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A HYPOTHESIS FOR THE MODE OF ACTION OF α-METHYLDOPA IN RELIEVING HYPERTENSION

BY M. D. DAY AND M. J. RAND

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

Received February 20, 1963

There is evidence that α-methyldopa can serve as the precursor of α-methylnoradrenaline in the body. The α-methylnoradrenaline so formed may enter noradrenaline storage sites and then be released as a false neuro-transmitter. Because of the lesser potency of α-methylnoradrenaline there is some loss of responsiveness to sympathetic nerve stimulation which can explain the lowering of blood pressure in hypertensive patients.

THE relief of hypertension by treatment with α-methyldopa was first reported by Oates, Gillespie, Udenfriend and Sjoerdsma (1960). Subsequently, many more clinical observations have been made (amongst others; Irvine, O'Brien and North, 1962; Dollery and Harington, 1962; Smirk, 1963). Clinical reports describe α-methyldopa as a moderately active hypotensive drug with fewer side effects than guanethidine; the greatest advantage that α-methyldopa offers over guanethidine is that it produces a significant lowering of pressure in both standing and supine positions (Oates and others, 1960; Irvine and others, 1962).

Inhibition of Dopa Decarboxylase

One of the stages in the formation of noradrenaline, the transmitter at sympathetic nerve endings, is the decarboxylation of the amino-acid dihydroxyphenylalanine (dopa) to form the amine, dopamine (Fig. 1). Sourkes (1954) showed that α-methyldopa was a powerful inhibitor *in vitro* of the enzyme dopa decarboxylase. Oates and others (1960) confirmed that α-methyldopa inhibited decarboxylation of amino-acids in man. Treatment with α-methyldopa leads to the depletion of noradrenaline from its stores in the tissues and it is presumed that it is this depletion which leads to lowering of blood pressure in hypertensive patients. However, noradrenaline depletion does not seem to be a result of inhibition of dopa decarboxylase, since α-methyldopa did not reduce the excretion of metabolites of noradrenaline (Cannon, Whitlock, Morris, Angers and Laragh, 1962), which suggests that the production of noradrenaline *in vivo* is not impaired. Furthermore, Hess, Connamacher, Ozaki and Udenfriend (1961) reported that inhibition of dopa decarboxylase by α-methyldopa was a transient phenomenon whereas the depletion of noradrenaline was prolonged, and Gillespie, Oates, Crout and Sjoerdsma (1962) found that other substances which are known to be very potent inhibitors of dopa decarboxylase in man did not lower blood pressure.

Depletion of Noradrenaline Stores

α-Methyldopa not only depletes noradrenaline from stores in the tissues, but it also impairs the noradrenaline storage capacity of the tissues (Stone, Ross, Wengler, Ludden, Blessing, Totaro and Porter, 1962; Hess and others, 1961). However it has been reported that the depletion of noradrenaline by α-methyldopa is not accompanied by any obvious failure of responses to sympathetic nerve stimulation in experimental animals (Stone and others, 1962) and we have found that responses to tyramine are not greatly affected, and in these respects α-methyldopa

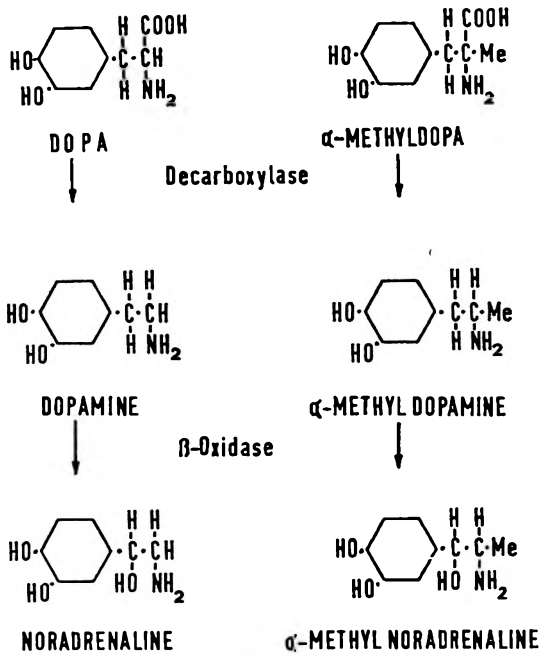

FIG. 1

differs from reserpine which causes depletion of noradrenaline and failure of responses to sympathetic nerve stimulation and to tyramine (Burn and Rand, 1958). Although Stone and others (1962) were unable to detect impairment of sympathetic nerve functioning after α-methyldopa in their experiments, the clinical findings indicate that in patients α-methyldopa causes at least partial sympathetic nerve blockade. Thus, postural hypotension and failure of ejaculation was reported by Gillespie and others (1962), and bradycardia and abolition of the overshoot in the Valsava manoeuvre was reported by Dollery and Harington (1962).

Responses to Sympathetic Nerve Stimulation after α-Methyldopa

We have confirmed the reports of others that α-methyldopa does not cause any striking effects indicative of sympathetic nerve blockage after

injection into conscious cats and rats; for example, it does not cause relaxation of the nictitating membrane. Nevertheless, in experiments in which we studied the contractions of the cat's nictitating membrane in response to stimulation of the postganglionic sympathetic nerves, we regularly observed an impairment of responses which was especially evident at low frequencies of nerve stimulation, although there was little or no impairment of responses to high frequencies of stimulation which produced maximal responses. Physiological rates of sympathetic nerve discharge are believed to be low. Therefore it seems likely that the impairment which we observed at low frequencies of stimulation can explain the clinical findings of sympathetic nerve impairment. The puzzling aspect of the pharmacological actions of α-methyldopa was that noradrenaline stores were depleted, yet responses to sympathetic nerve stimulation and to tyramine persisted.

Metabolism of α-Methyldopa

It has been shown that α-methyldopa can be decarboxylated to yield α-methyldopamine both in *in vitro* systems (Weisbach, Lovenberg and Udenfriend, 1960) and *in vivo* (Gillespie and others, 1962; Porter and Titus, 1963). There is evidence that the enzyme β-oxidase which converts dopamine to noradrenaline can convert α-methyldopamine to α-methylnoradrenaline (Fig. 1). Thus, Carlsson and Lindquist (1962) demonstrated the presence of α-methylnoradrenaline in the tissues of animals treated with α-methyldopa. Recently, Lauwers, Verstraete and Joossens (1963) found that in a patient treated with α-methyldopa there was what appeared to be an increased excretion of noradrenaline, but paper chromatography showed that there was another substance present, closely related but different to noradrenaline. Stott and others (1963) obtained high values for 3-methoxy metabolites resembling normetadrenaline in urine of patients on α-methyldopa and they showed that the high value was due to the presence of a substance having the properties of the 3-methoxy derivative of α-methylnoradrenaline.

We have obtained indirect evidence that α-methyldopa can be converted to α-methylnoradrenaline, and that the α-methylnoradrenaline formed can be utilised as a transmitter at sympathetic nerve endings. In animals treated with reserpine, noradrenaline stores are depleted and responses to sympathetic nerve stimulation or to indirectly acting sympathomimetic amines (such as tyramine) are greatly reduced or absent, then infusion of α-methyldopa causes a significant increase of these responses. Therefore α-methyldopa behaves like dopa, which also increases these responses in reserpine-treated animals (Burn and Rand, 1960), by increasing the noradrenaline stores (Pennefather and Rand, 1960). Partial restoration of responses to sympathetic nerve stimulation and to tyramine in reserpine-treated animals was also obtained after infusions of α-methyldopamine or of α-methylnoradrenaline. Our interpretation of these results is that after giving α-methylnoradrenaline, or its precursors, the noradrenaline storage sites are replenished with α-methylnoradrenaline, and that this substance then acts as the sympathetic transmitter.

Comparison of α-Methylnoradrenaline with Noradrenaline

That substitution of the false transmitter α-methylnoradrenaline for noradrenaline can lead to impairment of responses to nerve stimulation follows from its weaker activity. Ahlquist (1948) found that (±)-α-methylnoradrenaline had slightly less than half the potency of (±)-noradrenaline on the cat's nictitating membrane, and Goodman and Gilman (1955) state that corbasil ((±)-α-methylnoradrenaline) had slightly less than one quarter the potency of noradrenaline as a pressor amine. We have compared the pressor activity of (−)-α-methylnoradrenaline with (−)-noradrenaline and found it to have about half the potency in cats, about one-third in rats, and about one-sixth in guinea-pigs and rabbits.

Acknowledgements. We are indebted to the M.R.C. for a Scholarship in Research Methods held by one of us (M. D. D.). We gratefully acknowledge the gift, from Merck, Sharp and Dohme, of α-methyldopa (Aldomet) used in our experiments.

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RESEARCH PAPERS

A NEW METHOD FOR THE ESTIMATION OF HISTAMINASE ACTIVITY

BY P. S. J. SPENCER*

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Received November 5, 1962

The effects of enzyme and substrate concentrations on the rate of inactivation of histamine by histaminase of rat ileum have been investigated. The fact that the time taken for one-half of the histamine to be destroyed is inversely proportional to the enzyme content of the incubation mixture has been used as a basis for a new method for the estimation of histaminase activity in tissue extracts.

WICKSELL in 1949 described a simple biological method for estimating the histaminolytic activity of various tissues, and several workers have used this method (e.g. Carlsten and Wood, 1950; Haeger and Kahlson, 1952; Paratt and West, 1960). However, the rate at which histamine is destroyed by histaminase in rat tissues is not constant, as the relationship is exponential. To express activity in terms of the amount of histamine destroyed in unit time by a unit amount of tissue is therefore not accurate, and consequently activities determined in different laboratories by different workers vary widely.

The present paper describes experiments using ileum histaminase of the rat to obtain a linear relationship between histamine concentration and duration of incubation. During this work, the effect of changes both in enzyme and in substrate concentration on the rate of histamine destruction have also been investigated.

METHODS

Source of enzyme. Male Wistar albino rats, weighing 120–160 g. were used. The small intestine was removed from groups of 4 or 5 rats immediately after killing by a blow on the head. The tissue was washed with normal saline solution, the first 6 in. and the last 2 in. being discarded. It was then weighed.

Extraction of histaminase activity. The bulked tissue was ground in a glass mortar with a little sand. Tyrode solution (5 ml./g.) was added and extraction proceeded for 5 min. The suspension was then centrifuged at 2,800 r.p.m. for 10 min., and the resultant supernatant constituted the enzymic extract. Longer times of grinding, extraction and centrifugation did not increase the yield of enzyme.

Incubation. To 95 ml. of Tyrode solution in a 250 ml. conical flask, containing a known amount of histamine and equilibrated to 37°, was added 5 ml. of the histaminase extract. After shaking the flask to mix

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the contents, a first sample was immediately removed and the remainder incubated at 37° with frequent shaking. Further samples were removed at 10 min. intervals. Each sample was brought to the boil to arrest enzyme activity, cooled and stored at 4° until it was assayed for its histamine content. In experiments in which the effect of changes in enzyme or substrate concentration were investigated, different volumes or quantities of extract and histamine were used and the volume of Tyrode solution varied so that all incubation mixtures were initially 100 ml. in volume. Throughout the first 90 min. of incubation, the mixture remained within the limits pH 7·0 to 7·4.

Estimation of histamine. The samples were assayed directly on the atropinised ileum of the guinea-pig. All estimations were in duplicate and the means have been used to compile the Figures and Table I.

Results

Inactivation of histamine by ileum histaminase. Using an initial histamine concentration of 1·04 μg./ml. in the mixture and an extract from 1·0 g of rat ileum, the rate of inactivation of histamine was slowly reduced throughout the period of incubation. An exponential relationship between histamine concentration and time of incubation was produced (see Fig. 1). Experiments using initial histamine concentrations

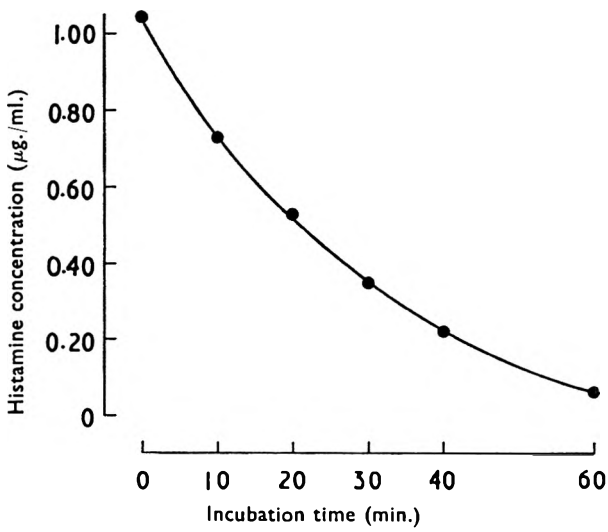

FIG. 1. Rate of inactivation of histamine by histaminase of rat ileum at 37°. Plot of histamine concentration (μg./ml.) against incubation time (min.).

of approximately 0·5, 1·5, 2·0, 2·5 and 5·0 μg./ml., or with histaminase activity from 0·5 to 2·0 g. of rat ileum tissue produced similar exponential relationships.

Huennekens (1953) has stated that observations on the rates of enzyme-catalysed reactions have disclosed two fundamental features: (*a*) at constant initial substrate concentration, the rate is proportional to the

enzyme concentration over a wide range, and (*b*) at constant enzyme concentration the rate increases (or decreases) with the concentration of the substrate in conformance with the curve of a rectangular hyperbola, eventually levelling off asymptotically. Providing the initial substrate concentration remains low, then at constant enzyme concentration the reaction should be a first order reaction with respect to the substrate. If the above destruction of histamine is a first-order (or pseudo first-order) reaction under the conditions of the experiment, then from the simplified equation for a first-order reaction,

$$k = \frac{2{\cdot}303 \log_{10} (x_o/x_t)}{t}$$

then a plot of $\log_{10} (x_o/x_t)$ against t should produce a straight line relationship. (x_o and x_t are the initial histamine concentration and the concentration after incubation for time t, respectively).

Fig. 2 shows the results from Fig. 1 expressed in this manner. A linear relationship exists for most of the reaction, and this fact has been utilised as a basis for the estimation of histaminase activity.

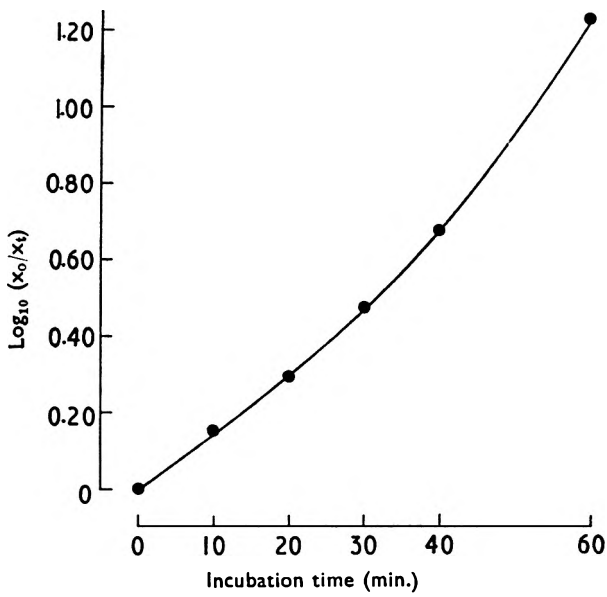

FIG. 2. Rate of inactivation of histamine by histaminase of rat ileum at 37°. Plot of $\log_{10} (x_o/x_t)$ against incubation time (min.).

Effect of changes in enzyme concentration. In a second series of experiments, the effect of varying the enzyme content using a constant initial histamine concentration was investigated. Results from a typical experiment are presented in Fig. 3.

Incubation mixtures containing 1·3 (A), 1·0 (B), 0·8 (C) and 0·5 g. (D) of ileum tissue and an initial histamine concentration of approximately 2·00 μg./ml. were sampled after 0, 20, 40 and 60 min. incubation

at 37°. By plotting $\log_{10}(x_o/x_t)$ against time, linear relationships are obtained in all incubations for at least 40 min., and in those mixtures containing the two lower enzyme concentrations (C and D) the graphs are still linear after 60 min. of incubation. In Fig. 3, a line has been drawn through the 50 per cent destruction level (where $\log_{10}(x_o/x_t) = 0{\cdot}3010$), and the times obtained at which 50 per cent of the histamine was destroyed (the DT50 values) (see Table I). By comparing incubations A, C and D with B (1·0 g. of tissue), time ratios of 1·27, 1·00, 0·82 and 0·53 were obtained, and these agree very closely with enzyme ratios in the four incubations of 1·30, 1·00, 0·80 and 0·50, respectively. Thus the time taken to reach 50 per cent destruction (or any other point on the linear part of the graphs) is inversely proportional to the enzyme concentration.

TABLE I

EFFECT OF TISSUE (ENZYME) CONTENT ON THE DT50 TIMES OF 4 INCUBATION MIXTURES, A, B, C AND D, WITH SIMILAR INITIAL HISTAMINE CONCENTRATIONS OF APPROXIMATELY 2 μG./ML.

Inbucation mixture	A	B	C	D
ml. of extract added	6·50	5·00	4·00	2·50
Amount of ileum tissue (g.) ..	1·30	1·00	0·80	0·50
DT50 values (min.)	22	28	34	53
Ratio of values (B = 1)	1·27*	1·00	0·82	0·53

* $\frac{\text{DT50 B}}{\text{DT50 A}} = \frac{28}{22} = 1{\cdot}27$

The relationship is to be expected theoretically. From the original statement by Huennekens (1953), the rate of reaction is proportional to the enzyme concentration, i.e. $k \propto E$. But in the equation, $k = (2{\cdot}303/t).\log_{10}(x_o/x_t)$, when $\log_{10}(x_o/x_t)$ is made a constant (for example 50 per cent destruction), then $k \propto 1/t$. Therefore E (enzyme concentration) $\propto 1/t$, and this is shown in Fig. 3.

Effect of changes in initial histamine concentration. The extraction of varying quantities of endogenous histamine during preparation of the enzyme extract resulted in a variable initial histamine content of an incubation mixture. The rate of inactivation of histamine in mixtures containing different amounts of initial histamine and constant amounts of enzyme, was therefore investigated.

Three incubation mixtures containing initial histamine concentrations of 2·49, 2·01 and 1·53 μg./ml. and enzyme from 1·0 g. of tissue, were followed for up to 60 min. incubation. Fig. 4 shows the conventional plot of histamine concentration against length of incubation, and three distinct rates of inactivation are seen. However, when the results are plotted by a different method (that is $\log_{10}(x_o/x_t)$ against t), three superimposable graphs are obtained, with a common DT50 time of 26 min. (Fig. 5).

When a wider range of initial histamine concentrations was investigated, the graphs were superimposable and only deviated markedly at incubation times beyond 40 min.

