RESEARCH PAPERS

THE EFFECTS IN RABBITS OF THYROIDECTOMY AND TREATMENT WITH TRIIODOTHYRONINE ON THE SENSITIVITY TO NORADRENALINE AND THE CONTENT OF NORADRENALINE IN AORTA AND SPLEEN

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Received February 7, 1962

The sensitivity of spiral strips of aorta and strips of isolated spleen to sympathomimetic amines and nicotine has been studied in the thyroidectomised rabbit and in rabbits treated with triiodothyronine. Aortic strips from hyperthyroid rabbits were less sensitive to noradrenaline and less able to contract than those from normal rabbits; they also usually failed to respond initially to nicotine or tyramine although the nicotine response could be restored by exposure of the tissues to noradrenaline. Strips from hypothyroid rabbits were slightly less sensitive to low doses of noradrenaline than the normal although their maximal responses to high doses was greater. The dose-response curves for noradrenaline of spleen capsules from hyperthyroid and hypothryoid rabbits were more shallow than the normal. While spleen capsules from both experimental groups responded to nicotine with tachyphylaxis, they failed to respond to tyramine. Exposure of the capsules to noradrenaline temporarily restored the nicotine response. The noradrenaline content of aorta and spleen from hyperthyroid and hypothyroid rabbits was determined and in each case was found to be reduced to about 50 per cent of normal. These observations indicate that not all sympathetically innervated tissues of the hyperthyroid rabbit develop an increased sensitivity to sympathomimetic amines. Changes in tissue noradrenaline levels during periods of altered thyroid activity do not explain the altered sensitivity of the aorta and spleen to noradrenaline.

SOME of the effects of increased amounts of thyroid hormones resemble the effects produced by injection of sympathomimetic amines. It is generally held that in hyperthyroid states there is an increased sensitivity to adrenaline and noradrenaline, while in hypothyroid states there is a decreased sensitivity. Some of the evidence for this hypothesis is reviewed by Brewster, Isaacs, Osgood and King (1956). However, the evidence from the literature does not unanimously support the hypothesis (see Discussion), nor do our own experiments.

Recent investigations have led to the suggestion that the depletion of tissue stores of noradrenaline by sympathetic denervation or by reserpine treatment leads to an increase in sensitivity to noradrenaline (Burn and Rand, 1959a). Moreover, some sympathomimetic drugs, such as tyramine and nicotine, depend on the presence or availability of tissue stores of noradrenaline for their action (Burn and Rand, 1958a, b;

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Macmillan, 1959). Therefore we have now investigated the effect of thyroidectomy and of treatment with triiodothyronine, in rabbits, on the sensitivity of isolated preparations of spleen and aorta to noradrenaline, tyramine and nicotine. We have also determined the content of noradrenaline in these tissues to see if the altered sensitivity of tissues from the hypothyroid and hyperthyroid animals to the sympathomimetic amines and nicotine was related to the tissue noradrenaline levels.

METHODS

Male rabbits of 2·5 to 3·5 kg. were selected at random from a colony fed on Purina Laboratory Chow. Thyroidectomies were performed with sterile precautions and the animals allowed to recover from the operation for 8–20 days; the drinking water of these rabbits contained 2 per cent of calcium gluconate to counteract possible parathyroid damage. Rabbits were made hyperthyroid by daily subcutaneous injections of 100 μg. of triiodothyronine, using a solution of 1 mg./ml. of triodothyronine in slightly alkaline saline. Injections were continued for 8 to 20 days until the day before the rabbit was killed. Reserpine treatment was given as described by Macmillan (1959).

Rabbits were killed by a blow on the head and then bled out. The entire thoracic aorta and the spleen were dissected out rapidly and washed in Locke's solution.

A spiral strip was prepared from the upper thoracic aorta (Burn and Rand, 1958a) and the remainder of the aorta was cleaned and stored at −8° for assay of its noradrenaline content. As far as possible the pitch of the spiral was the same in all experiments. The width of the final band of tissue was approximately 2 mm. It was arranged in a 9 ml. organ bath at 35° in Locke's solution gassed with O_2 so that its length was 7–7·5 cm. under a tension of 12 g. The spiral strip was allowed to remain untouched for 1 hr. Before each application of a drug the tension was reduced to 8 g., and the strip allowed to come to a new resting length. After the effect of the drug had been observed the bath was washed four times and the tension was again increased to 12 g. Noradrenaline was allowed to act for 2 min. and a further 6 min. elapsed before the next addition of noradrenaline. Tyramine and nicotine were allowed to act for 5 min. and a further 10 min. elapsed before the next addition of drug. Records were made on the smoked drum with a gimbal side writing lever with a magnification of 17 : 1.

The contractions of the isolated spleen capsule were observed by attaching threads to each pole of the spleen and cutting away the two lateral borders. The pieces removed from the spleen were stored at −8° for subsequent assay of their noradrenaline content. The central portion of the spleen was suspended in Locke's solution at 35° in a 40 ml. organ bath. Records were made with a frontal writing lever with a magnification of 17 : 1. The tension on the spleen capsule was 4·0 g.

The aorta and spleen fractions reserved for assay of noradrenaline were extracted and estimated using the methods of Burn and Rand (1959a).

Results

Treatment of Rabbits

The mean increase in weight of the thyroidectomised rabbits was 310 g. and the mean decrease in weight of the rabbits injected with triiodothyronine was 500 g. No other special observations were made on the efficacy of the treatments, but the general appearance of the animals indicated that the hypo- and hyperthyroid states had been achieved; for

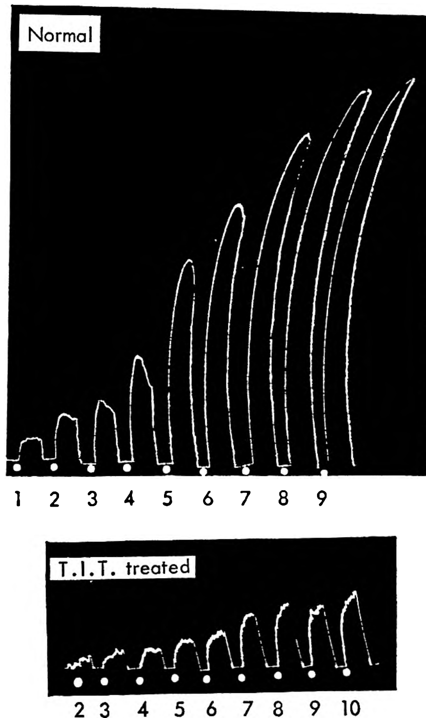

FIG. 1. Contractions of spiral strips of aorta to noradrenaline. Upper record, aorta strip from control rabbit; lower record, aorta strip from rabbit pretreated with triiodothyronine. The numerals refer to the dose of noradrenaline (base) in micrograms given into a 9 ml. bath. Contact time, 2 min.

example the triiodothyronine-treated rabbits were obviously hyperactive and their body temperature was higher than normal, while the opposite held for the thyroidectomised rabbits.

Noradrenaline on Aorta Strips

Typical records of the contractions to noradrenaline of isolated aorta strips from a hyperthyroid and from a normal rabbit are shown in Fig. 1, which illustrates the finding that the aorta strips from the hyperthyroid rabbits were less sensitive to noradrenaline and less well able to contract than those from normal rabbits. Strips from hypothyroid rabbits were slightly less sensitive to noradrenaline than normal as judged by the

higher mean threshold dose required, but they were able to give responses greater than the maximal responses obtained with normal strips. The results for all the experiments with noradrenaline on isolated aorta strips

TABLE I

MEAN RESPONSES OF RABBIT AORTA STRIPS TO NORADRENALINE

	Number in group	Threshold dose (μg./ml.). Geometric means	Dose for maximal response (μg./ml.). Geometric means	Maximal response (mm. contraction on kymograph). Means	Regression of response on log dose. Means
Normal	4	0·015	0·76	108	16·1
Treated with triiodothyronine	5	0·032	2·56*	40**	6·0*
Thyroidectomised ..	5	0·023	5·0**	149**	19·5

* Significantly different from normal ($P < 0·05$).
** Significnatly different from normal ($P < 0·01$).

are brought together in Table I. In each experiment the threshold dose for noradrenaline was determined by extrapolation to zero response from the calculated regression line relating response to the logarithm of the noradrenaline dose.

Nicotine and Tyramine on Aorta

Strips of aorta from normal rabbits responded with regular contractions to successive doses of nicotine bitartrate (60–250 μg./ml.) and of tyramine (25–200 μg./ml.) added to the bath, but strips from the hyperthyroid rabbits usually failed to respond to 10 times these doses of

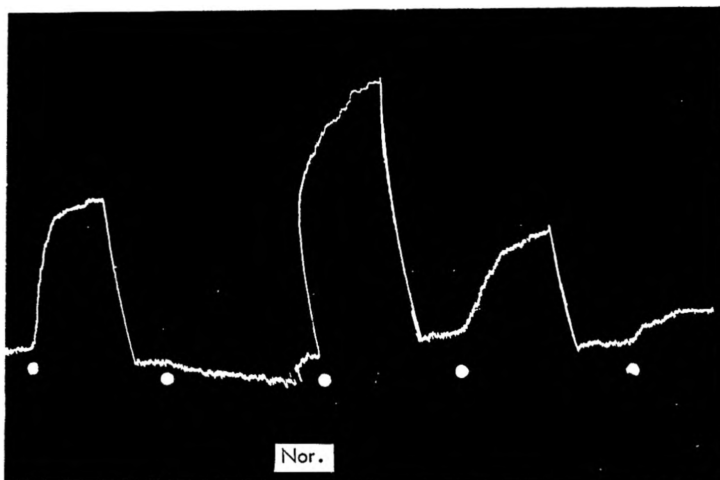

FIG. 2. Effect of nicotine on spiral strip of aorta from rabbit pretreated with triiodothyronine. At dots, 1 mg. of nicotine bitartrate was added to 9 ml. bath and allowed to act for 5 min. The second addition of nicotine did not cause a contraction, but after soaking the strip in noradrenaline (100 μg.) for 3 hr. (at Nor) and then washing out the noradrenaline the response to nicotine was restored.

nicotine or tyramine. However, in some experiments, a small contraction was produced by the first injection of nicotine, but no response was seen with subsequent doses (Fig. 2). When the response to nicotine was absent in an aorta strip from a hyperthyroid rabbit, it was restored after the addition of 10 to 100 μg. of noradrenaline to the bath for periods of time ranging from 5 min. to 3 hr. Fig. 2 shows an experiment in which there was a temporary restoration of responses to nicotine after soaking the aorta strip with noradrenaline. We were unable to restore the response of aorta strips to tyramine when it was absent as a result of

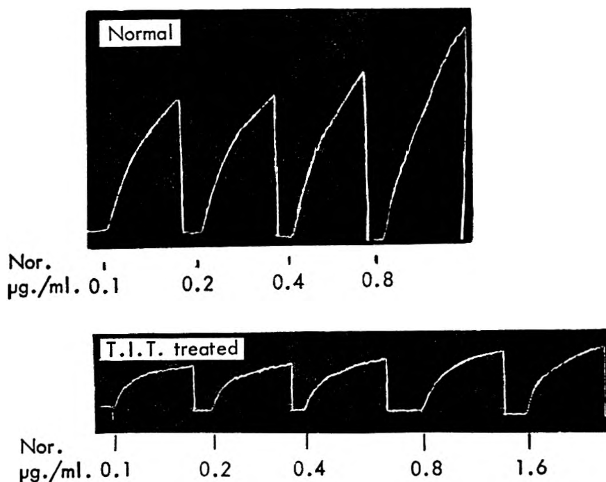

FIG. 3. Contractions of isolated spleen capsule produced by noradrenaline. Upper record from a normal rabbit, lower record from a rabbit pretreated with triiodothyronine. Contact time, 3 min.

pre-treatment of the rabbit with triiodothyronine. Aorta strips from thyroidectomised rabbits responded to nicotine and tyramine like strips from normal rabbits.

Noradrenaline on Spleen Capsule

The central part of the spleen capsule suspended in an isolated organ bath contracted in response to noradrenaline. Fig. 3 shows the contractions of spleen from a normal and a triiodothyronine rabbit to a range of concentrations of noradrenaline. The mean responses from all experiments are given in Fig. 4, which shows that the dose-response curves for noradrenaline obtained from the spleen capsules of both triiodothyronine-treated and thyroidectomised rabbits were more shallow than that obtained from the spleen capsules of the normal animals. The threshold doses of noradrenaline on the spleen capsule are given in Table II. Although the spleen capsules from the hyperthryoid rabbits were less contracted than the normal by large doses of noradrenaline, their sensitivity to low doses of noradrenaline was the most, and the capsules from the hypothyroid rabbits were the least sensitive. The

differences in threshold dose of noradrenaline between the three groups were shown to be significant by an analysis of variance at P less than 0·05.

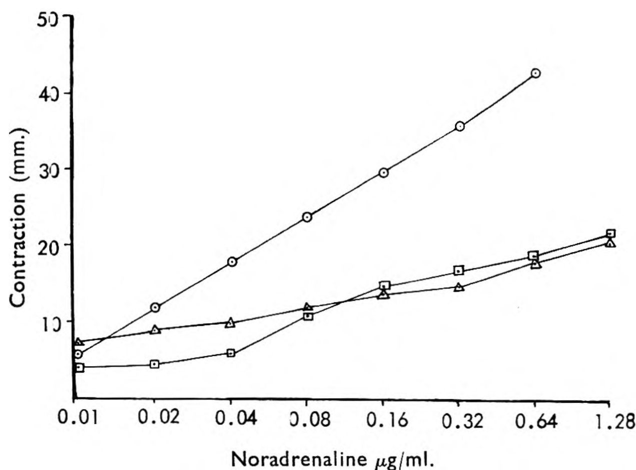

FIG. 4. Mean contractions of isolated spleen to noradrenaline. Ordinate, contractions of spleen in mm. on kymograph record (magnification of writing lever 17 : 1). Abscissa, concentration of noradrenaline in μg./ml. (log. scale). Mean results: from 3 normal rabbits, ⊙ ; from 6 rabbits treated with triiodothyronine, △ ; from 5 thyroidectomized rabbits, ⊡.

Nicotine and Tyramine on Spleen Capsule

The contractions of spleen capsules from normal rabbits in response to 10–80 μg./ml. of nicotine bitartrate were graded with dose and were reproducible. In spleens taken from both triiodothyronine-treated and thyroidectomised rabbits the responses to nicotine were less well shown and exhibited a pronounced tachyphylaxis, so that eventually even high

TABLE II
THRESHOLD CONCENTRATION (ng./ml.) NORADRENALINE REQUIRED TO PRODUCE CONTRACTION OF SPLEEN

	Normal	Triiodothyronine-treated	Thyroidectomised
	5	1	10
	2	0·3	4
	2	0·3	15
		0·05	6
		2	4
		1·5	
Means	3	0·8	8

doses were without effect. Fig. 5, shows an experiment in which increasing doses of nicotine were given to a strip of spleen capsule from a triiodothyronine treated animal; the responses became smaller. After noradrenaline was added to the organ bath (0·1 μg./ml. for 10 min.) and then washed out a further addition of nicotine produced a well marked

contraction, but this restoration was only temporary. Strips of spleen capsule taken from reserpine-treated rabbits behaved in a similar way to those from triiodothyronine-treated rabbits. They contracted in response

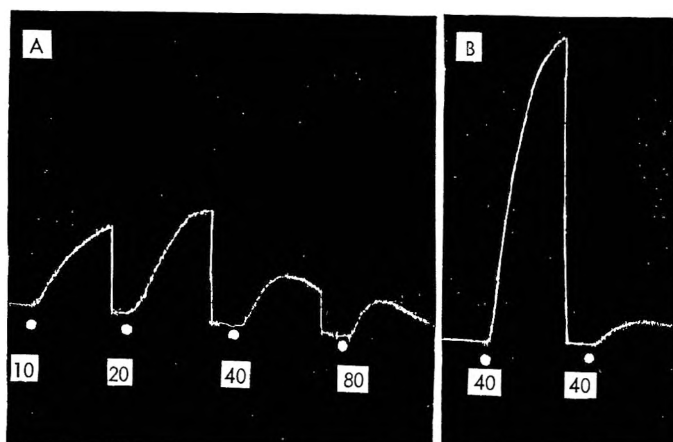

FIG. 5. Effect of nictotine on isolated spleen capsule from a triiodothyronine treated rabbit. At dots, nicotine bitartrate was added to the bath in the concentrations shown (μg./ml.). Panel A shows that there is marked tachyphylaxis to nicotine. Between A and B the spleen was soaked in noradrenaline (0·1 μg./ml.) for 10 min. and then noradrenaline was washed out. The next response to nicotine was potentiated.

to noradrenaline, but failed to contract with nicotine. After treating the reserpinised spleen in the organ bath with noradrenaline the response to nicotine was restored (Fig. 6). Tyramine did not contract the reserpinised spleen, nor the spleens from triiodothyronine-treated or thyroidectomised rabbits. We were unable to restore responses to tyramine by soaking the spleen with noradrenaline.

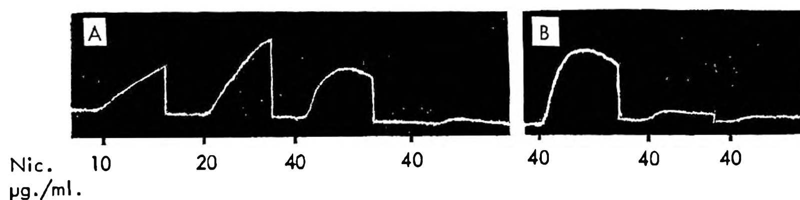

FIG. 6. Responses of isolated spleen strip from reserpine-treated rabbit to nicotine. Between A and B, noradrenaline (0·01 μg./ml.) was present in the bath fluid for 10 min. and then washed out.

Noradrenaline Content of Aorta and Spleen

The noradrenaline content of the aorta and spleen from normal, hyperthyroid and thyroidectomised rabbits was assayed by matching the pressor activity of tissue extracts with that of noradrenaline standards on the blood pressure of the pithed rat. In addition, we examined extracts

from the spleens of reserpine-treated rabbits. The results are shown in Table III.

TABLE III

SUBSTANCES PRESENT IN EXTRACTS OF RABBIT AORTA AND SPLEEN ESTIMATED AS NORADRENALINE (ng./g.)

	Aorta			Spleen			
	Normal	Triiodo-thyronine-treated	Thyroidect-omised	Normal	Triiodo-thryonine-treated	Thyroidect-omised	Reserpine-treated
	280	125		500	575		38
		129	138		206	425	75
	200	175	150	1500	550	700	180
	225	262	194	900	825	400	
	250	190	150	1140	500	310	
		200	125		335	500	
Means	239	180**	151***	1010	499*	467*	98*

* Significantly different from normal (P <0·05).
** Significantly different from normal (P <0·01).
*** Significantly different from normal (P <0·001).

DISCUSSION

The effect of treatment with triiodothyronine on the aorta and spleen resembles, in some respects, the effect of treatment with reserpine. Thus both treatments lead to a decrease in the noradrenaline content of these tissues and to a loss of the action of nicotine and tyramine. The threshold dose of noradrenaline to contract the cat spleen *in situ* is decreased after reserpine treatment (Burn and Rand, 1959a), and after treatment with triiodothyronine a decrease in threshold was seen in the spleen, but not in the aorta. Suprathreshold doses of noradrenaline were less effective in strips of aorta and of spleen taken from hyperthyroid rabbits than in strips from the normal controls. This decreased responsiveness may in part explain the diminished effects of nicotine and tyramine. However the observation that soaking the strip in noradrenaline temporarily restored the response to nicotine suggests that the decreased store of noradrenaline was also partly responsible for the impaired response to nicotine.

There was less noradrenaline stored in aorta and spleen taken from thyroidectomised rabbits than in those taken from normal rabbits. Isolated spleen strips from these rabbits did not respond to tyramine and to nicotine, but after soaking with noradrenaline the response to nicotine was increased. Therefore it can be concluded that the reduction in noradrenaline stores in the spleen was concerned in the loss of response of spleen strips to nicotine. However, aorta strips from thyroidectomised rabbits responded to nicotine and tyramine as did strips from normal rabbits. The threshold dose of noradrenaline on both the aorta and spleen strips was raised, and the responsiveness of the spleen to larger doses of noradrenaline was decreased. However, the responsiveness of aorta strips from thyroidectomised rabbits to larger doses of noradrenaline was greater than that of control strips.

These results show that an inverse relationship between tissue stores of noradrenaline and the sensitivity to exogenous noradrenaline does not

always hold. Such a relationship was demonstrated by Burn and Rand (1959a) when they investigated the hypersensitivity to noradrenaline of the cat's dilator iris muscle and spleen, produced by both sympathetic denervation and by reserpine. Fleming and Trendelenburg (1961) have pointed out that the time factor must also be considered, although this factor seemed to be more important for the appearance of supersensitivity of the nictitating membrane than of the cardiovascular system. Burn and Rand proposed that the supersensitivity was a result of the unmasking of more receptor sites when the normal transmitter output fell as a result of the depletion. Axelsson and Thesleff (1959) found that in denervated, hypersensitive, skeletal muscle, which has been deprived of its neurohormone by denervation, a greater acetylcholine sensitive area could be detected than in innervated muscle which is continually exposed to its neurohormone. On the other hand, if larger amounts of acetylcholine are applied to the end plate region, the receptors become refractory and the sensitivity of the muscle is decreased (Katz and Thesleff, 1957). Conceivably, a similar situation may exist in sympathetically innervated tissues with respect to the sympathetic transmitter, noradrenaline, and the receptors to noradrenaline.

A lack of correlation between depleted tissue stores of noradrenaline and increased sensitivity might occur for a variety of reasons. Macmillan (1959) suggested that if the exogenous noradrenaline was prevented from entering stores, for example, by cocaine, more would be available to act on receptors, and Vane (1961) suggested that if acidic non-specific binding sites in tissues were occupied by pharmacologically inert basic substances, then a greater portion of a subsequently injected dose of a pharmacologically active base would be available for receptors. In either case there would be an increased sensitivity without a decrease in the store. The converse possibility, decreased store without increased sensitivity might occur if the decreased store were a reflection of increased output of transmitter. The observation that infusion of noradrenaline leads to a loss of sensitivity to noradrenaline (Burn and Rand, 1959b) supports the possibility that bombardment of receptors by endogenous noradrenaline may lead to a decreased sensitivity to endogenous noradrenaline, as well as to a reduction in the size of the store of endogenous noradrenaline.

