RESEARCH PAPERS

PHARMACOLOGICAL AND BIOCHEMICAL EFFECTS OF 5-HYDROXYTRYPTAMINE IN ADRENALECTOMISED RATS

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In adrenalectomised rats 5-hydroxytryptamine induces a fall of temperature and blood pressure which is more marked than in intact animals. This hypersensitivity is not related to changes in tissue monoamine oxidase or serum ceruloplasmin. Administration of 5-HT raises the levels of tissue 5-HT in adrenalectomised rats to the same extent as in control rats.

IN adrenalectomised rats and mice 5-hydroxytryptamine (5-HT) is several times more toxic than in intact animals (Canal and Maffei-Faccioli, 1958; Garattini, Gaiardoni, Mortari and Palma, 1961; Munoz, Schuchardt and Ferwey, 1958). This enhanced toxicity is not shared by partially hepatectomised animals (Garattini and others, 1961). Furthermore adrenalectomy does not induce unspecific sensitivity to chemical treatment since tryptamine (Palma, unpublished results), methacholine and adrenaline (Loew and Woodman, 1956) are similarly toxic in intact or adrenalectomised animals.

This increased toxicity of 5-HT may be a direct effect, that is increased sensitivity to the pharmacological activities of the amine, or the result of an impaired metabolism of 5-HT.

To test these hypotheses several experiments have been carried out. The results show that the decrease of body temperature and blood pressure induced by 5-HT in adrenalectomised rats is not related to major changes in the metabolism of 5-HT.

MATERIALS AND METHODS

Female Sprague-Dawley rats of the weight of 150–180 g. fed with a balanced diet were used. Adrenalectomy was performed under ether anaesthesia (Bomskov and Bahnsen, 1935). Animals received saline for drinking; treatment was begun 72 hr. after the operation. Sham-operated rats were also employed; the effect of 5-HT in these animals was not significantly different from that observed in intact rats.

5-HT was given subcutaneously or intravenously at the doses indicated in the Tables. Body temperature was measured with a thermocouple inserted in the rectal cavity. Blood pressure was determined, after anaesthesia with ethylurethane 1 g./kg. subcutaneously, by cannulation of the carotid artery and using a Sanborn 150 electromanometer.

Brain, spleen, lung and kidney 5-HT were extracted by the method of Shore (1959) and measured spectrofluorimetrically by the method of Bogdanski, Pletscher, Brodie and Udenfriend (1956). Monoamine oxidase activity in brain, liver, lung and kidney was measured by the method recently described by Weissbach, Smith, Daly, Witkop and Udenfriend (1960) using as substrate kynureninamine (obtained from Regis Chemical Co., Chicago, Ill.).

Serum ceruloplasmin activity was determined by the method of Ravin (1956) with minor modifications using *p*-phenylendiamine as substrate (Garattini, Giachetti and Pieri, 1960).

TABLE I

EFFECT OF 5-HT ON BODY TEMPERATURE IN INTACT AND IN ADRENALECTOMISED RATS (TEMPERATURE OF UNTREATED CONTROLS, 37·1 ± 0·3°C)

No. of rats	Experimental condition	5-HT dose (mg./kg.)	5-HT route	Changes in body temperature (° C. ± S.E.) 1 hr.	3 hr.	6 hr.	24 hr.
10	intact	5	s.c.	−1·9 ± 0·2	−1·2 ± 0·2	−0·6 ± 0·1	−0·8 ± 0·2
17	adrenalectomised	5	s.c.	−3·7 ± 0·3	−1·8 ± 0·3	−2·0 ± 0·5	−0·8 ± 0·4
10	intact	10	s.c.	−2·8 ± 0·3	−1·0 ± 0·2	+0·3 ± 0·1	−0·9 ± 0·3
18	adrenalectomised	10	s.c.	−3·8 ± 0·3	−4·0 ± 0·3	−3·6 ± 0·4	−2·3 ± 0·3
10	intact	25	s.c.	−5·2 ± 0·3	−3·8 ± 0·5	−0·3 ± 0·3	−0·8 ± 0·1
34	adrenalectomised	25	s.c.	−6·0 ± 0·3	−9·7 ± 0·5	−10·1 ± 0·7*	−12·5 ± 1·0†
10	intact	3·75	i.v.	−0·5 ± 0·2	+0·4 ± 0·1	−0·3 ± 0·1	−0·9 ± 0·2
8	adrenalectomised	3·75	i.v.	−3·5 ± 0·4	−3·2 ± 0·6	−3·5 ± 0·7	−2·0 ± 0·2
9	intact	75	i.v.	−5·0 ± 0·5	−2·0 ± 0·6	−0·6 ± 0·4	−0·5 ± 0·2

* 11 rats died.
† 26 rats died.

5-Hydroxytryptamine creatinine sulphate was obtained through the generosity of Vister Co., Casatenovo (Como).

RESULTS

5-HT *on body temperature of intact and of adrenalectomised rats.* The effect of 5-HT on body temperature of intact and of adrenalectomised rats is summarised in Table I. The amine is more active in adrenalectomised than in intact animals in decreasing body temperature. This effect

TABLE II

EFFECT OF 5-HT ON BLOOD PRESSURE IN INTACT AND IN ADRENALECTOMISED RATS. READINGS TAKEN 1 HR. AFTER ADMINISTRATION

Group	No. of rats	Experimental condition	Subcutaneous dose of 5-HT (mg./kg.)	Blood pressure (mm. Hg ± S.E.) Max.	Min.	Difference Max.	Min.
1	12	intact	—	143 ± 4	101 ± 6	—	—
2	13	intact	10	104 ± 3	62 ± 4	39	39
3	7	sham-operated	—	144 ± 4	104 ± 2	—	—
4	7	sham-operated	10	112 ± 5	54 ± 5	32	50
5	6	adrenalectomised	—	130 ± 7	103 ± 6	—	—
6	11	adrenalectomised	10	66 ± 6	45 ± 5	64	58
Significance for groups 1–2				P < 0·01	P < 0·01		
2–6				P < 0·01	P < 0·02		
1–5				P > 0·05	P > 0·1		
5–6				P < 0·01	P < 0·01		

is not only more marked immediately after administration, but it is also longer lasting. When it is injected intravenously, the effect of 75 mg./kg. in intact animals corresponds to that of 3·75 mg./kg. in adrenalectomised rats.

5-HT *on blood pressure of intact and of adrenalectomised rats.* Table II shows the effect of 5-HT on the blood pressure of intact and adrenalectomised rats weighing about 300 g.

5-HT decreases the blood pressure of intact, sham-operated and adrenalectomised rats. However the effect is more marked in the latter group than in the first two experimental groups.

EFFECTS OF 5-HT IN ADRENALECTOMISED RATS

5-HT *on tissue* 5-HT *of intact and of adrenalectomised rats.* The content of 5-HT in brain, lung, spleen and kidney of intact and of adrenalectomised rats before and after 5-HT administration is reported in Table III.

The results show that the 5-HT content of tissues of adrenalectomised rats does not differ markedly from that of tissues of control animals. After 5-HT administration, the increases in the content of lung, kidney and

TABLE III

TISSUE 5-HT OF INTACT AND ADRENALECTOMISED RATS BEFORE AND 1 HR. AFTER ADMINISTRATION OF 5-HT

No. of rats	Experimental condition	Treatment mg./kg., s.c.	5-HT base (μg./g. ± S.E.)			
			Brain	Lung	Kidney	Spleen
8	intact	—	0·38 ± 0·01	0·56 ± 0·02	0·18 ± 0·01	3·03 ± 0·15
8	intact	5-HT 10	0·39 ± 0·01	1·61 ± 0·17	1·42 ± 0·11	5·27 ± 0·17
10	sham-operated	—	0·37 ± 0·03	1·26 ± 0·04	0·33 ± 0·02	2·48 ± 0·09
10	adrenalectomised	—	0·28 ± 0·01	0·85 ± 0·04	0·26 ± 0·01	2·40 ± 0·07
8	adrenalectomised	5-HT 10	0·28 ± 0·02	1·97 ± 0·19	1·09 ± 0·08	3·32 ± 0·03

TABLE IV

MONOAMINE OXIDASE (MAO) ACTIVITY IN TISSUES OF INTACT, SHAM-OPERATED, AND ADRENALECTOMISED RATS, MEASURED AS THE DIFFERENCE IN OPTICAL DENSITY AT 360 mμ AFTER INCUBATION AT 37° FOR 20 MIN.

No. of determinations	Experimental condition	MAO activity (difference ± S.E.)			
		Liver	Brain	Kidney	Lung
7	intact	152 ± 17	123 ± 10	34 ± 8	57 ± 9
7	sham-operated	154 ± 12	120 ± 12	49 ± 12	52 ± 8
7	adrenalectomised	132 ± 32	116 ± 6	48 ± 16	57 ± 4

spleen of the two groups are also similar. That the metabolism is not changed by adrenalectomy is shown by the results obtained for the activity of monoamine oxidase (see Table IV) and by the fact that ceruloplasmin activity in serum is also unchanged.

DISCUSSION

5-HT decreases the body temperature of rats and mice (Hoffman, 1958; Lessin and Parker 1957) but its effect is more marked after adrenalectomy. The data obtained in the present investigation largely agrees with that recently reported by Hoffman (1959). Further, the fall in blood pressure exerted in rats by 5-HT (Erspamer, 1954) is enhanced by adrenalectomy. Both effects may be due to alterations in the mechanisms regulating body temperature and blood pressure. It is not likely that 5-HT is more active in adrenalectomised rats because of an impaired degradation. In the present experiments, the brain, kidney, spleen and lung 5-HT levels were not changed after adrenalectomy. Other authors found a raised level in the tissues of adrenalectomised rats but only when these were maintained on drinking water (Hicks and West, 1958) instead of receiving saline as in our experiments. As far as the brain 5-HT is concerned these results are in agreement with data recently reported (Towne and Sherman, 1960), but at variance with those reported by De Maio (1959).

The administration of 5-HT induces an increase of lung, spleen and kidney 5-HT, but no changes in the brain concentration (Costa, Rinaldi and Himwich, 1957; Sjoerdsma, Weissbach and Udenfriend, 1956; Woolley and Shaw, 1957). In adrenalectomised rats this increase is less than in normal rats and this may be due to a replacement of the stores of blood 5-HT which have been lowered by adrenalectomy (Medaković and Spužić, 1959).

The measurement of monoamine oxidase activity, the major enzyme responsible for 5-HT degradation (Brodie, Spector and Shore, 1959; Zeller, Blanksma, Burkard, Pacha and Lazanas, 1959), confirms that liver, brain, lung and kidney of adrenalectomised animals should metabolise the amine at rates comparable with those of intact or sham-operated rats. Ceruloplasmin oxidase activity, recently suggested as important for the metabolism of 5-HT in blood (Blaschko, 1960; Martin, Eriksen and Benditt, 1958; Porter, Titus, Sanders and Smith, 1957), is also unchanged by adrenalectomy. The significance of these results in explaining the enhanced toxicity of 5-HT in adrenalectomised rats is consistent with the hypothesis that an impairment of 5-HT metabolism is not the major cause. It may be that there is an increased sensitivity to the pharmacological effects of 5-HT, probably because there is a lack of the compensatory mechanisms. The fall in blood pressure and particularly in body temperature may be sufficient to account for the increased toxicity of 5-HT.

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SPECTROFLUOROMETRIC ANALYSIS OF CARDIOTONIC STEROIDS

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The sensitive and specific fluorimetric methods introduced by Jensen (1952; 1953) for digitalis estimation were re-evaluated for the assay of cardiotonic steroids and other steroids of biological interest. Characteristics of the excitation and fluorescence spectra were determined. Using a modification of Jensen's original digitoxin method and a spectrofluorometer, a linear relation was demonstrated between fluorescence intensity and concentrations from 0·01 to 6 μg./ml. for both digitoxin and digoxin. Application of these methods to the assay of gitoxin and cholesterol would seem to be equally practicable. In addition, scillaren A and B and strophanthin K can be made to fluoresce weakly. On the other hand, ouabain, cholestanol, testosterone, and hydrocortisone showed no fluorescence. Possible mechanisms of the reactions are discussed.

The dehydration of the cardiotonic steroids by treatment with strong acids or oxidising agents has long been recognised (Jacobs and Collins, 1924; Smith, 1935; Smith, 1936). Petit (1950) showed that the unsaturated anhydrogitoxigenin resulting from phosphoric acid treatment of gitoxin emitted fluorescence under ultra-violet irradiation which could be utilised for the quantitative estimation of the glycoside. Sciarini and Salter (1951) described a fluorometric method for digitalis estimation using hot sulphuric acid. These methods were superseded by the hydrochloric acid methods for gitoxigenin (Jensen, 1952) and digitoxigenin (Jensen, 1953).

The present investigation was made to extend the methods of Jensen to other cardiotonic steroids, to increase the sensitivity to levels usually found in biological fluids and tissues, and to define the excitation and fluorescence spectra of the compounds investigated.

Experimental

Methods and Results

Reagents and standards. The reagents were of standard reagent grade. Only glycerol, which had a very high blank fluorescence, required additional purification. This was achieved by passage through a column of activated charcoal. The steroids were obtained from commercial sources and were checked for homogeneity using the following paper chromatography systems: I. methyl isobutyl ketone and isopropyl ether (4 : 1 v/v) with formamide as the stationary phase (Gisvold and Wright, 1957); II. dichloromethane with formamide as the stationary phase. Both systems were used with the descending technique for 2 to 4 hr. on Whatman No. 1 paper. System II was the only method of the many

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tested which moved gitoxin without tailing. Digitoxin and strophanthin K, which were found to contain appreciable amounts of impurities, were purified by chromatography of mg. quantities using the same solvent systems on large sheets.

Measurement and spectral analysis of fluorescence were made in the Aminco-Bowman Spectrofluorometer or the Aminco-Keirs Spectrophosphorimeter with fluorescence attachment. A few correlative measurements were made with the Farrand Fluorometer Model A. Slit arrangements were chosen for maximum sensitivity or maximum specificity.

Composition of the reaction mixture. Attention was focused on the digitoxigenin method of Jensen which depends on the action of hydrochloric acid in the presence of hydrogen peroxide and ascorbic acid in

TABLE I

EFFECT OF OMISSION OF COMPONENTS OF REAGENT ON SPECTRA AND ON FLUORESCENCE INTENSITY OF DIGITOXIN

Composition of reagent	Relative fluorescence (per cent)	Activation peak (mμ)	Fluorescence peak (mμ)
HCl, ascorbic acid, H_2O_2, MeOH	100	395	570
HCl, H_2O_2, ascorbic acid	42	395	580
HCl	10	400	580
HCl, H_2O_2, MeOH	11	395 380	570 535
HCl, H_2O_2	7	370	520
HCl, ascorbic acid	19	450	500

methanol. Table I summarises the effects of omitting one or more components of this mixture on the fluorescence of digitoxin. Acid alone is sufficient for the development of fluorescence. When acid was omitted no fluorescence could be detected. However, for maximum intensity, the other components are obviously important. The peak wavelengths fall into three general groups. In the experiment in which only ascorbic acid was omitted (Table I, row 4), there was a gradual shift of the activation and emission peaks to the lower wavelengths after about 10 min.

The effects of variation in the ascorbic acid and hydrogen peroxide concentration were also studied. As reported by Jensen (1953), variation in ascorbic acid concentration from 0·2 mg./ml. of final solution to over 1 mg./ml. had no effect on intensity or stability of fluorescence. However, in contrast to Jensen's optimum peroxide concentration of $3{\cdot}75 \times 10^{-4}$ M in the final reagent mixture, we found the concentration range of $7{\cdot}7 \times 10^{-5}$ to $1{\cdot}5 \times 10^{-4}$ M to yield maximum intensity. Therefore, a concentration of $1{\cdot}15 \times 10^{-4}$ M was used in all subsequent studies.

Increased temperature of incubation during the development of fluorescence (40°, 60° and 80°) was found to diminish the maximum fluorescence, in accordance with Jensen's results.

Deterioration from exposure to ordinary fluorescent and incandescent room light was ruled out by experiments in which samples prepared under a red darkroom safelight as the sole source of light were found to yield

the same intensity and spectral distribution of fluorescence as samples prepared under ordinary conditions. Nevertheless it was observed that constant exposure to the exciting radiation in the fluorometer did cause some deterioration as indicated by an initial fall off of 1 to 2 per cent in

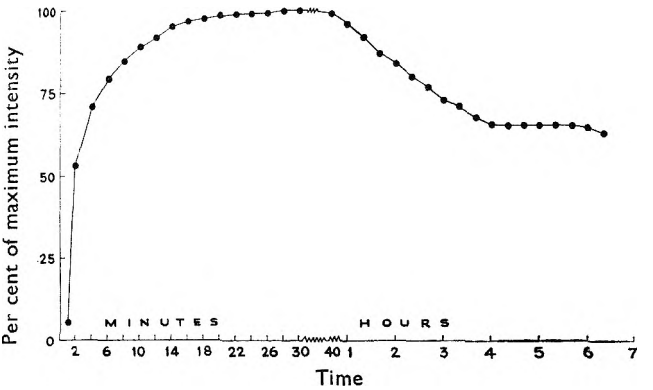

FIG. 1. Time course of fluorescence of digitoxin in the modified digitoxin reagent described in the text. Fluorescence intensity plotted as per cent of the maximum attained.

the galvanometer deflection during the first 2 min., followed by a slower decrease of 4 to 5 per cent over a period of 15 to 20 min. This deterioration was only partially reversible in the dark.

The development and decay of digitoxin fluorescence with time is shown in Fig. 1. Two stable periods were observed, one at the time of maximum fluorescence from 20 to 30 min. and a second after partial decay to approximately 70 per cent of maximum fluorescence in the 4 to 6 hr. period. The relatively earlier stable period compared to

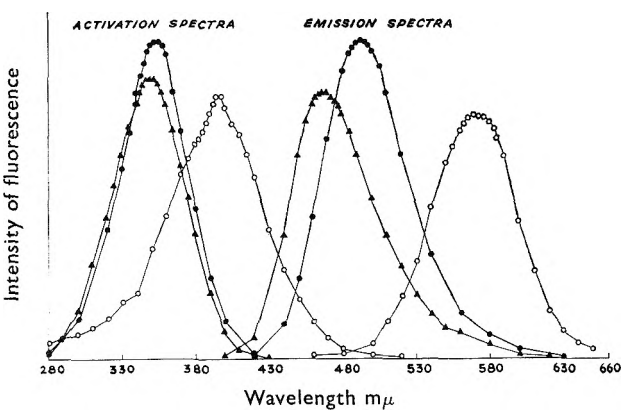

FIG. 2. Activation and fluorescence spectra of digitoxin, digoxin, and gitoxin using the modified digitoxin method. Activation spectra were measured with the fluorescence monochromator set at the previously determined fluorescence peak and vice versa. Fluorescence intensity in arbitrary units. Digitoxin, 10 μg./ml., ○—○; digoxin, 2·5 μg./ml., ●—●; gitoxin, 10 μg./ml., ▲—▲.

Jensen's period of 50 to 100 min. may be the consequence of the lower peroxide concentration which we used. Digoxin fluorescence, on the other hand, was found to remain stable for at least 24 hr.

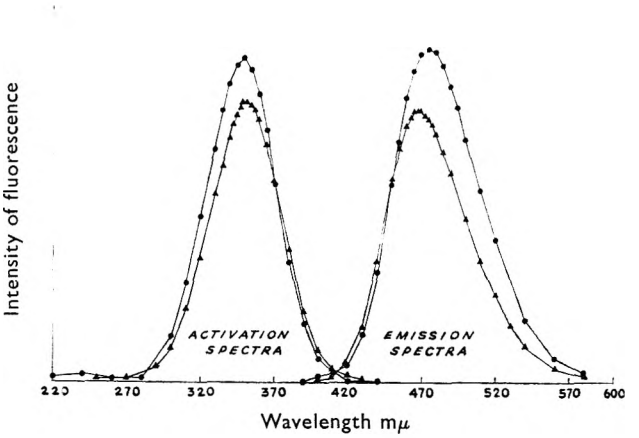

FIG. 3. Activation and fluorescence spectra of digoxin and gitoxin using Jensen's gitoxin method. Procedure as described in Fig. 2. Digoxin, 20μg./ml., ●—●; gitoxin, 10 μg./ml., ▲—▲.

