RESEARCH PAPERS

ANTAGONISM OF GUANETHIDINE BY DEXAMPHETAMINE AND OTHER RELATED SYMPATHOMIMETIC AMINES

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Dexamphetamine and certain other indirectly acting sympathomimetic amines prevent or reverse the sympathetic nerve blocking action of guanethidine in anaesthetised cats and dogs. Noradrenaline and dopamine do not antagonise the blocking action of guanethidine. These observations are discussed in relation to the mode of action of guanethidine and to a possible clinical significance of these findings.

Wilson and Long (1960) reported that bretylium did not reduce the blood pressure in hypertensive patients who were being treated with amphetamine to reduce obesity. In other patients, whose hypertension was successfully treated with bretylium, they found that amphetamine antagonised the hypotensive action of bretylium. Laurence and Rosenheim (1960) found in two patients that methylamphetamine abolished the postural hypotension due to treatment with guanethidine. Recently, Day (1962) has investigated the effect of a number of sympathomimetic drugs in antagonising the sympathetic nerve-blocking activity of guanethidine, bretylium and xylocholine. He found that when the responses of the rabbit's isolated ileum and of the cat's nictitating membrane to sympathetic nerve stimulation had been abolished by these sympathetic nerve blocking drugs the responses could be restored by dexamphetamine, ephedrine, mephentermine and other related sympathomimetic amines, whereas adrenaline, noradrenaline and phenylephrine did not restore responses.

We now wish to report further observations on the antagonism of guanethidine by dexamphetamine and related drugs.

Methods

Dogs and cats were anaesthetised with chloralose (80–100 mg./kg.) sometimes with an adjuvant dose of pentobarbitone (5–10 mg./kg.). The blood pressure was recorded, usually from a femoral artery, with a mercury manometer. Drugs were injected or infused into a suitable vein.

Sympathetic responses were elicited either directly, by stimulating the cervical sympathetic nerve and recording the contractions of the nictitating membrane, or reflexly, by bilateral occlusion of the carotid arteries or by electrical stimulation of the central end of a divided vagus nerve, and recording the effects on the blood pressure.

Results

Experiments on Dogs

The reflex pressor response to occlusion of the common carotid arteries was abolished after injection of guanethidine (Fig. 1). This phenomenon

has been observed previously by Maxwell, Plummer, Schneider, Povalski and Daniel (1960), Page and Dustan (1959) and McCubbin (1961). Fig. 1 also shows that at the same time as the response to sympathetic nerve stimulation was blocked the response to injection of noradrenaline was

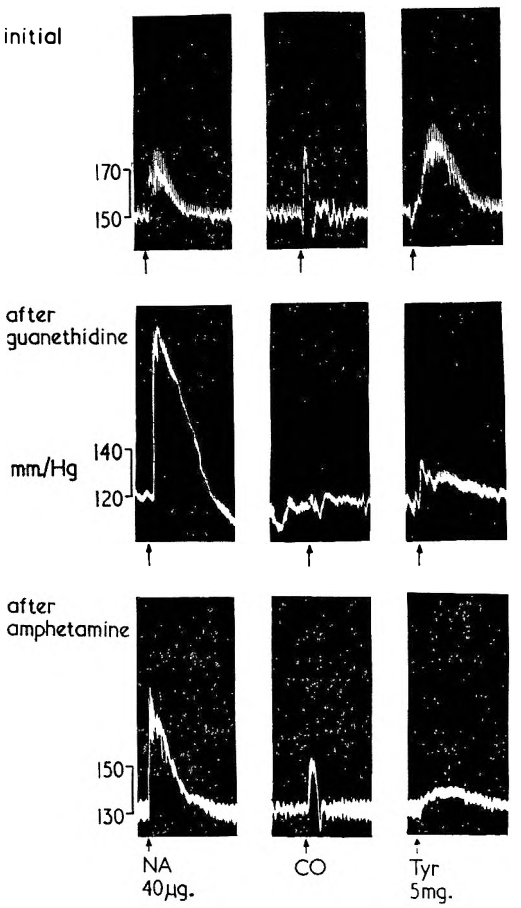

FIG. 1. Dog 12·0 kg., blood pressure recorded from femoral artery. Responses to noradrenaline 40 μg. (NA), bilateral carotid occlusion for 20 sec. (CO) and tyramine 5 mg. (Tyr.). Upper series of tracings: control observations. Middle series: after guanethidine 12 mg./kg. Lower series: after 1 mg./kg. dexamphetamine.

enhanced, confirming the findings of Maxwell and others (1960) and McCubbin (1961). These effects of guanethidine are very persistent; Maxwell and others (1960) and Page and Dustan (1959) using dogs found that the effects of a single, large dose of guanethidine were still present from 5 to 20 days later. However, as Fig. 1 shows, after the injection of a small dose of dexamphetamine the pressor response to occlusion of the carotid arteries was restored and the sensitivity to noradrenaline was reduced. Maxwell, Plummer, Povalski and Schneider (1960) found that guanethidine

antagonised the response to tyramine (and related compounds), and we have also observed this effect (Fig. 1). The injection of dexamphetamine, which caused the restoration of the pressor response provoked by occlusion of the common carotid arteries, did not restore the response to tyramine.

In other experiments it was observed that the sympathetically mediated pressor responses to stimulation of the central end of the vagus nerve were abolished by guanethidine (10 mg./kg.) and then partly restored after dexamphetamine (1 mg./kg.).

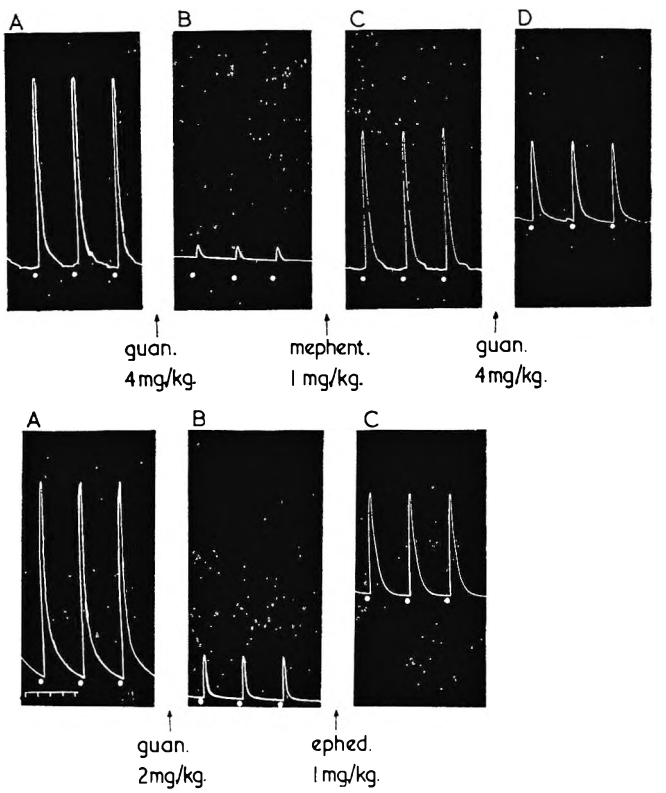

FIG. 2. Contractions of cat nictitating membrane to cervical sympathetic nerve stimulation. Stimulation at white dots with supramaximal voltage pulses of 2 msec. duration at 20/sec. applied for 10 sec. in upper records and for 20 sec. in lower records at 3 min. intervals. Upper and lower records are from two separate experiments. In the experiment shown in the upper records guanethidine (guan.) reduced the contraction, and mephentermine (mephent.) partly restored them. A second dose of guanethidine then had a lesser effect. The experiment in the lower series of records shows the block produced by guanethidine and the restoration of responses by ephedrine (ephed.).

We have investigated the effects of dexamphetamine alone on reflexly induced, sympathetic pressor responses in the anaesthetised dog. It was thought possible that the action of dexamphetamine in reversing guanethidine blockade might be due to a sensitisation of effector organs to the effects of sympathetic nerve stimulation. However, the results obtained

were not consistent with this view. Thus, when the blood pressure had returned to normal after an injection of dexamphetamine (1·6 mg./kg.) the reflex pressor responses to bilateral carotid artery occlusion and to central vagal stimulation were considerably reduced, whilst the sensitivity to noradrenaline was enhanced. It should be noted that these effects of dexamphetamine are qualitatively the same as the effects of guanethidine. When guanethidine was given after dexamphetamine there was no further blockade of the sympathetically induced responses. This observation is in accord with the findings of Day (1962) that dexamphetamine not only reverses a guanethidine blockade, but if given first will prevent its appearance.

Experiments on Cats

Day (1962) has shown in the cat that after the contractions of the nictitating membrane to cervical sympathetic nerve stimulation are blocked by guanethidine they can subsequently be restored by dexamphetamine or by related amines. The experiments illustrated in Fig. 2 show that mephentermine and ephedrine antagonise the action of guanethidine in blocking the response of the cat's nictitating membrane to sympathetic nerve stimulation. After mephentermine the same dose of guanethidine which previously produced a 94 per cent reduction in response to nerve stimulation now produced only a 45 per cent reduction.

Prolonged administration of guanethidine. Experiments in which the acute sympathetic nerve blocking action of guanethidine is reversed up to 4 hr. later by injection of dexamphetamine, are subject to the criticism that any delayed actions of guanethidine may not have had sufficient time to develop. One delayed action of guanethidine is the depletion of the noradrenaline content of tissues (Sheppard and Zimmerman, 1959; Cass, Kuntzman and Brodie, 1960; Cass and Spriggs, 1961). In this respect guanethidine resembles reserpine and it has been suggested that guanethidine may owe its sympathetic nerve blocking activity to the depletion of the noradrenaline stores. Therefore experiments were carried out on cats chronically treated with guanethidine.

The records illustrated in Fig. 3 are from an experiment on a cat which had been injected with large doses of guanethidine (12·5 mg./kg./day) for 7 days. The response of the nictitating membrane to stimulation of the cervical sympathetic nerve was a contraction of 3 mm. (on the kymograph). The reflex increase in blood pressure produced by occluding both the common carotid arteries was 40 mm. Hg. This cat was very sensitive to noradrenaline, 5 μg. injected intravenously produced a 100 mm. rise in blood pressure. An intravenous injection of 0·5 mg./kg. of dexamphetamine sulphate increased the blood pressure and contracted the nictitating membrane. These responses were smaller than those produced by the same dose of dexamphetamine in normal cats, but the initial effect of dexamphetamine on blood pressure was greater than that usually seen in guanethidine-treated animals (Maxwell, Mull and Plummer, 1959). However, the pressor response to dexamphetamine differed from that usually seen in normal cats in that the blood pressure did not return to the

pre-injection level during the next 2 hr. It appeared therefore that in addition to producing a pressor response, dexamphetamine had reversed the hypotension caused by guanethidine. Thus, before dexamphetamine, the mean blood pressure was 92 mm. but 2 hr. after the amine it was 104 mm. This reversal of the hypotension is even more impressive when it is considered that the blood pressure usually declines progressively during such experiments. That the sustained increase in blood pressure was, in fact, due to reversal of guanethidine-induced hypotension is borne out by the observation that the responses of the nictitating membrane to sympathetic nerve stimulation gradually increased, until finally the contraction of the nictitating membrane was 43 mm. (on the kymograph). The pressor response to occlusion of both common carotid arteries was increased by 50 per cent, whilst the response to noradrenaline was decreased by 20 per cent. Later in this experiment a further dose of 0·5 mg./kg. of dexamphetamine produced no more improvement of the responses of the nictitating membrane or of the carotid sinus pressor reflex. At this stage a further dose of guanethidine (12·5 mg./kg.) was given (which in normal cats rapidly and completely abolished sympathetic responses); it depressed but failed to abolish the contractions of the nictitating membrane or the pressor response to carotid occlusion. This experiment shows that dexamphetamine can reverse the effects of prolonged guanethidine treatment as effectively as it reverses the effects of an acute dose.

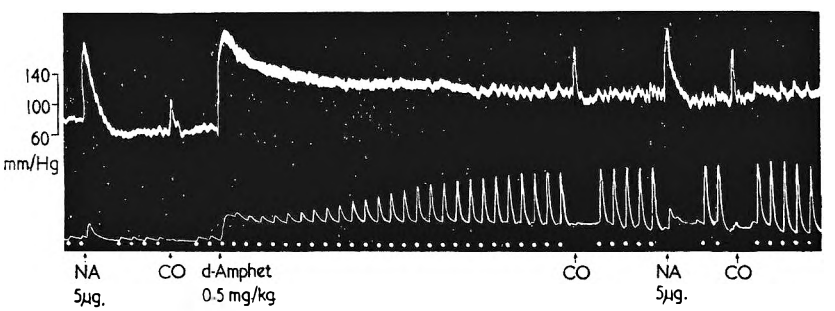

FIG. 3. Cat 3·2 kg., injected with 12·5 mg./kg./day guanethidine for 7 days. Upper record, blood pressure from left femoral artery. Lower record, contractions of right nictitating membrane. At white dots, right cervical sympathetic nerve stimulated with 5 V, 2 msec. pulses at 20/sec. for 8 sec. At NA, intravenous injections of 5 μg. noradrenaline bitartrate, at d-Amphet., 1·6 mg. of dexamphetamine sulphate, and at CO, both common carotid arteries were occluded for 30 sec.

Comparison with reserpine. In reserpine treated cats endogenous stores of noradrenaline are reduced. This leads to a failure of the responses to sympathetic nerve stimulation and of the effect on the blood pressure of tyramine, an amine which has been shown to produce its normal pressor action by releasing noradrenaline (Burn and Rand, 1958). When an infusion of noradrenaline, or one of its precursors, such as dopamine, is given, the responses to sympathetic stimulation and to tyramine are partly restored as a result of restoration of the noradrenaline

content of the tissues (Burn and Rand, 1958; Pennefather and Rand, 1960). In cats treated chronically with guanethidine some degree of noradrenaline depletion would be expected (Cass, Kuntzman and Brodie, 1960), but infusions of noradrenaline or of dopamine did not appreciably restore responses tc sympathetic nerve stimulation although they did slightly increase the responses of the nictitating membrane to tyramine. These results are shown in Fig. 4 in an experiment on a cat which had been pre-

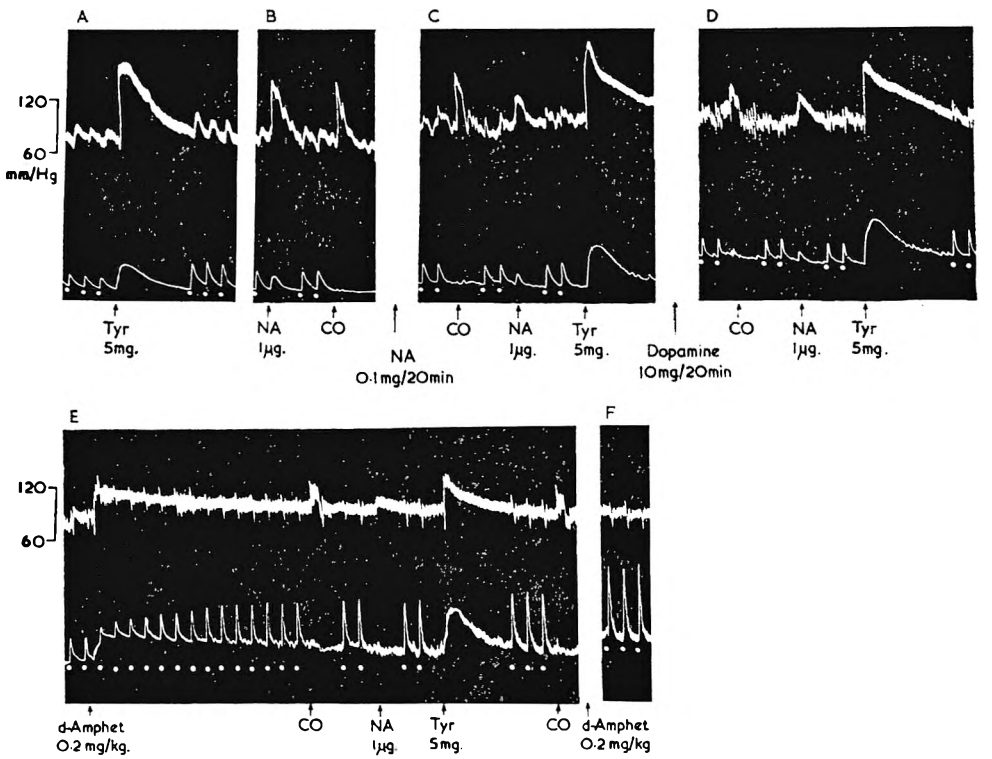

FIG. 4. Cat 2·2 kg., injected with 12·5 mg./kg./day of guanethidine for 4 days. Blood pressure from left carotid artery. At white dots, right cervical sympathetic nerve stimulated with 10 V, 2 msec. pulses at 20/sec. for 8 sec. At Tyr, tyramine hydrochloride, at NA, noradrenaline, at d-Amphet., dexamphetamine sulphate, given intravenously in the doses stated in the figure. At CO, right carotid artery occluded for 1 min.

treated with guanethidine (12·5 mg./kg./day) for 4 days; the responses of the nictitating membrane to cervical sympathetic stimulation and to tyramine (2 mg./kg.) were considerably depressed. After an infusion of noradrenaline the response of the nictitating membrane to tyramine was increased, but the responses to sympathetic nerve stimulation were not. Later, an infusion of dopamine further increased the response of the nictitating membrane to tyramine but did not increase responses to nerve stimulation. However, dexamphetamine increased the responses to sympathetic nerve stimulation, but decreased the responses to tyramine.

Our explanation of these results is that the decreased responses to sympathetic nerve stimulation after guanethidine treatment were not due solely to depletion of noradrenaline stores, although some degree of depletion may have accounted for the reduced responses to tyramine and for the ability of noradrenaline and dopamine to increase the response of the nictitating membrane to tyramine.

Discussion

Mode of Action of Guanethidine

Guanethidine has the property in common with bretylium and with reserpine of impairing the responses to sympathetic nerve stimulation. In addition guanethidine and reserpine prevent the action of sympathomimetic amines whose effects are mediated through release of noradrenaline. Tyramine and dexamphetamine serve as examples of this type of sympathomimetic amine.

However, there are marked differences between the actions of guanethidine and bretylium on the one hand and those of reserpine on the other. Thus, reserpine produces its effects mainly as a result of depletion of the transmitter substance (noradrenaline) from sympathetic nerve endings (Bein, 1953; Muscholl and Vogt, 1953; Burn and Rand, 1958; Burn, Leach, Rand and Thompson, 1959). When the store is replenished by an infusion of noradrenaline the responses to sympathetic nerve stimulation and to indirectly acting sympathomimetic amines are restored (Burn and Rand, 1958; Pennefather and Rand, 1960). The impairment in responses to sympathetic nerve stimulation induced by bretylium and by guanethidine are not reversed by noradrenaline (McCubbin, Kaneko and Page, 1961; Day, 1962). However, the blocking action of these agents is convincingly reversed by the injection of a small dose of dexamphetamine or of a related compound. For these reasons we suggest that the depletion of noradrenaline by guanethidine is not its main action and is not responsible for its hypotensive action.

There are minor differences between the actions of guanethidine and of bretylium. Thus, bretylium does not block the action of indirectly acting sympathomimetic amines such as tyramine (Boura and Green, 1959; Huković, 1960; Burn and Rand, 1960), and does not cause a significant depletion of noradrenaline stores (Cass and Spriggs, 1961).

However, Day (1962) has shown that dexamphetamine and related amines antagonise the sympathetic nerve blocking action of guanethidine and of bretylium, but not the block produced by reserpine. Therefore, from a practical as well as from a theoretical point of view, the action of bretylium and guanethidine may be classed together as quite distinct from the action of reserpine.

The action of guanethidine in potentiating noradrenaline at the time when sympathetic nerve responses are blocked, and the subsequent decrease in response to noradrenaline when sympathetic nerve responses are restored by dexamphetamine, suggest that there is a relationship between the functioning of the sympathetic nerves and the sensitivity of

tissues to noradrenaline. The potentiation of noradrenaline by guanethidine may be related to the potentiation of noradrenaline by denervation. After surgical section of the nerve the onset of supersensitivity occurs after degeneration of the distal portion of the nerve, but guanethidine "denervates" at the end of the nervous apparatus and so the supersensitivity is immediate in onset. Similar considerations apply to the hypersensitivity to noradrenaline produced by bretylium.

Antagonism of Guanethidine by Dexamphetamine

The exaggerated response to injected noradrenaline after guanethidine is decreased by dexamphetamine; therefore it is unlikely that the explanation for the restoration of responses to sympathetic stimulation by dexamphetamine is due to an increased sensitivity of the effector organs to the sympathetic nerve transmitter. Another explanation for the antagonism is that dexamphetamine may increase the amount of transmitter liberated by the nerves, but this explanation is unlikely because after dexamphetamine, given alone, the pressor responses to occlusion of the common carotid arteries and to central vagus nerve stimulation were impaired at the same time as the response to noradrenaline was potentiated and the response to tyramine reduced; these effects of dexamphetamine are, in fact, the same as the actions of guanethidine.

Day (1962) has proposed a mechanism to explain the way in which dexamphetamine antagonises the sympathetic nerve blocking action of guanethidine. He drew attention to the fact that dexamphetamine and guanethidine have a number of properties in common. Both possess sympathomimetic activity which depends on the presence of a store of noradrenaline at the sympathetic nerve ending, and both diminish the response to sympathetic nerve stimulation. Dexamphetamine is much less potent than guanethidine in diminishing the response to sympathetic stimulation. If dexamphetamine and guanethidine were acting at the same site, then the less potent blocking drug dexamphetamine may displace the more potent drug guanethidine. Day has recently made observations which show that the antagonism of guanethidine by dexamphetamine is probably competitive in nature.

The Use of Dexamphetamine to Terminate a Guanethidine or Bretylium Induced Hypotension

The intestinal absorption of bretylium is irregular and sometimes the blood pressure may fall precipitously (Dollery, Emslie-Smith and McMichael, 1960). Dexamphetamine could be used as an antidote for overdosage with either bretylium or guanethidine.

In experimental animals and in patients who have been treated with bretylium or guanethidine there is a marked hypersensitivity to noradrenaline (Laurence and Rosenheim, 1960). On this account and because of its transient action, noradrenaline is not a suitable drug for overcoming the hypotension.

There is no hypersensitivity to the pressor action of dexamphetamine after bretylium and guanethidine; instead dexamphetamine produces a

persistent increase in blood pressure of slow onset. The possession of a reliable and rapidly acting antagonist of bretylium and guanethidine, such as dexamphetamine which has the added advantage of being active by mouth, may extend their use to patients with occlusive vascular disease in whom unpredictable falls in blood pressure are dangerous (Dollery and others, 1960).

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DRUG-PLASMA BINDING MEASURED BY SEPHADEX*

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A method is described using the cross-linked dextran gel, Sephadex, for the study of drug binding. Although not quantitative in terms of per cent bound, this method gives an indication of the comparative strength of binding. The usefulness of this method in the separation of compounds with different plasma binding characteristics is suggested.

The availability of the cross-linked dextran gel, Sephadex, provides a promising system for the *in vitro* study of drug binding. Porath and Flodin (1959) first described its use and pointed out the possibility of separating molecules of different size by a method they refer to as "gel filtration". This cross-linked hydrophilic dextran polymer expands upon hydration but remains particulate and its high degree of cross linkage gives rise to a gel grain of low porosity which permits only molecules of small size to enter the internal volume of the grains. Molecules of larger size, such as protein, are excluded from the grains and appear in the early eluate of chromatographic columns. This principle has been used to separate bound and unbound fluorescein (Zwaan and Van Dam, 1961).

In the course of studies on the entry and accumulation of a number of isotopically labelled compounds in brain, we have investigated drug binding in plasma and tissue. Binding in plasma is important in assessing the relative quantity of a drug that is available for passage into the brain and cerebrospinal fluid. In addition, a number of our observations on regional or diffuse brain accumulation appears to be related to drug-tissue binding (Roth and Barlow, 1961). The nature of the binding of drugs in plasma and brain is unknown and the significance of this phenomenon is under study in various laboratories where the techniques of ultrafiltration, dialysis, differential centrifugation and electrophoresis have been employed in *in vitro* systems. This paper presents a method for the study of drug binding in plasma utilising the cross-linked dextran polymer, Sephadex.

Materials and Methods

Carbon-14 labelled phenytoin, thiopentone, phenobarbitone and barbitone were synthesised by Mr. L. Clark in the radiochemical laboratory of the Department of Pharmacology at the University of Chicago. These labelled drugs were shown by paper chromatography in multiple systems to be chemically and radiochemically pure. ^{14}C urea was purchased from Volk Radiochemical Company, Chicago, and sodium ^{35}S sulphate was purchased from Abbott Laboratories, Chicago. Normal human plasma was obtained from the blood bank of the University of Chicago and

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diluted to the desired concentration with 0·1M phosphate buffer of pH7·4. Sephadex G-25 was purchased from Pharmacia, Uppsala, Sweden.

Five g. dry gel were hydrated in 0·1M phosphate buffer pH 7·4, allowed to settle and the "fines" decanted. The hydrated Sephadex was then poured into a column 3 cm. in diameter and allowed to settle. A filter paper disc was placed on the top of the gel. Columns to be eluted with plasma were washed with 50 ml. of plasma just before application of the sample. Columns so prepared allowed a free flow of eluting fluid and could be used repeatedly.