Our experiments do not allow us to say by what mechanism the noradrenaline stores are depleted by triiodothyronine and by thyroidectomy, but it is probable that the mechanisms are different in each case. The effect of reserpine treatment (Thier and Gravenstein, 1960) and of guanethidine (Gaffney, Braunwald and Kahler, 1961) in abolishing those effects of hyperthyroidism which resemble the responses to sympathetic stimulation suggests that in the hyperthyroid state there is an increased output of noradrenaline. The decrease in noradrenaline stores in thyroidectomised rabbits may be due to impaired synthesis since Goodall (1951) found that the amino-acid precursor dihydroxyphenylalanine was present in the adrenals of thyroidectomised but not of normal sheep.

Our findings that artery strips from hyperthyroid rabbits were less sensitive to noradrenaline and that their ability to contract was considerably less than that of strips from normal rabbits confirms the earlier observations of Miculicich (1931), who investigated the effect of previous thyroid feeding on the vasoconstriction produced by injecting 20 μg. of adrenaline into the blood perfused leg of the dog. In legs from normal dogs the mean increase in pressure was 39 mm.; while legs from thyroid fed dogs were less sensitive with a mean increase in pressure of 35 mm. Smith (1954) found that isolated pig arteries stored for 18–24 hr. in a solution containing thyroxine were less sensitive to adrenaline than were control arteries stored for the same period. Smith (1953) also found that arteries from thyroidectomised pigs were insensitive to adrenaline, although thyroxine added to the perfusing fluid produced an immediate increase in sensitivity.

The effect of experimentally produced hyperthyroid states on the pressor response to adrenaline and noradrenaline has been investigated many times with somewhat contradictory results. In the dog it has been reported that the pressor response is less in the hyperthyroid than in the euthyroid state (Chamberlain, 1928; Hepler and Simonds, 1936; Riggs, Stanbury and Carr, 1951); on the other hand Brewster, Issacs, Osgood and King (1956) reported an increased pressor response after the hyperthyroid state had been produced. In the cat the pressor effect of adrenaline is reported as being greater in the hyperthyroid state (Blau and McNamara, 1930). In the hyperthyroid rabbit the pressor response to adrenaline has been reported as unchanged (Konig, 1928) or increased (Santesson, 1919; Spinks, 1952). To the extent that the responses of the isolated tissues we have chosen, the aorta and the spleen, may be taken as representative of the tissues which are responsible for pressor responses, our results indicate a decreased sensitivity to noradrenaline. However, in the whole animal the factors involved are certainly more complex. The conclusion we wish to draw is that not all effector organs show an increased sensitivity to noradrenaline in the hyperthyroid state.

Acknowledgements. It is a pleasure for M.J.R. to thank W.H.M. for his personal hospitality and Professor Durwood J. Smith and the Department of Pharmacology for their generosity, which made it possible to undertake these experiments.

This work was supported in part by the following grants from the United States Public Health Service: 2G–209, B–2673 and E–1203.

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THE URINARY EXCRETION OF TRITIATED BUTYLATED HYDROXYANISOLE AND BUTYLATED HYDROXYTOLUENE IN THE RAT

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Received February 5, 1962

The total radioactivity of rat urine after the administration of tritiated butylated hydroxyanisole and butylated hydroxytoluene has been measured. The results indicate that approximately 90 per cent of the dose of BHA is excreted within four days. With BHT, the total radioactivity excreted in the same period is equivalent to only 35 per cent of the dose, but some of the radioactivity of the labelled molecule is lost during metabolism and only an approximate estimation of the total urinary excretion is possible.

THE substances butylated hydroxyanisole (BHA) and butylated hydroxy toluene (BHT) are permitted antioxidants in certain foods in many countries so that their method of elimination from animals is a matter of interest. Dacre, Denz and Kennedy (1956) studied the chemical nature of the urinary excretory products of BHA as well as the rate of their excretion in rabbits. About 60 per cent of a single dose of 1 g. was excreted in the urine as glucuronide, ethereal sulphate and free phenol in 24 hr. The dose per cent recovered in the urine in a given time increased with diminishing doses. Dacre (1960) also reported that rats excreted 90 per cent of a single dose (0·08 to 0·1 g.) of BHA in urine. Astill, Fassett and Roudabush (1960) investigated the urinary excretion of BHA in rats at lower dose levels ranging from 2 to 100 mg./kg. and were able to account for 80–100 per cent of the dose as urinary metabolites over a period of about 3 to 6 days. The urinary excretory products of BHT in rabbits were also examined after high doses by Dacre (1961) who estimated that 54 per cent of a single dose could be recovered in the urine in the form of various metabolites over a period of 3 to 4 days.

By the use of BHA and BHT labelled with tritum we have now been able to compare the excretion of these substances in rat urine using a dose of 0·5 mg./kg. which approaches more closely the amounts of these antioxidants encountered in human diets than have previous experiments.

EXPERIMENTAL

Materials

Butylated hydroxyanisole is normally a mixture of two isomers, 85 per cent of 2-t-butyl-4-methoxyphenol and 15 per cent of 3-t-butyl-4-methoxyphenol. A sample of the pure 2-t-butyl isomer obtained from Nipa Laboratories Ltd., Cardiff, Wales, was used in the subsequent experiments. A pure sample of butylated hydroxytoluene, 2,6-di-t-butyl-4-methylphenol was obtained from the same source. Both substances were labelled by Dr. J. Garnett, Chemistry School, University of New South Wales, Sydney, by tritum exchange (Wilzbach, 1957). The tritiated

substances were purified by recrystallisation to constant specific activity, and the homogeneity of each checked by paper chromatography before use. R_F values BHA-^{3}H(2-t butyl isomer) 0·44; BHT-^{3}H 0·94. Descending method, using Whatman No. 1 paper, tanks equilibrated at 20–22°. Solvent systems: organic phase of iso-octane, methanol, water (10:8:2). The specific activity of the BHA-^{3}H(2-t-butyl isomer) was 35 μc/mg. and that of the BHT-^{3}H5 μc/mg.

Tritiated toluene (specific activity $1{\cdot}75 \times 10^6$ d.p.m./ml.) was obtained from the Packard Instrument Co. Inc., La Grange, Illinois, U.S.A. and used as internal standard in the counting of the samples.

Animal Experiments

2-t-Butyl-4-methoxyphenol (97 μg. $= 5{\cdot}44 \times 10^6$ d.p.m.) or 2,6-di-t-butyl-4-methylphenol (100 μg. $= 1{\cdot}29 \times 10^6$ d.p.m.) were injected in 50 per cent aqueous ethanol intraperitoneally into each rat (albino: average weight 200–250 g.). The rats were placed singly (BHA experiment) and in pairs (BHT experiment) in all-glass metabolic containers fitted with stainless steel mesh gauze to separate the urine from the faeces. The rats were fed with rat cubes once every 24 hr. and water was constantly available during the experiments; the urine was collected quantitatively from the containers every 24 hr. for a period of 4 successive days. The urine was filtered, counted adjusted to a convenient volume with water and three aliquots (0·10 ml.) were counted from each urine sample.

Counting Technique

All radioactive estimations were carried out using a Packard Tricarb Liquid Scintillation Spectrometer, Model 314.

The urine samples were pipetted into a scintillation solvent (10·0 ml.) which contained 3·0 g. 2,5-diphenyloxazole and 0·1 g. of 1,4-bis-2-(5-phenyloxazoyl)benzene dissolved in a litre of toluene-ethanol (4:1 v/v). Readings were taken after refrigeration of the samples to $-13°$ over an average time interval of 10 min. per vial with discriminator settings at 10–50 V, and 10–100 V.

Two assay samples of the injection solution were also taken, the volumes made up to 25·0 ml. with 50 per cent aqueous ethanol and subsequently three smaller aliquots (0·10 ml.) from the diluted solution were counted in the scintillation solvent described above (10·0 ml.).

Quenching due to the presence of urinary pigment and ethanol was compensated for by the addition of a fixed volume of the internal toluene-^{3}H standard (10 μl.) to urine vials, assay sample vials and blank vials (containing only scintillation solvent minus ethanol) and recounting all vials at the same high voltage and discriminator settings for the same time intervals.

Distribution of Tritium in 2,6-di-t-butyl-4-methylphenol (BHT-^{3}*H*)

A sample of the BHT-^{3}H used in the excretory experiments (47·3 mg.) was mixed with 2·0 g. of BHT and steam distilled to give a product which, when recrystallised from benzene-light petroleum to constant count, had

an activity of 6·0 × 10^7 d.p.m./mM (m.p. 70°). This product (2·0 g.) was oxidised with bromine in 80 per cent v/v acetic acid (Coppinger and Campbell, 1953) to yield 3,5-di-t-butyl-4-hydroxybenzaldehyde-^{3}H (0·8 g., m.p. 189°) which after recrystallisation from methanol to constant count had an activity of 4·1 × 10^7 d.p.m./mM.

Kuhn-Roth oxidation of this radioactive aldehyde (365 mg.) gave 146 mg. of acetic acid after distillation (estimated by titration with 0·1 N NaOH) which was converted to its *p*-bromophenacyl ester. The ester was recrystallised to constant count from light petroleum to give a product (216 mg.) with an activity of 0·087 × 10^7 d.p.m./mM (m.p. 86.°)

RESULTS AND DISCUSSION

A comparison of the urinary excretion of BHA and BHT based on the total radioactivity of the recovered urine is shown in Table I. With BHA the excretory levels obtained with the tritiated material are similar

TABLE I

URINARY EXCRETION OF BHA-^{3}H* (2-T-BUTYL ISOMER) AND OF BHT-^{3}H† BY RATS

Day	No. of expts. carried out	Mean per cent of radio activity excreted		Limits of error (P = 0·95)	
		BHA-^{3}H	BHT-^{3}H	BHA-^{3}H	BHT-^{3}H
1	6	86·0	12·0	77·0 –94·0	6·0–18·0
2	6	3·0	11·0	1·0 – 5·0	7·0–15·0
3	6	0·6	7·0	0·3 – 0·9	4·0–10·0
4	6	1·5	4·5	0·35– 2·5	3·0– 6·0
Total excretion		91·1	34·5	79·0–103·0	20·0–49·0

* Dose: 97 μg. (5·4 × 10^6 d.p.m.).
† Dose: 100 μg. (1·3 × 10^6 d.p.m.).

to those found by Astill and others (1960) using unlabelled material at dose levels extending from four to two hundred times as great, and indicate that BHA is most probably almost completely excreted in a short time at very low dose levels. These results support evidence for the safety of BHA as an antoxidant in foods.

Because of the difficulty of chemical estimation the excretion of BHT at very low dose levels has not been previously measured. Our results would indicate that the urinary excretion of BHT is less than that of BHA in rats. This is no doubt due to the lower polarity of BHT and the fact that the phenolic group is not available for conjugation with sulphate or glucuronic acid as it is in BHA (Dacre and others, 1956). However, it is possible to give only an approximate estimate of the urinary excretion of BHT from our results as it is not correct to assume that the total amount of tritium present in the urine after BHT-^{3}H administration is equivalent to the amount of BHT excreted.

Whereas BHA does not undergo metabolic oxidation before conjugation so that there will be no loss of labelled tritium, Dacre (1961) has shown that with BHT, oxidation of the 4-methyl group to the corresponding carboxylic acid and ω oxidation of one of the tertiary butyl groups to a primary alcohol occurs before conjugation. The tritiated BHT used in our

experiments has been shown to have approximately 48 per cent of the total radioactivity associated with the 4-methyl group so that oxidation of this group to carboxylic acid will result in the loss of this amount of radioactivity. The loss of one tritium atom of the tertiary butyl group due to ω oxidation will, however, produce a negligible loss of radioactivity as only 8·7 per cent of the total activity is shared by both tertiary butyl side chains. Thus, if the BHT administered is all oxidised to carboxylic acid during metabolism in the rat, the recovered radioactivity in the urine (Table I) would be equivalent to approximately half the total BHT excreted as metabolites. If, however, the main metabolic route is by ω oxidation of a tertiary butyl group, the radioactivity of the urine would approximate closely to the amount of BHT excreted. According to Dacre (1961) about one third of the urinary metabolites of BHT in the rabbit after high doses is ester glucuronide. If this should hold for the rat at low dose levels, the total radioactivity of the urine would be equivalent to approximately 50 per cent of the administered dose of BHT. This could be verified if ^{14}C-BHT were available and work is proceeding in this laboratory with this end in view.

Acknowledgements. This work is supported by grants from the National Health and Medical Research Council of Australia, the Australian Atomic Energy Commission and the Australian Food Technology Association.

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THE SPASMOLYTIC ACTIONS OF PYROGALLOL AND CATECHOL ON THE ISOLATED GUINEA-PIG ILEUM

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Received February 9, 1962

Pyrogallol and catechol, potent inhibitors of catechol-*o*-methyl transferase, have general non-specific spasmolytic actions on the isolated guinea-pig ileum. Spasmogens acting by the intramural nervous mechanism are inhibited by lower concentrations of pyrogallol and catechol than are spasmogens acting directly on the smooth muscle. Possible explanations and implications are discussed.

CATECHOL-*o*-METHYLTRANSFERASE is a principal pathway for the metabolism and inactivation of adrenaline and noradrenaline (Axelrod and Laroche, 1959; Axelrod, 1960) and pyrogallol and catechol are potent inhibitors of this enzyme (Axelrod and Laroche, 1959; Axelrod, 1960; Bacq, 1959; Wylie, Archer and Arnold, 1960). They have been shown to potentiate the actions of sympathomimetic amines (Axelrod, 1960; Wylie and others, 1960; Bacq, 1936; Izquierdo and Izquierdo, 1961) by the inhibition of the *o*-methyltransferase, but Barger and Dale (1910–11) concluded that catechol had no sympathomimetic effect but merely a direct tonic action on smooth muscle fibres.

Recently, completely different actions of pyrogallol and catechol have been described. Sjöstrand (1960; 1961) investigated the actions of catechol on the isolated terminal ileum of the guinea-pig; he reported that doses between 0·5 and 5·0 mg. (in a 15 ml. bath) usually produced a stimulation thought to be caused by the stimulation of ganglia in the walls of the intestine. Tachyphylaxis observed with consecutive doses was described as a secondary nicotine-like persistent depression of the ganglia. Higher doses of catechol inhibited contractures induced by acetylcholine, histamine, and substance P.

Izquierdo and Izquierdo (1961) found that pyrogallol produced an initial depressor followed by a stimulatory effect on the motility of the dog duodenum. They claimed the depressor action was adrenaline-like and thought that the stimulation was cholinergic.

Jaques and Doepfner (1959) showed that lysis of the spasm induced by different spasmogens on visceral smooth muscle was a general property possessed by phenol and many of its derivatives. Although pyrogallol and catechol were not investigated the 1,3- and 1,4-isomers of catechol were found to have spasmolytic actions.

It seems important to question the specificity of the *o*-methyl transferase activity of pyrogallol and catechol and to enquire into the site of other actions on smooth muscle.

METHODS

Adult female guinea-pigs of 600 to 750 g. were killed by a blow on the head and bled. The ileum was excised and 3 cm. segments from the

middle and terminal regions were removed and mounted with the oral end downwards in a 10 ml. bath containing Krebs's solution aerated with 95 per cent O_2 and 5 per cent CO_2. Longitudinal contractures were recorded isotonically. Pyrogallol or catechol dissolved in Krebs's solution replaced the bathing medium 10 min. before the dose-response curves were repeated.

The concentrations of pyrogallol were 1×10^{-6}, 5×10^{-6}, 1×10^{-5} and 5×10^{-5}, those of catechol were 1×10^{-4}, 5×10^{-4} and 1×10^{-3} but some intermediate concentrations were also used. The solutions were protected from light.

Dose-response curves were induced by the following spasmogens: acetyl-β-methylcholine chloride (methacholine), 5-hydroxytryptamine creatinine sulphate (5-HT), choline phenyl ether bromide (TMI), dimethylphenylpiperazinium iodide (DMPP) and histamine acid phosphate. All the drug concentrations were expressed as μg. base/ml.

Results

Pyrogallol

Pyrogallol, 1 μg. to 5 mg., did not contract the ileum. In doses of 1×10^{-6} pyrogallol had no effect on the dose-response curves of the spasmogens; doses of 5×10^{-6} usually reduced the responses to 5-HT, TMI and DMPP but did not modify the methacholine and histamine curves. 1×10^{-5} pyrogallol usually inhibited both methacholine and histamine at all doses, with the effect of displacing the dose-response curve, while the 5-HT, TMI and DMPP effects were almost abolished; 5×10^{-5} pyrogallol always abolished the 5-HT, TMI and DMPP effects and reduced the methacholine and histamine responses to such an extent that their former maxima were unattainable (Fig. 1).

In some experiments it was found that a concentration of 1×10^{-5} pyrogallol had very little effect whereas 5×10^{-5} had the effect described above, that is, abolition of the 5-HT, TMI and DMPP responses and a marked reduction of those to methacholine and histamine. In these experiments it was found that intermediate concentrations ($2{\cdot}5 \times 10^{-5}$) reduced only the 5-HT, TMI and DMPP responses.

In a single experiment, barium chloride and carbachol behaved in the same way as histamine and methacholine.

The preparation was washed with normal Krebs's solution for 15 to 30 min. after each concentration of pyrogallol and the normal responses were obtained again.

Catechol

When doses of catechol between 0·1 and 5 mg. were added to the bath no stimulation was recorded. An ED70 of histamine was injected in between each dose of catechol to monitor any possible persistent depressant action. But when the catechol Krebs's solution was used as the bathing medium, an initial longitudinal contracture was sometimes seen which returned to the baseline after one minute.

Qualitatively similar results to those with pyrogallol were obtained with catechol, the most important difference being that an approximately tenfold higher concentration of catechol was required to produce the same effects as pyrogallol. Thus a concentration of 1×10^{-4} catechol hardly affected the dose response curves to any spasmogen. At 5×10^{-4}, it reduced those of 5-HT, TMI and DMPP to the extent that their former maxima were unattainable; it inhibited the histamine effects, displacing the dose-response curve, but left the methacholine curve unchanged. 1×10^{-3} catechol abolished the 5-HT, TMI and DMPP contractures, greatly reduced the histamine maximum and displaced the methacholine dose-response curve leaving its maximum unchanged (Fig. 2).

The normal responses returned after washing with normal Krebs's solution for 30 min.

Pyrogallol and catechol had the same actions on both middle and terminal parts of the guinea-pig ileum.

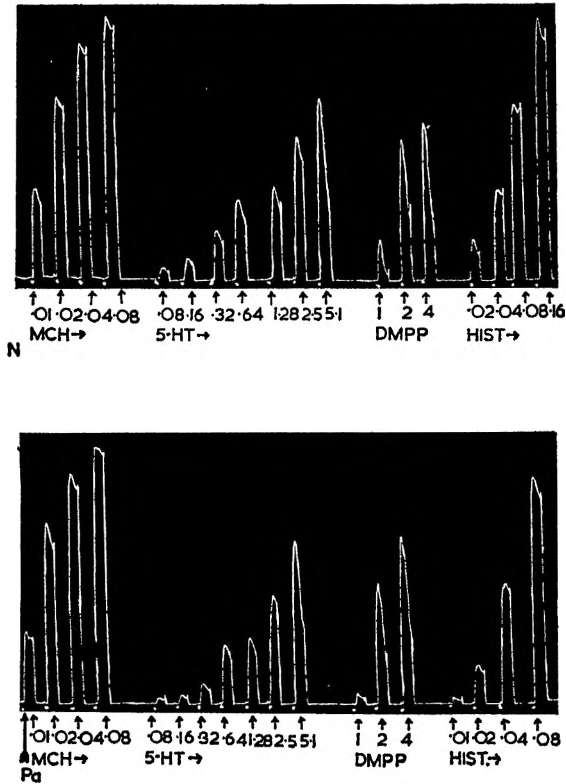

FIG. 1A. The spasmolytic actions of pyrogallol on the guinea-pig terminal ileum.
N = Dose response curves with normal Krebs's solution.
Pa = Dose response curves with Krebs's containing 1×10^{-6} pyrogallol.

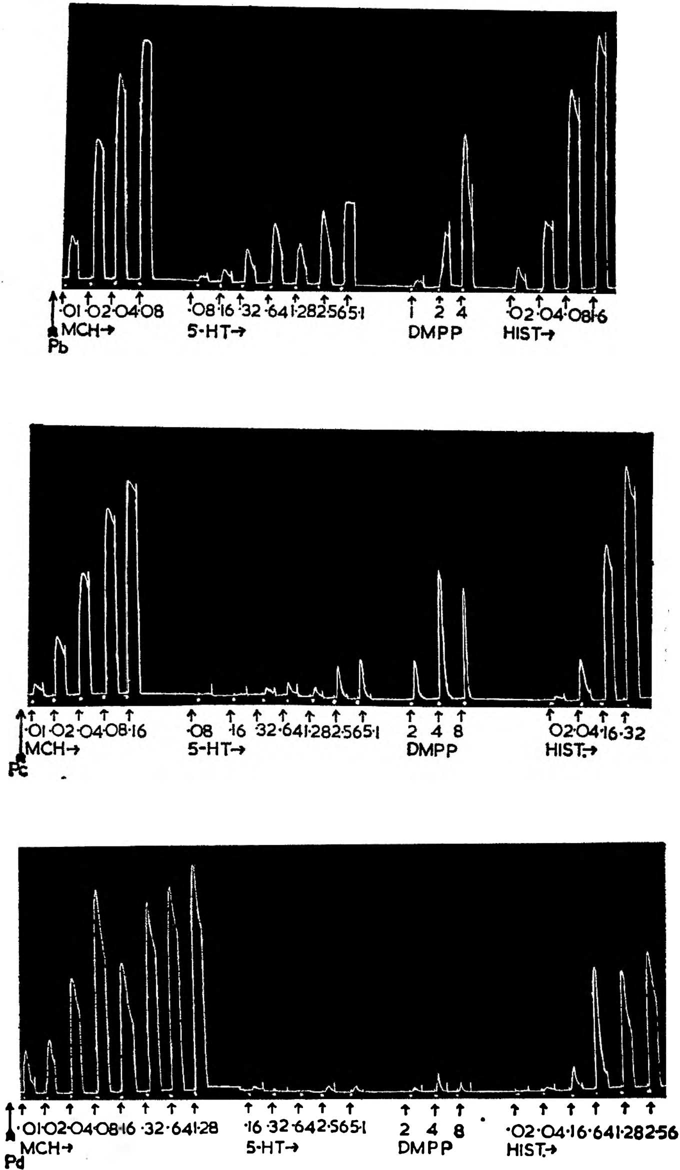

FIG. 1B. The spasmolytic actions of pyrogallol on the guinea-pig terminal ileum.
Pb = Dose response curves with Krebs's containing 5×10^{-6} pyrogallol.
Pc = Dose response curves with Krebs's containing 1×10^{-5} pyrogallol.
Pd = Dose response curves with Krebs's containing 5×10^{-5} pyrogallol.