From the preceding results the following optimal procedure was chosen. To the sample, dry or dissolved in a few μl. of methanol, are added 2 ml. methanol containing 2 mg. ascorbic acid, 3 ml. concentrated hydrochloric acid, and 0·2 ml. 0·003 M aqueous hydrogen peroxide. The tubes are immediately agitated to mix the contents and are then

TABLE II

FLUORESCENCE OF VARIOUS STEROIDS USING THE MODIFIED JENSEN TECHNIQUES

Steroid tested	Digitoxin method			Gitoxin method		
	Relative intensity (per cent)	Activation peak (mμ)	Fluorescence peak (mμ)	Relative intensity (per cent)	Activation peak (mμ)	Fluorescence peak (mμ)
Digitoxin	100	395	570	0	—	—
Digoxin	400	350	490	4	350	495
Gitoxin	125	350	470	100	350	470
Scillaren A	5	465	535	0	—	—
Scillaren B	0	—	—	4	465	535
Ouabain	0	—	—	0	—	—
Strophanthin K	0	—	—	1	350	470
Acetyl-strophanthidin	0	—	—	0·2	405	475
Cholesterol	21	470	530	0	—	—
Cholestanol	0	—	—	0	—	—
Testosterone	0	—	—	0	—	—
Hydrocortisone	0	—	—	0	—	—

allowed to stand at room temperature. After 20 to 40 min. the samples and a reagent blank are read in the fluorometer.

With the above procedure, activation and emission (fluorescence) spectra were determined for several cardiotonic steroids and for a few other steroids of biological interest. Spectra were similarly plotted for those compounds which were found to fluoresce with the gitoxin method of Jensen (1952) (equal volumes of glycerol and concentrated hydrochloric acid incubated with the sample at room temperature for 30 to 60 min.). Representative spectra are shown in Figs. 2 and 3. Table II summarises the results for all the steroids tested.

Application of the method to the assay of digitoxin and digoxin. Using the digitoxin method described above with activation and emission at the appropriate peak wavelengths, the concentration : fluorescence relation was found to be linear from 0·01 to over 6 μg. digitoxin per ml. of reaction mixture. A similar range of linearity was found for digoxin.

TABLE III

PRECISION OF THE MODIFIED DIGITOXIN METHOD

Steroid	Conc. (μg./ml.)	Mean fluorescence	Standard error n = 4
Digitoxin	0·02	5·1	0·23 (5 per cent)
	0·2	54·8	0·16 (0·3 per cent)
	2·0	514·0	2·12 (0·4 per cent)
Digoxin	0·02	24·4	0·48 (2 per cent)
	0·2	262·5	1·56 (0·6 per cent)
	2·0	2513·0	25·00 (1 per cent)

The reproducibility of the method was estimated from replicate determinations at three different glycoside concentrations and the results are shown in Table III.

The maximum sensitivity was arbitrarily defined as the least concentration of glycoside yielding a fluorescence intensity twice that of the reagent blank. Using the procedure outlined previously, with no special precautions, the least concentration meeting the above conditions was between 0·005 and 0·010 μg./ml. of reagent mixture for both digitoxin and digoxin.

Comparison of the fluorescence of digitoxigenin and digoxigenin with that of the parent glycosides demonstrated identical spectra and equivalent fluorescence intensity when calculated on a molar basis.

DISCUSSION

Of the non-isotopic digitalis assay methods in the literature, the fluorometric methods would seem to offer the maximum of both sensitivity and specificity. The modified Jensen method described in this paper offers a further increase in specificity if a spectrofluorometer is available. Thus, digitoxin and either digoxin or gitoxin can be measured separately in the same sample by selecting the appropriate activation and fluorescence

wavelengths. Jensen (1954) has described a simple and effective technique for the assay of glycosides on paper chromatograms. Application of the method to *direct* analysis of extracts of biological fluids and tissues is in progress but has been hampered by the presence of interfering fluorescent compounds.

Mechanism of the reaction. Bellet (1950) and Sasakawa (1959) have shown that the bluish-green fluorescent product of acid treatment of gitoxin is the Δ^{14}, Δ^{16}-dianhydro derivative of gitoxigenin. On the other hand, treatment of digitoxin and digoxin with concentrated hydrochloric acid yields the $\Delta^{8,(14)}$ anhydro compounds (Smith, 1935; Smith, 1936) which also fluoresce, according to our results. Thus it would appear that the orientation of the double bond at position 14 is not critical to the development of fluorescence. However, it may well be that the intensity and possibly the spectral characteristics of the fluorescence are related to the double bond position. From the three sets of peak wavelengths found for digitoxin by omitting various components of the reaction mixture (Table I), it would appear that there are at least three different fluorescent derivatives of digitoxin.

In the strongly hydrolytic conditions prevailing in the reaction mixture conversion of the glycosidic compounds to their genins is likely. That the sugars do not in any way influence the nature of the final product or its fluorescence is indicated by the identical spectra and intensities, mole for mole, which we obtained for two pairs of genins and glycosides. In the digitoxin method the fate of the hydroxyl group at position 3 after hydrolysis of the glycosidic linkage has not been established. Dehydration at this position with the formation of a Δ^2 or Δ^3 double bond may occur although we found no precedent in the literature for dehydration at this position under these conditions. This interpretation would seem to be supported by the observation that cholesterol which has only the single hydroxyl group at carbon 3 is converted in this reaction mixture from a non-fluorescent to a fluorescent compound whereas no fluorescence is produced in the gitoxin reagent which, as we have noted, dehydrates gitoxin at the 14 and 16 positions but not at position 3. However, other possibilities such as formation of an unusual complex at the 3 position remain to be ruled out. Isolation of the fluorescent products for subsequent structural analysis is in progress.

From the absence of fluorescence when cholestanol is treated with the digitoxin reagent mixture it would appear that more than one double bond is required in the final product. Every compound in this series which fluoresced had at least two hydroxyl groups or one hydroxyl group and a double bond in the steroid nucleus. However, some steroids with several hydroxyl groups or double bonds showed no fluorescence in either reagent mixture. In addition, since both squill and digitalis derivatives were capable of fluorescence, it is apparent that the number of double bonds in the lactone ring is not critical and may not contribute to the fluorescence of the final product at all.

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THE ANTITUSSIVE ACTIVITY OF GLYCYRRHETINIC ACID AND ITS DERIVATIVES

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The antitussive activity of 18β-glycyrrhetinic acid has been investigated using chemical stimulation in the unanaesthetised guinea-pig and electrical stimulation in the lightly anaesthetised cat. Derivatives of glycyrrhetinic acid were active in both types of experiment indicating a central antitussive effect. Several derivatives had approximately the same potency as codeine when given subcutaneously to guinea-pigs; one of these, dicholine glycyrrhetinic acid hydrogen succinate, exhibited the same degree of activity after oral administration.

LIQUORICE extracts have been included in cough mixtures for generations, although they are used to-day for their flavouring and demulcent properties rather than as active therapeutic constituents. An important constituent of liquorice is glycyrrhizin, the aglycone of which is glycyrrhetinic acid. We have examined 18β-glycyrrhetinic acid and a number of its derivatives for antitussive effect and here report activity of the same type and magnitude as that possessed by codeine.

MATERIALS AND METHODS

Materials

18β-glycyrrhetinic acid (G.A.), the ethanolamine salt of G.A., and the *N*-methylglucamine salt of G.A., were administered as suspensions in water. The choline salt of G.A. (choline G.A.), the piperazine salt of G.A., the disodium salt of G.A. hydrogen succinate, and the dicholine salt of G.A. hydrogen succinate (dicholine G.A.H.S.) were administered as solutions in water. G.A. and 3-keto G.A. were also administered as finely divided suspensions in 9 parts sesame oil : 1 part ethanol. Codeine was administered as the phosphate in solution in water and hydrocortisone hemisuccinate in solution in water or in suspension in water for high doses.

Method using Guinea-pigs

The compounds were investigated by a modification of the technique described by Winter and Flataker (1954). A guinea-pig was placed in a sealed glass chamber and coughs elicited by exposure to an aerosol of 3 per cent aqueous ammonia solution formed in a Riddostat inhaler (Riddell Products Ltd., London) using air at 10 lb./sq. in. Coughs were detected by means of a crystal microphone (Acos type Mic 35–1) connected by earthed co-axial cable to the upper beam of a Cossor double beam oscilloscope (Model 1049 Mk II). The lower beam was used for signals indicating the start and end of the period of exposure to the aerosol. Coughs could be recorded photographically with the aid of a standard camera attachment using a stationary signal spot and a film speed of 3 in./min. Examples of the records so obtained are shown in Fig. 1.

In all experiments, groups of ten animals were used for each dose of drug and the results were calculated as a percentage reduction in the mean cough counts obtained for 3 min. exposures to ammonia aerosol before and after drug administration.

Method using Cats

The technique used was that of Domenjoz (1952). Cats were lightly anaesthetised with pentobarbitone sodium intraperitoneally (usually 25 mg./kg.). Coughing was induced by electrical stimulation of the

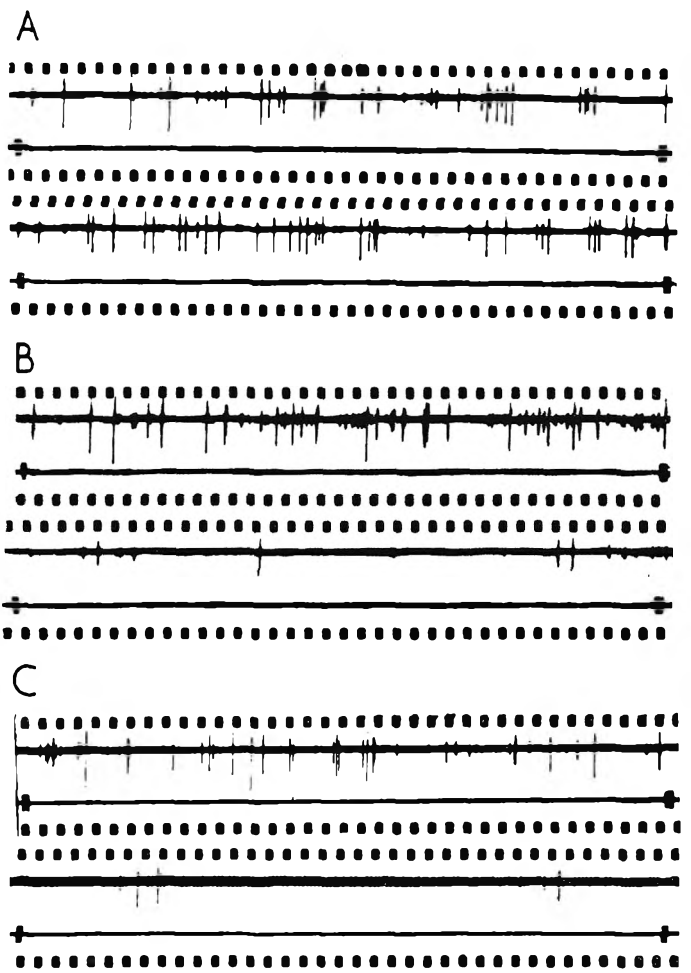

FIG. 1. Photographic records of antitussive effects in unanaesthetised guinea-pigs exposed to an ammonia aerosol.

(A) Normal saline s/c., (B) codeine 1 mg./kg. s/c., (C) Choline G.A. 1 mg./kg. s.c. In each example the upper record shows the coughs recorded before drug administration and the lower record the coughs recorded 1 hr. after drug administration. Coughs were recorded on the upper beam and the time interval on the lower beam of a standard double beam oscilloscope.

superior laryngeal nerve for 15 sec. at regular 3 or 5 min. intervals. The pulse width was fixed at 100 millisec., but the voltage (1 to 10 V.) and rate of stimulation (5 or 10 stimulations/sec.) were adjusted to obtain optimum responses from each animal. Coughing was recorded on a kymograph by a thread attached to the abdominal wall immediately below the inferior end of the sternum, and passed over pulleys to a Starling heart lever. Tension in the thread was adjusted so that cough responses were superimposed on a baseline of respiratory movement. Coughing in

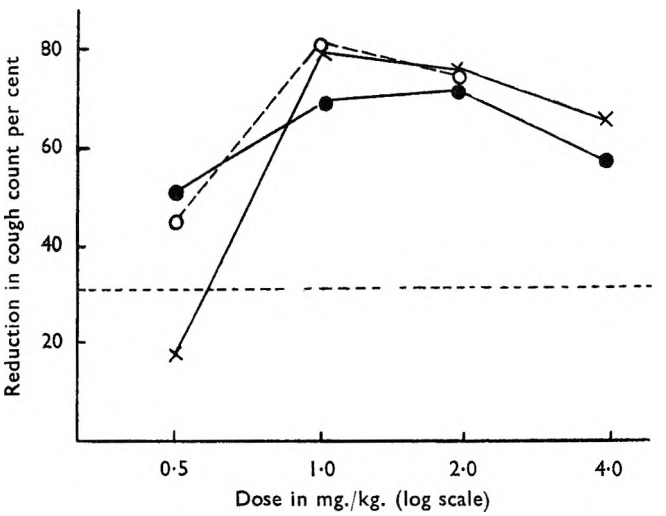

FIG. 2. The antitussive effects of codeine, choline G.A. and dicholine G.A.H.S. one hour after subcutaneous administration to unanaesthetised guinea-pigs. X—X Codeine. ●—● Dicholine G.A.H.S. ○- - -○ Choline G.A. The dotted line indicates the maximum effect (P = 0·95) expected after normal saline (see text).

response to stimulation culminated in an expiratory gasp recorded as a large upward excursion of the lever; its abolition was considered to be indicative of antitussive effect.

RESULTS

Subcutaneous Administration to Guinea-pigs

Control experiments. The effects of normal saline administration were first determined. In five groups of animals the highest reduction in cough count observed was 24 per cent. The mean value for these groups was 10·4 per cent with a standard deviation of 10·6 per cent and it was calculated that the maximum reduction in cough count to be expected at a probability level of 0·95 was 30·1 per cent. Only reductions in cough count in excess of this figure were subsequently considered indicative of antitussive activity.

Since the subcutaneous injection of the disodium salt of glycyrrhetinic acid hydrogen succinate had been reported by Finney and Tarnoky (1960)

to have a necrotic action in rat skin, two groups of animals were injected with a strongly necrotic solution which was not expected to have antitussive activity (tartar emetic 1 per cent in normal saline) to see whether or not the resultant irritation interfered with the cough counts of the animals. The observed reductions in cough count were 14 per cent and 1 per cent and well within the range obtained with normal saline.

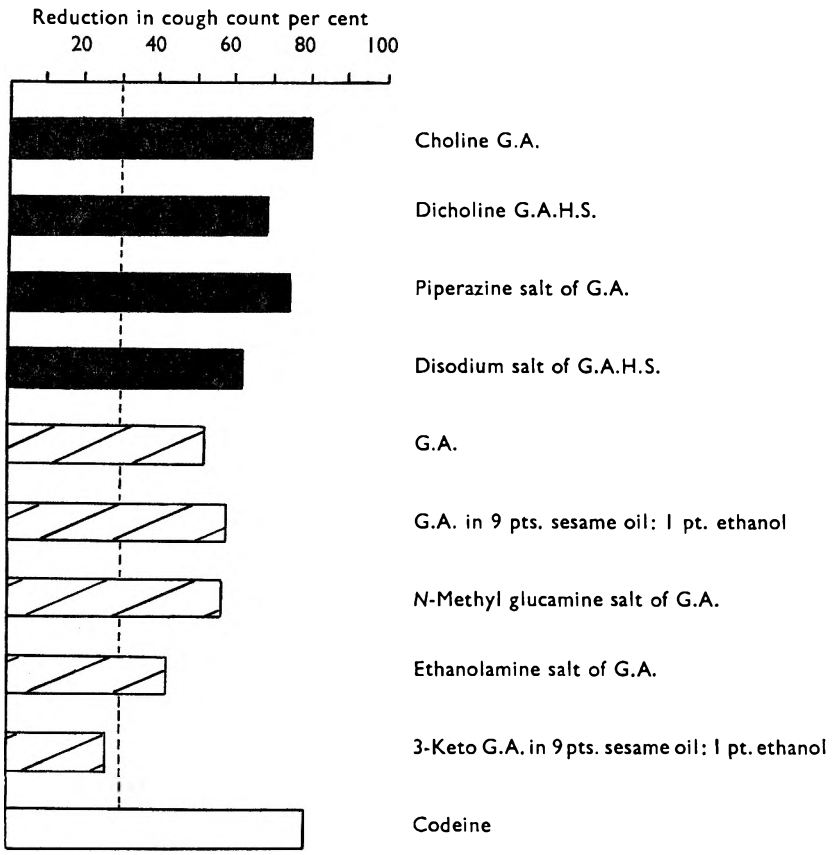

FIG. 3. The antitussive effect of a number of glycyrrhetinic acid derivatives one hr. after subcutaneous administration of 1 mg./kg. to unanaesthetised guinea-pigs. Solid columns indicate solution injected. Shaded columns indicate suspension injected. Last column—codeine 1 mg./kg. s/c. The dotted line indicates the maximum effect (P = 0·95) expected after normal saline (see text).

The activity of codeine, choline G.A., *and dicholine* G.A.H.S. These three substances were injected within the dose range of 0·25 to 4·0 mg./kg., and antitussive effects recorded 1 hr. later. Regression lines were obtained and are shown in Fig. 2.

Peak activity occurred with all three substances at about 1 mg./kg. At that dose all had a comparable activity.

The activity of other derivatives of 18*β-glycyrrhetinic acid.* A number of other derivatives as well as glycyrrhetinic acid itself were then examined

for activity at the 1 mg./kg. dose level. The results are shown in Fig. 3. All the compounds examined except 3-keto G.A. had activity in excess of the control level and several had an activity approaching that of codeine.

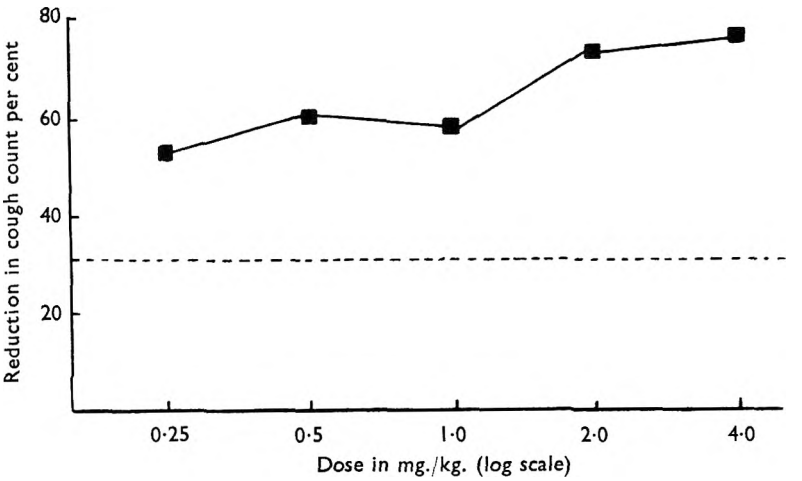

FIG. 4. The antitussive effect of hydrocortisone hemisuccinate one hour after subcutaneous administration to unanaesthetised guinea-pigs.
The dotted line indicates the maximum effect (P = 0·95) expected after normal saline (see text).

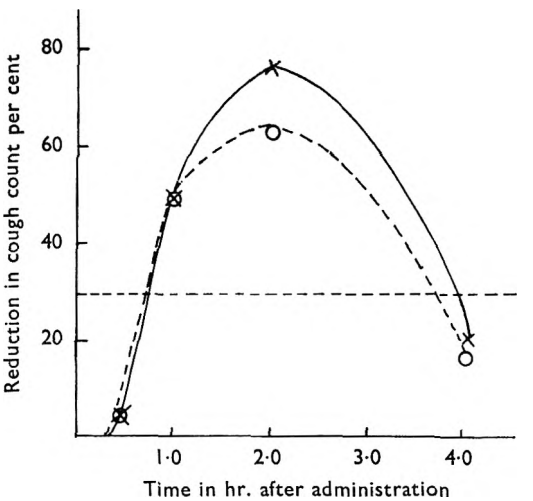

FIG. 5. The antitussive effect of codeine and dicholine G.A.H.S. at various times after oral administration of 5 mg./kg. to unanaesthetised guinea-pigs.
X—X Codeine. ○ – – ○ Dicholine G.A.H.S. The dotted line indicates the maximum effect (P = 0·95) expected after normal saline (see text).