Suggested method of calculating histaminase activity. In the experiments described above, the rate of histamine inactivation, expressed as μg./hr., varied not only with different enzyme concentrations, but also with different histamine concentrations. Thus the rate varied between

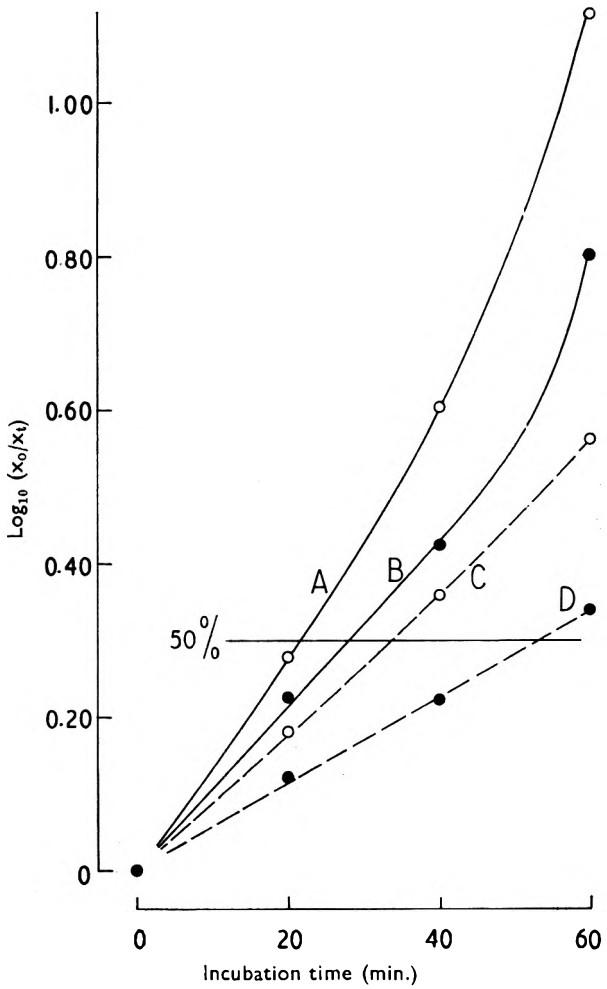

FIG. 3. Effect of changes in enzyme content on the rate of inactivation of histamine. A, B, C and D contain the enzyme activity from 1·30, 1·00, 0·80 and 0·50 g. of ileum tissue respectively. Plot of $\log_{10}(x_0/x_t)$ against incubation time (min.).

mixtures containing the same amount of enzyme, and also in the same mixture when examined at different times. To express enzyme activity as μg. of histamine inactivated in 1 hr. by 1 g. of tissue is therefore impossible under these conditions.

However, by plotting $\log_{10}(x_0/x_t)$ against t, then within a fairly wide range of initial histamine concentrations, the time taken for 50 per cent

of the histamine to be inactivated was a constant for a given amount of tissue.

that is, E (enzyme concentration) $\propto$ 1/DT50

The following method is proposed to determine enzyme activity. Histamine concentrations are determined at 0 min. and various time intervals afterwards and a plot of $\log_{10}(x_o/x_t)$ against t is made. A linear relationship should exist over most of the reaction, at least as far

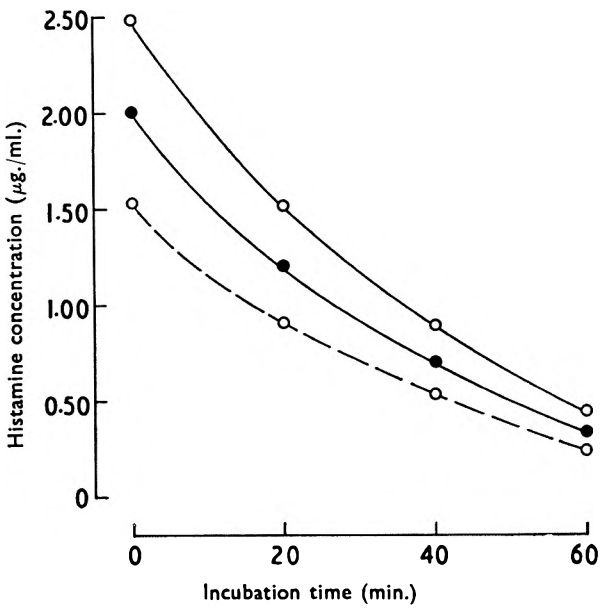

FIG. 4. Effect of changes in initial substrate concentration on the rate of inactivation of histamine. Plot of histamine concentration (μg./ml.) against incubation time (min.).

as 70–80 per cent inactivation. The time taken for 50 per cent (or another fixed percentage within the linear part of the graph) to be destroyed, is read; this is the DT50 value. The ratio of enzyme activity in different tissues is then:

$$\frac{E_1}{E_2} = \frac{DT50\ (2)}{DT50\ (1)}$$

As a percentage of control tissue activity,

$$E\ (\text{experimental}) = \frac{DT50\ (\text{control})}{DT50\ (\text{experimental})} \times 100$$

Using this method, maximum errors of $\pm$ 7 per cent have been obtained in experiments using known amounts of tissue-extract in different incubation mixtures. If an initial histamine concentration within the range

1·50–2·50 μg./ml. is used, then dilution before assay is possible throughout the range of samples obtained, and this excludes difficulties in assay due to interference by tissue proteins.

Enzyme activity in other tissues. Pilot experiments have been made with histaminase activity from rat lung. A similar exponential relationship exists between histamine concentration and length of incubation, and this becomes linear when plotting $\log_{10}(x_0/x_t)$ against t. Comparison by the method described above showed the lung to possess about 1/5th of the activity of the ileum. Mouse ileum and lung show similar exponential curves, and the ratio of activity is approximately the same as in the rat.

Initial experiments with 5-hydroxytryptamine and rat kidney as a source of amine oxidase suggest that a similar method may be used for estimating amine oxidase activity.

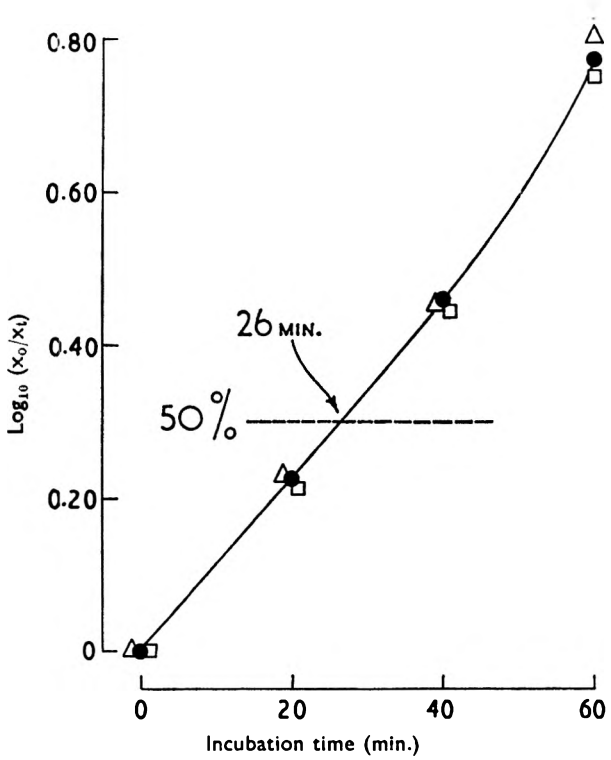

FIG. 5. Effect of changes in initial substrate concentration on the rate of inactivation histamine. Plot of $\log_{10}(x_0/x_t)$ against incubation time (min.) giving 3 superimposable graphs. Approximate initial concentrations: △ = 2·5, ● = 2·0 and □ = 1·5 μg./ml.

DISCUSSION

The results presented in this paper show that the rate, in μg./min. or μg./hr., at which histamine is inactivated by rat ileum histaminase is not constant, but decreases throughout the period of incubation. This is

not due to a progressive change in the pH of the medium, but appears to be the natural development of a pseudo first-order reaction. Examination of previous work describing biological (Wicksell, 1949) and biochemical methods (Kapeller-Adler, 1951) for estimating histaminase activity, reveals that an exponential curve is common to both types of procedure.

It is impossible to assess histaminase activity using an expression which is not constant for a particular incubation mixture, and it follows that such an assessment is a compromise and obtained under strictly controlled conditions. Wicksell (1949) for example stipulated that the rate should be calculated from data obtained when not less than 30 per cent and not more than 60 per cent of the initial histamine had been destroyed.

By converting the exponential relationship between histamine concentration and incubation time to the linear one of $\log_{10}(x_o/x_t)$ against time, a constant—the slope of the new graph—for a particular incubation mixture is obtained, and the time taken to obtain a certain proportion or percentage inactivation is dependent on the enzyme content of that mixture. This is the basis of the present method. Using this method, changes in histaminase activity after thyroxine treatment have been detected in the rat (Spencer and West, 1962a) and the mouse (Spencer and West, 1963).

The method offers the following advantages:

1. The DT50 time depends on a constant and does not change for a given concentration of enzyme.

2. The value is obtained from data from all of the samples assayed for histamine in a given mixture. Thus, one false assay only slightly affects the result, although the initial sample is perhaps the most important in any mixture.

3. Incubations containing different initial amounts of histamine can be compared, providing they fall within the range 75 per cent to 150 per cent of the mean initial histamine concentration. Varying amounts of endogenous histamine will not alter the answer.

4. A maximum experimental error of less than 10 per cent can be expected.

Acknowledgement. The author is indebted to Dr. G. B. West for his help and advice throughout the course of this work and in the preparation of the manuscript.

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POTENTIOMETRIC MEASUREMENTS IN SOLUTIONS OF NON-IONIC SURFACTANTS

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Received November 30, 1962

Potentiometric measurements made in deionized cetomacrogol solutions show that the surfactant does not affect the pH values of sodium hydroxide solution or hydrochloric acid. The pH in buffer solutions containing cetomacrogol is changed to an extent which depends on the buffer acid. Possible mechanisms of this action are discussed and analytical applications indicated.

Though electrochemical methods have been widely applied to solutions of cationic and anionic surfactants, there is a comparative paucity of literature dealing with the application of such methods to solutions of non-ionic surfactants (Moillet, Collie and Black, 1961).

The present introductory paper is concerned with the possibility of applying potentiometric techniques to investigations of the properties of non-ionic surfactant solutions.

No detailed studies of the behaviour of the glass electrode in the presence of surfactants are available, although potentiometric measurements have been made in the presence of surfactants and no fundamental difficulties reported (Sexsmith, 1959; Veis, 1960). The pH values measured in such solutions might differ from those measured in water because of change in asymmetry potential of the glass electrode or modification of the activity coefficients of the ions present.

Experimental

Cetomacrogol, B.P.C. (Evans Medical, Ltd.) was found to contain small traces of alkaline impurities. It was therefore deionised by passage through columns of ion-exchange resins (B.D.H., analytical grade, I.R.-120 and I.R.A.-120), a method of purification first applied to non-ionic surfactants by Ginn (1959). After regeneration, the resins were washed with distilled water till the washings were neutral. The purified cetomacrogol solution obtained from the column was collected under nitrogen. Physico-chemical data on the characteristics of the cetomacrogol used will be reported later.

All solutions were prepared from carbon dioxide-free distilled water. During pH measurements it was not possible to pass nitrogen into the solutions, to prevent contamination by carbon dioxide, because of foaming; nitrogen was therefore passed over the solution.

The concentration of cetomacrogol in the effluent solution obtained from the ion-exchange column was checked by measurement of refractive index which varied linearly with concentrations of cetomacrogol solutions between 1 and 20 per cent w/v. Both the titration cell used for the pH

measurements and the refractometer were thermostatically controlled to 25° ±0·1°.

The electrodes used for pH measurements were: reference-Cambridge saturated calomel; indicator E.I.L. GH533. Two pH meters were used: the Pye Dynacap (accuracy ±0·02 pH) and the E.I.L. Vibron Electrometer with unit 33B attached (accuracy ±0·002 pH under optimum conditions). The pH scale was standardised as usual on *aqueous* potassium hydrogen phthalate solution (0·05M), and the electrode response checked on this buffer after each run; no changes were observed after use with cetomacrogol solution. Periodic checks on linearity of response were made using borax buffer solution (0·01 M).

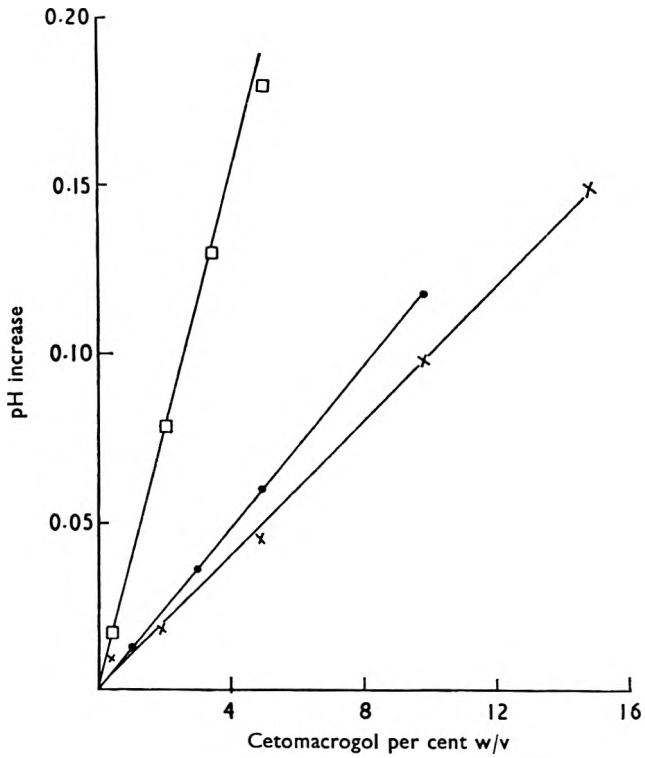

FIG. 1. Effect of cetomacrogol on buffer solutions.
□—□ Potassium hydrogen phthalate (0·05 M).
●—● Borax (0·05 M).
×—× Citric acid-sodium phosphate (McIlvaine buffer) pH 4·00.

RESULTS

Potentiometric curves of N/2,000 hydrochloric acid with N/300 sodium hydroxide solution and of the same hydrochloric acid containing 2, 4 and 10 per cent w/v cetomacrogol with the same sodium hydroxide solution were superimposable between pH 3 and 10, i.e. over the whole range of the titration.

The pH values of some common buffers in the presence of varying cetomacrogol concentrations were measured (Fig. 1). Increases in pH values were detected and in all instances the pH change appeared to be a linear function of the cetomacrogol concentration. The change in pH was greatest for the least water-soluble acid used, i.e. phthalic acid. This warranted further investigation into the effect of cetomacrogol upon such acids. As more information could be obtained from titration curves than from measurements on buffers, a series of titrations of varying concentrations of sodium benzoate (in the presence of cetomacrogol) with hydrochloric acid were performed. Some of the curves so obtained are shown in Figs. 2, 3 and 4. For comparison, similar titration curves of sodium benzoate in the absence of cetomacrogol are shown.

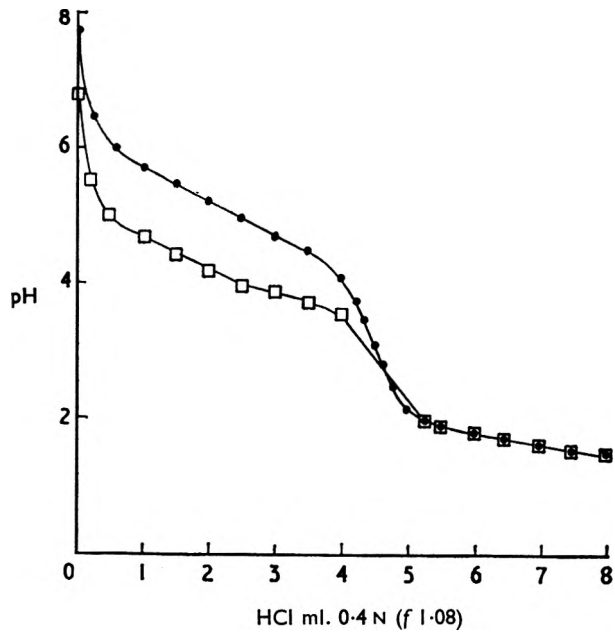

FIG. 2. Titration of sodium benzoate (77·8 mM) with hydrochloric acid (*f* 1·08) (25 ml. of solution containing $1{\cdot}95 \times 10^{-3}$ mole sodium benzoate).
●—● 20 per cent w/v cetomacrogol present.
□—□ No cetomacrogol present, precipitation occurred at about 2·5 ml.

DISCUSSION

Behaviour of Glass Electrode in Cetomacrogol Solution

The fact that the titration curves of hydrochloric acid against sodium hydroxide and of hydrochloric acid plus cetomacrogol against sodium hydroxide were superimposable, shows that the standard pH scale may be used in the presence of cetomacrogol (and probably equivalent amounts of other non-ionic surfactants). Thus the difference ($E_{cell}^{x} - E_{cell}^{s}$) between the measured cell e.m.f. values in the standard buffer solution, s, and any given hydrochloric acid/sodium hydroxide

mixture, x, is independent of the cetomacrogol concentration between pH 3 and 10. The pH values measured in cetomacrogol solution may therefore be described by the equation

$$pH_x = pH_s + \frac{E_{cell}^x - E_{cell}^s}{2{\cdot}303\ RT/F}$$

where pH_s is the pH value allocated to the standard *aqueous* buffer solution. Any pH value so described may be theoretically interpreted as for aqueous solutions, subject to evaluation of the correct activity coefficients. These appear to be very near unity in the hydrochloric acid/sodium hydroxide solutions.

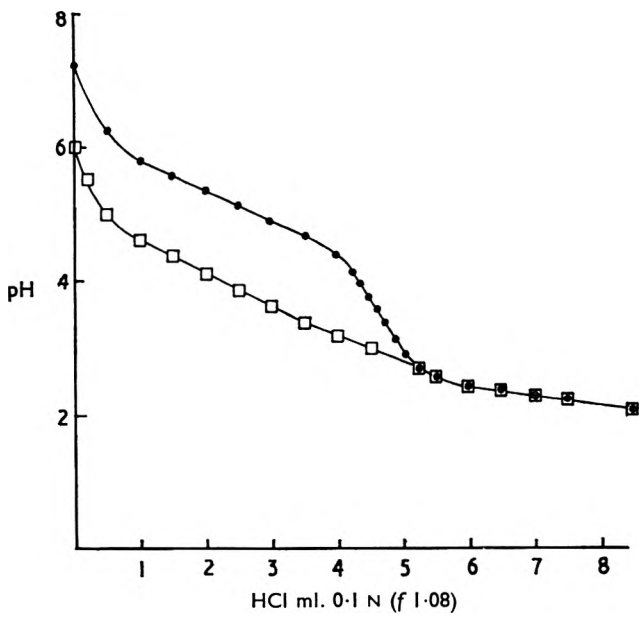

FIG. 3. Titration of sodium benzoate (19·46 mM) with hydrochloric acid (*f* 1·08) (25 ml. of solution containing 0·487 × 10^{-3} mole sodium benzoate).

●—● 20 per cent w/v cetomacrogol present.
□—□ No cetomacrogol present.

Mechanism of Surfactant-acid Interaction

It is apparent from Figs. 1 to 4 that the surfactant has reduced the activity of the various acid components of the solutions studied. The possibility that this is due to uptake of hydronium (H_3O^+) ions or of protons by the surfactant is remote, as no such effect was discernable in the hydrochloric acid/sodium hydroxide titrations. Were the pH changes due to alterations in asymmetry potential, such changes would have to be a linear function of cetomacrogol concentration with a different slope for each buffer; this is most unlikely.

The aqueous concentration of the buffers will change with cetomacrogol concentration if the micelles are considered as a separate phase. This

would not affect the buffer pH values to the extent found, as only the activity coefficients are modified and the calculated effect appears to be small.

It must therefore be concluded that the pH changes observed in the buffer solutions and in the sodium benzoate titration must arise from some other cause. Solubilisation studies by Dyer (1959), have shown that the ionised (or salt) form of a weak acid is not solubilised by surfactants and his results indicate that the pK_a values of such acids are not affected by the presence of surfactants. Although Dyer's results were obtained using ionic surfactants there seems no reason to anticipate that the same conclusions are not valid for non-ionic surfactants. Thus it would appear that the pH changes are due to selective solubilisation of the various acids present in the surfactant solutions.

