RESEARCH PAPERS

CURARE-LIKE DRUGS AND VAGAL SYNAPSES:
COMPARATIVE STUDY *IN VITRO* ON THE ISOLATED VAGUS-STOMACH PREPARATION OF THE RAT

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The anti-acetylcholine properties of curare-like drugs have been investigated on the motor responses of the electrically stimulated isolated vagus-stomach preparation of the rat. The synaptic site of the inhibitory action shown to varying degrees by the compounds tested has been located by comparing the modifications of the gastric responses to stimulation of preganglionic vagal fibres with the unmodified responses of the rat stomach strip preparation to acetylcholine.

PREVIOUS work on a series of curare-like drugs has uncovered a point of very considerable interest (Della Bella, Rognoni and Gopal, 1961). Much better correlation was found between *in vivo* neuromuscular blocking action than ganglion blocking action and the ability of these drugs to block the vagal ganglia in isolated vagus-heart preparations made from guinea-pigs. We have continued this investigation to determine whether this same correlation holds between *in vivo* neuromuscular blocking action and ability to block vagal ganglia at another site. We have chosen the rat stomach for this purpose and have used the intact stomach with vagi attached, prepared as recently described by Della Bella and Rognoni (1961). A parallel series of observations were made on strips of rat stomach stimulated by the addition of acetylcholine to the organ bath. The curare-like drugs examined were: (+)-tubocurarine, decamethonium, suxamethonium, succinyldisulphocholine, gallamine, hexafluorenium, laudexium and hexacarbacholine.

EXPERIMENTAL

Methods

White rats weighing 200 to 250 g. were used. The vagus-stomach preparation was made according to the method recently developed by Della Bella and Rognoni (1961). The whole stomach, isolated with vagus nerves intact, was suspended in a 100 ml. bath filled with oxygenated Ringer's solution at 30°. The gastric cavity, distended with 8 to 12 ml. of Ringer's fluid, was connected to a recording system consisting of a Marey tambour which, in turn, was connected to an isotonic lever having stops above and below. Thus it was possible to record the suitably amplified pressure variations occurring in the gastric cavity resulting from the muscle contractions caused by electrical stimulation of preganglionic vagal fibres. Stimulation was effected by means of platinum electrodes immersed in the bath. Rectangular pulses of 1 msec. duration

were applied at a frequency of 1–5/sec. in an alternating pattern of 3 min. of stimulation and 3 min. of rest. The solutions of the drugs to be tested were added directly to the organ bath 2 min. before electrical stimulation; the drug was removed by prolonged washing of the preparation.

The stomach strip preparation was made according to the method described by Vane (Vane, 1957): the strip was suspended in a 20 ml. bath kept at a temperature of 32° and connected to an isotonic lever, tension 2 g., with a 20-fold amplification of the contraction. Acetylcholine was used as a stimulant.

RESULTS

Experiments on the isolated vagus-stomach preparation of the rat have confirmed previous results obtained on cardiovagal synapses, showing clearly a marked anti-vagal property of some curare-like drugs. The results of a quantitative evaluation of the extent of inhibition exerted on the gastric motor responses to electrical stimulation are shown in Table I.

TABLE I

RELATIVE INHIBITORY POTENCY OF CURARE-LIKE DRUGS AND OF HEXAMETHONIUM ON THE RESPONSES OF THE ISOLATED VAGUS-STOMACH PREPARATION OF THE RAT TO ELECTRICAL STIMULATION

Drug	ED50 μg./ml.	Equipotent molar ratio (hexamethonium = 1·00)
Hexamethonium iodide	2·4	1
Hexafluorenium bromide	1	0·27
Gallamine iodide	10	2·1
Laudexium methylsulphate	5	0·9
Decamethonium iodide	12	3·9
Tubocurarine chloride	18	4·3
Hexacarbacholine bromide	20	7
Suxamethonium chloride	>500	>26·5
Succinyldisulphocholine iodide	>500	>17