The activity of hydrocortisone. Since glycyrrhetinic acid and some of the derivatives examined have been compared with hydrocortisone and reported to possess anti-inflammatory activity (Finney and Somers, 1958; Finney and Tarnoky, 1960), we examined hydrocortisone for antitussive

effect. It was observed to possess significant activity over the same dose range as codeine, choline G.A. and dicholine G.A.H.S. as shown in Fig. 4. But, the regression line obtained was different in slope from those obtained with the other three substances.

Oral Administration to Guinea-pigs

Preliminary experiments were conducted with codeine and dicholine G.A.H.S. to determine the time after oral administration that was coincident with maximum effect at the 5 mg./kg. dose level. The results are shown in Fig. 5. Maximum effect occurred 2 hr. after administration so this time interval between drug administration and measurement of antitussive effect was used in experiments with codeine, choline G.A. and dicholine

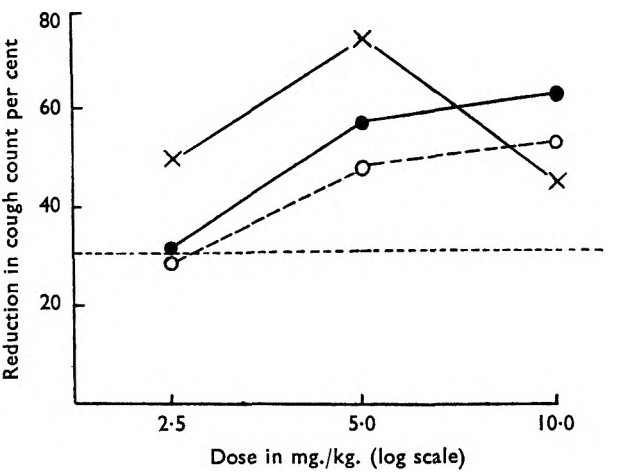

FIG. 6. The antitussive effects of codeine, choline G.A. and dicholine G.A.H.S. 2 hr. after oral administration to unanaesthetised guinea-pigs.
X—X Codeine. ●—● Dicholine G.A.H.S. ○ - - - ○ Choline G.A. The dotted line indicates the maximum effect (P = 0·95) expected after normal saline (see text).

G.A.H.S. The regression lines obtained are shown in Fig. 6. At the 5 mg./kg. dose level, the relative potency of dicholine G.A.H.S. to codeine was found to be the same as that observed after subcutaneous administration. Choline G.A., however, which had the same activity as codeine after subcutaneous administration showed only about half the activity of codeine when given orally. This might be due to poor absorption of choline G.A. from the gastrointestinal tract.

Intravenous Administration to Cats

The effects of hydrocortisone, codeine and choline G.A. in a single preparation are shown in Fig. 7. Doses of 1 mg./kg. and 2 mg./kg. of hydrocortisone were found to be inactive. After 1 mg./kg. of codeine the cough reflex was inhibited for 8 min. but returned after 11 min. A similar effect was observed with 1 mg./kg. of choline G.A. These

observations have been repeated in five preparations and found to be independent of the order in which the drugs were administered.

Fig. 8 shows the duration of the antitussive effects of codeine and choline G.A. within the dose range 1 to 4 mg./kg. Codeine and choline G.A. were active at the same dose levels but the duration of action of choline G.A. was approximately twice that of codeine.

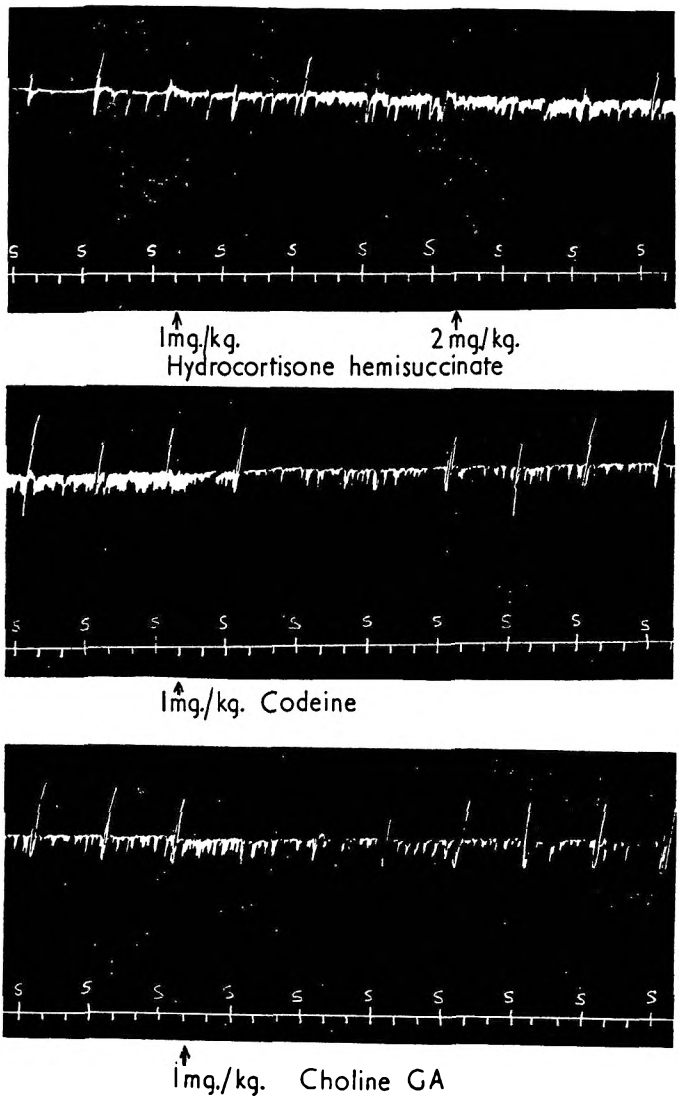

FIG. 7. A comparison of the antitussive effects of codeine, choline G.A. and hydrocortisone hemisuccinate after intravenous administration in the lightly anaesthetised cat. Coughs were recorded as large deflections upwards from a base line of respiratory movement. Time interval 1 min. S = stimulation.

Discussion

The evaluation of antitussive activity in a pharmacological laboratory presents certain difficulties. Although many methods are available and coughing can be induced by chemical, mechanical or electrical means, it is questionable whether the experiments imitate the type of cough stimulus for which antitussives are used in man. The methods used in the present study were selected because in our hands they proved both reliable and easy. In combination, they provided results from which it was possible to draw conclusions regarding the site of action of antitussive drugs. The guinea-pig technique is sensitive to peripheral actions on sensory

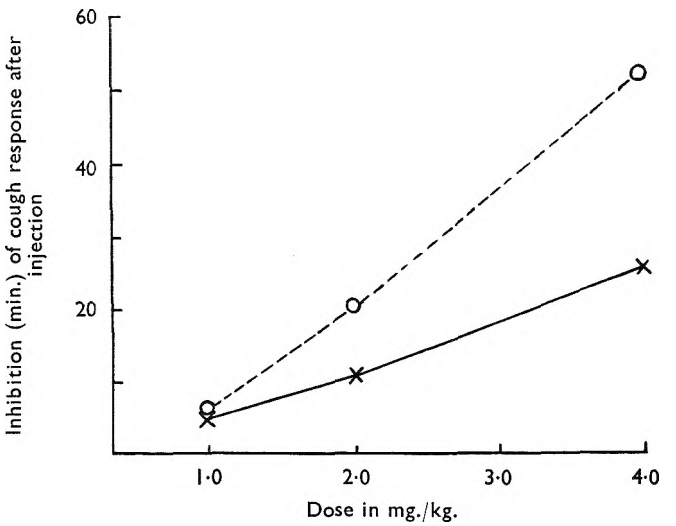

FIG. 8. A comparison of the duration of antitussive effect observed after various doses of codeine and choline G.A. administered intravenously to lightly anaesthetised cats. X—X Codeine. ○ - - - ○ Choline G.A.

nerve endings in the trachea as well as central actions on other parts of the cough reflex. In the cat method, sensory nerve impulses are derived from electrodes placed on the central portion of the sectioned superior laryngeal nerve so that only centrally acting antitussives can be detected. Thus hydrocortisone was found to be active in guinea-pigs but not in the cat, whereas codeine was active in both preparations. Choline G.A. was also active in both preparations, and it can therefore be concluded that it has central antitussive actions.

The guinea-pig technique could be criticised on the grounds that chemically induced coughing is artificial. Nevertheless it provides a rapid method of screening a number of compounds for antitussive activity if positive results are accepted with some reservation. The regression lines obtained with codeine, choline G.A. and dicholine G.A.H.S., using this technique are worthy of comment in that although antitussive activity increased with increasing dose, it then passed a peak value and declined. The reasons for this are not known, but the effect has been observed with

pholcodeine by Anderson and Smith (1959 unpublished) and opiate antitussives (Green, 1961, private communication). Although the fall in activity with high dosage might perhaps be indicative of an acute toxic response, no evidence for this has been found.

The discovery of antitussive activity in the glycyrrhetinic acid molecule is an interesting one. Some of its derivatives have activity of the same magnitude as codeine when given subcutaneously to guinea-pigs and one of these, dicholine G.A.H.S. is approximately equiactive when given orally. If dicholine G.A.H.S. has activity of this magnitude in man, the traditional use of liquorice for cough therapy can be considered to have some rational basis. Liquorice extracts, however, have too small a glycyrrhizin content for the full realisation of their antitussive potentialities.

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THE EFFECT OF PIPETTING ON THE CONCENTRATION OF HOMOGENEOUS SPORE SUSPENSIONS

PART II

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A concentrating effect produced by the pipetting of suspensions of spores of *B. subtilis* has been followed by using a spectrophotometer to determine optical densities of suspensions of spores and volumetric dilutions of such suspensions made by pipetting. The behaviour of suspensions of spores in capillary tubes has been investigated and used to explain in part the concentrating phenomenon.

METHODS of assessing the viable count of microorganisms involve at some stage the procedure of volumetric dilution. Gerrard (1959) has shown that serious errors can be introduced during the pipetting of spore suspensions of *P. spinulosum*, in preparation for roll tube counts. In a communication to the British Pharmaceutical Conference 1960, Gerrard and Porter reported that suspensions of spores of *P. spinulosum* became more concentrated on pipetting unless precautions were observed. This concentrating effect was studied by determining optical densities of the suspensions. The use of the spectrophotometer for this work was based on the observation that a linear relation existed between the gravimetric dilution factor and optical density for a given spore suspension at the wavelength employed. The phenomenon was attributed to the fact that in the pipetting of a spore suspension, a relatively spore-free film of liquid is retained on the pipette wall. Thus, during a successive series of pipettings, as in a serial dilution, the total volume of vehicle retained on the walls of the pipettes can cause a marked concentrating effect.

The importance of this effect in bacteriological work, led to the present investigation of the behaviour of spores of *Bacillus subtilis* during pipetting procedures.

There is a considerable dimensional difference between the fungal and bacterial spores; *P. spinulosum* spores are spherical in shape and have a diameter about 5μ, whereas, *B. subtilis* spores are ellipsoidal to cylindrical and measure approximately 1·5 μ by 0·8 μ.

EXPERIMENTAL AND RESULTS

Preparation of Spore Suspensions

10 day cultures of *B. subtilis* N.C.T.C. 3610 were prepared on nutrient Agar slopes (Oxoid Granules CM. 3) contained in 250 ml. flasks incubated at 37°. The spores were harvested in sterile water and washed three times. The washing process consisted of depositing the spores from aqueous suspension by centrifuging (R.C.F. 3,100 for 15 min.) and resuspending the packed cells in a fresh volume of sterile water. Suspensions prepared in this way were stored in the refrigerator to allow vegetative

forms to sporulate. Microscopic examination of stained preparations of samples from the suspensions indicated that the suspensions consisted of spores only.

Pipettes

Cleaning of pipettes. It was found that unless particular care was taken with the cleaning of pipettes the concentrating effect described was not apparent. The use of Teepol to aid the cleansing process had to be discontinued since, even after copious rinsings, sufficient of the surface-active agent remained on the walls of the pipettes to interfere with the practical work.

The method adopted was to rinse the pipettes thoroughly in chromic acid, and allow them to soak overnight in this acid. After repeated

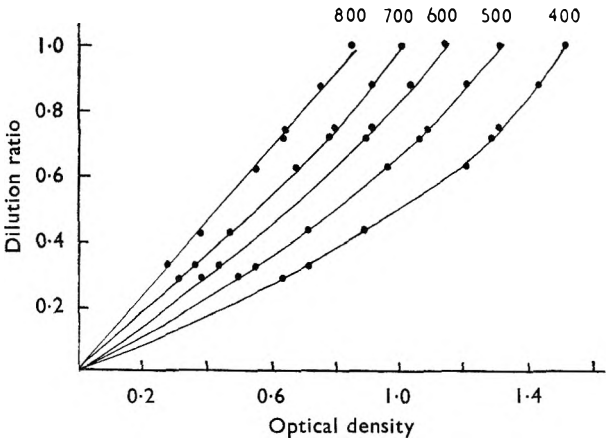

FIG. 1. The relation between dilution ratio and optical density. The figures at the top of the curves are wavelengths in mμ.

rinsing with distilled water in an automatic rinser, the pipettes were rinsed in absolute ethanol then ether and oven dried. The pipettes were finally sterilised in a hot-air-oven at 150° for 1 hr.

Choice and accuracy of pipettes. For this work which is essentially of a preliminary nature, Grade B pipettes designed to deliver their entire contents were used. These pipettes were graduated to 0·01 ml. and had an error of ± 0·02 ml. Pipettes of capacity 1 to 2 ml. were used.

Spectrophotometric Techniques

The Instrument. A Unicam S.P. 500 was used for the work described in this paper. All glass, optically matched 1 cm. cells were used with a Tungsten lamp as the light source.

The relationship between gravimetric dilutions of suspensions of B. subtilis *spores and optical density.* (*i*) *Choice of a suitable wavelength.* A heavy suspension of washed spores of *B. subtilis* was prepared, as previously described. The optical density of this suspension was measured. A series of gravimetric dilutions of this suspension were made in distilled

water and the optical densities of the diluted suspensions measured. Wavelengths in the range of 400 to 1,000 mμ were used for these measurements.

The relationship between dilution ratio (weight of master suspension/ weight of master suspension + water) and optical density became linear only at wavelengths of 800 mμ and over (Fig. 1). The direction of the non-linearity shown by the curves for wavelengths below 700 mμ indicates that lower optical densities are obtained than would be expected, at the higher concentration values. This would indicate that at wavelengths below 700 mμ, the more concentrated suspensions show a pronounced secondary scatter of light. The secondary scatter of light has the effect

TABLE I

OPTICAL DENSITY OF SUSPENSIONS AT VARIOUS DILUTION LEVELS

Wavelength in mμ	800	700	600	500	400	Dilution ratio
Original	0·855	1·022	1·150	1·375	1·530	—
1	0·760	0·930	1·0375	1·216	1·448	0·878
2	0·650	0·805	0·920	1·094	1·322	0·752
3	0·640	0·790	0·900	1·065	1·300	0·721
4	0·5575	0·695	0·8075	0·975	1·2125	0·629
5	0·382	0·480	0·665	0·720	0·895	0·428
6	0·290	0·370	0·445	0·560	0·721	0·330
7	0·2525	0·322	0·392	0·505	0·645	0·296

Specific gravity of suspension at room temperature = 0·9999.
Correlation of optical density at 800 mμ and dilution ratio — r = 0·998.

of redirecting the light, already scattered once out of the transmitted beam, so that some of it is returned to the original beam.

(ii) *Demonstration of linear relationship.* Having chosen a suitable wavelength range, a number of master suspensions of spores were prepared and the relationship between optical density (measure at 800 mμ) and gravimetric dilution ratio was found for each (Table I). In every case a positive linear relationship was found to exist between optical density of a suspension and its gravimetric dilution ratio. A statistical treatment of these results showed that a high degree of correlation was present between the two factors ($r = 0{\cdot}998$).

The relation between volumetric dilutions of spore suspensions of B. subtilis *made by pipette and the optical densities of such diluted suspensions. Experiment* (1). Five suspensions of washed spores of *B. subtilis* were prepared and their optical densities at 800 mμ determined. These suspensions will be referred to as master suspensions A, B, C, D and E (Table II).

Volumetric dilutions of the master suspensions were made by pipetting samples of suspension directly into a spectrophotometer cell and adding water from a burette (Class B 10 ml., tolerance ± 0·04 ml.).

The contents of the cell were thoroughly mixed by shaking and the optical density of the contained diluted suspension measured at 800 mμ.

The values obtained for replicate dilutions of the master suspensions are given in Table II. In every instance it will be seen that a concentrating effect is apparent in that the observed optical density of a diluted spore suspension is always in excess of the calculated value for that particular dilution.

Experiment (2). Two master suspensions of washed spores were prepared (F and G) and volumetric dilutions made with water. The procedure of making the dilutions differed from that used in Experiment (1)

TABLE II

VOLUMETRIC DILUTIONS OF SPORE SUSPENSIONS USING A 1 ML. PIPETTE
EXPERIMENT (1)

Suspension	Theoretical optical density	No. of replicate experiments	Observed optical density		Observed density of suspension × 100/ expected density
			Mean	Range	
Master A	—	—	0·88		
1 ml. (A) + 2·5 ml. H_2O	0·25	10	0·275	0·26–0·29	$\frac{0·275}{0·25} \times 100 = 110$
Master B	—	—	0·92		
1 ml. (B) + 2·5 ml. H_2O	0·26	6	0·285	0·28–0·29	$\frac{0·285}{0·26} \times 100 = 109$
Master C	—	—	1·20		
1 ml. (C) + 2·0 ml. H_2O	0·40	9	0·445	0·44–0·47	$\frac{0·445}{0·40} \times 100 = 110$
Master D	—		0·45		
1 ml. (D) + 1·25 ml. H_2O	0·20	3	0·245	0·24–0·25	$\frac{0·245}{0·200} \times 100 = 121$
1 ml. (D) + 1·50 ml. H_2O	0·18	3	0·215	0·21–0·22	$\frac{0·215}{0·18} \times 100 = 119$
1 ml. (D) + 1·0 ml. H_2O	0·23	3	0·265	0·26–0·27	$\frac{0·265}{0·23} \times 100 = 114$
Master E			0·88		
1 ml. (E) + 4 ml. H_2O	0·18	3	0·205	0·20–0·21	$\frac{0·205}{0·18} \times 100 = 114$
1 ml. (E) + 5 ml. H_2O	0·15	3	0·155	0·15–0·16	$\frac{0·155}{0·15} \times 100 = 103$
1 ml. (E) + 9 ml. H_2O	0·09	3	0·105	0·10–0·11	$\frac{0·105}{0·09} \times 100 = 116$

in that 2 ml. pipettes were used to measure the volumes of suspension and only the first ml. from each was delivered. As in Experiment (1) the dilutions were made directly into spectrophotometer cells and optical densities measured after thorough agitation of the contents of each cell. The values obtained for replicate dilutions of the master suspensions are given in Table III. It will be seen that no concentrating effect of the master suspension was obtained.

Study of Flow of Spores in a Capillary Tube

A capillary tube of elliptical cross section was attached vertically with plasticine to the stage of a microscope the optical axis of which was horizontal, the capillary tube having its major axis of cross section normal to the optical axis.

The spore suspension was introduced into the capillary tube through a polythene cannula and a needle and syringe barrel as shown in Fig. 2,A. The syringe acted as a reservoir the height of which could be varied by a

laboratory jack, and hence the rate of flow and position of meniscus in the capillary could be controlled.

When a descending column of suspension was observed through the microscope, stream line flow was seen, with peripheral spores moving more slowly and central spores more rapidly, than the meniscus.