One ml. of buffer or plasma containing drug and naphthol blue black dye as a protein indicator was placed on the column. When the sample had entered the column it was washed on by 1 ml. of elution fluid. The elution fluid (buffer or plasma) was then added in quantity and 3 ml. fractions were collected. One ml. aliquots of the radioactive eluates were plated on copper planchettes and counted in a Packard gas flow Geiger-Mueller counter. Protein was assayed colorimetrically (Gornall, Bardswill and David, 1949) and naphthol blue black was measured at 640 mμ in a Coleman Junior Spectrophotometer (Bailey and Heald, 1961).

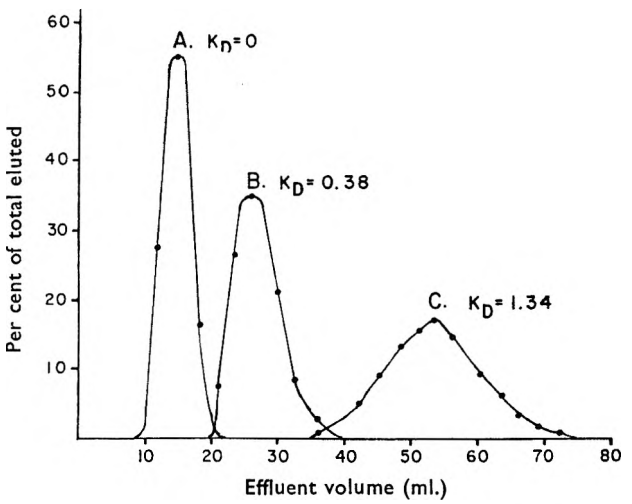

FIG. 1. Each point represents the per cent of total test substance eluted. Curve A represents the elution pattern of plasma protein eluted with 0·1M phosphate buffer pH 7·4 ($K_D = 0$). Curve B represents the elution pattern of phenytoin eluted with 50 per cent plasma, pH 7·4 ($K_D = 0{\cdot}38$). Curve C represents the elution pattern of phenytoin eluted with phosphate buffer 0·1M, pH 7·4 ($K_D = 1{\cdot}34$). One ml. of sample was applied in each case and the same column was used.

The partition ratio between the two aqueous phases in a Sephadex column is represented by the K_D value (Gelotte, 1960).

$$K_D = \frac{Ve - Vo}{Vi}$$

Vo is the water outside the gel grains, or "outer volume", while Vi is the water within the gel grains, or the "inner volume". Vi, the inner

volume, is calculated from the "water regain"* value (Granath and Flodin, 1961). Vo, the outer volume, is determined for each run and is the volume of eluate required to deliver the protein or protein dye complex in peak concentrations. Ve is the volume of eluate required to deliver the peak concentration of the experimental substance. When Ve and Vo are equal, as is the case if the test substance is of a molecular weight which restricts it to the outer volume, the K_D value is 0. A substance of low molecular weight which can diffuse freely into the grains and occupies both the inner and outer volumes will have a K_D value of 1. In practice, low molecular weight substances have a K_D value of 0·8 to 1·0, apparently because some of the water of hydration of the gel is bound (Gelotte, 1960). A K_D value greater than 1 indicates adsorption to the gel (Gelotte, 1960). The relationship between K_D values and effluent volume is represented in Fig. 1.

Results

In Table I, data are presented demonstrating that naphthol blue black (0·25 mg./ml. plasma) was eluted in the external volume, i.e., ($K_D = 0$) when the elution fluid was either phosphate buffer or plasma. If the dye,

TABLE I

Gel filtration of naphthol blue black in PO_4 buffer and plasma

Eluent	Naphthol blue black	K_D
PO_4 buffer pH 7·4 ..	0·25 mg. in 1 ml. plasma	0
Plasma 50 per cent ..	0·25 mg. in 1 ml. plasma	0
PO_4 buffer pH 7·4 ..	0·25 mg. in 1 ml. PO_4 buffer	4·12
PO_4 buffer pH 7·4 ..	0·5 mg. in 1 ml. 10 per cent plasma	peak I = 0 peak II = 4·17
Plasma 50 per cent ..	0·5 mg. in 1 ml. plasma	0

TABLE II

Gel filtration of drugs in PO_4 buffer and plasma

	Plasma concentration mg./ml.	Eluent					
		PO_4 buffer pH 7·4			50 per cent plasma		
		K_D*	Range	Runs	K_D*	Range	Runs
Phenytoin	0·01	1·22	(1·03–1·34)	5	0·35	(0·31–0·41)	6
Thiopentone	0·05	1·15	(1·15–1·15)	3	0·36	(0·32–0·38)	3
Phenobarbitone ..	0·05	1·30	(1·21–1·38)	4	1·02	(1·02–1·02)	2
Barbitone	0·05	0·84	(0·72–0·93)	4	0·94	(0·86–1·00)	4
Urea	Trace and 0·5	0·93	(0·83–1·00)	5	0·92	(0·90–0·93)	4
Sulphate	Trace and 0·5	0·83	(0·76–0·93)	7	0·92	(0·90–0·93)	5

* See text.

dissolved in buffer, was applied to the column and eluted with buffer then a K_D value of 4·12 was obtained indicating that the dye had been adsorbed on the gel. If an excess of dye (0·5 mg./ml. of 10 per cent plasma) was applied to the column, the dye was then eluted in 2 peaks by the buffer. One peak was associated with the plasma protein ($K_D = 0$) and the second peak was eluted at K_D 4·17, the value obtained with dye alone.

* Supplied by manufacturer with each batch.

As can be seen from Table II, phenytoin applied in plasma, was eluted at K_D 1·22 when phosphate buffer was the eluent indicating minimal binding to the gel. Similar K_D values were obtained when the drug was applied dissolved in buffer. When plasma was used as the eluent, however, the K_D of phenytoin was shifted toward the K_D of substances restricted to the external volume phase (K_D 0·35). Unlike the dye, phenytoin was never eluted at $K_D = 0$. Table II also shows that thiopentone behaved similarly. Phenobarbitone followed the same general trend, but the decrease in K_D with plasma as eluent was less marked. In experiments with phenytoin 10 per cent plasma eluted the drug with a K_D of 0·71, 25 per cent plasma with a K_D of 0·58, as compared to the K_D of 0·35 when 50 per cent plasma was employed. The use of undiluted plasma gave K_D values in the same range as 50 per cent plasma.

The K_D values for urea, barbitone and sulphate were similar whether these substances were eluted with buffer or plasma. These data indicate that these substances are not bound by plasma, a finding consistent with previous studies utilising other methods. In addition, these data serve as controls for possible changes in the gel related to the presence of plasma which could lead to a change in K_D unrelated to drug-plasma binding.

Discussion

Before discussing the results of this study, it is useful to consider the results obtained by accepted techniques. The plasma binding properties of the four drugs we have investigated here have been determined by ultrafiltration through a Cellophane membrane by the method of Lavietes (1937) or *in vivo* by comparison of the cerebrospinal fluid and plasma levels at equilibrium. By these methods, phenytoin and thiopentone were found by us (unpublished) to bind to plasma protein to the greatest extent (77 per cent), phenobarbitone to a lesser extent (37·7 per cent) (Domek, Barlow and Roth, 1960), while barbitone was found by others to exhibit little if any tendency to bind to plasma protein (2 per cent) (Brodie, Kurz and Schanker, 1960). Using ultrafiltration we have found that sulphate ion and urea remain unbound. Ultrafiltration of plasma containing up to 0·5 mg./ml. naphthol blue black yielded no dye in the ultrafiltrate. However, when an aqueous solution of the dye (0·5 mg./ml.) was subjected to ultrafiltration, we found a considerable fraction was adsorbed on the Cellophane resulting in 22 per cent recovery in the ultrafiltrate (data to be published). Clearly, ultrafiltration through a Cellophane membrane is not suitable to study binding of this dye and an error due to adsorption is introduced. This source of error should be considered whenever a membrane is utilised to study binding.

In the Sephadex system one might expect that the portion of test substance associated with protein molecules would be excluded from the gel grains and eluted in the early fraction ($K_D = 0$). The free or unbound drug should appear in a second elution fraction representing the internal volume ($K_D = 0·8–1·0$), or with a K_D greater than 1·0 if it is adsorbed to the gel. This situation applies when naphthol blue black is added

in excess as illustrated in Table I, where 0·5 mg. dye in 1 ml. 10 per cent plasma was eluted by buffer in 2 peaks. The first peak was associated with the protein and had a K_D value of 0 while the second had a K_D of 4·17, comparable to the dye alone. On the other hand, we were unable to saturate the phenytoin and thiopentone systems which were eluted as one fraction whether these drugs were applied in concentrations of pharmacologic interest or in great excess (2 mg./ml.), and whether they were placed on the column dissolved in plasma or buffer. However, the volume of eluent required to recover these drugs was found to be dependent upon the protein content of the eluent. When eluted by phosphate buffer they were recovered with a K_D value of approximately 1·2. When 50 per cent plasma was employed, they were brought down at $K_D = 0·35$. Phenobarbitone demonstrated a similar change, but to a lesser degree, while the elution volume of barbitone, urea and sulphate was unaltered regardless of the eluent. We conclude that binding to the plasma protein in the eluent was responsible for the reduction in the K_D of phenytoin, thiopentone and phenobarbitone. This change in K_D may therefore be used as a qualitative measure of binding. Barbitone, sulphate and urea, known not to bind appreciably to plasma protein, were unaffected by the protein concentration of the eluent and served as controls for nonspecific changes related to the composition of the eluting fluid and the gel itself.

The contrast between the plasma-dye interaction and the plasma drug interaction is of interest. The strength of the binding with the dye was such that naphthol blue black remained with the plasma in which it was originally dissolved as it passed down the column. The drugs studied did not do this, but apparently dissociated from the protein of the plasma in which they were dissolved. This difference between the dye and the drugs indicates a difference in the strength of binding to protein. If plasma protein was present in the eluting fluid, the presumed adsorption of the drugs to the gel was overcome and phenytoin, thiopentone and phenobarbitone were eluted with a K_D value distinctly lower than that found when buffer was employed as the eluent. Naphthol blue black was more firmly bound and was eluted at a K_D of zero with either buffer or plasma eluent.

Gel filtration with Sephadex is a useful addition to the procedures available for the study of drug binding *in vitro* because it avoids the use of an interposed potentially adsorbing membrane and gives some indication of strength of binding. It may be adaptable to the study of binding of drugs to various constituents of tissues such as brain by using eluents containing tissue components. Gel filtration with plasma eluent may also serve as a method for separation of mixtures of compounds which differ in the degree of binding to plasma; for example, barbitone from thiopentone, phenobarbitone from phenytoin.

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PHARMACOLOGICAL SCREENING OF SOME WEST INDIAN MEDICINAL PLANTS

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A systematic pharmacological examination has been made of 55 Jamaican plants, most of which have a local reputation as medicinals. A number of interesting, but no outstanding, activities were observed. The results are tabulated.

The use of local plants for medicinal purposes is still widespread in the West Indies and in view of the revived interest in medicinal plants over the past few years we have undertaken a pharmacological examination of some West Indian medicinal plants. Asprey and Thornton (1953, 1954, 1955) have reviewed the use of some 200 botanically identified plants in folk-medicine in Jamaica and we have used their lists as a guide to our work.

In Jamaica the medicinal plants are used as beverages ("bush teas") prepared by steeping either the leaves or the whole plant in hot water and this method of preparation influenced our choice of extraction procedure.

This report covers the first fifty-five plants examined.

Experimental

Plant Extractions

Two extracts (A and B) were used in the pharmacological tests, an aqueous extract (A) and an aqueous extract from which high molecular weight material had been precipitated with ethanol (B).

Extract (A). Freshly collected, botanically identified plant material (500 g. leaves and succulent stems) was macerated in a Waring Blendor and boiled with water (2·5 litre) for 1 hr. The extract was filtered through calico and the procedure was repeated with the residue. The filtrates were combined and evaporated under reduced pressure to 500 ml.

Extract (B). Extract (A) (500 ml.) was diluted with ethanol (1,500 ml.) and left to stand for 3 hr. at 4°. The precipitate was removed by filtration or centrifuging and the filtrate was evaporated under reduced pressure to a volume of *ca.* 100 ml. This solution was diluted with distilled water (*ca.* 400 ml.) to give Extract (B) (500 ml.).

Pharmacological Testing

The following tests were applied routinely to the extracts.

Acute Toxicity

The extract was injected intraperitoneally into mice weighing 20–35 g. Two mice were used at each dose level. The minimum dose which killed both the animals within 24 hr. was used to give a measure of the toxicity.

Effect on Isolated Organ Preparations

Guinea-pig ileum. The terminal ileum was suspended in oxygenated Ringer's solution at room temperature (28–30°) in a 30 ml. bath and the extract was introduced to test for spasmogenic effect and also for inhibition of the spasm induced by acetylcholine, histamine and barium. Test for ganglionic-blocking activity was also performed by testing the inhibitory effect of the extract against contraction induced by nicotine on guinea-pig ileum.

Rat uterus. A segment of uterus from adult rat was suspended in a bath similar to that used in a guinea-pig ileum preparation and under similar conditions. The extract was introduced to test for spasmogenic effect and also for inhibition of the spasm induced by oxytocin and 5-HT.

Rat hind limb flow. Both the descending aorta and inferior vena cava of the rat were cannulated with polythene tubes. The tips of the tubes were fastened just above the femoral bifurcations of these vessels. The arterial side was perfused with oxygenated Ringer's solution containing 0·5–4·0 μg./ml. of noradrenaline. Fluid was collected from the venous side and the volume was recorded through a phototransistor drop recording assembly.

Rat diaphragm phrenic nerve. The electrically stimulated diaphragm-phrenic nerve preparation of the rat described by Bülbring (1946) was used. The extract was tested for its effect on the muscular contraction initiated by the electrical stimulation of the diaphragm both directly or via the nerve.

Rat stomach fundus. The preparation of the rat fundus for 5-HT assay as described by Vane (1957) was used. The extract was introduced to test for its 5-HT like activity.

Rabbit duodenum. A segment of rabbit duodenum was suspended in 30 ml. of tyrode solution gassed with 95 per cent oxygen and 5 per cent CO_2 at 37° and the extract was tested for the effect on the spontaneous pendulum movements and the tone of the duodenum.

Isolated rabbit heart. The Langendorff preparation of the isolated rabbit heart was perfused with Ringer's solution. The extract was tested for its effect on the rate, the amplitude, and the rhythm of the heart.

Dog respiration and blood pressure. The dog was anaesthetised with pentobarbitone sodium intravenously. The trachea was cannulated and connected to a respiratory tambour. The carotid artery was cannulated and connected to a mercury manometer. The effect of the extract on respiration and main arterial pressure were recorded. The extract for testing was injected intravenously through the femoral vein. The weight of the dogs varied from 8 to 16 kg.

RESULTS

The results are tabulated in Table I with the following exceptions. Since no significant effects were found with any of the extracts in the tests for the neuromuscular blocking action and the 5-HT like activity, the results of these two tests were not included in the Table. Only extracts 41B, 13A, 21A, 46A, 11A and 53B showed some ganglionic

TABLE I RESULTS OF INVESTIGATION

Plant No.	Plant: Family	Plant: Botanical name	Ext.	Mouse toxicity	G.P. ileum	Rat uterus	Rat limb flow	Rabbit heart	Rabbit duodenum	Dog B.P.
1	Acanthaceae	*Andrographis paniculata* Nees	A	++	O	O		O	−−	O
			B	+	O	O	+++	O		O
2	Agavaceae	*Agave angustifolia* Haworth	A	+	++	O		−−−		O
			B	+	++	O	−−−	−−		O
3	Amaranthaceae	*Achyranthes indica* Mill.	A	++	++	O		−−		O
			B	O	++	O	++	O		O
4	Annonaceae	*Annona muricata* L.	A	+	+ + + +	+ + + +		+	−	P
			B	O	+++++	+++++	++++	O	−−	P
5	Apocynaceae	*Forsteronia floribunda* (Sw.) A.DC.	A	++	+++++	+++++		O		O
			B	++	+++++	+++++	−−−−	−		O
6	Araceae	*Dieffenbachia seguine* Schott	A	+	+++	+++++		O		O
			B	O	+++	O	−−	O		+
7	Bignoniaceae	*Spathodea campanulata* Beauv.	A	+	O	O				D
			B	O	O	O	++			D
8	Boraginaceae	*Cordia globosa* (L.) H.B.K.	A	+++	−−	O		?	−−	O
			B	++	−−	O	+++	O		O
9	Bromeliaceae	*Bromelia pinguin* L.	A	++	−−	O		−−		D
			B	+	−−	O	++	?		O
10	Burseraceae	*Bursera simaruba* Sarg.	A	++	−−	O		−		O
			B	O	−−	O	++	O		O
11	Cactaceae	*Rhipsalis cassutha* Gaertn.	A	+	+++	O				O
			B	+	+++	O	−−			O
12	Commelinaceae	*Zebrina pendula* Schnizl	A	+	++	O		O		O
			B	O	++	O	++	O		O
13	Compositae	*Pluchea carolinensis* (Jacq.) (G. Don	A	++	++	O		O		O
			B	+	++	O	+++	O		O
14	Compositae	*Eupatorium odoratum* L.	A	O	++	O		O	−−	
			B	O	+	O	?			
15	Convolvulaceae	*Cuscuta americana* L.	A	++	+++	O		−−−	+	O
			B	O	++	O	O			O
16	Crassulaceae	*Bryophyllum pinnatum* Kurz.	A	+	++++	O		−−	O	O
			B	+	+++	O	O	−		O
17	Cucurbitaceae	*Sechium edule* Sw.	A	O	++	O		+	O	O
			B	O	++	O	+++	O	O	D
18	Cucurbitaceae	*Cayaponia racemosa* Cogn.	A	O	+++	O		O	O	O
			B	O	+++	O	−−−−	O	O	
19	Euphorbiaceae	*Euphorbia hirta* L.	A	+	O	O		−	O	D
			B	O	−−	O	+++	+	O	D
20	Euphorbiaceae	*Euphorbia hypercifolia* L.	A	+	++	O				D
			B	O	+++	O	++	−−−	+	D
21	Euphorbiaceae	*Euphorbia lasiocarpa* Klotzsch	A	++	O	O		?	O	D
			B	++	O	O	−−			D
22	Euphorbiaceae	*Croton linearis* Jacq.	A	+	+++	+++		−	O	D
			B	O	+++	+++	−−−	O	+	
23	Gesneriaceae	*Rhytidophyllum tomentosum* Mart.	A	+	+++	O		O	O	O
			B	O	+++	O	++	O	O	
24	Gramineae	*Digitaria decumbens* Stent	A	+	++	O		O	O	D
			B	O	++	O	++	+	O	D
25	Labiatae	*Hyptis suaveolens* Poit	A	+	+	O		O	O	O
			B	O	+	O	++	O	O	
26	Labiatae	*Leonotis nepetaefolia* R.Br.	A	+	+++	O		+	O	
			B	O	+++	O	++++	O	+	D
27	Lauraceae	*Persea americana* Mill.	A	++	+++	++++		O	O	D
			B	+	++	+++	+++	−	−	
28	Papilionaceae	*Andira inermis* H.B.K.	A	O	+	O				
			B	O	++	O	O	O	O	O

TABLE I—*continued*

Plant No.	Plant: Family	Plant: Botanical name	Ext.	Mouse toxicity	G.P. ileum	Rat uterus	Rat limb flow	Rabbit heart	Rabbit duodenum	Dog B.P.
29	Caesalpiniaceae	*Cassia occidentalis* L.	A	O	++	++		+	O	D
			B	O	+	++	-----	+	−	D
30	Mimosaceae	*Samanea saman* Merrill	A	+++++	O	−−		−	−	D
			B	+++	O	−−	++++		+	D
31	Caesalpiniaceae	*Tarmarindus indica* L.	A	O	−	O		−−	O	O
			B	O	−−	O	+++			O
32	Loranthaceae	*Oryctanthus occidentalis* Eichl	A	++	O	++				
			B	++	O	++	++			D
33	Loranthaceae	*Phoradendron rubrum* Griseb.	A	+++++	+	+++		O	O	D
			B	+++++	+	+++	----	O	O	D
34	Loranthaceae	*Phoradendron wattii* Kr. and Urb.	A	++	+++	−−		−−	−	P
			B	++	+++	−−	-----	+++	−	P
35	Malvaceae	*Gossypium* Spp.	A	++	++++	++				
			B	+	++++	++	+++			
36	Meliaceae	*Cedrela odorata* L.	A	+++	O	O		O	O	
			B	+	O	O	+++	O	O	D
37	Menispermaceae	*Cissampelos pareira* L.	A	+	−−−	−−		+++	−−−	D
			B	+	−−−	−−	----			D
38	Moraceae	*Artocarpus incisa* L.	A	O	++	O				O
			B	O	++	O	++	O	O	O
39	Moraceae	*Cecropia peltata* L.	A	+++	+++	O		O	!	
			B	+	+++	O	?			
40	Palmae	*Cocos nucifera* L.	A	+++	++	O				D
			B	+	++	O	?			
41	Papaveraceae	*Argemone mexicana* L.	A	O	++	O		O	+	D
			B	O	++	O	+++	−−	+	D
42	Rhamnaceae	*Gouania lupuloides* Urb.	A	+	++	O				O
			B	O	++	O	++			
43	Piperaceae	*Piper amalago* L.	A	+	----	O				P
			B	+	----	O	----			P
44	Piperaceae	*Piper auritum* H.B.K.	A	O	+	+++				D
			B	O	+++	+++	++			
45	Portulacaceae	*Portulaca oleracea* L.	A	+	+++	++++		+++	−−−	D
			B	O	+	+++	----	+++	−−−	D
46	Rubiaceae	*Morinda royoc* L.	A	O	++	O		O	−	O
			B	O	+++	O	?			
47	Rutaceae	*Zanthoxylum flavum* Vahl.	A	+	+++	O				
			B	O	+++	O	+++			D
48	Sapindaceae	*Serjania mexicana* Willd.	A	++	++	O				O
			B	+	++	O	?			
49	Simarubaceae	*Picraena excelsa* Lindl.	A	++	++	−−				
			B	O	++	O	++			
50	Simarubaceae	*Picramnia pentandra* Sw.	A	+	++	O				
			B	+	+	O	+++			
51	Solanaceae	*Solanum ficifolium* Ortega	A	O	++	O		O	O	D
			B	O	+++	O	O	−−	O	D
52	Turneraceae	*Turnera ulmifolia* L.	A	O	+	O				
			B	O	++	O	++			
53	Verbenaceae	*Stachytarpheta jamaicensis* Vahl.	A	+++	+++	O		O	O	O
			B	+++	++	O	----	+	O	O
54	Verbenaceae	*Stachytarpheta mutabilis* Vahl.	A	+++	+++	O		O	O	O
			B	+++	++	O	----	+	O	O
55	Zygophyllaceae	*Tribulus cistoides* L.	A	+	++	O		----	+	D
			B	+	++	O	−−			D

blocking activity on the guinea-pig ileum. These results also were not included in the Table.

Expression of results. Since the activity observed was obtained from various dose levels rather than from a definite dose, the Table was compiled in a semi-quantitative manner by using pluses and minuses to indicate their relative activity. This system of expression also facilitates the reading of a lengthy tabulation.

Acute toxicity. The minimum dose (volume of extract per animal) required to kill all the animals was used as the toxic dose level. The relative toxicity of the extracts is expressed as follows:

$+ = 1{\cdot}0$ ml., $++ = 0{\cdot}5$ ml., $+++ = 0{\cdot}1$ ml., $++++ = 0{\cdot}05$ ml., and $+++++ = 0{\cdot}001$ ml.

Isolated organ preparation. The activity was expressed as the dose level (volume of extract per preparation) required to stimulate (plus) the spontaneous activities or to induce activities on a quiescent preparation by the extract, or to inhibit (minus) the spontaneous activities or to inhibit drug-induced activities of the preparation by the extract. In the case of rat hind limb flow, plus indicated the increase and minus indicated the decrease of the volume flow. The relative activity of the extracts is expressed as follows: 1·0 ml. (+), (−); 0·1 ml. (++), (−−); 0·01 ml. (+++), (−−−); 0·001 ml. (++++), (−−−−); 0·0001 ml. (+++++), (−−−−−).

Dog respiration and blood pressure. It is difficult to express the blood pressure response quantitatively without detail. It was decided that the effect of the extract on the blood pressure should be expressed simply as pressor (P) or depressor (D) when the extract (0·1 ml. or less of plant extract per kg. body wt.) gave a definite response. No significant effect on respiration was found with the extracts, and the results were not included in the Table.

Discussion and Conclusion

A pharmacological examination of 55 medicinal plants of Jamaica has been made. Although no outstanding pharmacological activity was observed, a number of plants did show interesting results, but it is too early to say whether the plants which have a local reputation as medicinals do show significantly different pharmacological properties as a group from plants without such a reputation. Guided by these results some plants have been selected for detailed chemical and pharmacological studies. In the following paper we present the isolation and identification of substances which have been responsible for the cardiovascular activities observed in some of the plants. Preliminary reports on the pharmacological activities of the crude alkaloids from the bark of No. 30 (Leonard, 1961) and the identification of (−)-noradrenaline in No. 45 (Feng, 1961) have been presented elsewhere. Further work on the isolation of substances of chemical or pharmacological interest, or both, is in progress.