Thus, the vagal blocking action of hexafluorenium appears to be approximately three times that of hexamethonium (Fig. 1). Also laudexium, the ganglion blocking action of which on the isolated guinea-pig intestine stimulated with nicotine was demonstrated by Collier and Macauley (1952), was found slightly more potent than hexamethonium. However, in contrast to observations made on the intestine, where laudexium has one-third to one-fourth the activity of tubocurarine, the relationship is reversed on the vagus-stomach preparation. Indeed laudexium proved approximately four times as potent as tubocurarine and even slightly superior to hexamethonium. Fig. 2 shows also that the inhibitory effect of laudexium is much more rapidly reversible than that of tubocurarine. Decamethonium was found virtually as active as tubocurarine and gallamine had even slightly greater activity. An estimate of the relative potency of the latter drug and hexamethonium is shown in Fig. 3. Hexacarbacholine showed only slight vagal blocking action. Suxamethonium and its disulphonium analogue proved even less active. Concentrations of the last three compounds somewhat lower

than those required markedly to antagonise responses to vagal stimulation, caused the appearance of a direct motor response of the organ, probably related to the known nicotine-like property of the two compounds (Bovet, Bovet-Nitti, Guarino, Longo and Marotta, 1949; Della Bella, Villani
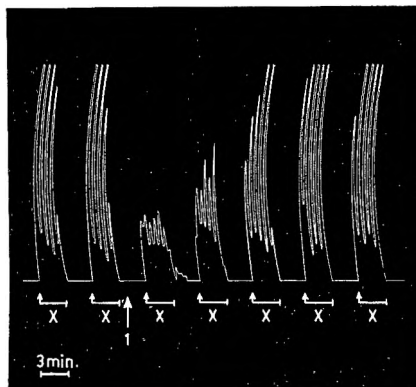

FIG. 1. The gastric motor responses after electrical stimulation of the vagal nervous supply. At X: electrical stimulation for 3 min. (frequency: 5 pulses/sec.; pulse duration: 1 msec.). The preparation was washed at the end of the stimulation period which was repeated at 3 min. intervals. At 1: hexafluorenium (1·5 μg./ml.) 2 min. before electrical stimulation.

and Zuanazzi, 1956); sometimes appreciable enhancement of the responses to electrical stimulation was also observed. Fig. 4 indicates one of the experiments effected with suxamethonium. In further experiments conducted on strips of rat stomach stimulated by acetylcholine, we investigated the influence exerted by the drugs tested on the effector structures.
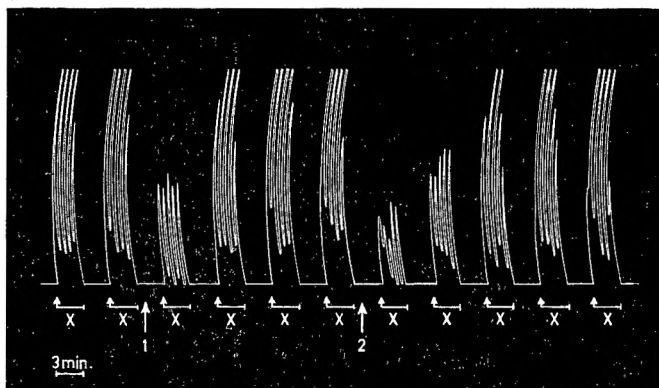

FIG. 2. The gastric motor responses after electrical stimulation of the vagal nervous supply. At X: electrical stimulation for 3 min. (frequency: 5 pulses/sec.; pulse duration: 1 msec.). The preparation was washed at the end of the stimulation period which was repeated at 3 min. intervals. Modification of the responses due to laudexium (5 μg./ml.) (at 1) and to tubocurarine (20 μg./ml.) (at 2) added to the bath 2 min. before electrical stimulation.

Since it is well known that curare-like drugs do not interfere with the liberation of the cholinergic mediator, the results of the above experiments should enable us to locate their site of action, in inhibiting the gastric motor responses to electrical stimulation of the preganglionic vagal fibres.
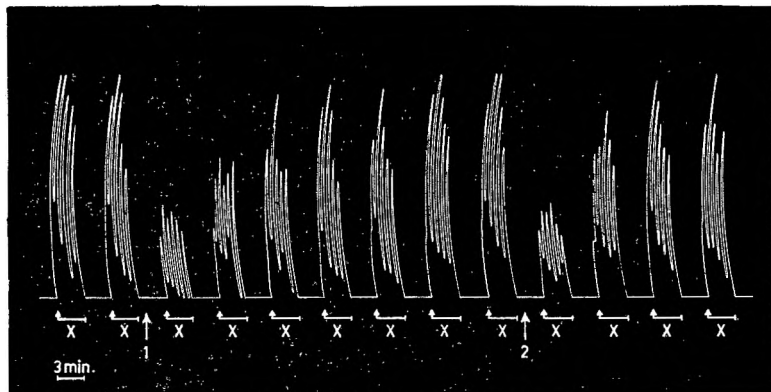