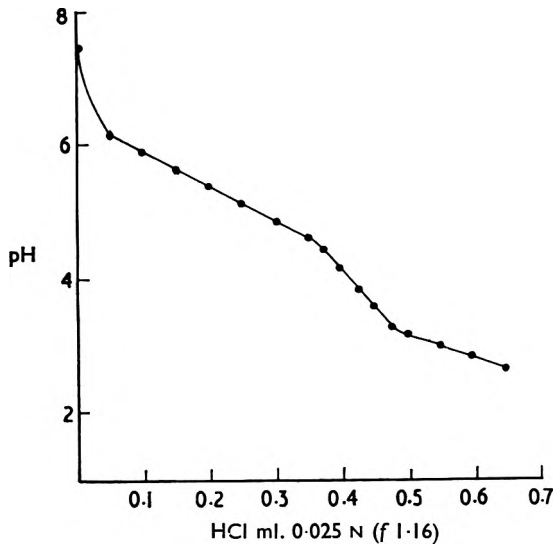

FIG. 4. Titration of sodium benzoate (4·87 mM) with hydrochloric acid (*f* 1·16) (25 ml. of solution containing $0{\cdot}1218 \times 10^{-3}$ mole sodium benzoate); 20 per cent w/v cetomacrogol present.

There are a number of possible mechanisms by which such solubilisation may occur. Ahsan (1960) and Barr (1957), have postulated complex formation between non-ionic surfactants and a variety of substances, and this may be involved in the case of the more water-soluble acids.

Alternatively, the acids may be adsorbed on to the surface of the micelle or dissolved in the palisade or central regions of the micelle. Solubilisation has been explained by a number of workers in terms of partition between two phases (McBain and Hutchinson, 1955, and Riegelman, 1958).

Pharmaceutical and Analytical Applications

Clearly the use of the usual phthalate standardising buffer solutions in the presence of cetomacrogol leads to error (e.g., if 5 per cent w/v cetomacrogol is present, the electrode standardisation will be 0·18 of a pH unit high). Small errors will also be introduced if citrate or borax buffers are used in the presence of cetomacrogol.

The formulation of buffered preparations containing surfactants may be more complicated than would at first appear, because of the possibility of preferential solubilisation of one of the constituents of the buffer system by the surfactant. The results obtained are consistent with observations by other workers on the reduction of activity of drugs and bactericides in systems containing surfactants (Allawala, 1953; Bean, 1950, 1951; Woodward, 1962).

The titration curves of sodium benzoate in the presence of cetomacrogol lie well above the curves for aqueous sodium benzoate up to the end-point, after which they coincide. This results in a sharper inflection being obtained at the end-point. The equivalence point may be determined potentiometrically in cetomacrogol solutions in very low concentrations of sodium benzoate (e.g. 1 mM), and good agreement is obtained with the present lengthy and somewhat cumbersome official method (B.P. 1958). Similar pH changes have been obtained in the titration of solutions of sodium salicylate and a number of other substances. Further details of analytical applications and the mechanism of solubilisation will be reported later.

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THE MYDRIATIC RESPONSE OF MICE TO ATROPINE

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The time after subcutaneous injection at which atropine produces most mydriasis in male mice increases with increased body-weight. If the response is measured when it is greatest, and is expressed as a percentage of the maximum mydriatic response obtainable in mice of the weight-range used, the mydriatic response to a dose of atropine is constant in male mice weighing from 16 to 50 g. The time-courses of the mydriatic response to atropine in mice of widely differing weights are parallel to the time-courses of the atropine concentration in the cardiac blood of the mice of the same weight-range given a high dose of atropine subcutaneously. The mydriatic response to atropine is found to be increased at times of poor ambient lighting (such as dusk): if however the mice are tested under conditions of artificial lighting (supplemented by daylight when present), the mydriatic response to a standard dose of atropine is nearly constant at all times from 8 a.m. to 2 a.m.

Since the dilatation of the pupil was first described as a qualitative test for the presence of the active principle of solanaceous plants by Runge (1824), many workers have used this response to assay atropine and atropine-like substances. The method, adapted for mice, was placed on a quantitative basis by Pulewka (1932), and subsequently slightly modified by Oelkers and Vincke (1935) and by Ing, Dawes and Wajda (1945).

Some factors influencing the accuracy of the bioassay method have been examined by Tonnesen (1948) and Huycke (1957). In general, mice within only a limited weight-range have been used, and doubts have been expressed as to the advisability of using the same mice repeatedly. Since mice over 25 g. are often readily available in the laboratory, being considered unsuitable for many pharmacological tests, and since the mydriatic assay provides a simple and direct means of investigating the relationship between dose, body-weight and biological response, it was decided to examine the mydriatic response to atropine in mice over a wide weight range. During this work, other factors were found which influenced the mydriatic response.

Experimental Methods

Measurement of mydriasis. Male albino mice, T.T. strain, 16–50 g., were used. The diameter of the pupil of the right eye was measured on a graticule set in one eye-piece of a binocular microscope, magnification × 17·5. During measurement, the mouse's head was held approximately 10 cm. from a 60 W bulb of an Anglepoise lamp which was partly masked to give a light aperture of 4 cm. The mouse was held in position for 8–15 sec., until the pupil size had become constant. Immediately after measurement of the initial pupil diameter, the mouse was injected subcutaneously between the shoulders with atropine sulphate solution. The pupil was remeasured at a selected time after injection, and the

mydriatic response expressed as the arithmetic difference between the two pupil diameters in units of the graticule scale (20 units = 1 mm.). In certain experiments, the diameter of the lens of each mouse's eye was measured also, and taken as the maximum pupil size possible. This ranged from about 38 units in smallest mice to 50 units in the largest mice used. The arithmetic difference between this value and the initial pupil size was taken as the *maximum mydriatic response possible.*

Atropine was calculated as the base, and injections were given in a dose volume of 0·10 ml./10 g. of mouse. Mice were kept during the test in groups of five, each group in a compartment about 12 cm. square.

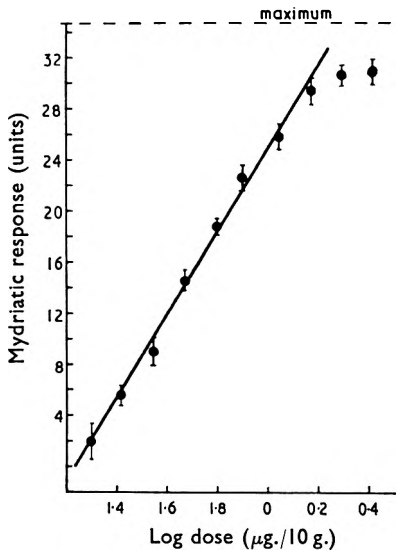

FIG. 1. Mydriatic response to atropine in 20–25 g. mice. Vertical lines represent 2 × s.e.

Determination of blood atropine content. Samples of cardiac blood were taken from mice anaesthetised 5 min. previously with pentobarbitone sodium (0·9 mg./10 g., i.p.) at 15, 30, 45, 60 and 120 min. after injection of atropine (1 mg./10 g., s.c.). The thorax was cut open and up to 1 ml. of blood withdrawn from the right ventricle into a syringe containing about 200 units of heparin in 0·05 ml. saline. The contents of the syringe were mixed and immediately reinjected subcutaneously, at a volume of 0·10 ml./10 g. weight of recipient mouse, into 26–30 g. mice, the initial size of whose pupils had been measured shortly beforehand. Usually enough blood could be withdrawn from each donor mouse to inject into 2 or 3 recipient mice, except for samples withdrawn 120 min. after dosage, when it was necessary to inject 0·20 ml./10 g. The pupils of the recipient animals were measured again 45 min. after injection and the mydriatic response used to calculate the concentration of atropine-like substance in the blood injected, by reference to a standard dose-response line. This line was determined in 26–30 g. mice for known amounts of atropine which

had been mixed immediately before injection with blood withdrawn from mice which had received pentobarbitone only. It was found that admixture with blood slightly depressed and delayed the mydriatic response to atropine after subcutaneous injection. A similar displacement of the atropine dose-response curve to the right when atropine was injected mixed with blood was reported by Godeaux and Tonnesen (1949).

Blood samples were withdrawn from 6–11 mice at each time interval after injection; a further 12–30 mice were used to assay these samples.

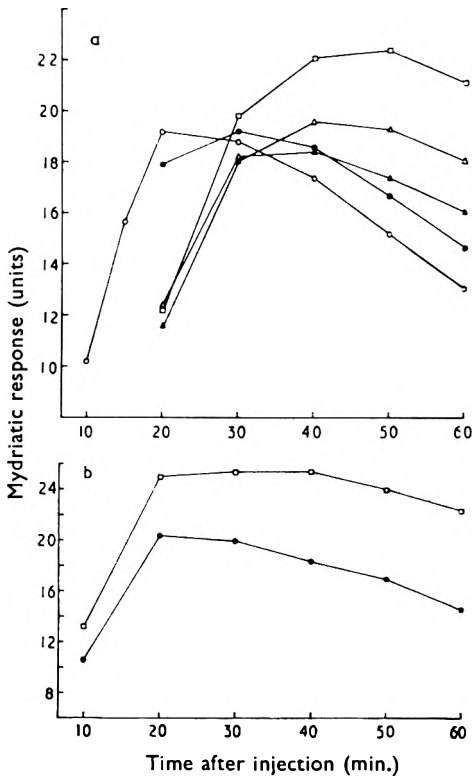

FIG. 2. Time course of the mydriatic response to:—(a) 0·625 μg./10 g. atropine injected subcutaneously in mice of different weights: ○——○ 16–20 g. ●——● 21–25 g. ▲——▲ 26–30 g. △——△ 31–40 g. □——□ 41–50 g. (b) 2 μg./10 g. atropine injected intraperitoneally into mice: ●——● 21–25 g. □——□ 41–50 g.

RESULTS

Dose-response Relationship

Mice of 20–25 g. were injected, in random order, with one of 10 different doses of atropine differing from each other by 0·1250 (log scale). 7–25 mice were injected with each dose, and the mydriatic response measured 30 min. after injection. Mean responses with their standard errors are represented in Fig. 1.

The log dose-response line, weighted to allow for the differing numbers of animals used at each dose-level, was calculated from all responses except those to the two largest doses (to which the mean responses were over 85 per cent of maximum). Deviation from linearity of regression was not significant ($P = 0{\cdot}10$). The slope of the calculated line was $32{\cdot}2 \pm 1{\cdot}4$ (s.e.).

Variation of Response With Body Weight

The mydriatic response to a selected dose of atropine (0·625 μg./10 g.) was measured in mice of various weights from 16 to 50 g. Since the time between injection and peak mydriasis was found to vary markedly with the size of the mice, the mydriatic response for each mouse was determined at 20, 30, 40 50 and 60 min. after injection (and at 10 and 15 min. in the 16–20 g. mice). 58–81 mice were used in each weight group. The time-courses of the responses for the various weight groups of mice are represented in Fig. 2.

It can be seen from the figure that the heavier the mice, the slower was the onset of mydriatic effect and the more delayed the peak response, to a standard dose of atropine. Mice of 16–20 g., for instance, showed peak mydriasis 20 min. after injection, at a time when the mydriatic response of 41–50 g. mice was only about half of that at the time of their peak effect. This peak occurred 50 min. after injection, by which time the response of the 16–20 g. mice had decreased by about 25 per cent.

The response appeared to be slightly greater in larger mice than in small. However, when this was expressed as a percentage of the maximal mydriatic response possible (by which means allowance was made for the slightly greater size of the eyes of larger mice), it was evident that the response was constant for all weight ranges of mice from 16–50 g. (Table I).

TABLE I

MYDRIATIC RESPONSE OF MICE TO ATROPINE (0·625 μg./10 g.)

Weight-range (g.)	16–20	21–25	26–30	31–40	41–50
(i) Maximum mydriatic response possible (units)	33·7	34·2	36·1	35·8	39·2
(ii) Time of peak effect (min. after injection)	20	30	40	40	50
(iii) Peak mydriatic response:					
(*a*) in units	19·2	19·3	18·6	19·7	22·4
(95 per cent limits)	(18·0–20·4)	(18·1–20·5)	(17·4–19·8)	(18·7–20·7)	(21·2–23·6)
(*b*) as per cent of (i)	57	56	51	55	57
(95 per cent limits)	(53–61)	(53–60)	(48–55)	(52–58)	(54–60)

In this experiment, the volume of injection was kept constant at 0·10 ml./10 g. of mouse. It was possible therefore that differences in the time-courses of mydriatic response in the various weight-ranges of mice could be at least partly attributable to differences in the volume of the injections. This was tested by injecting two groups of 26 mice (31–40 g.) with atropine, 0·625 μg./10 g., one group receiving the dose in a volume of 0·10 ml./10 g. and the other in 0·05 ml./10 g. The time-courses of their mydriatic responses are plotted in Fig. 3.

The time-courses of the mydriatic response for these two groups of mice were parallel, and their mean responses did not differ significantly ($P > 0{\cdot}2$). Furthermore, there was a marked difference between the time-course in these 31–40 g. mice given atropine in an injection volume of 0·05 ml./10 g. and that in the 16–20 g. mice which were given the same dose in an identical total volume of injection (Fig. 2a). It was evident therefore that the shape of the time-course curves for the mydriatic response to atropine was dependent upon the weight-range of the mice used and not upon the volume or concentration of the injection.

To confirm that the slopes of the regression lines for atropine mydriasis did not differ between mice of different weights, the mydriatic responses to a low dose (0·35 μg./10 g.) and a high dose (1·11 μg./10 g.) were determined in mice of two different weight-ranges, 21–25 g. and 41–50 g. These doses produced responses approximately 25 and 75 per cent of maximum. 10 mice were tested in each group. The slopes of the regression lines so obtained were virtually identical in both weight-ranges ($b = 32{\cdot}2$ and 33·2 respectively).

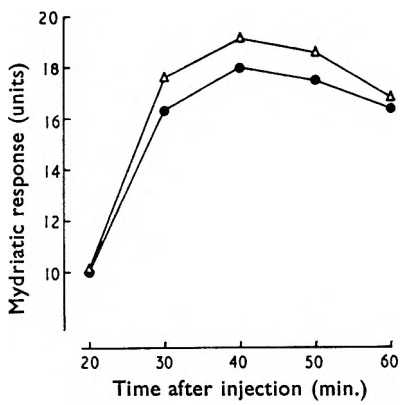

FIG. 3. Time course of the mydriatic response to atropine (0·625 μg./10 g.) in 31–40 g. mice: ●——● Injection 0·10 ml./10 g. △——△ Injection 0·05 ml./10 g.

Since many workers who have investigated the assay of atropine by its mydriatic response in mice have used the intraperitoneal route of injection (Ing, Dawes, and Wajda, 1945; Kroneberg, 1955; Huycke, 1957), the time-course of the mydriatic response to atropine given by this route was determined in mice of two different weight ranges, 21–25 g. and 41–50 g. The dose used for subcutaneous administration (0·625 μg./10 g.) was found to produce a barely significant mydriasis when injected intraperitoneally; therefore a dose of 2 μg./10 g. was used (Fig. 2b).

The onset of mydriasis was more rapid after intraperitoneal than after subcutaneous injection, especially in the heavier mice, in which the peak response was constant from 20 to 40 min. after administration. The 21–25 g. mice showed peak mydriasis at 20 min. At this time, the mydriatic responses, when expressed as percentages of the maximal possible in the respective weight-ranges, were not significantly different.

Comparison Between Responses in Mice Used Once or Repeatedly

The time-courses of the mydriatic response to atropine (0·625 μg./10 g.) of mice which had been used some 5–10 times previously during the preceding months were compared with those of hitherto unused mice. Mice of two weight-ranges were used (31–40 g. and 41–50 g.). A total of 242 mice were tested. The time-courses for the responses in the four groups of mice are plotted in Fig. 4.

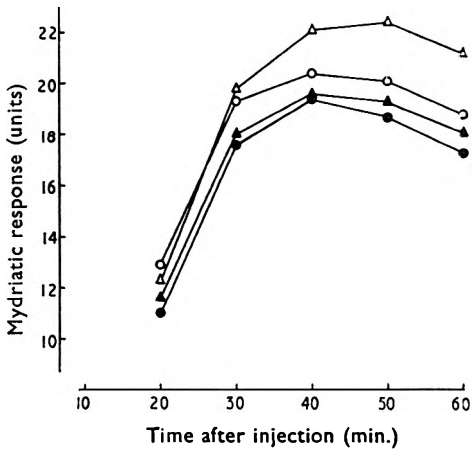

FIG. 4. Time-course of the mydriatic response to atropine (0·625 μg./10 g.) in mice of 2 weight-ranges which have or have not been tested beforehand:

	31–40 g.	41–50 g.
Hitherto-unused	▲——▲	△——△
Used frequently	●——●	○——○

Mice in the 31–40 g. weight-range showed nearly identical mydriatic responses whether or not they had been used repeatedly beforehand. This was not so with the larger mice. Hitherto unused mice of 41–50 g. body weight showed a significantly larger response ($P = 0{\cdot}01$–$0{\cdot}02$) than mice of a similar weight which had been used many times previously. This difference held true whether the responses were expressed directly as units or converted to percentages of the maximal mydriatic response possible in each group.

Determination of Atropine Content of Cardiac Blood in Mice at Various Times after Injection

To confirm that the difference in the time-courses of mydriatic response between mice of differing weight arose from differing rates of absorption of atropine from the subcutaneous site of injection (which seemed likely, since such differences were not evident after intraperitoneal injection), the time-course of the atropine content of cardiac blood was examined in mice of two weight ranges, 21–25 g. and 41–50 g., after subcutaneous injection of the alkaloid. To obtain assayable amounts of atropine in the blood

a dose of 1 mg./10 g. was given. This dose produced complete dilatation of the pupil, urination and slight "jumpiness", but no toxic symptoms.

The blood concentrations of atropine in the two groups of mice at the various times after injection are represented in Fig. 5. Comparison of these curves with those obtained for the time-course of the mydriatic response to atropine in mice of the same two weight-ranges reveals close parallelism. In both tests, the peak effect in the 21–25 g. mice was obtained about 30 min. after injection, whereas that in the 41–50 g. mice occurred at about 45 min. But there was a marked difference in the

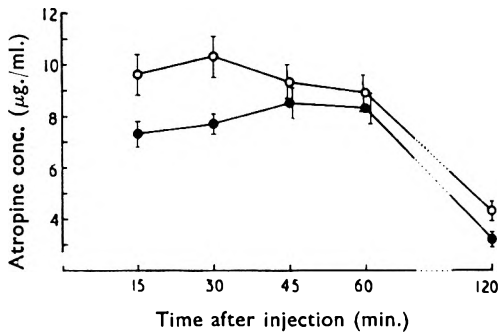

FIG. 5. Atropine concentration in the cardiac blood of mice injected subcutaneously with 1 mg./10 kg. atropine, withdrawn at various times after injection. Vertical lines represent 2 × s.e. ○——○ 21–25 g. mice. ●——● 41–50 g. mice.

relative size of the effects. In the mydriatic response the larger mice displayed a greater effect than the smaller, although there was no significant difference between the mean responses when they were expressed as percentages of the maximal mydriatic response possible in the two groups of mice. With cardiac blood concentration, however, the larger mice showed *less* atropine than the smaller mice at all times. The difference between the mean values for peak blood concentration of the two groups was not statistically significant ($P = 0{\cdot}05$–$0{\cdot}10$) but when values for all times were analysed, the difference in overall concentration between the two-weight ranges of mice was highly significant ($P < 0{\cdot}001$).

Variation of Mydriatic Response to Atropine with Time of Day

During the course of the investigations, it became apparent that variations occurred in the mydriatic response to a standard dose of atropine at different times of day. In an attempt to plot such variations, the mydriatic response to atropine (0·625 μg./10 g.) was determined in 21–25 g. mice at intervals of 3 hr. from 8 a.m. to 2 a.m. Since it was thought that these variations might be linked with the state of activity of the animals, this in turn being governed to some extent by the degree of ambient lighting, half the mice were tested under conditions of natural lighting (supplemented at night by the minimal amount of artificial lighting necessary to perform the test) and the other half under artificial light (plus daylight when present). The illumination of the mouse pupils by

shielded electric light during examination was kept constant throughout. A total of 653 mice were tested.