TABLE III

VOLUMETRIC DILUTIONS OF SPORE SUSPENSIONS USING A 2 ML. PIPETTE
EXPERIMENT (2)

Suspension	Theoretical Optical density	No. of replicate experiments	Observed optical density		Observed density of suspension × 100/ expected density
			Mean	Range	
Master F			0·80		
1 ml. + 3 ml. H_2O	0·20	6	0·195	0·19–0·20	$\frac{0·195}{0·200} \times 100 = 97·5$
Master G			1·12		
1 ml. + 3 ml. H_2O	0·28	6	0·268	0·26–0·28	$\frac{0.268}{0·280} \times 100 = 95·7$

Slower moving peripheral spores, on approach of the meniscus, were projected into the faster central stream and not apparently left behind on the capillary wall above the meniscus (Fig. 2,B).

Observation of suspension flow in a 5 mm. bore tube showed that in accordance with the Poiseuille equation, the meniscus moved at half the rate of a spore in the centre of a tube.

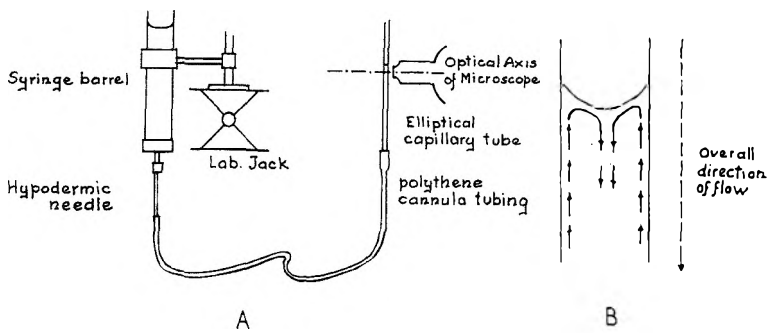

FIG. 2. A. Microscopical observation of behaviour of a *B. subtilis* spore suspension flowing down a capillary tube.
B. Relative movement of peripheral spores compared with that of meniscus.

Hence, if a delivery of 1 ml. is made from a full 2 ml. pipette, few or none of the spores originally at the periphery of the upper half of the 2 ml. of suspension will be ejected, and no concentrating effect should be apparent in the 1 ml. delivered.

DISCUSSION

It has long been recognised that the dilution procedures, involving the pipetting of suspensions of microorganisms, that form a part of any technique for determining viable count, can be a source of error. Ingram

and Eddy (1953) warned of the potential errors attendant upon the pipetting of suspensions of vegetative bacterial cells during dilution procedures, in preparation for viable count determinations. They found that the bacterial cells adhered to the walls of a pipette in such numbers that their dislodgement during the use of the same pipette for several successive dilutions could introduce serious errors. Errors introduced in this way may contribute to discrepancies in the final viable count of a given suspension, as reported by Fisher, Thornton and Mackenzie (1922). (See also Synder, 1947.)

To reduce this potential source of error, Bullock, Keepe and Rawlins (1949) have calibrated pipettes to deliver a determined volume of liquid between two calibration marks. Such pipettes will, indeed, give reproducible results in counts carried out with identical apparatus on a comparative basis. If, however, conditions for a series of dilution procedures are not identical, and this includes the precise position of the calibration marks on the stem of the pipettes, errors will become apparent.

When suspensions of spores of *B. subtilis* are placed in capillary tubes and examined with the microscope, the spores are seen to assume a definite uniform motion as the liquid column moves. A "funnelling movement" is seen to occur with the result that a fast flowing column of spores is formed in the centre of the capillary, leaving a relatively spore-free film of liquid on the wall of the capillary. This, in effect, means that at each pipetting the retention of spore-free liquid in the pipette has a concentrating effect on the spore suspension.

It has been shown that with pipettes which are designed to deliver their entire contents (i.e. not the volume between two fixed marks), the concentrating effect as judged by optical density readings can be about 7 per cent (see Table II). If, however, only a portion of the contents of such pipettes are delivered e.g. 1 ml. from a 2 ml. pipette, the concentrating effect can be negligible (see Table III).

This would suggest that one possible method of overcoming the concentrating effect, would be to calibrate pipettes to deliver a given volume of liquid between two points, the position of the points in relation to the entire length of the liquid column being a critical factor. The design and use of such pipettes for dealing with suspensions of spores of *P. spinulosum* has been described by Gerrard (1959).

As has been pointed out, the cleaning of glassware is a critical factor, in experiments of the type described. It was found that even minute traces of grease were sufficient to make the concentrating effect a variable factor. Surface-active cleaning agents, such as Teepol, left a fine film on the glassware which, also, detracted from the constancy of the concentrating phenomenon.

Earlier work described by Gerrard (1959), in which he followed the concentrating effect attendant upon pipetting of suspensions of spores of *Penicillium spinulosum*, showed the effect to be more marked and reproducible than the effect described here with bacterial spores. The reason for this might well lie in the comparative sizes of the two spores (viz. diameter about 3–5 μ for *P. spinulosum* and 1·5–2 μ for *B. subtilis*). It

has been found that a pipette of the type used in these experiments holding a liquid column of 16 cm. and having an internal diameter of 3 mm., will retain 30 mg. of liquid. This would correspond to a film of liquid along the interior of the pipette of 16 cm. length and 2 μ thick. A film of thickness of a similar diameter to a bacterial spore, might well be expected to retain these more readily than the much larger mould spores.

It is evident that many variable factors are at work and that detailed investigations will be necessary before the concentrating effects described can be followed on a truly quantitative basis.

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A NOTE ON THE ANTIDIURETIC EFFECT OF SMALL AMOUNTS OF ISOPRENALINE IN RATS

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Small subcutaneous amounts of isoprenaline, 0·5–1·0 μg./100 g. weight, caused reduction in the rates of excretion of sodium, potassium and water, both in sham operated and in adrenalectomised rats, and also in unoperated rats in the presence and absence of mannitol diuresis. These effects of isoprenaline were attributed to enhanced reabsorption of sodium and water in the proximal tubules.

Botting and Lockett (1961) demonstrated the antidiuretic action of as little as 0·25 μg. (±)-isoprenaline/100 g. weight injected subcutaneously into unanaesthetised rats. Later experiments (Botting, Farmer and Lockett, 1961) showed that this antidiuresis was accompanied by retention of sodium and chloride, a decreased excretion of ammonia and potassium and a rise in urinary pH; there was no demonstrable change either in glomerular filtration rate or effective renal plasma flow.

The extremely small quantities of isoprenaline required to elicit the urinary changes, suggested that they were brought about by change in the rate of secretion of neurohypophyseal or adrenal cortical hormones rather than by direct action of the amine on the kidneys. It is this hypothesis that we have sought to confirm or exclude by making examination of the effects of very small subcutaneous doses of isoprenaline during osmotic diuresis and in adrenalectomised animals.

Methods

Male rats of a single Wistar strain were fed diet 41 b. of Stein and were given water *ad libitum* unless otherwise stated. All were accustomed to stomach tubes and handling before use in experiments.

Water diuresis was induced, after absence of solid diet for 12–14 hr., by the oral administration of a standard water load equivalent to 5 per cent weight preceeded by an hydrating dose (Botting and Lockett, 1961). Combined mannitol and water diuresis was produced by the subcutaneous injection of 3 ml. 10 per cent mannitol per rat weighing 200–230 g. 30 min. before administration of the standard water load. Subcutaneous injections of 0·9 per cent NaCl, vasopressin and isoprenaline in 0·9 per cent NaCl were made immediately after the standard water load had been given. Urine was collected from individual animals for the succeeding period of 50 min.

Bilateral adrenalectomy and sham operations were performed through a middorsal incision on rats weighing 190–230 g. under pentobarbitone anaesthesia. Half the adrenalectomised animals were maintained by the addition of 0·4 per cent NaCl to their drinking water. The rest each received a daily injection of 0·1 ml. adrenal cortical extract and were

given the choice of tap or salt water to drink. Sham operated animals drank tap water.

All experiments were designed as cross-over tests in which treatments were assigned, day by day, to individual rats in an order predetermined by deliberate randomisation. Numerical values quoted are means $\pm$ the standard error of the mean followed by the number of animals within brackets. The significance of differences between means has been determined by *t* tests.

The concentrations of sodium and potassium in the urine were measured by means of an EEL flame photometer. Creatinine and diodone were estimated and their clearances were compared in cross over tests as described elsewhere (Botting, Farmer and Lockett, 1961).

Drugs. Isoprenaline sulphate (British Drug Houses Ltd.) Eucortone (Allen & Hanbury Ltd.) and pitressin (Parke Davis & Co. Ltd.) were obtained commercially.

Results

A comparison of the effect of a small subcutaneous injection of isoprenaline on water diuresis in adrenalectomised and sham operated rats. Comparison was made of the effect of subcutaneous injections of 0·5 μg. ($\pm$)-isoprenaline/100 g. weight and of saline on the rates of excretion of water, sodium and potassium by adrenalectomised and sham operated animals in the 50 min. after the administration of a water load and the making of these injections. This purpose was effected by means of a two day cross-over test made on the 4th and 6th postoperative days on one group of sham operated and two groups of adrenalectomised rats. One group of adrenalectomised animals had been treated with salt, the other with injections of adrenal cortical extract. In either case the treatment had been inadequate, for the rates of elimination of the water load were significantly lower ($P = <0{\cdot}01$) and those of excretion of $Na^+ + K^+$ higher ($P = <0{\cdot}01$) for the adrenalectomised than for the sham operated animals after control injections of saline (Table I). The Na^+/K^+ ratio in the urine was also higher in the adrenalectomised rats, 2·97 $\pm$ 0·21 (8), than in those which had had sham operations, 1·97 $\pm$ 0·52 (4). Despite the presence of adrenal insufficiency, isoprenaline, 0·5 μg./100 g. body weight, reduced the percentage of the water load and the μ-equivalents of Na^+ and K^+ excreted in the 50 min. after administration of the water load. significantly. Similar changes were induced by isoprenaline in the mock operated animals (Table I).

A comparison of the effect of subcutaneous injections of 1·0 μg. ($\pm$)-*isoprenaline*/100 *g. body weight on the excretion of sodium, potassium and water by rats in the presence and absence of mannitol.* Simultaneous investigation was made in 12 rats of the effect of subcutaneous injections of 0·9 per cent NaCl, 1·0 μg. ($\pm$)-isoprenaline/100 g. body weight and 1·5 mU. vasopressin/100 g. body weight both during water diuresis and during a combined water and mannitol diuresis. A six day cross over test was used for this purpose in which the subcutaneous injections were made at the time of administration of the water load, and the rates of

TABLE I

A COMPARISON OF THE EFFECTS OF SUBCUTANEOUS ISOPRENALINE, 0·5 μg./100 g. RAT, ON THE EXCRETION OF SODIUM POTASSIUM AND WATER BY SHAM OPERATED AND ADRENALECTOMISED RATS

Urine, per rat, excreted in 50 min.	Sham operated rats		Adrenalectomised rats			
			Salt maintained		Treated with adrenal cortical extract	
	Without amine	With amine	Without amine	With amine	Without amine	With amine
Per cent water load.. ..	50·3 ± 6·77 (4)	24·1 ± 3·66 (4)*	17·9 ± 1·41 (4)	8·7 ± 1·91 (4)*	18·6 ± 2·78 (4)	5·3 ± 4·15 (4)*
μ-equiv. Na^+	59·8 ± 13·62 (4)	18·0 ± 1·67 (4)*	165·8 ± 20·49 (4)	62·0 ± 19·11 (4)*	160·4 ± 16·14 (4)	79·3 ± 13·38 (4)*
μ-equiv. K^+	32·4 ± 5·27 (4)	14·4 ± 2·26 (4)*	57·8 ± 6·67 (4)	22·4 ± 6·90 (4)*	54·5 ± 3·07 (4)	16·3 ± 1·76 (4)*
Na^+/K^+	1·97 ± 0·52 (4)	1·59 ± 0·20 (4)	2·97 ± 0·27 (4)	4·3 ± 1·21 (4)	2·97 ± 0·25 (4)	3·7 ± 1·00 (4)

Significance of differences caused by isoprenaline was determined by *t* test and is indicated by an asterisk where P = <0·95.

TABLE II

A COMPARISON OF THE EFFECTS OF 1·0 μg. ISOPRENALINE AND OF 1·5 mU. VASOPRESSIN INJECTED SUBCUTANEOUSLY, EACH PER 100 g. RAT, ON THE EXCRETION OF WATER, SODIUM AND POTASSIUM IN THE PRESENCE AND ABSENCE OF MANNITOL

Urine per rat, excreted in 50 min.		Mannitol absent		Mannitol present		
	Saline subcut	Isoprenaline subcut	Vasopressin subcut	Saline subcut	Isoprenaline subcut	Vasopressin subcut
Per cent water load..	60·7 ± 1·9 (12)	27·8 ± 3·2 (12)*	34·3 ± 3·4 (12)*	57·9 ± 2·9 (12)	33·7 ± 1·7 (12)*	50·3 ± 2·4 (12)*
μ-equiv. Na^+ ..	84·6 ± 8·0 (12)	32·4 ± 2·7 (12)*	107·8 ± 10·5 (12)*	78·1 ± 11·4 (12)	40·2 ± 9·5 (12)*	111·7 ± 11·0 (12)*
μ-equiv. K^+ ..	81·8 ± 4·6 (12)	58·3 ± 1·5 (12)*	84·8 ± 1·9 (12)	79·5 ± 2·1 (12)	60·6 ± 2·7 (12)*	84·8 ± 3·6 (11)
Na^+/K^+	1·1 ± 0·12 (12)	0·6 ± 0·05 (12)*	1·2 ± 0·11 (12)*	1·2 ± 0·14 (12)	0·6 ± 0·13 (12)*	1·35 ± 00·04 (11)
Creatinine mg. ..	17·5 ± 0·4 (12)	16·5 ± 0·6 (12)	19·2 ± 1·0 (12)	18·9 ± 0·5 (12)	15·3 ± 0·4 (12)*	16·9 ± 0·6 (11)*
Diodone mg. ..	2·9 ± 0·08 (12)	2·8 ± 0·07 (12)	3·0 ± 0·13 (12)	3·0 ± 0·07 (12)	2·7 ± 0·08 (12)*	2·9 ± 0·08 (11)

Significance of difference caused by drugs has been determined by *t* test, and is indicated by an asterisk where P = <0·05.

excretion of Na^+, K^+ and water were measured over the succeeding period of 50 min. The combined results of these experiments are shown in Table II. The concentration of mannitol did not alter the rates of excretion of water, Na^+ and K^+ found during water diuresis but did, in very large part, antagonise the antidiuretic action of 1·5 mU. vasopressin/100 g. without influencing the augmented Na^+ output which resulted from the natural vasopressin used. Isoprenaline however, caused antidiuresis and reduction in the rates of excretion of Na^+ and K^+ almost equally effectively in the presence and the absence of the mannitol. The slight tendency to reduction in filtration rate and effective renal plasma flow caused by this dose of the amine during water diuresis became significant only during mannitol diuresis (Table II).

Discussion

The fact that subcutaneous injections of 0·5 μg. ($\pm$)-isoprenaline/100 g. body weight caused antidiuresis and reduced the rates of excretion of sodium and potassium similarly in sham operated and in partially deficient adrenalectomised rats (Table I) proves that these actions of isoprenaline are not caused by changes in the rate of secretion of adrenal hormones. Neither are they in part the consequence of change in the rate of secretion of vasopressin, for, whereas mannitol effectively antagonised the antidiuretic effect of 1·5 mU. vasopressin/100 g. rat, it did not antagonise that of isoprenaline, (Table II). Whereas both vasopressin and isoprenaline reduced the glomerular filtration rate, only the isoprenaline reduced the excretion of sodium and water (see Table II, mannitol diuresis).

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THE BIOLOGICAL ASSAY OF THYROACTIVE MATERIALS BY THE GOITRE PREVENTION TECHNIQUE

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The goitre prevention technique was employed to measure the biological activity of synthetic and natural thyroproteins as well as L-thyroxine and L-triiodothyronine. These studies indicated that L-thyroxine could not be used as a reference standard for natural thyroid preparations since the log dose response lines were not parallel. However, L-thyroxine was a suitable reference standard for the biological assay of the synthetic iodinated proteins.

The British Pharmacopoeia (1958) defines the potency of desiccated thyroid in terms of thyroxine iodine, while the United States Pharmacopeia XVI employs total organic iodine for measuring thyroid activity. However Stasilli and Kroc (1956) have reported recently that the total iodine content of desiccated thyroid and thyroglobulin is not necessarily correlated with biological activity. It was found that pork thyroid preparations were consistently more active in the goitre prevention assay than beef desiccated thyroid. In addition to the dried thyroid, some consideration must be given to synthetic thyroproteins such as iodinated casein (Protamone). The total organic iodine content of these compounds provides very little information about their biological activity because only a small percentage of the iodine is present as thyroxine (Reinecke, 1946).

In view of these apparent differences between biological effectiveness of thyroactive substances and their iodine content, a study was undertaken to examine the possibility of using L-thyroxine as the reference compound for the standardisation of thyroid preparations. The prevention of goitre in thiouracil-treated rats was employed as the criterion of response in the bioassay of the thyroactive substances. In addition to desiccated thyroid, iodinated proteins and L-triiodothyronine were included in the investigation. A comparison was also made of oral and parenteral potencies of the thyroid preparations.

Experimental

Oral Route of Administration

Adult female rats of an inbred Wistar strain, weighing 150-180 g. were used as the test animals. The goitre-prevention assay procedure employed was similar to that described by Stasilli and Kroc (1956). After random distribution into dose groups of 10 animals each, two rats were placed in each cage and fed a diet of ground Master Fox cubes containing 0·3 per cent thiouracil. Graded amounts of the thyroactive substances were mixed into the appropriate diets to provide two doses levels each of the standard and unknown preparations. The actual doses used were based on the total organic iodine concentration and expressed as μg. thyroid iodine per 100 g. diet. The rats were fed the test diets *ad libitum*

for 14 days. On the fifteenth day, the animals were killed and the thyroid glands removed and weighed. The response was expressed as the relative thyroid weight, i.e., mg. thyroid/100 g. of final body weight. For each assay two control groups were included, one providing the goitrogenic

TABLE I

IODINE CONTENT OF MATERIALS STUDIED

Preparation	Iodine per cent
Thyroxine pentahydrate (Sodium Salt) ..	57·3
Triiodothyronine (Sodium Salt)	58·9
Desiccated pork thyroid[1] (house standard) ..	0·202
Desiccated pork thyroid[2]	0·79
Thyroglobulin[3]	0·86
Iodinated protein[4]	6·90
Iodinated protein[5]	7·60

1. Wilson Laboratories. 2. Armour Pharmaceutical Co. 3. Warner-Chilcott Laboratories. 4. Protamone-AgriTech Ltd. 5. Lechavit-Pure. H.F. Cordes and Co. Hamburg, Germany.

effect of thiouracil alone on the thyroid gland, and the other the normal untreated thyroid weight.

Parenteral Administration

When the thyroactive substances were administered by intraperitoneal injection, they were dissolved or suspended in a 1 per cent (w/v) solution of sodium bicarbonate. A Potter-Elvehjem glass homogeniser was employed to make the suspensions when the material did not dissolve completely. The rats were distributed at random into dose groups and

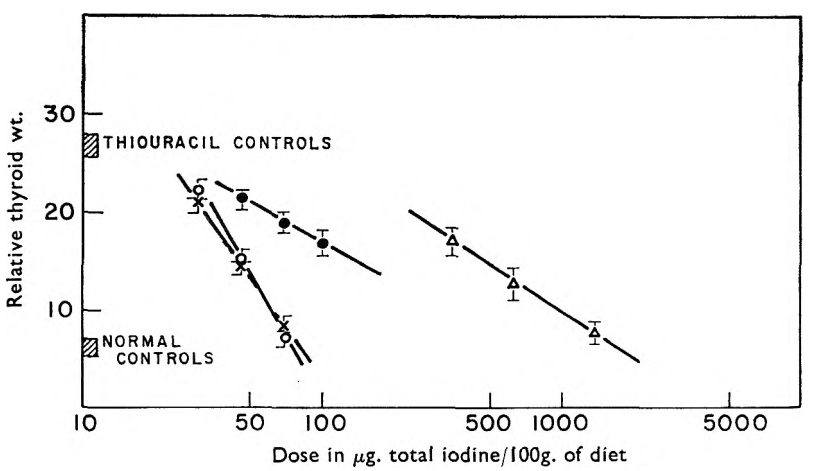

FIG. 1. Log. dose-response curves for thyroactive materials based on relative thyroid weights in thiouracilized rats. Each point represents the mean of 10 animals. Route of administration—oral.