Doses of spasmogens in μg./ml. bath volume.
Bath temperature 37°.
Contact time of the spasmogens 30 sec., doses repeated every 2 min.

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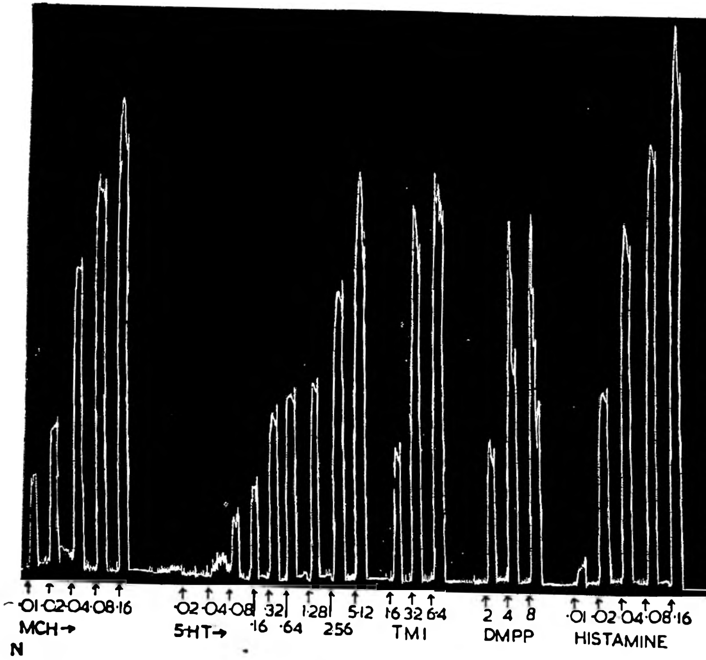


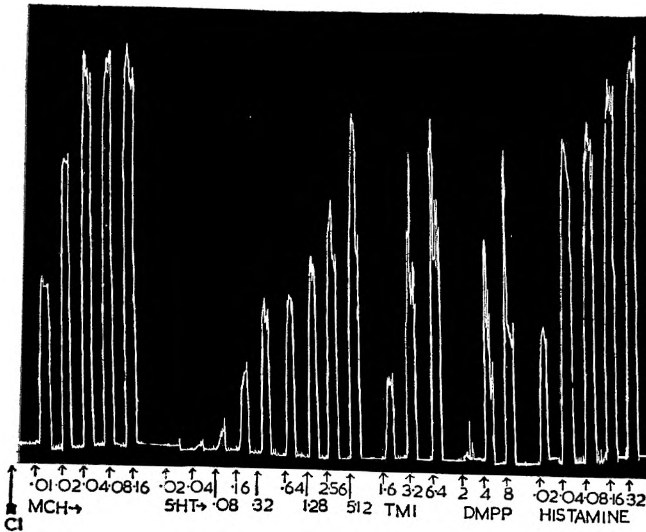
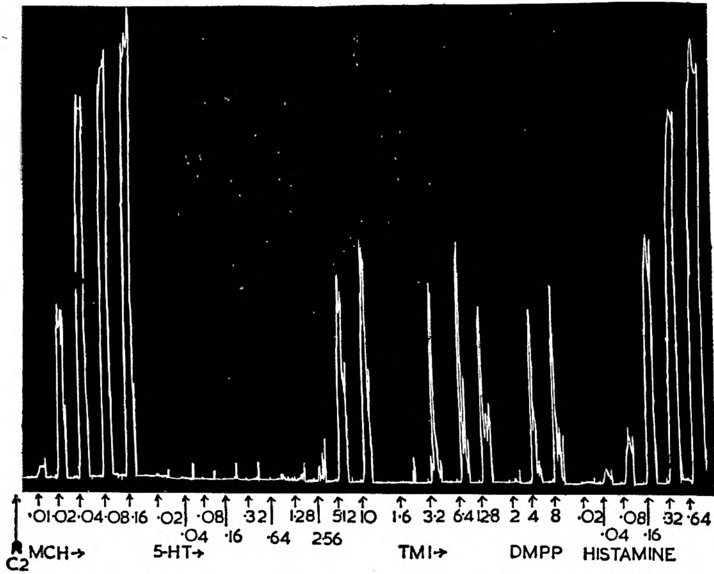


FIG. 2A. The spasmolytic actions of catechol on the guinea-pig terminal ileum.
N = Dose-response curves with normal Krebs's solution.
Cl = Dose-response curves with Krebs's containing 1×10^{-4} catechol.



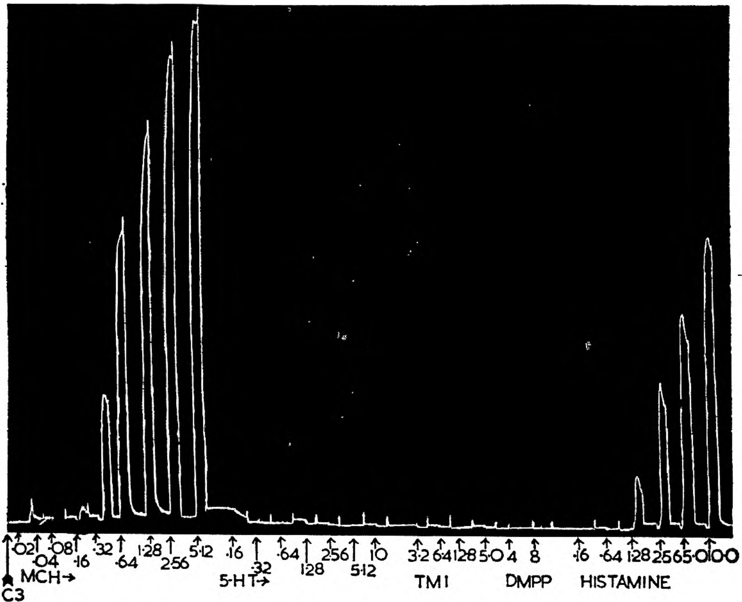


FIG. 2B. The spasmolytic action of catechol on the guinea-pig terminal ileum.
C2 = Dose-response curves with Krebs's containing 5×10^{-4} catechol.
C3 = Dose-response curves with Krebs's containing 1×10^{-3} catechol.

Doses of spasmogens in μg/ml. bath volume.
Bath temperature 37°.
Contact time of the spasmogens 30 sec., doses repeated every 2 min.

Discussion

Pyrogallol and catechol are derivatives of phenol and the discovery that these compounds produce lysis of drug-induced spasm is in agreement with the results of Jaques and Doepfner (1959) who investigated phenol and some of its derivatives. They concluded that phenol-like compounds possessed a general spasmolytic property on smooth muscle similar to that of papaverine.

The present experiments show that contractures induced by 5-HT, TMI and DMPP, which are believed to act by nervous pathways (Rocha e Silva, Valle and Picarelli, 1953; Gaddum and Picarelli, 1957; Hey, 1952; Leach, 1957; Ling, 1959), are inhibited by concentrations of pyrogallol and catechol which have little or no effect on contractures induced by histamine and methacholine. A possible explanation of this action resides in the local anaesthetic property common to phenols.

Because the action of pyrogallol and catechol in modifying the spasmogenic effects of all the spasmogens used, is reversible, they cannot be designated toxic but are more like the non-specific spasmolytic drug papaverine.

In experiments designed to investigate the possible stimulatory actions of catechol, no positive results were obtained and so the discovery of Sjöstrand (1960; 1961) that this compound acts by stimulating nicotinic receptors remains unconfirmed. The occasional stimulation seen with my experiments when normal Krebs's solution was replaced by Krebs's containing catechol may have been caused by the difference in temperature of the latter solution when first introduced into the bath. The inhibitory concentrations of catechol were in the same dose region as the inhibitory concentrations employed by Sjöstrand and his observations in this respect are confirmed.

Izquierdo and Izquierdo (1961) noted an initial depression of motility of the dog duodenum *in situ* with pyrogallol in the same dose ranges as those used in this report. It seems likely that part of this inhibition was caused by the general spasmolytic property of pyrogallol.

These experiments draw attention to the dangers of relying on pyrogallol and catechol as specific catechol-*o*-methyl transferase inhibitors. The general spasmolytic properties of these compounds can not be disregarded in assessing their actions on smooth muscle.

Acknowledgements. I am indebted to Professor G. Brownlee for his helpful advice and criticism. The choline phenyl ether bromide was a gift from Dr. P. Hey of Smith, Kline and French Research Institute, and is gratefully acknowledged. This work was supported by a Scholarship from the Medical Research Council.

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DIRECT COLORIMETRIC DETERMINATION OF SMALL QUANTITIES OF *m*-AMINOPHENOL IN SODIUM AMINOSALICYLATE

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Received October 30, 1961

A colorimetric method has been developed for the determination of *m*-aminophenol in sodium *p*-aminosalicylate by formation of a blue colour with phosphomolybdic acid. Although both sodium aminosalicylate and *m*-aminophenol form blue complexes with this reagent, prior isolation of the *m*-aminophenol is unnecessary because the extinction coefficient for the *m*-aminophenol complex is 90 times that of sodium *p*-aminosalicylate complex.

SINCE sodium *p*-aminosalicylate was introduced in therapy the amount of *m*-aminophenol, its most common impurity, has been of major interest to many authors. Although its toxic effect is not as great as initially believed, control of commercial sodium aminosalicylate is undoubtedly necessary because of the high toxicity of its oxidation products (Haberland, 1951; Boymond and Adatto, 1955; Kasalicky and Nejedly, 1945). According to various pharmacopoeias the limits of *m*-aminophenol vary from 0·1 to 1·0 per cent.

Most of the reported methods for the determination of *m*-aminophenol present as impurity in sodium aminosalicylate use an extraction or precipitation procedure for the preliminary separation (see, for example, Ettinger and Ruchloff, 1951; Faschalek, 1952; Simmonite, 1949). Others determine *m*-aminophenol directly. Haberland (1951) reported its direct determination by precipitation with formalin at pH 5·3. The drawback of this method is that after a time sodium aminosalicylate gives a precipitate with formalin. Ota, Nakajima and Nakagami (1956) elaborated a spectrophotometric method for the simultaneous determination of both substances, but co-existing substances seriously interfere in this determination. Franc (1955) reported a colorimetric method with 4-aminoantipyrine, which permits the direct determination up to 0·5 per cent of *m*-aminophenol in sodium aminosalicylate. The direct colorimetric method by the diazo method proposed by Pesez (1949) is unreliable because of the poor stability of the azo dye formed. A method based upon the formation of an indamine dye from *m*-aminophenol in the presence of oxidants has been described by Hrdý and Petíkova (1957).

We sought a simple, rapid and reliable method allowing the direct determination of small quantities of *m*-aminophenol in sodium aminosalicylate. As both substances are structurely very similar it was impossible to find a reagent which would react with the phenol but not with the aminosalicylate. From preliminary investigations only phosphomolybdic acid in the presence of ammonia gave promising results. Thus 20 mg. of the phenol gave an intense blue colour, while the same amount

of the aminosalicylate gave a pale blue colour. The extinction coefficient of the *m*-aminophenol complex was 90 times that of the sodium aminosalicylate complex. This observation suggested a simple colorimetric method.

EXPERIMENTAL

Reagents and Apparatus

m-Aminophenol, Bayer, redistilled, m.p. 122–123° (Koffler block), sodium *p*-aminosalicylate, $2H_2O$, Bayer, content of *m*-aminophenol less than 0·01 per cent, acetate buffer solution of pH 6·0 prepared from 0·2M sodium acetate and 0·2M acetic acid, phosphomolybdic acid, Carlo Erba, Reagent Grade.

Measurements were made with the Zeiss, Elko II, photometer, using a filter of wavelength 720 mμ and 1 cm. cells and a Jobin Yvon spectrophotometer, using 1 cm. quartz and glass cells respectively.

Methods of Assay

Reagent concentrations and reaction conditions were selected after evaluation of each factor over a range. The optimum conditions for the determination of *m*-aminophenol in sodium aminosalicylate are as follows:

To 50 mg. sodium aminosalicylate dissolved in 1 ml. of distilled water add 10 ml. acetate buffer and 7 ml. of 5 per cent aqueous solution of phosphomolybdic acid. Mix well and after 10 min. add 3 ml. of 2 per cent ammonia solution. After 5 min. measure the extinction against water of the blue reaction products of both the sample and a blank of 50 mg. "standard sodium aminosalicylate"* similarly treated, at a wavelength of 720 mμ. Correct the extinction of the sample by subtracting the reagent blank reading. The content of phenol is obtained by reference to a calibration graph.

Comparison of results obtained by this method with those using the official method of Sanz (1952) which was proposed by the firm of Bayer gave the following results.

m-Aminophenol per cent		
	Present method	Method of Sanz (1952)
1.	Not more	—
2.	than 0·01	—
3.	0·15	0·16
4.	0·30	0·28

Calibration Curve

1 ml. aliquots containing from 0·01 to 1 mg. *m*-aminophenol were added to 50 mg. amounts of standard sodium aminosalicylate. The solutions were treated with phosphomolybdic acid as above. After 10 min. extinction E, at 720 mμ of the blue reaction product was

* Sodium *p*-aminosalicylate Bayer containing less than 0·01 per cent *m*-aminophenol. Standards were used in all the experiments and gave constant extinction values of 0·091–0·093.

measured. A blank determination was also made using the standard sodium aminosalicylate with no added *m*-aminophenol and the extinction E_2 at 720 mμ also measured. $E_1 - E_2$ gave E due to the *m*-aminophenol complex above. The results of 86 determinations were plotted as E against mg. concentration of the *m*-aminophenol and a straight line obtained.

On statistical analysis of the results it was found that the limits of error for b = 0·2249, calculated according to Davies (1958), were between 95·1 and 104·9 per cent (P = 0·01).

RESULTS AND DISCUSSION

The method described is applicable to the determination of *m*-aminophenol in the concentration range 0·1–2·0 per cent in sodium aminosalicylate, the range in which it occurs most frequently commercially. The use of 4 cm. cells may improve the sensitivity of the method.

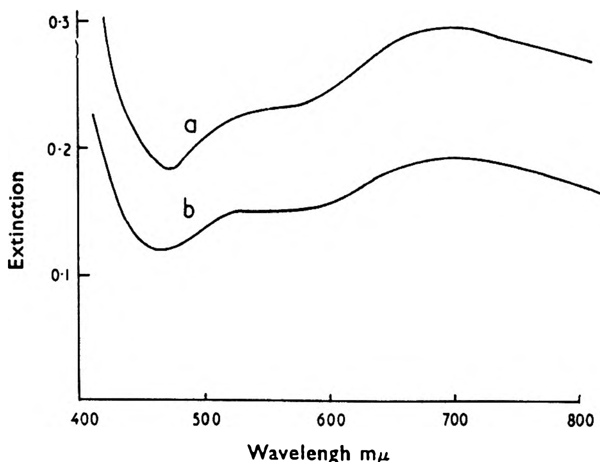

FIG. 1. Absorption curves for sodium aminosalicylate and *m*-aminophenol blue.
a. *m*-Aminophenol blue (prepared from *m*-aminophenol 1 mg./ml. solution).
b. Sodium aminosalicylate blue (prepared from 0·1 g./ml. solution).

The reaction of phenols with the phosphomolybdic acid reagent of Wavelet has been studied by Vignoli and Cristau (1952) and Cristau (1954). These authors have claimed that maximum absorption of the blue reaction product "molybden blue" occurs at 700 mμ, with maximum colour stability at pH 6·1.

Our preliminary investigations showed that a molybden-blue was formed from both *m*-aminophenol and sodium *p*-aminosalicylate. The absorption maximum for both was found to be between 680 and 700 mμ (Fig. 1) while maximum colour stability was attained at pH 6·2.

In the course of ultra-violet absorption studies of "molybden blues" obtained with various compounds Vaisberg and Ya Dain (1951) observed that the spectra of the "molybden blues" of the compounds examined between 200 and 450 mμ are similar to those of the original compounds.

From this they concluded that the original compound and the "Blue" produced therefrom had a similar structure.

The spectrum of *m*-aminophenol blue differs significantly from that of the *m*-aminophenol itself in showing no absorption in the region between 270 and 310 mμ. The ultra-violet spectrum of sodium aminosalicylate blue is identical to that of the original compound (Fig. 2).

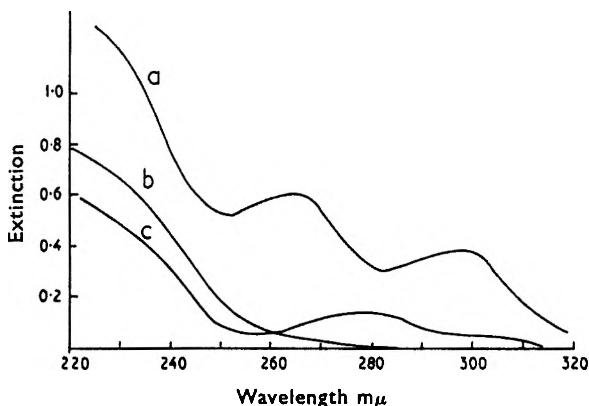

FIG. 2. Ultra-violet absorption curves of: a. Sodium aminosalicylate blue (0·02 mg./ml.) b. *m*-Aminophenol blue (0·002 mg./ml.) c. *m*-Aminophenol (0·08 mg./ml.) The curve for sodium aminosalicylate is identical with the curve for its blue complex.

Acknowledgements. The author is indebted to Dr. V. Vlahović for her help in the statistical analysis. *m*-Aminophenol and sodium *p*-aminosalicylate were kindly supplied by Bayer A.G. (Leverkusen).

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THE EFFECT OF ADRENERGIC BLOCKING AGENTS AND OF CHLORPROMAZINE ON BLOOD PRESSURE INCREASE BY VASOPRESSIN AND ANGIOTENSIN

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Received January 17, 1962

The influence of the adrenergic blocking agents hydergine, dibenamine and tolazoline and of chlorpromazine on the mean arterial pressure response to vasopressin, angiotensin and barium chloride in anaesthetised dogs was investigated. Adrenergic blocking agents potentiate significantly the pressure response to vasopressin while leaving the responses to angiotensin and barium chloride unaltered. Chloropromazine potentiates the responses to both vasopressin and angiotensin. This potentiation is not due to blood pressure lowering produced by adrenergic blockade or by chlorpromazine.

The experimental data on the influence of adrenergic blocking agents or of chlorpromazine on hypertensive effects of substances not belonging to the sympathomimetic drugs, are partly incomplete and partly contradictory. Nickerson, Bullock and Nomaguchi (1948) explained the potentiation of angiotensin after dibenamine as a result of a decrease of blood pressure caused by this adrenolytic drug. De Vleeschhouwer (1947) found an insignificant alteration of the effect of pituitrin after dibenamine. Youmans and Rankin (1947) could not demonstrate any influence of dibenamine on arterial pressure response of pitressin and angiotensin. According to Bianchi, De Schaepdryver, De Vleeschhouwer and Preziosi (1960) phentolamine does not change the pressor effect of synthetic angiotensin. We have made a controlled series of experiments to investigate the influence of adrenolytic drugs and of chlorpromazine on the hypertensive effect of vasopressin and angiotensin. Some of the results have already been briefly reported (Supek, Uroić, Gjuriš and Kečkeš, 1959).

Methods

Mongrel dogs were anaesthetised with chloralose (70 mg./kg.) dissolved in 25 per cent urethane solution (urethane solution 6·0 ml./100 mg. chloralose) and rabbits with urethane (1·25 g./kg.). Arterial blood pressure was taken through a cannula inserted into the carotid artery and recorded by a mercury manometer. The drugs studied were: vasopressin (Tonephin, Hoechst, 5 I.U./ml), angiotensin (2 Edman U./mg), barium chloride (analytical reagent), *NN*-dibenzyl-β-chloroethylamine chloride (Dibenamine, S.K.F.), tolazoline (Benizol, Krka), Hydergine (Sandoz), chlorpromazine (Largactil, Specia). Blood pressure was recorded before and after application of adrenolytic drugs or chlorpromazine.

In preliminary experiments no interference between the three vasopressor substances was found. Therefore all three substances were tested in the same experiment. The difference between the mean of blood

TABLE I

THE MEAN VALUES OF BLOOD PRESSURE INCREASE IN MM. HG OF VASOPRESSIN, ANGIOTENSIN AND BARIUM CHLORIDE BEFORE AND AFTER HYDERGINE, DIBENAMINE, TOLAZOLINE OR CHLORPROMAZINE IN ANAESTHETISED DOGS

	Number of dogs	Hydergine 0·3–0·9 mg./kg. i.v.			Number of dogs	Dibenamine 25 mg./kg. i.v.			Number of dogs	Tolazoline 20–40 mg./kg. i.v.			Number of dogs	Chlorpromazine 2–5 mg./kg. i.v.		
		Before	After			Before	After			Before	After			Before	After	
Vasopressin 0·1 I.U./kg. i.v.	14	8·9±1·1*	18·4±2·7	P<0·01	8	15·9±2·8	33·1±4·0	P<0·01	8	14·0±2·0	27·9±2·6	P<0·01	22	8·8±0·7	21·1±1·5	P<0·01
Angiotensin 0·07 mg./kg. i.v.	12	9·4±1·3	10·0±1·4	P>0·1	8	9·4±0·8	10·9±1·5	P>0·1	8	6·3±0·5	7·7±1·1	P>0·1	11	8·2±0·7	13·4±1·3	P<0·01
Barium chloride 0·5–1·0 mg./kg. i.v.	14	10·8±1·6	14·0±1·5	P>0·1	8	9·7±1·3	5·2±0·6	P<0·01	8	8·5±0·9	8·9±1·1	P>0·1	24	7·9±0·9	8·4±0·7	P>0·5

* Standard error of the mean.