The mean values for the initial and the final pupil diameters and the net mydriatic response to atropine (i.e., the difference between the final and initial pupil diameters) at various times of the day, under natural and artificial lighting, are represented in Fig. 6.

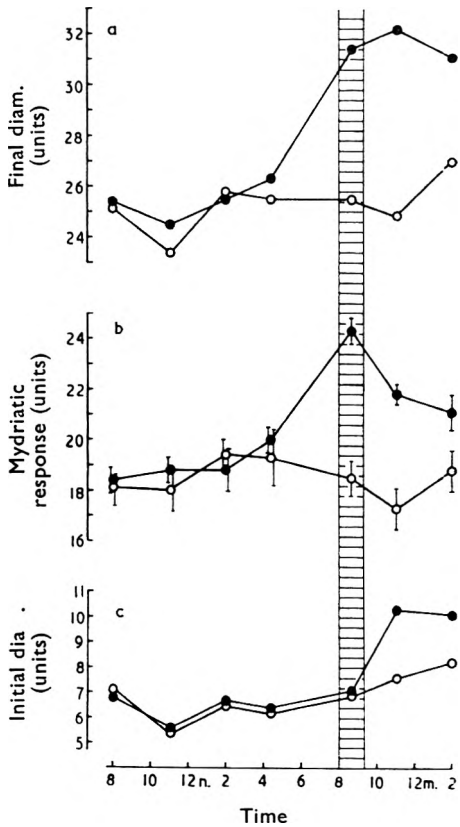

FIG. 6. (a) Final pupil diameter. (b) Net mydriatic response. (c) Initial pupil diameter of 21–25 g. mice given atropine (0·625 μg./10 g.) at various times of the day and night. Vertical lines indicate 2 × s.e. Hatched area represents dusk. ○——○ under artificial lighting (plus daylight when present). ●——● under natural lighting.

Under natural lighting conditions, the initial pupil diameter of the mice was fairly constant throughout the hours of daylight, apart from a slight fall at 11 a.m. It began to rise at dusk (8–9 p.m.). During the hours of darkness, the pupils were significantly larger than during the daytime. Similarly the final pupil size after atropine was constant from 8 a.m. to 5 p.m., but this rose at dusk. Since at this time the initial pupil size had increased little above daylight levels, the net mydriatic response to atropine

was significantly increased. For the readings during the hours of night (11 p.m. and 2 a.m.), the mean value for the final pupil size was the same as that at dusk; since the initial pupil size had now increased considerably, however, the net mydriatic response was reduced from its high value at dusk, but it was still significantly above mean daytime levels.

Under conditions of artificial light (supplemented by daylight when present), the mean values for initial pupil size agreed closely with those for mice under natural lighting, during the hours of daylight (8 a.m. to 8 p.m.), showing not only exactly the same overall mean (6·5 units) but also the same high values at 8 a.m. and 8 p.m., with a nadir at 11 a.m. During the hours of night, however, although the initial pupil size in mice under artificial lighting increased to a just significant degree, the increase was considerably less than that in mice under natural lighting conditions. The final pupil size measured after atropine under artificial lighting conditions changed little between 8 a.m. and 11 p.m., apart from a fall at 11 a.m.; the mean mydriatic response accordingly remained fairly constant throughout this time. At 2 a.m. the final pupil size rose in parallel to the increase in initial pupil size; the mean mydriatic response was therefore virtually unchanged.

Thus the net mydriatic response to a standard dose of atropine remained nearly constant between 8 a.m. and 2 a.m. if the animals were tested under conditions of artificial lighting (plus natural daylight when present). If however only natural lighting was used, the mydriatic response showed a marked increase at dusk, even though at this time lighting conditions appeared adequate to the experimenter. This increase at dusk was actually greater than that obtained during the hours of darkness, when the test would not normally be performed under the conditions of "natural" (i.e. minimum) lighting used here.

Discussion

The linearity of the regression of log dose with net response (in arithmetical units of increase of pupil diameter) has been confirmed here in the bioassay of atropine by its mydriatic effect in mice. Although Pulewka (1932) used a geometric ordinate scale, later workers have usually found the regression of log dose with arithmetic response to be reasonably linear (Oelkers and Vincke, 1935; Ing and others, 1945; Kroneberg, 1955; Huycke, 1957). In this work such a regression has been shown to be linear for responses from about 5 to 85 per cent of the maximum possible.

That the ordinate scale of mydriatic response can be expressed in terms simply of change in pupil diameter implies that the circular muscle of the iris sphincter may be considered to act pharmacodynamically in essentially a similar manner to a piece of longitudinal muscle, such as in a segment of isolated intestine. In such a tissue, equal log increments of atropine added to the organ bath produce equal degrees of depression of the contractile response of the muscle to acetylcholine. Similarly in the mouse eye *in vivo*, equal log increments of atropine injected into the animal may be considered to cause equal degrees of relaxation of the circular iris muscle (which is presumably contracted by endogenous acetylcholine)

since the length of the muscle is equivalent to the circumference, and hence related to the diameter, of the pupil.

Although exceptions have been reported (Rall and North, 1953; Gaddum, 1953; Angelakos, 1960; Russell, Emery and Bowers, 1960), adjustment of dose by a direct proportionality with the body weight of animals to be injected is generally considered adequate to ensure comparable blood levels and comparable pharmacological responses to drugs in animals of different weight, at least within fairly narrow ranges. It is not often however that the validity of this proportionality can be tested in large numbers of animals over wide weight ranges. The mydriatic response to a low dose of atropine in mice offers a simple means of doing so, and has been used here. In this test, providing the mydriatic response is measured at its time of peak effect (which varies with the body weight) and is expressed as a percentage of the maximal mydriatic response possible in the mice tested (to allow for slight differences in size of the eye in mice of grossly different body-weight), the response to a dose of atropine, given in terms of body-weight, is constant in male mice of all weights between 16 and 50 g.

The weight of the animals used by previous workers examining the mydriatic response of atropine in mice has been between 15 and 23 g. (Pulewka, 1932; Tonnesen, 1948; Ing and others, 1945; Huycke, 1957). Grewal (1951) and the last two mentioned workers all stated that mice of larger weight were less sensitive to the mydriatic action of atropine. They did not apparently increase the dose of atropine in proportion to the increased weight of the mice. In this work, where the dose has always been calculated in terms of body weight, no such diminution in response in larger mice has been noted.

That a pharmacological response—here mydriasis—is greater after administration of a drug by the subcutaneous than by the intraperitoneal route is uncommon, in view of the more rapid and complete absorption by the latter route. Presumably after intraperitoneal injection most of the atropine is absorbed into the portal circulation and passes directly to the liver (Werner and Schmidt, 1959) which is the major detoxicating organ for atropine in the mouse (Evertsbusch and Geiling, 1956). After subcutaneous injection, however, the alkaloid passes slowly into the systemic circulation and some reaches the eye without passing through the liver. The rate of onset of mydriasis after doses of atropine producing roughly similar peak responses is more rapid after intraperitoneal than after subcutaneous injection, and peak effects are obtained by 20 min. after injection, in larger as well as in smaller mice.

That the rate of pupil dilatation after a standard subcutaneous dose of atropine is markedly slower in larger than in smaller mice may be explained in terms of slower peripheral blood flow or slower absorption from the subcutaneous site of injection or both, as a result of fat deposition and increased fibrous tissue in older mice. Such an explanation receives support from the atropine blood concentrations at various times after subcutaneous injection of a large dose of the alkaloid into two groups of mice of differing weight. The time-courses of the blood concentrations

ran parallel to those of the mydriatic effect in the two groups of mice, the larger animals showing peak concentrations some 15–30 min. later than the smaller. The atropine levels in the blood of the larger animals were however slightly but significantly lower than those in the smaller mice. This may imply that, possibly because of its slower absorption, atropine is more rapidly metabolised in older mice, but, since mydriatic responses were at least as great in these animals as in the younger mice, presumably some compensatory mechanism is occurring also—such as increased binding of the alkaloid in sites within the eye, or increased sensitivity to its action as the result, say, of diminished central parasympathetic tone in older animals.

The finding that the time-course of the mydriatic response was the same whether the volume of solution injected was 0·05 or 0·10 ml./10 g. weight, confirmed the statement by Pulewka (1932) that no change in mydriatic response was caused by altering the volume of injection within the range of 0·3 to 0·8 ml.

Pulewka (1932) found no significant change in the mydriatic response to atropine in mice injected with 0·4 or 0·8 μg./10 g. daily, for 13 times in 15 days. Even less variation in response was obtained when the intervals between use were at least 2–3 days. Several other workers have used their mice several times, at 2–4 day intervals (Huycke, 1957; Ing and others, 1945; Oelkers and Vincke, 1935; Tonnesen, 1948; Nyman, 1949). The first two mentioned workers however limited the total number of tests to which any mouse was submitted to 8 and 3 respectively, stating that after that the mice became less sensitive (presumably because these workers found that larger mice were less sensitive than smaller). Here no significant difference could be found in the mydriatic response to atropine in mice of 31–40 g. between those used for the first time and those used many times over the previous few months. In 41–50 g. mice, however, the used animals were slightly less sensitive than the fresh mice. This may have been caused by poor absorption from the site of injection (back of the neck) as the result of formation of scar tissue produced by repeated injections in the same site. The larger mice, being older, would have received more injections. It was noted also that mice used many times before had slightly but significantly larger pupils before atropine injection than had unused mice.

The conclusions reached here from the investigations into variations in mydriatic response to atropine occurring at various times of the day and night may be of considerable practicable importance. In short, it would appear that, provided artificial lighting is always used (in addition to any natural light present), the net mydriatic response should be nearly constant at all times of the day and night (8 a.m. to 2 a.m.). If, however, only natural lighting is used, at times such as dusk (8–9 p.m. here) when such lighting is poor, the mydriatic response may be abnormally high. Normally assays would not be performed in the minimum lighting conditions used here to simulate "natural" lighting at night (when again high responses were obtained), but since absence of extraneous light facilitates observation of the mouse pupils, assays may often be performed in partly

darkened rooms. It is therefore essential that these lighting conditions are kept strictly constant, if reproducible results are to be obtained. No experiments were performed here to discover whether the mydriatic response is constant at all times of the day and night in standard conditions of reduced light. Pulewka (1932), who is the only other worker who appears to have examined this source of variation in the mydriatic response to atropine, worked in a darkened room, and chose the hours of 10.30 a.m. to 5 p.m. only, since he found that the mydriasis induced by atropine was greater in the evening than in the daytime. He took considerable precautions to exclude noise and excitement, since he found that any mice which were disturbed or excited by, for instance, hunger or "being on heat" gave abnormal results. It was noted in the present work that variations, especially in initial pupil size, sometimes occurred between groups of 5 mice apparently otherwise identical but kept in different sections of a "toxicity box". This was ascribed to a state of irritation or excitement engendered in all the animals in one group by one awkward individual in their number. Ing and others (1945) however, stated that initial pupil size was unaffected by excitement. Oelkers and Vincke (1935) agreed with Pulewka about the disturbing effect of noise, etc., and also mentioned a seasonal variation in response encountered. This latter phenomenon could not be confirmed by Tonnesen (1948).

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THE MORPHOLOGY AND ANATOMY OF THE LEAF OF *PODOPHYLLUM PELTATUM* L.*

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A brief review of the history of *Podophyllum peltatum* L. is given, together with an illustrated account of the macroscopical and anatomical structure of the leaf. The diagnostic characters of the powdered leaves are recorded and compared with those previously reported for *Podophyllum hexandrum* Royle. To distinguish the leaves of the two species in powder form, recourse must be made to palisade ratios: for *P. peltatum*, the values are 7·0–**8·5–12·16**–17, and for *P. hexandrum*, 4·0–**4·96–7·68**–11·25.

Podophyllum peltatum L. was first introduced into Britain as a horticultural plant and was listed in Evelyn's Kalendarium (1699) under the name *Anapodophyllum* Tournefort. This name, later shortened by Linnaeus (1737) to *Podophyllum*, was derived from πούς, a foot and φύλλουα, a leaf. Other common names were listed by Britten and Brown (1897) and Holm (1907) and include May Apple, Wild Lemon, Raccoon Berry, Duck's Foot and Wild or American Mandrake. Holm (1907) reported that the root and rhizome were used by the Cherokees as an anthelmintic and by the Osage Indians as a cure, by purgation, for poisoning; Lloyd (1910) noted that the Wyandottes regarded them as cathartics and emetics. The leaves appear to have been little used and Bentley (1861) reported that they contained a poisonous principle which was lost on drying. Duffield (1868) isolated only 0·3 per cent w/w of resin from the leaves whilst Husband (1869) reported resin absent but Carter (1886) obtained 6 per cent w/w of resin from leaves collected soon after flowering. Recent investigations by Hussain, Chaudhri, Muhammed and Wahhab (1954) of the resin content of *P. hexandrum* Royle indicated that further work was necessary and our preliminary estimations by the method previously described (Ellis and Fell, 1962) have established an average resin content of 4·2 per cent w/w in leaves of *P. peltatum*.

Holm (1907) described the anatomy of the leaf briefly together with that of the stem and underground organs and the present work was undertaken to expand this description and to note the diagnostic characters necessary for the identification of whole or powdered leaves and to distinguish them from those of *P. hexandrum* Royle. As it proved impossible to raise *P. peltatum* L. from seed under the conditions available and as the germination of the seeds and the variations in the morphology of the flowering plants have already been described by Holm (1899) and Porter (1877) no details of development are given.

* The subject-matter of this text forms part of a thesis submitted by one of us (S. E.) for the external degree of M.Pharm., University of London.

Material and Methods

The leaves were supplied (i) by Philadelphia College of Pharmacy and Science, Philadelphia (ii) Lafayette College, Easton, Pennsylvania, (iii) by Dr. T. E. Wallis, from plants grown in Kew Gardens, Surrey or (iv) were collected from plants supplied by various nurserymen and transplanted to a light peaty soil in West Yorkshire.

The techniques used in the examination of these leaves were similar to those described for *P. hexandrum* leaves (Ellis and Fell, 1962) excepting that the length of the maceration process with 5 per cent w/v potassium hydroxide solution was reduced to 10 to 15 min. in order to isolate the secretion cells in the whole condition as well as the lignified material.

Macroscopy

A high degree of variation occurs in the macroscopical appearance of radical and cauline leaves, but no significant differences were observed between the two types. The leaves (Fig. 1, *A–C*) measure from 10 to 25 cm. across and are peltate with a centric, or slightly eccentric, erect, cylindrical petiole. They are polygonal in outline, the lamina being palmatisect with 4 to 7 obovate, or ob-lanceolate, bifid lobes. The margin of the leaf is entire in the basal region and serrate near the tip. The leaf is curled around the petiole on emergence and the lobes are revolute. The acute apices of the lobes may retain this revolute margin and be permanently reflexed. The venation is palmate with one main vein per lobe arising from the apex of the petiole and running straight to the point of conjunction of the segments where it divides, each branch running to the apex of a segment. In each marginal tooth three ultimate veinlets unite to form the terminal network (Fig. 2, *A*). The veins, which are prominent on the under surface, are lighter in colour than the interneural tissue and are covered with long, silky, covering trichomes. Trichomes are also present along the margin and sparsely scattered over the interneural tissue. The lamina shows little or no mottling, but the petiole is mottled red and green and in some cases bears numerous covering trichomes at its upper end (Fig. 2, *A*, *B*, *D* and *G*; Fig. 6, *B*).

Anatomy

Lamina

Interneural Tissue (Fig. 2, *A–E* and Fig. 3, *B*).

The upper epidermis is covered by a thin, smooth cuticle. The epidermal cells are irregular in shape and measure about Lev L and B 55 to **84** to 119 μ and H 19 to **33** to 46 μ, with very wavy anticlinal walls. They contain prominent nuclei situated on the base of the cell. *Trichomes* are absent from this surface except along the margin and *stomata* are very rare. Within each marginal tooth 1 to 4 hydathodes, measuring about Lev L 37 to **40** to 44 μ and Lev B 25 to **32** to 35 μ, occur on a small proportion of the leaves examined (Fig. 2, *A* and *C*).

The mesophyll is not well differentiated and all the cells contain chloroplasts, which are spherical or subspherical. The *palisade* consists

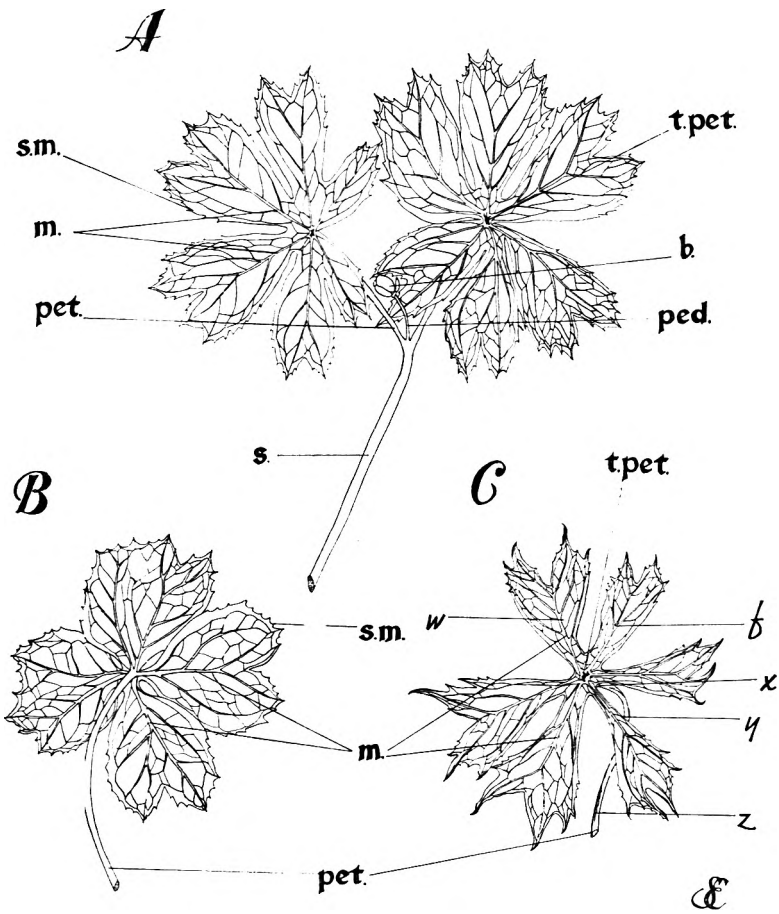

FIG. 1. Leaf of *Podophyllum peltatum* L. *A*, Flowering shoot; *B*, under surface of whole mature leaf; *C*, whole mature leaf of non-flowering plant. All × 1/5. "w," position of sections in Fig. 3, *A*; Fig. 4; Fig. 5, *A*; "x"–"z," position of sections in Fig. 5, *B-D*; "y" position of sections in Fig. 6; *b.*, flower bud; *m.*, main vein; *ped.*, pedicel; *pet.*, petiole; *s.*, stem; *s.m.*, serrate margin; *t.pet.*, top of petiole.

