. sclerophylla* (l, s, r, b); *Urophyllum blumeanum* (l, s, r); *U. glabrum* (l, s, r, frt); *U. griffithianum* (l, s, r); *U. trifurcum* (l, s, r, b, frt); *U. umbellatum* (l, s, r, b, frt); *Xanthophytum sp.* (l, s, r).

RUTACEAE—*Clausena harmandiana** (l); *Evodia latifolia* (l, s, r, b, frt); *E. cf. macrocarpa* (r); *Glycosmis sapindoides* (l, r); *G. chlorosperma* (l, s, r, b); *G. malayana* = *G. chlorosperma* (l, s, r); *Micromelum pubescens* (l, s, r, b, frt); *Paramignya lobata* = *P. andamanica* (r); *Tetractomia roxburghii* (l, s, b, frt); *Xanthophyllum obscurum* (l, s, r, b, frt).

SAPINDACEAE—*Lepisanthes cuneata* (l and frt, s); *L. sp.* (l, s, r); *Nephelium lappaceum* (l, s, r); *N. malaiense** (l, s); *N. mutabuie* (l, s, r, b); *Pometia pinnata* (l, s, r, b, frt); *Same sp.** (l, s); *Xerospermum sp.* (l, s, r, b).

SAPOTACEAE—*Chrysophyllum cainito** (l, s); *Madhuca longifolia** (l, s); *Manilkara kauki** (l, s); *Mimusops elengi** (l, s); *Palaquium hispidum* (l, frt); *Payena lucidia* (l, b, r, frt); *Same sp.** (l, s); *Pouteria sp.* (r, b, frt).

SCHIZAEACEAE—*Lygodium circinnatum* (l, s, r); *Schizaea digitata* (wp).

SCROPHULARIACEAE—*Curanga amara* (wp).

SIMARUBACEAE—*Eurycoma apiculata* (l, s, r); *E. longifolia* (l, s, r, wood).

SOLANACEAE—*Solanum torvum* (s, r); *S. verbascifolium* (l, s, r).

STERCULIACEAE—*Plerospermum jackianum* (B); *Scaphium macropodum* (b); *Sterculia laevis* (l, s, r); *S. macrophylla* (l, r, p, sd); *S. parviflora* (l, s, r, b, p, sd).

TACCACEAE—*Tacca cristata* (wp).

TERNSTROEMIACEAE—*Adinandra dumosa** (l, s); *Saurauja cauliflora* (l, r, b); *S. nudiflora* (l, s, r, b, flr); *S. tristyla* (l, s).

THEACEAE—*Pyrenaria acuminata* (l, s, r, b, frt).

THYMELAEACEAE—*Linostoma pauciflora* (r).

TILIACEAE—*Grewia antidesmifolia* (l, s, r, frt); *G. umbellata* (l, s, r); *Honckenya ficifolia* (l, s); *Trichospermum kurzii* (l, s, r, b, frt); *T. cymbiform* (l, s, r).

TURNERACEAE—*Turnera ulmifolia* (l, s, r).

ULMACEAE—*Gironniera nervosa* (l, s, r, b, frt); *Same sp.** (l, s); *Trema amboinensis* (l, s, r).

URTICACEAE—*Elatostema latifolium* (wp); *E. repens* (wp); *E. cf. integrifolia* (wp); *Laportea stimulans* (l, s, r); *Poikilospermum subtrinervium* (wp); *Pouzolzia viminea* (l, s, r); *Sloetia elongata** (l, s); *Streblus asper** (l).

VERBENACEAE—*Avicennia alba** (l); *Callicarpa furfuracea* (l, s, r, frt); *C. reeresii** (l, s); *Clerodendron fragrans* (l, s, r); *C. serratum* (l, s, r, flr, frt); *C. thomsonae** (l, s); *C. umbratile* (l, s, r); *C. villosum* (l, s, r); *Duranta repens** (l, s, frt); *Faradaya papuana** (l, s); *Gmelina arborea** (l, s); *Premma foetida* (l, s); *Stachytarpheta indica* (l, s, r); *Sphenodesme trifolia* (l, s); *Vitex heterophylla* (l, s, b, frt); *V. negundo* (l, s, r); *Same sp.** (l, s); *V. pubescens* (l, s, b, r); *V. vestita* (l, s, r, flr).

VIOLACEAE—*Alsodeia sp.* (l, s, r, b, frt); *Linoria sp.* (l, s, b, frt).

VITACEAE—*Leea sambusina* (s, b, frt); *L. sp.* (l, s, r, b); *Pterisanthes cissoides* (wp); *Vitis augustifolia* (l, s, b). *V. curtisii* (l, s, b, flr); *V. furcata** (l, s); *V. glaberrima* (wp); *V. kunstleri* (l, s, frt); *V. lawsoni* (l, s, frt); *V. lanceolaria* (l, s, frt); *V. mollissima* (l, s, r, frt); *V. novemfolia* (l, s, r); *V. penduncularis* (l, s, r, frt); *V. cf. scortechinii* (l, s, r, frt).

ZINGIBERACEAE—*Achasma sphaerocephalum* (l, s, r); *Alpinia rafflesiana* (s, r); *Amomum hastilabium* (l, s, r, p, sd); *Costus lucanngianus** *C. ridleyi* (l, s); *C. speciosa* (l,s, r); *Globba aurantica* (l, s); *Hornstedtia sp.** (wp); *Languas melanocarpa* (r); *Zingiber griffithii* (l, s, tuber).

TABLE II

PLANTS SOME PARTS OF WHICH HAVE GIVEN STRONG PRECIPITATES WITH EACH OF THE BUCHARDAT'S, DRAGENDORFF'S AND MAYER'S REAGENTS, AND WHICH ARE CONSIDERED TO CONTAIN ALKALOIDS

(Parts in parenthesis; abbreviations same as those in Table I.)

ACANTHACEAE—*Gendarusa vulgaris* (s, r).

ANONACEAE—*Cyathostemma hookeri* (r); *Fissistigma sp.* (s); *Miliusa longipes* (s, b, r); *Monocarpia marginalis* (r); *Oxymitra sp.* (l, s, r, b); *Phaenthus nutans* (l, b); *P. opthalmicus* (l, s, r, b); *Popowia pisocarpa* (l, b, s); *P. ramosissima* (l, s, r, b); *P. sp.* (l, s, r, b); *Trivalvaria macrophylla* (s, r); *T. pumila* (r).

APOCYANACEAE—*Alstonia spathulata* (b); *Chilocarpus costatus* (r); *Chonemorpha penangensis* (l, s); *Leuconotis griffithii* (l, s, r); *Lochnera rosea* (l, s, r); *Tabernaemontana coronaria* (l, s); *T. malaccensis* (l, s, r); *T. peduncularis* (l, r).

BORAGINACEAE—*Heliotropium indicum* (s).

CAMPANULACEAE—*Isotoma longiflora* (l, frt, r).

CONNARACEAE—*Connarus sp.* (flr).

DIOSCORACEAE—*Dioscorea hispida* (s, r).

EBENACEAE—*Diospyros subrhomboidea* (s, frt).

EUPHORBIACEAE—*Baccaurea motleyana* (frt).

ICACINACEAE—*Gomphandra affinis* (l, s, r); *Phytocrene bracteata* (frt); *P. oblonga* (frt).

LAURACEAE—*Actinodaphne sesquipedalis* (l, s, r, b); *Alseodaphne peduncularis* (l, s, r); *A. petiolare* (l, s, r, b, frt); *Lindera pipericarpa* (r); *L. spathacea* (r); *L. spathacea var. tomentosa* (r); *Phoebe opaca* (l, s, r, b); *P. taroyna* (l, s, r); *Stemmatodaphne perakensis* (l, s, r, b, frt).

TABLE II—*continued.*

LEGUMINOSAE—*Draccena conferta* (l); *Enterolobium saman* (l, s); *Indigofera teysmanii* (s); *Pterocarpus indicus* (l).
LOGANIACEAE—*Strycanos ovalifolia* (s, r).
MENISPERMACEAE—*Arcangelisia loureiri* (l, s, r); *Coscinium wallichianum* (l, s); *Cyclea laxiflora* (wp); *Fibraurea chloroleuca* (b, r); *Limacia oblonga* (r); *Tinomiscium petiolare* (s, r).
PALMAE—*Plectocomiopsis geminiflorus* (frt).
PIPERACEAE—*Piper porphyrophyllum* (l, s, r).
RUBIACEAE—*Coptosapelta flavescens* (r); *Hedyotis capitellata* (r); *Nauclea maingayi* (s, r); *Pavetta graciflora* (l, s, frt); *P. indica* (l); *Psychotria montana* (l, s, r); *Randia stenopetala* (r); *Uncaria gambir* (s, r); *U. parviflora* (l, s, r); *U. pteropoda* (l, s, r); *U. umbellatum* (l).
RUTACEAE—*Evodia lutifolia* (r); *Glycosmis sapindoides* (l, r); *G. malayana* (r).
SIMBARUBACEAE—*Eurycoma apiculata* (s, r).
SOLANACEAE—*Solanum verbascifolium* (r).