TABLE II

1. THE CORRELATION OF BLOOD PRESSURE LEVEL AFTER HYDERGINE, DIBENAMINE, TOLAZOLINE OR CHLORPROMAZINE AND PRESSOR EFFECT OF VASOPRESSIN, ANGIOTENSIN AND BARIUM CHLORIDE
2. THE CORRELATION OF PERCENTAGE FALL OF BLOOD PRESSURE CAUSED BY THESE ADRENOLYTICS OR CHLORPROMAZINE AND PRESSOR EFFECT OF VASOPRESSIN, ANGIOTENSIN AND BARIUM CHLORIDE

	1. Blood pressure after adrenolytics and chlorpromazine				2. Percentual fall of blood pressure after adrenolytics and chlorpromazine			
	Hydergine	Dibenamine	Tolazoline	Chlorpromazine	Hydergine	Dibenamine	Tolazoline	Chlorpromazine
Vasopressin	n = 13 r = −0·486 P > 0·05	n = 6 r = −0·366 P > 0·1	n = 6 r = −0·585 P > 0·1	n = 18 r = −0·162 P > 0·1	n = 13 r = −0·422 P > 0·1	n = 6 r = −0·368 P > 0·1	n = 6 r = −0·695 P > 0·05	n = 18 r = −0·316 P > 0·1
Angiotensin	n = 10 r = −0·501 P > 0·05	n = 6 r = 0·906 P < 0·01	n = 6 r = −0·792 P < 0·02	n = 8 r = 0·376 P > 0·1	n = 10 r = −0·378 P > 0·1	n = 6 r = 0·941 P < 0·01	n = 6 r = −0·917 P < 0·01	n = 8 r = 0·251 P > 0·1
Barium chloride	n = 12 r = −0·195 P > 0·1	n = 6 r = 0·603 P > 0·05	n = 6 r = −0·535 P > 0·1	n = 22 r = 0·615 P < 0·01	n = 12 r = −0·276 P > 0·1	n = 6 r = 0·613 P > 0·1	n = 6 r = 0·531 P > 0·1	n = 22 r = 0·311 P > 0·1

pressure effects before and after tested substances was compared by a "*t*" test. The correlation coefficients were calculated according to Fisher (1950).

Results

The results summarised in Table I show that the blood pressure increase produced by vasopressin is significantly potentiated by all the drugs. Moreover chlorpromazine also potentiates the pressor effect of angiotensin. Barium chloride used as an unspecific vasopressor control agent showed no potentiation. Correlations of blood pressure level, before and after adrenergic blockade or chlorpromazine, and blood pressure effect of investigated substances are presented in Tables II and III.

TABLE III

THE CORRELATIONS OF HYPERTENSIVE EFFECT OF VASOPRESSIN, ANGIOTENSIN AND BARIUM CHLORIDE AND INITIAL BLOOD PRESSURE LEVEL BEFORE AND AFTER HYDERGINE, DIBENAMINE, TOLAZOLINE OR CHLORPROMAZINE

	Before adrenolytics and chlorpromazine	After adrenolytics and chlorpromazine			
		Hydergine	Dibenamine	Tolazoline	Chlorpromazine
Vasopressin	n = 50 r = −0·160 P > 0·1	n = 13 r = −0·053 P > 0·1	n = 6 r = −0·006 P > 0·1	n = 6 r = 0·233 P > 0·1	n = 18 r = 0·145 P > 0·1
Angiotensin	n = 36 r = −0·325 P < 0·05	n = 9 r = −0·246 P > 0·1	n = 6 r = 0·179 P > 0·1	n = 6 r = 0·427 P > 0·1	n = 9 r = 0·457 P > 0·1
Barium chloride	n = 28 r = −0·307 P > 0·1	n = 12 r = 0·518 P > 0·05	n = 6 r = −0·229 P > 0·1	n = 6 r = 0·147 P > 0·1	n = 22 r = 0·336 P > 0·05

Initial blood pressure level, blood pressure after adrenolytics or chlorpromazine, as well as blood pressure fall caused by these agents are not related to the above described phenomenon. Between the hypertensive effect of angiotensin and the initial blood pressure level there is a significant negative correlation ($P<0·05$; Table III).

Discussion

Braun-Menendez, Fasciolo, Leloir and Munoz (1940) found that the pressor effects of angiotensin were inhibited by previous administration of vasopressin. Our experiments did not confirm this finding.

Page and Taylor (1949) could not show any relationship between blood pressure level and pressor effect of angiotensin in anaesthetised dogs. Our results showed a statistically significant negative relation between these two values.

The most interesting finding in our experiments is the potentiation of vasopressin and angiotensin by chlorpromazine and the potentiation of vasopressin by three different adrenergic blocking agents. It is well known that chlorpromazine is a very potent adrenolytic drug. Therefore it is likely that the potentiation of vasopressin is related to the adrenergic blocking properties of the drugs investigated. The additional potentiation

of angiotensin by chlorpromazine seem to be ascribable to other pharmacological activities of this drug which are, as it is well known, numerous. The described potentiations are neither correlated with blood pressure level (before and after blockade) nor with the blood pressure decrease caused by the investigated blocking agents. This is evident from our correlation data.

Up to now we have no satisfactory explanation for the described effect. The mechanisms of this phenomenon are under investigation. Preliminary experiments showed that pure synthetic substances (isoleucin[5]-angiotensin octapeptide and lysin[8]-vasopressin) behave identically to the crude preparations.

Acknowledgements. The sample of crude angiotensin was kindly supplied by Dr. I. H. Page, Cleveland, U.S.A. and dibenamine by Smith, Kline and French Labs., Philadelphia, U.S.A.

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DITERPENE ALKALOIDS. ISOLATION AND STUDY OF TWO NEW ALKALOIDS

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Received November 21, 1961

Two new alkaloids tentatively named as "denudatine" and "denudatidine" have been isolated in pure form from an indigenous crude drug "Judwar" which has been provisionally identified as the roots of *Delphinium denudatum* Wall. (Fam. Ranunculaceae). The purity of these alkaloids has been checked by paper chromatographic analysis and they have been characterised. Denudatine is the major alkaloid and has been isolated in pure form in a yield of 0·05 per cent. On the basis of microanalysis for the elements of the base, m.p. 248–249° and its derivatives; the determination of the active hydrogen, =N–Me, ≡C–Me and infra-red and ultra-violet absorption spectra, denudatine has been tentatively classified as a diterpene alkaloid of atisine group. Pharmacological actions of denudatine have been investigated. The normal rhythmic contractions of isolated rabbit duodenal strip are inhibited. The isolated guinea-pig uterine strip is stimulated by the base solution. It decreases the tone and inhibits the peristaltic movements of the intestine but does not show any effect on the blood pressure and respiration of the anaesthetised dog. It is non-toxic and lacks curariform activity. Denudatidine, m.p. 273° (yield, 0·003 per cent) has not been studied further.

THE tuberous roots of a large number of *Aconite* and *Delphinium* species are exploited commercially in India and have been used in the indigenous system of medicine for treating numerous ailments. The Indian species of these plants have largely remained uninvestigated except for the preliminary reports on chemical investigations by Dunstan and others (c.f. Stern, 1954, 1960; Henry, 1949; Chopra, Chopra, Handa and Kapur, 1958), although considerable interest has been shown by foreign workers in the elucidation of the structures of the diterpene alkaloids present in the various locally available species (Stern, 1954, 1960; Wiesner and Valenta, 1958). We have undertaken to investigate commercial samples with the object of elucidating the structures of the alkaloids present.

Herein we report the results of a preliminary study of a root "Judwar" which has been provisionally identified as the root of *Delphinium denudatum*. Wall. (Fam. Ranunculaceae). The crude drug has reported uses in some diseases of the blood, in insanity and as stimulant in conditions of debility. It is also used against painful piles and toothache, and as antidote to snake and scorpion venoms (Kirtikar and Basu, 1933). No previous investigation of any part of this plant has been reported.

EXPERIMENTAL

Materials

The tuberous roots were obtained from the Amritsar crude drug market under the name "Judwar" (it is also known as "Nirbishi"). Roots were

conical, dark brown to blackish in colour, 1·5 to 7·0 cm. long, 1 to 2·5 cm. in diameter at the crown, occurring largely singly. Internally the root was yellowish to dark brown marked by a central light portion with an angular line representing cambium. The taste was initially bitter and was then followed by sensation of numbness. The root has been provisionally identified by Mr. S. N. Sobti of the Regional Drug Research Laboratories, Jammu (India).

Extraction and Isolation of Denudatine and Denudatidine

Root powder (7 kg.) was extracted with ethanol (95 per cent) in a soxhlet apparatus (liming the drug before extraction did not improve the yield or facilitate extraction) until exhausted of alkaloids (8 hr.). The ethanol was recovered from the extract and the dark viscous residue was dissolved in hydrochloric acid (5 per cent) and the acid liquor extracted with ether (10 × 35 ml.) to remove non-basic impurities. The insoluble residue that separated at the interface was removed and washed free of alkaloids with dilute acid and the acid washings were added to the acid liquor. The acid liquor was made alkaline with ammonia solution and alkaloid extracted with ether (18 × 45 ml.). The ether solution was dried over exsiccated sodium sulphate and the ether was removed to yield a residue (17·6 g.), which was again dissolved in hydrochloric acid (60 ml. 5 per cent), made alkaline with ammonia solution and extracted with ether (15 × 35 ml.). The solvent was removed to yield a crude base "A" (12·9 g.). The crude base "A" was purified by washing the resinous colouring impurities with ethanol (10 ml. 60 per cent) and recrystallisation from benzene (or absolute ethanol) to yield light white needles (3·5 g. 0·05 per cent), m.p. 246–47°. Two recrystallisations raised the m.p. to 248–49°. The base was provisionally named "denudatine" and was proved to be a single chemical entity by paper chromatography.

The alkaline liquor left after extraction of base "A" with ether, was extracted with chloroform (15 × 30 ml.). The chloroform solution was dried over exsiccated sodium sulphate, the solvent removed and the residue (4·9 g.) dissolved in hydrochloric acid (30 ml. 5 per cent), made alkaline with ammonia solution and extracted with chloroform (15 × 20 ml.). The solvent was dried and removed to yield a residue (1·3 g.) of crude base "B", which was purified by adsorption on an alumina (Merck) column from acetone solution and eluting it with the same solvent, followed by crystallisation from absolute ethanol to yield shining light brown prismatic crystals (0·2 g., 0·003 per cent), m.p. 273°. This base was provisionally named "denudatidine" and was found to be a single chemical entity by paper chromatographic analysis.

Paper Chromatographic Analysis

The ascending strip paper and circular paper methods were employed. Whatman No. 1 filter paper buffered at pH 4·0 by sodium dihydrogen-citrate was used. The solvent system consisted of butanol : water : citric acid (50 : 50 : 1). The organic layer was employed as the mobile phase. Samples for analysis consisted of the ether and the chloroform residues,

the crude bases "A" and "B" and the pure bases, denudatine and denudatidine. Development was at 20° for 6–7 hr. The chromatograms were air-dried and dipped in Munnier's modified Dragendorff's reagent (Block, Le Strange and Zweig, 1952) and R_F values were calculated. Denudatine and denudatidine were the only two bases indicated to be present in the root with their average R_F values of 0·80 and 0·63 respectively. Both alkaloids gave orange spots on a yellow background.

Chemical Characterisation of Denudatine. Analyses by Drs. Weiler and Straus

Denudatine was very soluble in absolute ethanol; slightly less soluble in methanol, benzene and carbon disulphide; sparingly soluble in chloroform, ethyl acetate and dioxan; practically insoluble in water and carbon tetrachloride. The base solution in hydrochloric acid (1 per cent) gave positive tests with most of the precipitating alkaloidal reagents. Found: C, 75·7; H, 10·0; N, 4·3 per cent; Mol. wt. 294 (Cryoscopic method in benzene); $[\alpha]_D^{21}$ (Ethanol) = +0·154. Calc. for $C_{21}H_{33}NO_2$: C, 76·1; H, 10·0; N, 4·2 per cent. Mol. wt., 331.

Denudatine hydriodide. This was prepared by adding potassium iodide solution (10 per cent) in a slight excess to a saturated solution of the base in dilute hydrochloric acid and recrystallisation of the precipitate produced from ethanol (60 per cent), m.p. 267–268°. Found: C, 54·4; H, 7·5; I, 28·0; N, 3·2 per cent. Calc. for $C_{21}H_{33}NO_2HI$: C, 54·9; H, 7·4; I, 27·7; N, 3·2 per cent.

Denudatine picrate. This was prepared by adding a saturated solution of picric acid in water in a slight excess to a saturated solution of the base in dilute hydrochloric acid and recrystallisation of the yellow precipitate from methanol, m.p. 215°. Found: C, 57·5; H, 5·8; N, 9·8 per cent. Calc. for $C_{27}H_{35}N_4O_9$: C, 57·9; H, 6·0; N, 10·0 per cent.

Denudatine-gold chloride complex was prepared by adding gold chloride solution (2 per cent w/v) to a saturated solution of the base in dilute hydrochloric acid and recrystallisation of the product from alcohol or acetone, m.p. 158–59°. Found: C, 40·3; H, 5·0; N, 2·4 per cent. Calc. for $C_{21}H_{33}NO_2.AuCl_3$: C, 39·7; H, 5·2; N, 2·2 per cent.

Denudatine reineckate was prepared by adding ammonium reineckate solution (1 per cent w/v) to a solution of the base in dilute hydrochloric acid as microcrystalline power, m.p. 202–203°. Found: C, 44·3; H, 6·4; N, 13·7 per cent. Calc. for $C_{25}H_{40}N_7O_2S_4$ Cr.$2H_2O$: C, 43·7; H, 6·4; N, 14·3 per cent.

Diacetyldenudatine. Denudatine (100 mg.), dry pyridine (1 ml.) and acetic anhydride (0·2 ml.) were refluxed together on a sand bath for 4 hr., cooled, poured into water (25 ml.) and ammonia solution was added to precipitate the product. It was separated and recrystallised from absolute ethanol as microcrystalline powder, m.p. 128–30°. Found: C, 72·3; H, 8·7 per cent. Calc. for $C_{21}H_{31}NO_2(COMe)_2$: C, 72·3; H, 8·9 per cent.

Trichloroacetyl denudatine was prepared by heating a saturated aqueous solution of trichloroacetic acid with a saturated solution of the base in

dilute hydrochloric acid solution (1 per cent) and recrystallisation from absolute ethanol, m.p. 235°. Found: C, 46·5; H, 4·8; N, 2·0 per cent. Calc. for $C_{21}H_{31}NO_2(COCCl_3)_2.H_2O$: C, 46·9; H, 5·2; N, 2·2 per cent.

Active hydrogen. Found: 0·5 per cent. Calc. for 2 active hydrogens: 0·6 per cent.

Tertiary carbon-methyl group. Found: 5·7 per cent. Calc. for one ≡ C–Me: 8·2 per cent.

N-*Methyl group.* On heating the base with soda-lime, vapours having an ammoniacal odour were evolved and when these were passed into a solution of hydrochloric acid in ether, a precipitate was formed, which on recrystallisation melted at 225° and was identified as methylamine hydrochloride by mixed melting point determination. On treating the base

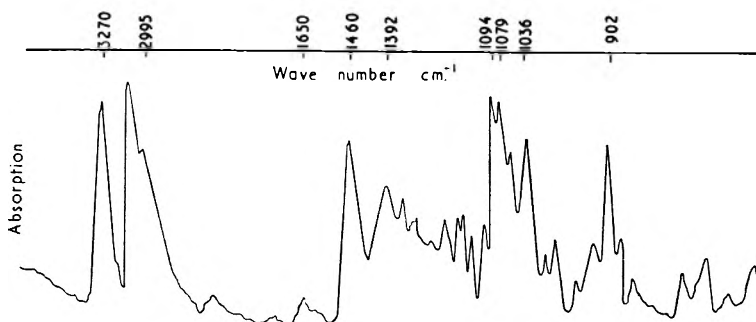

FIG. 1. Infra-red spectrum of denudatine.

with ice-cold nitrous acid, a gas was evolved, but it did not give Liebermann's test and a dye test, Rimnis' test and the carbon-disulphide reagent test were also negative. Found: 8·7 per cent. Calc. for one = *N*–Me; 8·8 per cent.

Ultra-violet spectrum. The ultra-violet spectrum was determined on a DU-spectrophotometer (Beckman) equipped with fused silica prisms. Denudatine in methanol (0·0045 per cent) exhibited only one absorption maximum at 210 mμ. Molar extinction coefficient ϵ = 4,925.

Infra-red spectrum. The infra-red spectrum of denudatine in Nujol indicated the presence of hydroxyl group (3,270 cm.$^{-1}$), i.e., vibration frequency of H–O bond supported by the vibration frequencies at 1,094 and 1,079 cm.$^{-1}$ due to C–O bond; exomethylene group, C=CH_2 (2,995 cm.$^{-1}$, C–H stretching frequency as shoulder on the Nujol band; 1,650 cm.$^{-1}$, C=C stretching frequency and 902 cm.$^{-1}$, C–H deformation frequency; =C–Me (C–H deformation frequencies at 1,460, 1,392 and 1,368 cm.$^{-1}$) (Fig. 1).

These groups were confirmed by the infra-red spectra of the base hydriodide in KCl mulls done on a Grubb-Parsons infra-red spectrophotometer. OH (3,436, 1,092, 1,075 cm.$^{-1}$); N-Me (2,900 cm.$^{-1}$); C=CH_2 (2,941, 1,653, 905 cm.$^{-1}$); C–Me (1,481, 1,449, 1,394 and 1,370 cm.$^{-1}$). There was no evidence for the presence of a carbonyl group or a lactam

group. It further indicated that the salt was not anhydronium salt and thus denudatine is not a carbinol base nor is the base a carbinolamine.

Chemical Characterisation of Denudatidine

Denudatidine was found to be soluble in ethanol, methanol, acetone, pyridine, and dioxan while sparingly soluble in ethyl acetate, light petroleum and carbon tetrachloride. The base solution in hydrochloric acid (1 per cent) gave positive tests with all the general precipitating alkaloidal reagents. Found: C, 67·6; H, 8·6; N, 3·3 per cent; Titration equivalent 400 $\pm$ 20 (Electrometric method); $[\alpha]_D^{21}$ (ethanol) $= +31{\cdot}56$. Calc. for $C_{23}H_{35}NO_5$: C, 68·1; H, 8·6; N, 3·5 per cent; Mol. wt. 405; for $C_{23}H_{37}NO_5$: C, 67·8; H, 9·1; N, 3·4 per cent.

Denudatidine reineckate was prepared by adding ammonium reineckate solution (2 per cent) to a saturated solution of the base in hydrochloric acid (1 per cent). The reineckate was purified by passing its solution in acetone through an alumina column; it was then crystallised from acetone as microprisms, m.p. 190°. Found: C, 44·3; H, 6·0; N, 13·4 per cent. Calc. for $C_{27}H_{42}CrN_5O_5S_4$: C, 44·8; H, 5·8; N, 13·5 per cent; for $C_{27}H_{44}CrN_5O_5S_4$: C, 44·6; H, 6·1; N, 13·5 per cent.

Denudatidine picrate was prepared by adding a saturated ethanolic solution of picric acid to a saturated solution of the base in absolute ethanol; the product was recrystallised from methanol as yellow microneedles, m.p. 100°. Found: C, 51·0; H, 6·1 per cent. Calc. for $C_{29}H_{40}N_4O_{12}$: C, 52·0; H, 6·6 per cent.

PHARMACOLOGICAL

Blood pressure and respiration studies were made on healthy dogs (2–4 kg.) anaesthetised with phenobarbitone (150 mg./kg. i.p.). The effects were recorded by direct cannulation of the carotid artery and trachea respectively. Denudatine was administered through the femoral vein. With a dose of 0·5 to 5 mg./kg., blood pressure and respiration remained unaffected.

The alkaloid given at a dose of 0·5–5 mg./kg. produced a decrease of tone and inhibition of peristaltic movements of the dog intestine *in situ*. At a concentration of 2×10^{-5}M it produced a marked inhibitory effect on the movements of the rabbit isolated duodenal strip. The contractions induced by acetylcholine, 2×10^{-5}M, and by histamine, 8×10^{-6}M, in guinea-pig intestinal strips, were not antagonised by the alkaloid, 2×10^{-5} M. But at this concentration it stimulated isolated guinea-pig uterine strips. Atropinisation did not alter the effect of drug.

Curariform activity was determined in dogs anaesthetised with phenobarbitone. The gastrocnemius nerve-muscle preparation was set up according to the technique of Hoppe (1950) for observing the action of the drug on the neuromuscular transmission. Denudatine in doses of 200 mg./kg. given i.v., did not produce any curariform effect on the muscle.

For these studies, denudatine was dissolved in a mixture of ethanol, propyleneglycol and distilled water (1 : 1 : 2) and the mixture adjusted to

pH 7·2. In all experiments proportionate amounts of the solvent were given as controls.

Discussion

The microanalytical data of the alkaloid, denudatine and its salts and derivatives show that its molecular formula is $C_{21}H_{33}NO_2$. Both the oxygen atoms are present as alcoholic hydroxyl groups as confirmed by the infra-red spectra and active hydrogen determination. The nitrogen atom is tertiary and carries a methyl group. An exomethylene group is indicated by the infra-red spectra. The presence of a methyl group attached to a tertiary carbon atom is indicated by analysis. Denudatine is, therefore, most probably a diterpene alkaloid of atisine group with pentacyclic structure bearing one unsaturated linkage. The ultra-violet spectrum indicates that one of the two hydroxyl groups is an allylic alcoholic group in relation to an exomethylene group. Further work on the constitution of denudatine has been reported by Nazar Singh (1961). Se-dehydrogenation studies recently reported (Singh, Singh and Malik, 1961) confirm the constitution, 1-methyl-6-ethylphenanthrene and 1-methyl-6-ethyl-3-azaphenanthrene being obtained.

Acknowledgements. The spectra on the base were prepared through the courtesy of Dr. G. Englinton, Chemistry Department, Glasgow University; and on the base hydriodide through the courtesy of Prof. P. L. Pauson, the Royal College of Science and Technology, Glasgow. The authors are grateful to Dr. Kartar Singh, of the Regional Drug Research Laboratories, Jammu, for his assistance in the Pharmacological study, and to Mr. S. N. Sobti for his provisional identification of the crude material.