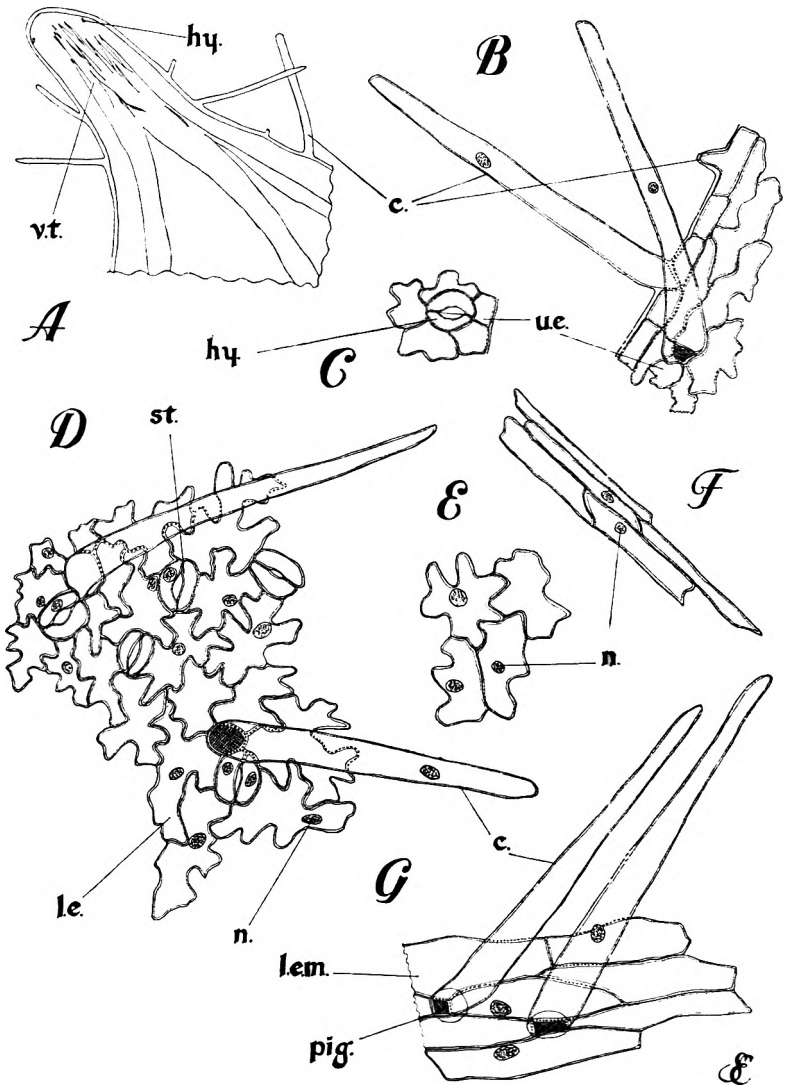

FIG. 2. Leaf of *P. peltatum* L. *A*, marginal tooth of leaf; *B*. upper epidermis of lamina; marginal region; *C*, upper epidermis of lamina with hydathode; *D*, lower epidermis of lamina, interneural region; *E*, upper epidermis of lamina; *F*, upper epidermis of main vein; *G*, lower epidermis of main vein. *A* × 30; *B–G* × 150. *c.*, covering trichome; *hy.*, hydathode; *l.e.* lower epidermis; *l.e.m.*, lower epidermis of main vein; *n.*, nucleus; *pig.*, pigment; *st.*, stoma; *u.e.*, upper epidermis; *v.t.*, veinlet termination.

of an ill-differentiated single layer of irregularly cylindrical cells measuring about Lev L and B 15 to **27** to 46 μ and H 27 to **43** to 78 μ. The *spongy mesophyll* is aereolate and consists of five or six layers of trabeculate parenchyma cells about 11 to **32·5** to 78 μ in diameter, and occasional rounded cells of the same diameter, with brown amorphous contents staining red with 50 per cent v/v nitric acid, with large intercellular spaces. *Calcium oxalate* occurs only rarely in the mesophyll but in some leaves rosette crystals are found near the veins (Fig. 3, *B* and *C*).

The LOWER EPIDERMIS is covered by a thin, smooth cuticle. The epidermal cells are irregular in shape, measuring about Lev L and B 55 to **91** to 137 μ and H 15 to **25** to 35 μ, with very wavy anticlinal walls. Anomocytic *stomata* are numerous, raised slightly above the level of the epidermis, ellipitical, or circular, in outline and measure about 36 to **45·5** to 61 μ in length and 25 to **34** to 40 μ in breadth, or 32 to **40** to 46 μ in diameter (Fig. 2, *D*; Fig. 3, *B*). *Covering trichomes* occur over the whole surface of the lamina but are most numerous near the larger veins and in the marginal region. Those of the interneural tissue measure about 200 to **310** to 470 μ in length and 29 to **37** to 47 μ in diameter at the base whereas those of the marginal region show a much wider variation in size measuring about 14 to **330** to 865 μ in length and about 14 to **32·5** to 61 μ in diameter at the base. They are thin walled, cellulosic and unicellular with a blunt, rounded apex (Fig. 2, *B* and *D*).

The lamina has a smooth margin with a few scattered acutely pointed teeth near the apices of the lobes. The ultimate veinlets extend to within about 0·1 mm. of the teeth apices and terminate in several small spiral elements. Three ultimate veinlets unite to form this terminal group about 0·6 mm. from the tip (Fig. 2, *A*).

Main Vein (Fig. 2, *F* and *G*; Fig. 3, *A* and *D*; Fig. 4; Fig. 5, *A*).

The central veins of each lobe and its segments are similar in construction. The transverse section, in the basal half of the lobe, shows three unequal bundles embedded in a central, cordate shaped mass of collenchyma (Fig. 5, *A*). The extreme tip of the lobe shows three, small, equal bundles which unite and subsequently redivide to give three unequal bundles after the point of conjunction of the two segments has been reached. The size of the central bundle increases gradually as small secondary veins enter and the amount of collenchyma increases proportionately. The entry of larger secondary veins alters the arrangement of the bundles temporarily, but it always returns to three. No unification of the main veins occurs before they enter the petiole.

The UPPER EPIDERMIS is composed of polygonal straight, or slightly wavy walled cells elongated along the axis of the lobe (Fig. 2, *F*; Fig. 3, *A*). They measure about Lev L 68 to **127** to 200 μ, Lev B 10 to **18** to 25 μ and H 23 to **31** to 39 μ. The cuticle is thicker than on the lamina but the nuclei are similar to those already described. *Trichomes* and *stomata* are absent.

The CORTEX is divided into two main regions. The upper hypodermal region is composed of thick-walled, collenchymatous cells measuring

about L 82 to **160** to 277 μ and R and T 19 to **38** to 74 μ throughout, but the lower hypodermal region becomes collenchymatous only in the basal half of the lobe, where it is large-celled, measuring about L 82 to **160** to 277 μ and R and T 21 to **39** to 58 μ. Both hypodermal regions contain scattered spherical, or sub-spherical chloroplasts. The remaining cortex is parenchymatous, the cells measuring about L 93 to **135** to 190 μ and R and T 20 to **38** to 75 μ above the stele and L 62 to **109** to 292 μ. R and T 45 to **76** to 117 μ below the stele. Rosette crystals of *calcium oxalate* occur scattered throughout the cortex in the larger cells, measuring about 15 to **28** to 43 μ in diameter.

The ENDODERMIS is not well differentiated but a continuous band of cells, which contain starch grains, measuring about 4 to **6** to 10 μ in diameter, can be traced around the central collenchymatous tissue surrounding the stele. As the parenchyma of the cortex does not contain starch this layer may be regarded as a starch sheath (Fig. 3, *A*).

The MERISTELE consists of three well defined bundles embedded in a mass of relatively thin walled collenchyma (Fig. 3, *A*; Fig. 5, *A*), measuring about L 117 to **195** to 320 μ and R and T 9 to **25** to 55 μ. The cells frequently contain nuclei and occasionally brown amorphous material, which stains red with 50 per cent v/v nitric acid.

The PHLOEM consists of sieve tissue with well defined companion cells and patches of small celled phloem parenchyma. The sieve tubes measure about R and T 11 to **17** to 31 μ, the individual segments being about 177 μ in length, with transverse, or oblique sieve plates. The companion cells are narrow being only 2 to **6·3** to 12 μ in diameter and 66 to **96** to 140 μ in length. There are distinct medullary rays between the three bundles, composed of cellulosic parenchyma, slightly elongated radially, measuring about R 19 to **31** to 43 μ and T 11 to **16** to 23 μ. There is no radial arrangement within the bundles, but phloem parenchyma cells, measuring about R and T 17 to **26** to 39 μ and L 78 to **130** to 200 μ and occasionally containing brown amorphous contents similar to those of the collenchyma, occur in small groups. Large secretory structures, which are unicellular with brown, thin, cellulosic cell walls and brown cell contents, staining red with 50 per cent v/v nitric acid, grey with ferric chloride solution and a faint pink with Sudan III, and measuring about R and T 19 to **33** to 51 μ and L 225 to **452** to 875 μ occur in small numbers in the phloem. In addition, in the main veins of larger leaves, in the region of the petiole apex, thick walled, non-lignified fibre-like cells occur in the pericyclic region. They measure about R and T 15 to **26** to 35 μ and L 70 to **84** to 175 μ and occur scattered in the outer region of the vascular collenchyma (Fig. 3, *A*; Fig. 4).

The *cambium* consists of an ill defined layer of thin walled, tangentially elongated cells.

The XYLEM consists of irregularly arranged vessels and tracheids with patches of xylem parenchyma. The vessels are lignified with spiral, annular or rarely reticulate thickening and measure about 11 to **25** to 39 μ in diameter. The tracheids and tracheidal vessels are of similar diameter and measure about 245 to **391** to 665 μ in length and show spiral, annular,

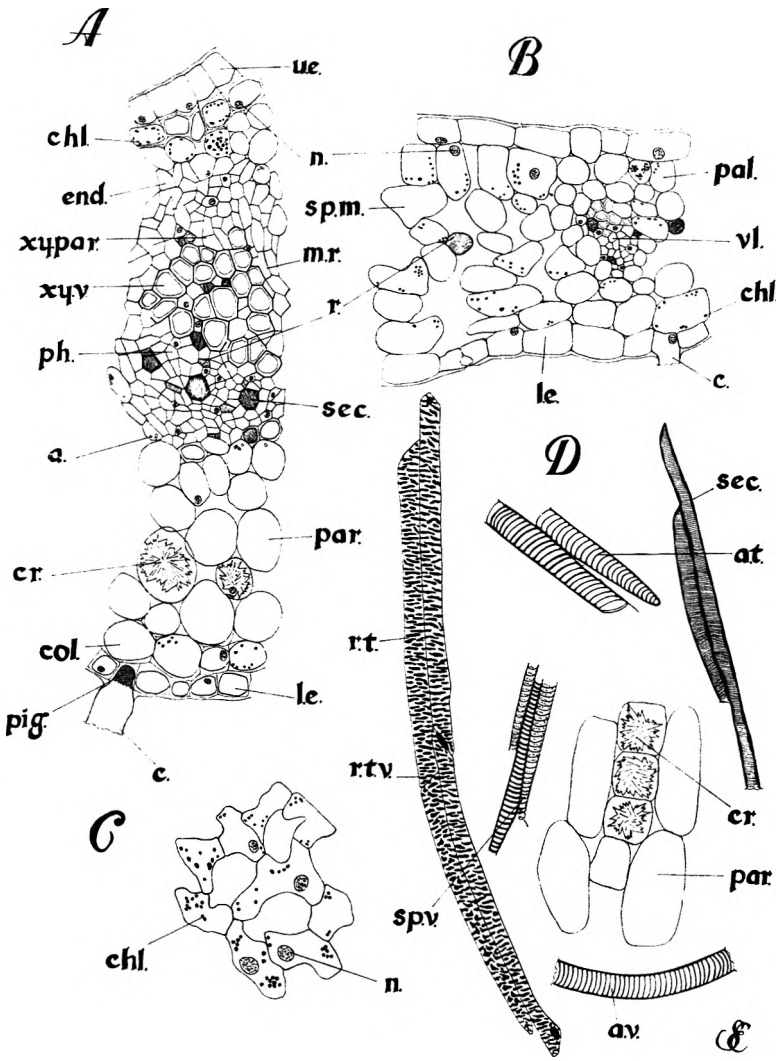

FIG. 3. Leaf of *P. peltatum* L. *A*, transverse section of the main vein cut at position "w" (see Fig. 1, *C*); *B*, transverse section of the lamina; *C*, spongy mesophyll; *D*, isolated elements of the main vein obtained by maceration. *A*, *B* and *C* × 150; *D* × 75. *a.*, starch; *a.t.*, annular tracheid; *a.v.*, annular vessel; *chl.*, chloroplast; *col.*, collenchyma; *cr.*, crystal of calcium oxalate; *end.*, endodermis; *l.e.*, lower epidermis; *m.r.*, medullary ray; *n.*, nucleus; *pal.*, palisade; *par.*, parenchyma; *ph.*, phloem; *pig.*, pigment; *r.*, resinous material; *r.t.*, reticulate tracheid; *r.t.v.*, reticulate tracheidal vessel; *sec.*, secretion cell; *sp.m.*, spongy mesophyll; *sp.v.*, spiral vessel; *st.g.c.*, stoma guard cell; *u.e.*, upper epidermis; *v.col.*, vascular collenchyma; *vl.*, veinlet; *xy.par.*, xylem parenchyma; *xy.v.*, xylem vessel.

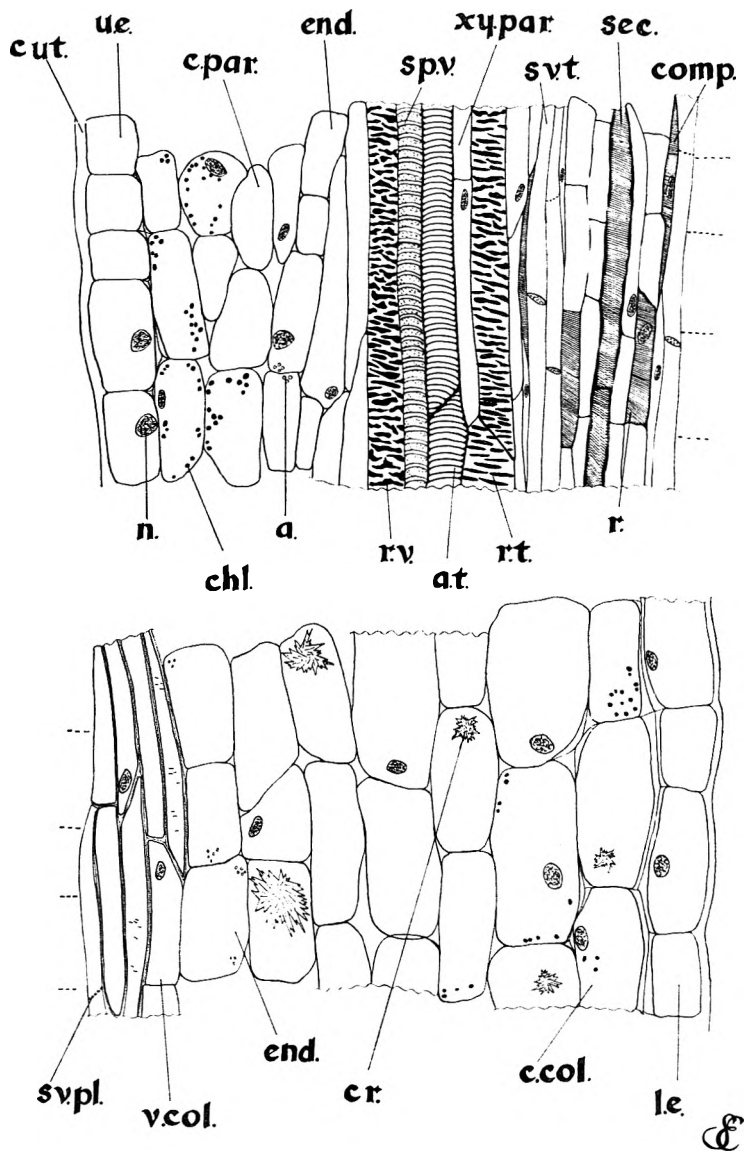

FIG. 4. Leaf of *P. peltatum* L. Longitudinal section of the main vein cut approximately at position "w" (see Fig. 1, *C*). × 150. *a.*, starch; *a.t.*, annular tracheid; *chl.*, chloroplast; *c.col.*, cortical collenchyma; *comp.*, companion cell; *c.par.*, cortical parenchyma; *cr.*, crystal of calcium oxalate; *cut.*, cuticle; *end.*, endodermis; *l.e.*, lower epidermis; *n.*, nucleus; *r.*, resinous material; *r.t.*, reticulate trachieid; *r.v.* reticulate vessel; *sec.*, secretion cell; *sp.v.*, spiral vessel; *sv.pl.*, sieve plate; *sv.t.*, sieve tube; *u.e.*, upper epidermis; *v.col.*, vascular collenchyma; *xy.par.*, xylem parenchyma.

reticulate or pitted thickening. The number of tracheids increases towards the tip of the lobe. The xylem parenchyma is thin walled, cellulosic; its cells have similar cell contents to those of the phloem parenchyma.

The LOWER EPIDERMIS (Fig. 2, *G*; Fig. 3, *A*; Fig. 4) is composed of large polygonal cells elongated along the axis of the lobe, measuring about Lev L 29 to **44** to 60 μ, Lev B 21 to **31** to 43 μ and H 27 to **40** to 63 μ with straight anticlinal walls. *Stomata* are absent but numerous unicellular *covering trichomes* occur. They measure about 288 to **550** to 820 μ in length and 25 to **38** to 54 μ in diameter at the base. The walls are thin and cellulosic and the trichomes have a blunt apex (Fig. 2, *G*).

PETIOLE (Fig. 5, *B—D*; Fig. 6).

The petiole is smoothly cylindrical, from 5 to 10 cm. long in cauline leaves and from 8 to 20 cm. long in radical leaves and from 3 to 6 mm. in diameter. The upper region, for a length of about 1 to 2 cm., bears long, silky covering trichomes similar to those of the main vein and the lower half of the petiole is frequently mottled red and green.

The vascular tissue occurs in two regions: (1) the outer ring of 15 to 25 bundles situated near the periphery and containing pericyclic fibres, phloem, cambium and xylem embedded in a mass of collenchyma and surrounded by a starch sheath. At the distal end of the petiole the pericyclic fibres are few, or absent, but the amount of lignified tissue gradually increases towards the base (Fig. 5, *B–D*). About 4 cm. below the leaf, the pericyclic fibres form an arc extending from cambium to cambium around the phloem and, 4 cm. further down, the whole of the vascular collenchyma within the starch sheath has been replaced by lignified fibres. Near the junction of the petiole with the stem, or rhizome, lignification of the interfascicular parenchyma in the pericyclic region occurs in a small proportion of leaves. In none of the leaves examined had this lignification become a complete ring (Fig. 5, *D*). (2) The central group of 6 bundles are asymmetrically placed in the pith parenchyma. These bundles contain a smaller number of pericyclic fibres, phloem, cambium and xylem embedded in collenchyma and surrounded by a starch sheath, one or two cells in breadth. The amount of lignified material increases towards the base, but in none of the leaves examined did the arc of pericyclic fibres extend to the cambium and the interfascicular parenchyma does not become lignified. The medullary bundles are irregularly arranged in the upper half of the petiole but tend to form a second, eccentric circle in the basal half (Fig. 5, *B–D*).

Tracing the entry of the primary leaf veins into the petiole shows that each vein divides on entry, part of the vascular tissue forming bundles in the outer ring and part entering the central bundles.

The EPIDERMIS consists of straight walled cells elongated longitudinally and measuring about Lev L 165 to **310** to 440 μ, Lev B 18 to **29** to 41 μ and H 19 to **28·5** to 39 μ. The cell walls are thin and cellulosic and the outer surface is covered by a thin cuticle. Anomocytic *stomata* measuring about 63 μ in length and 48 μ in breadth occur infrequently over the whole

of the petiole surface and unicellular, *covering trichomes*, closely resembling those of the main vein and measuring about 170 to **584** to 957 μ in length and 24 to **36** to 50 μ in diameter at the base, occur near the apex. Many cells contain red-brown pigment (Fig. 6; Fig. 7, B).