TABLE III

LIST OF PLANT MATERIALS FROM WHICH CRUDE ALKALOIDS HAVE BEEN ISOLATED

Alkaloids which have been isolated in crystalline form are mentioned in the remarks column. Some investigations (†) have been carried out by Dr. I. J. Pachter in the Smith, Kline and French Laboratories in Philadelphia. Abbreviations: wb = weak bases (extracted at pH ca. 9); sb = strong bases (extracted at pH >10); tb = total bases = wb + sb; qb = quaternary bases; rb = reduced bases. Abbreviations for parts of plants are the same as those in Table I).

Plant	Part	Per cent alkaloids	Remarks
ANONACEAE			
Desmos chinensis	r	0·05 wb	Paper chromatogram shows 8 to 10 bases present†
Popowia pisocarpa	l and s	0·02 tb†	
	l and s	0·71 qb†	
Trivalvaria pumila	s and r	0·09 wb	Paper chromatogram shows 2 to 3 bases present†
APOCYNACEAE			
Chonemorpha penangensis	s	0·08 wb	
	s	0·33 sb	
Hunteria corymbosa	l	0·15 wb	
	l	0·02 sb	
Tabernaemontana malaccensis ..	l	0·11 wb	Crystalline alkaloid $C_{21}H_{26}N_2O_2$, m.p. 220°, isolated (Chan, 1959)
	s	0·18 wb	
LAURACEAE			
Andrographis paniculata	l and s	0·02 wb	
Lindera pipericarpa	r	0·03 wb	
LEGUMINOSAE			
Enterolobium saman	l	1·54 sb	
LOGANIACEAE			
Fagraea fragrans	s	0·03 wb	
	s	0·07 rb	
MAGNOLIACEAE			
Michelia champaca	l	0·07 wb	
	l	0·035 sb	
	s	0·10 wb	
	frt	0·10 wb	
OLACACEAE			
Phlebocalymna pyriforme	s	0·003 wb	
PALMAE			
Plectocomiopsis geminiflorus	frt	0·45 sb	Principal alkaloid is plectocomine, $C_{11}H_{12}N_2O$, m.p. 294° (new alkaloid). Crystalline salts: acetate, m.p. 295°; picrate, m.p. 240°; hydrochloride and sulphate, m.p. >300° (Chan, 1959)
RUBIACEAE			
Coptosapelta flavescens	r	0·04 wb	
	r	0·03 sb	
	r	trace qb	
Nauclea maingayi	r	0·024 wb	
Pavetta indica	l	0·25 tb	
Randia macranta	l	0·08 tb	
Uncaria pteropoda	r	0·2 wb	Crystalline alkaloids isolated $C_{21}H_{24}N_2O_4$, m.p. 202–203°, $[\alpha]_D^{29} - 14°$; $C_{22}H_{28}N_2O_4$, m.p. 215–216°, $[\alpha]_D^{29} - 76°$
SIMARUBACEAE			
Eurycoma longiflora	r	0·03 wb	Paper chromatogram gave 5 spots under ultra-violet and 2 spots with Dragendorff's reagent
	s	0·045 wb	

Acknowledgements. The authors wish to thank the Director of Chemistry, the Adviser on Aborigines, the Chief Research Officer of Forest Research Institute, the Director of the Department of Agriculture, and Miss B. E. Copeland of St. John's Ambulance, Federation of Malaya, for the collection of plant materials; Professor H. B. Gilliland and Mr. Wong Phui Weng of the Botany Department, University of Malaya in Singapore, the Director of the Botanic Gardens, Singapore, Mr. K. M. Kochuman of the Forest Research Institute, Kepong, for the identification of plant specimens; Messrs. Smith, Kline and French Laboratories, Philadelphia, for a financial grant; and Professor R. A. Robinson (now at the University of New England, Australia) for his interest in this work.

References

Arthur, H. R. (1954). *J. Pharm. Pharmacol.*, 6, 66–72.

Bisset, N. G. (1957). *Proc. Symposium on Phytochemistry*, p. 125. UNESCO Science Co-operation Office for South East Asia, Djakarta.

Burkill, I. H. (1935). *Dictionary of Economic Products of the Malay Peninsula*, Vol. I and II, Government Printing Press.

Chan, K. C. (1959). M.Sc. thesis, University of Malaya in Singapore.

Douglas, B., and Kiang, A. K. (1957). *Malayan Pharm. J.*, 6, 1–16.

Kiang, A. K., and Douglas, B. (1957). *Proc. Third Congress, Pan Indian Ocean Science Assocn.*, Section G, 19–24.

Webb, L. J. (1949). *Australian Phytochemical Survey, Part I, Bulletin No.* 241, *C.S.I.R.O.; Part II, Bulletin No.* 268, *C.S.I.R.O.*

ANALGESIC AND ANTIPYRETIC PROPERTIES OF SOME ASPIRIN DERIVATIVES

By L. C. Weaver, Alice B. Richards and H. E. Martin

From the Pharmacology Department, Research Center, Pitman-Moore Co., Indianapolis 6, Indiana, U.S.A.

Received October 7, 1960

Several derivatives of acetylsalicylic acid have been tested for analgesic and antipyretic properties in mice and rats. *o*-Diphenylacetoxy benzoic acid (*O*-(diphenylacetyl)salicylic acid; DPA) is a superior analgesic but an inferior antipyretic to aspirin. DPA has a low toxicity: it failed to show significant anti-inflammatory or tranquillising properties. In dogs it produced only slight and transient falls in blood pressure and there was no appreciable effect on the direct or reflex muscle preparation.

The first synthetic ester of salicylic acid to be introduced into medicine was phenyl salicylate (Nencki, 1886). Later acetylsalicylic acid was prepared by Dreser (1899). The compound was named aspirin and has been perhaps the most successful synthetic drug in the whole field of medicinal chemistry. Since aspirin is one of the oldest remedies still occupying an important place in modern therapeutics, some of its analogues (Raczynski, 1943) were examined (see Table I).

Methods

Male albino mice of the Swiss-Webster strain, male albino rats of the Harlan-Wistar strain, and adult mongrel dogs, of both sex, were used. Animals had food and water except during the period of testing. All drugs were given either as aqueous solutions or as suspension in 1 per cent cellulose (CMC-120, high viscosity, Hercules Powder Co.) gum solution.

Analgesic Experiments

A thermal method was used for determining analgesic activity in mice (Chen and Beckman, 1951). All drugs for analgesic testing were administered intragastrically (i.g.) except morphine sulphate, which was given intraperitoneally (i.p.). Controls of distilled water (i.p.) and morphine were run simultaneously. Six animals were used at each dose level. The ability of drugs to block hydrochloric acid-induced writhing in mice was also determined (Vander, Wende and Margolin, 1956, and Eckhardt, Cheplovitz, Lipo and Govier, 1958). Simultaneous controls with hydrochloric acid are necessary.

Antipyretic Experiments

The effect of compounds given i.g. on normal body temperature and yeast-induced fever (Maren, 1951) was evaluated in rats. For temperature determinations, thermistor probes were inserted rectally into male rats confined in wire mesh tubes. Two control temperature readings at 30 min. intervals were made before drug injection and served as controls for each group of four animals; saline controls were run simultaneously.

Temperature readings were made at hourly intervals. For experiments with fevered rats, control readings were taken before yeast injection (3 ml. of a 15 per cent brewer's yeast suspension in saline subcutaneously) and again before drug administration. The temperature of the room was 24 ± 1°.

Acute Toxicity

The i.p. and i.g. lethal doses for 50 per cent of mice were determined (Litchfield and Wilcoxon, 1949) for *o*-diphenylacetoxybenzoic acid (*O*-(diphenylacetyl) salicylic acid; DPA).

TABLE I

TOXICITIES AND BLOCKADE OF IRRITANT-INDUCED WRITHING IN MICE

Compound	Writhing in mice	
	Dose mg./kg.	No. writhing / No. tested
Hydrochloric acid	10	84/90
Acetylsalicylic acid (*o*-hydroxybenzoic acid) (Aspirin)	50	24/30
	100	11/20
	200	1/10
2-Acetoxy-4,5, dimethylbenzoic acid (I)	50	20/20
	100	19/20
2-diphenylacetoxybenzoic acid (*O*-(diphenylacetyl)salicylic acid; DPA)	50	9/10
	100	19/20
	200	13/20
Methyl 2-diphenylacetoxybenzoate (II)	200	9/10
Ethyl 2-diphenylacetoxybenzoate (III)	200	10/10
Pentyl 2-diphenylacetoxybenzoate (IV)	200	8/10
Isopentyl 2-diphenylacetoxybenzoate (V)	200	10/10
Phenyl 2-diphenylacetoxybenzoate (VI)	200	9/10
Benzyl 2-diphenylacetoxybenzoate (VII)	200	10/10
Methyl 2-dibenzylacetoxybenzoate (VIII)	200	9/10
Ethyl 2-dibenzylacetoxybenzoate (IX)	200	10/10

Other Studies

Tests for antigranulomatous activity were made in male rats (Meier, Schuler and Desaulles, 1950). Tranquillising activity was assessed in rats by the method of Hughes and Kopmann (1960). Mongrel dogs were used to evaluate the effects of DPA on cardiovascular and neuromuscular systems.

RESULTS

Only two compounds, aspirin and possibly DPA at the highest dose, blocked irritant-induced writhing (Table I); aspirin was the most effective compound by this test. When groups of animals were challenged at

various intervals after drug administration of a dose of 200 mg./kg. i.g., DPA had a peak activity of 30 min. whereas aspirin was equally effective over a period of 2 hr. Of the salicylate derivatives listed in Table I only DPA showed significant analgesic effect by the thermal method. It can be seen in Fig. 1 that DPA at 200 mg./kg. i.g. was approximately equivalent to morphine sulphate at 5 mg./kg. i.p. All reaction times greater than 5 sec. were significantly greater ($P < 0.05$) than control.