-X-	desiccated pork thyroid	b = − 39·6 ± 1·04	λ = 0·065
-○-	pork thyroglobulin	b = − 40·0 ± 0·97	λ = 0·093
-●-	thyroxine	b = − 14·2 ± 1·27	λ = 0·161
-△-	iodinated casein (*Protamone*)	b = − 18·7 ± 1·24	λ = 0·161

fed a diet containing 0·3 per cent thiouracil *ad libitum* during the assay period. Each animal received a daily intraperitoneal injection (0·2 ml.) of the material under test for a period of 14 consecutive days. The doses

TABLE II

BIOLOGICAL ASSAY OF A SYNTHETIC IODINATED PROTEIN AGAINST SODIUM L-THYROXINE

Preparation	No. of animals	Dose µg. total iodine / 100 g. diet	Relative thyroid weight ± S.E.M.*
Standard			
Na L-thyroxine	10	66·6	19·0 ± 0·8
	10	100	15·6 ± 0·5
	10	150	10·3 ± 0·7
Unknown			
Protamone	10	345	20·3 ± 0·7
	10	690	12·6 ± 1·5
	10	1380	7·2 ± 0·6
	Thiouracil	Controls	28·8 ± 2·2
	Normal	Controls	5·5 ± 0·6

s = 2·64 b = −22·8 λ = 0·115
Relative potency 17·0 per cent
Confidence limits (P = 0·95) 15·0 − 19·4 per cent

* mg. thyroid/100 g. final body weight ± standard error of the mean.

were not administered on the basis of body weight because of the narrow range (150–160 g.) of the initial body weights. The control groups of rats were fed either the untreated diet or that containing 0·3 per cent thiouracil, and were given intraperitoneal injections of a 1 per cent solution of sodium bicarbonate for the period of the assay. On the fifteenth day of the test,

TABLE III

BIOLOGICAL ASSAY OF A DESICCATED PORK THYROID PREPARATION AGAINST A PURIFIED PORK THYROGLOBULIN

Preparation	No. of animals	Dose µg. total iodine / 100 g. diet	Relative thyroid weight ± S.E.M.*
Standard			
Thyroglobulin	10	45·0	15·2 ± 1·1
	10	67·5	6·3 ± 1·1
Unknown			
Desiccated thyroid	10	45·0	14·7 ± 1·7
	10	67·5	8·2 ± 0·1
	Thiouracil controls		26·8 ± 1·4
	Normal controls		6·5 ± 0·4

s = 3·72 b = −43·49 λ = 0·085
Relative potency 96·4 per cent
Confidence limits (P = 0·95) 85·0 − 109·2 per cent

* mg. thyroid/100 g. final body weight ± standard error of the mean.

the animals were killed, and the thyroid glands removed and weighed. The results were expressed as the relative thyroid weight.

The total iodine content of the compounds tested in this study are shown in Table I.

RESULTS

Oral Route of Administration

Fig. 1 shows typical log dose-response lines obtained for a number of thyroactive substances by plotting the relative thyroid weight against the

log of the dose expressed as μg. of total iodine/100 g. of diet. The slopes for thyroxine and iodinated casein (Protamone) were significantly smaller than those for desiccated pork thyroid and pork thyroglobulin. This significant difference in slopes clearly indicates that thyroxine cannot be used as a reference standard for the bioassay of dried thyroid by the goitre-prevention technique, at least by the method of parallel lines.

However this method can be used for estimating the biologically available thyroxine content of iodinated proteins, and the results of such an assay are given in Table II. On the basis of the total organic iodine, the potency of Protamone is approximately 17 per cent relative to that of thyroxine. Since 17 per cent of the total iodine is assumed to be thyroxine, it is estimated that Protamone contains approximately 1·2 per cent biologically available thyroxine. The thyroxine content of Protamone was

TABLE IV

BIOLOGICAL ASSAY OF DESICCATED PORK THYROID (AGAINST ANOTHER DESICCATED PORK THYROID PREPARATION)

Preparation	No. of animals	Dose μg. total iodine / 100 g. diet	Relative thyroid weight ± S.E.M.*
Standard			
Wilson	10	40	20·5 ± 0·7
Desiccated thyroid	10	60	12·7 ± 0·9
Unknown			
Armour desiccated	10	40	19·3 ± 0·4
Thyroid	10	80	8·0 ± 0·7
Thiouracil controls			24·3 ± 1·0
Normal controls			6·8 ± 0·8

s = 1·83 b = −39·3 λ = 0·047
Relative potency 103 per cent
Confidence limits (P = 0·95) 95–112 per cent

* mg. thyroid/100 g. body weight ± standard error of the mean.

measured by the method described in the British Pharmacopoeia 1958 and it was found to be 1·2 per cent, showing excellent agreement with the value obtained by the bioassay procedure.

Since thyroxine could not be used as the reference standard for determining the physiological activity of dried thyroid, it was necessary to use a thyroid preparation as a reference standard. Tables III and IV show the results of two bioassays in which a pork thyroglobulin and a desiccated pork thyroid were employed as the reference standards. All the samples were derived from pork and displayed similar biological activity on the basis of total organic iodine in the goitre-prevention assay.

Intraperitoneal Route of Administration

Fig. 2 shows that the slopes of the log dose-response lines for desiccated thyroid, administered by intraperitoneal injections is significantly steeper than those obtained under similar conditions for triiodothyronine, thyroxine, and the two iodinated proteins, Protamone and Lechavit. These findings agree with those observed when the thyroactive substances were given orally in the diet.

As the slopes for triiodothyronine, Lechavit and Protamone did not differ significantly from thyroxine, it was possible to get an approximate indication of the physiological activity by measuring the horizontal difference between the log dose-response lines. By using this method thyroxine had 19 per cent of the biological activity of triiodothyronine, Protamone had 16 per cent of the potency of thyroxine, while Lechavit

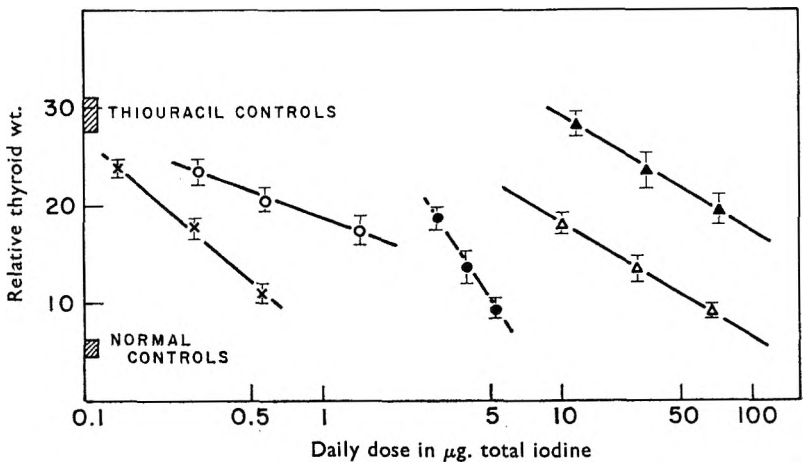

FIG. 2. Log. dose-response curves based on relative thyroid weights of thiouracilized rats receiving thyroactive substances by intraperitoneal injection. Each point represents the mean of 10 animals.

-X-	triiodothyronine	$b = -20{\cdot}79 \pm 1{\cdot}42$	$\lambda = 0{\cdot}099$
-○-	thyroxine	$b = -15{\cdot}07 \pm 2{\cdot}82$	$\lambda = 0{\cdot}176$
-●-	desiccated pork thyroid	$b = -42{\cdot}01 \pm 1{\cdot}91$	$\lambda = 0{\cdot}072$
-△-	iodinated casein (*Protamone*)	$b = -14{\cdot}67 \pm 0{\cdot}30$	$\lambda = 0{\cdot}162$
-▲-	iodinated protein (*Lechavit*)	$b = -15{\cdot}19 \pm 0{\cdot}90$	$\lambda = 0{\cdot}265$

was only 2 per cent as potent as thyroxine and 19 per cent as active biologically as Protamone. These estimates are all in good agreement with data obtained by oral assay.

DISCUSSION

Numerous workers have commented on the existence of greater biological activity in dried thyroid preparations than their thyroxine content would indicate. The extensive earlier literature on this subject has been summarised by Frieden and Winzler (1948). Since the majority of the studies antedate the discovery of triiodothyronine by Gross and Pitt-Rivers (1953), the presence of this hormone might explain the enhanced physiological response of the natural dried thyroid products.

Nevertheless the results presented here suggest that one characteristic of the biological response elicited by thyroxine and triiodothyronine differs from that found with desiccated thyroid, namely, the slope of the log dose-response line. Although few investigators have published actual estimates of the slopes of the linear relationship between log of the dose and the relative thyroid weight, examination of their data provides an

approximate assessment of the probable values. In general, the results of Cortell (1949), Dempsey and Astwood (1943), Gross and Pitt-Rivers (1953), Hemming and Holtcamp (1953), Kroc, Phillips, Stasilli and Malament (1954), and Mussett and Pitt-Rivers (1957) yielded log dose-response lines with slopes which were of the same order of magnitude as those shown in Figs. 1 and 2. Cortell (1949) found that the slope of the log dose-response line was much steeper for thyroglobulin than it was for thyroxine, whereas the results of Kroc and others (1954) indicate that the log dose-response lines for these two materials were essentially parallel. In a later study, however, Stasilli and Kroc (1956) reported that the slopes of log dose-response lines in a large series of bioassays of desiccated thyroid preparations, ranged from −23·8 to −58·3 with a mean of −40·5 for 15 assays. These values do not differ significantly from those obtained in the present study. Ferguson and Warson (1953) found that the log dose-response lines for thyroxine, triiodothyronine and desiccated thyroid varied from −6 to −36 with an average value of −16.

The results obtained in the goitre-prevention assay suggest that thyroid preparations must be assayed against a standard derived from glandular material in order to obtain a meaningful estimate of biological potency. Chemical methods which determine total iodine or thyroxine content may not be indicative of physiological activity.

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AFRICAN *RAUWOLFIA* SPECIES

PART I. THE STRUCTURE OF THE ROOT AND STEM OF *RAUWOLFIA VOLKENSII* STAPF

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A possible substitute or adulterant for the roots of *R. vomitoria* Afz. is the shrubby East African species *R. volkensii* Stapf. The anatomy of the roots and stems is described and illustrated, and compared with published data about other African species.

R. volkensii was first described by Schumann (1895) as *Tabernaemontana volkensii* and recorded as such in Supplement I, Kew Index (1886–95). Later Stapf described the shrub as *R. volkensii* (Thiselton-Dyer, 1904), the Kew Index being amended in Supplement III (1901–1905). Markgraf (1923) described *R. oreogiton* but this species is regarded as doubtfully distinct from *R. volkensii* (Willan, 1960). Pichon (1947), in his classification of the genus *Rauwolfia*, grouped the species together in the section Ophioxylanthus but was unable to examine specimens of *R. oreogiton* as no authentic specimens are available (Greenway, 1955; Willan, 1960).

R. volkensii is a shrub with slender branches, growing to a height of 6 ft., which occurs in Tanganyika in the West Usambara Mountains, the Pare Mountains, the Ulugura Mountains and on Kilimanjaro at elevations in excess of 4,000 ft., and in Mozambique. Although occasionally encountered in dry forest areas, *R. volkensii* normally occurs in typical moist montane forest dominated by *Ocotea usambarensis* and *Podocarpus spp.* (Thiselton-Dyer, 1904; Willan, 1960).

Plant Material

The following material was used in this investigation:—

1. *R. volkensii* roots and stems collected by the Forest Department, Lushoto, Tanganyika and vouched for by the East African Herbarium, Nairobi, from botanical specimens sent under collector's number "Semsei 2827" (March 10, 1959).

2. Further supply from Lushoto under collector's number "Semsei 2948" (January 20, 1960).

MACROSCOPY

Root

The roots occur as somewhat contorted cylindrical or conical, occasionally branched pieces of varying lengths up to about 70 cm. and diameters up to 4 cm. Most samples possess a few side rootlets varying from about 1 to 6 mm. diameter (Fig. 1, A). Externally the soft, buff or yellow brown cork is longitudinally striated. Occasionally, especially on the outer aspect of bends, the cork has rubbed away to reveal the inner

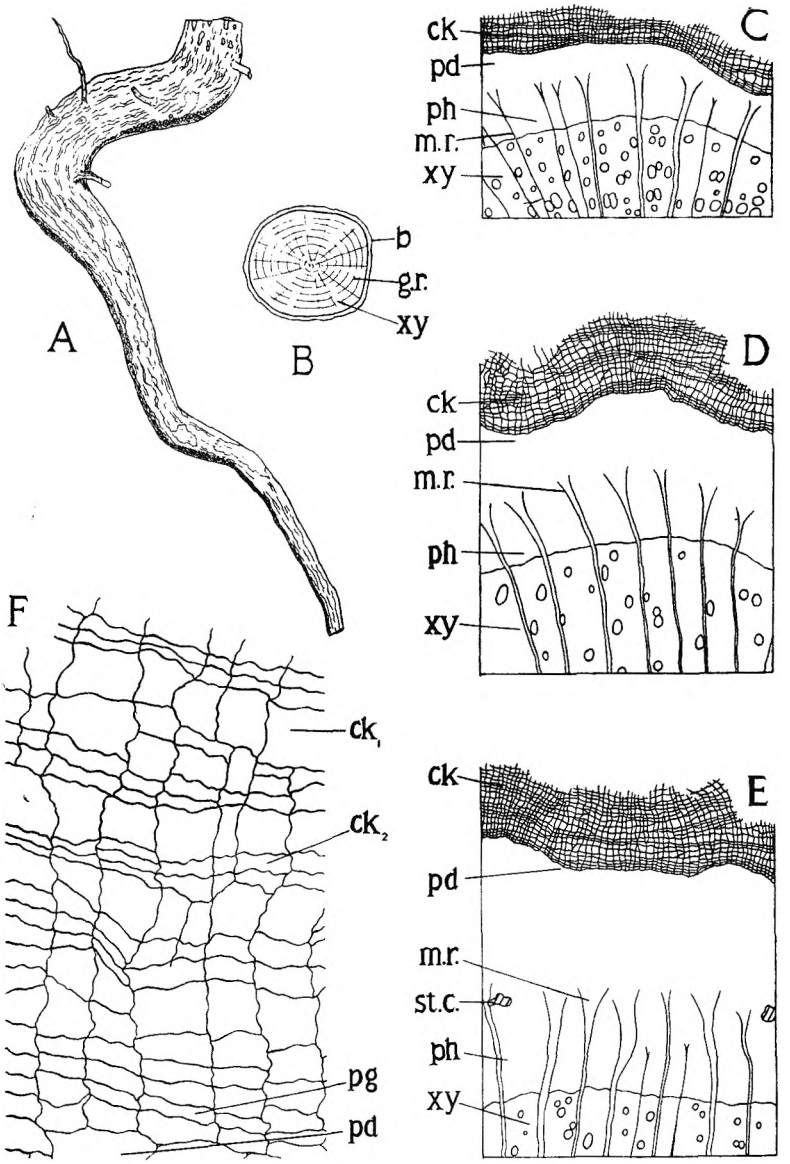

FIG. 1. *Rauwolfia volkensii* Stapf. Root. A, external appearance, × 1/3; B, smoothed transverse surface of root, × 2/3; C, transverse section, root diameter 6 mm., × 25; D, transverse section, root diameter 18 mm., × 25; E, transverse section, root diameter 45 mm., × 25; F, outer tissues, root diameter 17 mm., × 200; b, bark; ck, cork; ck_1, large lignified cork cells; ck_2, small unlignified cork cells; g.r., growth ring; m.r., medullary ray; pd, phelloderm; pg, phellogen; ph, phloem; st.c., sclereid group; xy, xylem.

yellowish phloem tissue or even the yellowish-brown wood. Rootlet scars occur at intervals, but no glistening points could be detected on the outer surface. The cork of the rootlets flakes off readily and longitudinal striations are apparent only on the larger rootlets.

Smoothed tranverse surfaces of the roots show a very narrow bark seldom exceeding 1·5 mm. in diameter. The buff or yellowish finely radiate porous wood possesses a few distinct growth rings, specimens showing up to 16 rings having been examined (Fig. 1, B).

Stem

The stems occur as slender, cylindrical branched pieces up to 1 m. in length and 4 cm. diameter. Externally the yellow-brown or greyish-brown cork is longitudinally striated and bears greyish patches and pale-brown tangentially elongated lenticels. Smoothed transverse surfaces of the stems show a narrow bark 0·25 to 1·0 mm. thick, a cylinder of secondary xylem with up to 12 growth rings and a small central or somewhat eccentric pith or a cavity due to contraction of the pith.

Sensory Characters

Dried stems and roots are odourless. The bark of root or stem is intensely bitter, whereas the wood of each is almost tasteless; the fracture of the bark is short whilst that of the wood is splintery. Exposed fractured surfaces and powdered samples of root or stem exhibit a bluish-green fluorescence in screened ultra-violet light; aqueous extracts fluoresce similarly.

Microscopy

In the following description the symbols R, T, and L refer to measurements made in the radial, tangential and longitudinal directions respectively of material mounted usually in Berlese mountant.

Root

The cork is stratified and consists of alternating layers of larger and smaller cells. The smaller suberised, but unlignified, cells measure about 7 to **11** to **18** to 22 μ radially and the larger suberised and lignified cells measure about 22 to **30** to **44** to 104 μ radially; the other measurements of both kinds of cells are T = 22 to **30** to **48** to 67 μ and L = 22 to **30** to **44** to 56 μ (Fig. 1, F, ck_1, ck_2). In surface view, the cork cells are polygonal (Fig. 2, B).

The cork is followed by a layer of thin-walled phellogen cells. The phelloderm consists of up to 12 rows of cells, those near to the phellogen being arranged in regular radial rows whilst the innermost cells are more oval in shape with intercellular spaces between them (Fig. 2, A). Sclereids are absent. For the phelloderm cells R = 11 to **18** to **26** to 37 μ, T = 30 to **44** to **59** to 96 μ and L = 26 to **37** to **48** to 81 μ. Starch and scattered twinned prisms of calcium oxalate occur in the phelloderm. Starch grains occur singly or occasionally 2 to 4 compound. The single grains are rounded and individual grains measure 1 to 3 to **7** to 30 μ in diameter,

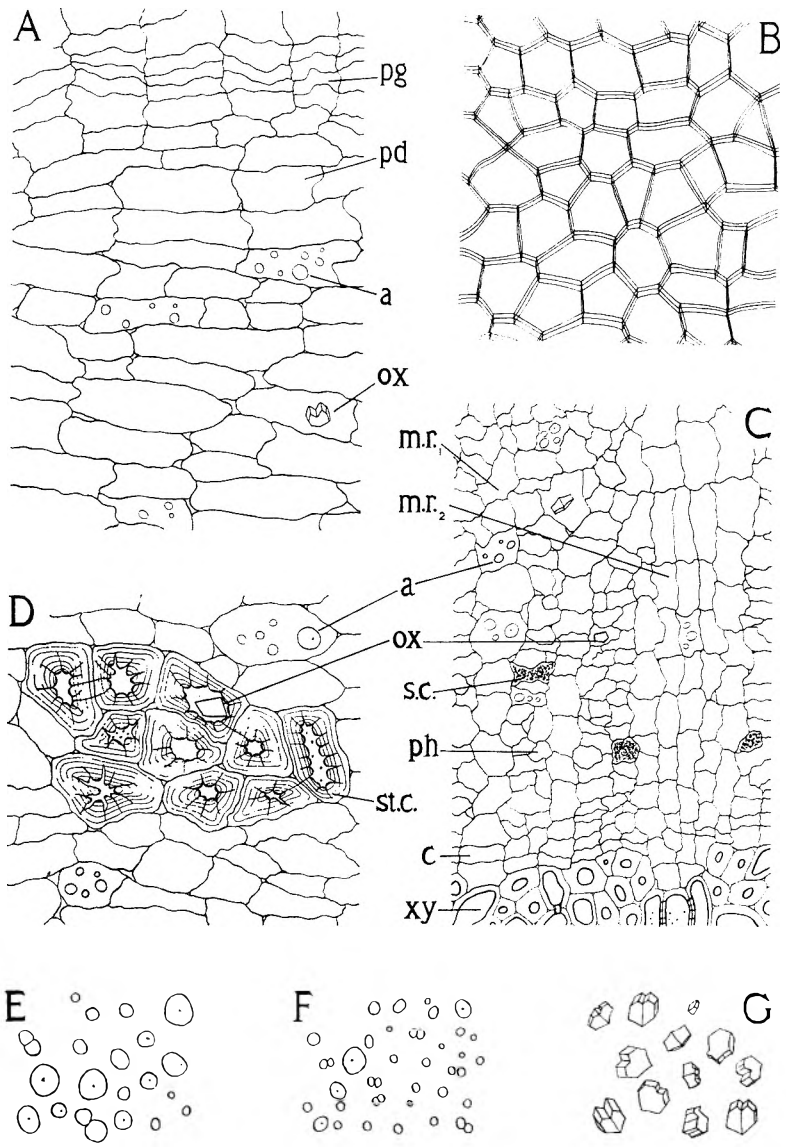

FIG. 2. *Rauwolfia volkensii* Stapf. Root. A, transverse section of the outer tissues; B, cork cells in surface view; C, transverse section of the inner phloem; D, transverse section of the outer phloem; all from 45 mm. diameter root; E, starch grains from the wood; F, starch grains from the bark; G, calcium oxalate crystals from the bark. All × 200. a, starch; c, cambium; $m.r_1$, uniseriate medullary ray of upright cells; $m.r_2$, multiseriate medullary ray of procumbent cells; ox, calcium oxalate crystal; pd, phelloderm; pg, phellogen; ph, phloem elements; s.c., secretion cell; st. c., sclereid; xy, xylem.

the hilum normally appearing as a point or is replaced by a tri-radiate cleft (Fig. 2, F).