The CORTEX, like that of the main vein, consists of two layers of tissue. The outermost layer is a band of collenchyma several cells wide with thick cellulosic walls, measuring about R and T 15 to **29** to 39 μ and L 66 to **114** to 195 μ. The cells contain lenticular chloroplasts and in the outermost layers red-brown pigment. The remaining cortex is parenchymatous, occasionally becoming lignified and pitted in the pericyclic region of the basal half of the petiole. The cells are similar in size to the collenchyma being about R and T 15 to **39** to 63 μ and L 70 to **106** to 148 μ and frequently contain rosette crystals of calcium oxalate measuring about 27 to **36** to 59 μ in diameter (Fig. 6).

The ENDODERMIS takes the form of a starch sheath of one, or rarely two, layers of cells surrounding each bundle. The cells are similar in size and shape to the surrounding parenchyma but they contain numerous starch grains about 2 to **5** to 8 μ in diameter.

The PERICYCLIC FIBRES increase in number from the apex to the base of the petiole and eventually replace the whole of the vascular collenchyma in the outer bundles. The fibres are extremely long, measuring about L 580 to **1050** to 1,450 μ and R and T 5 to **16** to 31 μ, with thick, lignified, pitted walls and acute apices (Fig. 5, *B–D*; Fig. 7, *A*).

The *vascular bundles* of the inner and outer rings differ only slightly, the main difference being in the relative proportions of lignified fibres and collenchyma to conducting tissue; the inner ones have more conducting tissue and collenchyma and fewer fibres. In both cases at the apex of the petiole the bundles are embedded in collenchyma, the cells of which measure about L 97 to **215** to 390 μ and R and T 11 to **24** to 43 μ and have thin cellulosic walls.

The PHLOEM consists of groups of sieve tubes about 11 to **17** to 27 μ in diameter, the individual segments being about 78 to **162** to 280 μ in length; irregular groups of phloem parenchyma occur, individual cells measuring about L 46 to **107** to 226 μ and R and T 11 to **19·5** to 31 μ, and frequently containing brown cell contents similar to those of the main vein.

The XYLEM is well defined, the elements being irregularly arranged. The conducting elements resemble those of the main vein with a higher proportion of vessels to tracheids and a larger amount of reticulate and pitted thickening (Fig. 7, *A*). A little xylem parenchyma, measuring about 7 to **15** to 28 μ in diameter and 58 to **107** to 195 μ in length occurs close to the indistinct cambium and associated with the groups of vessels. The parenchyma frequently contains nuclei and, less often, brown cell contents similar to those of the phloem. The vessels are larger than those of the main vein being about 11 to **30** to 47 μ in diameter. The tracheids and tracheidal vessels have diameters within this range and measure about L 400 to **655** to 1260 μ (Fig. 7, *A*).

The PITH is composed of large celled parenchyma in which little or no lignification occurs. The cells are thin walled and measure about L 39 to

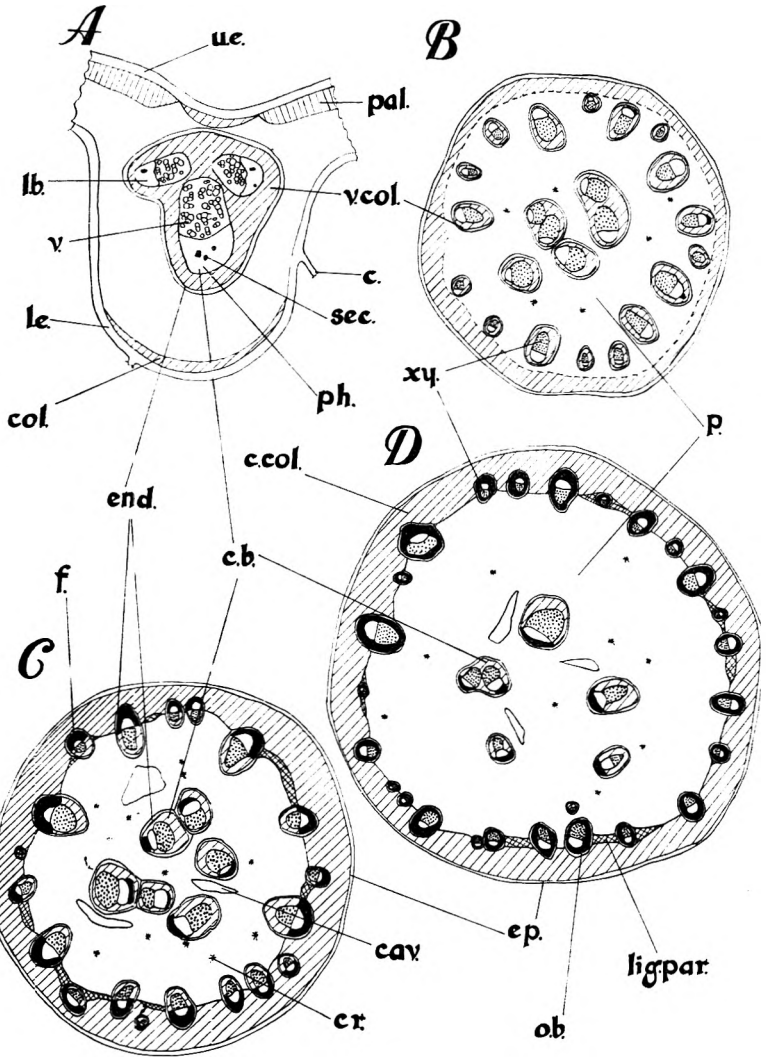

FIG. 5. Leaf and Petiole of *P. peltatum* L. *A*, transverse section of the main vein cut at position "w"; *B–D*, transverse sections of the petiole cut at positions "x," "y" and "z" (see Fig. 1, *C*). *A* × 40; *B–D* × 10. *c.*, covering trichome; *cav.*, cavity; *c.b.*, central bundle; *c.col.*, cortical collenchyma; *col.*, collenchyma; *cr.*, crystal of calcium oxalate; *end.*, endodermis; *ep.*, epidermis; *f.*, fibre; *l.b.*, lateral bundle; *l.e.*, lower epidermis; *lig.par.*, lignified parenchyma; *o.b.*, outer bundle; *p.* pith; *pal.*, palisade; *ph.*, phloem; *sec.*, secretion cell; *v.*, vessel; *v. col.*, vascular collenchyma. *xv.*, xylem.

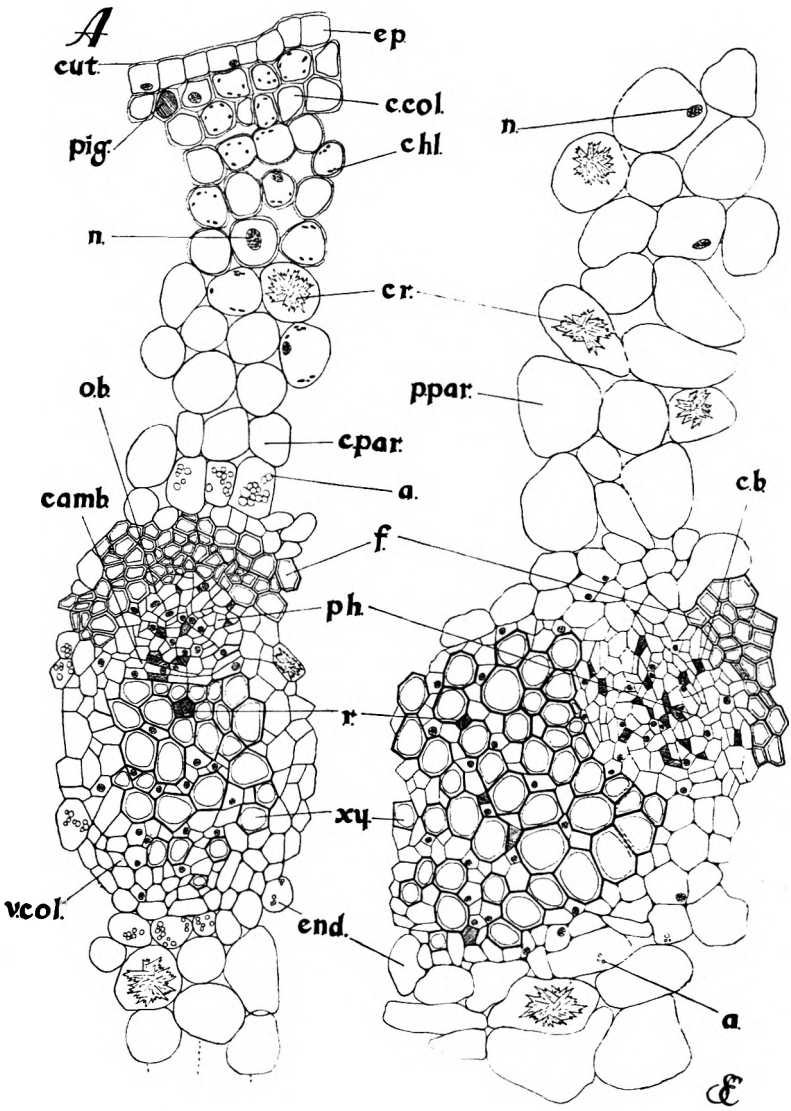

FIG. 6. Petiole of *P. peltatum* L. Transverse section of petiole cut at position "y" (see Fig. 1, *C*). × 150. *a.*, starch; *camb.*, cambium; *c.b.*, central bundle; *c.col.*, cortical collenchyma; *chl.*, chloroplast; *c.par.*, cortical parenchyma; *cr.*, crystal of calcium oxalate; *cut.*, cuticle; *end.*, endodermis; *ep.*, epidermis; *f.*, fibre; *n.*, nucleus; *o.b.*, outer bundle; *ph.*, phloem; *pig.*, pigment; *p. par.*, pith parenchyma; *r.*, resinous material; *v.col.*, vascular collenchyma; *xy.*, xylem.

85 to 183 μ and R and T 23 to 77 to 175 μ. Large rosette crystals of *calcium oxalate* measuring about 27 to 36 to 59 μ in diameter occur in vertical files of more or less cylindrical cells scattered throughout the pith but more common near the vascular bundles (Fig. 6; Fig. 7, *A*).

POWDER

The colour of a No. 40 powder varied from light green to brown, according to the time of collection of the leaves; it has a slightly acrid odour and a bitter taste. When a small amount of powder is mixed with (a) 5 per cent w/v copper acetate solution a bright green colour develops and (b) with 5 per cent w/v ferric chloride solution, a gradual darkening of the fragments of tissue is observed.

To examine the structural features of the powder mounts were prepared using 50 per cent v/v glycerol solution, solution of chloral hydrate or phloroglucinol and hydrochloric acid. The characters of the powder (Fig. 8) are:

1. Very numerous whole, or fragmented unicellular *covering trichomes.*

2. Fragments showing, in surface view, the wavy walled cells of the *upper epidermus of the interneural lamina* and usually the underlying palisade and, in the marginal region, covering trichomes of varying lengths or an occasional cicatrix.

3. Fragments of the *lower epidermis* showing, in surface view, the very wavy walled epidermal cells, anomocytic *stomata* and the bases or cicatrices of covering trichomes.

4. Less frequent particles showing the straight walled cells of the *upper epidermis of the main vein* or the elongated straight walled cells of the *lower epidermis of the main vein*, with numerous trichomes or cicatrices.

5. Fragments of lamina in transverse sectional view, about 160 μ wide with a single ill-differentiated palisade.

6. Small spiral and annular vessels from the veins; large annular tracheids and vessels associated with secretory cells, which are thick walled and have orange-brown resinous contents, from the main veins; reticulate vessels with associated collenchyma and large sieve tubes from the petiole.

7. Lignified acutely pointed *fibres* with pitted walls from the petiole.

8. Large thin walled parenchyma cells, frequently containing rosette crystals of *calcium oxalate* from the pith of the petiole, the cortex of the main vein or more rarely the mesophyll of the leaf.

9. Scattered rosette crystals of *calcium oxalate.*

Proportion of Petiole. Investigations on samples available showed that the percentage of petiole present was about 18 to 24 per cent by weight of the dry leaf.

DISCUSSION

Although it is comparatively easy to distinguish the whole leaves of *P. peltatum* L. and *P. hexandrum* Royle by the degree of incision, the number and shape of the lobes and the eccentricity of the petiole in relation to the lamina, they show a close similarity in broken or powdered condition.

These two species of the genus *Podophyllum* are characterised by the presence of long, unicellular, *covering trichomes* with cellulosic walls and blunt, rounded apices; long, lignified, pitted *fibres* with acute apices, frequently associated with sieve tissue or collenchyma; epidermal tissue from the *lower epidermis* of the *interneural lamina* composed of very wavy walled cells and having anomocytic *stomata*; rosette crystals of *calcium oxalate*; brown amorphous material staining orange-red with 50 per cent v/v nitric acid and the development of a green colour on the addition of 5 per cent w/v copper acetate solution. Only three structures can be considered of diagnostic value in distinguishing the two species *P. peltatum* and *P. hexandrum* by microscopical examination alone.

1. The presence of long, cellulosic, *secretory cells* with brown cell walls and orange-brown cell contents from the phloem parenchyma of *P. peltatum*.

2. The character of the *upper epidermis* of the *interneural lamina*, which is wavy walled in *P. peltatum* and almost straight walled in *P. hexandrum*; stomata are absent from this surface in both species.

3. The presence in *P. hexandrum* of fragments of lamina in transverse sectional view which show a *double palisade*. The palisade of *P. peltatum* is single and ill-differentiated.

Other differences which occur are merely a matter of degree. *P. peltatum* contains a large number of lignified *fibres*, but *lignified parenchyma* is rare, whilst in *P. hexandrum* fibres are fewer and frequent fragments of lignified parenchyma occur. The amount of lignified parenchyma will depend upon the number of long petioles included in the sample and hence may vary widely.

P. peltatum contains a larger number of *calcium oxalate* crystals than *P. hexandrum* but the amount of calcium oxalate in either species varies according to the location of the plant and may not be constant.

TABLE I

DIAMETER OF THE CALCIUM OXALATE CRYSTALS

Leaf	Range of diameters in μ from 50 readings	Mean diameter	Standard deviation	t	t (from tables) P = 0·01	t (from tables) P = 0·05	Minimum number of readings
P. peltatum	17·5–26·98–38·51–50·04–63	38·51	11·53	4·205	2·571	1·970	21·3 adjusted to 22
P. hexandrum	11·5–20·91–29·84–38·77–52·5	29·84	8·95				

Note. $t = \dfrac{m_p - m_h}{\sqrt{\dfrac{s_p^2 + s_h^2}{N}}}$ Minimum number of readings $= \dfrac{\left(\dfrac{s_p + s_h}{2}\right)^{t^2}}{\left(\dfrac{m_p - m_h}{2}\right)^2}$

There is a variation in size between the crystals in the leaves of the two species and the examination of powders, prepared from leaves gathered in two different locations for each species, gave an average diameter of crystal of 38·5 μ for *P. peltatum* and 29·8 μ for *P. hexandrum* (see Table I).

The ranges of diameters overlap considerably but the variation in the means can be shown to be highly significant at the P = 0·05 and P = 0·01 probability levels. Further statistical calculations indicate that the size of the calcium oxalate crystals can be used as a diagnostic character provided 22 or more measurements are made.

P. peltatum also contains more trichomes, or trichome fragments, than *P. hexandrum* although they are of similar size, the lengths lying between 150 and 960 μ in the case of *P. peltatum* and 150 and 825 μ in *P. hexandrum*. The number of trichomes present on the whole leaf of *P. hexandrum*,

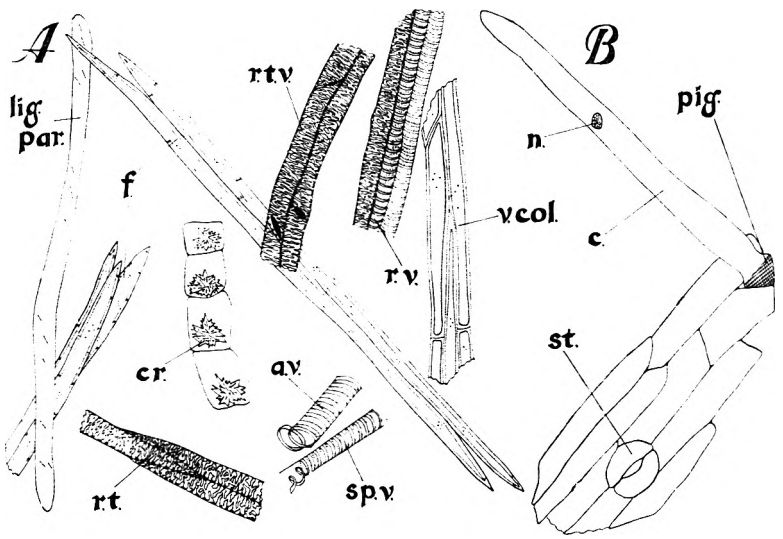

FIG. 7. Petiole of *P. peltatum* L. *A*, isolated elements from the petiole obtained by maceration; *B*, epidermis of petiole. *A* × 75; *B* × 150. *a.v.*, annular vessel; *c.*, covering trichome; *cr.*, crystal of calcium oxalate; *f.*, fibre; *lig.par.*, lignified parenchyma; *n.*, nucleus; *pig.*, pigment; *r.t.*, reticulate tracheid; *r.t.v.*, reticulate tracheidal vessel; *r.v.*, reticulate vessel; *sp.v.*, spiral vessel; *st.*, stoma; *v.col.*, vascular collenchyma.

especially in the marginal regions, has been observed to vary with the age of the leaf. Hence the proportion of trichomes present in a powder will vary according to the time of collection and any numerical differences between the two species based on this character can not be diagnostic.

The identification of these powders as belonging to the genus *Podophyllum* is straightforward, but there is insufficient differential evidence to assign them to their specific rank. The significance of palisade ratio and stomatal index of these species was examined in an attempt to effect a clearer distinction. The values were determined using the methods of Wallis and Dewar (1933) and Salisbury (1927) respectively and the figures from 20 positions on each of 20 leaves were subjected to statistical analysis.