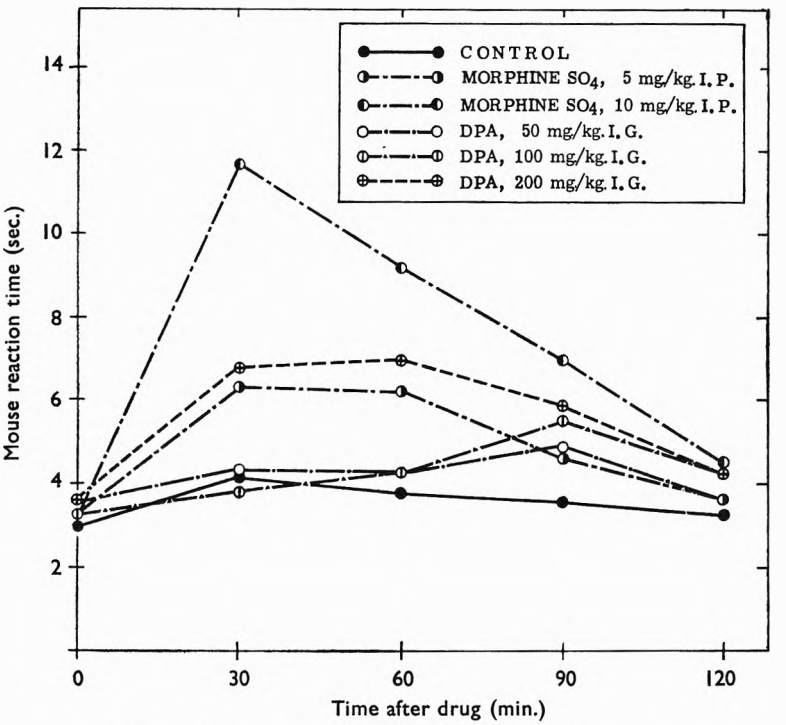

FIG. 1. Analgesic effects of morphine and DPA in mice.

Aspirin failed to show analgesia even at a dose of 400 mg./kg. i.g. DPA appeared to enhance the effects of morphine (Fig. 2) either when given in combination simultaneously or given separately 1 hr. apart. Aspirin used in combination with morphine did not show similar activity.

Antipyretic Activity

None of the compounds tested (DPA, II, VI, VIII) at 400 mg./kg. i.g. produced a decrease in normal body temperature of rats. However, DPA and aspirin were both effective in lowering the rectal temperature of rats with yeast-induced fever (Fig. 3).

Acute Toxicities

In mice the LD50 for DPA was 316(277·2–360·2) mg./kg. i.p. and 1,010 (878–1,162) mg./kg. i.g. Toxic signs appeared within 7 min.

by either route and consisted of tremors or shaking with arched back which proceeded to clonic convulsions and death within 30 min. to 24 hr. There was some moderate depression remaining at 24 hr.

Other Studies

DPA in doses of 50 and 200 mg./kg./day i.g. for 7 days failed to reduce the weights of granulomas in rats below those of controls.

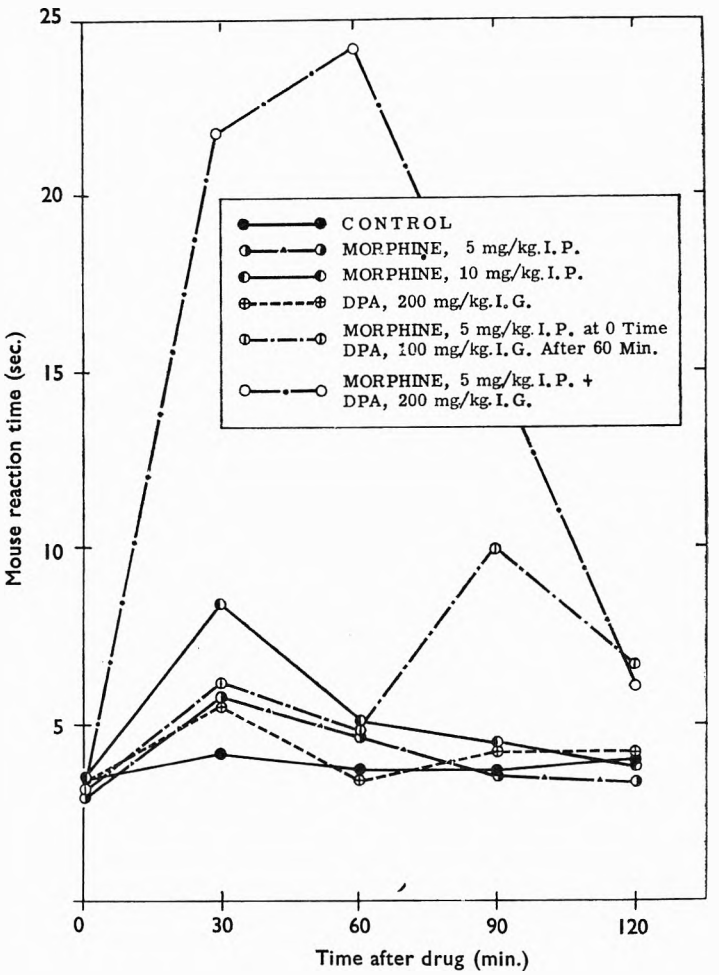

FIG. 2. Analgesic effects of morphine and DPA alone and in combination in mice.

DPA at 400 and 600 mg./kg. i.g. and morphine at 5 mg./kg. i.p. did not exhibit desirable tranquillising properties.

DPA in doses of 32 mg./kg. i.v. in dogs produced only slight and transient falls in blood pressure. Similarly there was no appreciable effect on the direct or reflex muscle preparation.

Discussion

Burger (1951) has noted the analgesic and antipyretic properties of several derivatives of salicylic acid were usually not as active as the parent compound. Similarly, the compounds reported in this study seem to follow this trend; the exception being DPA.

Most investigators agree that present day analgesic methods are inadequate for assessing the analgesic properties of aspirin; the writhing test being an exception to this general conclusion. However, the type of "pain" being measured by the writhing test is not completely understood.

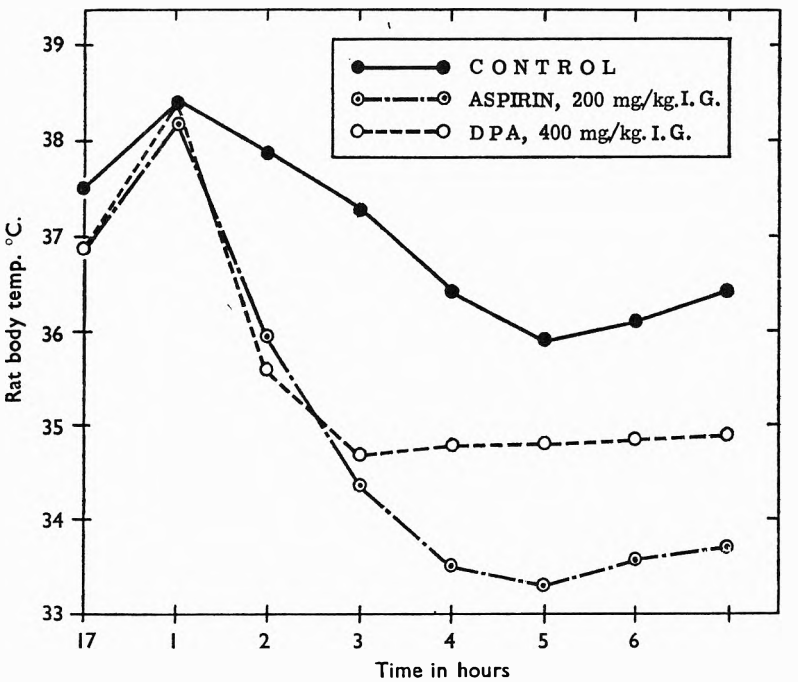

Fig. 3. Effect of aspirin and DPA on yeast-induced fever in rats.

It would seem logical, then, that when a derivative of aspirin is shown to possess analgesic properties by a thermal method, that compound might be a more potent analgesic than aspirin. DPA exhibited this ability in a dose of 200 mg./kg. i.g.; the analgesic effects were approximately equal to morphine at 5 mg./kg. i.p. (Fig. 1). Further, DPA added to, or enhanced, the analgesic properties of morphine sulphate when given simultaneously or separated by a 1-hr. interval (Fig. 2). Nevertheless, DPA was inferior to aspirin in its ability to counteract fevers in rats. Finally, DPA exhibited relatively little antigranuloma, or tranquillising activity in rats and, with dogs, there were negligible effects on blood pressure and direct and reflex muscle preparations The low toxicity exhibited suggests that it should be evaluated further.

Acknowledgements. We are indebted to Dr. Elva G. Shipley, Director, The Endocrine Laboratories, Madison, Wis., for antigranuloma testing, to Jessa Tempke for technical assistance.