The phloem contains secretion cells and is traversed by conspicuous medullary rays. The heterogeneous medullary rays consist of groups of small procumbent cells often with wavy walls, 2 to 4 cells wide and up to 17 cells high, with upper and lower uniseriate extensions consisting of 1 to 4 larger cells (Fig. 3, C). For the smaller cells R = 15 to **19** to **30** to 52 μ, T = 18 to **26** to **37** to 48 μ and L = 18 to **22** to **30** to 48 μ and for the larger cells R = 11 to **19** to **30** to 37 μ, T = 26 to **37** to **59** to 93 μ and L = 30 to **44** to **59** to 100 μ.

Occasional small groups of up to 10 sclereids arranged in 1 or 2 rows were observed in transverse sections of the outer phloem of specimens exceeding 4 cm. diameter (Fig. 1, E). Such specimens represented the rootstock region of the shrub. Individual sclereids measured R = 18 to **30** to **52** to 92 μ, T = 30 to **37** to **52** to 96 μ and L = 30 to **44** to **74** to 255 μ. The maximum length of sclereids isolated by maceration using chromic-nitric acid reagent was 577 μ. All the sclereids are lignified and possess stratified walls with funnel shaped pits (Fig. 2, D).

Vertical rows of calcium oxalate crystals are frequently evident in radial and tangential longitudinal sections through the secondary phloem (Fig. 3, B, C). These crystals consist of monoclinic prisms usually twinned on one of the hemipyramid faces and frequently exhibiting in polarised light a bicolouration effect. Length of prisms = 15 to **22** to **26** to 33 μ; breadth = 7 to **11** to **15** to 19 μ. Starch grains similar to those in the phelloderm occur uniformly although not abundantly throughout the phloem parenchyma and medullary rays. Secretory cells occur in the phelloderm and more frequently in the inner phloem region; their amorphous contents stain with Sudan III, Tincture of Alkanna and iodine solution. No latex canals were observed in the root.

The completely lignified secondary xylem consists of vessels, fibres and wood parenchyma and is traversed by medullary rays (Fig 4, A, B, C) and the primary xylem is hexarch. In transverse sections, the rounded or somewhat oval vessels are frequently inconspicuous and occur singly or very occasionally in pairs. The relatively thin, lignified vessel walls bear numerous alternately arranged bordered pits. For the vessels in transverse section R = 26 to **44** to **56** to 96 μ and T = 26 to **37** to **48** to 78 μ. Vessel segments isolated by chromic-nitric acid maceration show transverse and oblique perforation plates and peg-like prolongations. For isolated segments, length = 95 to **392** to **730** to 1,031 μ (Fig. 5, G).

The abundant xylem fibres possess thick lignified walls with spirally arranged slit-like pits (Fig. 5, F). The length of the individual fibres is about 774 to **1,322** to **1,806** to 2,225 μ and the breadth 15 to **22** to **30** to 48 μ.

The apotracheal xylem parenchyma, as seen in transverse sections, is not abundant and occurs in short uniseriate rows connecting the vessels and medullary rays (Fig. 4, A). In longitudinal sections these cells appear in vertical files of up to 12 cells, the cell walls bearing simple or half bordered pits, dependent on the nature of the adjacent cell structure.

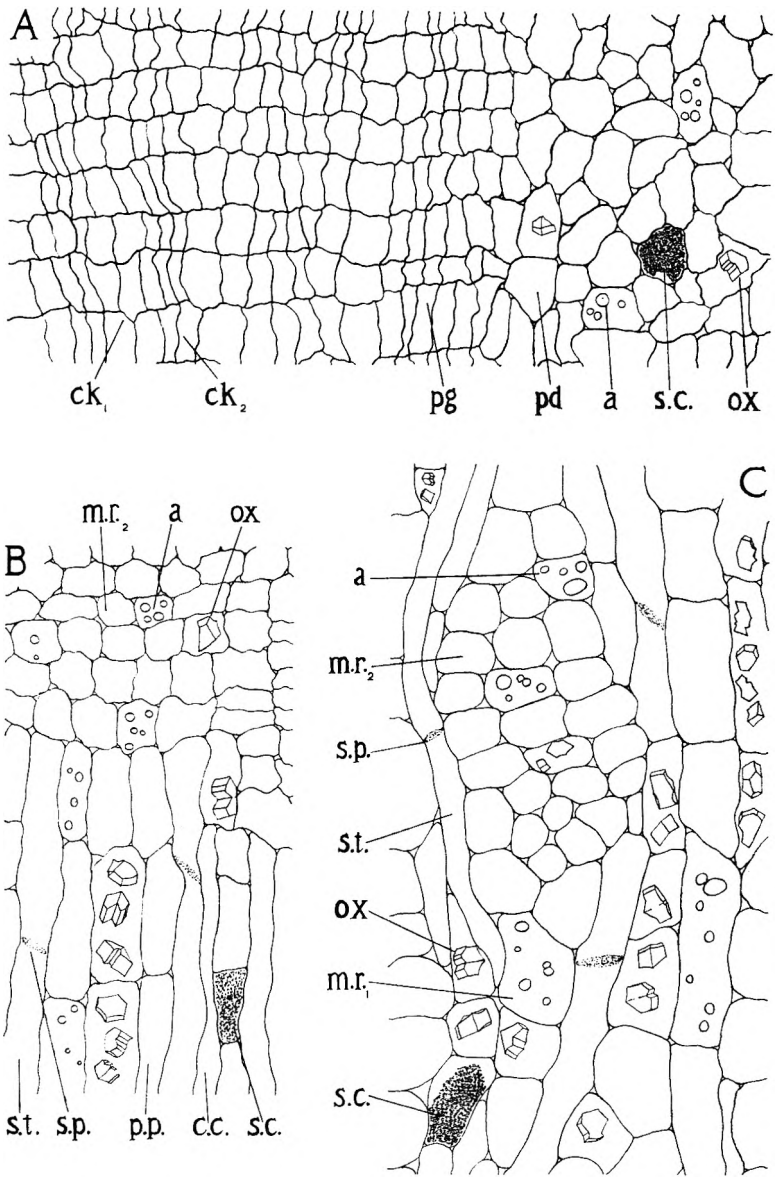

FIG. 3. *Rauwolfia volkensii* Stapf. Root. Longitudinal sections of the bark. A, radical section of outer tissues, root diameter 30 mm.; B, radial section of inner phloem, root diameter 30 mm.; C, tangential section of inner phloem, root diameter 17 mm. All × 200. a, starch; c.c., companion cell; ck_1, large lignified cork cells; ck_2, small unlignified cork cells; ox, calcium oxalate crystal; $m.r_1$, upright medullary ray cell; $m.r_2$, procumbent medullary ray cell; pd, phelloderm; pg, phellogen; p.p., phloem parenchyma; s.c., secretion cell; s.p., sieve plate; s.t., sieve tube.

R = 11 to **22** to **30** to 41 μ; T = 15 to **22** to **30** to 37 μ; L = 37 to **67** to **85** to 133 μ.

The heterogeneous medullary rays resemble the rays in the phloem but are completely lignified. Typical medullary rays in tangential longitudinal section show a central core of procumbent cells usually 2 to 4 cells wide and up to 23 cells high with uniseriate upper and lower extensions of

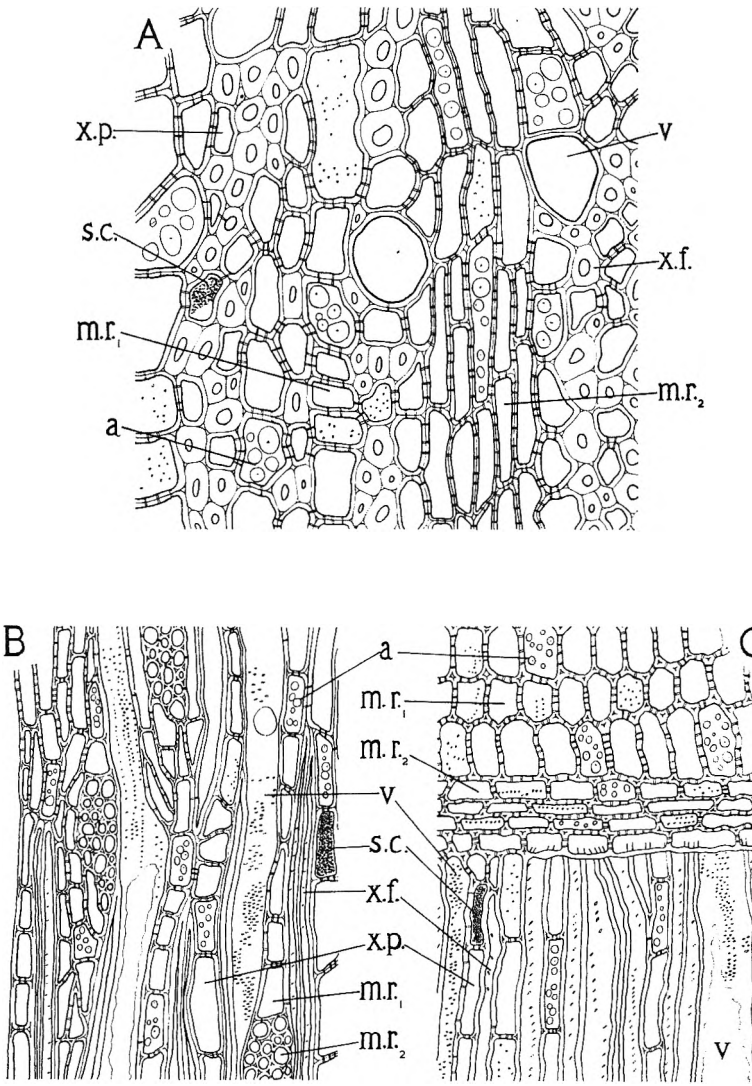

FIG. 4. *Rauwolfia volkensii* Stapf. Root. Secondary Wood. A, transverse section, root diameter 17 mm. × 200; B, tangential longitudinal section, root diameter 10 mm. × 100; C, radial longitudinal section, root diameter 30 mm. × 100. a, starch; $m.r_1$, upright medullary ray cells; $m.r_2$, procumbent medullary ray cells; s.c., secretion cell; v, vessel; x.f., xylem fibre; x.p., xylem parenchyma.

1 to 6 larger upright cells. In transverse sections the uniseriate rays are predominant, the cells often appearing larger than adjacent vessels. For the procumbent cells R = 41 to **67** to **82** to 126 μ, T = 11 to **15** to **19** to 37 μ and L = 11 to **15** to **26** to 48 μ; for the upright cells R = 18 to **30** to **48** to 74 μ, T = 22 to **30** to **48** to 67 μ and L = 30 to **44** to **59** to

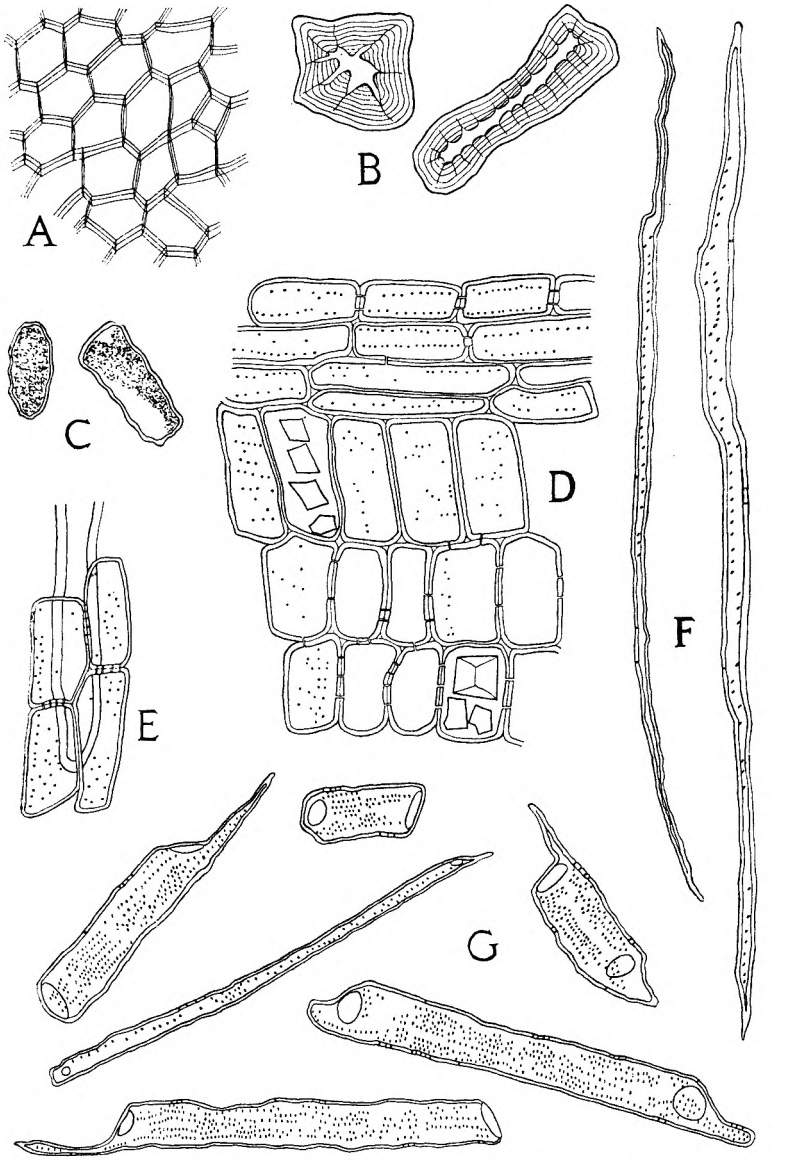

FIG. 5. *Rauwolfia volkensii* Stapf. Isolated elements of the root. A, cork cells, × 200; B, sclereids, × 200; C, secretion cells, × 200; D, lignified medullary ray cells, × 200; E, xylem parenchyma × 200; F, xylem fibres × 100; G, vessel segments, × 100.

96 μ. In tangential longitudinal sections intercellular spaces are apparent especially between the rounded procumbent cells (Fig. 4, B).

Starch grains, 3 to 7 to 11 to 30 μ in diameter, similar to those in the bark, occur in the parenchyma of the xylem and in the medullary rays. Occasional cells contain material staining with iodine and Sudan III and more infrequently calcium oxalate prisms are found in the ray cells.

Stem

In general, the tissue distribution and cell dimensions of the stem resemble those of the root. The soft cork layer, which is not extensive consists of up to about 30 rows of cells showing the alternation of large celled and small celled zones as observed in the root. Internal to the phelloderm and cortex, which is a narrow layer of up to 10 radial rows of

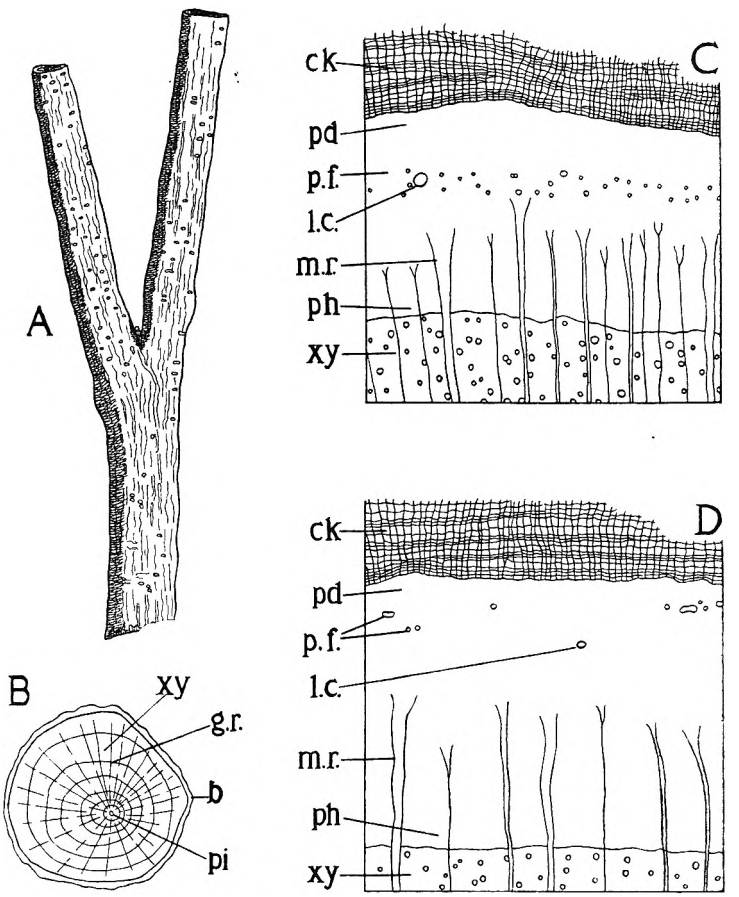

FIG. 6. *Rauwolfia volkensii* Stapf. Stem. A, external appearance, × 1/3; B, smoothed transverse surface of stem, × 1; C, transverse section, stem diameter 16 mm., × 25; D, transverse section, stem diameter 29 mm., × 25. b, bark; ck, cork; g.r., growth ring; l.c., latex canal; m.r., medullary ray; pd, phelloderm; p.f., unlignified fibre; ph, phloem; pi, pith; xy, xylem.

cells, is a zone of highly refractive unlignified fibres. In specimens of small diameter the fibres form an almost continuous layer and appear uniform in shape and measure 11 to **15** to **33** to 48 μ diameter (Fig. 7, B). In larger older specimens the fibres are more widely scattered and, after isolation by alkaline maceration, many show pronounced swellings, 30 to **44** to **96** to 163 μ in diameter (Fig. 8, D); hence their appearance in transverse section is variable (Fig. 7, C). The length of the fibres exceeds 13 mm.

Occasional latex canals containing granular matter which stains with iodine and Sudan III occur in the outer phloem usually associated with the fibres. In transverse section the latex canals appear rounded or oval, R = 26 to 53 μ; T = 37 to 113 μ. Fragments isolated by alkaline maceration measured 26 to **37** to **64** to 128 μ in width and exceeded 13 mm. in length. Secretion cells are scattered throughout the phloem tissue.

Sclereids rarely occur in the stem except in the region of the stem base, where groups of about 60 cells occur arranged in 1 or 2 rows in the secondary phloem.