The *Palisade Ratios* show a highly significant variation and this value could be used to distinguish the leaves of the two species in both broken

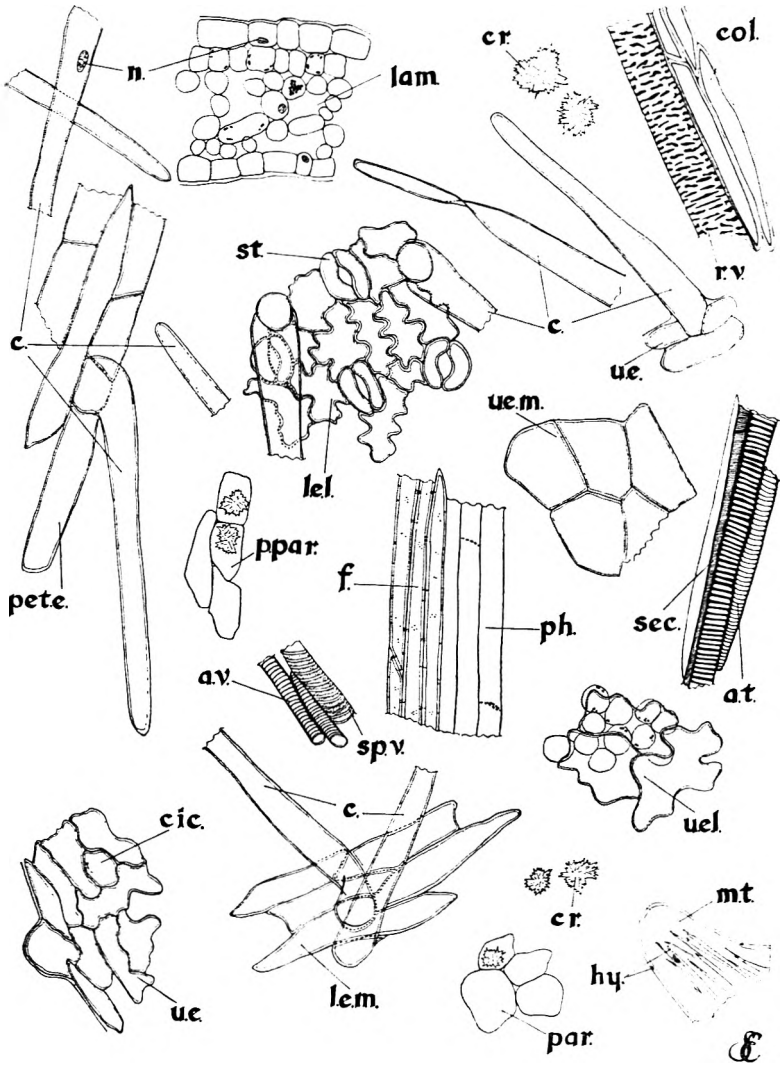

FIG. 8. Powder of the Leaf and Petiole of *P. peltatum* L. Marginal tooth × 30; other fragments × 150. *a.t.*, annular tracheid; *a.v.*, annular vessel; *c.*, covering trichome; *cic.*, cicatrix; *col.*, collenchyma; *cr.*, crystal of calcium oxalate; *f.*, fibre; *hy.*, hydathode; *lam.*, lamina; *l.e.l.*, lower epidermis of lamina; *l.e.m.*, lower epidermis of main vein; *m.t.*, marginal tooth; *n.*, nucleus; *par.*, parenchyma; *pet.e.*, petiole epidermis; *ph.*, phloem; *p.par.*, pith parenchyma; *r.v.*, reticulate vessel; *sec.*, secretion cell; *sp.v.*, spiral vessel; *st.*, stoma; *u.e.*, upper epidermis; *u.e.l.*, upper epidermis of lamina; *u.e.m.*, upper epidermis of main vein.

or powdered condition (see Table II). The *Stomatal Indices* show a statistically significant variation of the means but the high standard deviations cause the ranges to overlap to such an extent that 350 readings would be necessary. To attempt a distinction of the two species *P. peltatum* and *P. hexandrum* by this method would be impracticable.

TABLE II

RESULTS FROM PALISADE RATIOS AND STOMATAL INDICES OF THE TWO SPECIES

Leaf	Range of values from 400 readings	Mean ratio	Standard deviation	*t*	*t* (from tables) P=0·01	*t* (from tables) P=0·05	Minimum number of readings
Palisade Ratios							
P. peltatum	7·0–8·5–12·16–17·0	10·25	1·93	33·3	2·571	1·97	4·6 adjusted to 5
P. hexandrum	4·0–4·96–7·68–11·25	6·32	1·36				
Stomatal Indices							
P. peltatum	9·09–15·57–23·65–33·33	19·61	4·04	4·03	2·571	1·97	335
P. hexandrum	4·0–14·76–22·24–30·78	18·5	3·74				

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THE DETERMINATION OF EPITETRACYCLINE AND TETRACYCLINE BY ION-EXCHANGE PAPER CHROMATOGRAPHY AND ITS APPLICATION TO HUMAN URINE AND SERUM

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A simple quantitative chromatographic method is described for the separation of epitetracycline and tetracycline in pharmaceutical preparations. The method is applicable qualitatively to human serum and quantitatively to the biological determination of tetracycline in urine. Whatman's modified cellulose phosphate cation-exchange paper is developed by the descending technique using 0·1 per cent w/v ammonium chloride solution. The spots are located as yellow fluorescent spots under ultra-violet light and ammonia vapour, eluted in 10 per cent w/v ammonium chloride, and the extinction determined at 356 mμ. The method is not suitable for the separation of chlortetracycline or oxytetracycline from tetracycline.

THE tetracyclines undergo a reversible isomerisation between the pH range 2 to 6 (Doerschuk, Bitler and McCormick, 1955). This has been shown to be an epimerisation at Carbon 4, giving rise to a new series of compounds, the epitetracyclines or quatrimycins. Their preparation and properties have been described by McCormick and others (1957).

Commercial tetracycline normally contains 5 per cent or less of its epimer. Liquid suspensions and solutions of tetracycline may contain appreciable amounts of epitetracycline. A simple quantitative method for its estimation is desirable, since epitetracycline has only about 5 per cent of the *in vitro* biological activity of tetracycline.

Previous methods of chromatographic separation involve the use of paper saturated with McElvaine's buffer at pH 3·5 and developing with nitromethane-chloroform-pyridine (Selzer and Wright, 1957), or nitromethane-benzene-collidine mixtures (Coppi, 1960). These solvent systems are toxic and unpleasant to use and in both, a third band between the epi-band and the main band has been noted on standard preparations of tetracycline. This, on rechromatographing, is resolved into epitetracycline and tetracycline, showing that some conversion of tetracycline to epitetracycline may take place on the paper. In addition, variable results can be obtained due to the difficulty of standardising the degree of dampness of the buffered paper. These systems proved unsatisfactory for resolving epitetracycline from tetracycline in urine and serum samples.

Kelly and Buyske (1960) described a method for the separation of epitetracycline in urine using Whatman No. 1 paper impregnated with ethylenediaminetetra-acetic acid, and developing with the organic phase of n-butanol : ammonium hydroxide : water system, in the ratio 4 : 1 : 5. At the loading necessary for the quantitative determination of epitetracycline in bulk tetracycline and broths, appreciable quantities of

anhydrotetracycline are formed. This does not occur with the other systems mentioned.

The present paper describes a simple system for the separation of epitetracycline from tetracycline. It is suitable for quantitative determinations in tetracycline destined for therapeutic use, for quantitative determination in urine and qualitative separation in blood serum samples.

Experimental and results

General

All tetracyclines used were commercial samples with the exception of the epitetracycline ammonium salt, and the anhydrotetracycline, which were prepared in this laboratory according to the method of McCormick and others (1957). Other reagents were of analytical grade.

Whatman modified cellulose phosphate cation-exchange paper has many of the characteristics of a strongly acidic cation-exchange resin. In the acid form it is essentially cellulose dihydrogen phosphate and thus contains both strong and very weak acidic groups. Using this paper and water as developing solvent, epitetracycline and tetracycline gave variable R_F values. Anhydrotetracycline was not separated from epitetracycline. The former compound is the product of the acidic degradation of tetracycline. As acidic conditions prevail at various stages in the production of tetracycline, anhydrotetracycline is a possible impurity. Satisfactory separation with reproducible R_F values could be achieved by development of the chromatogram with 0·1 per cent w/v ammonium chloride solution. The R_F values of various tetracyclines in this system are given in Table I.

TABLE I

R_F VALUES OF TETRACYCLINE ON MODIFIED CELLULOSE PHOSPHATE CATION EXCHANGE PAPER USING 0·1 PER CENT W/V AMMONIUM CHLORIDE FOR DEVELOPMENT

Sample	R_F value
Tetracycline	0·59
Epitetracycline	0·36
Chlortetracycline	0·61
Oxytetracycline	0·61
6-Demethylchlortetracycline	0·53
Epi-6-demethyl-chlortetracycline	0·32
6-Demethyl-6-desoxy-tetracycline	0·42
Epi-6-demethyl-6-desoxy-tetracycline	0·21
6-Methylene oxytetracycline	0·46
Anhydrotetracycline	0·14

Attempts to elute epitetracycline and tetracycline from the paper with 0·01N hydrochloric acid gave low recoveries. Better recoveries were obtained if the acid strength was increased to 0·5N, but the formation of anhydrotetracycline during elution rendered this procedure unacceptable. Elution with 10 per cent w/v sodium chloride had a similar disadvantage due to the formation of isotetracycline. 10 per cent w/v ammonium chloride was finally selected for the elution. Tetracycline was stable in this solution over several hours.

Recovery from the paper was 93·1 $\pm$ 1·5 per cent based on 26 experiments using commercial samples of tetracycline base and hydrochloride.

This is comparable with previous methods described for this estimation. A typical series of results is shown in Table II.

TABLE II

TETRACYCLINE AND EPITETRACYCLINE CONTENTS OF COMMERCIAL SAMPLES OF TETRACYCLINE

Tetracycline	Sample	Total tetracyclines by direct U.V. measurement, per cent	Tetracycline ex chromatography, per cent	Epitetracycline ex chromatography, per cent	Recovery total tetracyclines, per cent	Moisture, per cent
Base	1	90·2	80·8	2·9	92·8	9·1
	2	90·3	82·0	2·9	94·0	9·1
Base	1	91·9	82·4	2·4	92·3	7·5
	2	92·0	84·1	2·5	94·1	7·5
Base	1	89·5	80·1	2·3	92·1	9·7
	2	89·6	80·7	2·8	93·2	9·7
Hydrochloride	1	99·5	90·5	2·2	93·2	0·15
	2	99·6	89·6	2·5	92·5	0·15
Hydrochloride	1	99·7	92·1	2·4	94·8	0·28
	2	99·5	91·9	2·3	94·7	0·28
Hydrochloride	1	99·4	90·2	2·7	93·5	0·31
	2	99·3	90·1	2·6	93·4	0·31

Tetracycline hydrochloride (125 mg.) was dissolved in 0·01N hydrochloric acid to 25 ml.

Tetracycline base (125 mg.) was dissolved in a minimum of 0·1N hydrochloric acid (not greater than 5 ml.) and diluted to 25 ml. with water.

A starting line was marked on a sheet of Whatman modified cellulose phosphate cation-exchange paper about 10 cm. from one end of the sheet and 2 cm. from the edges. 0·2 ml. (≡1 mg.) of the tetracycline solution, or a suitably diluted aliquot of other preparations was streaked on the line. The sheet was placed, together with a blank, in a chromatography tank and developed with 0·1 per cent w/v ammonium chloride by the descending technique. After the solvent front had travelled about 25 cm. the papers were air dried for some hours, preferably overnight.

The yellow fluorescent bands were located under Wood's light in the presence of ammonia vapour. They were cut into 1 cm. squares, taking equivalent areas from the "blank sheet" for the blank determinations. The main band (tetracycline) was eluted by shaking in 100 ml. of 10 per cent w/v ammonium chloride solution and the epitetracycline band in 20 ml. of 10 per cent w/v ammonium chloride solution for 30 min. Alternatively the mixtures could be stood for 2 hr. with occasional shaking. If only small amounts of epitetracycline were present the bands from two or more sheets were combined in one elution. The suspensions were filtered and the optical densities measured in a 1 cm. cell at 356 mμ. E (1 per cent, 1 cm.) at 356 mμ for tetracycline and epitetracycline in 10 per cent w/v ammonium chloride was 325.

Separation in Urine

Initial experiments were designed to demonstrate any interference that urine constituents might exert on the chromatographic system.

Tetracycline and epitetracycline, both separate and in admixture, were dissolved in urine (100 μg./ml.), a level comparable to that found in urine during tetracycline therapy. The solutions were spotted on the paper in 0·02 ml. aliquots (≡2 μg. antibiotic) and run according to the described method. The chromatogram, inspected under Wood's light and ammonia vapour, showed that tetracycline and epimer could be separated under these conditions with no interference from urine salts. The separation was confirmed by bio-autography.

To show that the method would separate the epi-compound in urine of patients taking tetracycline, three groups of patients were dosed with tetracycline tablets at low, medium, and high dose levels, and the urine collected for 4 hr. after dosing. In all instances, compact spots of tetracycline were revealed by Wood's light and ammonia, and at the highest dose, a faint epitetracycline spot was detectable. Bio-autographs showed the same picture as those obtained when the tetracycline and epitetracycline were added to the urine, and R_F values were comparable.

Kelly and Buyske (1960) reported that a particular failure of many of the earlier chromatographic systems was their tendency to give very diffuse spots at loadings greater than 5 μg. Using the described method, tetracycline, 100 μg. in 0·02 ml., gave a main spot 3 cm. diameter and a spot of epitetracycline 1·5 cm. diameter without streaking.

Spectrophotometric estimation of eluates was not possible due to the high urine blank. Using a microbiological assay (Grove and Randall, 1955) tetracycline was estimated in samples of urine containing 100 μg./ml. of added tetracycline. 0·04 ml. of the sample was chromatographed and the tetracycline spot eluted in 10 ml. of 10 per cent w/v ammonium chloride solution, centrifuged, and determined by a microbiological plate assay using *Bacillus cereus* as the test organism. Recovery was comparable to that obtained for the standard procedure described above. Results are shown in Table III.

TABLE III

ESTIMATION OF TETRACYCLINE IN URINE MICROBIOLOGICALLY AFTER CHROMATOGRAPHY AND ELUTION

Urine sample	Added tetracycline, μg./ml.	Recovered tetracycline after chromatography, μg./ml.
blanks	Nil	Nil
1	100	91·3
2	100	101·3
3	100	88·7
4	100	88·8
5	100	92·6
6	100	93·7

Separation in Blood Serum

The normal level of tetracycline in blood serum after tetracycline therapy is about 1–1·5 μg./ml., but preliminary experiments were made on

samples of serum containing added tetracycline and epitetracycline (100 μg./ml.), to detect the spots by visual location. Serum (0·01 ml.) $\equiv$ 1 μg. of epitetracycline and 1 μg. of tetracycline gave a satisfactory separation. At loadings up to 5 μg. separation was still satisfactory and no streaking occurred. The R_F values were: epitetracycline, 0·31; tetracycline, 0·46.

Serum from each of the 3 groups of patients in the experiment described above was examined by the described method. In each instance, 0·1 ml. was streaked on the paper and 0·02 ml. on a thin strip suitable for bio-autography. No bands were detected by visual examination, but a zone of epitetracycline was observed on the bio-autographs at the medium and high dose levels and a zone for tetracycline in all dose levels. The R_F values were comparable to those quoted above.

Discussion

In the separation and estimation of epitetracycline in tetracycline where admixture with chlortetracycline or oxytetracycline is not important, the above method has several advantages over previous methods described. No buffering of the chromatographic paper is required. Simple aqueous non-toxic reagents are used. Saturation of the tank is not critical. No detectable epimerisation takes place on the paper. The method is applicable to urine and serum, and, coupled with a microbiological assay after elution, can be used for the quantitative determination in urine.

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DIOSCOREA BELIZENSIS LUNDELL AS A SOURCE OF DIOSGENIN

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Twenty-nine species of wild *Dioscorea* from British Honduras were screened for saponins. The tubers of *D. belizensis* Lundell afforded yields of steroids of about 2 per cent of the dry weight, calculated as the acetate of the predominant genin, diosgenin. A routine assay for diosgenin in these tubers is given and the effects of comminution, chemical disintegration, fermentation, refrigeration, autoclaving, and drying of the tubers are described. The yield is controlled by an endogenous enzyme system.

Dioscorea tubers are an important source of steroids for the pharmaceutical industry. In 1959, Dr. S. S. Bampton of the Tropical Products Institute, London collected 29 species of wild yams from British Honduras. These were subsequently grown in the hot house at Nottingham and screened for steroids. One species, *Dioscorea belizensis* Lundell, identified and described by Blunden, Hardman and Trease (1963) was chosen because it had a steroid content of potential commercial interest and was readily propagated (Blunden and Hardman, unpublished) for future selection of high yielding strains.

Steroidal assays of yams are usually done by acid hydrolysis of the glycosides *in situ* and extraction of the sapogenins by a hydrocarbon solvent. The gravimetric method of Morris, Roark and Cancel (1958) is of this kind. It was modified to give a reliable routine procedure appropriate to the form and lignified nature (Blunden, Hardman and Trease, 1963) of the tuber of *D. belizensis* and enabled us to study the effect of various preliminary laboratory treatments of this species on its yield of diosgenin.

Experimental

It was unnecessary to extract the yams to screen them for saponins. A fragment of fresh or dried tuber was placed on a blood agar plate at about 19° and any haemolysis noted in 3 to 6 hr., before the influence of any contaminants was evident.

Tubers of *D. belizensis*, received by air mail from British Honduras, deteriorated rapidly unless they were repacked on arrival, in vermiculite in well filled sealed tins and kept at 17 to 20°. They then remained sound for at least 1 year.

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Assay: Standard Procedure

The tuber branches occur in lengths of up to 1 metre and from 2·5 to 5 cm. in diameter. A clean (free of soil and fungal attack) piece (70 g.) was sliced transversely, two thick (about 9 mm.) slices being taken for the sapogenin estimation and the next thin (about 3 mm.) slice for the moisture content. This sampling was continued for the entire length of the tuber. It was arranged that about 30 g. of the tuber was used for each of two sapogenin determinations and about 5 g. for each of two moisture values. The latter were obtained by drying the slices at 95–105° for 12 hr. The tuber slices for the sapogenin determination were cut into thin strips before being disintegrated for 5 min. in a Townson and Mercer top drive macerator in the presence of 100 ml. water. The mixture, with water rinsings (100 ml.), was incubated at 25 or 37° for 5 days in a plugged flask. After concentrated hydrochloric acid had been added to make the acid concentration 2N, the mixture was boiled for 2 hr., and cooled. The acid-insoluble material collected at the pump, was neutralised by washing with water, 20 per cent sodium carbonate solution and again with w[illegible].

TABLE I

DUPLICATE DETERMINATIONS, (*a*) AND (*b*) OF SAPOGENIN ACETATE BY THE STANDARD ASSAY PROCEDURE

Fresh tuber Piece No.	Yield*		Fresh tuber Piece No.	Yield*	
	(*a*)	(*b*)		(*a*)	(*b*)
1	2·26	2·21	4	0·43	0·42
2	1·40	1·42	5	2·31	2·33
3	2·07	2·07	6	1·62	1·64

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

The residue was dried at 80° overnight, powdered and the sapogenins extracted with light petroleum (b.p. 40–60°) in a Soxhlet apparatus for 24 hr. The petroleum-soluble material (0·1–0·2 g.) was acetylated using 2 ml. acetic anhydride and the procedure of Wall, Eddy, McClennan and Klumpp (1952) and the benzene-soluble material assayed in carbon disulphide at a concentration of 0·7 to 2 per cent in the 1·0 mm. cells of a Hilger H 800 infra-red spectrophotometer. The estimation was based on the band at 982 cm.$^{-1}$ at which pure diosgenin acetate had the extinction value of 7·685.

This assay procedure was used to study the effect on the sapogenin yield of various pre-assay treatments of the tubers. The paper chromatography method of Sannié and Lapin (1952) was used to examine the light petroleum-soluble extract for sapogenins, the latter being detected by spraying with antimony trichloride in hydrochloric acid (Nakao, Hirai and Yoshizawa, 1958).

RESULTS

Tubers of four of the 29 species gave a positive test for saponins. One of these was *D. belizensis*; its root, stem, and leaf also caused haemolysis. The fresh tuber contained 73 to 85 per cent moisture. The sapogenin

content is expressed as diosgenin acetate per cent of the moisture free tuber. Chromatographic examination (Sannié and Lapin, 1952; Nakaɵ and others, 1958) of the petroleum-soluble material obtained in the above assay of the tuber, disclosed four sapogenins with diosgenin predominating.