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THE EFFECTS OF PROLONGED ADMINISTRATION OF SOME ADRENOCORTICAL STEROIDS IN THE RAT

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Received December 20, 1961

An approximation has been made of the relative potency of some adrenocortical steroids to retard body growth and cause adrenocortical atrophy in chronically treated rats. The site of cortical atrophy and depletion of sudanophilic lipid varies with the nature of the steroid administered. The implications of these findings have been discussed with particular reference to the pituitary control of the zona glomerulosa. Corticosteroid induced adrenocortical atrophy is reversible on cessation of treatment. The effect of prolonged administration of cortisone on the weight of organs and other endocrine glands has also been studied.

THE prolonged administration of adrenocortical steroids to laboratory animals causes inhibition of normal growth (Silber and Porter, 1953; Shewell and Long, 1956; Hansen, Blivaiss and Rosenzweig, 1957: Goodlad and Munro, 1958) and adrenocortical atrophy (Ingle, 1938; Sayers and Sayers, 1949; Stebbins, 1950; Winter, Silber and Stoerk, 1950). The chronic administration of these steroids to man may suppress adrenocortical function resulting in the absence of a normal response to stress (Salassa, Bennett, Keating and Sprague, 1953; Nabarro, 1960; Stevens, 1960), and in particular to that of surgical trauma (*Lancet*, 1957; Bayliss, 1958; Shneewind and Cole, 1959). Severe shock and collapse from adrenal failure may occur in patients, who had ceased corticosteroid treatment weeks or months before the time of surgery (Lewis, Robinson, Yee, Hacker and Eison, 1953; Salassa and others, 1953; Hayes and Kushlan, 1956).

It was therefore thought of interest to determine whether there were any quantitative or qualitative differences between the effects of prolonged administration of the more commonly used corticosteroids on the adrenal glands of rats, and to determine whether adrenals which had atrophied as a result of such dosage regained their normal weight and histology when treatment ceased.

Organs and glands, other than the adrenals, were routinely examined, and the results included in this report.

Some preliminary results of this work have already been published (D'Arcy, 1958; D'Arcy and Howard, 1958a, 1958b, 1960, 1961).

Materials and Methods

Male albino rats, from the Agricultural Research Council were used. The weights were 100–120 g.; they were kept in a thermostatically controlled room at 68–70° F, and were maintained on a cubed diet and tap water provided *ad lib.*

Adrenocortical steroids were injected intramuscularly, suspended in saline, with the exception of large doses of deoxycorticosterone acetate, which were injected subcutaneously because of the density of the suspension. Except where specifically mentioned, corticosteroids were

injected on alternate days, thrice weekly for 6 weeks; in each experiment, control groups of rats received injections of saline alone. The weight of each rat was recorded at regular intervals. At the end of each experiment, rats were killed with ether and the adrenal glands and other tissues were removed to formol saline. Subsequently these tissues were dissected from fat and other extraneous matter, dried between filter papers and weighed; adrenal glands were also examined histologically. Frozen sections of the adrenals were stained with Sudan III to show the distribution of sudanophilic lipid.

Results

Effect on Body Weight and Adrenal Weight

The intramuscular injection of cortisone acetate (1·25 mg./rat with twofold increases in dose to 10 mg./rat), hydrocortisone acetate (1·25 to 5·0 mg./rat), prednisone acetate (0·63 to 2·5 mg./rat), prednisolone (0·63 to 2·5 mg./rat) and fludrocortisone acetate (0·31 to 2·5 mg./rat) retarded body growth and reduced the weights of the adrenal glands. Both effects increased with an increase in the dose of the steroid. An example of the effect of prolonged administration of adrenal steroids on body weight is shown in Fig. 1, which shows the effects of graded doses of cortisone acetate. Some deaths from infection occurred with high doses. Post-mortem examination of these animals revealed lesions of the lungs and liver and the presence of Gram-positive bacteria in the heart blood.

Deoxycorticosterone acetate (DCA) in doses of 1·25 to 5·0 mg./rat intramuscularly failed to influence either body or adrenal weights;

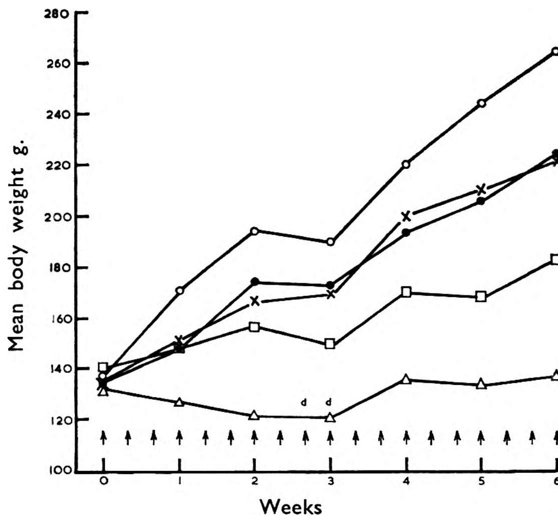

FIG. 1. The effect of prolonged administration of cortisone acetate on body weight. The steroid was injected 3 × a week, i.m. (at arrows) for 6 weeks to groups of 6 male rats at doses of 1·25, (●——●); 2·5, (X——X); 5·0, (□——□), and 10 mg./rat (△——△). The control group (○——○), received saline injections. Mortality is indicated by "d."

however, larger doses of this steroid (10 to 50 mg./rat subcutaneously) retarded growth and caused adrenal atrophy. The effects of other steroids on body weight and adrenal weight are compared with the effects of cortisone acetate in Table I.

An attempt has been made to evaluate the approximate potencies of these steroids, relative to cortisone acetate, in causing adrenal atrophy, retardation of body growth and also for their relative toxicity (Table II). Relative toxicity was assessed by comparing the maximum tolerated doses of each steroid, when dosage was increased by twofold stages. Fludrocortisone is the most active and DCA the least active; also with increase in potency there is an accompanying increase in toxicity.

TABLE I

THE EFFECT OF PROLONGED ADMINISTRATION OF ADRENAL STEROIDS ON BODY AND ADRENAL WEIGHTS

Steroid	Dose*	No. of rats	Adrenal atrophy		
			Mean body weight per cent of control	Mean adrenal weight (mg.) per cent of control	Mean adrenal weight (mg./100 g. body wt.) per cent of control
Cortisone acetate	1·25	6	84·6	65·8	80·4
	2·5	6	83·9	66·3	79·0
	5·0	6	69·2	40·3	59·4
	10·0	6	51·7	25·5	57·2
Hydrocortisone acetate ..	1·25	6	89·2	68·4	77·3
	2·5	6	62·5	34·8	55·5
	5·0	6	55·8	31·6	60·9
Prednisone acetate	0·63	6	93·1	78·5	81·1
	1·25	6	89·7	76·0	82·9
	2·5	6	47·2	47·3	103·7†
Prednisolone	0·63	6	76·7	74·0	98·5
	1·25	6	54·1	37·8	67·4
	2·5	6	33·5	25·8	68·8
Fludrocortisone acetate ..	0·31	6	71·5	58·7	79·3
	0·63	6	65·9	28·2	43·1
	1·25	6	42·3	29·1†	69·1†
	2·5	6	40·8	29·8†	73·4†
Deoxycorticosterone acetate	1·25	6	101·1	102·1	80·4
	2·5	6	104·2	100·0	99·1
	5·0	6	98·4	75·6	103·7
	10·0	6	81·9	34·2	40·8
	25·0	6	61·1	26·9	40·8
	50·0	6	49·1	28·6	57·7

* mg./rat, i.m. × 3 per week for 6 weeks.
† Apparent increase in adrenal weight from early mortality at these doses.

TABLE II

RELATIVE POTENCIES OF ADRENAL STEROIDS IN CAUSING ADRENAL ATROPHY, RETARDATION OF BODY GROWTH, AND DEATH DURING CHRONIC ADMINISTRATION*

Steroid	Adrenal atrophy	Retardation of growth	Toxicity (death)
Cortisone acetate	1·0	1·0	1
Hydrocortisone acetate	2·2	2·2	2
Prednisone acetate	1·6	2·8	4
Prednisolone	3·9	6·7	8
Fludrocortisone acetate	11·5	9·9	16
Deoxycorticosterone acetate ..	0·5	0·3	Non toxic at doses of 50 mg./rat × 3 per week.

* Intramuscular injections × 3 per week for 6 weeks.

PLATE 1

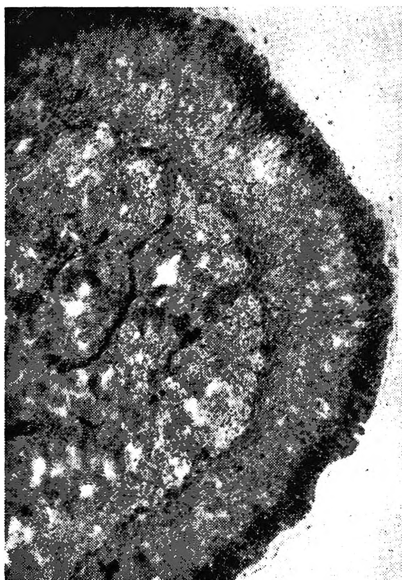

FIG. 1.

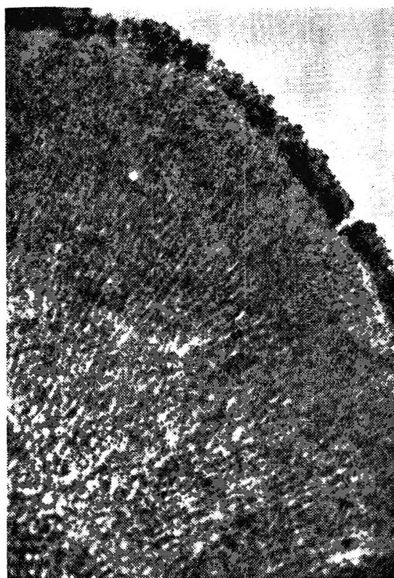

FIG. 2.

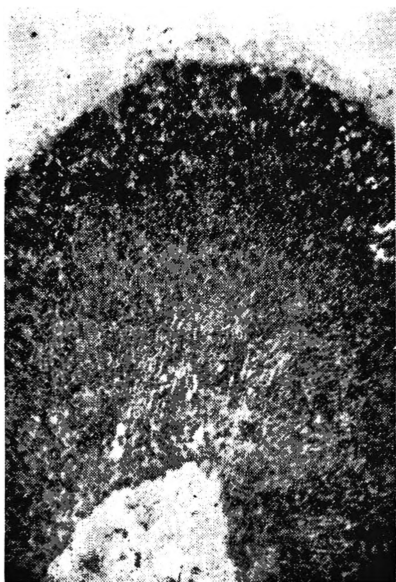

FIG. 3.

FIG. 1. Adrenal gland of rat treated with cortisone acetate (10 mg./rat 3 × a week, i.m., for 6 weeks). Marked atrophy of the zona fasciculata, but the zona glomerulosa is normal and contains abundant sudanophilic lipid. × 110.

FIG. 2. Adrenal gland of saline treated control rat. Normal morphology and distribution of sudanophilic lipid. × 110.

FIG. 3. Adrenal gland of rat treated with DCA, (5 mg./rat 3 × a week, i.m., for 6 weeks). Marked depletion of sudanophilic lipid in the zona glomerulosa; no evidence of cortical atrophy. × 110.

PLATE 2

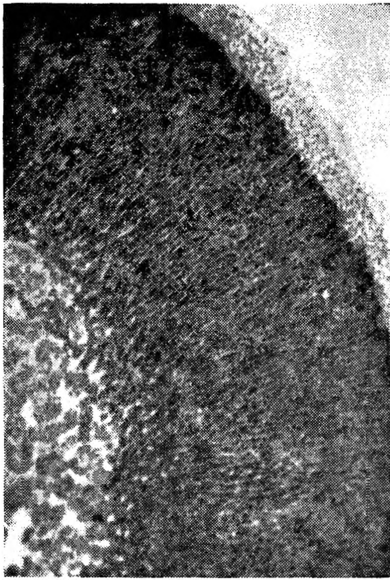
FIG. 1.

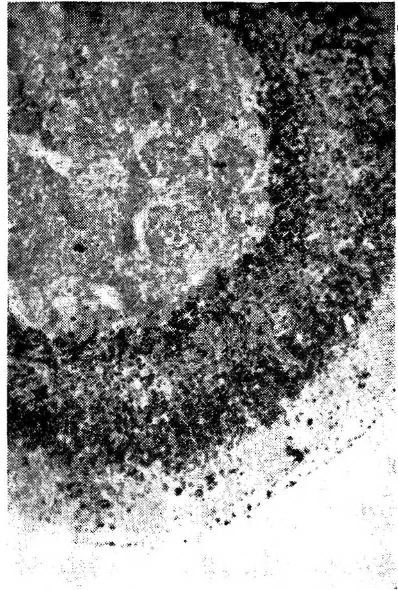
FIG. 2.

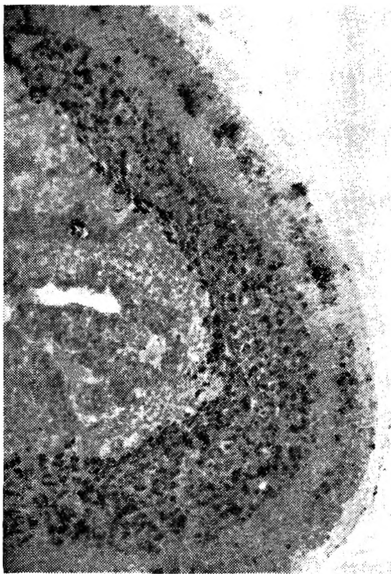
FIG. 3.

FIG. 1. Adrenal gland of rat treated with fludrocortisone acetate (0·31 mg./rat 3 × a week, i.m., for 6 weeks). Marked depletion of sudanophilic lipid in the zona glomerulosa: slight evidence of cortical atrophy. × 110.

FIG. 2. Adrenal gland of rat treated with DCA (50 mg./rat 3 × a week, s.c., for 6 weeks). Depletion of sudanophilic lipid in the zona glomerulosa and in outer region of the zona fasciculata, together with extensive atrophy of cells in the middle and inner regions of the zona fasciculata. × 110.

FIG. 3. Adrenal gland of rat treated with fludrocortisone acetate (0·63 mg./rat 3 × a week, i.m., for 6 weeks). Depletion of sudanophilic lipid in the zona glomerulosa and in outer region of the zona fasciculata, together with extensive atrophy of cells in the middle and inner regions of the zona fasciculata. × 110.

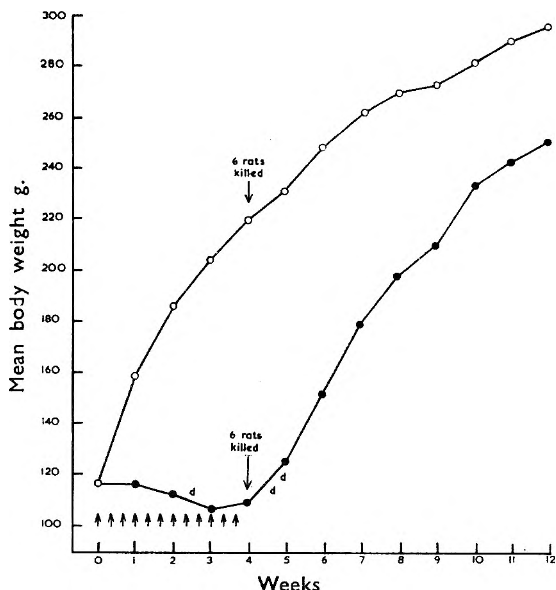

FIG. 2. The effect of prolonged administration of cortisone acetate and the sudden cessation of this treatment on body weight. The steroid was injected at a dosage level of 10 mg./rat 3 × a week, i.m. (at arrows) for 4 weeks to a group of 12 male rats (●——●), control animals received injections of saline (○——○). Mortality is indicated by "d."

Effect on Histology of the Adrenal Glands

Prolonged administration of cortisone acetate (10 mg./rat) caused marked atrophy of the zona fasciculata but the zona glomerulosa remained unaffected (Plate 1, Fig. 1); this is shown in comparison with a normal adrenal gland from a saline treated control rat (Plate 1, Fig. 2). The prolonged administration of hydrocortisone acetate (5 mg./rat), prednisone acetate (2·5 mg./rat) and prednisolone (2·5 mg./rat) resulted in qualitatively similar patterns. However, relatively low doses of DCA (5 mg./rat) and fludrocortisone (0·31 mg./rat) caused a specific and marked depletion of sudanophilic material in the zona glomerulosa (Plate 1, Fig. 3, and Plate 2, Fig. 1 respectively); with the latter steroid there was, in addition, a slight cortical atrophy. Larger doses of DCA (50 mg./rat subcutaneously) and slightly larger doses of fludrocortisone (0·63 mg./rat) caused a depletion of Sudan III staining material in the zona glomerulosa and in the outer region of the zona fasciculata, together with an extensive atrophy of cells in the middle and inner regions of the zona fasciculata (Plate 2, Figs. 2 and 3 respectively).

Regeneration of Adrenal Glands after Cortical Atrophy

Cortisone acetate (10 mg./rat) was injected intramuscularly thrice weekly to a group of 12 rats; a similar control group was injected with the same volume of saline. Cortisone treatment was stopped after 4 weeks and 6 animals were killed from each group. The adrenals from these

PLATE 3

FIG. 1.

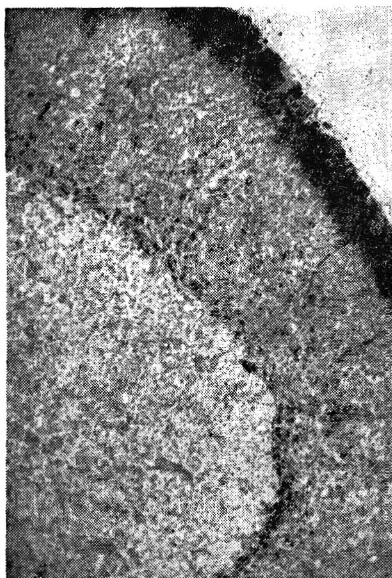

FIG. 2.

FIG. 3.

FIG. 1. Adrenal gland of saline treated control rat, killed 12 weeks after start of experiment. Normal morphology and distribution of sudanophilic lipid. × 110.

FIG. 2. Adrenal gland of rat treated with cortisone acetate (10 mg./rat 3 × a week, i.m.), for 4 weeks and then killed. Extensive and typical cortical atrophy; depletion of sudanophilic lipid. × 110.

FIG. 3. Adrenal gland of rat treated with cortisone acetate, 10 mg./rat, 3 × a week, i.m.), for 4 weeks and then allowed to live until 12th week without any further treatment. Normal morphology and distribution of sudanophilic lipid. × 110.

rats were weighed and examined histologically. The remaining rats in the treated and control groups were then kept without further treatment for an additional 8 weeks; at this time they were killed and the adrenals weighed and sectioned.

The effect of this régime of treatment on body weight is shown graphically on Fig. 2. Cortisone caused the familiar retardation of body growth and death of some animals.

When this treatment was stopped two rats died but the remaining animals continued to grow rapidly. However, the mean body weight of

TABLE III

THE REGENERATION OF ADRENAL GLANDS AFTER THE CESSATION OF CHRONIC DOSES OF CORTISONE ACETATE

Treatment	No. of rats	Mean adrenal weight (mg.) ± S.E.	Mean adrenal weight (mg./ 100 g. body weight) ± S.E.
Controls, saline i.m. × 3 per week for 4 weeks. No further treatment, killed at 12 weeks	6	31·8 ± 2·0	10·7 ± 0·5
Controls, saline i.m. × 3 per week for 4 weeks then killed ..	6	22·0 ± 1·7	9·6 ± 0·6
Cortisone acetate, 10 mg./rat i.m. × 3 per week for 4 weeks. No further treatment, killed at 12 weeks	6	25·7 ± 2·3*	10·2 ± 0·7*
Cortisone acetate, 10 mg./rat i.m. × 3 per week for 4 weeks then killed	6	6·5 ± 0·2	6·3 ± 0·7

* Value from 3 surviving animals.

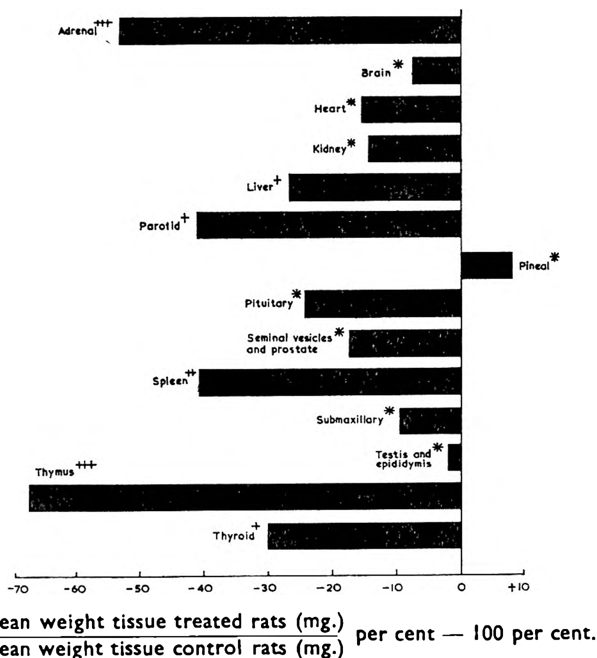

FIG. 3. The effect of prolonged administration of cortisone acetate (5 mg./rat 3 × a week, i.m., for 6 weeks) on the absolute weight (mg.) of specific tissues in groups of 6 rats. The symbols denote the levels of significance of the differences between mean weights of tissues from control and treated rats; +++ = P < 0·001, ++= P < 0·01, += P < 0·05, * = P > 0·05.

the survivors did not equal that of the controls at any time during the remaining 8 week period of the experiment. Table III shows the mean adrenal weight for each group of rats expressed both in terms of absolute weight and as adrenal weight per 100 g. body weight.