FIG. 3. The gastric motor responses after electrical stimulation of the vagal nervous supply. At X: electrical stimulation for 3 min. (frequency: 5 pulses/sec.; pulse duration: 1 msec.). The preparation was washed at the end of the stimulation period which was repeated at 3 min. intervals. Modifications of the responses due to hexamethonium (2·5 μg./ml.) (at 1) and to gallamine (10 μg./ml.) (at 2) added to the bath 2 min. before electrical stimulation.

It is of interest to describe the behaviour of stomach strips to various drugs: while no direct stimulant action was observed for tubocurarine and gallamine, this was particularly evident for decamethonium, hexafluorenium and for suxamethonium; the stimulation caused by laudexium and hexacarbacholine was of no constant pattern.
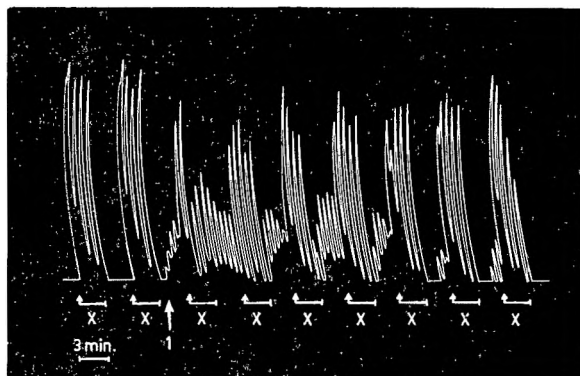

FIG. 4. The gastric motor responses after electrical stimulation of the vagal nervous supply. At X: electrical stimulation for 3 min. (frequency: 5 pulses/sec.; pulse duration: 1 msec.). The preparation was washed at the end of the stimulation period which was repeated at 3 min. intervals. Nicotine-like effect and modifications of the responses due to suxamethonium (0·35 mg./ml.) (at 1) added to the bath 2 min. before electrical stimulation.

As shown in Figs. 5 and 6, none of the compounds examined caused reduction of the gastric motor responses to the stimulation by acetylcholine.

Moreover, while decamethonium did not interfere with responses of the organ to acetylcholine, these were found markedly increased after a pretreatment with hexafluorenium. This sensitisation to acetylcholine might be attributed to the anticholinesterase activity shown by hexafluorenium both *in vitro* and *in vivo* (Rizzi, 1957; Della Bella, Rognoni and Gopal, 1961).
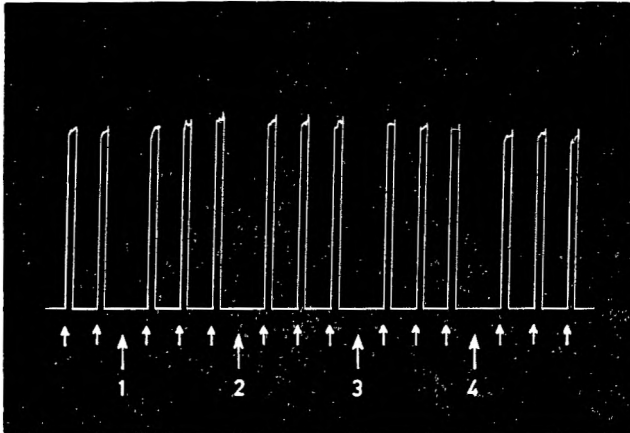