The secondary phloem is traversed by the medullary rays and resembles the secondary phloem of the root. The medullary rays are usually 2 to 5 small cells in width and 11 to 23 small cells high with upper and lower uniseriate extensions 1 to 4 cells high (Fig. 7, E). Calcium oxalate prisms and starch grains resemble those present in the root bark.

The stem wood is similar to the root wood. Calcium oxalate prisms are occasionally present in the medullary ray and xylem parenchyma cells.

The large celled parenchymatous pith is characterised by peripheral groups of small-celled perimedullary phloem tissue, latex canals and occasional sclereids and non-lignified fibres (Fig. 8, A). A few starch grains and calcium oxalate prisms also occur in the pith.

The Powdered Root

The principle features of the powdered root are:—

1. Thin walled yellow cork cells of two types—lignified cells and radially compressed unlignified cells, the former being more frequent in occurrence.

2. Thin walled cellulosic elements of the phelloderm and phloem; many cells often containing starch grains and sometimes calcium oxalate crystals or yellowish granular material.

3. Rounded, ovoid or plano-convex starch grains 1 to **3** to **11** to 30 μ in diameter; occasional 2 to 4 compound grains.

4. Single or twinned monoclinic prisms and irregular masses of calcium oxalate.

5. Very occasional isodiametric, elongated or irregularly shaped lignified sclereids either singly or in small groups.

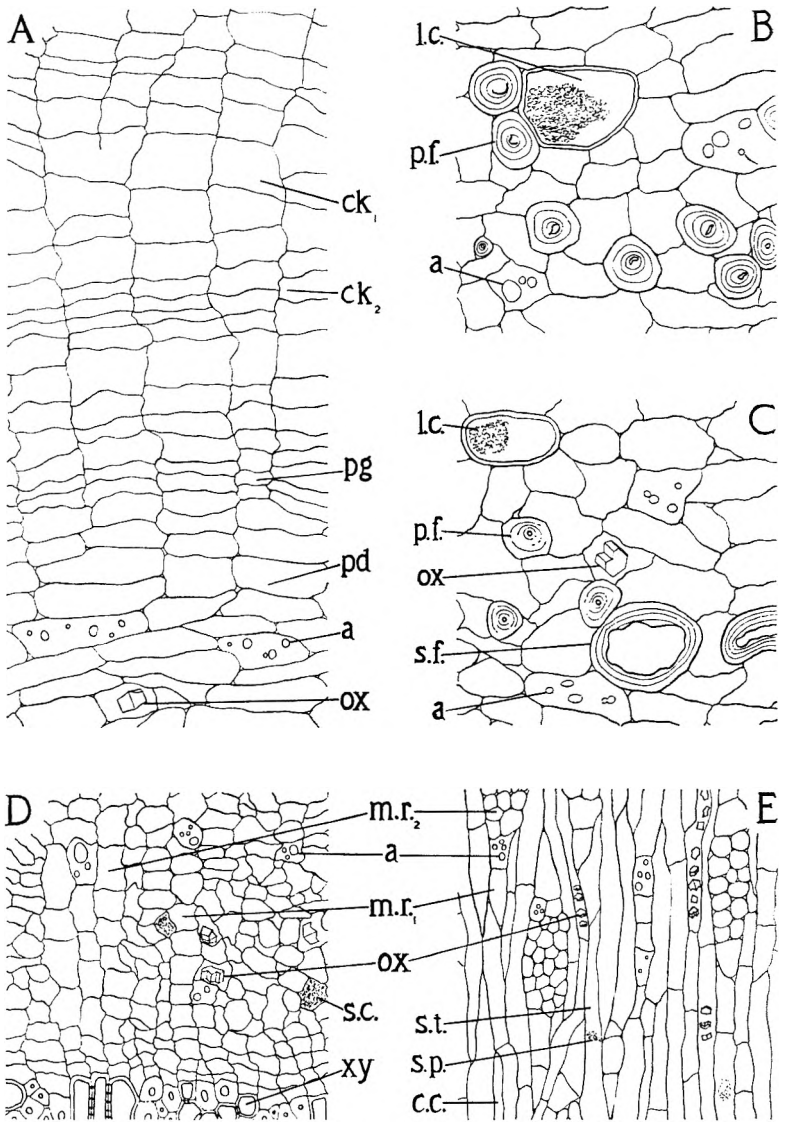

FIG. 7. *Rauwolfia volkensii* Stapf. Stem bark. A, transverse section of outer tissues, stem diameter 29 mm., × 200; B, transverse section in region of outer phloem, stem diameter 8 mm., × 200; C, transverse section in region of outer phloem, stem diameter 16 mm., × 200; D, transverse section of inner phloem, root diameter 16 mm., × 200; E, tangential longitudinal section of inner phloem, stem diameter 16 mm., × 100. a, starch; c.c., companion cell; ck_1, large lignified cork cells; ck_2, small unlignified cork cells; l.c., latex canal; $m.r_1$, uniseriate upright medullary ray cells; $m.r_2$, multiseriate procumbent medullary ray cells; ox, calcium oxalate crystal; pd, phelloderm; p.f., unlignified fibre; pg, phellogen; s.c., secretion cell; s.f., swollen fibre; s.p., sieve plate; s.t., sieve tube; xy, xylem.

6. Abundant fragments of lignified xylem elements comprising xylem fibres, thin walled vessels with alternately arranged bordered pits and elongated xylem parenchyma and medullary ray cells usually containing starch grains.

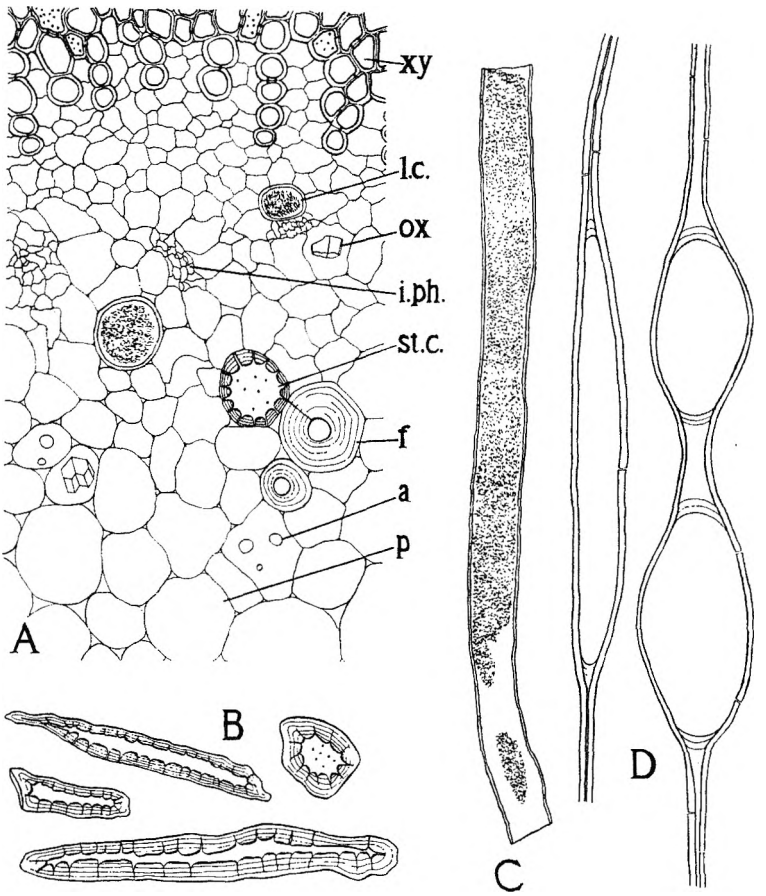

FIG. 8. *Rauwolfia volkensii* Stapf. Stem. A, transverse section of pith, stem diameter 16 mm., × 200; B, isolated sclereids of stem, × 100; C, isolated latex canal, × 100; D, isolated fibre segments, × 100. a, starch; f, fibre; i.ph., internal phloem; l.c., latex canal; ox, calcium oxalate crystal; p, large celled parenchyma; st.c., sclereid; xy, xylem.

DISCUSSION

The structure of the root and stem of *R. volkensii* exhibits the typical features of the family Apocynaceae and of the genus *Rauwolfia* (Metcalfe and Chalk, 1950). The stem structure is clearly differentiated from the root structure by the presence of unlignified fibres in the outer phloem (pericycle), latex vessels and a central pith. The absence of marked sclereid development and the small vessel diameters are related to the herbaceous habit of the plant.

The presence of well defined sclereid groups distinguishes the roots and stems of *R. vomitoria* (Evans, 1956), *R. caffra Sond.* (Court, Evans and Trease, 1957), *R. macrophylla* Stapf (Paris, Dillemann and Chaumelle, 1957) and *R. mombasiana* Stapf (Delourme-Houdé, 1944) from those of *R. volkensii*. The structure of *R. obscura* K. Schum, a species indigenous to the Congo, closely resembles the structure of *R. volkensii*; neither species shows marked sclereid development and the reported vessel diameters are identical (Paris and Dillemann, 1956). Until more information is available concerning *R. obscura* roots, differentiation will remain difficult.

Summarising, the adulteration or substitution of *R. vomitoria* roots with *R. volkensii* roots could be readily detected, but the precise identification of the adulterant or substitute by anatomical methods presents a complex problem, which it is hoped to investigate further when other East African species have been examined.

Acknowledgement. The author is grateful to Mr. T. Yates for technical assistance.

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TREATMENT OF POISONING BY ANTICHOLINESTERASE INSECTICIDES IN THE RAT

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The effects of an oxime cholinesterase reactivator, 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (pralidoxime methanesulphonate, P2S) or iodide (pralidoxime iodide, PAM, P2AM), and atropine, given separately or together, on poisoning by ten organophosphate or carbamate anticholinesterase insecticides are reported on rats. Atropine alone was beneficial with all materials. Oxime therapy alone was effective to varying degrees with isolan, phosdrin, gusathion, ethyl-gusathion, demeton, thimet, phosphamidon and possibly dimetilan, but ineffective with sevin and morphothion. With the organophosphates, these findings are related to the proportion of reversible enzyme inhibition predicted. No potentiation between atropine and oxime was found when the insecticide was given orally, and the combination was less effective than atropine alone with gusathion, ethyl-gusathion, demeton and morphothion. Beneficial potentiation between oxime and atropine did occur with intraperitoneal morphothion.

Previous work by Sanderson and Edson (1959) on oxime therapy for poisoning by organophosphorus insecticides in the rat showed that the relative effectiveness of therapy by oxime cholinesterase reactivators could be related to the proportion of reversible cholinesterase inhibition present, as predicted from the structure and duration of action of the organophosphate. With the slower acting indirect inhibitors of cholinesterase now commonly used as insecticides, repeated doses of oxime are necessary. Oxime injections were beneficial in poisoning by parathion, parathion-methyl, phenkaptone and, to a lesser extent, diazinon, but ineffective with dimefox and dimethoate.

Some studies have been published of oxime therapy for poisoning by other commercial organophosphate insecticides. Work summarised by Davies and Green (1959) showed that oxime therapy is ineffective with schradan poisoning, due to irreversibility of inhibition, though a conflicting report by Wills (1959) does suggest some beneficial effect. Fournel (1957a, b) showed that pralidoxime iodide therapy reduced mortality with endothion, and to a lesser extent with demeton-methyl. Wills (1959) also reports that pralidoxime iodide is effective against EPN and dipterex, but not against malathion or diazinon, while Bergner (1959) reports that it can reactivate tissue cholinesterase after diazinon poisoning. With the exception of the findings of Wills (1959) on schradan and possibly diazinon, these observations are consistent with expectations from predicted proportions of reversible cholinesterase inhibition.

A number of carbamate anticholinesterase insecticides are now available. The only known reports on oxime therapy of poisoning by carbamate insecticides are those by Smyth, Carpenter, Nair, Palm, Rogers,

TABLE I

EFFECT OF IMMEDIATE ATROPINE AND OXIME THERAPY ON RATS GIVEN ORAL INSECTICIDE

Compound	Rat sex	LD50, mg./kg.	Dose, mg./kg.	Therapy	Deaths	Time of onset, min.	Time of death	Remarks
Isolan	M	12	20	Nil	5/6	1	4–29′	
				Atropine	1/6	2	18′	Effective, animals dry.
				P2S	2/6	2	22′	No effect till convulsions, then reduced mortality.
				P2AM	1/6	2	24′	No effect till convulsions, then reduced mortality.
				Atropine/P2S	0/6	2	--	Effects as atropine group.
				Atropine/P2AM	1/6	7	16′	Effects as atropine group.
Sevin	F	400	700	Nil	6/6	6	45′–23 hr.	
				Atropine	1/6	10	25′	Effective, animals dry.
				P2AM	6/6	8	10′–26 hr.	No benefit. Deaths apparently faster than control.
				Atropine/P2AM	2/6	14	22 hr.–3d.	Effects as atropine group.
Dimetilan	F	25	50	Nil	6/6	2	4–33′	
				Atropine	2/6	1	26′	Effective, animals dry.
				P2AM	5/6	2	14–30′	Slight benefit.
				Atropine/P2AM	1/6	5	20′	Effects as atropine group.
Phosdrin	M	5	8	Nil	5/6	5	7–16′	
				Atropine	1/6	11	12′	Effective, animals dry.
				P2S	1/6	2	19′	No effect till convulsions, then reduced mortality.
				P2AM	2/6	5	35′	No effect till convulsions, then reduced mortality.
				Atropine/P2S	0/6	5	--	Effects as atropine group.
				Atropine/P2AM	0/6	6	--	Effects as atropine group.
Gusathion	F	7	12	Nil	4/6	6	22–35′	
				Atropine	0/6	9	—	Effective, animals dry.
				P2AM	0/6	6	—	Effective, symptoms reduced.
				Atropine/P2AM	1/6	14	28′	Effects as atropine group, but recovery slower.

TABLE I—*continued*

Compounds	Rat sex	LD50 mg./kg.	Dose, mg./kg.	Therapy	Deaths	Time of onset, min.	Time of death	Remarks
Ethyl-gusathion	F	9·5	14	Nil	6/6	10	50′–2d.	
				Atropine	0/6	17	—	Effective, animals dry.
				P2AM	3/6	5	50′–20 hr.	Lower mortality, symptoms unaffected.
				Atropine/P2AM	3/6	15	35′–4d.	Effects as atropine group, greater mortality.
Morphothion*	M	200	300	Nil	6/6	50	2½–23 hr.	
				Atropine	2/6	29	2d.	Effective, animals dry.
				P2AM	5/6	12	20′–25 hr.	No benefit.
				Atropine/P2AM	6/6	3	7′–28 hr.	Effects faster than control group.
Demeton*	F	2·7	3·5	Nil	5/6	30	1½–2¼ hr.	
				Atropine	0/6	35	—	Effective, animals dry.
				P2AM	1/6	20	24′	Effective, symptoms reduced.
				Atropine/P2AM	3/6	10	20′	Effects more severe than atropine group.
Thimet*	M	3	4	Nil	6/6	36	3–23 hr.	
				Atropine	3/6	40	6 hr.–2d.	Effective, animals dry.
				P2AM	5/6	53	5 hr.–2d.	Deaths slower, symptoms otherwise unaffected.
				Atropine/P2AM	3/6	50	5 hr.–2d.	Effects as atropine group.
Phosphamidon*	F	15	20	Nil	6/6	10	25–35′	
				Atropine	0/6	14	—	Effective, animals dry.
				P2AM	0/6	17	—	Effective, symptoms still severe.
				Atropine/P2AM	0/6	10	—	Effects as atropine group.

* Therapeutic injections repeated subcutaneously after 4 hr.

Weil and Woodside (1958) and by Carpenter, Weil, Palm, Woodside, Nair and Smyth (1961) who found pralidoxime iodide ineffective against sevin poisoning in dogs and rats.

It is generally accepted (Davies and Green, 1959) that where oxime therapy is effective, there is a beneficial potentiating effect between the oxime and atropine, so that the two drugs given together produce greater benefit than either separately. Sanderson and Edson (1959) found indications that supplementing atropine by pralidoxime iodide in rats poisoned by oral dimethoate could reduce the beneficial effect of the atropine and give a higher mortality. Some of the results of Fournel (1957a) on mice poisoned orally by parathion, endothion or demeton-methyl are open to similar interpretation.

It thus seemed desirable to study, on others of the many commercially available anticholinesterase insecticides, the effectiveness of oxime therapy with and without atropine.

Materials and Methods

Of the 10 insecticides tested (whose structures are included in Table III), phosdrin, gusathion, ethyl-gusathion, morphothion, demeton and phosphamidon were commercial liquid formulations, administered undiluted;

TABLE II

Effect of atropine and pralidoxime iodide therapy subcutaneously on male rats given 150 mg./kg. intraperitoneal morphothion

Therapy*	Mortality	Time of onset, min.	Time of death, hr.	Remarks
Nil	2/6	35	4¼–22	
Atropine	0/6	30	—	Effective, animals dry
Pralidoxime iodide	3/6	35	3–22	No benefit
Atropine + pralidoxime iodide	0/6	50	—	Animals much less affected than atropine group, and dry

* Given subcutaneously immediately and repeated after 4 hr.

isolan and thimet were liquid technical samples, administered undiluted; sevin and dimetilan were technical samples administered in glycerol formal (Sanderson, 1959). A pure sample of morphothion was also used, in glycerol formal, for intraperitoneal injection. 2-Hydroxyiminomethyl-*N*-methylpyridinium iodide (pyridine-2-aldoxime methiodide, pralidoxime iodide, P2AM, PAM), obtained from Messrs. L. Light and Company and the methanesulphonate (pyridine-2-aldoxime methyl methanesulphonate, pralidoxime methanesulphonate, P2S), obtained from Aldrich Chemical Co. Inc., and B.P. grade atropine sulphate were all administered in aqueous solution.

Rats were semi-adult (150–250 g.) animals of Wistar strain, maintained and fed under standard conditions. Administration techniques were orthodox, and animals were observed for 7 days.

In most experiments, the insecticide was given orally, followed immediately by intraperitoneal injections of the appropriate therapeutic drugs. With the relatively slower acting insecticides morphothion, demeton,

phosphamidon and thimet, further subcutaneous therapeutic injections were given 4 hr. later. Doses of the therapeutic drugs were standardised at 100 mg./kg. for pralidoxime iodide and methane-sulphonate, and 17·4 mg./kg. for atropine sulphate, these doses alone having previously been shown to be non-toxic. Oral insecticide doses were chosen to be just above the previously determined LD50.

Observations were based on appearance of the animals and rate of development of effects, as well as on mortalities. Mortality differences of one animal between groups were ignored, and differences of two, regarded arbitrarily as borderline. The results did not permit statistical examination.

Results

The results of tests on the effects of oximes and atropine on oral poisoning by the ten insecticides in groups of six rats are summarised in Table I. In a further test, summarised in Table II, the effects of subcutaneous therapeutic injections immediately and after 4 hr. on groups of six rats poisoned by intraperitoneal morphothion, pure, in glycerol formal, were examined.

All of the insecticides caused typical anticholinesterase effects, but at different rates. All showed reduced mortality with atropine therapy,

TABLE III

Relationship between chemical structure and effectiveness of oxime therapy
(Insecticide given orally)

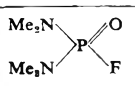
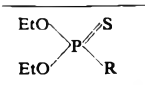
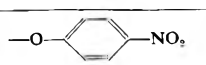
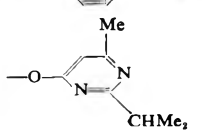
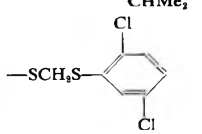
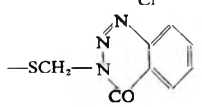
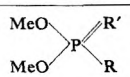
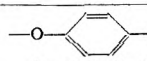
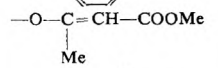
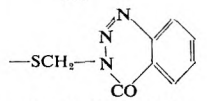
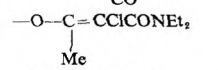
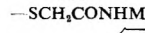
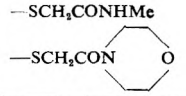
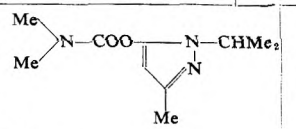
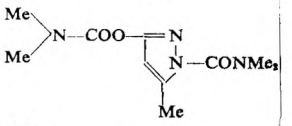
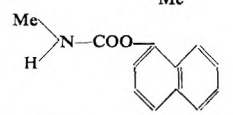
Compound	Structure	Speed of toxic action	Relative benefit from oxime
Dimefox		Slow	None
			
	R		
Parathion		Moderate	Marked
Diazinon		V. slow	Moderate
Phenkaptone		Slow	Marked
Ethyl-gusathion		Moderate	Moderate
Demeton	—OCH_2CH_2SEt	Moderate	Marked
Thimet	—SCH_2—SEt	Slow	Slight

TABLE III—*continued*

Compound	Structure		Speed of toxic action	Relative benefit from oxime
				
	R	R′		
Parathion-methyl		S	Rapid	Marked
Phosdrin		O	Rapid	Marked
Gusathion		S	Rapid	Marked
Phosphamidon		O	Moderate	Marked
Dimethoate		S	Slow	None
Morphothion		S	Slow	None
Isolan			V. rapid	Marked
Dimetilan			V. rapid	Slight
Sevin			Rapid	None

accompanied by reduced symptom intensity and particularly by suppression of salivation, lachrymation and urinary incontinence.