References

Burger, A. (1951). *Medicinal Chemistry*, vol. 1, p. 201, New York: Interscience.
Chen, J. Y. P., and Beckman, H. (1951). *Science*, **113**, 631.
Dreser, H. (1899). *Arch. ges. Physiol.*, **76**, 306–318.
Eckhardt, E. T., Cheplovitz, F., Lipo, M., and Govier, W. M. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 186–188.
Hughes, F. W., and Kopmann, E. (1960). *Arch. int. Pharmacodyn.*, **126**, 158–170.
Litchfield, J. T., Jun., and Wilcoxon, F. (1949). *J. Pharmacol.*, **96**, 99–113.
Maren, T. H. (1951). *Ibid.*, **101**, 313–316.
Meier, R., Schuler, W., and Desaulles, P. (1950). *Experientia*, **6**, 469–471.
Nencki, M. (1886). *Arch. exp. Path. Pharmacol.*, **20**, 396.
Raczynski, W. (1943). Doctoral Thesis, Indiana University, Bloomington, Ind.
Vander Wende, C., and Margolin, S. (1956). *Fed. Proc.*, **15**, 494.

A NOTE ON BACTERIOLOGICAL TOXICITY TESTS OF SILICONE RUBBERS FOR MEDICAL AND PHARMACEUTICAL USES

By I. H. Riley and H. I. Winner

From Midland Silicones Ltd., Barry, Glam., and the Department of Clinical Pathology, Charing Cross Hospital, London

Received September 9, 1960

Tests of the effect on the growth of a number of organisms of several silicone rubbers has been made. The toxicity of suitably compounded rubbers which could be used in pharmacy, medicine and in contact with foodstuffs is very low.

Properly formulated and processed silicone rubbers have been found useful in medicine and pharmacy because of their general inertness, non-toxic and non-irritant properties. Typical applications have been blood transfusion tubes, drainage tubes, baby bottle teats and pharmaceutical vial stoppers. The rubbers used in these applications have generally been formulated to contain, when processed, only silicone polymer, usually a polydimethyl siloxane, and a fine particle size filler, either an amorphous precipitated or a fume silica.

The elastic properties of silicone rubbers are obtained by making a cross-linked structure between the polymer and the filler by means of curing agents which are usually organic peroxy compounds, for example, benzoyl peroxide. In the past it has been felt necessary to ensure removal of the decomposition products of these curing agents and silicone rubbers intended for medical and pharmaceutical applications have, therefore, usually been given long cures in air at high temperatures, for example, 24 hr. at 250°.

For some applications like baby bottle teats, it would be advantageous if a lesser cure than 24 hr. at 250° could be given, since better physical properties, particularly tear strength, would then be obtained. Accordingly it was decided to arrange a series of bacteriological toxicity tests of such rubbers, to assess suitable cures and formulations for rubbers for medical use.

Details of Rubber Formulations

Most modern general purpose silicone rubber stocks are formulated with siloxane polymers having predominantly methyl side groups with a small proportion of unsaturated groups, usually vinyl. One advantage of these vinyl groups is that they enable a wider range of peroxy compounds to be used as curing agents.

Since it was thought that any toxic effects of the cured rubbers were likely to arise from decomposition products of the curing agents, the effects of using several curing agents at various states of cure in a base mix consisting of a polymethyl vinyl siloxane polymer and a fume silica filler, were investigated.

Three curing agents were chosen for the tests, 2,4-dichlorobenzoyl peroxide, dicumyl peroxide and di-tertiary-butyl peroxide. The 2,4-dichloro compound was used because it is the curing agent for most silicone rubber stocks. Dicumyl peroxide was included as an example of the newer curing agents used with polymers containing vinyl groups and so was di-tertiary-butyl peroxide. This latter compound was also thought to have highly volatile decomposition products and might, therefore, show a lower toxicity.

For comparison purposes some tests were also included on two proprietary grades, Silastomer 156 and DP.2452 (Midland Silicones Ltd.). Silastomer 156 is a translucent silicone rubber based on a dimethyl polymer and fume silica filler which has been widely used in medical and pharmaceutical applications and DP.2452 is a more recent development based on a polymethyl vinyl siloxane polymer.

Various formulations were made from a rubber masterbatch containing 100 parts by weight of a polymethyl vinyl siloxane gum and 37·5 parts by weight of a fume silica. This was prepared by mixing 10 parts by weight of Aerosil K3 (Bush Beach and Segner Bayley, Ltd.), with 100 parts by weight of Polysil 2432 (Midland Silicones Ltd.).

Formulation	A	B	C
Rubber masterbatch	100	100	100
2,4-Dichlorobenzoyl peroxide*	0·65	—	—
Dicumyl peroxide	—	0·68	—
Di-tertiary-butyl peroxide	—	—	0·9

* Added as a 40 per cent dispersion of the peroxide in a dimethyl silicone fluid.

Toxicity Tests

There is no generally accepted test of toxicity for medical rubbers. For transfusion rubbers, the toxicity test in common use tests the inhibitory action of the rubber on the growth of a single species of bacteria, *Streptococcus pyogenes*, on a solid medium. This test is described and recommended in the appropriate British Standard (B.S. 2463 : 1954, Appendix B, page 12). Inhibition of bacterial growth by the substance under test is taken as an indication of its unsuitability for transfusion purposes.

The toxicity test used by us was an extension of the B.S. test for transfusion rubbers, by testing the samples against a number of different bacterial species, instead of against one species only.

Test Method

Samples of the various rubbers were prepared by moulding sheets of nominal thickness 1/16 in. in a press at a temperature appropriate to the curing agent. Subsequent cures in a standard air circulating oven varied from 1 hr. at 150° to 24 hr. at 250°. Pieces of sheet 0·5 × 0·5 cm. were used in the tests. These samples were subjected to one of two types of preliminary treatment: (1) washing in acetone and water, and drying between strips of blotting paper, or (2) sterilising in a domestic pressure cooker for 20 min. at 15 lb./sq. in. steam pressure.

Blood agar plates were heavily inoculated over the entire surface with young glucose broth cultures of the particular bacterium. Pieces of silicone rubber sheeting, after preliminary treatment, were placed at intervals over the surface of the plates. The plates were then incubated at 37° for 24 hr., and then examined. Any inhibition of bacterial growth around the rubber samples were noted. The bacteria used were *Streptococcus pyogenes*, *Staphylococcus pyogenes*, *Bacterium coli*, *Pneumococcus sp.* (except with some washed samples of rubber), *Streptococcus viridans* (with autoclaved, and with a few washed samples) and *Pseudomonas aeruginosa* (with washed samples only).

Results

The results indicate an extremely low toxicity. Even where some inhibition of bacterial growth has been observed, the effect is very small.

TABLE I

THE EFFECT OF DIFFERENT RUBBER FORMULATIONS ON THE GROWTH OF FIVE DIFFERENT BACTERIA. THE SAMPLES WERE AUTOCLAVED FOR 20 MIN. AT 15 LB./SQ. IN. STEAM

Formulation	Oven cure	*Strep. pyogenes*	*Staph. pyogenes*	*B. coli*	*Pneumo coccus*	*Strep. viridans*
A	Nil	0	0	0	0	0
	4 hr./150°	0	0	0	0	0
	4 hr./200°	0	0	0	0	0
	4 hr./250°	0	0	0	0	0
B	Nil	0	0	0	0	0
	4 hr./150°	0	0	0	0	0
	4 hr./200°	0	0	0	0	0
	4 hr./250°	0	0	0	0	0
C	Nil	0	0	0	0	*
	4 hr./150°	0	0	0	0	0
	4 hr./200°	0	0	0	0	0
	4 hr./250°	0	0	0	0	0
Silastomer 156 ..	Nil	0	0	0	*	*
	4 hr./150°	0	0	0	0	*
	4 hr./200°	0	0	0	*	*
	4 hr./250°	0	0	0	0	*
DP.2452	Nil	0	0	0	0	0

0 indicates no detectable inhibition of growth. * indicates inhibition of growth.

The results of some of the tests on autoclaved samples are given in Table I which includes nearly all the positive results but only a small proportion of the negative ones.

Formulations A and B had no detectable effect on the growth of any of the organisms tested both with washed and autoclaved samples and using rubbers at all states of cure. Formulation C gave similar good results in most tests but slight inhibition of the growth of *Streptococcus viridans* was detected with two samples (press cured and oven cured 1 hour/150°) prepared by autoclaving.

Of all the rubbers tested Silastomer 156 gave the greatest number of inhibitory results though in all tests the effect was small. In addition to those noted in Table I, a washed sample of Silastomer 156 oven cured for 4 hr./250° and one of press cured DP.2452, caused slight inhibition of the growth of *Streptococcus pyogenes*. These are the only two of the

samples tested which would have been found unsuitable for transfusion purposes according to the test recommended in the British Standard for transfusion rubbers.

Conclusions

Suitably compounded and processed rubbers have a very low toxicity which makes them suitable for use in pharmacy, medicine and in contact with food stuffs. For the most stringent applications a silicone rubber compounded from a polymethyl vinyl siloxane, a fume silica and either dicumyl or 2,4-dichlorobenzoyl peroxide is to be recommended.

POLYHYDROXY (CATECHOLIC) PHENOLIC ACIDS—STUDIES OF THEIR METABOLISM IN MAN

By S. L. Tompsett

From the Biochemical Laboratory, Northern General Hospital, Edinburgh

Received October 28, 1960

The urinary excretion of glycine and glutamine conjugates has been studied after the oral ingestion of some phenolic acids. The stability of such conjugates and also of the parent substances to various forms of acid hydrolysis has been made. Further observations on the 4-hydroxy-3-methoxy- and *m*-hydroxyphenolic acids present in human urine have been made.