FIG. 5. Rat stomach strip preparation. At the arrows: the preparation is stimulated by adding to the bath acetylcholine of 0·05 μg./ml. (after 30 sec. contact, prolonged washing out). At 1: tubocurarine (25 μg./ml.) added to the bath, 1 min. before acetylcholine. At 2: gallamine (20 μg./ml.) added 1 min. before acetylcholine. At 3: laudexium (5 μg./ml.) added 1 min. before acetylcholine. At 4: hexacarba-choline (15 μg./ml.) added 1 min. before acetylcholine.
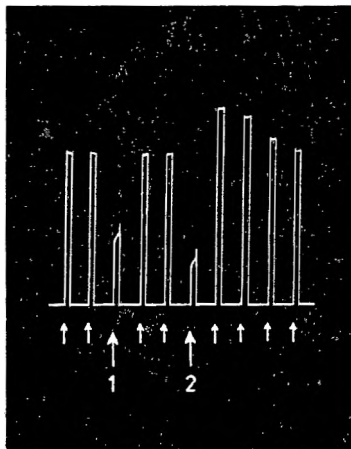

FIG. 6. Rat stomach strip preparation. At the arrows: the preparation is stimu-lated by adding to the bath acetylcholine (0·05 μg./ml.) (after 30 sec. contact, pro-longed washing out). At 1 and 2: effect of direct stimulation by decamethonium (10 μg./ml.) (1) and by hexafluorenium (5 μg./ml.) (2) (after 30 sec. contact, prolonged washing out).

Discussion

The results described above clearly show the ability of the curare-like drugs tested to antagonise cholinergic transmission in vagal ganglia. The inhibitory effects produced by the compounds on the electrically driven stomach preparation were similar to those obtained at the cardiovagat synapses and, as observed in this latter case, bear little or no relation to their ganglion blocking activity *in vivo* (Della Bella, Rognoni and Gopal, 1961). The ganglionic site of action of the effects studied was evidenced, indirectly, by the experiments showing that the responses of the stomach strip to acetylcholine treatment were not modified by the same compounds.

Now, although both experimental and clinical evidence of the influence of curare-like drugs on gastrointestinal motility is scanty and does not cover all the compounds examined: tubocurarine (Gross and Cullen, 1945), gallamine (Riker and Wescoe, 1951), laudexium (Collier and Macauley, 1952), nevertheless, the existence of a marked behavioural difference between the activity displayed *in vivo* and that *in vitro* cannot be doubted. As previously reported (Della Bella, Rognoni and Gopal, 1961) it is not possible at the present moment to give an adequate explanation for these differences, but a working hypothesis would appear the one advanced by Cavallito, suggesting that the selectivity of action exhibited by the above drugs might be due to their different physicochemical properties. On these properties, in fact, depend both the distribution of the drugs in the body and the possibility and ease of their reaching various sites of action (Cavallito and Gray, 1960).

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THE RETENTION OF AQUEOUS SUSPENSIONS ON LEAF SURFACES

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A method has been devised, using lycopodium, for studying the contribution of leaf surface characteristics to the wetting of leaves and the retention of particles applied as suspensions. Plants have been classified according to wettability, in relation to particle distribution and surface roughness. The recession of liquid on leaves of *Rumex obtusifolius* was studied in detail and the contributing factors analysed.

PREVIOUSLY, leaf surfaces have been classified as waxy (unwettable), or non-waxy (wettable) according to the behaviour of liquid, applied as agricultural sprays. No attempts at further classification have been made despite the considerable variation in surface characteristics of leaves. It is probable that the degree of wetting varies with the type of surface and it seemed desirable that a further classification of leaves should be attempted. Visual studies of spray particles on leaf surfaces have been made by Martin (1952, 1960) using replica techniques, but such methods suffer from the disadvantage that complete removal of particles by the replica is uncertain and the quality of replicas varies. Fluorescent tracer techniques have been used by Staniland (1960) but a range of fluorescent tracers is essential to give sufficient choice of chemicals which contrast with the natural fluorescent background.

A more suitable technique was sought, which could be used to study the detailed microscopic roughness of leaf surfaces and at the same time the deposition of standard particles. Lycopodium spores proved suitable as standard particles and were examined by direct microscopy, together with leaf surfaces, using illumination specially designed for opaque surfaces.