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SIMPLE HYPOTENSIVE AND HYPERTENSIVE PRINCIPLES FROM SOME WEST INDIAN MEDICINAL PLANTS

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Examination of those plant extracts which were previously found (Feng and others, 1962) to have transient depressor activity has led to the isolation of γ-aminobutyric acid, from the leaves of *Artocarpus incisa* L. and of *Piper amalago* L. and to the demonstration by paper chromatography of its presence in several extracts. Extracts showing pressor activity were also examined and tyramine has been isolated from *Phoradendron wattii* Kr. and Urb. and dopamine has been shown by paper chromatography to be present in *Piper amalago* L. and *Stachytarpheta jamaicensis* Vahl.

Several extracts obtained from West Indian medicinal plants were found to affect the blood pressure when injected into a dog anaesthetised with pentobarbitone sodium (Feng and others, 1962). These extracts have been examined in more detail to determine what substances were responsible for the effects: we now report the identification of γ-aminobutyric acid, tyramine and dopamine in various extracts.

Results

γ-Aminobutyric acid. Several of the extracts showed quite marked transient depressor effect on the blood pressure of the pentobarbitone anaesthetised dog. In two such experiments, the extracts from the leaves of *Artocarpus incisa* L. ("breadfruit") and from those of *Piper amalago* L. ("pepper elder"), the factor responsible was isolated by using ion-exchange resins and thick paper chromatography. It was identified as γ-aminobutyric acid by paper chromatography, infra-red spectrum, mixed melting point, and by preparation of derivatives. This substance has been shown to have a transient depressor effect when injected into laboratory mammals (Iino, 1955, Takayasu, 1956) and man (Elliott and Hobbiger, 1959).

Other extracts which showed qualitatively similar transient depressor effects were examined by two dimensional paper chromatography for the presence of γ-aminobutyric acid. By this procedure, extracts from the following plants were found to contain it.

Agave angustifolia Haworth Var. Marginata.
Alchornea latifolia Sw. ("loblob", "sweet wood").
Andira inermis H.B.K. ("Cabbage bark").
Annona muricata L. ("Soursop").
Cassia occidentalis L. ("Wild dandelion").
Cayaponia racemosa Cogn. ("Wild cerassee").
Cocos nucifera L. ("Coconut")—mature liquid endosperm.
Croton linearis Jacq. ("Wild rosemarie", "Spanish rosemarie").
Cuscuta americana L. ("Love bush").

Forsteronia floribunda (Sw.) A.DC.
Momordica charantia L. ("Cerassee").
Oryctanthus occidentalis Eichl ("Mistletoe").
Phoradendron rubrum Griseb. var. gracile ("Mistletoe").
Piper auritum H.B.K.
Samanea saman Merrill ("Guango").
Sechium edule Sw. ("Cho'cho").
Stachytarpheta jamaicensis Vahl. ("Vervine").
Solanum torvum Sw. ("Susumba").
Tribulus cistoides L. ("Police macca").
Zebrina pendula Schnizl. ("Red water grass").

Tyramine. The strong pressor effect on the blood pressure of the pentobarbitone anaesthetised dog observed in extracts of *Phoradendron wattii* Kr. and Urb. ("mistletoe") was found to be associated with a substance which behaved similarly to tyramine on paper chromatography. The substance was isolated by extraction, alumina chromatography, and crystallisation. It was identified as tyramine by infra-red spectrum and mixed melting point. Tyramine has been reported to be present in other *Phoradendron* species (Crawford and Watanabe, 1914 and 1916).

Dopamine. Strong pressor effects were also observed in extracts of *Piper amalago* L. and *Stachytarpheta jamaicensis* Vahl. These extracts were shown to contain a substance which was identical with dopamine on paper chromatography and the pressor activity is almost certainly caused by the presence of dopamine.

Experimental

Isolation of γ-Aminobutyric Acid from Artocarpus incisa *L.*

Freshly picked mature leaves (2 kg.) were macerated in a Waring Blendor with water (10 litres) then boiled for 1 hr. and filtered through fine cloth. The residue was again extracted with water (8 litres) and the combined filtrates were concentrated under reduced pressure to a volume of 1·2 litres. Ethanol (3·6 litres) was added to precipitate protein and the solution left standing for 2 hr. at 4° then filtered. The filtrate after concentration under reduced pressure to 1 litre was passed through a column of Amberlite IR 120(H^+) (780 g.). The resin was washed with water until the eluate no longer gave a colour with ninhydrin and then with 0·1N ammonium hydroxide. The ammoniacal eluate but not the aqueous showed hypotensive activity.

Evaporation of the active solution to dryness under reduced pressure gave a brown solid (7·9 g.) which on paper chromatography in butanol : acetic acid : water (4 : 1 : 5) was found to contain eight ninhydrin positive spots. The activity was associated with the spot running with R_F 0·36 and the material (305 mg. from 2·7 g. crude solid) was isolated by elution of the appropriate zone of chromatograms run on Whatman 3 MM and Whatman Seed Test (Brownell, 1957) papers in the same system. Three crystallisations from ethanol using charcoal gave colourless needles (52 mg.) m.p. 192–197°. The infra-red spectrum was identical with that

of authentic γ-aminobutyric acid, with which no depression was observed on mixed m.p. determination. Chromatographic behaviour was also identical with an authentic sample and no differences were found on two dimensional chromatography on Whatman No. 1 paper in phenol : water (4 : 1) and collidine : lutidine : water (1 : 1 : 1); on circular chromatography (Saifer, 1956) in butanol : acetic acid : water (4 : 1 : 5) and in phenol : water (4 : 1); and on dusting the origin of chromatograms developed in phenol : water (4 : 1) with basic copper carbonate (Crumper, 1949) which forms complexes with α-amino-acids but does not affect γ-aminobutyric acid. A *p*-toluene sulphonyl derivative was prepared, m.p. 137–139° undepressed on admixture with authentic *N*-(*p*-sulphonyltoluene) γ-aminobutyric acid.

Isolation of γ-Aminobutyric Acid from Piper amalago *L.*

γ-Aminobutyric acid was isolated from the leaves of this plant by the method described in the previous section. Additional criteria for its identity were: the comparative behaviour on paper chromatography on Whatman No. 1 paper in the systems: methyl ethyl ketone : isopropanol : water (2 : 2 : 1); t-butanol : 2N ammonium hydroxide (2 : 3); phenol (25 g.) : *m*-cresol (25 g.) : borate buffer pH 9·3 (7 ml.); and in the two dimensional systems of Levy and Chung (1953) which showed the isolated material to be identical with an authentic sample. Further evidence for this identity was given by the behaviour on electrophoresis by the horizontal technique of Grassman and Hannig (1950). On Whatman No. 1 paper, at pH 3·5 and pH 6·5 (pyridine acetate buffer) with a current of 25 mA at 250 V no difference in mobility was observed. A 2,4-dinitrophenyl derivative m.p. 146–147° was prepared, identical with 2,4-dinitrophenyl γ-aminobutyric acid.

Paper Chromatographic Screen for γ-Aminobutyric Acid

3 ml. of every standard solution which showed a transient hypotensive response was shaken with Dowex-50-X8, 100 mesh, H^+ form (3 g.) for 30 min. The resin was filtered off, washed with water (3 × 5 ml.) and the amino-acids eluted with 4 × 3 ml. of 3N ammonia solution. The solution was brought to dryness under reduced pressure, made up to 3 ml. with water and applied to one corner of Whatman No. 1 paper (20 cm.^{2}) as (5–10) × 5 μl. spots as the hydrochloride. Papers were run in pairs, to one of which 25 μg. each of proline and γ-aminobutyric acid were added, and accompanied by a marker paper with proline (which provides a useful guide to the γ-aminobutyric acid position) and γ-aminobutyric acid. These were run with occasional full marker sheets with one of two standard mixtures of amino-acids. Mixture A: leucine, proline, phenylalanine, valine, tryptophan, threonine, glycine, aspartic acid and lysine. Mixture B: isoleucine, methionine, tyrosine, alanine, glutamic acid, serine, cystine, histidine and arginine. Ascending runs were carried out in two dimensions [first system: butanol : acetic acid : water (4 : 1 : 5); second system: phenol (25 g.) : *m*-cresol (25 g.) : borate buffer pH 9·3 (7 ml.)]. After the papers had been dried thoroughly, colours were

developed by dipping the papers into 0·25 per cent ninhydrin in acetone then heating to 80° for 5 min.

Isolation of Tyramine from Phoradendron wattii *Kr. and Urb.*

Ten Whatman 3 MM papers ($18\frac{1}{4} \times 22\frac{1}{2}$ in.) were loaded with standard deproteinised solution (circa 1 ml. each) in a narrow (0·6 mm.) band and run in butanol : acetic acid : water (4 : 1 : 5). Horizontal strips were arbitrarily cut, eluted with warm water and tested in a pentobarbitone anaesthetised dog. The strip corresponding to R_F 0·60–0·62 showed pressor activity and the material from this zone gave a positive coupling test with diazotised sulphanilic acid for phenols and a positive fluorescein chloride test for primary amines.

280 g. of dried, milled plant was extracted with methanol in a Soxhlet apparatus for 24 hr. The solvent was evaporated under reduced pressure and the dry residue digested with 10 per cent hydrochloric acid (1 litre) for 24 hr. The solution was continuously extracted with chloroform for 15 hr. to remove neutral material, then made just basic with ammonia and extracted further with fresh chloroform. Evaporation of the solvent gave crude base (0·3 g.) which was purified by adsorption on an alumina column (30 g.) from which it was eluted with acetone. The resulting material ran with a single spot (R_F 0·61) on Whatman No. 1 paper in the *n*-butanol : acetic acid : water (4 : 1 : 5) system, and formed a crystalline hydrochloride with an infra-red spectrum identical with that of authentic tyramine hydrochloride and giving m.p. and mixed m.p. 269°.

Paper Chromatographic Identification of Dopamine in Piper amalago *L. and* Stachytarpheta jamaicensis *Vahl.*

The pressor activity in these plants was associated with bands of R_F circa 0·31 in the butanol : acetic acid : water (4 : 1 : 5) system. The reaction of the materials in these bands with potassium ferricyanide in buffer pH 7·8 (James, 1948) gave a colour characteristic of the sympathomimetic catecholamine type.

1·38 litres of either plant extract prepared by boiling fresh crushed plant material (650 g.) with water (2·38 litre), was introduced to a column of Dowex-50-X4 (Bertler, Carlsson and Rosengren, 1958) (H^+ form, 100–200 mesh) (30 × 4 cm.) and allowed to percolate (drip rate 500 ml./hr.). The column was then washed with N hydrochloric acid and the initial eluate (circa 2·2 litres) was discarded. The next fraction (total volume 600 ml.) which gave a positive reaction with the potassium ferricyanide reagent was collected, concentrated under reduced pressure at 45° to 100 ml. and examined by paper chromatography. Spots (50 μl.) were applied to Whatman No. 1 paper and chromatography in the following systems:

	R_F
Phenol : water (atmosphere of HCl)	0·49
Butanol : acetic acid : water (4 : 1 : 5)	0·31
Methyl ethyl ketone : water	0·71
Methanol : pentanol : benzene : water (2 : 1 : 1)	0·57
Methanol : water (1 : 1)	0·16

The R_F values of the spot the spot obtained with potassium ferricyanide reagent, are identical to those given by authentic dopamine, which was run as marker. Dopa, Epinine, Corbasil, adrenaline and noradrenaline were also run as markers.

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ULTRASONIC IRRADIATION OF SOME PHOSPHOLIPID SOLS

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Ultrasonic irradiation of turbid sols of egg lecithin and of highly unsaturated synthetic lecithins, breaks down the large particles in the sols into micelles of micellar weight of about 5×10^6. The irradiated sols are clear and do not show any great increase of turbidity over a period of several days; they are capable of solubilising large quantities of cholesterol. The size and shape of micelles in the irradiated sols has been studied by means of viscosity and diffusion measurements and by determination of sedimentation coefficients in the ultracentrifuge.

Lecithin sols of concentration above 5 per cent readily separate into two layers on centrifugation at low speeds (Saunders, 1960). The lower layer contains all the phospholipid, has a concentration of about 15 per cent (w/w) and has the appearance of a thick emulsion. Microscopic examination of these concentrated sols shows that they contain large structures, as shown in Fig. 1. Attempts to clarify the 15 per cent emulsion by the addition of surface-active agents were not successful, but we have found that ultrasonic irradiation at 20 kc/sec. breaks down the structures and gives optically clear sols containing slightly asymmetric micelles of molecular weight about 10^7. Providing oxygen is excluded, the lecithin does not undergo any drastic chemical change.

The clarified sols are not flocculated by electrolytes and they are able to solubilise concentrations of lipids such as cholesterol, to give clear sols. This effect has been noted by Fleischer and Brierley (1961) who have pointed out the potential value of these sols in biochemistry. They may also be of use in preparing injection solutions of water insoluble lipids.

Experimental

Ultrasonic dispersion was done with a 60 watt Mullard ultrasonic generator using titanium probes. The tip of the probe was immersed to 2 mm. in the surface of the liquid contained in a glass tube of diameter about 1 cm. greater than that of the probe and cooled in an ice bath. The generator was tuned to give maximum cavitation which is mainly effective in breaking down structures in the liquid (Thomas, 1959; Littlewood, 1962). The irradiation was made in an atmosphere of nitrogen.

Materials. Egg lecithin was prepared and purified by chromatography and crystallisation, as described in previous papers (Saunders, 1957; Perrin and Saunders, 1960). The synthetic phospholipids were the gift of Professor van Deenan and Dr. de Haas of Utrecht University.

Preparation of sols. Egg lecithin sols were formed by adding water to an ethereal solution, evaporating the ether under reduced pressure until

only a small volume of liquid remained, then adding water to give the final required concentration. The concentrations are in weight per cent.

Optical density measurements. Measurements were made with a Spekker absorptiometer using a 0·25 cm. cell and a mercury vapour lamp with a neutral density filter.

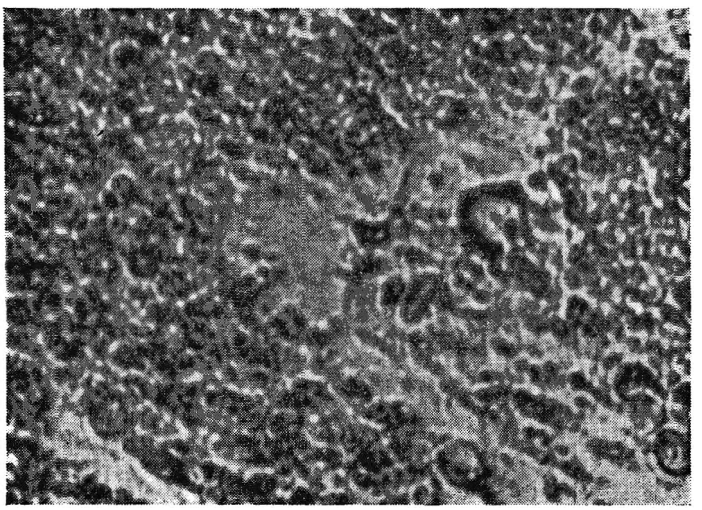

FIG. 1. A concentrated lecithin sol before irradiation (x 700).

TABLE I

VARIATION OF OPTICAL DENSITY WITH TIME FOR A 5 PER CENT EGG LECITHIN SOL AFTER ULTRASONIC IRRADIATION

Measurements made using a mercury light source, a neutral density filter and a 0·25 cm. cell

Time (hr.)	Optical density
0	0·097
1	0·098
2	0·098
4	0·100
6	0·101
17	0·101
43	0·099
67	0·105
77	0·112

Twenty min. of ultrasonic irradiation of lecithin sols gave a constant low optical density. On stopping the irradiation, the optical density decreased further and then remained constant over a period of several days showing that there is no spontaneous reversion of the sol to its original form; the initial drop in optical density could be eliminated by centifuging the sol. Results for a 5 per cent dispersion are given in Table I. The original dispersion could only be regained by evaporating to dryness and redispersing by the ether and water method; the residue on evaporation was completely soluble in ether showing that little lysolecithin was formed in the ultrasonic treatment.

A study of the effect of dilution on the clarified sol indicated that the optical density of a diluted sol usually was slightly higher than that of a sol of the same concentration irradiated directly, but this effect was small and the diluted sols showed no change of density with time.

Viscosity measurements. Concentrated dispersions of egg lecithin in water are thixotropic and give ratios of specific viscosity to volume fraction (zero concentration and shear) of 26·5 at 25° indicating highly asymmetric particles (cf. Robinson, 1960). The ultrasonically dispersed sols, however, showed no variation of viscosity with shear rate and no fall in deflection was noted on shearing a 5 per cent sol at 7·1 rev./sec. in a Couette viscometer, also the plot of deflection against shear rate was linear. In view of the Newtonian behaviour of these sols, the intrinsic viscosity was determined by measurements in a Cannon-Fenske capillary viscometer. The results gave a ratio of specific viscosity to volume fraction, extrapolated to zero volume fraction, of 3·7. This differs from the theoretical value for spheres sufficiently to suggest that the micelles in these sols are asymmetric.

Light scattering. Some preliminary measurements indicate that the scattering of very dilute ultrasonically irradiated sols is of the same order as the very dilute sols dispersed by normal methods (Robinson, 1960).

Sedimentation studies. These were carried out using a Spinco model E ultracentrifuge fitted with a schlieren phase plate. Sedimentation coefficients (S) were computed from plots of ln(x) against time at constant speed, where x is the distance of the sedimenting boundary from the centre of rotation. The observed sedimentation coefficients were corrected to water at 25°.

The sedimentation of a 5 per cent sol of egg lecithin which had not been treated ultrasonically, was first examined. This emulsion gave a clear separation into two phases at low speeds and when a centrifugal field of up to 250,000 *g* was applied only a slight compression of the lower layer occurred. At 30,000 *g* the concentration of lecithin in the lower layer was 12 per cent, at 250,000 *g* it was increased to 18 per cent. There was no evidence of any phospholipid remaining in the upper layer.

Three separately prepared, ultrasonically irradiated sols were next examined. These were all of a concentration of 1 per cent of egg lecithin in water. The schlieren patterns were all similar to those shown in Fig. 2. No splitting of the peaks was observed but they are clearly asymmetric with a trail away from the centre of rotation, indicating some degree of polydispersity. The rate of sedimentation was low but this is due to the small density difference between lecithin and water. To obtain a mean micellar weight estimate from the sedimentation data the partial specific volume of the phospholipid must be measured with great accuracy. This was done by measuring densities of the sols and gave a value for egg lecithin of 0·9833 ml./g. (cf. Elworthy (1959), density of dry lecithin 1·016 g./ml.).

The values of S corrected to water at 25° obtained for the three samples were 3·12, 2·60 and 3·45.

Diffusion. Rates of diffusion of the ultrasonically dispersed lecithin micelles into water and into more dilute sols were measured by the Gouy

interference method (Brudney and Saunders, 1955; Thomas and Saunders, 1959) at 25°. The patterns obtained indicated solute heterogeneity and the mean diffusion coefficient which was very low, decreased with time. Results obtained for diffusion from a 1·4 per cent sol into a 0·4 per cent sol gave more regular patterns than the diffusion into water and gave a diffusion coefficient of $1{\cdot}8 \times 10^{-7}$ cm.^{2}/sec. which fell after 24 hr. to a value of 8×10^{-8} remaining constant over a four day period. It is clear from these results that a range of micellar sizes is present in the ultrasonically dispersed sols.

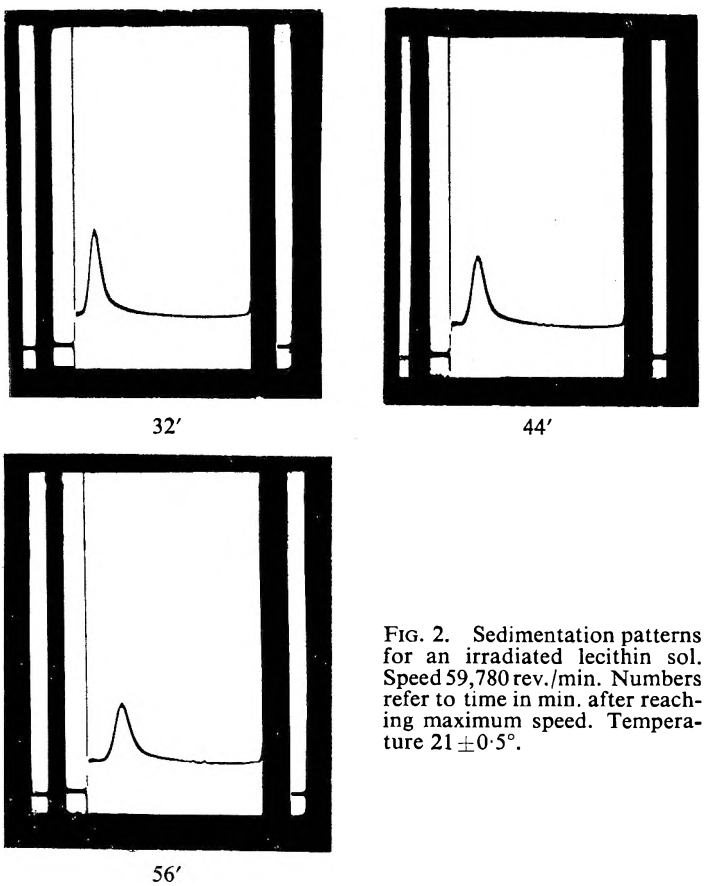

FIG. 2. Sedimentation patterns for an irradiated lecithin sol. Speed 59,780 rev./min. Numbers refer to time in min. after reaching maximum speed. Temperature $21 \pm 0{\cdot}5°$.

Synthetic phospholipids. Synthetic lecithins containing only long chain saturated fatty acids cannot be dispersed in water (Saunders, 1957) (dilauryl)lecithin being the longest chain lecithin which gives stable aqueous dispersion. The work of de Haas and van Deenan (1961) has led to the synthesis of lecithins containing unsaturated acyl groups and also to the preparation of mixed acid lecithins containing both saturated and unsaturated fatty acids; these should show properties similar to the natural substances.

We have attempted to disperse (stearoyl-oleyl)- and (di-oleyl)lecithin in water. Some difficulty was found in obtaining the initial dispersion; this was finally made by adding water to an ethanol solution of the phospholipid, evaporating to small bulk under vacuum adding water and re-evaporating and then finally making up to weight with water. Turbid 0·5 per cent sols were obtained, the (di-oleyl)lecithin sol was cleared by sonic irradiation, but unlike the natural material this clearing was reversible and after 1 hr. the sol was again turbid. Attempts to disperse (di-oleyl)cephalin in water were unsuccessful. (Di-linoleyl)lecithin did, however, give dispersions in water which could be cleared irreversibly by the ultrasonic irradiation and which were capable of solubilising cholesterol.

Solubilisation of cholesterol. When cholesterol is dissolved in an ethereal solution of egg lecithin, the lipids can be dispersed together in water. If, after evaporating off the ether, the sol is irradiated ultrasonically, an optically clear dispersion can be obtained. A concentration of up to 10 per cent of cholesterol in water can be achieved in the presence of 20 per cent lecithin, and on standing this concentrated sol forms a clear gel. At a slightly lower concentration (16 per cent lecithin, 8 per cent cholesterol) the sol remains liquid and is stable over periods of a week or more. The lecithin cholesterol sols are precipitated by salts, unlike the irradiated sols containing lecithin alone.

Discussion

Ultrasonic irradiation of concentrated egg lecithin and highly unsaturated synthetic lecithin sols breaks down the microscopically visible structures present, into micelles of molecular weight of about 10^7. The viscosity results indicate some asymmetry and if the micelles are considered as disc shaped entities they would correspond to oblate ellipsoids of axial ratio about 3·5. The diffusion and viscosity results together indicate a range of micellar weights of from 4 to 50×10^6, the sedimentation and diffusion results give a range of 2 to 7×10^6.

If the oblate ellipsoid representing the micelle is considered to have a thickness equal to that of a bimolecular lecithin layer, then the theoretical molecular weight assuming the above axial ratio would be 2×10^6, a doubled bimolecular layer would give a micellar weight of 16×10^6.

The ultrasonic irradiation method permits the preparation of phospholipid sols containing relatively high concentrations of lipids such as cholesterol. It therefore offers a new approach to the study of dispersions of bio-colloids from which membranes, resembling cell membranes, can be precipitated.

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THE MORPHOLOGY AND ANATOMY OF THE LEAF OF *PODOPHYLLUM HEXANDRUM* ROYLE

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A brief review of the history and medicinal uses of *Podophyllum hexandrum* Royle (= *P. emodi* Wall.) is given, together with an illustrated account of the macroscopy and anatomical structure of the leaf. The diagnostic characters of the powdered leaves are recorded and illustrated.

Podophyllum hexandrum Royle, although esteemed by the natives of India for its "bile expelling properties", was not introduced into Western Medicine until the late nineteenth century. Dunstan and Henry (1898), Thompson (1890) and Umney (1892) had reported a resin content equal to, or greater than, that of the American plant, *P. peltatum* L., and Hooper (1913) recommended the cultivation of *P. hexandrum* in India in view of its inclusion in the *Indian and Colonial Addendum to the British Pharmacopoeia* (1900). In recent years, interest in podophyllum as a purgative and cholagogue has declined although it still occurs as an active ingredient in Compound Tablets of Colocynth and Jalap, B.P.C. and various proprietary "liver pills". During this same period, interest has grown because of its reported activity as a mitotic poison and carcinoclastic, and from Kaplan (1942), who pioneered the use of podophyllin in condyloma acuminata, to Kern and Franger (1950), who investigated its use in cutaneous carcinoma, numerous successful treatments of dermatological conditions have been reported and reviewed by Nelson (1953). Further clinical applications have been reviewed by Kelly and Hartwell (1954).