The metabolism of aromatic substances in man is increasingly studied since it is believed that deviations from the normal may be associated with certain diseases. The metabolism of catecholic phenolic acids in man has been previously discussed (Tompsett, 1958a, 1959, 1960) and the present paper is an extension of these studies.

The following problems have been investigated. The effect of hot acid hydrolysis upon the stability of a number of phenolic acids. The evidence for conjugation with glycine or glutamine, or both, after the oral administration of a number of phenolic acids. The fractionation of methoxy phenolic acids present in urine. The presence of *m*-hydroxybenzoic and vanillic acids in urine and the metabolism of caffeic acid.

Experimental

Hydrolysis of urinary conjugates. (*a*) 10 ml. of urine and 1 ml. of 10 N hydrochloric acid were placed in boiling water bath for 1 hr., or (*b*) 10 ml. of urine and 10 ml. of 10 N hydrochloric acid were heated under a reflux condenser for 1½ hr.

Extraction of phenolic acids. Urine which had been treated as (*a*) or (*b*) above was extracted three times with 4 vol. of ether, and the extracts were evaporated to dryness.

The Determination of Conjugated Glycine and Glutamine

Glycine and aspartic and glutamic acids were measured in urine (1) untreated and (2) after hot acid hydrolysis. It was assumed that the difference between (2) and (1) would represent conjugation with glycine and glutamine respectively. To effect hydrolysis of the conjugates, 10 ml. of urine and 10 ml. of 10 N hydrochloric acid were boiled under a reflux condenser for 1½ hr. The mixture was then evaporated to dryness *in vacuo* in an all-glass still. The residue was dissolved in water, and the volume made up to 10 ml.

(i) *The determination of glycine.* The method of Smith (1953) was used. 1 ml. of urine [(1) before or (2) after hydrolysis with 5 N hydrochloric acid] was distilled in the presence of buffer pH 6·5, and ninhydrin. The formaldehyde content of the distillate, which is representative of the original amount of glycine present, was measured colorimetrically with

chromotropic acid. It has been shown that hippuric acid does not react until after hydrolysis in the presence of hot 5 N hydrochloric acid.

(ii) *The determination of aspartic and glutamic acids.* Aspartic and glutamic acids may be conveniently separated from the neutral and basic amino-acids by electrophoresis.

Apparatus: EEL electrophoretic apparatus. Whatman filter paper No. 1—34 × 5 cm. Reagent: phosphate buffer, pH 7·0/0·05 M.

Method. Examinations were duplicated. 20 μl. of urine [(1) before or (2) after hydrolysis with 5 N hydrochloric acid] were applied to the centre of each paper located in the electrophoresis apparatus. A potential difference of 2 mA per paper strip was applied for 6 hr. After drying, the positions of the three amino-acid fractions were located by means of the ninhydrin reaction on one strip. The dicarboxylic amino-acid fraction was then determined in the duplicate strip by the method of Smith and Tompsett (1954).

Paper Chromatography of Phenolic Acids

The Kawerau Unit was used and the developing solvent was benzene: acetic acid: water (Tompsett, 1958b).

Ether extracts of urine hydrolysed in the presence of 5 N hydrochloric acid (*b*) were prepared and alcoholic solutions of the residues applied to the paper. Extract equivalent to 2·5 ml. of urine was applied to the paper.

(i) *The fractionation of methoxy phenolic acids.* Developed paper chromatograms were dried and divided into 10 equal strips within the limits R_F 0·0 to 1·0. The strips were extracted with ethanol which was removed by evaporation and the methoxyl content of the residue determined (Tompsett, 1959). Owing to the low sensitivity of this reaction, the extracts from six separate chromatograms were combined.

(ii) m-*Hydroxybenzoic and vanillic acids.* The chlorimide reaction (Tompsett, 1958b; 1959; 1960) was applied to extracts obtained from strips corresponding to an R_F 0·1 to 0·5 for *m*-hydroxybenzoic acid, and an R_F 0·5 to 0·7 for vanillic acid.

Results and Discussions

The Hydrolysis of Conjugates

Although phenolic substances can be detected in urine in the free state, they are for the most part excreted as conjugates with sulphate, glucuronic acid, glycine and sometimes glutamine. The mode of conjugation can be of interest, yet confusion can result in the identification and determination of individual phenolic substances unless these are examined in the free state. Adequate methods of hydrolysis are however necessary.

Hydrolysis in the presence of N hydrochloric acid generally liberates phenolic substances from conjugation with sulphate, acetic acid and glucuronic acid. Conjugates containing glycine are quite resistant to such treatment, hydrolysis in the presence of 5 N hydrochloric acid being required. It is assumed that glutamine conjugates require the same

method of hydrolysis. The methoxyl group is resistant to both hydrolytic treatments, heating with concentrated sulphuric acid being essential.

It was considered of importance to examine the effects of acid hydrolysis upon the stability of a number of parent substances. The following substances were found to be unaffected by hot acid hydrolysis in the presence of 5 N hydrochloric acid:

o-, *m*- and *p*-Hydroxybenzoic acids, *o*-, *m*- and *p*-hydroxyphenylacetic acids, 3,4-dihydroxybenzoic acid, vanillic acid and *o*- and *p*-aminobenzoic acids. Substituted cinnamic acids, e.g., caffeic, ferulic and *o*-hydroxycinnamic acids, are completely destroyed by such treatment and cannot be

TABLE I

THE URINARY EXCRETION OF HIPPURATES AFTER THE ORAL INGESTION OF SOME PHENOLIC ACIDS AND RELATED COMPOUNDS

	Hippurates (mg. glycine/8 hr. urine)
Control	125
After 1 g. salicylic acid	210
Control	110
After 1 g. salicylic acid	225
Control	98
After 1 g. salicylic acid	235
Control	96
After 1 g. *m*-hydroxybenzoic acid	198
Control	88
After 1 g. *m*-hydroxybenzoic acid	215
Control	115
After 1 g. *m*-hydroxybenzoic acid	218
Control	92
After 1 g. *p*-hydroxybenzoic acid	238
Control	82
After 1 g. *p*-hydroxybenzoic acid	242
Control	78
After 1 g. 3,4-dihydroxybenzoic acid	198
Control	82
After 1 g. 3,4-dihydroxybenzoic acid	225
Control	110
After 1 g. caffeic acid	210
Control	82
After 1 g. caffeic acid	240
Control	115
After 1 g. tryptophan	238
Control	98
After 1 g. tryptophan	184

Combined glutamic acid (glutamic conjugates)—not detectable.

recognised by the usual reactions. These substances appear, however, to be stable to hot acid hydrolysis in the presence of N hydrochloric acid.

Armstrong and Shaw (1955) have reported the instability of *m*-hydroxyphenylhydracrylic acid when heated in the presence of strong mineral acid.

Glycine and Glutamine Conjugation

An assessment was made of glycine and glutamine conjugation as the result of the oral administration of some phenolic acids. Examinations were made on the night urine (11 p.m. to 7 a.m.) to minimise the effect of diet. Assessment was made on changes in the quantity of combined glycine or the dicarboxylic amino-acid (glutamic + aspartic) fraction. The results of this investigation are shown in Table I. In each experiment there was evidence of glycine conjugation but none for conjugation with glutamine.

The Fractionation of the Methoxy Phenolic Acids of Urine

The object of the investigation was to identify the principal methoxy phenolic acids of urine and in particular whether dimethoxyphenolic acids existed in any quantity. An assessment of the ferulic and 4-hydroxy-3-methoxymandelic acid contents were excluded by the drastic method of

TABLE II

THE DISTRIBUTION OF METHOXY PHENOLIC ACIDS FROM A URINE EXTRACT ON A PAPER CHROMATOGRAM (BENZENE : ACETIC ACID : WATER)

	R_F (average)	Per cent of the total
Homovanillic acid fraction	0·50	52
Vanillic acid fraction	0·75	38
Methoxyphenylacetic acid fraction	0·90	8
	Colorimetric reactions. Folin-Ciocalteu reaction	2,6-Dichloroquinone chlorimide reaction
Homovanillic acid	+	–
Vanillic and ferulic acids	+	+
p- and *m*-Methoxyphenylacetic and veratric acids	–	–

acid hydrolysis employed which results in the destruction of these substances.

Results of this investigation are shown in Table II. It will be noted that the greatest part of the methoxy phenolic acids exist as vanillic and homovanillic acids. Dimethoxy phenolic acids, if present, constitute a minor fraction.

m-*Hydroxybenzoic and Vanillic Acids in Urine*

Determinations have been made on 10 urines and the results are shown in Table III. The ranges of excretion are very wide. This is to be expected since these substances have a dietary origin. Urinary vanillic

TABLE III

VANILLIC AND *m*-HYDROXYBENZOIC ACIDS IN HUMAN URINE. THE RESULTS ARE EXPRESSED IN MG./DAY

	Vanillic acid	*m*-Hydroxybenzoic acid
1	12·6	12·6
2	23·8	31·8
3	19·2	43·6
4	30·8	18·2
5	45·6	46·8
6	28·4	18·2
7	41·6	35·2
8	35·8	41·6
9	49·2	78·6
10	128	310

acid is undoubtedly derived from two distinct sources, from the metabolism of 3,4-dihydroxyphenolic substances, e.g., caffeic acid and from ingested 4-hydroxy-3-methoxyphenolic substances, e.g., vanillin.