These results suggest that the adrenal atrophy induced by cortisone is a reversible process. After treatment was stopped, the glands of the surviving rats gained in weight and at 12 weeks from the start of the experiment they did not differ appreciably in weight from those of

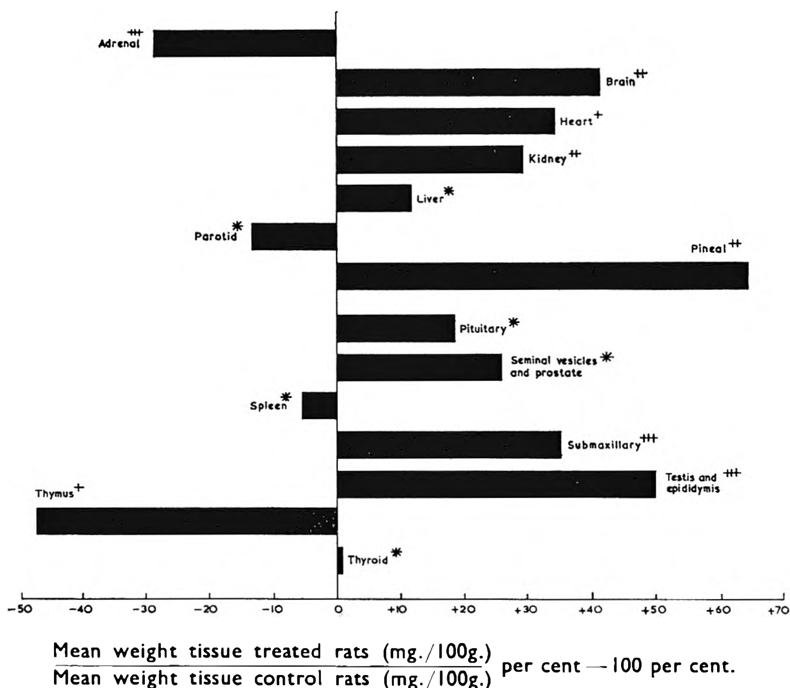

FIG. 4. The effect of prolonged administration of cortisone acetate (5 mg./rat 3 × a week, i.m., for 6 weeks) on the weight (mg./100 g. body weight) of specific tissues in groups of 6 rats. The symbols denote the levels of significance of the differences between mean weights of tissues from control and treated rats; $+++ = P < 0{\cdot}001$, $++ = P < 0{\cdot}01$, $+ = P < 0{\cdot}05$, $* = P > 0{\cdot}05$.

the untreated controls. When examined histologically, the adrenal glands from rats after 4 weeks treatment showed extensive and typical atrophy (Plate 3, Fig. 2). However, the adrenals from rats surviving the 8 week period after the cessation of treatment (Plate 3, Fig. 3) did not differ in morphology and distribution of sudanophilic lipid from those of the control animals (Plate 3, Fig. 1).

Effect on Weights of Other Tissues

Since the chronic administration of corticosteroids to rats caused such marked changes in the weight and histology of the adrenal glands, it was thought of interest to examine the effect of such treatment on the weights

of various organs and tissues and also on endocrine glands other than the adrenals. These tissues were brain, heart, kidney, liver, parotid salivary gland, pineal, pituitary, seminal vesicles and prostate, spleen, submaxillary salivary gland, testis and epididymis, thymus and thyroid.

In this experiment, cortisone acetate (5 mg./rat) was injected intramuscularly thrice weekly for 6 weeks. Fig. 3 compares the absolute weights of the tissues with those from the control animals; all tissues from the treated rats were smaller than those from the controls; the pineal gland was the sole exception, this appeared to be slightly larger in the treated rats although the difference was not statistically significant ($P>0{\cdot}05$). However, since cortisone treatment also affected the body weight of the rats, the tissue weights were expressed in terms of proportion of body weight to exclude the influence of the steroid on the animals' general body growth. The presentation of experimental results in this manner (Fig. 4) suggests that a significant atrophy is present with the adrenal glands ($P<0{\cdot}001$) and the thymus ($P<0{\cdot}05$) but not with the spleen or the parotid gland. The pineal gland showed the most marked relative increase in weight while the submaxillary gland, the testis and epididymis, the brain, heart and kidney showed a less marked but still significant increase in weight over corresponding tissues from the control rats.

Discussion

All the corticosteroids examined caused an inhibition of the animals' normal rate of growth. For example with high doses of cortisone acetate (10 mg./rat) this inhibition was almost complete. Fludrocortisone, the most potent of the steroids tested, is more active in producing adrenocortical atrophy than in causing a retardation of body growth. Prednisone and prednisolone, on the other hand, appear to be more potent in their effects on body weight than on adrenal weight, whilst hydrocortisone has equal activity on both.

Continued high dosage of some steroids caused mortality, from the sudden flare up of a dormant infection. Post-mortem examination of these animals revealed lesions of the lungs and liver and the presence of Gram-positive bacteria in the heart blood. Selye (1955) has reported a similar pattern of infection in rats given large doses of cortisone and Ingle, Prestrud and Li (1951) have described a severe infection in rats in which hypercorticalism was induced by a continuous injection of adrenocorticotrophic hormone. In man, also, there have been many reports of a decreased resistance to infection accompanying continued treatment with large doses of cortical steroids or corticotrophin (Kass and Finland, 1953; *British Medical Journal*, 1954; Robinson, 1956).

The present studies indicate that adrenocorticoids may be divided into two classes from their effects on the adrenal cortex. Firstly, steroids like cortisone, hydrocortisone and their $\triangle^1$- analogues, which produce extensive atrophy of the zona fasciculata, without affecting the cellular histology or lipid distribution within the zona glomerulosa, even when administered repeatedly at high dosage. Secondly, those like fludrocortisone and DCA which in low doses, produce a specific depletion of the

sudanophilic material in the zona glomerulosa with little or no accompanying adrenocortical atrophy. It would seem likely that the first group of steroids act by reducing the secretion of corticotrophin from the anterior pituitary and so produce adrenocortical atrophy, whereas the second group influence the secretion of lipid in the zona glomerulosa by a predominant effect on the animals' electrolyte balance. At the same time this latter group of steroids may also have an effect on the pituitary output of corticotrophin since higher doses of both fludrocortisone and DCA cause atrophy of the zona fasciculata as well.

Deane and Greep (1946) and Greep and Deane (1949) observed that the zona glomerulosa of the hypophysectomised rat maintains its morphological integrity and a readily demonstrable lipid content while the zonae fasciculata and reticularis atrophy, observations which led these and other workers to suggest that the zona glomerulosa of the rat adrenal cortex is independent of the pituitary gland (Greep and Deane, 1949; Yoffey and Baxter, 1949). The results of this present series of experiments are in agreement with this concept since steroids administered in doses which inhibit the anterior pituitary and produce cortical atrophy do not affect the zona glomerulosa, whereas steroids, known to have potent effects on electrolyte metabolism, specifically deplete the zona glomerulosa at doses which do not produce cortical atrophy. On the other hand, exogenous corticotrophin has been reported to cause marked activity in the zona glomerulosa of the rat adrenal (Cater and Stack-Dunne, 1953; Wexler and Rinfret, 1955), an observation that has been confirmed in our own unpublished experiments; it may be therefore that this zone is not entirely autonomous.

The present studies also indicate that the steroid induced adrenocortical atrophy is a reversible process, since when treatment ceased the glands gradually recovered their normal weight, histology and distribution of sudanophilic lipid, although the body weight of the treated rats did not attain that of the controls.

During these studies, it was also observed that prolonged treatment with cortisone acetate had a marked effect on the weight of various tissues other than the adrenals. Cortisone has a marked effect on body growth and also inevitably a general, but non-specific, catabolic effect on many of the tissues of the body. The change in weight of a tissue may be due to a specific effect on that tissue, alternatively it may reflect the general catabolic effect of the steroid on body growth. For this reason comparison between the weights of tissues from control and treated rats was made in terms of the absolute weights of the tissues and also in terms of the weight of the tissue per 100 g. body weight.

In the absence of a more reliable index, specific atrophy has been assumed when a tissue shows a statistically significant decrease in weight when expressed either as absolute weight or weight related to body weight. A true hypertrophy exists when the steroid exerts a direct effect on the tissue and increases its weight; however, hypertrophy may also seem to be present when the weight of the particular tissue is independent of the catabolic effect of the steroid on general body growth. Histology is

perhaps the only simple way of distinguishing between true and apparent hypertrophy.

The adrenal glands, the thymus but not the spleen shcwed specific atrophy. Shewell (1957) reported that thymic involution was a more sensitive index of the biological activity of corticosteroids than was atrophy of the spleen. Only the pineal gland was larger in the treated animals, and when its weight was related to the animal's body weight there was a greater difference between pineal weight from treated and control rats than there was for any other tissue examined. The significance of this finding is the subject of current investigation.

Acknowledgements. The early experiments in this study were made in the Department of Pharmacology, School of Pharmacy, University of London. We wish to thank Professor G. A. H. Buttle for his constant enthusiasm and helpful advice in this work. We would also like to thank Mr. D. Boxall for preparing the photomicrographs, and Mr. J. J. Grimshaw for his statistical treatment of the experimental results.

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SOME DERIVATIVES OF 1,2,3,6-TETRAHYDROPYRIDINE

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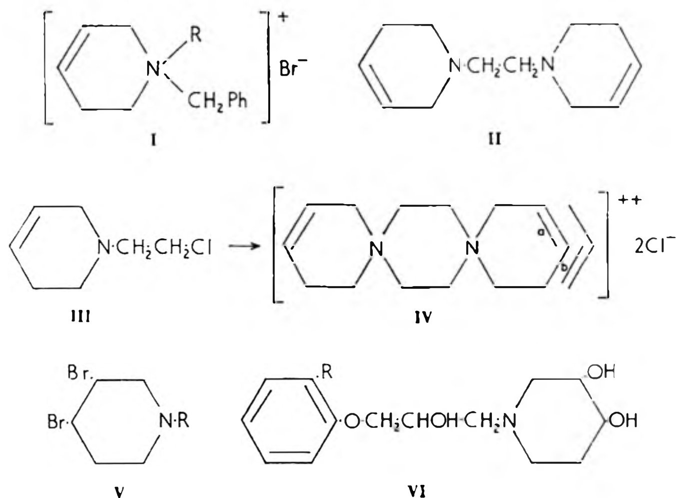
Received January 12, 1962

The preparation of some derivatives of 1,2,3,6-tetrahydropyridine and hydroxylation of certain of these to *cis*- and *trans*-piperidine-3,4-diols is described.

1-Benzyl-1,2,3,6-tetrahydropyridine reacted with benzyl bromide and with phenethyl bromide to yield the quaternary salts (Ia, R = $\cdot CH_2\cdot Ph$) and (Ib, R = $\cdot CH_2\cdot CH_2\cdot Ph$) respectively. The latter compound, more readily prepared by reaction of 1,2,3,6-tetrahydro-1-phenethylpyridine with benzyl bromide, was only obtained in one isomeric form (compare Beasley, Petrow and Stephenson, 1958). 1,2,3,6-Tetrahydropyridine reacted slowly with 1,2-dichloroethane at reflux temperature to yield the dihydrochloride of 1,2-di(1,2,3,6-tetrahydropyridino)ethane (II). With ethylene oxide the tetrahydropyridine gave a high yield of 2-(1,2,3,6-tetrahydropyridino)ethanol, smoothly converted by reaction with thionyl chloride [compare Chabrier, Najer, Giudicelli and Joannic (1957)] into the hydrochloride of the chloride (III). The last compound was converted into the free base, which dimerised on prolonged heating in ethanolic solution to give the di*spiro*piperazine derivative (IVa or IVb), which again was obtained in only one form.



Bromination of 1,2,3,6-tetrahydropyridine hydrobromide in acetic acid gave a high yield of the hydrobromide of the 3,4-dibromo-compound, smoothly converted into the urea (Va, R = $\cdot CO\cdot NH_2$). Corresponding 3,4-dibromoderivatives were prepared from 1-benzoyl- (Vb, R = $\cdot CO\cdot Ph$), and 1-benzyl-1,2,3,6-tetrahydropyridine (Vc, R = $\cdot CH_2\cdot Ph$), the former

yielding two isomers, one of which passed slowly into the higher-melting form on storage. Neither of the isomers proved suitable for conversion into piperidine-3,4-diols by means of reaction with potassium acetate-acetic acid.

Only one mention of piperidine-3,4-diols has been found in the literature. McElvain and Safranski (1950) obtained 1-methyl-4-phenylpiperidine-3,4-diol by hydrolysis of 3-bromo-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a reaction involving conversion of the bromine atom into a hydroxyl group followed by hydration at the double bond.

1-Benzoyl-1,2,3,6-tetrahydropyridine was readily converted by peracetic acid into *trans*-1-benzoylpiperidine-3,4-diol in good yield. With ethereal perphthalic acid, the benzoyl derivative gave an epoxide, obtained only in the form of a *gum*, which yielded the foregoing *trans*-diol on hydration. Hydrolysis of *trans*-1-benzoyl-piperidine-3,4-diol with ethanolic hydrochloric acid gave *trans*-piperidine-3,4-diol hydrochloride, subsequently obtained more readily by hydrolysis of *trans*-1-acetylpiperidine-3,4-diol, though the acetyl derivative itself could not be obtained pure. Hydrolysis of *trans*-1-benzoylpiperidine-3,4-diol with hot alkali, followed by reaction of the resultant base *in situ* with 1,2-epoxy-3-phenoxypropane gave *trans*-1-(2-hydroxy-3-phenoxypropyl)piperidine-3,4-diol (VI; R = H). The *o*-tolyl analogue (VI; R = Me) was similarly prepared. *trans*-1-Carbamoylpiperidine-3,4-diol was obtained by reaction of the diol hydrochloride with aqueous sodium cyanate.

cis Hydroxylation of 1-acetyl- and 1-benzoyl-1,2,3,6-tetrahydropyridine was readily accomplished using aqueous sodium chlorate-osmium tetroxide at 50–60°, a procedure used by Böeseken and van Giffen (1920) and later by Clarke and Owen (1949). The *cis*-diol was also obtained, albeit in poor yield, using iodine-silver acetate in moist acetic acid as described by Ginsberg (1953), Gunstone and Morris (1957) and Woodward and Brutcher (1958). Hydrolysis of the 1-acetyl- or -benzoyl- *cis*-diols with ethanolic hydrochloric acid yielded *cis*-piperidine-3,4-diol hydrochloride, which reacted with benzoyl chloride in the presence of alkali to yield the original benzoyl derivative and with sodium cyanate to give *cis*-1-carbamoylpiperidine-3,4-diol. *cis*-Piperidine-3,4-diol was converted by 1,2-epoxy-3-phenoxypropane into *cis*-1-(2-hydroxy-3-phenoxypropyl)-piperidine-3,4-diol.

Hydroxylation of 4-phenyl-1-toluene-*p*-sulphonyl-1,2,3,6-tetrahydropyridine with peracetic acid gave small yields of both the *cis*- and *trans*-3,4-diols. The former isomer alone was obtained using sodium chlorate-osmium tetroxide in aqueous ethanol.

Finally, the hydroxylation of 1-benzyl-1,2,3,6-tetrahydropyridine was studied. Possible complications due to the presence of a basic nitrogen centre were avoided by using the sulphate of the base in the sodium chlorate-osmium tetroxide procedure when *cis*-1-benzylpiperidine-3,4-diol was readily obtained. Reaction of this compound with methyl iodide furnished a difficultly separable mixture of isomeric methiodides. Their conversion into methochlorides permitted facile separation. 1-Benzyl-1,2,3,6-tetrahydropyridine was converted by peracetic acid into a mixture

of isomeric *trans*-1-benzyl-3,4-dihydroxypiperidine *N*-oxides which were readily separated as their hydrochlorides. Conversion of the corresponding *cis*-1-benzylpiperidine-3,4-diol to the *N*-oxides using acetone-hydrogen peroxide led to the isolation of only one pure isomer, which proved to be unstable on storage.

Pharmacological study of many of the above compounds by Dr. A. David and his colleagues gave no results worthy of comment.

EXPERIMENTAL

1-*Benzyl*-1,2,3,6-*tetrahydropyridine*. A solution of 1,2,3,6-tetrahydropyridine (91·5 g.) in water (400 ml.) containing potassium hydroxide (61·6 g.) was heated with stirring to 40° and benzyl chloride (126·5 g.) added during 10 min. Reaction was completed by heating to 70° for 2 hr. The *product* (151 g.), isolated with benzene, had b.p. 68° at 0·1 mm. Found: C, 83·6; H, 8·4; N, 7·8. $C_{12}H_{15}N$ requires C, 83·2; H, 8·7; N, 8·1 per cent.

1-*Benzyl*-3,4-*dibromopiperidine hydrobromide*. A solution of the foregoing base (86·5 g.) in acetic acid (200 ml.) was treated with stirring and water-cooling with 50 per cent w/v hydrogen bromide in acetic acid (90 ml.) followed by a solution of bromine (80 g.) in acetic acid (100 ml.) added during 20 min. The mixture was concentrated at reduced pressure and the residue dissolved in ethyl acetate (200 ml.) to yield the *product* (172 g.) m.p.184° (decomp.) (from ethanol-ethyl acetate). Found: C, 35·1; H, 4·0; Br, 57·5; N, 3·3. $C_{12}H_{16}Br_3N$ requires C, 34·8; H, 3·9; Br, 57·9; N, 3·4 per cent.

1,1-*Dibenzyl*-1,2,3,6-*tetrahydropyridinium bromide*, prepared by reaction of 1-benzyl-1,2,3,6-tetrahydropyridine with benzyl bromide in benzene, had m.p. 188–190° (from ethanol-ethyl acetate). Found: C, 66·5; H, 6·4; Br, 23·0; N, 3·8. $C_{19}H_{22}BrN$ requires C, 66·2; H, 6·4; Br, 23·2; N, 4·1 per cent. The *picrate* had m.p. 153–154° (from ethanol). Found: C, 60·8; H, 4·8; N, 11·1. $C_{25}H_{24}N_4O_7$ requires C, 60·9; H, 4·9; N, 11·4 per cent. A solution of the *bromide* (1·0 g.) in acetic acid (10 ml.) treated with the theoretical amount of bromine in acetic acid yielded 1,1-di-*benzyl*-3,4-*dibromopiperidinium bromide* (1·5 g.) as light orange needles, (from ethanol). Found: C, 45·1; H, 4·3; Br, 47·2; N, 2·9. $C_{19}H_{22}Br_3N$ requires C, 45·2; H, 4·4; Br, 47·6; N, 2·8 per cent. 1,2,3,6-*Tetrahydro*-1-*phenethylpyridine*, had b.p. 70° at 0·1 mm. Found: C, 83·5; H, 9·1; N, 7·4. $C_{13}H_{17}N$ requires C, 83·4; H, 9·2; N, 7·5 per cent.

1-*Benzyl*-1,2,3,6-*tetrahydro*-1-*phenethylpyridinium bromide* prepared (*a*) by reaction of the foregoing base with benzyl bromide at room temperature or in lower yield by (*b*) reaction of 1-benzyl-1,2,3,6-tetrahydropyridine with phenethyl bromide at 100° for 8 hr., had m.p. 195–197° (from ethanol-ethyl acetate). Found: C, 67·2; H, 6·7; Br, 22·3; N, 3·9. $C_{20}H_{24}BrN$ requires C, 67·0; H, 6·8; Br, 22·3; N, 3·9 per cent. Fractional crystallisation of a sample did not show the presence of an isomer. The *iodide* had m.p. 181–182° (from ethanol-ethyl acetate). Found: C, 59·0; H, 5·8. $C_{20}H_{24}IN$ requires C, 59·2; H, 6·0 per cent. The *picrate*, bright yellow crystals (from ethanol), had m.p. 136–138°. Found: C, 62·0;

H, 5·2; N, 10·8. $C_{26}H_{26}N_4O_7$ requires C, 61·6; H, 5·2; N, 11·1 per cent. A solution of the bromide in acetic acid treated with bromine in the same solvent yielded 1-*benzyl*-3,4-*dibromo*-1-*phenethyl-piperidinium bromide*, orange crystals (from ethanol), m.p. 92–94°. Found: C, 46·1; H, 4·9; Br, 46·3; N, 3·0. $C_{20}H_{24}Br_3N$ requires C, 46·3; H, 4·7; Br, 46·3; N, 2·7 per cent. 1,2-Di-(1,2,3,6-*tetrahydropyridino*)*ethane*. To a solution of 1,2,3,6-tetrahydropyridine (83 g.) in benzene (300 ml.) was added sodium carbonate (74·2 g.) followed by 1,2-dichloroethane (49·5 g.) and the mixture was heated under reflux for 20 hr. The *product* was obtained as an oil, b.p. 74° at 0·2 mm. Found: C, 74·9; H, 10·6; N, 14·8. $C_{12}H_{20}N_2$ requires C, 74·9; H, 10·5; N, 14·6 per cent. The *dihydrochloride* had m.p. 316° (decomp.) (from ethanol). Found: C, 54·6; H, 8·4; Cl, 26·8. $C_{12}H_{22}Cl_2N_2$ requires C, 54·3; H, 8·4; Cl, 26·8 per cent.

1,2,3,6-*Tetrahydro*-1-(2-*hydroxyethyl*)*pyridine* obtained in 72 per cent yield by reaction of 1,2,3,6-tetrahydropyridine with ethylene oxide in ethanolic solution, had b.p. 46° at 0·2 mm. Found: C, 65·8; H, 10·5; N, 10·8. $C_7H_{13}NO$ requires C, 66·1; H, 10·3; N, 11·0 per cent.

1-(2-*Chloroethyl*)-1,2,3,6-*tetrahydropyridine hydrochloride*. A solution of the foregoing base (100 g.) in benzene (500 ml.) was treated with thionyl chloride (141 g.), added during 30 min. with shaking and water cooling. The mixture was then heated under reflux for 1 hr. The *product* (141 g.) had m.p. 236° (decomp.) (from ethanol-ethyl acetate). Found: C, 46·4; H, 7·1; Cl, 38·6; N, 7·7. $C_7H_{13}Cl_2N$ requires C, 46·2; H, 7·2; Cl, 38·9; N, 7·7 per cent.

1,2,3,6-*Tetrahydropyridinium*-1-*spiro*-1′-*piperazine*-4′-*spiro*-1″-1″,2″,3″ (or 5″), 6″-*tetrahydropyridinium dichloride*. A solution of the foregoing hydrochloride (18·2 g.) in water (15 ml.) was treated with a solution of sodium hydroxide (5·5 g.) in water (10 ml.). The base was isolated with chloroform and the solvent distilled off. The residual oil was dissolved in ethanol (50 ml.) and the solution heated under reflux for 6 hr. The *product* which separated (10·1 g.) had m.p. 338° (decomp.) (from aqueous ethanol). Fractional crystallisation of a sample from the same solvent mixture yielded no sign of isomeric product. Found: C, 57·4; H, 8·3; N, 9·7. $C_{14}H_{24}Cl_2N_2$ requires C, 57·7; H, 8·3; N, 9·6 per cent.