Hussain, Chandri, Muhammad and Wahhab (1954) reported on the resin content of various plant organs of *P. hexandrum*, and recorded a resin content of 7–9 per cent w/w for the leaves, and indicated that they may serve as a more economic source of the resin than the rhizome and root. We have made preliminary estimations of the podophyllin content of the leaves of *P. hexandrum* grown in England and Norway, by the method of the *British Pharmaceutical Codex* (1959) modified so that the final purification by filtration and solution in alcohol was replaced by solution of the precipitated resin in chloroform and treatment with dilute ammonia solution to separate the resin from the chlorophyll—the resin was obtained by evaporation of the ammoniacal solution.

The present investigation was undertaken to describe the anatomical structure of the leaf and to note the diagnostic characters necessary for the identification of whole or powdered leaf and to distinguish it from the leaf of *P. peltatum*, to be described later.

MATERIAL

All the material was supplied as *P. emodi* Wall. but the literature suggests that *P. emodi* and *P. hexandrum* Royle are synonymous and comparison

with material at the Natural History Museum, London, S.W.7, confirmed its identity as *P. hexandrum* Royle. The leaves were obtained from three sources: (*a*) various nurserymen supplied plants which were transplanted to a shady position in a light peaty soil in West Yorkshire; (*b*) the botanical garden of Bergen University and (*c*) plants grown by Dr. T. E. Wallis at Mill Hill, London.

Methods of Investigation

Chloral hydrate solution was used to prepare fragments for examination of epidermises, but no heating was necessary with material which had been previously stored in 60 per cent ethanol and the epidermis of the petiole was best mounted directly in 50 per cent v/v glycerol solution. Serial sections were prepared from material embedded in polyethylene glycols 4,000, 20 per cent and 1,500 GEN, 80 per cent w/w (Fell and Rowson, 1955) and were mounted in 50 per cent v/v glycerol solution. Fresh material was used in attempts to locate the resin, which is soluble in ethanol and in polyethylene glycols. Phloroglucinol and hydrochloric acid were used to detect lignified material and chloral iodine and 0·02N iodine solution to establish the presence of starch in the endodermis. The aceto-carmine staining technique (Brown, 1951) was used to establish the presence of chromosome material in the commonly occurring granular bodies and hence to confirm their identity as nuclei. The transient bright orange-red colour produced by 50 per cent v/v nitric acid with the resinous material was characteristic. The extracted resin and commercial podophyllin behaved similarly.

Macroscopy

The characters of the leaf of *P. hexandrum* vary according to the age of the plant. The cotyledons, which are epigeal (Fig. 1, *A–F*) and persist until the first-year leaf emerges through the cotyledonary stalk, are fused along their bases and are continuous with the cotyledonary stalk. The plumule emerges from a slit at one side of the base of the cotyledonary stalk and stands opposite to the cotyledons. There is no hypocotyl, and, since the cotyledons may, in these circumstances, be regarded as a combined "cotyledon", *P. hexandrum* provides a good example of pseudo-monocotyledonous germination, similar to certain other species within the Berberidaceae and Ranunculaceae (McLean and Ivimey-Cook, 1956). The cotyledons are ovate, with a single main vein running from the stalk to the emarginate apex and anastomosing secondary veins (Fig. 1, *C*). The first-year leaf has three broadly ovate, or obovate, irregular lobes with five main veins emerging from the petiole apex and anastomosing secondary veins. The margin is irregularly serrate (Fig. 1, *D*). The leaves fall each winter; the leaves in the succeeding two years differ from their predecessors only in their increased size. Growth proceeds sympodially, and the flowering shoot is produced in the fourth or fifth year, emerging enclosed in a sheath which persists for several weeks. On bursting it reveals a terminal flower-bud surmounting two coiled leaves (Fig. 1, *B*). The pedicel arises from the petiole of one leaf, or less frequently from the junction of the two petioles, but the leaves do not

uncoil until the petals have fallen. Only the flowering shoot has two leaves on a common stalk, all other leaves occur singly and are radical. The leaves of the flowering shoot are often more divided than the radical ones and the lobes are more lanceolate (Fig. 1, *A*). Both types of leaf are mottled red-brown and green, the amount of red decreasing as the leaf matures.

The mature leaves of *P. hexandrum* measure from 12 to 25 cm. across and are peltate with an eccentric, erect, cylindrical petiole. They are pentagonal in outline, the lamina being palmatisect with 3 to 5 lobes.

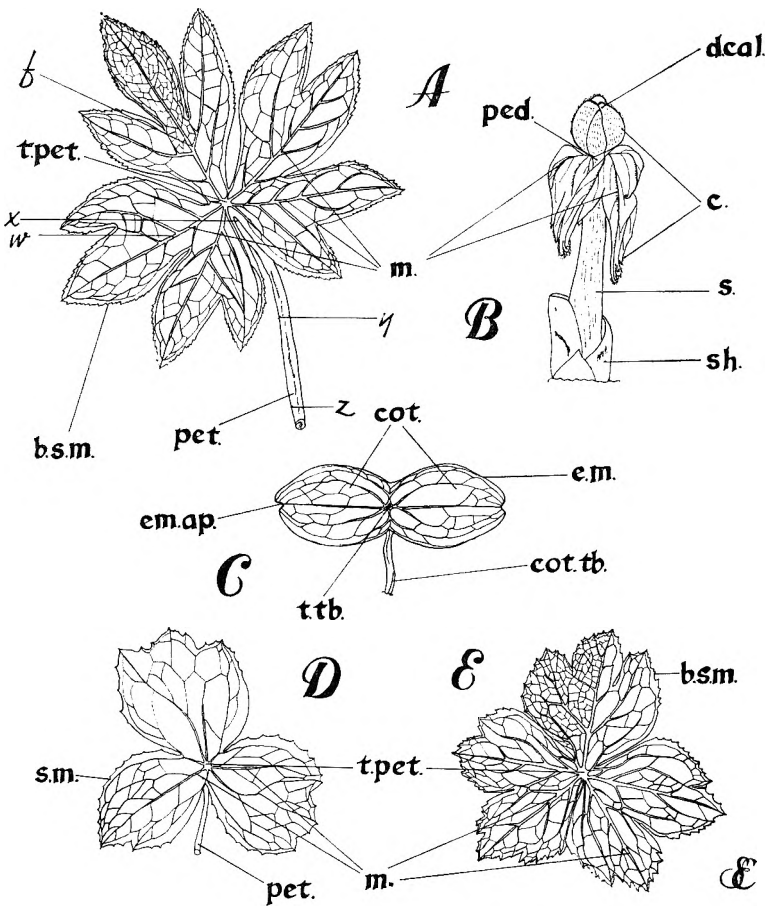

FIG. 1. Leaf of *Podophyllum hexandrum* Royle. *A*, complete mature leaf from flowering plant; *B*, flowering shoot; *C*, young seedling, showing fused cotyledons; *D*, first year, immature leaf; *E*, mature leaf from non-flowering plant. *A* and *E* × 1/5; *B* and *D*, × ½; *C* × ⅔. *b.s.m.*, biserrate margin; *c.*, covering trichome; *cot.*, cotyledon; *cot. tb.*, cotyledonary tube; *d. cal.*, deciduous calyx; *e.m.*, entire margin; *em ap.*, emarginate apex; *m.*, main vein; *ped.*, pedicel; *pet.*, petiole; *s.*, stem; *sh.*, sheath; *s.m.*, serrate margin; *t. pet.*, top of petiole; *t. tb.*, top of cotyledonary tube; w, position of sections 3, *A*, and 4, *A*; x, y and z, position of sections 4*B–D*; f, position of first main vascular junction.

Each lobe is subdivided, the lateral ones into two and the terminal ones into three ovate, or ovate-lanceolate, segments; the margin is entire in the lower half of the lobes and serrate, or irregularly biserrate, in the apical half. The venation is palmate with five main veins arising from the apex of the petiole and running to the acute tip of each main lobe. The secondary veins branch from them alternately at an acute angle and run towards the tip of the lobe segments. In each case, at the conjunction of the two lobes, or segments, a single vein, arising in the case of the lobes from the apex of the petiole and in the case of the segments from the main veins, divides and its branches run along the margins of the two lobes, or segments, formed (Fig. 1, *A* and *E*). The tertiary veins leave the secondary veins at a wide, or even obtuse, angle and terminate near the margin. In each marginal tooth three ultimate veinlets unite to form the terminal network (Fig. 2, *A*). The veins are prominent on the under-surface, are lighter in colour than the interneural tissue and are covered with long, silky, covering trichomes. In some leaves similar trichomes are visible along the margin (Fig. 2, *B*, *F* and *G*). The interneural tissue is glabrous except near the larger veins.

Anatomical Structure

Lamina

Interneural Tissue (Fig. 2, *B*, *C*, *E*, *H* and *I*; Fig. 3, *B*)

The Upper Epidermis is covered with a relatively thin cuticle which is smooth over most of the surface of the leaf but slightly granular at the margin. The epidermal cells are polygonal and measure about Lev L and B 37 to **47** to 71 μ and H 26 to **33** to 45 μ with wavy anticlinal walls; in occasional cells, red-brown pigment occurs (Fig. 2, *E*; Fig. 3, *B*). *Trichomes* are absent from this surface and *stomata* are very rare. Upon each marginal tooth 4 to 10 *hydathodes* are often present; they measure about Lev L 48 to **57** to 78 μ and Lev B 32 to **39** to 45 μ (Fig. 2, *A* and *C*).

Mesophyll. The *palisade* consists of one layer of regular cylindrical cells measuring about Lev L and B 15 to **26** to 60 μ and H 44 to **53** to 75μ, and a second layer of shorter cylindrical cells measuring about Lev L and B 22 to **38** to 67 μ and H 25 to **45** to 63μ, which is discontinuous at irregular intervals. The *spongy mesophyll* consists of 5 or 6 layers of trabeculate parenchyma cells about 18 to **41** to 74 μ in diameter, with occasional transversely elongated cells, measuring about 14 to **41** to 74 μ in diameter and 59 to **91** to 126 μ in length with large intercellular spaces (Fig. 2, *H*; Fig. 3, *B*). *Calcium oxalate* crystals occur rarely; they measured from 14 to 22 μ in diameter and were located in the spongy mesophyll, always very close to a vein.

The Lower Epidermis is covered by a thin cuticle, striated in small patches near some stomata (Fig. 2, *I*). The epidermal cells are polygonal, measuring about Lev L and B 30 to **55** to 94 μ and H 15 to **25** to 38 μ, with very wavy anticlinal walls. Anomocytic *stomata* are numerous, raised slightly above the level of the epidermis, elliptical, or circular, in outline, and measure about 35 to **45** to 52 μ in length and 29 to **37** to 45 μ in breadth, or 35 to **39** to 45 μ in diameter (Fig. 2, *I*; Fig. 3, *B*).

Covering trichomes occur on this surface over, and near, the veins and in the marginal region. They are thin-walled, cellulosic and unicellular in both regions with a blunt and rounded apex. Near the veins, the trichomes are of one type only with a smooth cuticle and measuring about

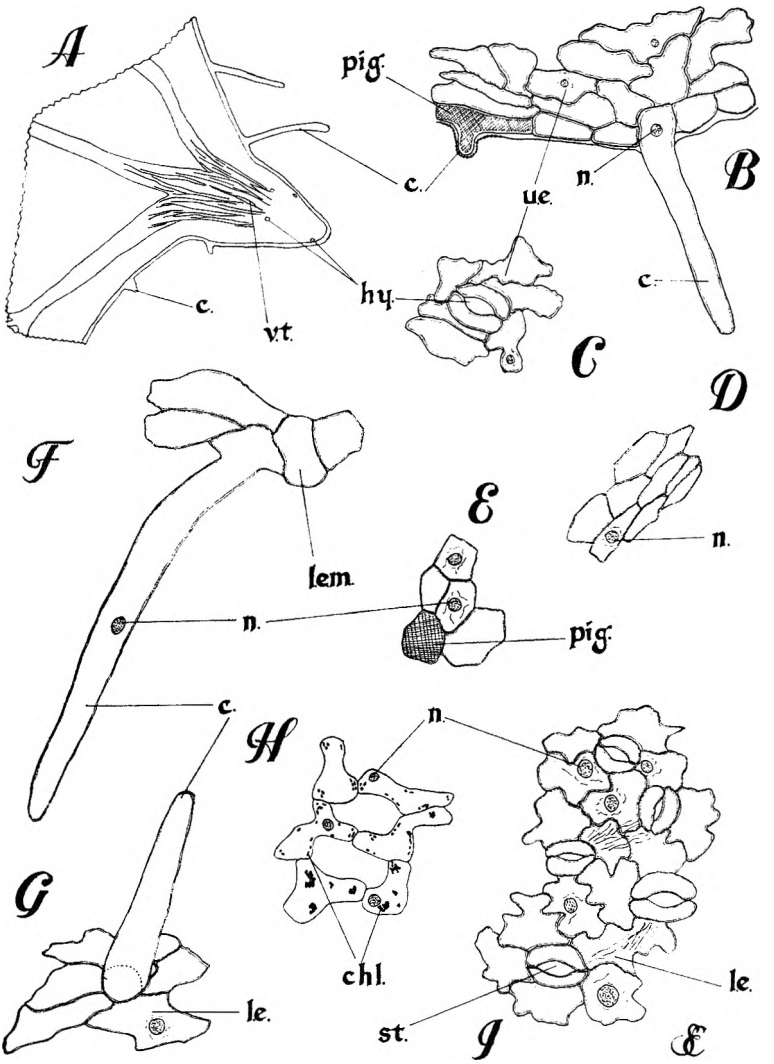

FIG. 2. Leaf of *P. hexandrum* Royle. *A*, marginal tooth of leaf; *B*, upper epidermis of leaf, marginal region; *C*. upper epidermis of leaf with hydathode; *D*, upper epidermis of main vein; *E*. upper epidermis of lamina; *F*, lower epidermis of main vein; *G*, lower epidermis of lamina, neural region; *H*, spongy mesophyll; *I*, lower epidermis of lamina, interneural region; *A* × 30; *B–I*, × 150. *c.*, covering trichrome; *chl.*, chloroplast; *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.

150 to **425** to 825 μ in length and about 22 to **40** to 60 μ in diameter at the base. In the marginal region the number of trichomes varies, being very rare in some leaves and forming a dense covering in other specimens examined. The majority resemble those of the neural region in appearance (Fig. 2, *B*), but are slightly smaller, being about 150 to **272** to 520 μ in length and 26 to **35** to 53 μ in diameter at the base. In addition, a small number of shorter trichomes with a slightly warty wall occur near the teeth (Fig. 2, *A* and *B*). These are of the same width but are only about 20 to **36** to 55 μ in length.

The lamina has a serrate margin, individual teeth being acutely pointed. The tertiary veins extend to within about 0·3 mm. of the teeth apices and terminate in several small spiral elements. Three ultimate veinlets unite to form this terminal network about 0·6 mm. from the apex (Fig. 2, *A*).

MAIN VEIN (Fig. 2, *D* and *F*; Fig. 3, *A* and *C*; Fig. 4, *A*)

The leaf, which is deeply lobed, has palmate venation and hence the following description applies to the central vein of each lobe, which runs from the petiole to the apex (Fig. 1, *A*). The veins from each lobe, from several leaves, were examined and showed no significant variation from the following description. The transverse section shows three unequal bundles embedded in a central, cordate shaped mass of collenchyma (Fig. 4, *A*). The extreme tip of each leaf segment shows one bundle only, where a side vein enters there are two bundles, and there are three, 2 cm. from the tip. The amount of central collenchyma increases proportionately. At the first main junction (Fig. 1, point *f*), the main vein contains three bundles and side vein two. These unite to produce five bundles which are gradually rearranged to form three once more. The entrance of further secondary veins disturbs the pattern temporarily but it always returns to three. No unification of the bundles of the primary veins occur before they enter the petiole.

The UPPER EPIDERMIS is composed of polygonal, straight-walled cells, elongated along the axis of the lobe (Fig. 2, *D*). They measure about Lev L 36 to **74** to 104 μ, Lev B 12 to **33** to 56 μ and H 20 to **36** to 60 μ. The cuticle is thicker than on the interneural tissue; *trichomes* and *stomata* are absent.

The CORTEX is divided into two main regions. The upper hypodermal region is composed of thick-walled collenchyma, the cells measuring about L 100 to **187** to 260 μ and R and T 20 to **29** to 45 μ, but the lower hypodermal region becomes collenchymatous only in the basal half of the lobe, where it is large-celled, measuring about L 75 to **135** to 225 μ and R and T 26 to **63** to 108 μ. The remaining cortex is parenchymatous, the cells measuring about L 92 to **142** to 203 μ, R and T 8 to **15** to 24 μ above the stele and about L 80 to **154** to 240 μ, R and T 12 to **40** to 75 μ below the stele. The outer cortical cells contain a few elliptical chloroplasts, similar to those of the interneural tissue, and rare rosette crystals of *calcium oxalate*; only four of these were measured, the diameters were between 30 and 45 μ (Fig. 3, *A*).

The *endodermis* is not well differentiated, but a continuous band of

cells, which contain starch grains measuring about 4 to 6 to 12 μ in diameter, surrounds the collenchyma of the pericycle. The cells do not differ in size, shape or structure of the cell wall, from the surrounding cortical parenchyma, but in view of their contents may be regarded as a starch sheath (Jackson, 1953).

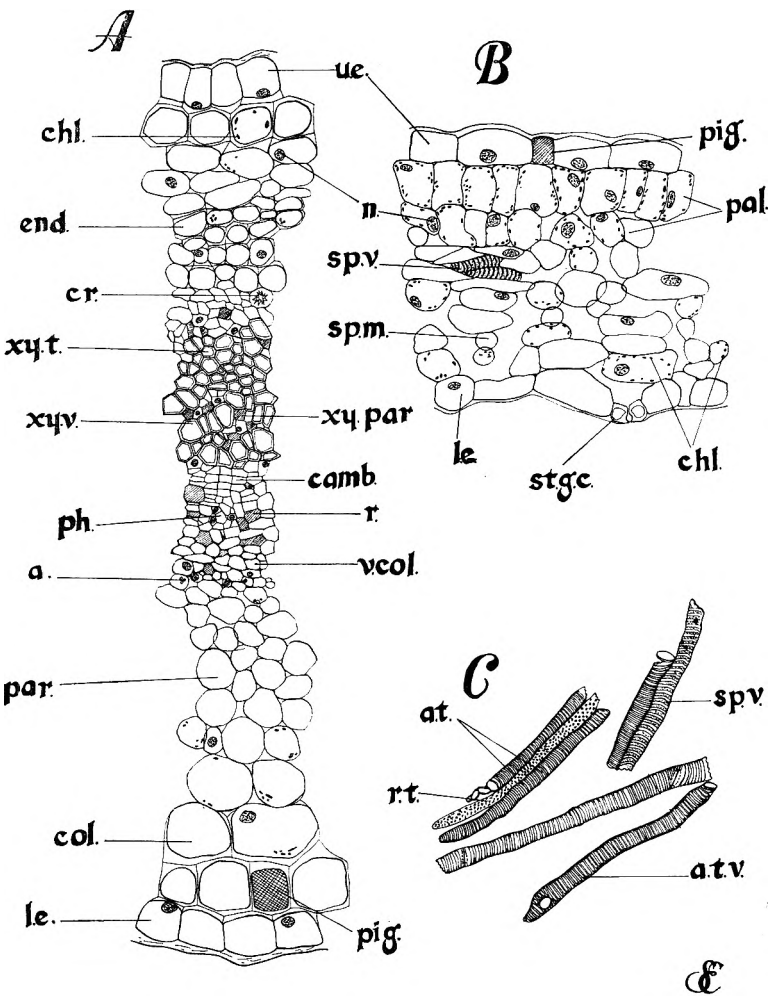

FIG. 3. Leaf of *P. hexandrum* Royle. *A*, tranverse section of the main vein cut at position *w* (see Fig. 1, *A*); *B*, transverse section of the lamina, interneural region; *C*, isolated elements obtained by maceration. *A* and *B*, × 150; *C* × 75. *a.*, starch; *a.t.*, annular tracheid; *a.t.v.*,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

The MERISTELE consists of three well-defined bundles embedded in a mass of relatively thin-walled collenchyma, measuring about L 135 to **186** to 265 μ and R and T 18 to **33** to 52 μ; occasional cells contain brown contents, which do not stain with 0·02N iodine solution or 5 per cent w/v ferric chloride solution, but give a reddish-brown colour with 50 per cent v/v nitric acid (Fig. 3, *A*).

The *phloem* consists of sieve tissue with well-defined companion cells and small-celled phloem parenchyma. The sieve-tubes measure about R and T 11 to **25** to 45 μ, the individual segments being about L 90 to **150** to 200 μ with transverse or oblique sieve plates. The companion cells are narrow, being about R and T 2 to **7·5** to 18 μ and L 96 to **148** to 207 μ and usually the nuclei completely fill the width of the cells. There are two distinct medullary rays between the three bundles, composed of cellulosic parenchyma; there is no radial arrangement of cells within the bundles. Patches of phloem parenchyma, the cells measuring about L 77 to **153** to 222 μ and R and T 5 to **15** to 30 μ with brown cell contents occur which stains red with 50 per cent v/v nitric acid and orange with 0·02N iodine solution.

The *cambium* consists of a well-defined layer of thin-walled, tangentially elongated cells.

The *xylem* consists of irregularly arranged vessels and tracheids with patches of cellulosic xylem parenchyma (Fig. 3, *A*). The vessels are lignified with spiral or annular thickening and a diameter of about 5 to **12** to 22 μ. The tracheids and tracheidal vessels are similar, having spiral, annular or pitted thickening and a diameter of about 5 to **12** to 20 μ. The tracheids measure about L 120 to **280** to 430 μ and the tracheidal vessels about L 240 to **390** to 585 μ (Fig. 3, *C*); the number of tracheids increases towards the tip. The xylem parenchyma is thin-walled, cellulosic and some cells have similar brown contents to the phloem parenchyma.

The LOWER EPIDERMIS (Fig. 2, *F*; Fig. 3, *A*) is composed of large polygonal cells about Lev L 60 to **82** to 120 μ, Lev B 16 to **37** to 80 μ and H 28 to **41** to 60 μ, with slightly wavy anticlinal walls. *Stomata* are absent but numerous unicellular *covering trichomes* occur. They measure about 150 to **425** to 825 μ in length and 22 to **40** to 60 μ in diameter at the base. The walls are thin and cellulosic, and the trichomes have a blunt and rounded apex (Fig. 2, *F* and *G*).