Effects of oxime therapy varied from one compound to another, as shown in Table I. With some compounds symptoms and mortality were both reduced, with some, mortality was reduced or delayed with little effect on symptoms until that stage, with some, there was no apparent effect, while with one, sevin, there was a suggestion of faster deaths.

Atropine and oxime given together never showed a greater benefit than atropine alone after oral insecticide, but did so after intraperitoneal morphothion. With some insecticides the combination was less beneficial than atropine alone, this being most marked with oral morphothion where the combination produced more severe effects than no therapy at all. There was no question of direct toxic effects from the therapeutic injections, these being readily distinguishable and excluded at these doses by

control tests. In the two compounds with which pralidoxime methanesulphonate was tested, no difference was detected between its beneficial effects and those of the same dose of the iodide.

Discussion

As might be expected, atropine therapy was beneficial with all the compounds tested.

Oxime therapy alone was beneficial with the diethyl phosphate insecticides ethyl-gusathion, demeton and thimet, and with the quicker-acting dimethyl phosphates phosdrin, gusathion and phosphamidon, but not with the slow-acting dimethyl phosphate morphothion, either orally or intraperitoneally. These findings are thus in accord with predicted proportions of reversible cholinesterase inhibition, since of these materials only morphothion would be expected to give largely irreversible inhibition. These and previous results (Sanderson and Edson, 1959) are related to chemical structure in Table III.

Of the carbamate insecticides tested, oxime therapy alone was beneficial with isolan, probably slightly with dimetilan, and not with sevin. Of these, sevin is an *N*-monomethyl carbamate, while isolan and dimetilan are *NN*-dimethyl carbamates. The result with sevin agrees with those of Smyth, Carpenter, Nair, Palm, Rogers, Weil and Woodside (1958), and Carpenter, Weil, Palm, Woodside, Nair and Smyth (1961). Effects due to the *NN*-dimethyl carbamate anticholinesterase neostigmine and some of its derivatives were reversed by oxime administration (Grob and Johns, 1958). Insufficient work has yet been done on reactivation of carbamoylated cholinesterase to enable any reasonable theories relating structure and ease of oxime reactivation to be propounded.

It is noteworthy that none of the tests of Table I showed any increased benefit when oxime was given as well as atropine after oral administration of insecticide. Most workers who have noted potentiation between the two therapeutic drugs (Davies and Green, 1959), however, have administered their anticholinesterases by injection. In these tests, potentiation of beneficial effect was, in fact, seen after intraperitoneal morphothion (Table II). In the tests of Table I, it was found that pralidoxime iodide apparently reduced the beneficial effect of atropine with gusathion, ethyl-gusathion, demeton and morphothion given orally, the toxic effects with morphothion being more severe and faster than with no therapy at all. With demeton and ethyl-gusathion, the apparent deleterious effect of the combination occurred where pralidoxime iodide alone was beneficial. Similar apparent deleterious effects when oral anticholinesterase poisoning is treated with oxime and atropine have been noted by Sanderson and Edson (1959) with dimethoate, and possibly unwittingly by Fournel (1957a) with parathion, endothion and demeton-methyl. This finding was at first sight difficult to account for except possibly in terms of an interference by the combined therapeutic drugs with activating or detoxifying enzyme systems, thus indirectly altering the proportion of reversible cholinesterase inhibition. A further possibility, though most unlikely, is that the effect may be partly due to formation of a more toxic

compound between inhibitor and oxime, as has been reported with sarin (Hackley, Steinberg and Lamb, 1959). However, it has recently been suggested (F. Hobbiger, personal communication) that the combination might effect absorption rate of the insecticide from the gut after oral administration, partly by reducing pyloric peristalsis, giving a higher and earlier tissue concentration of inhibitor, and greater persistence also. It is not easy to explain all the effects observed on this basis, but it could well account for the deleterious effect apparently only occurring after poisoning by some compounds given orally, as is suggested by these results.

It is thus apparent that oxime therapy alone is not beneficial with poisoning by all anticholinesterases, and that with organophosphates it is usually possible to predict whether oximes will be beneficial on the basis of expected proportion of reversible cholinesterase inhibition. Even if oxime therapy is not beneficial, it is not harmful alone or, in many cases, in the presence of atropine. It is suggested that any occasional adverse effects of combined therapy might occur only immediately after oral poisoning. If Hobbiger's suggestion of an effect on gut absorption is correct, any adverse effect of giving oxime with atropine should be reduced if absorption of the poison from the gut is largely complete before therapy is commenced, and absent after poisoning by skin absorption. Some further exploration of factors affecting the combined effects of oxime and atropine therapy for anticholinesterase poisoning under different conditions seems desirable.

Acknowledgments. The author wishes to thank Drs. F. Hobbiger and E. F. Edson for valuable discussions and encouragement, and Miss L. Townsend and Miss P. Humphries for technical assistance.

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BOOK REVIEWS

PHOSPHORUS METABOLISM OF BRAIN. P. J. Heald. Pp. vii + 195 (Including Index). Pergamon Press, Oxford, 1960. 42s.

Progress in the biochemistry of brain has been gaining momentum continuously over the last ten years, and Dr. Heald's book is a valuable contribution to existing knowledge on the relation between phosphates and the functioning of the brain. In presenting this subject Dr. Heald divides the book into two parts, the first dealing with metabolism *in vivo* and the second an examination of information relating to changes occurring *in vitro*.

Part I begins with a critical assessment of phosphorus metabolism in the normal functional state, outlining the phosphorus compounds found in the brain and their mode of entry and relative rates of exchange of both acid-soluble and acid-insoluble phosphates. The pathways by which phosphorus enters the brain, mainly followed by measuring uptake of radioactive phosphorus, have been critically examined, and Dr. Heald gives a lucid account of the available knowledge.

To assess the significance of the relative rates of exchange of phosphorus by phosphate derivatives the author describes two techniques that have been used: firstly, alteration of the normal functioning of the brain and an examination of this changed cerebral activity on phosphate metabolism. Alterations in the cerebral activity such as those induced by anaesthesia, hypoglycaemia, hypoxia, hypothermia, the administration of electro-shock and convulsive agents, have all provided changes in cerebral phosphate levels. Secondly, use is made of changes taking place in metabolism during brain development when signs of its co-ordinated functioning are appearing in the whole animal.

Observations on preparations of cerebral tissue slices, homogenates and particulates *in vitro* discussed in Part II show the usual limitations brought about by a change in many characteristics when cellular structure is disrupted. Nevertheless, a much more detailed examination of the metabolic potential of the tissue and its response to different conditions can be made by elucidation of the synthesis, degradation and interactions of the various cellular constituents *in vitro*. Studies of general metabolism *in vitro* of tissue preparations under a variety of conditions have provided close parallels with changes taking place *in vivo*. Whenever possible, the difficult task of correlating and interpreting the studies of phosphorus metabolism *in vivo* with observations *in vitro* is achieved, and most convincingly.

Factors affecting the metabolism in cerebral tissue slices *in vitro* which bear close relation to those factors similarly active *in vivo* are dealt with in two groups. In the first group are included different oxidisable substrates and metabolic inhibitors, and in the second group are factors whose activity is linked to physical changes, for example, electrical stimulus and concentration changes in inorganic ions. Dr. Heald also discussed levels of inorganic phosphate and phosphate acceptors which increase oxygen uptake in the different brain preparations he examines.

A chapter of particular interest is devoted to the effect of therapeutic agents on phosphate metabolism in brain preparations observed *in vitro* and correlated to effects found *in vivo*. Dr. Heald examines the effects of substances known as tranquillising agents and depressants on the level of energy-rich phosphates and phosphate acceptors, and on the efficiency of the phosphorylating systems and the oxidative phosphorylation process; the account is extended to electrically

stimulated systems. In this field it is not possible to generalise on the parallelism between results *in vivo* and *in vitro*; in some instances it exists, whilst in others it is absent or insufficient evidence is available.

The book ends with a short discussion on the analytical methods employed in the determination of phosphorus in cerebral tissue.

We are indebted to Dr. Heald for his valuable contribution to the understanding of phosphate metabolism in cerebral tissue, and his book is thoroughly recommended to workers in biochemistry, pharmacology and medicine who are interested in this field of research. N. ROBINSON.

OFFICIAL METHODS OF ANALYSIS, A.O.A.C., NINTH EDITION, 1960. Pp. xx + 831 (including Index). Published by the Association of Official Agricultural Chemists, Inc., Washington, D.C., U.S.A. U.S.A., $17.50; elsewhere, $18.00.

The latest edition of this well-known volume has changed in comparison with its predecessors. In previous editions the increasing number of pages had been kept in check by the abbreviation of words and by shortened scientific nomenclature. In the present volume, however, the increase in new material has necessitated a larger page with two columns of type.

This growth in the number of analytical methods closely reflects the increasing importance of analysis in the application of legislation to pharmacy, food and agriculture. The present volume includes a number of analytical methods directed towards the enforcement of the pesticides amendment to the Federal Food, Drug and Cosmetic Act which requires a method for the determination of a given pesticide residue on a treated crop. Of the methods tested by the A.O.A.C. collaborative procedure, eight: aramite, benzene hexachloride, captan, malathion, methoxychlor, piperonyl butoxide, sulphenone and tetramethylthiuram disulphide have been found to be suitable for inclusion. Nevertheless, it is obvious that it is becoming increasingly difficult for methods of analysis to keep pace with the necessary regulations.

Most of the methods given in the book are essentially the same as in previous editions, but certain changes should be mentioned. The chapter on soils has been omitted although the classical scheme of elementary analysis has been retained elsewhere. Bacteriological methods for disinfectants now constitute a separate chapter, and methods for the analysis of additives in animal feeding stuffs are given.

Many new analytical procedures are included. Chromatography is used for the detection of commercial glucose in honey, and to differentiate technical benzene hexachloride from lindane. A radioactive tracer method using ^{36}Cl for the determination of benzene hexachloride in pesticide formulations is included and there is an infra-red method for the identification of gums in foods. Microbiological methods for vitamins constitute a useful chapter and a biological test is given for paralytic shell-fish poison.

There are those analysts who hold that the increasing complexity of modern analytical methods makes so-called "standard" methods undesirable. And, indeed, methods are likely to become both more specific and more complex with the wide variation of analytical problems particularly in the fields of food and agriculture. Nevertheless, the existence of a method which has been tried and found satisfactory by a collaborative trial such as the A.O.A.C. organises, is of great value to analysts who, when confronted with a new analytical problem, are often at a loss to decide which of several published methods is most likely to be satisfactory. This volume is an essential for the food and agricultural analyst and for many others as well. R. E. STUCKEY.

LETTERS TO THE EDITOR

A Sensitive Method for the Assay of Acetylcholine

SIR,—Several methods have previously been described for the detection or assay of minute amounts of acetylcholine. Unfortunately most of these methods are either very laborious or the preparations used are unstable and vary in sensitivity throughout the assay. We have developed a method, based on that described by Paton (1957), with which we were able to assay amounts of acetylcholine of about 1 picogram (pg.) or less.

The terminal ileum of large guinea-pigs, weighing between 750 g. and 1 kg., was removed, washed in saline solution and cut into segments of approximately 3 cm. in length. The ileum from smaller guinea-pigs proved to be insensitive.

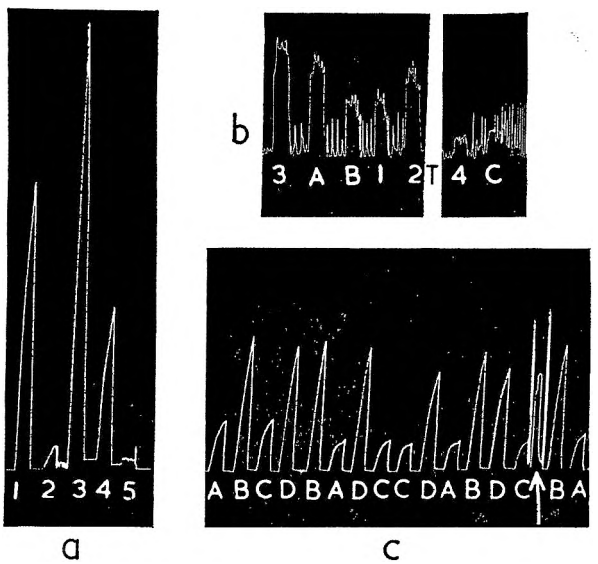

FIG. 1a. Guinea-pig ileum suspended in double-glucose Tyrode solution, sensitised to acetylcholine. 1, 20 ng. histamine; 2, 0·5 μg. 5-hydroxytryptamine; 3, 0·05 pg. acetylcholine chloride; 4, 0·025 pg. acetylcholine chloride; 5, 0·2 ml. double glucose Tyrode solution.

b. Guinea-pig ileum suspended in Hanks' balanced salt solution sensitised to acetylcholine. The numbers denote the amount of standard acetylcholine chloride solution in pg. Doses of test solution A, 0·2 ml.; B, 0·1 ml. and C, 0·4 ml. In period T, atropine sulphate (10^{-8} g./ml.) was added to the bath for 5 min.

c. Guinea-pig ileum suspended in double-glucose Tyrode solution, sensitised to acetylcholine. Four point Latin square assay of two acetylcholine chloride solutions. A = C = 0·1 pg.; B = D = 0·15 pg. Dose interval 90 sec., contact time 30 sec. At the arrow the tissue went into spasm.

The segments of ileum were placed in about 1 litre of a suitable aerated physiological salt sclution which contained 10^{-5}M di-isopropylphosphorofluoridate and allowed to stand at 37° for 1 hr. A segment of this ileum was then cleared of mesentery and mounted in a 2·0 ml. organ bath at 28–32°; the sensitivity of the tissue to acetylcholine did not appear to be affected by slight changes in temperature but reduced temperature minimized the spontaneous movements. The physiological salt solution in which the ileum was mounted varied in composition according to the tissue from which extracts containing acetylcholine were collected but always contained 5 mg./l. of morphine sulphate. Paton (1957 has shown that this concentration of morphine sulphate reduces the violent intermittent spasms of the ileum which usually occur after cholinesterase inhibition. In different experiments the ileums were suspended in Tyrode solution containing twice the usual quantity of glucose and in Hanks' balanced salt Solution. Similar sensitivities to acetylcholine were obtained in each solution. Standard acetylcholine solution and test solutions were diluted with the same solution as that in which the ileum was suspended; the dose volume did not exceed 0·2 ml.

The threshold amount of acetylcholine varied with the preparation and was occasionally as low as 0·0125 pg. which represents a bath concentration of $6{\cdot}25 \times 10^{-15}$ g./ml; more usually the threshold amount fell between 0·1 and 1 pg. The sensitivity to other common spasmogens such as histamine and 5-HT was similar to that in untreated ileum (Fig. 1a); Atropine 10^{-8} was effective in blocking the action of acetylcholine and a typical response is shown in Fig. 1b.

Fig. 1c shows a four point Latin square assay. The dose response curve was steep and a dose ratio of 1 : 1·5 was used. The index of precision (λ) calculated for this assay was 0·024 and the fiducial limits (P = 0·05) of the potency ratio were 94·5 to 105·8 per cent.

The difficulties encountered with the preparation were (i) that when mounted in double-glucose Tyrode solution the tissue often goes into spasm at 20–30 min. intervals making it almost impossible to perform a complete latin square assay—see Fig. 1c; and (ii) that in Hanks' solution the spontaneous movements were troublesome. A small proportion of the guinea-pigs proved to be completely insensitive to all concentrations of acetylcholine after the sensitising treatment, some showing a slow rhythmical contraction which could be abolished by atropine.

We hope that this method may prove suitable for assaying minute amounts of acetylcholine liberated from tissues. The method is suitable for use with the two physiological salt solutions described, and possibly a variety of other mammalian physiological salt solutions can be used.

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Effect of Hersaponin and Acorus oil on Noradrenaline and 5-Hydroxytryptamine Content of Rat Brain

SIR,—The neuro-pharmacological actions of hersaponin, the active principle from *Herpestis monniera*, Linn, have been reported earlier (Malhotra and Das, 1959; Malhotra, Das and Dhalla, 1960). The barbiturate hypnotic potentiating action of the drug was shown to be antagonised by lysergic acid diethylamide (LSD). Dandiya, Cullumbine and Sellers (1959) reported that the volatile oil (acorus oil) from the rhizomes of *Acorus calamus*, Linn, potentiated barbiturate hypnosis and this was antagonised by LSD. Acorus oil (Dhalla, Malhotra and Sastry, 1961) and hersaponin (Dhalla, Sastry and Malhotra, 1961) have both been found to depress rat brain respiration *in vitro*. The depressant effects were enhanced by 5-HT and decreased by LSD. Recent studies from our laboratory showed that the barbiturate hypnotic potentiating action of hersaponin, acorus oil and reserpine is antagonised by dibenzyline while that of chlorpromazine is unaffected (Malhotra, Das and Dhalla, unpublished observations). These investigations indicated that hersaponin and acorus oil might also affect the 5-HT and noradrenaline contents of brain, as does reserpine.

Hersaponin, isolated from *Herpestis monniera* (Sastry, Dhalla and Malhotra, 1959), was suspended in 10 per cent propylene glycol, and acorus oil was suspended in 1 per cent Tween 80. 5-HT and noradrenaline contents of whole brain of adult albino rats were estimated after the intraperitoneal administration of hersaponin, acorus oil, reserpine or chlorpromazine. Control animals were treated with solvents. Noradrenaline was extracted by the method of Vogt (1954) and assayed on rat blood pressure by the method of Crawford and Outschoorn (1951). 5-HT was extracted and assayed on oestrous uterus of rat by the method of Parratt and West (1957).

The results of the investigations (Table I) show that hersaponin and acorus oil deplete the rat brain of its noradrenaline and 5-HT contents, as does reserpine.

TABLE I

EFFECT OF HERSAPONIN, ACORUS OIL, RESERPINE AND CHLORPROMAZINE ON THE NORADRENALINE AND 5-HT CONTENTS OF RAT BRAIN

(Mean ± Standard Errors and Probability (P) from Student's "*t*" test)

Drug and dose	Time interval in hr.	No. of rats	Noradrenaline µg./g.	No. of rats	5-HT µg./g.
Control	—	10	0·320 ± 0·018	10	0·196 ± 0·010
Hersaponin 20 mg./kg.	½	8	0·115 ± 0·004 (P <0·01)	8	0·130 ± 0·013 (P <0·05)
Acorus oil 100 mg./kg.	1	8	0·145 ± 0·012 (P <0·01)	8	0·156 ± 0·012 (P <0·05)
Reserpine 1 mg./kg.	3	5	0·140 ± 0·009 (P <0·01)	5	0·102 ± 0·003 (P <0·01)
Chlorpromazine 2 mg./kg.	2	6	0·270 ± 0·010 (P >0·05)	6	0·204 ± 0·012 (P >0·05)

Chlorpromazine, had no significant effect. It appears, therefore, that the active constituents of the two Indian indigenous drugs *Herpestis monniera* and *Acorus calamus*, which have been widely used in the Ayurvedic System of medicine for different nervous and mental diseases (Nadkarni, 1954), may prove to have a mechanism of action similar to that of reserpine.

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