TABLE II

YIELD OF SAPOGENIN. COMPARISON OF YIELD BY INFRA-RED ANALYSIS OF LIGHT PETROLEUM-SOLUBLE MATERIAL AFTER ACETYLATION, WITH THE GRAVIMETRIC YIELD OF THIS MATERIAL BEFORE AND AFTER ACETYLATION

Fresh tuber Piece No.	Infra-red*	Gravimetric†	
		Acetylated	Unacetylated
7	1·91	2·92	2·80
8	2·26	3·58	3·46
9	1·40	3·50	3·25
10	1·82	4·11	3·88

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.
† Per cent of moisture free tuber.

The latter crystallised from the light petroleum solution when it was concentrated and on re-crystallisation from ethanol had m.p. and mixed m.p. 204–206°; its acetate, from methanol, had m.p. and mixed m.p. 198°. Moreover the infra-red spectrum obtained from the acetate of the predominant sapogenin was identical with that obtained from diosgenin acetate.

TABLE III

THE EFFECT OF CONTAMINANTS ON THE YIELD OF DIOSGENIN ACETATE WHEN DETERMINED FROM THE INFRA-RED SPECTRA

Weight of contaminant, g.	Weight of diosgenin acetate added, g.	Yield of diosgenin acetate, per cent
0·1622	0·0278	102·3
0·0814	0·0508	100·9
0·0812	0·0493	98·4
0·0972	0·0411	100·1
0·1102	0·0436	100·4

The nature of the tuber of *D. belizensis* is such that it is difficult to remove it from the ground, free it of soil and transport it, without the branches of the tuber being broken. Small pieces (70 g.) were commonly received, for example pieces Nos. 1–23 (see Tables); large pieces (165 g.) for example Nos. 24 and 25 (Table V), were rare. This limited the design of the experiments.

The validity of the method of subsampling of the tuber branches was demonstrated by the close agreement of duplicate determinations of sapogenins (Table I). An infra-red determination was necessary as seen from a comparison of the value so obtained with the gravimetric yield of crude sapogenin acetate (Table II). The contaminants did not interfere with the infra-red assay; known weights of pure diosgenin acetate were mixed with known weights of benzene-soluble material obtained by

extracting the powdered dry tuber with benzene (Table III). Addition of known weights of diosgenin to the disintegrated tuber at the onset of the standard assay resulted in the expected yield.

The yields of sapogenin obtained from duplicate subsamples of tuber, one of which had received the standard assay procedure and the other had not been fermented, are shown in Table IV. Small increases were

TABLE IV

COMPARISON OF SAPOGENIN YIELDS* FROM FRESH TUBER WHEN UNFERMENTED AND WHEN FERMENTED

Fresh tuber Piece No.	Unfermented	Fermented (standard assay procedure)	Increase, per cent
11	1·92	2·21	15
12	2·07	2·27	10
13	1·37	1·44	5
14	2·23	2·39	7
15	0·86	0·92	7

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

always achieved by fermentation; the results were similar whether the temperature was 25 or 37°. In a single experiment the deliberate addition of soil (air dried, 2 g.) received on the tubers from British Honduras, to soil-free fresh tuber (30 g.) resulted in a marked loss of diosgenin on fermentation at 25° for 5 days (diosgenin; 1·39 per cent compared with 2·02 per cent without soil).

TABLE V

SAPOGENIN YIELD* AFTER DRYING AT 80°

Fresh tuber Piece No.	Standard assay procedure (fermented)	Sliced, dried, and powdered		Homogenised, dried, and powdered (unfermented)	Increase (per cent) on fermentation (relative to column 3)
		Unfermented	Fermented		
16	0·76	0·57			33
17	1·23	0·85			45
18	0·87	0·41			112
19	2·63	1·24			112
20		1·12	2·59		131
21		0·43	1·33		209
22		0·63	1·82		189
23		0·80	1·77	(*a*) (*b*)	121
24	2·27	1·82	2·21	2·20 2·19	23
25	1·51	0·78	1·53	1·48 1·47	95

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

A large piece of tuber (165 g.) (pieces Nos. 24 and 25 of Table V) was cut into alternate thick and thin transverse slices. The thin slices afforded the moisture content in duplicate. Each thick slice was cut in turn into four approximately equal segments and each segment was allocated in rotation to one of five samples. One sample was used for the assay by the standard procedure. Two samples were bulked, dried at 80° for 16 hr., and powdered in a hand-mill until all the particles passed through a No. 60 sieve. After mixing the powder well and removing portions for moisture determinations, the rest was divided into two equal parts. One part was mixed with water (250 ml.) and acid hydrolysed, and the other

was fermented in water (250 ml.) before acid hydrolysis as in the standard assay procedure. The remaining two samples were treated separately as follows (assay in duplicate): The segments were first cut into small pieces and then homogenised with water portionwise, in a Potter-Elvehjem homogeniser during 5 hr. and using up to 400 ml. water. The resultant suspension was dried at 80° for 24 hr. and the residue assayed without being fermented. The results of this and related experiments are given in Table V. In such an experiment chromatographic examination (Sannié and Lapin, 1952) (Nakao and others, 1958) of the petroleum-soluble material from the fermented tuber via the standard assay method and that from tuber dried at 80° and not fermented, gave the same four sapogenins.

TABLE VI

COMPARISON OF SAPOGENIN YIELDS* FROM DISINTEGRATED (STANDARD ASSAY PROCEDURE) AND HOMOGENISED TUBER, BEFORE AND AFTER FERMENTATION

Fresh tuber Piece No.	Standard assay procedure (fermented)	Homogenised	
		Unfermented	Fermented
26	1·31	1·29	1·27
27	0·89	0·86	0·89

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

Similar subdivision of single pieces of tuber afforded this result: homogenised tuber when fermented at 37° for 5 days gave the same sapogenin yield as when the tuber was homogenised, dried and assayed without being fermented (Table VI).

The yield by the standard assay procedure was also compared with the yield obtained when the tuber slices were first autoclaved at 115° for 30 min. before subjecting to the standard procedure, and when the fermentation stage of this procedure was omitted after autoclaving (Table VII).

TABLE VII

COMPARISON OF SAPOGENIN YIELDS* BEFORE AND AFTER AUTOCLAVING

Fresh tuber Piece No.	Standard assay procedure (fermented)	Autoclaved, then disintegrated		Increase (per cent); column 2 relative to column 4
		Unfermented	Fermented	
28	2·27	0·88	0·95	139
29	2·26	1·40	1·37	65
30	2·59	1·83	1·76	41
31	1·65	0·88	0·89	85

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

The results obtained after storage of the tuber slices at 5° for 1 week before the standard assay procedure and such storage and assay but without fermentation, are shown in Table VIII.

The sliced tuber when disintegrated by treating with boiling 5 per cent potassium hydroxide solution for 20 min. gave a low sapogenin yield

(0·95 per cent) when compared with slices given the standard assay process (1·60 per cent).

None of the specimens of acid insoluble material in the above experiments gave a positive haemolysis test on blood agar, thus showing absence of saponins.

TABLE VIII

COMPARISON OF SAPOGENIN YIELDS* BEFORE AND AFTER STORAGE AT 5° FOR 7 DAYS

Fresh tuber Piece No.	Standard assay procedure (fermented)	Stored at 5°, then disintegrated		Increase (per cent) on fermentation (Column 4 relative to column 3)
		Unfermented	Fermented	
32	1·65	0·56	1·66	196
33	0·53	0·38	0·57	50
34	2·41	1·36	2·39	76

* Per cent of moisture free tuber calculated as diosgenin acetate from infra-red spectra.

DISCUSSION

The tubers of species of saponin-holding yams vary markedly in their texture, content of petroleum-soluble material, and the ease with which they afford diosgenin.

Rothrock, Hammes, and McAleer (1957) and Morris, Roark and Cancel (1958) have described gravimetric assays of yams for diosgenin. Their procedures were modified to give a reliable routine assay of small weights of *D. belizensis* tuber. Its moisture content was about 80 per cent; the method of this estimation, as described by Morris and others (1958), was found unsatisfactory on the weight of tuber involved. When water alone was used for washing the acid-insoluble material it was found that the filtrate could be neutral to indicator paper but the particles when squeezed between the paper still gave an acid reaction. A loss of diosgenin occurred when the acid-insoluble material was dried in an acid state and then stored for 2 months, but not when the material was left in a slightly alkaline condition. The final light petroleum extract of the acid-insoluble residue contained sufficiently high and variable quantities of contaminants to make a gravimetric procedure unreliable.

Yields of diosgenin are reported to be increased when comminuted tubers of dioscorea are fermented (Schering Corporation, 1956). Krider, Cordon and Wall (1954) and Rothrock Stoudt, and Garber (1955) have shown that fungi are capable of hydrolysing steroidal saponins to sapogenins. Only clean tubers, free of mould attack and soil, were normally used in our experiments. When a large amount of soil was deliberately introduced a low yield of diosgenin was obtained after fermentation.

Diosgenin yield may be expected to vary with tissue maturity. This was taken into account in the method of subsampling in the individual experiments. The results fall into two groups: Low yield of genin as given by the autoclaved tuber and high yield as obtained when the tuber is homogenised. Low yields of similar quantities were obtained when the fresh tuber slices were autoclaved; or dried at 80°, powdered and not

fermented; or stored at 5° for 1 week, disintegrated and not fermented; or treated with boiling 5 per cent potassium hydroxide solution. High yields of similar quantities were obtained when the fresh slices were disintegrated and fermented; or dried at 80°, powdered and fermented; or homogenised; or homogenised and dried; or stored at 5° for 1 week, disintegrated and fermented. The high yield was 40 to 200 per cent more than the low one. The results indicate the presence of an endogenous enzyme system capable of producing much of this increase in diosgenin during only 5 min. of cell disintegration of the fresh tuber. While the optimum cell damage for this increase may be that caused by the homogenisation procedure used, this took 5 hr. to achieve with 30 g. fresh tuber. Both the vascular tissue which ramifies throughout the tuber, and the cork, are lignified. Five min. distintegration afforded only about 10 per cent less diosgenin which could be made up by fermentation of the aqueous tuber mash containing particles with edges up to 6 mm. Dried sliced tuber if powdered and fermented gave the same yield as the homogenised tuber. The results after chilling (5°) or drying (80°), common procedures for glycoside studies on medicinal plants, show an apparent loss of genin if, before the assay, the appropriate conditions are not given for the formation of steroids. This is of obvious importance where dried yam is being assayed for diosgenin or is the starting material for its isolation.

Unknown to us, Roark and Morris (1961) and Roark, Cruzado, Delpin and Morris (1961) made similar experiments using the tubers of *Dioscorea composita* and *D. floribunda*. Our results with *D. belizensis*, the tuber of which has a different form from that of the other two yams, are in agreement with and extend those of Roark and his colleagues. Our chemical studies on *D. belizensis* begun in 1960 were completed before the work of Roark and others (1961) was seen and before our studies on the morphology and propagation of *D. belizensis* were made. All of our work on *D. belizensis*, including that reported in this paper, was published by one of us in March, 1962 in thesis form (Blunden, 1962). No doubt had Roark and Morris (1961) fermented their tubers which had been "sliced, dried at 85° and ground to a fine dust" they would have got the high steroid yield afforded by homogenising the tubers. They did not study the effect of chilling of the tubers.

Heftman, Bennett, and Bonner (1961) report unexpected and very low (0·013 per cent) incorporation into diosgenin of ^{14}C from labelled acetate and even less from labelled mevalonic acid, when these precursors of sterols, were used with homogenised tubers of *D. floribunda* under their conditions. While they did not say how the tubers were stored before homogenisation the latter step was done in the presence of cracked ice. This might have contributed to the low uptake of acetate since diosgenin is not immediately synthesised when the tuber of *D. belizensis* is disintegrated in water at 17–20° after the tuber has been stored at 5°. Roark and Morris (1961) have also used 1-^{14}C-labelled acetate with homogenised tubers of *D. floribunda* and obtained labelled sapogenins. Unfortunately, they give no indication of the amount of acetate so incorporated.

Much of the diosgenin which can be obtained from the tubers of *D. belizensis* is not present as such nor as its glycoside in the harvested tuber but is very rapidly produced by an endogenous enzyme system on disintegration of the sound tissue. All sapogenin yielding yams may behave in this manner and plants which are outside the family Dioscoreaceae but yield steroidal or triterpenoid compounds may also exhibit the phenomenon. This is being investigated.

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LETTERS TO THE EDITOR

The Extraction of Acetylcholine in Small Samples of Cerebral Tissue

SIR,—The extraction of acetylcholine (Ach) and acetylcholine-like substances (Florey, 1961) can be made by one of several methods (Abdon, 1944; Bentley and Shaw, 1952; Crossland, 1961; Lewis and Smallman, 1958; Smallman and Fisher, 1958; Birks and MacIntosh, 1961.

The substance is assayed usually either on the frog rectus abdominis or on the cat blood pressure preparation with the precautions indicated by Felberg (1945). Also, the guinea-pig ileum may be used (Blaber and Cuthbert, 1961; Birmingham, 1961).

With some of these procedures it is not always possible to assay extracts with low concentrations of Ach, since they are prepared from small samples of tissue, such as certain parts of the central nervous system of, for example, rats or guinea-pigs. These animals, on the other hand, are useful for the analysis of the zonal distribution of Ach and the modification brought about in this by drugs or experimental procedures.

We now describe our method of overcoming the problem of estimation.

Guinea-pigs of either sex, weighing 270–330 g., are decapitated and the skull is opened with scissors. 50–100 mg. of cerebral tissue (even 20–30 mg. if necessary) is rapidly excised, weighed and immersed for 2–3 min. in 3 ml. of McIlvaine citric acid-disodium phosphate buffer, ph 4; 0–014 *M*, at 98°–99° contained in glass homogeniser tubes in boiling water. The standards are prepared by adding known amounts of Ach (30–100-300 ng.) to 50–100 mg. (or 20–30 mg.) of nervous tissue, previously boiled for 10 min. at pH 9·5–10. After cooling, the tissue is carefully homogenised under ice and kept at 18° for 15 min. Then the pestles and the side of tubes are washed with 3 ml. of Tyrode solution without glucose and bicarbonate, but with a double concentration of salts, to obtain an isotonic medium. The supernatant is diluted 2:5 or 1:5 with the normal Tyrode solution and assayed (1–2 ml.) on the guinea-pig ileum against either the standards or freshly prepared solutions of Ach.

The terminal ileum is set up in 3 ml. of the normal Tyrode, oxygenated at 30°, containing diphenhydramine 2×10^{-8} to improve the selectivity, and morphine 5×10^{-8} to reduce the motility and increase the discrimination within smaller responses. The lower sensitivity limit is 1×10^{-10}, as Ach final concentration. Our experimental conditions differ slightly from those of Blaber and Cuthbert (1961) and Birmingham (1961); they used the guinea-pig ileum sensitised with organophosphorus anticholinesterases, to assay amounts of Ach smaller than those present in our extracts.

The assay is done at intervals of 90–120 sec. The contractions are recorded with an isotonic lever, 1:20 magnification, loaded at 0·40–0·50 g., writing on a smoked drum. At the end of the assay the usual controls are made: alkaline hydrolysis of the sample and treatment of the terminal ileum with atropine 2×10^{-8}. The amount of Ach in the extract is obtained by bracketing its response between that of 2 known doses of Ach and of 2 standards.

The average recovery of the standards, against known solutions of Ach, was 101·3 $\pm$ 17 per cent (s.d.): so it is clear that our extraction method releases only small amounts of interfering substances (Feldberg, 1945) and does not cause loss.

In the Table, the Ach content detected in the olfactory bulbs, in the posterior part of the parietal area and in the anterior part of verme cerebellaris is compared using the above method and also that of Lewis and Smallman (1956).

Also the extracts prepared according to the second method were assayed on the terminal ileum. The average recovery of the standard is 90 ± 22 per cent (s.d.).

TABLE I

ACETYLCHOLINE CONTENT OF GUINEA-PIG BRAIN
(ACH CHLORIDE μG./G. FRESH TISSUE ± S.D.)

Method	Animals No.	Olfactory bulbs	Cerebral cortex	Cerebellum
Lewis and Smallman (1956) ..	16	2·017 ± 0·447*	2·207 ± 0·425*	0·498 ± 0·172
Citric acid disodium-phosphate bufler	16	2·053 ± 0·419†	2·454 ± 0·490-	0·485 ± 0·132

* = The difference between these two values is not statistically significant.
† = The difference btween these two values is statistically significant. (P = 0·01.)

Although the results do not differ significantly, only with our extraction procedure is the Ach content of the cerebral cortex significantly higher than that of olfactory bulbs. (P = 0·01.)

It is probable that the immediate treatment with heat at pH optimum for the stability of Ach and the use of a strongly hypotonic medium, favour both the inactivation of the esterases and the diffusion of the neural hormone from the tissue.

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Banana and Experimental Peptic Ulcer

SIR,—Recently attention has been drawn by Sanyal, Das, Sinha and Sinha (1961) and Sinha, Sanyal and Sinha (1961) to the beneficial effects of ripe banana in the prevention of histamine-induced gastric hyperacidity, ulcerations and perforations in guinea-pigs. These observations were confined to acute types of peptic ulcers. They also reported a reduction in histamine-induced acidity when banana extract was applied on the gastric mucosa, and this was considered to be due to the high 5-hydroxytryptamine (5-HT) content of banana (West, 1958; Wealkes and others, 1958).

For the present study, chronic gastric ulcers were produced in adult guinea-pigs by phenylbutazone. This drug has been reported to produce various types of gastric complications, including ulcerations, perforations, and haemorrhages, in man (Kirsner, 1957). In experimental animals, oral and parenteral administration of phenylbutazone has also been found to produce peptic ulcerations (Watt and Wilson, 1959; Zaidi, Singh and Bajpai, 1961).

TABLE I

EFFECT OF BANANA AND ALUMINIUM HYDROXIDE ON PHENYLBUTAZONE INDUCED PEPTIC ULCERS

Drug	Dose/kg.	No. of experiments	Ulcer present	Survival in days, mean ± s.e. (range)	Other features
Phenylbutazone	100 mg.	40	36 (90 per cent)	21 ± 8 (8–40)	Haemorrhage in 4. Adhesion in 5. Perforations in 3. Acute dilatation in 2
Phenylbutazone + Banana powder	100 mg. + 1 g.	24	14 (48·3 per cent) $P < 0.01$	24 ± 9 (7–42) $P > 0.05$	Ulcers were either healed or were attempting to heal as shown by more fibrosis and thickening
Phenylbutazone + Aluminium hydroxide	100 mg. + 1 g.	14	8 (47·1 per cent) $P < 0.05$	18 ± 5·94(7–29) $P > 0.05$	Same as above

In the present series, phenylbutazone, 100 mg./kg., as a 10 per cent suspension was administered daily with the help of a fine catheter orally to the adult guinea-pigs. Unripe banana was selected for the present study as it was reported to contain the maximum amount of 5-HT. The pulp was sun-dried, powdered and administered daily in the dose of 1 g./kg. Control experiments were made with a proprietary preparation of aluminium hydroxide (Aludrox) (1 g./kg.). Post-mortem and histological examinations were made whenever any animal died during the study. The results are summarised in Table I.

The results clearly indicate that banana helps in the prevention and healing of the phenylbutazone induced ulcers. The results not only compare well with the antacid, but the results are highly significant. The life span of the guinea-pigs under phenylbutazone treatment was not significantly altered by either banana or antacid treatment but it has to be remembered that the deaths after phenylbutazone might also arise from other toxic effects of the drug.

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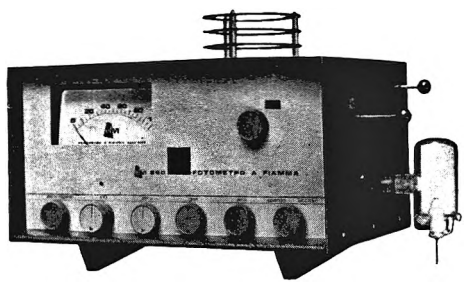

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