MATERIALS AND METHODS

Smooth Surfaces

To test the suitability of lycopodium, the deposition of spores from a suspension was first studied on glass slides in the presence and absence of a surface-active agent. Slides, previously cleaned with chromic acid, were stored in absolute ethanol and when required dried with hot air. Slides were dipped in 1 per cent aqueous suspensions of lycopodium alone or containing sodium lauryl sulphate 0·1, 0·01, 0·05 or 0·005 per cent. Slides were laid flat, allowed to dry under a bell jar, then solitary and clumped spores in ten fields were counted under the microscope. For all suspensions duplicate counts were made. Suspensions were shaken for 5 min. immediately before use. The suspension which showed maximum dispersion of spores was used for dipping tests with leaves.

Tests with Leaves of Rumex obtusifolius

Initially, dock leaves (*Rumex obtusifolius*) were dipped vertically either in aqueous suspensions of lycopodium 1 per cent alone or containing 0·01 per cent sodium lauryl sulphate. After withdrawal the leaves were laid flat lower surface downwards on a sheet of glass. The wettability of the upper surface was immediately noted visually and classified as: (i) unwettable, with no visible film of liquid; (ii) partially wettable, with a discontinuous film of liquid; (iii) completely wettable, with persistent film of liquid. As soon as the water had dried, the surfaces were examined microscopically for distribution of lycopodium, using 32 mm. and 16 mm. objectives, with a "Beck-Chapman Illuminator."

Leaves of different age, grown under glass, and leaves which had been washed with ether were also tested. The cuticle structure of untreated leaves was studied by direct microscopical examination using a 4 mm. objective and by means of cellulose acetate impressions (method of Martin, 1952). Transverse sections of leaves were treated with Sudan III and Ruthenium red solution as tests for cutin and mucilage respectively.

Tests with Leaves of Other Species

Leaves of some common weeds and plants of economic importance, were collected from the Chelsea Physic Garden and the Myddelton House Estate, Enfield, and the wettability of upper surfaces assessed, using aqueous suspensions of lycopodium alone. The surfactant was excluded from this general work as the chief object was to study the contribution of natural factors to particle retention. The cuticle structure was examined using the methods already described for *Rumex obtusifolius*. In addition the presence or absence of a waxy bloom on leaves was determined microscopically by examination before and after rubbing the surface with lens tissue. For hairy leaves, both surfaces were studied and the type and distribution of trichomes was determined from transverse sections and pieces of leaves cleared in chloral hydrate solution.

RESULTS

Experiments with Smooth Surfaces

With the preliminary experiments using smooth surfaces, and suspensions of lycopodium in water, 51 per cent of the lycopodium was found as solitary spores and the remainder in clumps. The concentration of sodium lauryl sulphate giving the maximum dispersion of spores was 0·01 per cent, this gave 90 per cent solitary spores. Higher concentrations were too frothy and produced an uneven distribution of spores on the slides.

Tests with Leaves of Rumex obtusifolius

Immediately after the withdrawal of leaves from suspensions of lycopodium in water complete coverage of the surface was seen. Subsequently recession of the liquid film occurred and was accompanied by drifting of the particles. The deposit viewed microscopically was found to be

patchy. This also occurred in the presence of 0·01 per cent sodium lauryl sulphate but although patchy deposits were produced on drying, clumping of spores was considerably reduced. Similar results were obtained with leaves of different age; on both surfaces (differing in vein prominence); with ether dipped leaves and with leaves grown in a pollution free atmosphere. Aqueous 1 per cent suspensions of kaolin were similarly tried, but the distribution of particles was difficult to assess microscopically.

Recession of water on leaves did not occur after a single application of spray drops (50–110–170 μ diameter) when applied from a hand operated glass atomiser, but did occur after several applications of spray, when complete coverage with a film of water had been achieved. Drops of different size (230 μ to 2 mm. diameter, from a micrometer pipette) placed at different positions on the leaf, spread uniformly and recession did not occur, but drops (2 mm. diameter) allowed to fall on the surface from a height of 100 cm. spread on impact and recession quickly followed on both upper and lower surfaces.

Microscopical examination of leaves of different age, using cellulose acetate replicas and by direct observation showed that the interneural epidermal cells in addition to cells overlying veins are roughened due to ridges and projections, mucilage and cutin were not detectable in epidermal cells treated with staining reagents.