Since a large number of phenolic acids have been identified in urine, some reference to substances estimated by the chlorimide method is merited. The drastic form of acid hydrolysis employed, eliminates those

phenolic acids possessing a cinnamic acid structure and prevents interference by hippurates in the paper chromatographic procedure. It is believed that vanillic acid measured after separation by paper chromatography does represent a specific determination.

An extract obtained after paper chromatography would contain, in addition to *m*-hydroxybenzoic acid, such substances as *o*- and *m*-hydroxyphenylacetic acids, both of which react to produce blue colours. *m*-Hydroxyphenylacetic acid behaves similar to *m*-hydroxybenzoic acid in that the blue colour is not extractable by butanol. Since both appear to have a similar metabolic origin, this should produce little confusion in the interpretation of investigations concerning the formation of *m*-hydroxyl compounds. Under normal conditions, the urinary excretion of *o*-hydroxyphenylacetic acid is about 1 mg./day (Armstrong and others, 1955). This substance reacts to produce a blue colour which is extractable by butanol, hence little interference should result from its presence.

The Metabolism of Caffeic Acid

The metabolism of caffeic acid in man is of interest since in the form of the conjugate, chlorogenic acid, it has a wide distribution in natural

TABLE IV

URINARY EXCRETION OF METABOLITES AFTER THE ORAL INGESTION OF 1 G. CAFFEIC ACID. URINE WAS COLLECTED FOR 8 HR. AFTER INGESTION

Catecholic phenolic acids*	117 mg.
m-Hydroxybenzoic acid†	184 mg.
Vanillic acid†	110 mg.
Other methoxy phenolic acids‡	210 mg.

* Identified by paper chromatography to consist almost entirely of 3,4-dihydroxybenzoic acid. Expressed in terms of 3,4-dihydroxybenzoic acid.

† Determined by means of the chlorimide reaction.

‡ Calculated by difference between total methoxy phenolic and vanillic acid content. Expressed in terms of vanillic acid.

products. Chlorogenic acid, a conjugate of caffeic acid with quinic or isoquinic acid, is readily hydrolysed by treatment for 1 hr. in a boiling water bath in the presence of N hydrochloric acid. In the Mitchell reaction, chlorogenic acid produces a yellow colour and caffeic acid the typical purple colour.

An examination has been made of the urinary excretions of some metabolites after the ingestion of 1 g. of caffeic acid. The night urine was used and a correction has been applied by the examination of a control. Three determinations were made, total methoxy phenolic acids (Tompsett, 1959), phenolic acids reacting in the Mitchell reaction (Tompsett, 1958a), and vanillic and *m*-hydroxybenzoic acids. Acid hydrolysis in the presence of 5 N hydrochloric acid was made as a preliminary to these examinations.

Typical results are shown in Table IV. Naturally only some of the varied products would be measured. The catecholic phenolic fraction would not include caffeic acid since this substance is destroyed in the initial hot acid hydrolysis. For similar reasons, ferulic acid cannot be identified individually. By means of paper chromatography, it was

found that the catecholic phenolic fraction consisted very largely of 3,4-dihydroxybenzoic acid.

Human urine has been shown to contain a variety of phenolic acids. The majority of these may be described as metabolites, the result of methylation, de-hydroxylation, oxidation, reduction, or conjugation. Against such a complex background, the detection and determination of individual phenolic acids may prove a difficult and laborious process. Many investigations are concerned mainly with the examination of a particular mode of metabolism and such, the patient's health permitting, are simplified by the study of the fate of an administered substance.

References

Armstrong, M. D. and Shaw, K. N. F. (1957). *J. biol. Chem.*, **225**, 269–278.
Armstrong, M. D., Shaw, K.N.F. and Robinson, K. S. (1955). *Ibid.*, **213**, 797–811.
Smith, D. C. (1953). *J. Med. Lab. Technol.*, **11**, 205.
Smith, D. C. and Tompsett, S. L. (1954). *J. Clin. Path.*, **7**, 79–80.
Tompsett, S. L. (1958a). *J. Pharm. Pharmacol.*, **10**, 157–161.
Tompsett, S. L. (1958b). *Clin. Chem. Acta*, **3**, 149.
Tompsett, S. L. (1959). *J. Pharm. Pharmacol.*, **11**, 32–35.
Tompsett, S. L. (1960). *Ibid.*, **12**, 62–64.

NOTES ON THE ORIGIN AND SIGNIFICANCE OF THE PEROXIDE VALUE OF ANHYDROUS LANOLIN

By E. W. Clark and G. F. Kitchen

From Westbrook Lanolin Company, Argonaut Works, Laisterdyke, Bradford 4

Received December 12, 1960

Wool fat on the fleece has a high peroxide value because of autoxidation, this value falls greatly during washing of the wool and grease-recovery as a result of biological or chemical reduction. It increases again if anhydrous lanolin refined from this grease is bleached by oxidation. Peroxide values are useful as a measure of autoxidation only if values of the surface and lower layers are compared.

Anhydrous lanolin (referred to hereafter as "lanolin") when newly-refined and before oxidative bleaching processes, is invariably of low peroxide value as is also the crude wool fat from which lanolin is refined. But ample evidence has been published (Lifschutz, 1924; Drummond and Baker, 1929; Freney, 1940; Gillam, 1948) of the extensive oxidation of wool fat on the fleece and which would be expected, therefore, to have a high peroxide value. No peroxide values of such wool fat have been published therefore we have investigated the apparent anomaly.

Experimental

Three types of wool, between 1 and 2 years old, were treated with hot diethyl ether to extract most of the wool fat, the ethereal solutions being filtered and evaporated to dryness on a water bath as quickly as possible under a stream of nitrogen. Whilst these wools were being washed at the woolcombing factory, and the grease recovered by the normal centrifugal processes, further samples of fat were obtained at different stages of the processing, in one instance the sampled scouring liquor, after being allowed to stand for 24 hr. at room temperature, was then boiled for 1 hr. before extracting further quantities of fat with ether.

Peroxide values of all the wool fat samples were determined using the simplified Lea (1938) method and expressing results as ml. of 0·002N thiosulphate per g. of sample.

Results

The results are given in Table I.

TABLE I

Peroxide values of all wool fat samples

Sample	Peroxide value
Fat from 66–70^s wool	46·5
Fat from 64^{s}B Australian wool	73·5
Fat from 74^s Australian wool	49·5
Average of the 3 wools	56·5
Fat from wash-bowl liquor	15·5
Fat from liquor after standing 24 hr.	12·0
Fat from liquor after boiling 1 hr.	5·7
Crude wool grease from centrifuges	3·5

Discussion

The results show that the wool fat on the fleece had a high peroxide value, but that when the fat was emulsified in the scouring liquor (containing soap and sodium carbonate) the value rapidly fell, reaching a very low level in the grease recovered from the centrifuges. It seems probable that there was a reduction of the peroxides by chemical or biological reducing agents from the wool and activated by conditions in the emulsion. Under the relatively anhydrous conditions on the fleece these agents were presumably inactive or their effect out-paced by aerial oxidation.

Unbleached lanolins produced from centrifugally-recovered wool fat have been found to have peroxide values between 1 and 12 depending on processing methods. A higher peroxide value of lanolin, however, is not indicative of autoxidation unless the peroxide value of the surface is significantly higher than that of the underlying bulk, since autoxidation of lanolin during storage affects only a thin surface layer (Clark and Kitchen, 1961). A high peroxide value for the whole of lanolin is a normal result of oxidative bleaching processes which are used because of the call for lanolin of the palest possible colour. A peroxide value produced in this way is not accompanied by the large changes in acidity, unsaponifiables and cholesterol content which accompany a similar peroxide value resulting from gradual autoxidation. Odour improves rather than deteriorates during bleaching, confirming that the odour of highly autoxidised lanolin is not directly attributable to peroxides but to their breakdown products. Our results suggest that these develop no more rapidly in a bleached lanolin than in one of low original peroxide value.

That a high peroxide value in bleached lanolin is no detriment to its general application is made obvious by the preference which has always been shown for such lanolins by users. Only recently has an isolated disadvantage been reported, here batches of penicillin ointment prepared from lanolin of high peroxide value were found to have poor shelf life (Diding and Sandell, 1949).

Thus, the recent tendency to regard lanolin as being autoxidised or rancid because of a high peroxide value is based upon an unsound comparison with other fats and oils. If the specification of a maximum limit for peroxide value should be contemplated, it should logically only be considered for special circumstances which arise such as that of penicillin ointment.

References

Clark, E. W. and Kitchen, G. F. (1961). *J. Pharm. Pharmacol.*, **13**, in the Press.
Diding, A. and Sandell, E. (1949). *Svensk farm. Tidskr.*, **53**, 617–621.
Drummond, J. C. and Baker, L. C. (1929). *J. Soc. chem. Ind., Lond.*, **48**, 232*T*–238*T*.
Freney, M. R. (1940). *Bull. sci. ind. Res. Org. Melbourne*, No. 130, 1–52.
Gillam, N. W. (1948). *J. Aust. chem. Inst.*, **14**, 361–372.
Lea, C. H.. (1938). *Rep. Dep. sci. industr. Res.*, No. 46, 108.
Lifschutz, I. (1924). *Hoppe-Seyl. Z.*, **141**, 146–152.