PETIOLE (Fig. 4, *B*, *C* and *D*; Figs. 5 and 6)

The petiole is smoothly cylindrical, from 10 to 35 cm. long and from 3 to 5 mm. in diameter and sometimes showing an eccentric hollow in the basal portion of the petiole of older leaves. The vascular tissue occurs in two regions: (1) An outer ring of 15 to 25 bundles situated near the periphery and containing pericyclic fibres, phloem, cambium and xylem. Each is encircled by a mass of collenchyma bounded on its outer edge by a starch sheath. At the upper end of the petiole pericyclic fibres are few, or occasionally absent, but the amount of lignified material gradually increases towards the base and near the junction of the petiole with the stem, or rhizome, the interfascicular parenchyma in the pericyclic region

becomes lignified forming a complete ring of lignified tissue. (2) A V-shaped arrangement of larger bundles asymmetrically placed in the pith parenchyma. These bundles contain smaller numbers of pericyclic fibres, phloem, cambium and xylem all enclosed by an area of collenchyma and a starch sheath. The amount of fibrous tissue increases towards the base, but the interfascicular parenchyma does not become lignified. In the hollow petioles this V-shaped structure persists as a projection into the hollow. In some longer petioles this second arrangement of bundles tends to become circular giving two rings of bundles, the inner bundle ring

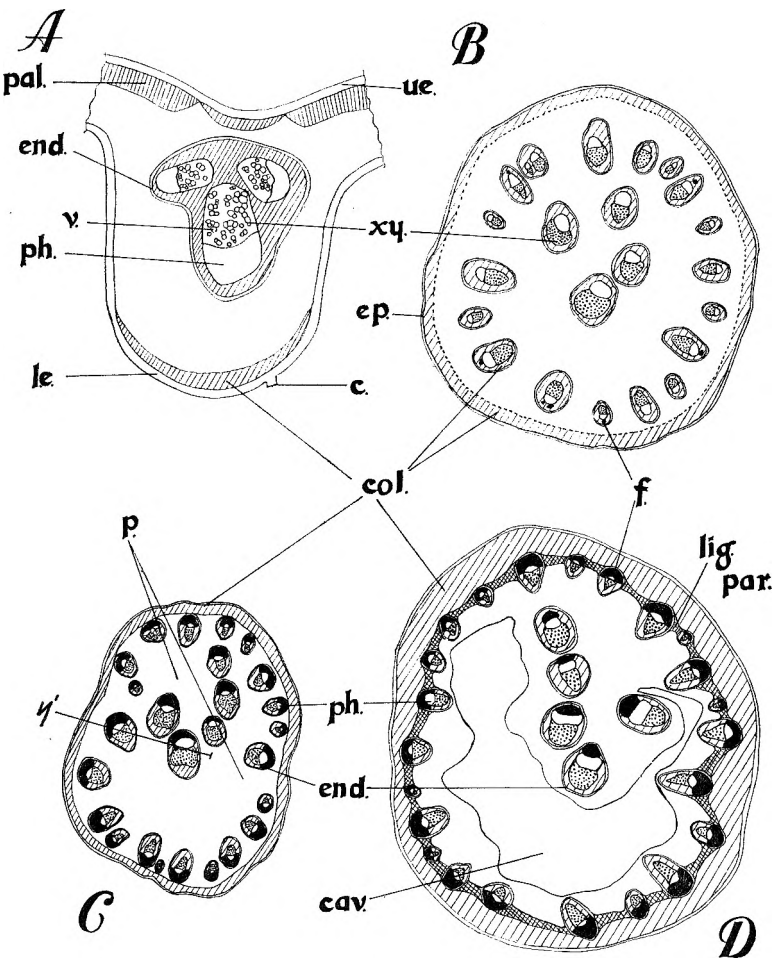

FIG. 4. Leaf and petiole of *P. hexandrum* Royle. *A*, transverse section of main vein cut at position w (see Fig. 1, *A*); *B–D*, transverse sections of petiole cut at positions *x*, *y* and *z* respectively (see Fig. 1, *A*). *A*, × 30; *B–D*, × 10. *c.*, base of covering trichome; *cav.*, cavity; *col.*, collenchyma; *end.*, endodermis; *ep.*, epidermis; *f.*, fibres; *l.e.*, lower epidermis; *lig.par.*, lignified parenchyma; *p.*, pith; *pal.*, palisade; *ph.*, phloem; *u.e.*, upper epidermis; *v.*, vessel; *xy.*, xylem; y', position and extent of section 6.

being eccentric (Fig. 4, *B*, *C* and *D*). This arrangement of the vascular system shows some affinities with some species of the Ranunculaceae (cf. *Glaucidium*, Metcalfe and Chalk, 1957); Kumazawa (1930) has suggested

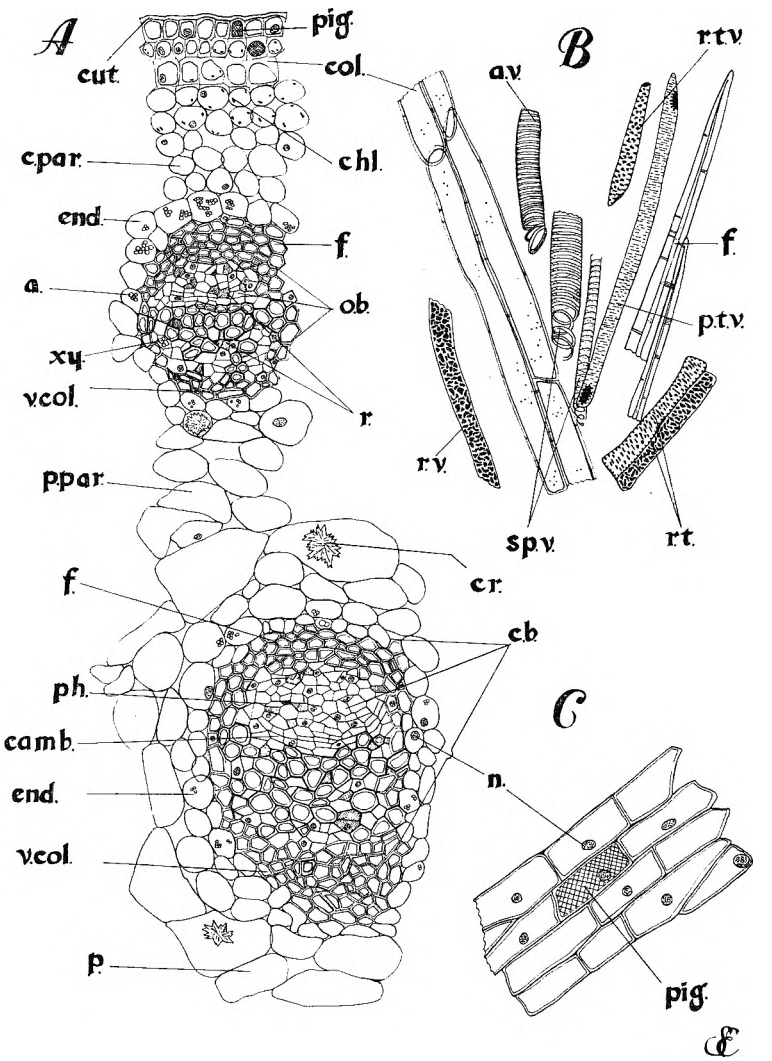

FIG. 5. Petiole of *P. hexandrum* Royle. *A*, transverse section of petiole cut at position *y* (see Fig. 1, *A*) showing an outer bundle and a central bundle; *B*, isolated elements obtained by maceration; *C*, epidermis of petiole. *A* and *C*, × 130; *B*, × 70. *a.*, starch; *a.v.*, annular vessels; *camb.*, cambium; *c.b.*, central bundle; *chl.*, chloroplasts; *col.*, collenchyma; *c.par.*, cortical parenchyma; *cr.*, crystal of calcium oxalate; *cut.*, cuticle; *end.*, endodermis; *f.*, fibre; *n.*, nucleus; *o.b.* outer bundle; *p.*, pith; *ph.*, phloem; *pig.*, pigment; *p.par.*, pith parenchyma; *p.t.v.*, pitted tracheidal vessel; *r.*, resinous material; *r.t.*, reticulate tracheid; *r.t.v.*, reticulate tracheidal vessel; *r.v.*, reticulate vessel; *sp.v.*, spiral vessel; *v.col.*, vascular collenchyma; *xy.*, xylem.

on anatomical grounds that certain genera of Berberidaceae and Ranunculaceae should be removed from their respective families and merged into a single family, the Podophyllaceae.

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 80 to **172** to 280 μ, Lev B 13 to **19** to 34 μ. The cell walls are thin and cellulosic and the outer surface is covered by a thin cuticle (Fig. 5, *C*).

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 very thick walls. The cells measure about L 100 to **204** to 480 μ and R and T 11 to **23** to 37 μ and contain lenticular chloroplasts. The remaining cortex is parenchymatous, frequently becoming lignified and pitted in the pericyclic region of the lower part of the petiole. The cells are similar in size to those of the collenchyma (Fig. 5, *B*).

The *endodermis* is even less distinct than in the main veins as the starch is not confined to a single layer but forms a sheath 2 or 3 cells wide around the collenchyma. The starch grains average about 6 μ in diameter.

PERICYCLIC FIBRES increase in number from apex to base of the petiole, and eventually, as seen in a transverse section, form a crescent-shaped group external to the phloem, extending on either side of the phloem tissue to the cambium. The fibres are extremely long, measuring about L 690 to **1,030** to 1,400 μ and R and T 3 to **11** to 19 μ, with thick, lignified, pitted walls and acute apices (Fig. 5, *B*; Fig. 6).

The *vascular bundles* of the outer and inner rings differ only slightly, the main difference being that the inner bundles are larger but have fewer pericyclic fibres. Both types of bundle are embedded in collenchyma, the cells of which measure about L 156 to **433** to 759 μ and R and T 7 to **19** to 34 μ. The *phloem* consists of groups of sieve tubes about 11 to **18** to 22 μ in diameter, each individual segment being about 160 to **285** to 340 μ long, associated with narrow companion cells measuring about 3 to **7** to 15 μ in diameter and 248 to **309** to 407 μ in length. There is no radial arrangement of cells within the bundles, but irregular groups of phloem parenchyma occur, the individual cells measuring about L 110 to **235** to 335 μ and R and T 11 to **22** to 37 μ, and frequently containing brown amorphous 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 some reticulate thickening (Fig. 5, *A* and *B*; Fig. 6). A little xylem parenchyma occurs close to the distinct cambium and associated with the larger vessels; some cells of the parenchyma contain brown cell-contents similar to those already described. The vessels are somewhat larger than those of the main veins, being about 7 to **26** to 60 μ in diameter. The tracheids and tracheidal vessels have diameters within this range and measure about 207 to **418** to 700 μ in length.

The PITH is composed of large-celled parenchyma, which frequently

breaks down to form an eccentric hollow at the base of the petiole. The cells are thin-walled and measure about L 110 to **275** to 420 μ and R and T 35 to **82** to 152 μ. Occasional simple starch grains measuring about 5 to **15** to 26 μ in diameter occur in the cells. In cells adjacent to the vascular bundles rosette crystals of *calcium oxalate*, measuring about 14 to **26** to 35 μ in diameter, occur (Fig. 5, *A*; Fig. 6).

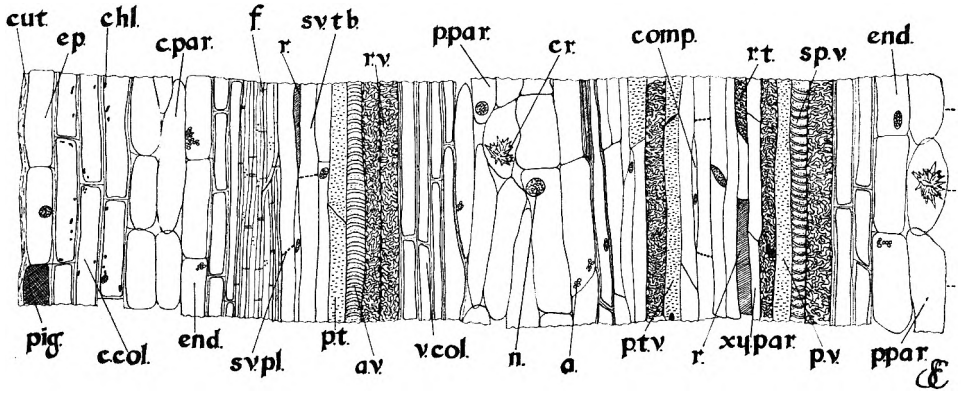

FIG. 6. Petiole of *P. hexandrum* Royle. Longitudinal section of the petiole cut at position *y'* (see Fig. 4, *C*) × 150. *a.*, starch; *a.v.*, annular vessel; *chl.*, chloroplast; *c.col.*, cortical collenchyma; *comp.*, companion cell; *c.par.*, cortical parenchyma; *cr.*, crystal of calcium oxalate; *cut.*, cuticle; *end.*, endodermis; *ep.*, epidermis; *f.*, fibre; *n.*, nucleus; *pig.*, pigment; *p.par.*, pith parenchyma; *p.t.*, pitted tracheid; *p.t.v.*, pitted tracheidal vessel; *p.v.*, pitted vessel; *r.*, resinous material; *r.t.*, reticulate tracheidal vessel; *r.v.*, reticulate vessel; *sp.v.*, spiral vessel; *sv.pl.* sieve plate; *sv. tb.*, sieve tube; *v. col.*, vascular collenchyma; *xy. par.*, xylem parenchyma.

POWDER

The colour of a No. 40 powder varied from yellowish-green to dark-green, but all had a heavy odour and bitter taste. When a small amount of the powder is mixed with a 5 per cent w/v solution of ferric chloride a gradual darkening of the particles of tissue is observed and when mixed with a 5 per cent w/v solution of copper acetate an emerald-green colour gradually develops on the particles.

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 diagnostic characters of the Powder (Fig. 7) are:

1. Numerous whole and fragmented unicellular *covering trichomes*, from the lower epidermis of the lamina, with thin cellulosic walls and blunt and rounded apices.

2. Fragments showing, in surface view, the sinuous walled cells of the *upper interneural epidermis* and usually the underlying palisade and, in the marginal region, infrequent covering trichomes.

3. Fragments of the *lower interneural epidermis* showing, in surface view, the wavy-walled epidermal cells and anomocytic *stomata*.

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.

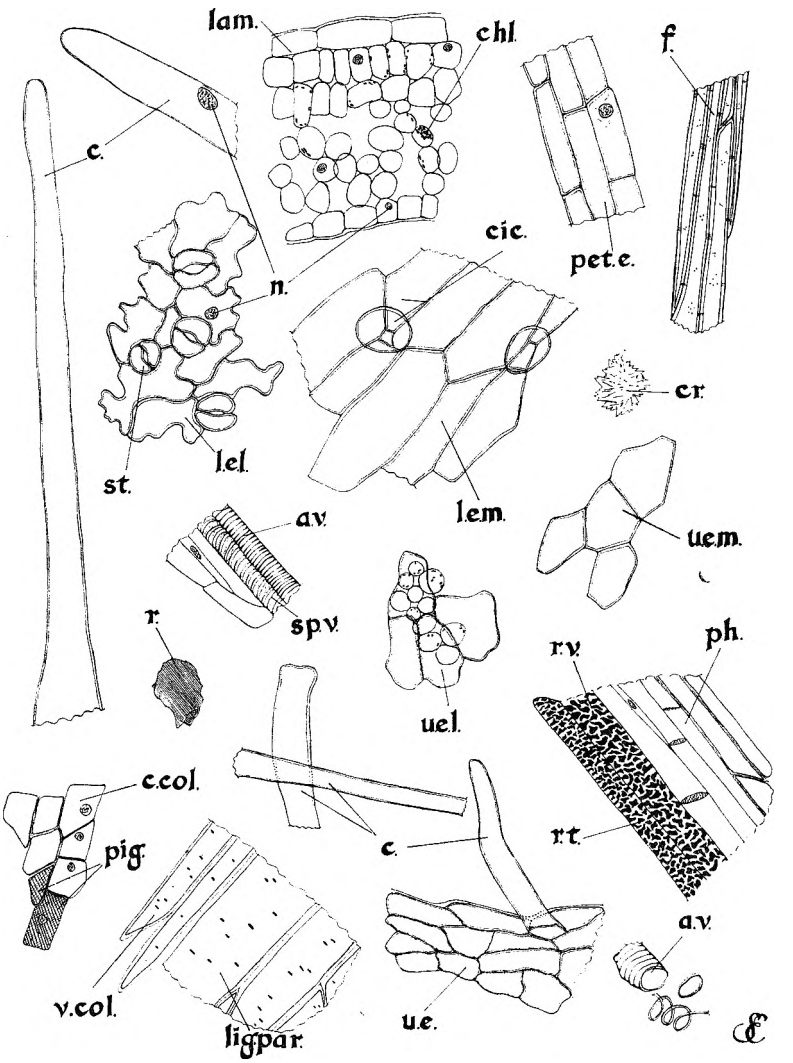

FIG. 7. Powdered leaf and petiole of *P. hexandrum* Royle. All × 150. *a.v.*, annular vessel; *c.*, covering trichome; *c.col.*, cortical collenchyma; *chl.*, chloroplast; *cic.*, cicatrix; *cr.*, crystal of calcium oxalate; *f.*, fibre; *lam.*, lamina; *l.e.l.*, lower epidermis of lamina; *l.e.m.*, lower epidermis of main vein; *lig. par.*, lignified parenchyma; *n.*, nucleus; *pet.e.*, petiole epidermis; *ph.*, phloem; *pig.*, pigment; *r.*, resinous material; *r.t.*, reticulate tracheid; *r.v.*, reticulate vessel; *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; *v.col.*, vascular collenchyma.

5. Fragments of lamina in transverse sectional view, about 200 μ wide with a double, or single, palisade.

6. Small spiral and annular vessels from the veins; reticulate vessels associated with large sieve-tubes, and large annular and spiral vessels from the petiole.

7. Lignified, acutely pointed *fibres* with pitted walls; collenchyma with simple pits associated with *lignified parenchyma* from the inter-fascicular region of the petiole.

8. Cortical collenchyma from the main veins or petiole, with thick cellulosic walls and frequently containing nuclei and red pigment.

9. Infrequent cluster crystals of *calcium oxalate* from main veins or petiole, and occasionally from the spongy mesophyll.

10. Occasional brown *resinous fragments*, non-cellular in nature, which gradually dissolve to give a green solution in 5 per cent w/v copper acetate solution and an orange-red colour with 50 per cent v/v nitric acid.

Proportion of petiole. Investigations on samples available showed that the percentage of petiole present was about 14 to 22 per cent by weight of the dry leaf.

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THE SYNTHESIS OF *ORTHO*-SUBSTITUTED 2-DIETHYLAMINOETHYL BENZOATES AS POTENTIAL LOCAL ANAESTHETICS

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A series of mono-*ortho*- and di-*ortho*- substituted benzoic esters of 2-diethylaminoethanol has been synthesised. Improved methods for the preparation of 2,6-difluorobenzoic acid and 2,6-dimethylbenzoic acid are reported. The preparation of esters of di-*ortho*- substituted benzoic acids is discussed and an efficient method of esterification of these acids is described.

THOMAS and Stoker (1961) demonstrated that *ortho* substitution in benzoylcholine could lead to increased stability of the ester group towards both hydroxide-ion- and cholinesterase-catalysed hydrolysis and that the *ortho* substituents did not prevent the formation of an enzyme–substrate complex. Two enzymes, acetylcholinesterase and cholinesterase, were used. Thomas and Buckley (1962) examined the effects of *ortho* substitution in benzoylcholine on some of the pharmacological actions of the ester. Neuromuscular block of the rat diaphragm–phrenic nerve preparation and stimulation of the frog rectus abdominis preparation were examined. It was found that *ortho* substitution did not necessarily reduce the activity from that of benzoylcholine and in some instances an increase in potency was noted. The conclusion from these studies is that *ortho* substitution of benzoic esters is, in principle at least, a method of decreasing the rate of both *in vivo* and *in vitro* hydrolysis of the ester group without removing the biological actions of such esters.

To examine a further application of *ortho* substitution of benzoic esters in medicinal chemistry a series of mono-*ortho*- and di-*ortho*-substituted 2-diethylaminoethyl benzoates has now been synthesised for testing for their ability to block axonal nervous transmission.

A number of *ortho*-substituted benzoic esters with local anaesthetic activity have been reported. Dvoretzky and Richter (1953) prepared ethyl and propyl 4-amino-2,6-dimethylbenzoate and reported them to have a longer duration of local anaesthetic action than benzocaine. They postulated that this was due to the decreased rate of hydrolysis. Childress and others (1954) reported that 2-diethylaminoethyl 4-amino-2,6-dichlorobenzoate had an intradermal local anaesthetic activity 3·5 times as great as procaine, and that the duration of action was considerably longer. Rabjohn and others (1955) prepared a number of 2-diethylaminoethyl esters of sterically hindered alkyl-substituted benzoic acids and found them to show considerably longer duration of anaesthetic action than equipotent concentrations of procaine. Mono-*ortho*-substituted benzoic esters with local anaesthetic activity have also been examined. Foldes and Rhodes (1953) have reported that chloroprocaine is an excellent local anaesthetic. A wider range than previously reported of both mono-*ortho*

and di-*ortho*-substituted benzoic esters with local anaesthetic activity has now been prepared.

EXPERIMENTAL

Mono-ortho-*Substituted esters*

All the mono-*ortho*-substituted benzoic acids and some of the acid chlorides were obtained commercially. The method used to prepare 2-diethylaminoethyl *o*-methoxybenzoate is typical of the general method used to prepare the mono-substituted esters.

o-*Methoxybenzoyl chloride.* *o*-Methoxybenzoic acid (15·2 g. 0·1 mole) was refluxed with thionyl chloride (11·9 g. 0·1 mole) in a 50-ml. flask on a steam-bath for 2 hr. after which no further fumes of hydrogen chloride were evolved. The reaction mixture was distilled under reduced pressure and *o*-methoxybenzoyl chloride was collected as a colourless liquid, b.p. 131° at 12 mm. Yield 15 g.

2-*Diethylaminoethyl* o-*methoxybenzoate hydrochloride.* *o*-Methoxybenzoyl chloride (5·5 g. 0·05 mole) was dissolved in dry benzene (25 ml.) in a 100-ml. two-necked flask fitted with a reflux condenser and a dropping funnel. The mixture was refluxed and 2-diethylaminoethanol (5·8 g. 0·05 mole) was added dropwise over 30 min. The mixture was refluxed for a further 1 hr. and the contents allowed to cool. The precipitate was filtered, washed with dry benzene, dried, and recrystallised from an acetone-ethanol mixture to give a white crystalline solid, m.p. 136°. Yield 55 per cent. $C_{14}H_{22}ClNO_3$ requires: C, 58·4; H, 7·7; Cl, 12·35. Found: C, 58·28; H, 7·9; Cl^-, 12·36.

A list of mono-*ortho*-substituted compounds prepared by this method and details of the preparation of each are given in Table I.

TABLE I

MONO-ORTHO-SUBSTITUTED 2-DIETHYLAMINOETHYL BENZOATE HYDROCHLORIDES

Compound	Acyl halides		2-diethylaminoethyl benzoates hydrochloride									
							Analysis					
							Found			Required		
2-diethyl-aminoethyl X-benzoates X =	Reflux time hr.	b.p.	Reflux time hr.	Solv. for recryst.	m.p. °C	Yield per cent	C	H	Cl	C	H	Cl
o-Methyl	1	87–8° at 15 mm.	1½	Acetone	133–5	66	61·5	7·9	13·02	61·8	8·1	13·08
o-Ethoxy	2	130° at 27 mm.	2	Acetone-ethanol	138	63	59·3	8·1	11·97	59·7	8·0	11·78
o-Iodo	2	(*a*)	1½	Acetone-ethanol	155	32	40·5	4·8	9·28	40·7	5·0	9·26
o-Nitro	1	(*a*)	1½	Acetone-ethanol	165	68	51·8	6·0	11·68	51·6	6·3	11·74
o-Bromo	(*b*)		1½	Acetone-ethanol	130	67	46·2	6·1	10·71	46·4	5·7	10·56
o-Chloro	(*b*)		1½	Acetone-ethanol	127–8	73	53·3	6·4	12·16	53·4	6·5	12·16
	(*b*)		1½	Acetone-ethanol	123–4	63	60·3	7·7	13·92	60·5	7·8	13·80

(*a*) Not isolated. (*b*) Acyl chloride obtained commercially.

Di-ortho-*Substituted Esters*

6-*Nitro*-o-*toluidine.* 2,6-Dinitrotoluene (50 g.) was dissolved in ethanol (100 ml. 95 per cent) in a 500-ml. three necked flask fitted with a reflux

condenser, mechanical stirrer and dropping funnel. The solution was stirred and refluxed while an ammonium sulphide solution (280 ml. 16 per cent) was added dropwise over 30 min., refluxed for a further 15 min. and then cooled. The buff coloured precipitate which appeared was filtered off, washed with water and transferred to a beaker containing hydrochloric acid (36 ml.) and water (300 ml.). The mixture was boiled, filtered hot and the residue extracted a second time with acid. The acidic filtrates were mixed and the 6-nitro-*o*-toluidine hydrochloride crystallised out overnight in a refrigerator. The free base was obtained by treating an aqueous solution of the salt with sodium bicarbonate, collecting the precipitate, washing it with water and drying the yellow product at 60°, m.p. 89–91° (Brady and Taylor, 1920; m.p. 91·5°). Yield 39·2 g. 94 per cent.

2-*Methyl*-3-*nitrobenzenediazonium fluoroborate.* 6-Nitro-*o*-toluidine (38 g.) was suspended in hydrochloric acid (60 ml.) in a 1 litre polythene beaker. An aqueous solution of sodium fluoroborate (65 g. in 50 ml.) was added and the mixture cooled to 0°. A cold (0°) aqueous solution of potassium nitrite (25 g. in 25 ml.) was added dropwise over 1 hr. while the mixture was stirred with a copper rod. The mixture was maintained at 0° for a further hr. to allow the diazotisation to go to completion and then cooled to −5° for another hr. to complete the precipitation of 2-methyl-3-nitrobenzenediazonium fluoroborate. The fawn-coloured product was filtered off on a sintered-glass filter and then washed in turn with (*a*) sodium fluoroborate solution (40 ml. 5 per cent), (*b*) ethanol (25 ml. 95 per cent) and (*c*) ether (2 × 50 ml.), the solid being sucked as dry as possible after each washing. The white product was dried at 40°. Yield 88 g.

2-*Fluoro*-6-*nitrotoluene.* 2-Methyl-3-nitrobenzenediazonium fluoroborate (78 g.) was mixed with an equal weight of pure dry sand and placed in a 500 ml. borosilicate round-bottomed flask. The flask was connected, by means of a glass tube ($\frac{1}{2}$ inch internal diameter) and a double-surface condenser, to two 250 ml. Erlenmeyer flasks which were cooled by a freezing mixture. The apparatus was evacuated and the diazonium compound heated gently to start decomposition. Heat was applied to maintain a steady rate of decomposition and finally the temperature of the mixture was raised to 300° to ensure complete reaction. The product, which was a brown oil, collected in the first Erlenmeyer flask and the fumes of boron trifluoride which were evolved were trapped in sodium hydroxide solution. Heating was stopped when fumes ceased to be produced. The distillate was washed in turn with sodium hydroxide solution (2 × 25 ml. 10 per cent) and water (2 × 25 ml.), then dried over calcium chloride and redistilled under reduced pressure to give a pale yellow oil, b.p. 209–210°, m.p. 6° (Lock, 1936; m.p. 6·7–7°). The presence of fluorine was established by a Lassaigne test. *Note:* When an attempt was made to decompose larger batches of 2-methyl-3-nitrobenzenediazonium fluoroborate (200 g.) an explosion occurred.