Tests with Leaves of Other Species

These results are summarised by classifying the surfaces according to wettability as already defined; in relation to the distribution of lycopodium spores and in relation to the chief cause of surface roughness. Upper surfaces only were studied except where it is mentioned otherwise. Common names of weeds are those given by Clapham, Tutin and Warburg (1958) and varieties of cultivated plants quoted if known.

1. *Unwettable*

Cuticle waxy (surface bloom removable by rubbing with tissue paper), spores not retained, e.g. cabbage *Brassica oleracea var capitata* (Fig. 1A and 1B), clover *Trifolium repens*, opium poppy *Papaver somniferum var album*, rose (the Doctor), sowthistle *Sonchus oleraceus*, strawberry (Royal Sovereign), lower surface.

2. *Partially Wettable*

(*a*) Cuticle granular (no surface bloom), patchy retention of spores, e.g. apple (Worcester Permain), cherry-laurel *Prunus lauroceracus*, rhododendron *Rhododendron ponticum*, rose (Peace), strawberry (Royal Sovereign).

(*b*) Cuticle ridged, patchy retention of spores, e.g. bindweed *Polygonum convolvulus*, bistort *Polygonum bistorta*, cleavers *Galium aparine*, dandelion *Taraxacum officinale*, dock *Rumex obtusifolius* (Fig. 1C), groundsel *Senecio vulgaris*, horse-chestnut *Aesculus hippocastanum* (Fig. 1D), redshank *Polygonum persicaria*, tomato (Sunrise).

(c) Abundant trichomes ("closed" pattern, see Fig. 2), good particle retention, e.g. apple (Worcester Permain), lower surface, blackberry *Rubus fruticosus*, lower surface, elecampane *Inula helenium*, lower surface, raspberry *Rubus idaeus*, lower surface (Fig. 2C), chrysanthemum (Brenda Talbot), lower surface (Fig. 2D).
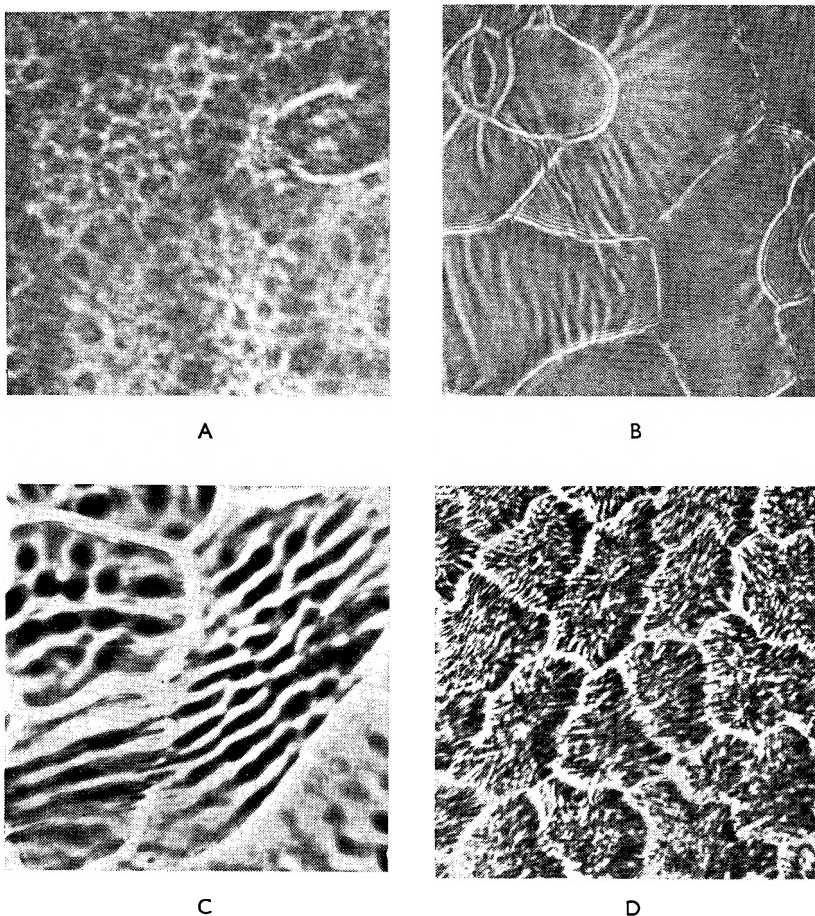
FIG. 1. Photomicrographs of replicas prepared from upper surfaces of leaves (all × 550).