LETTERS TO THE EDITOR

The Effects of Tubocurarine, Decamethonium, Suxamethonium, Edrophonium and Neostigmine upon Flux of Calcium-47 in Frog Skeletal Muscle

SIR,—The importance of calcium ions (Ca^{++}) in maintaining muscle and nerve in a state of normal excitability is well established (Hodgkin, 1951; Brink, 1954; Frankenhaeuser and Hodgkin, 1957; Frankenhaeuser, 1957; Shanes, 1958).

We have investigated the effects of tubocurarine, decamethonium, suxamethonium, edrophonium and neostigmine upon $^{47}Ca^{++}$-uptake and release in paired resting frog sartorius muscles and have compared this with their effects upon uptake and release of $^{42}K^{+}$ and $^{24}Na^{+}$ uptake.

Uptake experiments were carried out by suspending the isolated sartorius muscles in oxygenated frog Ringer's solution (NaCl, 0·65; KCl, 0·014; $CaCl_2 6H_2O$, 0·012; $NaHCO_3$, 0·02; $NaH_2PO_4 2H_2O$, 0·001, and glucose 0·2 per cent) in which part of the stable Ca^{++}, K^{+} or Na^{+} was replaced by $^{47}Ca^{++}$, $^{42}K^{+}$ or $^{24}Na^{+}$. At intervals of 30 min. the muscles were removed, washed, blotted dry and counted in a thallium-activated, sodium iodide scintillation

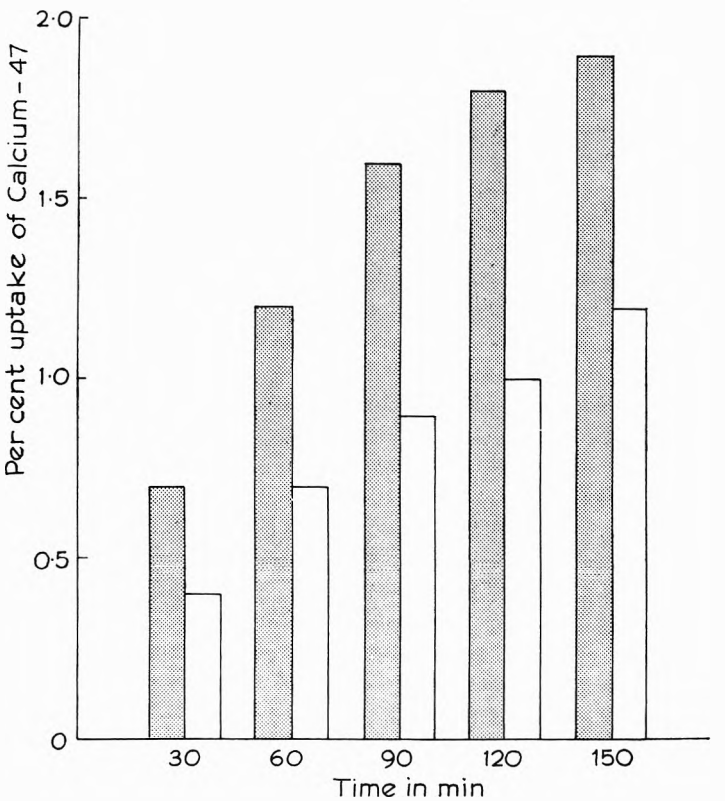

FIG. 1. Effect of suxamethonium chloride dihydrate (5 mg./ml.) (stippled areas) on the uptake of $^{47}Ca^{++}$ by resting frog sartorius muscle. Unstippled area = control muscle.

crystal (Ekco type N597) connected through a photomultiplier to an automatic scaler (Ekco type N530D).

For studying $^{47}Ca^{++}$-release muscles were soaked for a period of from 4 to 6 hr. in oxygenated frog Ringer's solution in which part of the stable Ca^{++} was replaced by $^{47}Ca^{++}$. The muscles were then passed along two parallel series of 7 tubes each containing 10 ml. of non-radioactive, Ca^{++}-free, oxygenated frog Ringer's solution, one series being used for control purposes only. The muscle

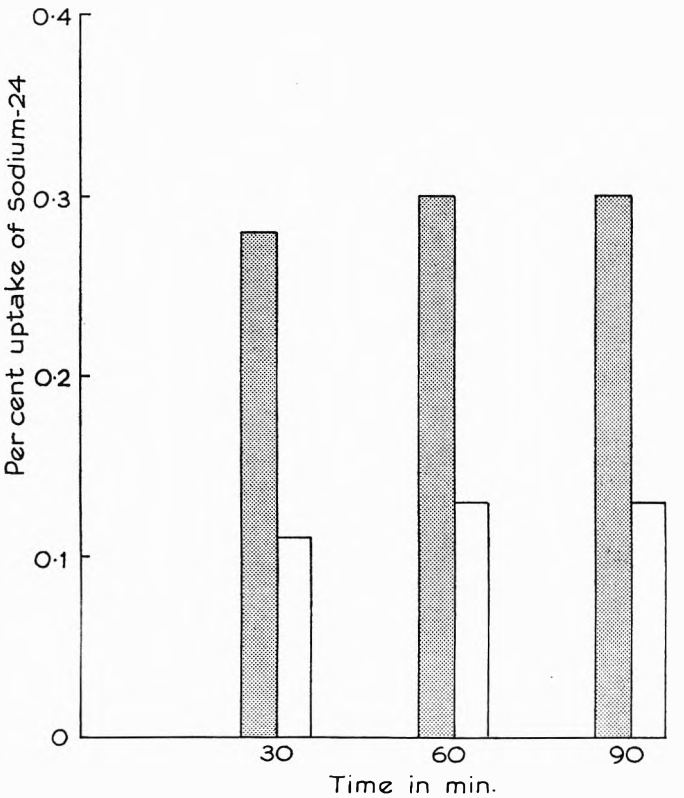

FIG. 2. Effect of neostigmine methyl sulphate (100 μg./ml.) (stippled areas) on the uptake of $^{24}Na^{+}$ by resting frog sartorius muscle. Unstippled areas = control muscle.

was kept in each tube for 10 min., and the drug added to the fourth tube in the test series. After the muscles were transferred to the next tube in the series, the fluid remaining was counted, as before.

For estimation of $^{42}K^{+}$-release, the procedure was similar, but the muscles were labelled by injecting the frogs with ^{42}KCl into the dorsal lymph sac. They were killed and the sartorius muscles removed after an equilibration period of 2 hr. The muscles were passed through two series of tubes each containing non-radioactive, K^{+}-free, oxygenated frog Ringer's solution in the manner used for estimating $^{47}Ca^{++}$-release. The fluid remaining was counted by means of a Geiger-Müller liquid counter (type M6) connected through a probe unit to an automatic scaler (Ekco type N530D).

Decamethonium (50 μg./ml. to 0·5 mg./ml.) and suxamethonium (1 to 10 mg./ml., Fig. 1) caused increased uptake of $^{47}Ca^{++}$ while tubocurarine (50 μg./ml. to 1 mg./ml.) and edrophonium (0·5 to 2 mg./ml.) depressed this. In some experiments neostigmine (25 to 150 μg./ml.) increased $^{47}Ca^{++}$-uptake, while in others this was unchanged or decreased. Decamethonium, suxamethonium, edrophonium and neostigmine all depressed $^{42}K^{+}$-uptake but tubocurarine had no apparent effect. Decamethonium, suxamethonium and neostigmine (Fig. 2)

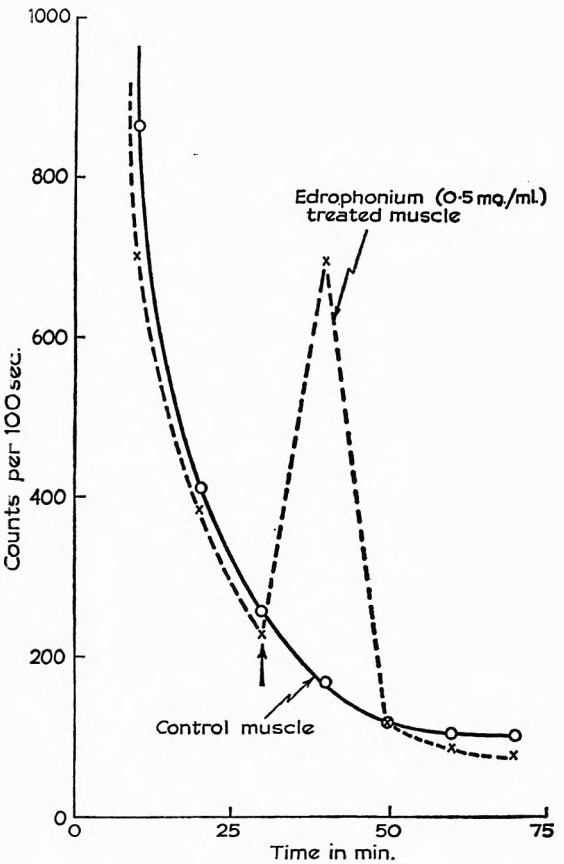

FIG. 3. Effect of edrophonium (0·5 mg./ml.) on the release of $^{47}Ca^{++}$ from resting frog sartorius muscle. Arrow indicates point of exposure of the test muscle to the drug.

increased $^{24}Na^{+}$-uptake but tubocurarine and edrophonium had no apparent effect on this. Suxamethonium, edrophonium (Fig. 3) and neostigmine also caused release of $^{47}Ca^{++}$. Large doses of tubocurarine (0·5 to 1 mg./ml.) caused release of $^{47}Ca^{++}$ but decamethonium had no effect. Decamethonium (Fig. 4), suxamethonium, and neostigmine all caused release of $^{42}K^{+}$. The latter confirms the observations of Klupp and Kraupp (1954) and Kraupp and his colleagues (1960) on decamethonium and suxamethonium. Tubocurarine and edrophonium had no effect.