3,4-*Dibromopiperidine hydrobromide* was obtained in 86 per cent yield by reaction of 1,2,3,6-tetrahydropyridine hydrobromide in acetic acid with a solution of bromine in the same solvent. It had m.p. 207° (decomp.) (from ethanol). Found: C, 18·9; H, 3·1; Br, 73·3; N, 4·4. $C_5H_{10}Br_3N$ requires C, 18·5; H, 3·1; Br, 74·1; N, 4·3 per cent.

3,4-*Dibromo*-1-*carbamoylpiperidine*. A solution of the foregoing hydrobromide (3·2 g.) in water (20 ml.) was treated with a solution of sodium cyanate (0·8 g.) in water (5 ml.) and the mixture warmed to 60–65° for 10 min. The product (2·8 g.) was collected and washed with a little cold water. It had m.p. 182–183° (decomp.) (from ethyl acetate). Found: C, 25·6; H, 3·5; N, 10·2. $C_6H_{10}Br_2N_2O$ requires C, 25·2; H, 3·5; N, 9·8 per cent.

1-*Benzoyl*-3,4-*dibromopiperidine*. A solution of 1-benzoyl-1,2,3,6-tetrahydropyridine (37·4 g.) in acetic acid (100 ml.) was treated with

stirring and water cooling with a solution of bromine (32 g.) in acetic acid (50 ml.). Dilution with water furnished a *product* (30·5 g.) having m.p. 86–89° after crystallisation from light petroleum (b.p. 60–80°) containing a small amount of ethyl acetate. Found: C, 41·3; H, 3·7; N, 4·2. $C_{12}H_{13}Br_2NO$ requires C, 41·5; H, 3·8; N, 4·0 per cent. The m.p. of the material after several weeks was 86–106°. Concentration of the mother liquors from the first crystallisation yielded a second *product* (12 g.), m.p. 106° (from aqueous methanol). Found: C, 41·5; H, 3·7; N, 3·7 per cent.

trans-1-*Benzoylpiperidine*-3,4-*diol* (*a*) 1-Benzoyl-1,2,3,6-tetrahydropyridine (37·4 g.) was melted and added with stirring to a solution of peracetic acid prepared from acetic acid (150 ml.) and 30 per cent hydrogen peroxide (46 ml.) and the mixture was heated at 85–90° for 10 hr. It was then concentrated to about one third bulk and neutralised with a concentrated solution of potassium hydroxide. The oil was isolated by extraction with chloroform and the extract washed with saturated salt solution when the chloroform was boiled off. The residue was dissolved in ethanol (150 ml.), sodium carbonate (10 g.) added and the mixture heated under reflux for 2 hr. It was filtered, concentrated to about 50 ml. and diluted with ethyl acetate. The product (29·2 g.) had m.p. 148–150° (from ethyl acetate containing a trace of methanol). Found: C, 65·2; H, 7·0; N, 6·5. $C_{12}H_{15}NO_3$ requires C, 65·1; H, 6·8; N, 6·3 per cent.

(*b*) A solution of 1-benzoyl-1,2,3,6-tetrahydropyridine (9·35 g.) in ether (20 ml.) was treated with a solution of perphthalic acid in ether (100 ml., 0·05 M.). The mixture was allowed to stand for 12 days when chloroform was added and the solution, after washing free from acid and peroxide, was concentrated. The resultant gum failed to crystallise. It was heated with acetic acid (10 ml.) at 95° for 3 hr. when the acid was distilled off at reduced pressure and the product dissolved in ethanol (30 ml.) and the solution heated under reflux with sodium carbonate (2 g.) for 3 hr. The solution was filtered and concentrated. Crystallisation of the residue from ethyl acetate furnished the product (2·2 g.) m.p. 147–149°. The melting point was not depressed on admixture with the compound prepared in (*a*). Reaction of the diol (1·5 g.) with two equivalents of benzoyl chloride in pyridine yielded trans-1-*benzoyl*-3,4-*benzoyloxypiperidine*, m.p. 140–141° [from ethyl acetate-light petroleum (b.p. 60–80°)]. Found: C, 72·5; H, 5·1; N, 3·1. $C_{26}H_{23}NO_5$ requires C, 72·7; H, 5·4; N, 3·3 per cent.

trans-*Piperidine*-3,4-*diol hydrochloride*. A solution of *trans*-1-benzoylpiperidine-3,4-diol (50 g.) in concentrated hydrochloric acid (80 ml.) was boiled under reflux for 6 hr. after which excess of acid was removed under reduced pressure. The residual solid was crystallised from ethanol-ethyl acetate to yield the product (28·6 g.) m.p. 138–140°. Found: C, 39·3; H, 7·8; Cl, 23·6; N, 9·4. $C_5H_{12}ClNO_2$ requires C, 39·1; H, 7·9; Cl, 23·1; N, 9·1 per cent.

trans-1-*Carbamoylpiperazine*-3,4-*diol*, prepared by reaction of the foregoing hydrochloride with sodium cyanate in concentrated aqueous solution, had m.p. 131–133° (from ethanol-ethyl acetate). Found:

C, 44·7; H, 7·5; N, 17·2. $C_6H_{12}N_2O_3$ requires C, 45·0; H, 7·6; N, 17·5 per cent.

trans-1-(2-p-*Acetamidophenoxyethyl)piperidine*-3,4-*diol hydrochloride.* A mixture of *trans*-piperidine-3,4-diol hydrochloride (6·14 g.), 2-*p*-acetamidophenoxyethyl bromide (10 g.)and sodium carbonate (4·7 g.) in ethanol (50 ml.) was heated under reflux for 8 hr. and filtered. Treatment of the filtrate with hydrogen chloride yielded the *product*, m.p. 192° (from ethanol-ethyl acetate). Found: Cl, 10·8; N, 8·4. $C_{15}H_{23}ClN_2O_4$ requires Cl, 10·7; N, 8·5 per cent.

trans-1-(2-*Hydroxy*-3-*phenoxypropyl)piperidine*-3,4-*diol.* 1-Acetyl-1,2,3,6-tetrahydropyridine (25 g.) was added to a solution of peracetic acid prepared from 30 per cent hydrogen peroxide (46 ml.) and acetic acid (120 ml.) and the solution was heated at 90° for 2 hr. It was then concentrated to about one-fifth volume at reduced pressure and just basified with 25 per cent aqueous potassium hydroxide. The solution was saturated with sodium chloride and extracted with t-butanol. The extract was concentrated and the residual oil hydrolysed by heating with a solution of potassium hydroxide (11·2 g.) in water (20 ml.) for 2 hr. Ethanol (50 ml.) was then added, followed by 1,2-epoxy-3-phenoxypropane (16 g.) and the mixture heated under reflux for 30 min. The solution was concentrated at reduced pressure and the residual solid crystallised from ethyl acetate containing a trace of ethanol to yield the *product* (10·4 g.) m.p. 166–168°. Found: C, 62·9; H, 7·6; N, 4·8. $C_{14}H_{21}NO_4$ requires C, 62·9; H, 7·9; N, 5·2 per cent.

trans-1-(2-*Hydroxy*-3-o-*tolyloxypropyl)piperidine*-3,4-*diol*, had m.p. 150–152° (from ethyl acetate). Found: C, 63·5; H, 8·1; N, 5·1. $C_{15}H_{23}NO_4$ requires C, 64·0; H, 8·2; N, 5·0 per cent.

cis-1-*Acetylpiperidine*-3,4-*diol.* 1-Acetyl-1,2,3,6-tetrahydropyridine (87·6 g.) was dissolved in water (700 ml.) containing sodium chlorate (97 g.) and a solution of osmium tetroxide (0·25 g.) in water (25 ml.) added at once with stirring. The temperature of the mixture rose spontaneously to 48° over about 45 min. and was then kept at 45–50° for 1 hr. The mixture was cooled, extracted with benzene and the aqueous layer evaporated to dryness at reduced pressure. The residual solid was extracted with four 250 ml. portions of boiling chloroform, the chloroform extracts were concentrated, and diluted with ethyl acetate. The *product* (83·5 g.) had m.p. 119–120° after crystallisation from ethyl acetate containing a little methanol. Found: C, 52·9; H, 8·2; N, 9·0. $C_7H_{13}NO_3$ requires C, 52·8; H, 8·2; N, 8·8 per cent.

cis-1-*Benzoylpiperidine*-3,4-*diol*, was prepared by hydroxylation of 1-benzoyl-1,2,3,6-tetrahydropyridine (37·4 g.) in water (500 ml.) containing sodium chlorate (34 g.) and osmium tetroxide (0·2 g.) at 50° for 5 hr. The *product* (30 g.) had m.p. 151–152° after crystallisation from ethyl acetate containing a trace of methanol. Found: C, 64·7; H, 6·8; N, 6·5. $C_{12}H_{15}NO_3$ requires C, 65·1; H, 6·8; N, 6·3 per cent. A mixture of the diol (2·21 g.) and *p*-nitrobenzaldehyde (1·51 g.) in benzene (40 ml.) and dioxan (20 ml.) was treated with toluene *p*-sulphonic acid (50 mg.) and heated on the steam-bath for 1 hr., to yield a p-*nitrobenzylidene*

derivative (2·1 g.) m.p. 138–139° (from aqueous methanol). Found: C, 64·6; H, 5·6; N, 7·8. $C_{19}H_{18}N_2O_5$ requires C, 64·4; H, 5·1; N, 7·9 per cent. Treatment of the diol with 2 mole equivs. of benzoyl chloride in pyridine yielded a *gum* which failed to crystallise.

cis-*Piperidine*-3,4-*diol hydrochloride.* A solution of *cis*-1-acetylpiperidine-3,4-diol (79·5 g.) in ethanol (200 ml.) containing concentrated hydrochloric acid (100 ml.) was heated under reflux for 5 hr. and the mixture evaporated to dryness at reduced pressure. The *product* (63 g.) had m.p. 224–225° (from methanol-ether). Found: C, 39·1; H, 7·8; Cl, 23·5; N, 9·2. $C_5H_{12}ClNO_2$ requires C, 39·1; H, 7·9; Cl, 23·1; N, 9·1 per cent. A solution of the hydrochloride (7·7 g.) in water (40 ml.) was stirred and treated successively with 40 per cent caustic soda solution (10 ml.) and benzoyl chloride (7 g.). After 4 hr. the product was isolated with chloroform to yield *cis*-1-benzoylpiperidine-3,4-diol, m.p. 150–151°, identical with the compound described earlier.

cis-1-*Carbamoylpiperidine*-3,4-*diol*, obtained in 70 per-cent yield, had m.p. 176° (from methanol). Found: C, 44·6; H, 7·8; N, 17·9. $C_6H_{12}N_2O_3$ requires C, 45·0; H, 7·6; N, 17·5 per cent.

cis-1-(2-*p-Acetamidophenoxyethyl*)*piperidine*-3,4-*diol*, was prepared by reaction of *cis*-piperidine-3,4-diol hydrochloride (6·14 g.) with 2-*p*-acetamidophenoxyethyl bromide (10 g.) in ethanol (50 ml.) containing sodium carbonate (4·7 g.) at reflux temperature for 8 hr. It (8·6 g.) had m.p. 139–140° [from ethanol-light petroleum (b.p. 60–80°)]. Found: C, 61·5; H, 7·5; N, 9·6. $C_{15}H_{22}N_2O_4$ requires C, 61·2; H, 7·5; N, 9·5 per cent.

cis-1-2-*Hydroxy*-3-*phenoxypropyl*)*piperidine*-3,4-*diol.* A mixture of *cis*-piperidine-3,4-diol hydrochloride (15·36 g.) and 1,2-epoxy-3-phenoxypropane (15 g.) in methanol (25 ml.) was treated with a solution of potassium hydroxide (5·8 g.) in methanol (25 ml.) and the mixture heated under reflux for 30 min. It was then concentrated, diluted with water and extracted with chloroform. The extract was washed with salt solution, concentrated, and diluted with light petroleum (b.p. 60–80°) to yield the *product* (19·3 g.), m.p. 123–124° (from ethanol). Found: C, 63·0; H, 7·8; N, 5·1. $C_{14}H_{21}NO_4$ requires C, 62·9; H, 7·9; N, 5·2 per cent.

trans-1-*Benzylpiperidine*-3,4-*diol* N-*oxide hydrochloride.* 1-Benzyl-1,2,3,6-tetrahydropyridine (31·8 g.) was added to a solution of peracetic acid prepared from 30 per cent hydrogen peroxide (46 ml.) and acetic acid (150 ml.) and the mixture heated at 75–80° for 5 hr., when it was concentrated to about one fifth of its volume at reduced pressure. The viscous residue was neutralised with concentrated aqueous sodium carbonate, saturated with salt and extracted with chloroform. The extracts were concentrated, the residue dissolved in ethanol (50 ml.) and treated with a slight excess of hydrogen chloride. Dilution with ether furnished two products, (A) 8 g. m.p. 152–154° (decomp.) and (B) 1·5 g., m.p. 140–142°. Repeated crystallisation from ethanol-ether yielded *isomer* (A), m.p. 158° (decomp.) Found: C, 55·4; H, 6·8; N, 5·2 and *isomer* (B), m.p. 168–170°. Found: C, 55·3; H, 6·8; N, 5·6. $C_{12}H_{18}ClNO_3$ requires C, 55·5; H, 7·0; N, 5·4 per cent. Mixtures of the two isomers melted at 140–146°.

cis-1-*Benzylpiperidine*-3,4-*diol.* (*a*) A solution of 1-benzyl-1,2,3,6-tetrahydropyridine (34·6 g.) in water (400 ml.) was brought to pH 7 by addition of concentrated sulphuric acid (6·5 ml.). The solution was stirred and treated with sodium chlorate (34 g.), followed by osmium tetroxide (0·2 g.), and the mixture left at 30–35° for 2 hr. and then heated to 75° for 10 min. It was cooled, extracted with benzene and the aqueous fraction saturated with solid sodium carbonate. The resultant oil was isolated with chloroform and distilled at reduced pressure to yield unchanged material (14·3 g.), b.p. 75–80° at 0·5 mm., along with *product* (21·1 g.) b.p. 150–155° at 0·5 mm. The latter had m.p. 98–100° [from ethyl acetate-light petroleum (b.p. 60–80°)]. Found: C, 69·7; H, 8·2; N, 6·6. $C_{12}H_{17}NO_2$ requires C, 69·5; H, 8·3; N, 6·8 per cent.

(*b*) A mixture of *cis*-piperidine-3,4-diol hydrochloride (30·72 g.), benzyl chloride (25·3 g.) and sodium hydroxide (20 g.) in water (110 ml.) was stirred at 35° for 6 hr. The *product* (31·5 g.) isolated with chloroform had b.p. 150–153° at 0·5 mm. and m.p. 98–100° [from ethyl acetate-light petroleum (b.p. 60–80°)].

cis-1-*Benzylpiperidine*-3,4-*diol* N-*oxide hydrochloride.* A solution of the foregoing diol (10·35 g.) in acetone (50 ml.) was treated with 30 per cent hydrogen peroxide solution (8·5 ml.) and kept at 20–25° for 4 days, when the mixture was evaporated to dryness at reduced pressure. The residual gum, treated with ethanolic hydrogen chloride, yielded the product (7·2 g.), m.p. 145–146° (from ethanol-ethyl acetate). Found: C, 55·7; H, 7·3; Cl, 13·6; N, 5·6. $C_{12}H_{18}ClNO_3$ requires C, 55·5; H, 7·0; Cl, 13·7; N, 5·4 per cent. The compound decomposed slowly on storage.

cis-1-*Benzyl*-3,4-*dihydroxy*-1-*methylpiperidinium chloride.* A mixture of *cis*-1-benzylpiperidine-3,4-diol (20·7 g.) and methyl iodide (17 g.) in ethanol (60 ml.) was heated under reflux for 1 hr., when dilution with ethyl acetate furnished the *methiodides* (30 g.). Conversion into the *methochlorides* yielded two readily separable isomers, the less-soluble *isomer* (A) (5·1 g.) had m.p. 239–240° (from ethanol). Found: C, 60·6; H, 8·3; Cl, 13·8; N, 5·3. $C_{13}H_{20}ClNO_2$ requires C, 60·5; H, 7·8; Cl, 13·8; N, 5·4 per cent. The more-soluble *isomer* (B) (4·9 g.) had m.p. 184–186° (from ethanol-ethyl acetate). Found: C, 60·5; H, 8·0; Cl, 13·8; N, 5·5 per cent.

1,2,3,6-*Tetrahydro*-4-*phenyl*-1-*toluene*-p-*sulphonylpyridine* was obtained in 90 per cent yield by reaction of 1,2,3,6-tetrahydro-4-phenylpyridine with toluene-*p*-sulphonyl chloride in pyridine. It had m.p. 203–205° (from benzene). Found: C, 68·7; H, 5·8; N, 4·3; S, 10·5. $C_{18}H_{19}NO_2S$ requires C, 69·0; H, 6·1; N, 4·5; S, 10·2 per cent.

cis-4-*Phenyl*-1-*toluene*-p-*sulphonylpiperidine*-3,4-*diol.* A suspension of the foregoing tetrahydropyridine (10·45 g.) in ethanol (300 ml.) was treated with a solution of sodium chlorate (5·33 g.) in water (100 ml.). Aqueous osmium tetroxide (20 ml., 1 per cent w/v) was then added and the mixture stirred at 60–65° for 4 hr. The insoluble solid (8·6 g.; unchanged material) was filtered off, the filtrate concentrated to remove ethanol and extracted with four 50 ml. portions of chloroform. Concentration of the chloroform extract furnished the *product* (2·0 g.) m.p.

168–169° [from ethyl acetate-light petroleum (b.p. 60–80°)]. Found: C, 62·1; H, 6·1; N, 4·2; S, 9·5. $C_{18}H_{21}NO_4S$ requires C, 62·2; H, 6·1; N, 4·0; S, 9·2 per cent.

trans-4-*Phenyl*-1-*toluene*-p-*sulphonylpiperidine*-3,4-*diol.* A suspension of 1,2,3,6-tetrahydro-4-phenyl-1-toluene-*p*-sulphonylpyridine (6·26 g.) in acetic acid (40 ml.) was added to a solution of peracetic acid prepared from acetic acid (15 ml.) and 30 per cent hydrogen peroxide (5 ml.) and the mixture (homogeneous after 1 hr.) heated at 90–95° for 2 hr. The solution was diluted with water, the viscous material isolated with chloroform, the chloroform distilled off and the residual gum refluxed in ethanol (50 ml.) containing sodium carbonate (2 g.) for 1 hr. The ethanolic suspension was filtered, concentrated and diluted with light petroleum (b.p. 60–80°). The crystalline material which separated (1·0 g.) had m.p. 196–198° (from aqueous methanol). Found: C, 62·4; H, 5·9; N, 4·2; S, 9·1. $C_{18}H_{21}NO_4S$ requires C, 62·2; H, 6·1; N, 4·0; S, 9·2 per cent. The mother-liquors from the crystallisation were concentrated, the residual gum dissolved in ethanol (30 ml.) containing concentrated hydrochloric acid (5 drops) and the solution heated under reflux for 30 min. After removal of the ethanol, the product was isolated with chloroform. It (0·7 g.) had m.p. 168–169° [from ethyl acetate-light petroleum (b.p. 60–80°)]. The m.p. was not depressed on admixture with the *cis*-isomer described earlier.

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LETTERS TO THE EDITOR

Ectopic Ventricular Arrhythmia after Coronary Occlusion in the Indian Domestic Pig

SIR,—Sudden marked narrowing or a complete ligation of the anterior descending branch of the coronary artery in the pig is reported to result in 100 per cent mortality within 15 min. (Blumgart, Zoll, Freedberg and Gilligan, 1950). The only purposeful effort made on the genesis of arrhythmia from coronary occlusion in the pig was that of Winbury, Lorraine, Nicholas and Zitowitz (1960). Their preliminary pilot study on "miniature pigs" did not favour even a remote possibility of using the pig for such studies, because no ectopic ventricular rhythm was demonstrable; but the myocardium was sensitised to the ectopic provoking action of adrenaline. In a searching analysis of the problem of coronary artery disease, Sir H. Florey (1960) in his Jephcott lecture, declared that it might not be going too far to believe that the same factors were operating to produce the lesion in the pig as the one present in man, and that it may now be possible to elaborate observations made on man in a more comprehensive manner on animals. A reappraisal of the feasibility of using the domestic pig was, therefore, deemed pertinent. The present study set out to examine the possibility of developing the animal for use in a study of ectopic ventricular arrhythmia by a 2-stage coronary artery ligation. To our knowledge this is the first time that successful experimental induction of a delayed development of ectopic ventricular arrhythmia in the pig has been reported.

Twenty domestic pigs, raised and maintained in our laboratory, aged 6 to 8 months and weighing 15–25 kg., were chosen for the experiment. The two-stage ligation technique of Harris (1950) was adapted to occlude aseptically the anterior descending branch of the left coronary artery; the size of the probe over which the first ligature was tied was of 0·45 mm. diameter. Intravenous pentobarbitone sodium (35 mg./kg.) was used for general anaesthesia. No parenteral antibiotics were used; neomycin-bacitracin-sulphacetamide powder was dusted on before closure of the chest in layers.

The site of the ligature at which the ectopic ventricular arrhythmia developed in the pig is shown below to be much lower down on the artery than in the dog.

Group	No. of pigs used	Distance from the ostium of the Lt. coronary artery	Result
I	5	1·6–2·0 cm.	Immediate fatal ventricular fibrillation
II	5	2·1–2·5 cm.	Reversible delayed ventricular fibrillation
III	5	2·6–3·0 cm.	Delayed ectopic ventricular arrhythmia
IV	5	3·1–4·0 cm.	A few ectopic beats

The incidence of the development of an immediately fatal ventricular fibrillation was directly proportional to the proximity of the ligature to the origin of the left coronary artery.

After recovery from the anaesthesia, ventricular ectopic arrhythmia developed gradually in 2–3 hr. after a complete occlusion in Group III, as recorded on a 4-channel ink-writing electrocardiograph. Intense ectopic activity was maintained from about 6 to 28 hr., after which it was observed to be interpolated more and more frequently with a sinus rhythm. The normal sinus rhythm was almost always evident by about 48 hr. after ligature. The latent period of

development of the ventricular ectopic rhythm with tachycardia was unrelated to the arousal of the animal from anaesthesia.

While the spontaneous ventricular arrhythmia gradually disappeared over a period of 3 days, the exaggerated ectopic responses to noradrenaline persisted for about 7 days as shown in Fig. 1.