A. cabbage (showing waxy "bloom"). B. cabbage (wax removed). C. dock. D. horse-chestnut.

3. Completely Wettable

(a) Cuticle smooth, good distribution of spores, e.g. chickweed *Stellaria media*, creeping butter-cup *Ranunculus repens*, dahlia (Zonnegoud), daisy *Bellis perennis*, fat hen *Chenopodium album*, french bean (Canadian Wonder), forget-me-not *Myosotis arvensis*, lucerne *Medicago sativa*,

plantain *Plantago major*, raspberry *Rubus idaeus*, speedwell *Veronica officinalis*, tormentil *Potentilla erecta*.

(*b*) Abundant trichomes ("open" pattern, see Fig. 2), good spore retention, heavy deposit, e.g. foxglove *Digitalis purpurea*, lower surface, hoary cinqefoil *potentilla argentea*, lavender *Lavandula intermedia* (Fig.
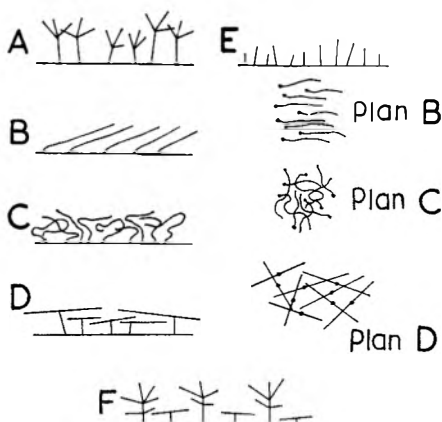

FIG. 2. Diagrams of trichome patterns, elevation and plan.
A. Lavender. B. Hoary cinqefoil. C. Raspberry. D. Chrysanthemum. E. Digitalis. F. Buddleia.

2A), lady's mantle *Alchemilla vulgaris*, mouse-ear chickweed *Cerastium vulgatum*, mullein *Verbascum thapsus*, primrose *Primula vulgaris*, lower surface, witchazel *Hamamelis virginiana*, lower surface.

The leaves of buddleia *Buddleja davidii*, lower surface (Fig. 2F) were easier to wet than those of chrysanthemum (lower surface) and the arrangement cf trichomes resembled the "open" also "closed" patterns, both "T" shaped and candelabra trichomes being present.

DISCUSSION

Preliminary Work

Lycopodium appears to be a satisfactory material for studying the influence of leaf surface roughness on the behaviour of particles when applied in the form of a suspension, as the spores are easily suspended in water and easily recognised by direct microscopical examination of the surface (Fig. 3A and 3B). The only fault is that clumping of spores occurs, but this difficulty can be overcome by the addition of sodium lauryl sulphate 0·01 per cent to the suspensions. From the tests with dock leaves, the distribution of spores does not differ from the picture obtained when no surface-active agent is incorporated provided that the concentration of 0·01 per cent is not exceeded. Although kaolin would be more appropriate for retention studies, since it is used as a pesticide diluent, lycopodium spores are more easily recognised on leaf surfaces

than kaolin particles, and hence a more reliable assessment of distribution can be made.