6-*Amino*-2-*fluorotoluene.* 2-Fluoro-6-nitrotoluene (31 g.) was treated portionwise with a solution of stannous chloride (152 g.) in hydrochloric

acid (160 ml.) in a 1-litre three-necked flask fitted with a mechanical stirrer, dropping funnel and reflux condenser. The fluoronitrotoluene, together with 50 ml. of the stannous chloride solution, was heated on a steam-bath and the mixture stirred continuously. The remainder of the stannous chloride solution was introduced in 20 ml. portions and the flask heated for a further 1 hr. after all the stannous chloride had been added. The reaction mixture was cooled, made alkaline with sodium hydroxide and then the free base steam-distilled off. The distillate was saturated with sodium chloride and extracted with ether. The ether was distilled off, the residue dried over sodium hydroxide and redistilled to give a pale brown oil, b.p. 204–5°. Yield 22·7 g., 91 per cent.

6-*Amino*-2-*fluorotoluene picrate* was prepared, m.p. 194–5°. $C_{13}H_{11}FN_4O_7$ requires: C, 44·1; H, 3·12. Found: C, 44·0; H, 3·2.

3-*Fluoro*-2-*methylbenzenediazonium fluoroborate.* This was prepared from 6-*amino*-2-*fluorotoluene* (31·2 g.) by the same method as was used for 2-methyl-3-nitrobenzenediazonium fluoroborate. Yield 67 g.

2,6-*Difluorotoluene.* This was prepared from 3-fluoro-2-methylbenzenediazonium fluoroborate (67 g.) by a similar method to that used for 2-fluoro-6-nitrotoluene, the only differences being that no sand was required as a diluent and decomposition occurred at 120–130°. The product was a colourless liquid, b.p. 114–115° (Lock, 1936; b.p. 112° corrected). Overall yield from 6-amino-2-fluorotoluene 34 per cent, $n_D^{20°}$, 1·4338.

2,6-*Difluorobenzoic acid.* This acid was prepared from 2,6-difluorotoluene (8·0 g.) by the method described for the preparation of 2,6-dichlorobenzoic acid from 2,6-dichlorotoluene by Norris and Bearse (1940). The product was crystallised from water, m.p. 155–6° (Lock, 1936; m.p. 157·5° corrected). Yield 6·1 g. 62 per cent. Found: C, 52·9; H, 2·7. Calc. for $C_7H_4F_2O_2$: C, 53·2; H, 2·5.

2,6-*Dinitrobenzoic acid.* This acid was prepared from 2,6-dinitrotoluene by oxidation with acid permanganate by the method described by Sirks (1908) but carrying out the reaction at 100°. The use of a higher temperature increased the yield. The product was crystallised from cold hydrochloric acid to give a white crystalline compound, m.p. 204–5° (Sirks; m.p. 201–3°). The yield based on the 2,6-dinitrotoluene consumed was 48 per cent. Found: C, 40·0; H, 2·2. Calc. for $C_7H_4N_2O_6$: C, 39·6; H, 1·89.

2,6-*Dimethylbenzoic acid.* 2-Iodo-*m*-xylene (14 g.), ethyl bromide (5·2 g.), magnesium turnings (10 g.), sodium-dried ether (15 ml.) and a small crystal of iodine were introduced into a two-necked 500 ml. flask which was fitted with a dropping funnel and reflux condenser, to both of which were attached calcium chloride guard tubes. The reaction commenced immediately and a solution of 2-iodo-*m*-xylene (27·4 g.) and ethyl bromide (12·8 g.) in dry ether (120 ml.) was run in over a period of 30 min. and then the mixture refluxed for a further 1 hr. The mixture was poured onto crushed solid carbon dioxide (80 g. approximately) in a beaker and stirred until the dry ice had evaporated. A mixture of crushed ice (200 g.) and dilute hydrochloric acid (60 ml.) was added with stirring and

the ether layer separated. The ethereal solution was washed with water (4 × 20 ml.) and then extracted with sodium hydroxide solution (2 × 50 ml. 5 per cent). The alkaline solution was decolourised with charcoal, acidified with hydrochloric acid and the precipitated 2,6-dimethylbenzoic acid filtered off. It was recrystallised from water, m.p. 115° (Jacobs and others, 1951; m.p. 115·5°). Yield 22·7 g. 91 per cent. Found: C, 71·96; H, 6·67. Calc. for $C_9H_{10}O_2$: C, 72·0; H, 6·65.

2,6-*Dichlorobenzoic acid.* This acid was prepared by the method of Norris and Bearse (1940), m.p. 143–4° (Norris and Bearse, 1940; m.p. 143°). Yield 12·8 g. 54 per cent. Found: C, 43·8; H, 2·4. Calc. for $C_7H_4Cl_2O_2$: C, 43·95; H, 2·1.

2,4,6-*Tribromobenzoic acid.* The acid was prepared from *m*-aminobenzoic acid by the method of Robinson and Robinson (1956), m.p. 192° (Robinson and Robinson, 1956; m.p. 192·5 to 194·5°). Found: C, 23·1; H, 0·80. Calc. for $C_7H_3Br_3O_2$: C, 23·42; H, 0·98.

2,6-Dimethoxybenzoic acid, 1-naphthoic acid and 2-naphthoic acid were obtained commercially.

2-*Diethylaminoethyl chloride hydrochloride.* 2-Diethylaminoethanol (58·5 g.) dissolved in dry benzene (750 ml.) was introduced into a 2-litre three-necked flask fitted with a reflux condenser, stirrer and a dropping funnel. The mixture was stirred and cooled while thionyl chloride (59·5 g.) was added dropwise over 2 hr. and then the mixture refluxed for 2 hr. The reaction mixture was cooled and the precipitate filtered off, washed with dry benzene, dried and recrystallised from an acetone-ethanol mixture, m.p. 211° (Slotta and Benisch, 1935; m.p. 212°). Yield 67 g. 78 per cent. Found: C, 41·9; H, 8·4; Cl, 20·75. Calc. for $C_6H_{15}Cl_2N$: C, 41·86; H, 8·7; Cl^-, 20·65.

2-*Diethylaminoethyl chloride.* 2-Diethylaminoethyl chloride hydrochloride (21·5 g.) was placed in a 500 ml. two-necked flask fitted with a vacuum-tight stirrer and a condenser set for distillation. Sodium hydroxide (10 g.) was ground to a fine powder, then added to the flask and mixed with the amine hydrochloride. The mixed powders were stirred and reduced pressure (50 mm.) applied to the apparatus. The flask was heated gently and a colourless liquid distilled over and was collected in a receiving flask cooled in an ice-bath. The product was dried over anhydrous magnesium sulphate in a refrigerator, filtered off and stored below 0°, b.p. 146° (Slotta and Benisch, 1935; b.p. 146–7°). Yield 13·5 g. 80 per cent.

The di-*ortho*-substituted esters were prepared by one of two methods:

Method A

2-*Diethylaminoethyl 2,6-dimethoxybenzoate hydrochloride.* 2,6-Dimethoxybenzoic acid (9·1 g.) and isopropanol (75 ml.) were introduced into a 250-ml. two-necked flask fitted with a reflux condenser and a dropping funnel. The solution was heated to 50° and 2-diethylaminoethyl chloride was added dropwise over 15 min. The reaction mixture was maintained at 50° for a further 15 min. and then refluxed for 12 hr.

The mixture was cooled to 0°, whereupon a buff coloured solid was precipitated, filtered off, washed with cold isopropanol and recrystallised from isopropanol, m.p. 186°. Yield 9·6 g. 60 per cent. $C_{15}H_{24}ClNO_4$ requires: C, 56·69; H, 7·6; Cl, 11·18. Found: C, 56·6; H, 7·6; Cl, 11·37.

Method B

2-*Diethylaminoethyl* 2,6-*difluorobenzoate hydrochloride.* This ester was prepared from 2,6-difluorobenzoic acid and 2-diethylaminoethyl chloride as described in Method A. However, as the ester did not crystallise readily from the reaction mixture, it was isolated in the following manner. The reaction mixture was evaporated to dryness under reduced pressure and the residue was dissolved in the minimum quantity of water, the solution made alkaline with sodium bicarbonate, and the free base which was liberated extracted with chloroform. The solution was dried with calcium chloride, the chloroform removed and the residue dissolved in dry benzene. Dry hydrogen chloride was passed through the solution and the resulting precipitate was filtered off, washed with benzene and recrystallised from an ethyl methyl ketone-ethanol mixture to give a white crystalline solid, m.p. 144°.

A list of di-*ortho*-substituted esters prepared by these two methods is given in Table II.

Discussion

Esterification of Di-ortho-*Substituted Benzoic acids*

The general methods of esterification either fail or are very slow and produce poor yields with hindered acids such as di-*ortho*-substituted benzoic acids. Esterification by these methods involves an intermediate stage in which the acyl carbon atom has a tetrahedral configuration and the presence of two groups *ortho* to the carboxyl group restricts the space available for the formation of such a bulky intermediate. This is the primary steric effect of *ortho* substitutents and is the main reason why di-*ortho*-substituted benzoic esters are stable. (For a full discussion of the *ortho* effect see Stoker, 1959). In synthesising esters of di-*ortho*-substituted benzoic acids, therefore, a method of esterification which does not involve an attack on the acyl carbon atom is required. One such method which has been used is the silver salt method (Thomas and Stoker, 1961; Rabjohn and others, 1955.) The reactions involved in the preparation of amino-esters by this method are given in Fig. 1.

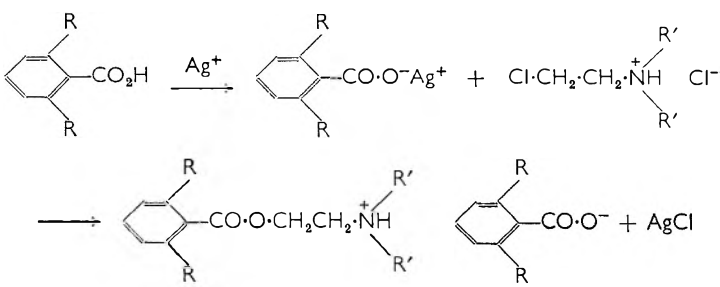

FIG. 1

TABLE II

DI-ORTHO-SUBSTITUTED BENZOIC AND 1- AND 2-NAPHTHOIC ESTERS OF 2-DIETHYLAMINOETHANOL HYDROCHLORIDE

2-Diethylaminoethyl hydrochloride ester of:	Method	Quantity of Reactants		Reflux time hr.	Solv. for recryst.	m.p. °C	Yield per cent	Analysis					
		Acid	Amino-chloride					Found			Required		
								C	H	Cl	C	H	Cl
2,6-Difluorobenzoic acid ..	B	3·16 g. 0·02 mole	2·71 g. 0·02 mole	7	Ethyl methyl ketone	144	55	53·5	6·3	12·03	53·2	6·1	12·10
2,6-Dichlorobenzoic acid ..	A	9·55 g. 0·05 mole	6·77 g. 0·05 mole	10	Isopropanol	182	63	47·6	5·8	10·81	47·8	5·5	10·88
2,4,6-Tribromobenzoic acid ..	B	3·59 g. 0·01 mole	1·35 g. 0·01 mole	8	Acetone	197–8	42	31·2	3·7	7·19	31·5	3·4	7·18
2,6-Dinitrobenzoic acid	A	5·30 g. 0·025 mole	3·39 g. 0·025 mole	8	Acetone	158	62	45·0	5·3	10·25	44·9	5·2	10·22
2,6-Dimethylbenzoic acid ..	B	3·75 g. 0·025 mole	3·39 g. 0·025 mole	8	Ethyl methyl ketone	164–5	56	63·0	8·4	12·42	63·0	8·4	12·44
2,6-Dimethoxybenzoic acid ..	A	9·10 g. 0·05 mole	6·77 g. 0·05 mole	12	Isopropanol	186	60	56·6	7·6	11·37	56·7	7·6	11·18
1-Naphthoic acid	B	6·90 g. 0·04 mole	5·42 g. 0·04 mole	10	Acetone	162–4	58	66·5	6·9	11·59	66·3	7·2	11·55
2-Naphthoic acid	B	4·30 g. 0·025 mole	3·39 g. 0·025 mole	10	Acetone	163–4	62	66·0	7·4	11·62	66·3	7·2	11·55

The reaction is slow because it is heterogeneous. Other disadvantages of this method are that silver is expensive, and that the highest theoretical yield based on the acid is only 50 per cent since the insolubility of silver chloride causes one molecule of the acid to become the anion of the amino-ester salt.

A method of esterifying di-*ortho*-substituted benzoic acids has now been developed without the disadvantages of the silver salt method. It consists of reacting the acid with the alkylamine chloride (in the present case 2-diethylaminoethyl chloride) in a suitable solvent such as isopropanol. The reaction is given in Fig. 2.

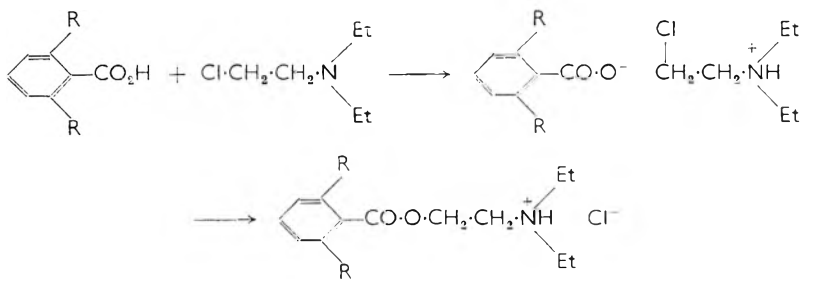

FIG. 2

The present method is based on the use of the carboxylate ion as a nucleophillic reagent in a normal aliphatic nucleophillic substitution reaction of a primary alkyl chloride. One possible problem of this method is the dimerisation of the chloroalkylamine. However, 2-diethylaminoethyl chloride is reasonably stable below 0° and as soon as the substituted benzoic acid is mixed with the base the proton from the acid is co-ordinated by the basic nitrogen atom to form the amino-conjugate acid, which is stable and cannot dimerise. The presence of a positive charge in the chloroalkylamine will favour a nucleophillic substitution of the chlorine atom by its inductive effect.

The reaction is of general application and is limited only by the tendency of any particular chloroalkylamine to self-condense. Probably the most reactive chloroalkylamine from this point of view is 2-dimethylaminoethyl chloride and even this may be used diluted with an equal volume of an inert solvent such as toluene and kept below 0° before mixing it with a substituted benzoic acid.

Preparation of 2,6-*Dimethylbenzoic Acid*

Three general methods of preparing this acid have been reported in the literature. Hufferd and Noyes (1921) prepared it from mesitylene by a method which was long and produced low yield. Fuson and others (1940) reported a better method of synthesis which involved the hydrolysis of the nitrile obtained from *m*-2-xylidine in a Sandmeyer reaction. However, the hydrolysis of 2,6-dimethylbenzonitrile proved difficult and a yield of 20 per cent was the best obtained. Häring (1960) recently modified the hydrolysis procedure and claimed a yield of 55 per cent.

Berger and Olivier (1927) converted 2,6-dimethylbenzonitrile to 2,6-dimethylbenzoic acid in a two-step procedure, going through 2,6-dimethylbenzamide as an intermediate, with an overall yield of 70 per cent for the hydrolysis. The method found most satisfactory in the present work for the hydrolysis of 2,6-dimethylbenzonitrile was that of Berger and Olivier (1927) but the overall yield of 2,6-dimethylbenzoic acid from *m*-2-xylidine was only 25 per cent.

Faber and Nauta (1951) have prepared 2,6-dimethylbenzoic acid from 2-bromo-*m*-xylene by carbonation of the corresponding Grignard reagent, and Jacobs and others (1951) carried out a similar reaction starting from 2-iodo-*m*-xylene. The latter authors claimed a yield of 73 per cent but they gave few experimental details. We have been unable to obtain a yield of this order using standard Grignard conditions. The main problem appeared to be the difficulty of forming a Grignard reagent. Grignard (1934) developed the "entrainment" method to overcome the difficulty. This involves adding an equimolar proportion of ethyl bromide, or some similarly active alkyl halide, to the reaction mixture. The effect of the ethyl bromide is a constant "cleaning" of the magnesium surface, thus permitting the reaction of acyl halide and magnesium to proceed at a satisfactory rate.

A comparison of the standard Grignard technique and the "entrainment" method for the preparation of benzoic acid and 2,6-dimethylbenzoic acid from bromobenzene and 2-iodo-*m*-xylene respectively has now been made. The quantities of organic reactants and the yield of products obtained are summarised in Table III. Using the "entrainment" method, 2,6-dimethylbenzoic acid was produced in very good yield.

TABLE III

A COMPARISON OF TWO METHODS OF THE GRIGNARD REACTION FOR THE PREPARATION OF BENZOIC ACID AND 2,6-DIMETHYLBENOZIC ACID

Compound	Quantity of organic reactants		By-products isolated	Yield of product	
	Bromobenzene	Ethyl bromide		Wt.	Per cent
Benzoic acid	26·5 g. $\frac{1}{6}$ mole	Nil	2 g.	13·2 g.	64
	26·5 g. $\frac{1}{3}$ mole	18 g. $\frac{1}{3}$ mole	—	20·5 g.	99·5
	2-Iodo-*m*-xylene	Ethyl bromide	*m*-Xylene		
2,6-Dimethylbenzoic acid	38·7 g. $\frac{1}{6}$ mole	Nil	1·7 g.	10·5 g.	42
	38·7 g. $\frac{1}{3}$ mole	18 g. $\frac{1}{3}$ mole	0·1 g.	22·7 g.	90·7

Preparation of 2,6-*Difluorobenzoic Acid*

There are two general methods by which fluorine may be introduced into an aromatic ring using the Schiemann reaction. One is to diazotise the amino-group by the usual method and then form the diazonium fluoroborate by adding the fluoroborate ion. The second method is to carry out the diazotisation reaction in the presence of the fluoroborate ion. Both these methods have now been examined for the preparation of 2,6-difluorobenzoic acid and it was found that the second method was preferable because less phenolic by-products were formed; the reaction could be carried out at 0–5° and hence the reaction time was shorter; the

yield of aromatic fluoro-compound was higher and more consistent when the diazonium fluoroborate prepared by the second method was decomposed.

Acknowledgement. The authors wish to thank Miss J. Savage for the microanalyses.

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THE CHEMICAL DETERMINATION OF LIOTHYRONINE AND THYROXINE IN ENZYMIC HYDROLYSATES OF PORK THYROID

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Maximum amounts of liothyronine and thyroxine were released from pork thyroid by the proteolytic action of erepsin and trypsin after incubation for 96 hr. at 39°. The iodinated thyronines were extracted from the hydrolysate with butanol, separated chromatographically, eluted from the paper and measured quantitatively by a chemical method in which the reduction of ceric ion by arsenious acid is catalysed by the iodine containing compounds. The iodine found in liothyronine and thyroxine accounted for 18 to 25 per cent of the total organic iodine in pork thyroid. It is postulated that pork thyroglobulin contains approximately 1 molar residue of liothyronine and 2 molar residues of thyroxine.

Thyroid preparations are standardised chemically by measuring either the total organic iodine (Pharmacopeia of the United States XVI) or "thyroxine" iodine (British Pharmacopoeia 1958, Addendum 1960). The latter method is based on a procedure originally described by Harington and Randall (1929) and assumes that thyroxine in an alkaline hydrolysate of desiccated thyroid is precipitated quantitatively at pH 3·5, while the former method presumes a quantitative relationship between biologically active iodine and the total iodine. However, recent publications have cast some doubt on the adequacy of these chemical assays (Stasilli and Kroc, 1956; Taylor, 1961; Sturnick and Lesses, 1961; *Pharm. J.*, 1961).

Since thyroid contains liothyronine (3,5,3′-triiodothyronine) in addition to thyroxine (Gross and Pitt-Rivers, 1953) any chemical procedure for determining the potency of desiccated thyroid should provide a quantitative measure of both of these biologically active iodinated thyronines.

Mandl and Block (1959) described a method for the quantitative determination of the various iodinated compounds in a commercial sample of thyroglobulin. Since there appeared to be some variation between the estimates of liothyronine and thyroxine reported by Mandl and Block, modifications of their conditions of enzymic hydrolysis and extraction were investigated in our laboratory. In addition, a study was made of methods of chromatographic resolution and quantitative measurement of liothyronine and thyroxine employed by others (Leffler, 1954; Maclagan, Bowden, and Wilkinson, 1956; Pind, 1957). We have made a comparison of the rate of release of thyroxine and liothyronine from pork thyroid powder by the action of trypsin and erepsin both alone and in combination. Evidence is presented which suggests that under the conditions described, the amounts of liothyronine and thyroxine released by enzymic hydrolysis of thyroid approach maximum values. Recovery experiments have shown that losses which may occur during the determination have been reduced to a minimum.

EXPERIMENTAL

Reagents

All reagent solutions were prepared from analytical grade chemicals and water distilled from an all-glass apparatus over alkaline potassium permanganate.

Borate buffer. 0·05M adjusted to pH 8·5.

Arsenious acid solution. Sodium arsenite (13·0 g. $NaAsO_2$) was dissolved in 500 ml. water and the volume made up to 1000 ml. with 30 per cent sulphuric acid. In this solution was dissolved 0·5 g. recrystallised sodium chloride.

Stock ceric sulphate solution. (Leffler, 1954.) In a beaker, were placed 6·5 g. ceric sulphate ($Ce(SO_4)_2$), 6·0 ml. concentrated sulphuric acid and 5·0 ml. water. The mixture was heated to just below boiling for 30 min., then cooled and made up to a volume of 70 ml. The solution was filtered and stored in a refrigerator.

Ceric sulphate solution. The stock ceric sulphate was diluted (usually about 1 : 20) by the addition of 2N sulphuric acid so the reagent control tube described under "quantitative measurement" would give an absorbance between 0·700 and 0·800 at 420 mμ in a spectrophotometer cell of 1·0 cm. light path.

Ceric sulphate arsenious acid reagent for staining. The stock ceric sulphate was diluted with two volumes 2N sulphuric acid and mixed with an equal volume of a 1 : 3 dilution of the arsenious acid solution immediately before use.

Ammoniacal alcohol. 5 per cent concentrated ammonia in methanol.

Standard solutions. An accurately weighed amount of the sodium salt of either liothyronine or thyroxine was dissolved in a 1 : 10 dilution of 20 per cent acetic acid in methanol to provide a concentration of 100 μg. of the free dehydrated form per ml. Although liothyronine and thyroxine are not readily soluble in this solvent, they will dissolve completely when shaken gently for an hr. or more. Once in solution, they remain stable for at least 7 days when kept at 4°.

Mercuric nitrate. (Strickland and Maloney, 1957.) Two g. mercuric nitrate were dissolved in 100 ml. water to which a few drops of concentrated nitric acid had been added.

Enzyme Hydrolysis

In a 15 by 150 mm. test-tube were placed approximately 25 mg. of thyroid, accurately weighed, 10 mg. trypsin (Difco 1 : 250), 10 mg. erepsin (Nutritional Biochemicals Corporation), and 2 ml. borate buffer containing one drop of thiomersal (Eli Lilly and Company—solution No. 45). The suspension was thoroughly mixed and placed in the dark at 38–40° for 96 hr. The mixture was shaken occasionally. After the first 4 hr. the pH was adjusted to 8·5 with 0·2N NaOH. At daily intervals thereafter, 5 mg. erepsin were added, with gentle shaking.

Extraction

At the end of the incubation period, the pH was adjusted to 3·0 with N sulphuric acid. The acidified digest was extracted four times with

3 ml. volumes of n-butanol which had been previously equilibrated with 0·1N sodium thiosulphate. The extracts were combined and the solvent removed under reduced pressure at 60°. The dried residue may be stored for at least 1 week in the cold under nitrogen.

Paper Chromatography

The dried residue was dissolved in sufficient ammoniacal alcohol (usually less than 1·0 ml.) to permit the application of an aliquot containing about 4 μg. total organic iodine to each of three 1 in. Whatman No. 1 paper strips, assuming for this calculation that all of the iodinated compounds have been extracted by the butanol. The first strip was used to locate the liothyronine and thyroxine areas, and the other two strips for duplicate measurement of each of the thyronines. On respective strips of Whatman No. 1 paper were spotted 0·2, 0·5, and 1·0 μg. liothyronine, and 0·5, 1·0 and 2·0 μg. thyroxine. A marker strip was included for each standard. These quantities of standard and hydrolysate have been chosen so that calibration and measurement may be made on eluates which have been treated in an identical manner. This allows compensation for any possible deterioration of liothyronine and thyroxine in the ammoniacal alcohol. Chromatographic examination has not indicated any observable deterioration of these compounds when dissolved in ammoniacal alcohol for up to 2 hr. The chromatograms were allowed to develop overnight in a descending t-pentanol : N-ammonia (2 : 1) solvent system (Gleason, 1955).* The solvent fronts were marked and the strips were stained by the method of Bowden, Maclagan, and Wilkinson (1955). As soon as the strip was removed from between the glass plates, it was drawn once through 2N ammonia and then dried in an oven. This treatment provides a stained chromatogram which, if kept in the dark, remains in good condition for several weeks.

Quantitative Measurement

The areas on the unstained chromatograms corresponding to liothyronine and thyroxine were cut out and placed in 25 ml. glass stoppered flasks containing 5 ml. ammoniacal alcohol. The flasks were shaken gently for 30 min. to elute the iodinated thyronines.