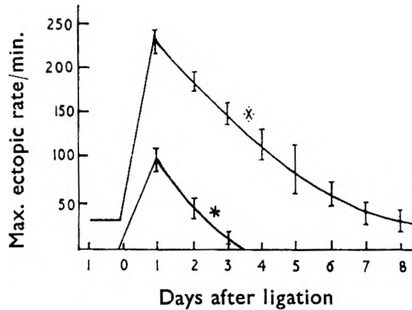

FIG. 1. Ectopic ventricular arrhythmia after coronary occlusion. Upper curve after noradrenaline, 8 μg./kg. i.v. Lower curve—spontaneous.

The 5 pigs developing the delayed ectopic ventricular arrhythmia in Group III survived, and were killed for histological study of the hearts. In the hearts of these animals, a month after ligation, the corresponding areas of the left ventricle were observed to have undergone a massive necrosis leaving a fibrotic thinned out part of the ventricular wall.

Acknowledgement. Part of this work was financed by the Indian Council of Medical Research, New Delhi-16.

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February 19, 1962

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Observations on Conditioned and Unconditioned on- and off- Behavioural Responses to a Buzzer

SIR,—Responses occurring at the initiation of an afferent stimulation are called on-responses, and those occurring at its cessation, off-responses. Conditioned on- and off- responses have been studied at the behavioural level by Galeano, Roig, Segundo and Sommer-Smith (1959), Sommer-Smith, Galeano, Piñeyrúa-Payssé, Roig and Segundo (1962), Izquierdo and Östman (1961a,b) and Izquierdo (1962a). Further observations on the subject are added here.

The experiments were made on 17 adult albino rats. The noise of a buzzer acting during randomly variable periods separated by randomly variable silent

intervals, was the first indifferent, later conditioned stimulation. After a period of habituation, a shock to a metallic grid on the floor of the training box (square 0·1 m-sec./100 V pulses, 150/sec. during 0·5 to 2 sec.) was delivered 2–4 sec. after cessation of the buzzer as an absolute stimulus. After a number of such reinforcements, buzzes were again presented alone to extinguish the conditioned reflexes developed. Experiments were made in acute conditions (a single session lasting 60–130 min.; 10 buzzes for control; 50–80, reinforced with the shock; 10–30 for extinction) or in chronic conditions (daily sessions over a period of 6–14 weeks, at 10 or 20 buzzes per session; 100–300 for habituation; 200–500 reinforced; 50–300 for extinction).

Unconditioned on-responses to the buzzer were of three kinds. (i) Startle-defensive movements; (ii) investigative, orienting ones, and (iii) arrest, attention. Habituation was always difficult to achieve. In one experiment, after 1,000 buzzes in a chronic animal, no habituation occurred for any of these three kinds of responses. Group (i) reflexes were by far the most numerous; the defensive component (retraction, crouching) always habituated quickly but the startle seldom did so. Unconditioned off-responses to the buzzer were mostly group (iii); other off-responses were occasional and their habituation was extremely slow.

In chronic-treated rats, after the first few pairings of stimuli, dishabituation invariably occurred which lasted throughout the rest of the training schedule. Abortive, ill-defined, irregular and diverse on- and off-conditioned responses occurred at this early stage. Soon after, a clear-cut on-conditioned reflex occurred. When reinforcements were continued, however (2–10 sessions), only an off-conditioned reflex remained, and all other conditioned phenomena disappeared.

In acute-treated rats, the fate of unconditioned on- and off-responses was not studied in detail. Two kinds of conditioned reflexes appeared: an on- and an off- one (Izquierdo and Östman, 1961a).

Conditioned responses usually consisted of one or more of the following: squealing, running about, or standing up. These responses were qualitatively related but never equal to the absolute response to shock: shouting, running, tonic thrust, clonic movements. The percentage of conditioned responses never exceeding 60–80 in chronic and 80–90 in successive tenths in acute rat experiments.

Criteria for considering a particular response as conditioned were the following: (i) appearance during the reinforcement stage and not before; (ii) qualitative relationship to the absolute response; (iii) susceptibility to external inhibition and disinhibition; (iv) appearance after the conditioned signals; (v) internal inhibition by extinction. Of course, in the abortive early responses, these criteria were seldom completely considered.

Ten to 20 min. after the intraperitoneal injection of chlorpromazine (2–2·5 mg./kg.), both on- and off-conditioned reflexes were inhibited in 7 of 8 acute rats. Six of the chronic animals received 2 mg./kg. once and 2·5 mg./kg. twice, at intervals of four days between injections. In the 18 trials, the off-conditioned reflex was inhibited only twice. Difference between both groups was highly significant (P>, 0·005).

In a discussion of these data, it should be considered that both conditioned signals (on and off) reach the auditory cortex by a double route: one, via the classic primary sensory pathway; the other, via the reticular formation and its connections of the non-specific subcortical system. When employed at doses comparable to those used by us, chlorpromazine selectively blocks the collateral afferent inflow into the reticular formation (Bradley, 1959). As such blockade

affects early or acute conditioned reflexes but not chronic ones, it could reasonably be supposed that some sort of a "memory" mechanism develops by continued training at the acoustic cortex, which enables it to emit a conditioned message towards the motor area without receiving the reticular information; this, on the other hand, would be necessary in acute circumstances (Izquierdo, 1962b).

The existence of both an on- and an off-conditioned reflex in acute animals, and the final persistence of only an off- reflex in chronic ones, suggests that the acute experiments are equivalent to an early stage of the chronic one.

Acknowledgement. The valuable technical assistance of Mr. R. Östman is appreciated.

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Peroxide Value of Anhydrous Lanolin

SIR,—Anderson and Wood (1962) deduced that short periods of heating at 100° were sufficient to provide lanolin of low peroxide value for special purposes. We have found longer periods to be necessary for refined Wool Fat B.P., with concomitant increases in acid value and colour which make the method of little practical value. Another disadvantage is that the removal of peroxides

TABLE I

CHANGES IN WOOL FAT B.P. HEATED AT 105°

Heating time hr.	Sample A				Sample B + 0·05 per cent BHA			
	Peroxide value (ml. 0·002N per g.)	Acid value	Colour (Lovibond)		Peroxide value	Acid value	Colour (Lovibond)	
			Yellow	Red			Yellow	Red
0	57·5	0·78	4·3	0·4	63·7	0·84	4·1	0·4
3	48·1	0·82	4·4	0·4				
4					46·5	0·88	4·5	0·5
6	24·6	0·86	4·9	0·5				
10·5					13·4	0·98	5·6	0·7
13	8·4	0·90	6·4	0·7				
17	5·7	0·92	7·2	0·8				
17·5					4·9	0·98	6·6	0·7
22·5					4·0	1·00	7·7	0·8
25	4·2	0·94	7·7	0·8				
30	5·1	0·96	8·3	0·9				
30·5					2·8	1·04	8·8	0·9
36	4·4	1·00	9·1	0·9				
37·5					2·9	1·06	9·9	1·0
44	3·8	1·04	10·0	1·0				
47					4·5	1·12	10·7	1·1

by heating alone does not eliminate the permanganate-decolorising substances present in oxidised lanolin (Clark and Kitchen, 1961b). Table I shows typical results obtained with two different samples of Wool Fat B.P. in open glass beakers heated at 105°, one sample containing butylated hydroxyanisole as antioxidant.

To achieve a peroxide value of less than 5, heating for approximately 20 hr. without, and 17 hr. with an antioxidant was needed and increased both colour and acid value. About 12 hr. were needed to give values less than 10.

Other tests made at 55·5° showed that the destruction of peroxides was accelerated by introducing a strip of metal into each sample (Table II), the effects of

TABLE II

PEROXIDE VALUES OF WOOL FAT B.P. HEATED AT 55·5° IN CONTACT WITH METAL

Heating time (days)	Type of metal strip inserted in sample					
	None	Aluminium	Brass	Copper	Galvanised iron	Mild steel
Sample A						
0	50·0	50·0	50·0	50·0	50·0	—
7	50·0	48·5	31·7	37·0	43·5	—
14	38·5	38·0	3·9	7·9	27·3	—
21	37·6	37·5	4·0	5·0	24·5	—
28	33·0	37·5	4·0	3·6	19·8	—
Sample B						
0	72·2	—	72·2	72·2	—	72·2
7	63·7	—	60·6	58·1	—	59·7
11	63·2	—	52·3	54·6	—	46·7
14	53·7	—	41·9	30·7	—	34·1
18	46·9	—	29·9	19·0	—	33·6
25	46·2	—	4·0	4·1	—	27·4
33	43·5	—	3·8	4·3	—	19·5

iron and copper confirming the findings of Janecke and Senft (1957). Colours of the samples measured during the tests showed similar darkening to those reported in Table I.

The rates of fall in peroxide value shown in Table I are low compared to our earlier results on wool-scouring liquor (Clark and Kitchen, 1961a), which showed that even when standing at room temperature the peroxide value of wax in wool-scouring liquor fell by 3·5 in 24 hr. Such a fall does not occur after the wax has been removed from the liquor, and these facts support the suggestion that the peroxides in the liquor are largely chemically or biologically reduced. A catalase-like action may be involved although heavy metal impurities derived from the wool (Janecke and Senft, 1957) could be implicated in a rapid breakdown of peroxides, perhaps accelerated by the finely-emulsified state of the liquor. Relatively high contents of trace-metals might account for the different findings of Anderson and Wood, especially in the wool wax which they solvent-extracted from the fleece.

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Cardiotonic Action of Hamycin

SIR,—A new antifungal antibiotic, hamycin, was recently prepared by Hindustan Antibiotics Ltd. A pharmacological investigation of its properties shows it to be a potent cardiotonic agent.

In 12 experiments on perfused frog hearts, failure was induced by raising venous pressure in steps of 1 cm. (Burn, 1952). Cardiac output was simultaneously recorded through a cannula in the aorta. The drug was then perfused in a concentration of 2×10^{-7} g./ml. of amphibian Ringer-Locke solution. A marked increase in cardiac output as well as a marked increase in the amplitude of contraction was noted. This was followed by a gradual increase in diastolic tone and a decrease in cardiac output terminating in systolic arrest of the ventricles. A concentration of 10 ml. of tincture of digitalis per litre of amphibian Ringer-Locke solution produced similar effects, both qualitatively as well as in time course.

In another 6 experiments on perfused frog hearts failure was induced with pentobarbitone sodium, 0·1 mg./ml. of amphibian Ringer-Locke solution. After 10 min. of perfusion with pentobarbitone sodium, a concentration of 2×10^{-7} g./ml. of hamycin along with 0·1 mg./ml. of pentobarbitone sodium was perfused. This produced a marked increase in the amplitude of contraction, terminating finally in systolic arrest. The effect was comparable qualitatively and in time course with a concentration of 10 ml. of tincture of digitalis per litre of amphibian Ringer-Locke solution.

In perfused rabbit hearts (Burn, 1952) (Langendorff's preparation), a concentration of hamycin 2×10^{-7} g./ml. of mammalian Ringer-Locke solution increased the amplitude of contraction in 7 out of 8 experiments. In another 7 experiments on perfused rabbit heart, failure was induced by perfusing pentobarbitone sodium, 0·1 mg./ml. of Ringer-Locke solution. After 10 min. of perfusion with pentobarbitone sodium, Ringer-Locke solution containing hamycin, 2×10^{-7} g./ml. and pentobarbitone sodium 0·1 mg./ml. was perfused. In 4 of these experiments an increase in the amplitude of contraction occurred; in 2 experiments the ventricles rapidly passed into systolic arrest while in one experiment there was a decrease in the amplitude of contraction. In 4 control experiments with tincture of digitalis, 10 ml./litre of Ringer-Locke solution, a similar increase in amplitude of contraction was noted in 2 while in the other 2 experiments only systolic arrest was noted. Control experiments with propylene glycol, which was used as a solvent for hamycin failed to exhibit any of the actions of hamycin.

This compound thus appears to be more potent than digitalis and offers new avenues in the search for cardiotonic drugs. Further studies are in progress.

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The Stability of Aqueous Solutions of Picrotoxin to Light

SIR,—It is generally believed that picrotoxin and solutions of picrotoxin are affected by light, but during an examination of some of the physico-chemical properties of substances which possess analeptic activity, it was of interest to observe that aqueous solutions of picrotoxin do not exhibit significant ultra-violet light absorption above 200 mμ. In the monograph on "Picrotoxin Injection" in the 1958 British Pharmacopoeia, and the International Pharmacopoeia 1951 it is stated that "Picrotoxin Injection should be protected from light"; similar statements may be found in *Remington's Practice of Pharmacy* Martin and Cook, 1956 and the *United States Dispensatory* (1955).

After exposing aqueous solutions of picrotoxin to intense tungsten light and to ultra-violet light we have failed to find any evidence to support this belief.

0·1 ml. of different solutions of picrotoxin were injected into the tail vein of unfasted, randomly bred, male albino mice weighing 20–25 g. In addition to measuring the time interval between injection and the first stage of convulsions, another yet less obvious response of the mouse to picrotoxin was employed. With small doses ($>$7·5 μg.) the mouse will assume a flaccid posture in which it lies full length with its head lowered and stretched out onto the front paws; the hind legs are not properly co-ordinated and are often placed awkwardly in relation to the body. This posture will be described by us more fully elsewhere. There is a linear relationship between the logarithm of the dose and the response time for both "end points".

Solutions of picrotoxin prepared in distilled water (25 mg. and 200 mg./100 ml.) were exposed to intense tungsten light in acid-washed pyrex glassware or to ultra-violet light (254 and 366 mμ) in silica cells, for varying time intervals.

The results of a typical experiment are illustrated in Table I; there was no significant difference in the potency between solutions kept in the dark (controls)

TABLE I

Dose of picrotoxin μg. injected in 0·1 ml.	Flaccid mouse posture (time, sec.)		Convulsion time (sec.)		P
	Controls ± S.E.	Experimental ± S.E.	Controls ± S.E.	Experimental ± S.E.	
	Tungsten light				
	(a) 24 hr.				
25	86 ± 2·63	96 ± 5·30	—	—	N.S.
200	—	—	45 ± 1·64	51 ± 7·23	N.S.
	(b) 96 hr.				
25	92 ± 3·53	98 ± 6·49	—	—	N.S.
200	—	—	49 ± 4·10	54 ± 3·72	N.S.
	Ultra-violet light (254 and 366 mμ)				
	(a) 24 hr.				
25	100 ± 3·76	98 ± 5·33	—	—	N.S.
200	—	—	61 ± 3·56	66 ± 5·14	N.S.
	(b) 48 hr.				
25	100 ± 3·76	101 ± 3·39	—	—	N.S.
200	—	—	68 ± 4·42	64 ± 3·64	N.S.

N.S. indicates value for P not less than 0·1. Between 7 and 10 animals per group were used.

and those exposed to light. Since we have failed to detect a decrease in potency of picrotoxin solutions under these conditions, we would like to suggest an alternative explanation for changes in strength on storage. This is that solutions which are stored in glassware which has not been acid-washed may become alkaline. As shown by Bryan and Marshall (1948) and confirmed by our

unpublished results, the potency of picrotoxin solutions diminishes with increase in alkalinity above pH 7.

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BOOK REVIEWS

MEDICAL PHARMACOLOGY. Principles and Concepts. By Andres Goth. Pp. 551 (including Index). Henry Kimpton, London, 1961. 82s. 6d.

This book is written primarily for medical students and practitioners. Its aim is to present the current pharmacological knowledge with particular reference to principles and concepts and not to include all drugs related to each important compound used in medicine. This means that many chapters are short (e.g. antihistaminic drugs, 6 pages) but nevertheless they are concise and do not worry the practitioner with a mass of chemical formulae. The elements of pharmacology are to my mind clearly set out and I enjoyed reading the volume which is well printed and strongly bound. At the end of each chapter, a short set of important references directs the reader to the original studies. The main sections are devoted to General Aspects of Pharmacology, Drug Effects on the Nervous System and Neuroeffectors, Psychopharmacology, Depressants and Stimulants of the Central Nervous System, Anaesthetics, Drugs used in Cardiovascular Disease, Drug Effects on the Gastrointestinal Tract, Drugs Influencing Metabolic and Endocrine Functions, Chemotherapy, Poisons and Antidotes, and Prescription Writing. There are few mistakes (the formula for Regitine on page 113 is incorrect), but some chapter titles are open to objection, e.g. Adrenergic drugs, Cholinergic drugs and Nonnarcotic analgesic drugs, besides a section on Antidiarrheal agents. The book may be thoroughly recommended to those who are not particularly keen in finding out the mechanism of action of drugs, though its high price is a great drawback. G. B. WEST.

PHARMACOGNOSY. 4th Edition. By Edward P. Claus. Pp. 565 (including 227 illustrations, 1 plate in colour, and Index). Henry Kimpton, London, 1961. 93s. 6d.

The author states that the term Pharmacognosy literally means (p. 9) a "cognizance of pharmaceuticals" and (p. 10) defines it as "an applied science which deals with the biological, biochemical and economic features of natural

drugs and their constituents". He goes on to say "modern aspects of the science include not only the crude drugs, but also their natural derivatives" and then explains that the glycoside digoxin, the alkaloid reserpine, the hormone thyroxin and other derivatives of crude drugs are "all part of the subject matter of pharmacognosy". He also includes (p. 11) "economic substances affecting the health of man and other animals" such as "allergens, allergenic extracts, antibiotics, immunising biologicals, flavouring agents, condiments, beverages, insecticides, rodenticides, and herbicides".

A general introduction deals in an interesting way with commerce and the relation of the study to Pharmacopoeias and other official publications. Evaluation is described (p. 23) as identification and determination of quality and purity. A useful discussion is given of drug adulteration and its detection, but little or nothing is said about attack by insects and other pests during storage and their identification. The method of classification adopted is based upon the chemical nature of the constituents of the drugs described, giving chapters on Carbohydrates, Glycosides, Tannins, Lipids, Volatile oils, Resins, Alkaloids, Endocrine products, Vitamins, Enzymes, Proteins, Antibiotics, Immunising Biologicals, Allergens, Pesticides. Two appendices are included, comprising (1) A Taxonomic list of Important Drugs, and (2) Powdered Drugs with Key for Identification".

For many of the more important drugs no attempt is made to give a description of the commercial article or of its powder. The matter is avoided by a brief paragraph worded as follows:—"Structure and Powder—See figs. x and y, and the U.S.P. page k. or N.F. page m.". This seems to omit one of the most important functions of a textbook; the author of such a book is expected to lead the way instead of following official writings. Good original descriptions help to guide the Committees concerned with official publications and their members naturally look to standard textbooks for assistance in formulating official requirements.

The book has many good photomicrographs of the crystals of alkaloids and their salts and of other drug constituents, but no indication is given of the magnification of the pictures. There are also good photographs of some of the drug yielding plants, but sometimes the drugs derived from them are not figured, thus two pages, pp. 122 and 123, are given of good photographs of the plants yielding rhubarb, but there is no illustration of the drug itself. The drawings of the anatomical structure of drugs are often semi-diagrammatic and no magnification is given. The drawings of powders leave much to be desired; for example, powder of cascara bark (p. 116) shows stone cells and fibres bearing no resemblance to those of the drug, and the crystal cells accompanying the fibres are at a different magnification. The drawing of a transverse section of a tea leaf on p. 374 is entirely misleading; the drawing is upside down and the two epidermises are incorrectly named, while the mesophyll bears no resemblance to that of the leaf. These matters are of great importance in this book, because verbal descriptions are often omitted and are replaced by reference to the photographs and drawings.

In the section concerned with glycosides the cyanophoric glycoside linamarin (p. 182) is not named amongst the constituents of linseed although this constituent has been responsible for poisoning of cattle. When antibiotics are being described, no description or drawings are given of the organisms producing the antibiotics. It is, however, good propaganda to introduce chapters on, for example, antibiotics, and allergens, which come legitimately within the province of pharmacognosy.

The book is well produced and is printed on good paper, which enhances the appearance of the 227 illustrations. The frontispiece is a beautiful coloured photograph of the inflorescence and leaves of *Rauwolfia serpentina* Benth.

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QUANTITATIVE METHODS IN PHARMACOLOGY. Edited by H. De Jonge. Pp. xx + 391. North-Holland Publishing Company, Amsterdam, 1961. 84s.

This volume contains the papers presented and the discussions arising at the Symposium on Quantitative Methods in Pharmacology which was sponsored by the Biometric Society and held at Leyden during May, 1960. It contains some 26 papers on quantitative, statistical methods used in pharmacological testing which are grouped under five main headings, namely, sequential analysis, standardisation of drugs, parametric and non-parametric statistical methods, drug screening and the effects of mixtures of drugs. The scope of the papers is very wide, the treatment of the subject matter is largely, but not entirely mathematical, and deals not only with routine laboratory methods, but also with clinical trials. Most of the contributions deserve separate detailed comment and it is difficult to select individual papers for special attention. Among the papers found more interesting by the reviewer, is one by J. Hajnal on sequential trials of analgesics in rheumatoid arthritis in which the author in the clearest possible style sets out the value (and limits) of sequential analysis in clinical trials. M. A. Schneiderman gives a clear and interesting description of a multistage sequential method for the screening of large numbers of natural and synthetic anti-cancer agents against a combination of the three mouse tumours; sarcoma S-180, mammary carcinoma CA-755 and leukemia L-1210. J. J. Grimshaw and P. F. D'Arcy discuss some of the difficulties met with in the biological assay of suxamethonium and other muscle relaxants, the screening of drugs which potentiate or diminish barbiturate anaesthesia, of compounds with anti-inflammatory activity, analgesics and local anaesthetics. This is a very useful contribution, the more so because it is rare for experimental laboratory workers to publish details of the manner in which they have overcome their practical difficulties—there must be a vast amount of useful information of this kind in innumerable laboratory notebooks and reports. N. Brock and B. Schneider point out the value in the assessment of drugs of using the ratio LD5/CD95 as the therapeutic index. E. J. Ariens and A. M. Simonis make a characteristic contribution on the analysis of drug-receptor interactions, and C. W. Dunnett presents an interesting theory of drug screening applicable to drugs which are either active or inactive and involving a truncated sequential method, which actually takes into account the cost of testing the drugs and of making an incorrect decision.

The book will repay reading and, for a work of its kind, is easy to read. It should find its way on to the shelves of most biologists and statisticians interested in, and there are few who can afford not to be, quantitative methods. It is unfortunately marred by a number of spelling errors and misprints, but these apart, is well produced with good figures and line diagrams.

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