It has been suggested by Frankenhaeuser and Hodgkin (1957) that depolarization acts by removing Ca^{++} from combination with a sodium carrier and our results tend to support this view and are not unexpected in view of the report by Robertson (1960) that acetylcholine increases $^{45}Ca^{++}$-uptake in depolarised smooth muscle.

It seems therefore that the application of depolarising drugs is associated with increased uptake of $^{47}Ca^{++}$ and $^{24}Na^{+}$ and increased release of $^{42}K^{+}$. The non-

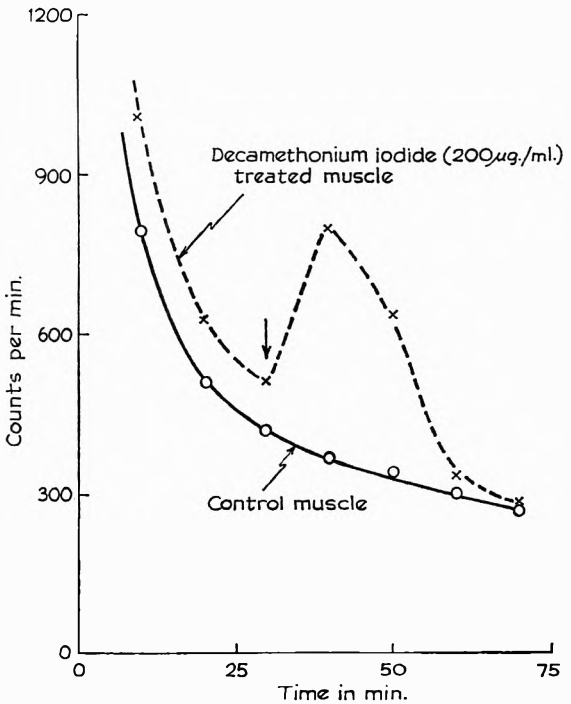

FIG. 4. Effect of decamethonium iodide (200 μg./ml.) on the release of $^{42}K^{+}$ from resting frog sartorius muscle. Arrow indicates point of exposure of the test muscle to the drug.

depolarising drug, tubocurarine, depresses uptake of $^{47}Ca^{++}$, causes no change in the uptake of $^{24}Na^{+}$ and does not release $^{42}K^{+}$. We hope to report our findings in more detail later.

K. AHMAD.*
J. J. LEWIS.

Experimental Pharmacology Division,
Institute of Physiology,
University of Glasgow.
December 13, 1960.

* Govt. of Pakistan Scholar.

REFERENCES

Brink, F. (1954). *Pharmacol. Rev.*, **6**, 243–298.
Frankenhaeuser, B. and Hodgkin, A. L. (1957). *J. Physiol.*, **137**, 218–244.
Frankenhaeuser, B. (1957). *Ibid.*, **137**, 245–260.
Hodgkin, A. L. (1951). *Biol. Rev.*, **26**, 339–409.

Klupp, H. and Kraupp, O. (1954). *Arch. int. Pharmacodyn.*, **98**, 340–354.
Kraupp, O., Pillat, B., Stormann, H., Bernheimer, H. and Schnetz. (1960). *Ibid.*, **124**, 82–104.
Robertson, P. A. (1960). *Nature, Lond.*, **186**, 316–317.
Shanes, A. M. (1958). *Pharmacol. Rev.*, **10**, 59–164, 165–273.

Tolerance to Tremorine

SIR,—The tremor-producing drug 1,4-dipyrrolidino-2-butyne, Tremorine, is becoming increasingly used in the screening of anti-Parkinsonism substances. In this connection it may be of some interest to record the observation made in this laboratory, that a surprisingly rapid tolerance to tremorine develops in the mouse. The tolerance is easily observed as soon as the third treatment of 6 to 18 mg./kg. intraperitoneally or subcutaneously is given, and is essentially complete after five to six administrations made at two-days intervals. Tolerance comprises all of the three main central effects of the drug, namely, tremor-producing action, analgesic action and anaesthesia-prolonging action. Sensitivity to tremorine returns after discontinuing of drug administration for 2 or 3 weeks.

The property of tremorine to cause tolerance should be taken into account when used in routine pharmacological screening.

L. DECSI.
M. VÁRSZEGI.
J. MÉHES.

Institute of Pharmacology,
Medical University,
Pécs, Hungary.
December 5, 1960.

Antifertility Agents

SIR,—Petrow (1960) has recently proposed the generic term, "claudogens," for steroidal antifertility agents.

This term appears less general, less descriptive, and (to me) less euphonious than the increasing importance of this new class of pharmacologic agents would seem to require. For this reason, I wish to propose the more general, alternative term, "genotropic agent," which has been in use for some time in this laboratory and has met with acceptance by all who have had occasion to use it; in informal usage, "genotropic agent" often becomes simply "genotrope."

The adjective, "genotropic" (soft g), was coined from the Greek roots $\gamma\epsilon\nu\omega\varsigma$ = population (or $\gamma\epsilon\nu\nu\alpha\nu$ = reproductive) and $\tau\rho\omega\pi\sigma\varsigma$ = changing, affecting, altering. The resulting word expresses precisely what is intended and also conveys the sociologic connotation that is fundamental to the problem of fertility control.

The term, "genotropic," need not imply any specific site, mode, direction, or degree of action by any limited type of agent upon the reproductive process, but requires, for its proper usage, only that the agent in question (steroidal or non-steroidal) have a net effect upon the number of normal progeny produced by an individual of the species under study.

MARTIN L. BLACK.

The Research Laboratories,
Parke, Davis and Co.,
Ann Arbor, Michigan, U.S.A.
December 14, 1960.

REFERENCE

Petrow, V. (1960). *J. Pharm. Pharmacol.*, **12**, 704.

Hypoglycaemic Agent from Onions

SIR,—Many indigenous plants of India have been claimed to contain anti-diabetic principles, and Mukerji (1957) listed those claimed to have anti-diabetic properties after oral administration.

Allium cepa, the common onion, was investigated by Collip (1923), Janot and Laurin (1930) and Laurin (1931) and reported to contain a hypoglycaemic agent, a claim confirmed by Laland and Havrivold (1933) and Kreitmar (1936). A detailed study of various onion extracts was therefore undertaken in an attempt to isolate an orally effective hypoglycaemic principle from this important vegetable.

Onion bulbs were cut into pieces, thoroughly dried and completely extracted with different solvents. These extracts were dried and fed to groups of fasting normal male rabbits weighing 2 kg. and having a fasting 18 hr. blood-sugar level of 100–120 mg./100 ml. Only light petroleum extracts of the dried powder were found to have hypoglycaemic action which was compared with that produced by a standard dose of tolbutamide. The potency of these extracts was

TABLE I

BIOLOGICAL ASSAY OF ORALLY EFFECTIVE HYPOGLYCAEMIC FRACTIONS FROM *Allium cepa* COMPARED WITH TOLBUTAMIDE

Substance administered	Dose	Blood sugar response mg./100 ml.		Mean reduction per cent	Hypoglycaemic potency as per cent of tolbutamide
		Initial mean values for six rabbits	4 hr. pool. Mean value for six rabbits		
Tolbutamide	0·5 g.	100	74·98	25·0 ± 2·1	—
Light petroleum 60°–80° extract.	0·5 g.	117·2	98·99	15·5 ± 1·2	62·1
Ethyl ether 34°–36° extract of dried residue from above.	0·5 g.	115	82·97	19·2 ± 1·6	76·6

expressed as percentages of the standard substance, tolbutamide, according to the procedure laid down by Marks (1926) for the biological assay of insulin. Blood sugar was determined by the micro method of Folin and Malmros (1929).

The results, Table I, prove *Allium cepa* to contain light-petroleum-soluble material with a hypoglycaemic action. Further, ethyl ether extracts most of the hypoglycaemic fraction from a powder prepared from the petroleum extract.

Further work on the separation and purification of orally effective hypoglycemic compounds from these extracts is in progress.

H. D. BRAHMACHARI.
K. T. AUGUSTI.

Dept. of Biochemistry,
Birla College, Pilani,
India.
December 21, 1960.

REFERENCES

Collip, P. (1923). *J. biol. Chem.*, **56**, pp. 65, 163, 513.
Folin, O. and Malros, H. (1929). *Ibid.*, **83**, 115–120.
Janot, M. M. and Laurin, J. (1930). *C.R. Acad. Sci. Paris*, **191**, 1098–1099.
Kreitmair, H. (1936). E. Merk's Jahr-b., **50**, 102–104.
Leland, P. and Havrivold, O. W. (1933). *Z. Physiol. Chem.*, **221**, 180–196.
Laurin, J. (1931). *C.R. Acad. Sci. Paris*, **192**, 1289–1291.
Marks, M. (1926). League of Nations report on Insulin standardisation.
Mukerji, B. (1957). *J. Sci. Ind. Research* (India), **16a**, No. 10 Suppl., 1–12.