For the determination of liothyronine, 3 ml. aliquots were withdrawn from both the standard and hydrolysate eluates, transferred to 10 × 125 mm. test-tubes,† and taken to dryness under a stream of nitrogen. For the measurement of thyroxine, 1 ml. aliquots of the appropriate eluates were taken to dryness in a similar manner. To all tubes and one reagent control tube were added 3 ml. of water and 2 ml. arsenious acid solution. The contents were mixed thoroughly and the tubes were placed in a water bath at 30° for 15 min. At carefully timed intervals, 1·0 ml. of the ceric sulphate solution was added to each tube,

* When developed at room temperature (21–23°) the solvent front advanced about 10 in. from the line of application, giving R_F values for iodide, thyroxine and liothyronine of 0·15, 0·25 and 0·53 respectively.

† These tubes were cleaned with hot nitric acid to remove traces of mercury salt, and were discarded after several weeks' use (Strickland and Maloney, 1957).

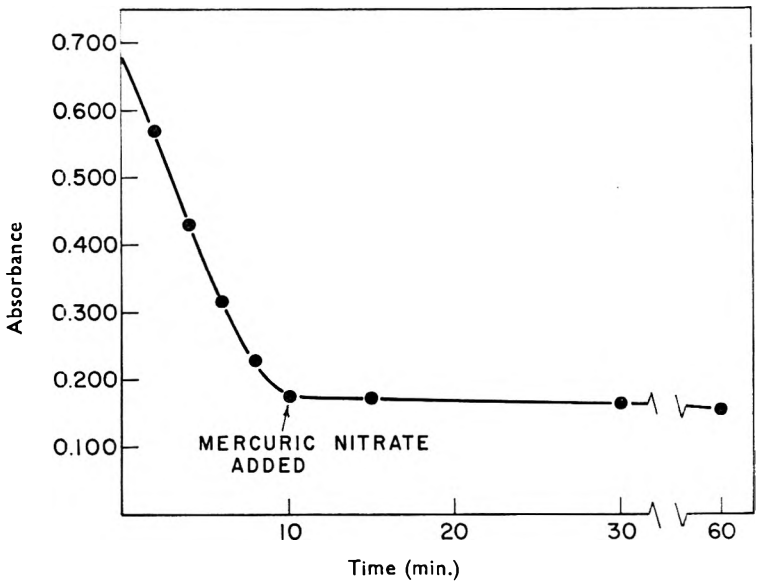

FIG. 1. The effect of the addition of mercuric nitrate on the rate of reduction of ceric ion by arsenious acid catalyzed by iodine.

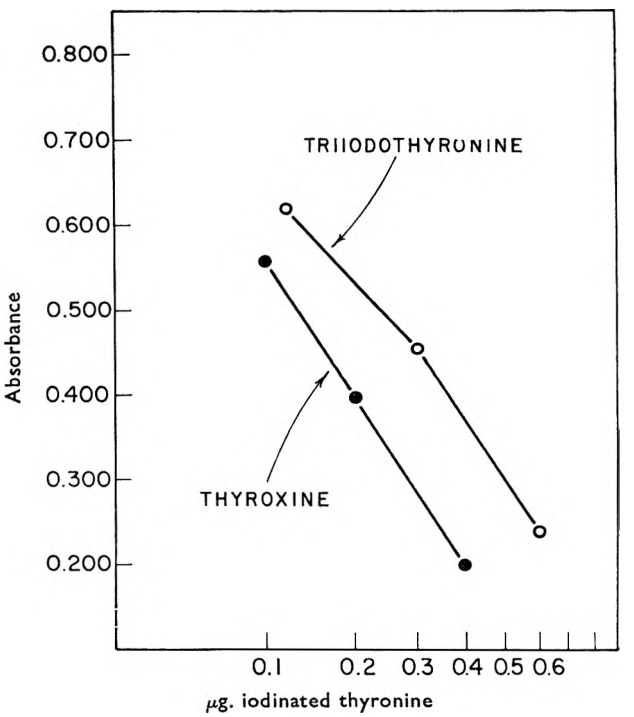

FIG. 2. The calibration curves for liothyronine and thyroxine.

and exactly 10 min. later, the reaction was stopped by the addition of 2 drops of mercuric nitrate solution (Strickland and Maloney, 1957). Fig. 1 illustrates the effect of mercuric nitrate on the rate of ceric ion reduction under these conditions. The absorbance of each solution at 420 mμ was recorded promptly against a water blank. Fig. 2 shows the standard curves obtained by plotting absorbances of the reference materials on semilogarithmic paper, employing the relation described by Acland (1957). The amounts of liothyronine and thyroxine in the hydrolysates were estimated directly from the calibration curves. It has been found advisable to calibrate for each set of determinations.

The arsenious acid and ceric sulphate reagents employed for the final measurement were of high purity. The reagents were considered satisfactory if the absorbance decrease of the reagent control at 420 mμ did not exceed 0·050 during the 10 min. catalytic reduction period of ceric sulphate.

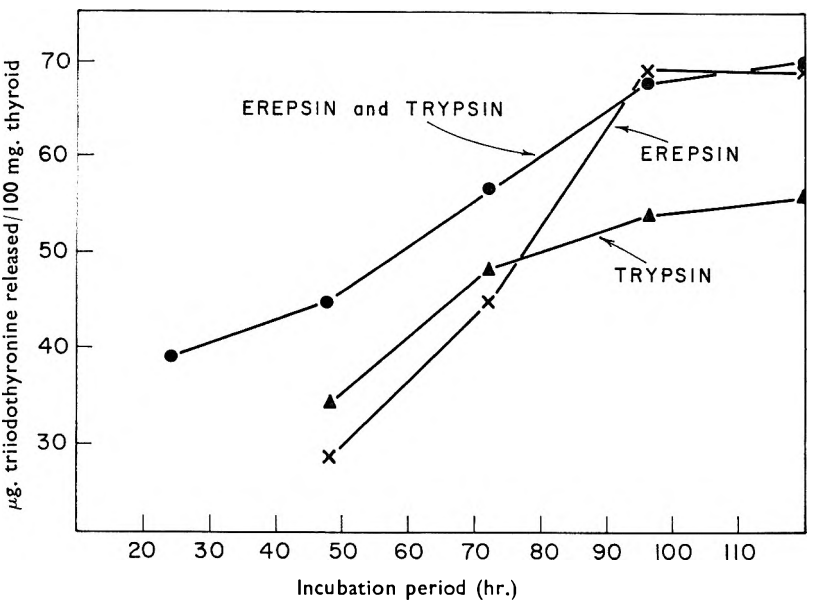

FIG. 3. The rate of release of liothyronine from pork thyroid by proteolytic hydrolysis.

Erepsin vs. Trypsin Hydrolysis

Although erepsin alone usually released maximum amounts of liothyronine and thyroxine (Figs. 3 and 4) over 96 hr., trypsin was added at the beginning of the digestion period because more consistent results were thus obtained. Crude trypsin alone, under the same conditions, was not capable of hydrolysing the maximum amounts of liothyronine and thyroxine from thyroid and was markedly inferior to erepsin in releasing thryoxine (Fig. 4). The trypsin activity appears to have more access to the liothyronine linkage with thyroglobulin than to the thyroxine

linkage. A similar result was obtained with a pancreatin preparation (Pancreatin N.F.—Fisher Scientific Co.). Increased enzyme concentration did not affect the rate of release. In erepsin digests, it has been noted that the drop in buffer pH was gradual over the first 48 hr. whereas when trypsin was present, the increase in acidity was rapid and required adjustment to pH 8·5 after 4 hr. digestion.

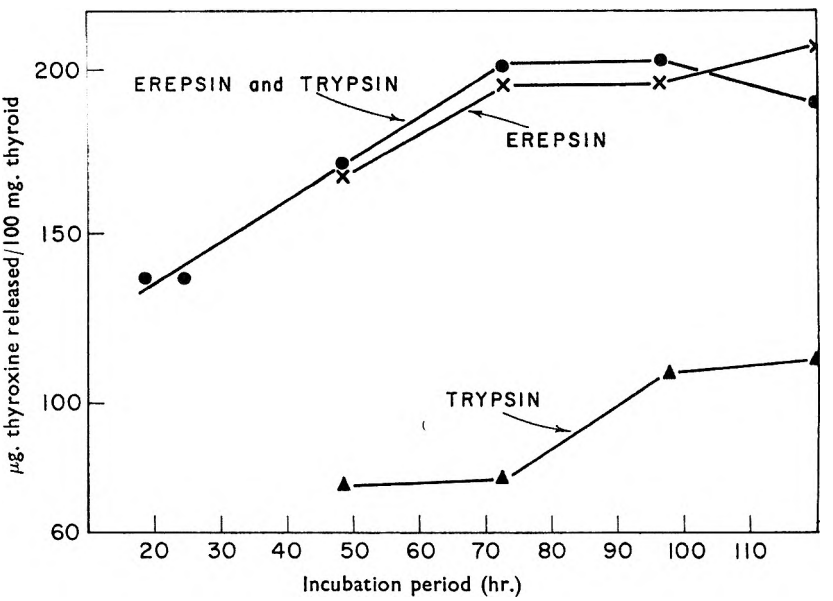

FIG. 4. The rate of release of thyroxine from pork thyroid by proteolytic hydrolysis.

TABLE I

RECOVERY OF LIOTHYRONINE AND THYROXINE FROM THYROID HYDROLYSATES

	Liothyronine				Thyroxine			
Digest	From thyroid μg.	Added standard μg.	Measured total μg.	Per cent recovery	From thyroid μg.	Added standard μg.	Measured total μg.	Per cent recovery
1	0·133	0·20	0·343	105	0·502	1·0	1·408	91
2	0·136	0·20	0·338	101	0·514	1·0	1·428	91
3	0·134	0·20	0·347	107	0·563	1·0	1·565	100

Recoveries

To demonstrate recovery values, quantities of liothyronine and thyroxine were added to digests of thyroid at the beginning of the incubation period to provide final concentrations which would approximate those obtained when thyroid alone was processed. The values shown in Table I indicate that extraction with butanol provided satisfactory recovery of liothyronine and thyroxine. In addition, other amino-acids or peptides released during hydrolysis did not appear to interfere with the quantitative measurement of the iodinated thyronines. The slight increase in per cent recovery

of liothyronine suggests the possibility that some of the thyroxine may be converted to liothyronine at some stage during the analysis.

Liothyronine and Thyroxine in Thyroid

The per cent composition of five samples of pork thyroid is listed in Table II. Samples A, B and E were desiccated thyroid while C and D

TABLE II

PER CENT BY WEIGHT COMPOSITION OF PORK THYROID

Thyroid	Iodine per cent	Moisture per cent	Thyroxine per cent	Liothyronine per cent
A	0·23	6·4	0·047 ± 0·002*	0·019 ± 0·001
B	0·79	8·8	0·212 ± 0·007	0·073 ± 0·002
C	0·86	6·5	0·230 ± 0·005	0·108 ± 0·005
D	1·02	7·2	0·243 ± 0·004	0·090 ± 0·002
E	0·63	7·8	0·146 ± 0·003	0·075 ± 0·002

*S.E.

TABLE III

THE RELATION OF LIOTHYRONINE, THYROXINE AND "THYROXINE" IODINE (B.P.) CONCENTRATION IN THYROID TO TOTAL IODINE

Thyroid	mg. T−4 per 100 mg. I	mg. T−3 per 100 mg. I	Molar ratio T−3: T−4	$\frac{I_{T-4} + I_{T-3}}{I_{Total}} \times 100$	$\frac{\text{"Thyroxine" iodine}}{I_{Total}} \times 100$
A	20·4	8·3	0·48	18·2	27·4
B	26·8	9·2	0·42	22·9	32·4
C	26·7	12·5	0·57	24·8	33·6
D	23·8	8·8	0·44	20·8	33·0
E	23·2	11·9	0·61	22·2	30·2

T−4 = thyroxine. T−3 = liothyronine.

were commercial pork thyroglobulin preparations. The samples were of North American origin with the exception of E which was obtained from the United Kingdom. The per cent total iodine was determined by the oxygen flask method (Johnson and Vickers, 1959), which gives values that are usually slightly higher than those obtained by the procedure described in the Pharmacopeia of the United States XVI.

In Table III, the concentrations of liothyronine and thyroxine are expressed as mg. of thyronine per 100 mg. of thyroid iodine. The variation in the per cent of the total iodine found in the liothyronine plus thyroxine fraction shown in Table III, suggests that the total iodine is related to the biologically active iodinated thyronine content in an approximate manner only. In addition, this relation is complicated by the fact that the ratio of liothyronine to thyroxine varies significantly.

Also from Table III, the per cent of the total iodine found in the "thyroxine" iodine fraction as measured by the method in the British Pharmacopoeia was consistently higher than that estimated from the liothyronine plus thyroxine content. When the material precipitated from an alkaline hydrolysate of thyroid at pH 3·5 was chromatographed on paper, using the t-pentanol : N-ammonia system, thyroxine as well as liothyronine and other unidentified iodinated compounds were detected.

Molar concentrations of Liothyronine and Thyroxine

The samples available for this investigation were obtained from four different sources and it is assumed that each sample was taken from a pool collected from large numbers of animals. In Table III it is noted that pork thyroid is iodinated to a degree where the molar ratio of liothyronine to thyroxine for all samples is close to 0·5. The molar residues for the two pork thyroglobulin samples, C and D, have been calculated and are shown in Table IV. Apparently, for each mole of thyroglobulin, assuming a molecular weight of 650,000 (Derrien and others, 1949) there is approximately 1 mole of liothyronine and 2 moles of thyroxine.

TABLE IV

MOLAR RESIDUES OF LIOTHYRONINE AND THYROXINE IN COMMERCIAL HOG THYROGLOBULIN

Thyroid	Residues thyroxine per mole thyroglobulin	Residues liothyronine per mole thyroglobulin
C	2·1	1·2
D	2·2	1·0

Acknowledgements. Chemical assays for thyroxine iodine were made by Mr. A. Bayne of this laboratory. Generous gifts of thyroid preparations were received from Warner Chilcott Laboratories, Wilson Laboratories, Armour Pharmaceutical Company and Burroughs Wellcome & Co. We wish to thank Smith, Kline and French Corporation for providing generous amounts of sodium-L-thyroxine and sodium liothyronine with accompanying chemical assays of purity.

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STUDIES ON THE KINETICS OF FUNGICIDAL ACTION

PART II. THE EFFECT OF TEMPERATURE ON THE VIABILITY OF *PENICILLIUM NOTATUM* SPORES IN WATER AND SOLUTIONS OF PHENOL

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Received May 24, 1962

The mean Q_{10} value for the mortality of *Penicillium notatum* spores in water from 50 to 55° was 881. In 1 to 1·25 per cent solutions of phenol from 15 to 40° it was 3·4; higher values were obtained for 0·75 and 0·875 per cent phenol.

THE effect of temperature on the reaction velocity of fungicidal action has not been greatly studied, yet for the practical use of fungicidal solutions it is a parameter worthy of as much consideration as the concentration of the solutions.

This paper reports the effect of temperature on the viability of *Penicillium notatum* spores in aqueous suspensions with and without phenol.

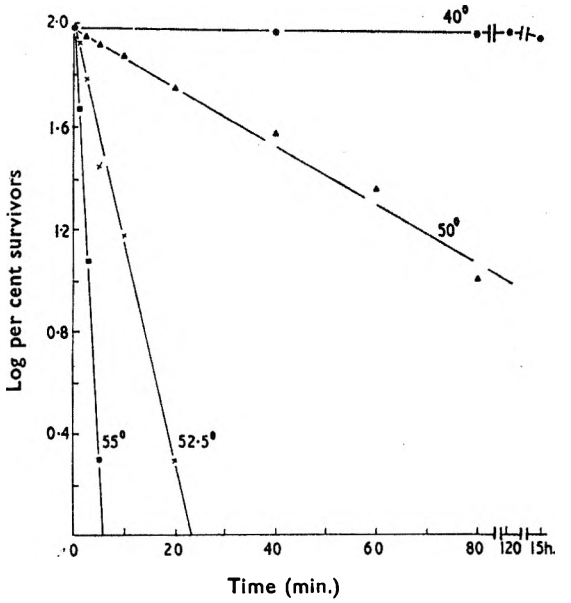

FIG. 1. Effect of temperature on the viability of *P. notatum* spores in water.

EXPERIMENTAL

The method of preparation of *P. notatum* spore suspensions and the slide-germination technique used were the same as those described previously (Chauhan and Walters, 1961).

The viability of spores in water was determined by adding 0·5 ml. of

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an aqueous suspension containing 10^{10} spores per ml. to 9·5 ml. of water at the required temperature. 1 ml. quantities were withdrawn at intervals and added to 9 ml. of water at 25° to stop the reaction. One drop aliquots were then mixed with equal volumes of double-strength Horowitz fluid medium. Counts were made after 12 hr. incubation and up to 48 hr. when there were few or no survivors. This was necessary since spores damaged by heat were found to germinate after longer lag phases than phenol treated spores.

To examine the effect of temperature on spores in solutions of phenol, 10 ml. of phenol solutions of twice the required concentration were pipetted into 10 ml. suspensions containing 10^8 spores per ml. The solutions and suspensions were previously equilibrated to the required temperature in the range 15 to 40° ± 0·1°. The reactions were allowed to proceed for 10 min. and in the case of 1·125 per cent phenol for various time intervals. Differential counts of 100 spores were made in duplicate afer 12 to 24 hr.

Results

The effect of temperature on the viability of spores in water from 40 to 55° is shown in Fig. 1. At 60° there were less than 1 per cent survivors after 30 sec., and after 60 sec., following incubation for 67 hr., only 3 spores in approximately 10^6 germinated.

The rate of germination and the percentage viability of spores exposed for 10 min. to 0·5 per cent phenol from 15 to 40° were the same as for spores in water whereas the activity of 0·75 to 1·25 per cent solutions increased markedly with temperature (Figs. 2, 3 and 4).

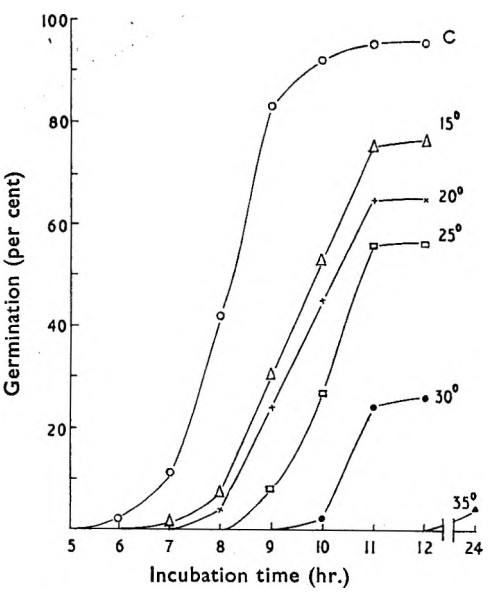

Fig. 2. Effect of temperature on the viability and rate of germination of *P. notatum* spores exposed to 1 per cent phenol for 10 min.

C = Control (spores in water at 25°).

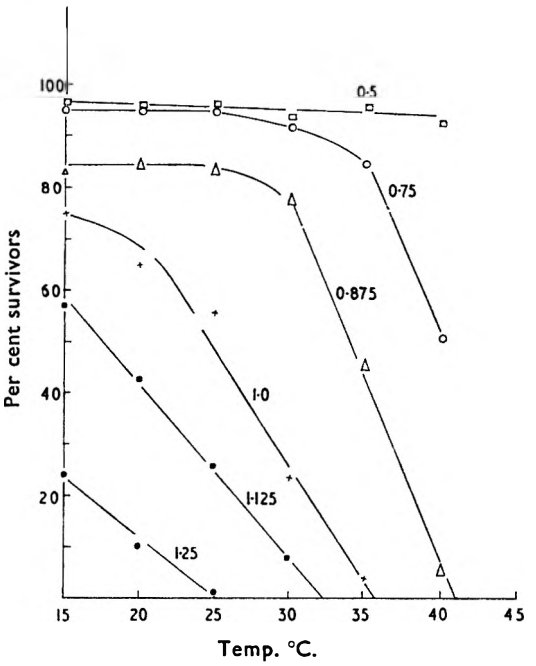

FIG. 3. Per cent survivor-temperature curves for *P. notatum* spores exposed to 0·5–1·25 per cent solutions of phenol for 10 min.

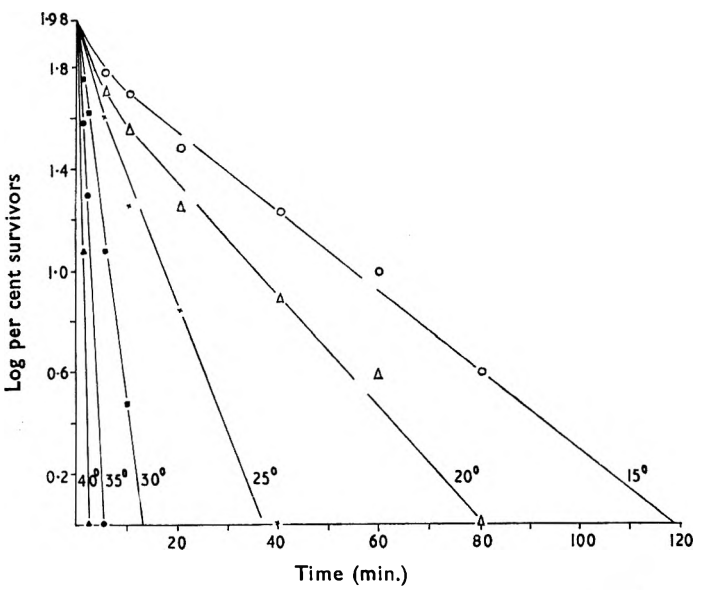

FIG. 4. Log per cent survivor-time curves for *P. notatum* spores in 1·125 per cent phenol. Viability before addition of phenol = 96 per cent.

Discussion

The destruction of *P. notatum* spores in 2 min. in water at 60° is typical of fungal spores. Relatively few species possess high resistance to moist heat, a notable example being the conidia of *Micromonospora vulgaris* which can withstand 100° for 45 min. (Cochrane, 1958; Erikson, 1952, 1955; Hull, 1939).

The times required for 50 per cent mortality of *P. notatum* spores in water (Fig. 1) were substituted in Phelps' (1911) equation to obtain the values of θ and Q_{10} given in Table I. These results show that when the sensitivity of an organism to a lethal agent is confined to a narrow temperature range, high Q_{10} values are obtained. High values were also obtained by Chick (1910) and Smith (1923).

TABLE I

TEMPERATURE COEFFICIENTS FOR THE MORTALITY OF *P. notatum* SPORES IN WATER

Temperature	θ	Q_{10}
50 –52·5°	2·24	3177
52·5–55°	1·74	254
50 –55°	1·97	881

TABLE II

TEMPERATURE COEFFICIENTS FOR THE MORTALITY OF *P. notatum* SPORES IN SOLUTIONS OF PHENOL

Phenol concentration (per cent)	Temperature (°C)	θ	Q_{10}
0·75	30–35	1·23	7·9
	35–40	1·39	27·0
0·875	25–30	1·09	2·4
	30–35	1·29	12·8
	35–40	1·30	13·8
1·0	15–20	1·10	2·6
	20–25	1·07	2·0
	25–30	1·21	6·7
	30–35	1·18	5·2
1·125	15–20	1·09	2·4
	20–25	1·10	2·6
	25–30	1·14	3·7
1·25	15–20	1·10	2·6
	20–25	1·15	4·0

The rate of death of *P. notatum* spores in water and 1·125 per cent phenol solution follows a first order reaction except for an initial higher death-rate in the phenol solution at lower temperatures (Figs. 1 and 4). These results differ from those of Smith (1921, 1923) who obtained mainly sigmoidal curves for *Botrytis cinerea* spores.

The temperature coefficients (Table II) calculated from reaction velocities (Fig. 3) are in good agreement for phenol concentrations of 1 to 1·25 per cent. Below 30° and 25° respectively, for 0·75 and 0·875 per cent phenol, the effect of temperature was negligible. Above these temperatures

the effect increased markedly, confirming the existence of a minimum temperature for the activity of low concentrations of phenol (Jordan and Jacobs, 1946b). Berry and Michaels (1950) also obtained higher Q_{10} values for the weaker concentrations of monoalkyl ethers of ethylene glycol examined. The values of n calculated from the results in Fig. 3 increased slightly with increase in temperature (Table III).

TABLE III

CONCENTRATION EXPONENTS FOR THE MORTALITY OF *P. notatum* SPORES IN SOLUTIONS OF PHENOL

Phenol concentration (per cent)	Temperature (°C)	n
1 to 1·25	15	7·9
1 to 1·25	20	8·0
1 to 1·25	25	9·6
0·875 to 1·125	30	9·9
0·75 to 1·0	35	11·3
0·75 to 0·875	40	9·8

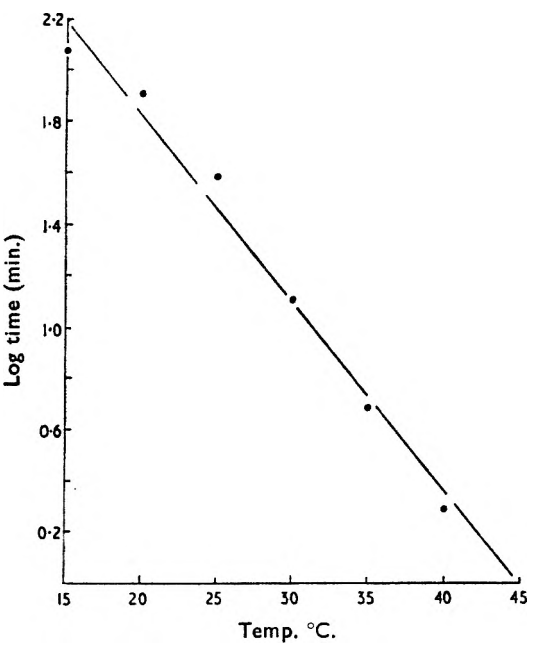

FIG. 5. Log time-temperature relationship for 99 per cent mortality of *P. notatum* spores in 1·125 per cent phenol.

The times required for 99 per cent mortality of *P. notatum* spores in 1·125 per cent phenol at temperatures of 15 to 40° were calculated from the results shown in Fig. 4. The calculated log. time-temperature regression for this percentage mortality was linear (Fig. 5) and its slope, log θ, gives mean values of 1·19 and 5·7 for θ and Q_{10}. These values compare favourably with those shown in Table II. Similar values of Q_{10} have

been obtained for phenol with bacteria (Chick, 1908; Jordan and Jacobs, 1946a; Tilley, 1942).

The marked difference in the Q_{10} values obtained for the death of *P. notatum* spores exposed to moist heat and phenol indicates an essential difference in the mode of action of these two lethal agents.

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A NOTE ON THE ANALYSIS OF OIL OF PEPPERMINT BY AN ALUMINIUM OXIDE-SILICIC ACID DOUBLE COLUMN

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Received March 20, 1962

The separation and determination of the constituents of oil of peppermint have been effected using two columns in succession, one consisting of aluminium oxide and the other of silicic acid.

The separation of the constituents of oil of peppermint has been improved by using the behaviour of the constituents on aluminium oxide and silicic acid columns.

Experimental

Five g. of aluminium oxide is packed in column 1 (C_1) of the apparatus (Fig. 1), (0·7 cm. in internal diameter and 30 cm. in length) and 10 g. silicic acid in column 2 (C_2) (1·0 cm. in internal diameter and 30 cm. in length). Funnel (F_1) is filled with light petroleum (b.p. 30–50°) and Funnel (F_2) with a mixture of 5 per cent ether in light petroleum.

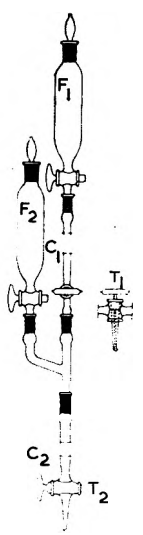

Fig. 1. Two Column System for the Evaluation of Oil of Peppermint.

About 100 mg. of oil of peppermint is accurately weighed and transferred quantitatively to the top of column 1 and light petroleum is allowed to percolate through. The three-way tap (T_1) is regulated so that the surface of the silicic acid in column 2 is always covered with about 1 cm. of the light petroleum effluent from column 1.

140 ml. of light petroleum is collected from the second column. At this stage the constituents of oil of peppermint are located as follows:

1. The hydrocarbons are present in the light petroleum effluent and are evaluated gravimetrically after allowing the solvent to evaporate at low temperature.
2. Menthone and menthyl acetate are on column 2.
3. Menthol is left on column 1.

The 5 per cent ether in light petroleum mixture is then allowed to pass through the silicic acid column immediately following on the light petroleum layer. Twenty ml. of this ether light petroleum mixture is collected, evaporated at 40° and assayed by the hydroxylamine hydrochloride method of the Egyptian Pharmacopoeia (1953) using 0·1N ethanolic potassium hydroxide to determine the eluted menthone.

Elution is continued with the same solvent mixture; 70 ml. of pure solvent is collected before the appearance of menthyl acetate. The following 20 ml. will elute all the menthyl acetate which is then determined by evaporating the solvents at 40° and using the Egyptian Pharmacopoeial method with 0·02N ethanolic potassium hydroxide and refluxing on a boiling water bath for 2 hr.

Menthol which is held by aluminium oxide column is then washed out by 15 ml. of chloroform and collected through the three-way tap (T_1).

The chloroformic effluent is then made up to 100 ml. by chloroform, and menthol is determined colorimetrically by the method of Masamune (1933) using an Ogal colorimeter. The results are shown in Table I.

TABLE I

ANALYSIS OF OIL OF PEPPERMINT ON ALUMINIUM OXIDE AND SILICIC ACID COLUMNS

Wt. of oil in mg.	Eluted constituents per cent			
	Hydrocarbons	Menthyl acetate	Menthone	Menthol
109	11·30	15·90	21·72	50·85
120	11·45	16·00	21·42	50·60
115	11·75	15·85	21·70	50·88
106	11·00	15·92	21·56	50·51
Mean	11·33	15·91	21·60	50·71

Small quantities of the oil (about 100 mg.) can be analysed by this technique, while about 15 g. of the oil is required for the pharmacopoeial methods.

Acknowledgement. The authors acknowledge with cordial thanks the valuable help and advice they have had from Dr. S. R. El-Deeb, Professor of Chemistry of Crude Drugs, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, U.A.R.

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LETTERS TO THE EDITOR

The Identification of Digoxin Metabolite B (Digitoxin Metabolite C) with Digoxigenin Di-Digitoxoside

SIR,—The cardiac glycosides digoxin and lanatoside C are converted by the rat and by man to *metabolite B*, a derivative of digoxigenin (Brown and Wright, 1956; Ashley and others, 1958). Digitoxin (Brown and Wright, 1956) and acetyldigitoxin (Martin and Wright, 1960) also undergo metabolism in the rat to give *metabolite C* which could not be separated from *metabolite B* on several paper chromatography systems (Ashley and others, 1958) and which is therefore formed by β-hydroxylation of the steroid nucleus of digitoxin at position 12. Using paper chromatography Brown and Wright (1956) could not separate *metabolite B* from *metabolite X* obtained from rat urine after digoxigenin administration and therefore believed that *metabolite B* did not contain digitoxose. Repke, Roth and Klesczewski (1959) however obtained a metabolite (compound 8) after digitoxin administration to rats which behaved similarly to *metabolite B* (= *metabolite C*) of Brown and Wright (1956) on paper chromatograms. This metabolite gave a positive xanthydrol test for digitoxose and could not be separated from digoxigenin di-digitoxoside by paper chromatography.

Confirmation that *metabolite B* obtained from digoxin is identical with digoxigenin di-digtoxoside has now been obtained by the use of radioactively labelled ^{14}C digoxin.

Biosynthetically labelled ^{14}C-digoxin (specific activity $3{\cdot}55 \times 10^5$ c.p.m./mg.) 0·19 mg. diluted with 0·3 mg. of non-radioactive digoxin was injected into a femoral vein of each of four anaethetised rats and the bile collected through the cannulated bile ducts for 5 hr. The metabolites in the bile were separated by paper chromatography (Cox and Wright 1959) and the *metabolite B* area on the chromatogram eluted with methanol. To the eluate was added 21 mg. of digoxigenin di-digitoxoside prepared by the method of Haack, Kaiser and Spingler (1956). The recovered digoxigenin di-digitoxoside was recrystallised to constant specific activity from acetone-light petroleum (b.p. 60–80°) and specific activities of 377, 394, 404, 409 c.p.m./mg. were obtained in four separate recrystallisations. The melting point of the recovered digoxigenin di-digitoxoside (219–222°) was undepressed by a sample of this substance kindly supplied by Dr. E. Haack.

It may be concluded that lanatoside C and digoxin undergo progressive loss of sugar residues in the rat and in man to give digoxigenin di-digitoxoside. The same metabolite is produced from digitoxin and acetyl digitoxin by C(12)-hydroxylation and the loss of one molecule of digitoxose or acetyl digitoxose. The nature of *metabolite X* found in rat urine after digoxigenin administration is being re-investigated.

The author wishes to acknowledge the careful technical assistance of Mr. W. Golder in this investigation and a gift of ^{14}C-digoxin from Burroughs Wellcome & Co. (U.S.A.) Inc.

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July 6, 1962

S. E. WRIGHT.

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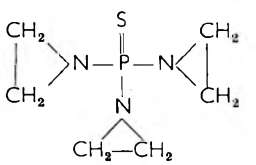
Estimation of Thiotepa in Urine

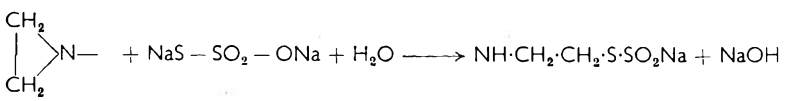
SIR,—When given in doses sufficient to suppress malignant tumours, Thiotepa has been shown to cause a severe, and sometimes fatal, depression of the bone marrow (Cree, 1960). For this reason topical and localising methods of treatment have been sought and applied with some success (Abel, 1960). In order to assess the possible systemic effects of treating bladder tumours by instilling Thiotepa directly into the bladder, it was necessary to estimate the quantity of the drug introduced, and that obtained by washing out the bladder, usually three hours later (Jones and Swinney, 1961). Because variations in technique and application may require a reassessment of this factor, and since the chemistry of the analytical reaction is unusual, the method used in these estimations is briefly reported.

Principle of Method

Thiotepa is *NN'N''*-triethylene thiophosphoramide.



Whilst information on its chemistry is largely unpublished, the ethylene-imino ring group is known to be rapidly attacked by nucleophilic reagents, of which thiosulphate is particularly suitable for reactions in aqueous solution (Golumbic, Fruton and Bergmann, 1946). The reaction which is catalysed by an acid medium (Ross, 1950, see. p. 2269), results in the liberation of sodium hydroxide equivalent to the number of ethylene imino rings attacked.



The estimation is therefore made by dissolving the drug in thiosulphate and titrating the liberated NaOH with HCl, adding an excess to bring the mixture to approximately pH 4 (methyl orange). The reaction is then allowed to go to completion (30 min.) and the amount of acid still unneutralised estimated by titration with NaOH to pH 8 (phenolphthalein).

Method

The concentrations of Thiotepa used in this study were about 1–2 mg./ml., and samples of 2–5 ml. were taken for analysis. The sample was extracted three times with 2 ml. of chloroform in a 10ml. stoppered tube. In the

presence of urine it was usually necessary to separate the layers by centrifugation. The combined extracts were then evaporated to dryness *in vacuo* at room temperature, and to the residue, 10 ml. of 20 per cent sodium thiosulphate solution, and one drop of methyl orange were added. The solution was then titrated with 0·1N HCl until the indicator remained red for 10–15 sec. (V_1). 30 min. later two drops of phenolphthalein were added, and the solution titrated to a pink colour with 0·1N NaOH (V_2). A blank estimation was made by treating 10 ml. of thiosulphate in a similar way (B_1 and B_2). Then $(V_1 - V_2) - (B_1 - B_2)$ = volume of 0·1N HCl equivalent to the NaOH liberated by the reaction. Since each molecule of Thiotepa (mol. wt. 189·3) contains 3 ethylene imine rings, multiplication of this volume by 18·93/3 gives the number of mg. of Thiotepa in the sample taken.

As pure crystalline Thiotepa was not available it has not been possible to check every aspect of the analytical procedure which must therefore form the basis for further study. However, estimations of the drug in standard ampoules were consistent with the stated content and several analyses of a solution kept at −15° over a week gave reproducible results.

I am greatly indebted to Lederle Laboratories through the courtesy of Dr. J. R. Wilson for unpublished information on which this method was based. I also wish to acknowledge the help of Dr. B. E. Tomlinson of the Department of Pathology, The General Hospital, Newcastle upon Tyne, in whose department these studies were made.

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July 3, 1962

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The Action of Aryloxyaliphatic Acids on the Permeability of Blood Vessels

SIR,—It has been shown recently that certain aryloxyaliphatic acids have anti-inflammatory properties (Northover and Subramanian, 1961) and it was of interest, therefore, to study the correlation of chemical structure and biological activity in this series.

The method for measuring the effect of drugs on the permeability of peritoneal blood vessels has been described in detail elsewhere (Northover, 1962). Briefly, the method consists of following the movements of azovan blue dye from the circulation into the peritoneal fluid of mice given 4 ml. of 0·9 per cent saline solution intraperitoneally. The concentration of dye in the peritoneal fluid at the end of 1 hr. is a measure of the permeability of the peritoneal blood vessels to plasma albumin. Male mice weighing between 25 and 30 g. were arranged in groups of 12 animals each, and the groups were treated with graded doses of the substances under test. 0·1 ml. of a neutralised solution or suspension of the substance under investigation was administered subcutaneously to each animal. 30 min. later, 4 ml. of 0·9 per cent saline solution at 38° were given intraperitoneally to each mouse. Immediately afterwards each animal was

given 0·2 ml. of a 0·5 per cent solution of azovan blue in 0·9 per saline through a lateral tail vein. The peritoneal fluid was withdrawn 1 hr. after it had been administered, centrifuged, and its optical extinction measured.

The optical extinction for the group is expressed as a per cent of the value obtained with the untreated control group and this is termed the per cent permeability for the group. The test is performed with various doses of the substance under investigation, hence it is possible to calculate the dose which would produce a per cent permeability of 50 per cent, and this is termed the ED50 dose.

TABLE I

PERMEABILITY-INHIBITING ACTION OF ARYLOXYALIPHATIC ACIDS

Name	ED50 dose mg./kg.	Per cent permeability with 200 mg./kg.
Phenoxyacetic acid	—	70
p-Acetylphenoxyacetic acid	—	62
o-Aldehydophenoxyacetic acid	Inactive*	—
p-Carboxyphenoxyacetic acid	—	58
Resorcinoxydiacetic acid	Inactive	—
Vanilloxyacetic acid	Inactive	—
o-Chlorophenoxyacetic acid	42	35
p-Chlorophenoxyacetic acid	173	44
o-Cresoloxyacetic acid	—	62
p-Cresoloxyacetic acid	158	41
Eugenoxyacetic acid	—	72
Thymoxyacetic acid	40	13
4-n-Hexylresorcinoxyacetic acid	78	36
α-Naphthoxyacetic acid	37	13
β-Naphthoxyacetic acid	104	43
α-(*p*-t-Butylphenoxy) propionic acid	50	22
2-(*p*-(1,1-Dimethylpropyl)phenoxybutyric acid	51	27

* Indicates that the per cent permeability was not significantly different ($P > 0.05$) from the control All quoted figures are significantly different ($P < 0.05$) from the controls.

A number of aryloxyaliphatic acids were tested in this way and the results are given in Table I. Phenoxyacetic acid itself has little activity, whereas some of its substituted derivatives are active. Phenolic, aldehydic, acyl, and carboxylic substituent groups produce little activity. The greatest activity seems to be associated with alkyl and aryl substituent groups, although chlorine substitution also produces some activity. It appears that by increasing the size of the alkyl substituent group the activity is increased.

None of the compounds tested was acutely toxic to mice in doses up to 200 mg./kg. which was the highest dose tested. With one exception, all the compounds tested were either available commercially or were synthesised in the laboratory and the melting points checked against those in the literature. For the oxyacetic acid of n-hexylresorcinol we find no record in the literature of its preparation and properties so its identity is only provisionally established. Its equivalent weight as an acid was 252, and on analysis it gave 14 per cent carbon, 9 per cent hydrogen and 17 per cent oxygen.

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June 20, 1962

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Effects of Orally Effective Hypoglycaemic Agents from Plants on Alloxan Diabetes

SIR,—Five hypoglycaemic agents have so far been extracted by us from different plant sources, all of which are orally effective and are capable of reducing the blood sugar level of fasting as well as of glucose fed rabbits. The biological assay of these five products against tolbutamide as standard has been already reported (1961–62). The present communication describes the effects of three of these drugs on the blood sugar of alloxan diabetic rabbits.

Normal healthy rabbits weighing 2 kg. with fasting 18 hr. blood sugar levels of 100–125 mg./100 ml. were given intravenous injections of 180 mg./kg. of alloxan monohydrate in distilled water to make them moderately diabetic. After allowing 7 days for the fasting blood sugar levels to stabilise, the effects of oral administration of the drugs were observed in groups of 18 hr. fasted animals for a period of 4 hr. and the hypoglycaemic effects compared with that produced by tolbutamide, under similar conditions. Blood sugar was determined by the micromethod of Folin and Malmros (1929). The results are given in Table I and indicate the possibility of the extracts from *Ficus*

TABLE I

EFFECTS OF HYPOGLYCAEMIC AGENTS FROM PLANTS ON THE BLOOD SUGAR OF ALLOXAN DIABETIC RABBITS COMPARED WITH TOLBUTAMIDE

Drug tested (dose/kg.)	Average blood sugar values for six rabbits (mg./100 ml.)					Max. fall in fasting blood sugar per cent
	Initial	1 hr.	2 hr.	3 hr.	4 hr.	
Ficus bengalensis stem bark (95 per cent ethanol) 1·25 g.	214·4	202·6	194·8	226·4	230·4	9·14 ± 1·1
Ficus religiosa root bark (water) 1·25 g.	181·9	186·9	211·0	154·22	152·2	15·22 ± 1·48
Allium cepa bulbs (light petroleum, 60–80°) 0·25 g.	179·08	143·7	158·8	179·08	172·32	19·76 ± 1·78
Tolbutamide (standard) 0·25 g. ..	180·3	151·2	128·4	132·4	133·5	28·78 ± 2·98
Distilled water (control) 10 ml. ..	190·4	196·9	186·3	188·4	194·0	2·20

bengalensis, *F. religiosa* and *Allium cepa*, being useful as substitutes for tolbutamide in controlling alloxan diabetes in rabbits.

Preliminary experiments in this laboratory have also indicated that neither tolbutamide nor the above three drugs are capable of reducing the fasting blood sugar level of completely depancreatised rabbits in 4 hr. after oral administration. The mechanism of action of these drugs may, therefore, be expected to be similar to that of tolbutamide.

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Function of Mast-Cells

SIR,—Both heparin (Jorpes, 1946) and histamine (Riley and West, 1953) are now known to be concentrated in tissue mast-cells, but the physiological function of each still remains uncertain. The release of histamine as a result of damage to the mast-cells might be expected to be accompanied by the release of heparin and a consequent increase in the clotting time of blood. However, this dual release has been observed in only one species, the dog, in which an intravenous injection of peptone or of a chemical histamine liberator such as compound 48/80 may release sufficient heparin to render the blood incoagulable. In contrast, compound 48/80 releases histamine from the mast-cells of the rat but the clotting time of the rat's blood remains unchanged. Where does the released heparin disappear to in the rat?

Some time ago it was found (Riley, Shepherd, West and Stroud, 1955) that the almost complete release of histamine from the subcutis of the rat by compound 48/80 is accompanied by a loss of only half of the associated heparin. At that time, it was suggested that some of the metachromatic material from the disrupted mast cells is diposed of locally by macrophages, some adheres to connective tissue cells and some is bound to the basic histamine-liberator itself. The function of heparin was thought to be concerned rather with events in the tissues than with the coagulability of the circulating blood.

Now, Riley (1962) has shown that both histamine and heparin can and do act primarily on the connective tissues and that they act in sequence, histamine stimulating mesenchymal cells to phagocytose and digest metachromatic material released from nearby mast-cells. The connective tissue cells are thereby stimulated to produce on their own account fresh and specific mucopolysaccharide and contribute in this way to the formation of extracellular ground-substance. Once this has served its purpose, it may, in turn, be broken down, rebuilt, and stored in sulphated form in the granules of tissue mast-cells. Riley has proposed, in this way, a place for histamine in the cycle of the mast-cell. But perhaps of even greater importance is the fact that 5-hydroxytryptamine exerts a more powerful phagocytocic action than does histamine (Northover, 1958) and the former amine is located in, and is released from, some mast-cells in the rat and mouse (Parratt and West, 1957). This may also explain why the recovery rate of mast-cells and tissue histamine is more rapid after treatment with compound 48/80 (which releases histamine and some 5-hydroxytryptamine) than after treatment with polymyxin B (which releases only histamine). Further, low doses of most histamine-liberators have been shown to release as much as 30 per cent of the tissue histamine without affecting the histological appearance of the mast-cells (West, 1956), such tissues then regaining their normal complement of histamine within a short interval of time (about 3 days).

Mention must also be made of the lipaemic-clearing property of heparin since the concentrations required for this effect are at least one-fifth those needed for an anticoagulant action. Here again it appears that heparin is being produced not for its anticoagulant action but for a local more important effect on connective tissue whereby the repair of injured tissue is speeded up. Preliminary studies on wound healing in rats using the method of Boyd and Smith (1959) shows that intense activity in the mesenchyme may be produced locally by injecting a mixture of histamine and heparin but not by histamine alone. Wound healing as measured by tensile strength is stimulated by locally administered heparin which among other things may neutralise the action of local adrenal corticoids.

This evidence supports Riley's hypothesis that the primary action of histamine,

first released when the mast-cell is injured, is to prepare many more connective-tissue cells than are normally available in the reticuloendothelial system to receive the heparin that will follow. Another action of the released histamine may be to cause the appearance in the lymph of an enzyme capable of producing the nonapeptide, bradykinin, from one of the plasma globulins (Edery and Lewis, 1962); this peptide has already been shown to possess a potent vasodilator property, to increase capillary permeability, to cause the accumulation of leucocytes, and to produce pain.

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BOOK REVIEWS

MEDICINAL PLANTS IN NIGERIA. By Bep Oliver. Private Edition. Pp. 138. Nigerian College of Arts, Science and Technology, Ibadan, 1960.

This book is a preliminary review of the more important plant material used in native medicine in Nigeria. The object in view is "to provide a critical study of the scattered information that exists on drugs growing in Nigeria". It is hoped that this study will lead the way to the undertaking of research work to discover the active principles (if any) present in the drugs and to sort out drugs possessing useful medicinal properties from those which are inert. The introduction points out how much mumbo jumbo is associated with many native remedies.

Chapter II gives a tabulated list (Table I) of 94 plants yielding drugs which are, or have been, recognised in European medicine as therapeutic agents. The plants are arranged alphabetically according to their generic names, and particulars are arranged in six columns headed Name of Plant, Family, Part Used, Constituents, Medicinal Use, Other Uses.

For each plant in the Table an indication is given of the publication in which the plant or a product from the plant is or has been described, e.g., B.P., B.P.C., Indian Pharmacopoeia, International Pharmacopoiea, etc.

Chapter III records the "Chemical Constituents of Vegetable Drugs" and Chapter IV lists "Plants Used in Local Medicine" with particulars of their constituents, medicinal uses and a note of any other commercial or possible uses to which they might be put. 362 drugs are included in this list (Table II). Chapter V gives brief botanical descriptions of a selection (247 plants) of Nigerian Medicinal Plants, based largely on Hutchinson and Dalziel's *Flora of West Tropical Africa* and the revision (Vol. I, parts 1 and 2) made by R. W. J. Keay.

The descriptions are accompanied by 24 full-page plates of drawings of 46 of the more important plants. Chapter VI gives very brief notes on collecting and drying, and Chapters VII and VIII classify the plants according to their pharmacological action or therapeutic use. The book closes with an alphabetical index of the plants included in the text and a general bibliography.

The book is based upon a series of four lectures given in April, 1959, in the Pharmacy Department of the Nigerian College; it forms a very useful preliminary attempt to sort out the very numerous native medicinal products, and should be of much value to assist future research workers in choosing material for further investigation.

T. E. WALLIS.

ORGANOPHOSPHORUS POISONS. ANTICHOLINESTERASES AND RELATED COMPOUNDS. By D. F. Heath. Pp. vi + 403 (including Indices). Pergamon Press, Oxford, 1961. 80s.

The number of research papers on organophosphates (with insecticidal and anticholinesterase properties) which have appeared since Schrader carried out his pioneer work 30 years ago is tremendous and reviews of the subject turn up at short intervals. These usually deal with selected topics and thus there is great need for a book which presents the more important aspects on a broad basis and which bridges the gap between various disciplines. This the author has set out to do. He reviews fundamental aspects of the chemistry, biochemistry and pharmacology of organophosphates. At the same time he tries to cater for the novice as well as the expert in an individual field who wishes to know something about related work. To do this in the space available it was necessary to use some drastic surgery regarding references to published work, to restrict severely the presentation of details and to acquaint the reader with a large number of basic concepts.

The presentation of the material is lucid and the sections on chemistry and biochemistry contain a large number of figures and tables summarising neatly a wealth of information which the author has extracted with great patience from a large number of individual papers. Pharmacological aspects are frequently more difficult to tackle than chemical and biochemical aspects if one aims at putting everything into nice little packages and one can't help having the feeling that in the sections on pharmacology the main theme is at times obscured by details which should either have been put into small print or which should have been omitted as was done in some other sections. Some of the terminology, interpretations and conclusions in the sections on pharmacology might not find the full approval of purists amongst pharmacologists and physiologists.

References to published work are numerous and only in very few cases is original work referred to incorrectly or misleadingly. One of these exceptions can be found on page 325 where it is stated that "by mouth the LD_{50}'s to man of a few organophosphates are about as follows. . . ." This might shake anyone who does not know the original paper from which this information was taken and which only lists "lethal doses" calculated from the inhibition of acetylcholinesterase by low doses of organophosphates.

The book is well produced, with the exception of some kymograph records, well indexed and cheap considering the wealth and quality of the information which it contains. It is undoubtedly a credit to the author and can be recommended to all who work on organophosphates or intend to do so, particularly if they are younger pharmacologists who are interested in biochemistry but have only limited or no biochemical training. F. HOBBIGER.