Experiments with dock leaves show that liquid recession occurs on surfaces which are not contaminated by dirt or grease from the atmosphere and this phenomenon is not due either to the presence of extruded wax
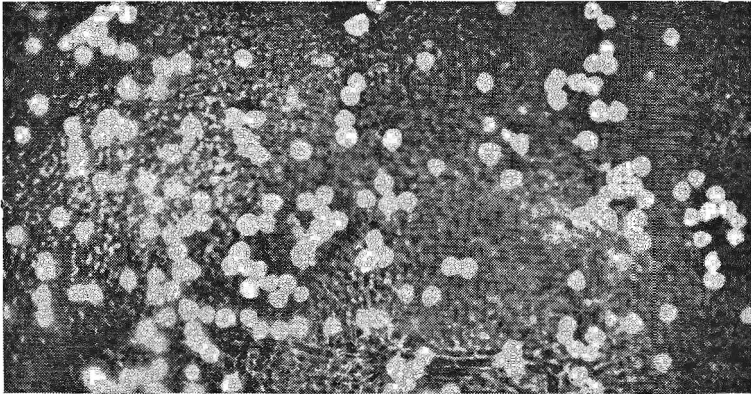
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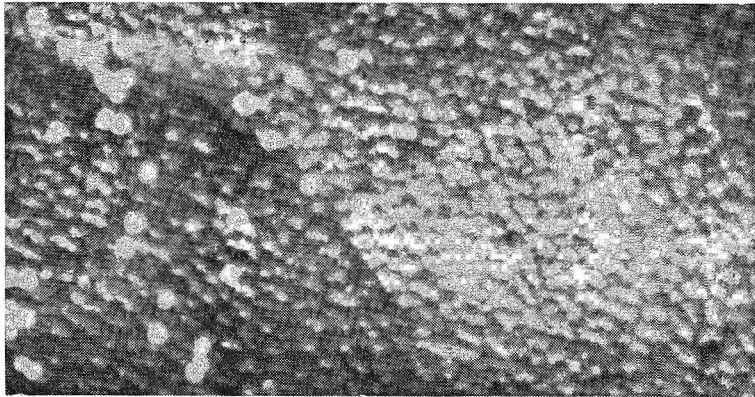
B

FIG. 3. Photomicrographs of leaf upper surface (× 100), showing distribution of lycopodium.
A. French bean. B. Bindweed.

or to mucilage secreted from glandular trichomes. Although histochemical tests for cutin proved negative it cannot be assumed that the epidermal walls are entirely free from it as a thin deposit would not be detected because of the limited resolving power of the miscroscope. The presence of natural water repellant surface chemicals (even a monomolecular layer) could account for recession of liquid which would be

enhanced by roughness in the form of projections (Fig. 1C). Tests made to compare the dipping technique with the effect of spraying show that recession only occurs when water is present on the surface as a film and that drops carefully applied at a position close to the surface remain stationary. It was also evident that drops of water applied some distance from the surface, on impact, spread to give a thin film, followed by recession. Recession does not appear to be related to vein prominence as the phenomenon occurs on both surfaces of leaves despite differences in macroscopical appearance.

Different Species

The value of a better classification of leaf surfaces in terms of wettability is borne out by the results obtained with different types of leaves. For instance the leaves of one variety of rose have no waxy bloom, are only partially wettable and particle retention is poor (cf. variety with waxy bloom, unwettable). It has been assumed by Juniper (1960), because of the surface characters of dock and horse-chestnut leaves, seen by electron microscopy, that there would be no difficulty in wetting these surfaces. The latter conclusions do not appear to be adequate as it is essential to differentiate between "spreading" and "wetting" especially when recession of the liquid occurs. The latter was demonstrated with tests on horse-chestnut and dock leaves, where wetting and particle distribution was poor, despite initial spreading, which was good. Juniper (1958) also states that the surface of dock leaf is smooth, but direct and replica investigation of the surface shows that it is ridged and also roughened by projections. This roughness could be overlooked when using the limited field of view of electron microscopy. Juniper (1959) further believes that the ultra microscopic roughness of the waxy extrusions of cabbage and other species largely determine wettability. The influence of waxy extrusions cannot be neglected but the present work shows that cuticle which is roughened by ridges is only partially wettable. It can be seen from (Fig. 1B) that the cuticle of cabbage leaf is ridged and consequently it is difficult to judge the contribution of either microscopic or ultra-microscopic roughness or both, to water repellency.

The differences in behaviour of water on hairy leaves of the "open" and "closed" pattern suggests that chemicals applied as solutions would be retained and distributed efficiently on the first type of surface but not on the second type (without a wetting agent being used). Leaves with trichomes exhibiting the "open" pattern illustrate a form of surface roughness, which actually enhances wetting, possibly due to the influence of capillarity. As the upper surface of chrysanthemum leaf is less hairy than the lower, better wetting can be achieved on the upper surface; there being less tendency for air pocket formation. Water repellency may therefore vary between varieties of chrysanthemum depending on the density of trichomes per unit area. With all but one class of leaf surface, spores were distributed according to the distribution of water. Where recession occurred spores were carried with the receding film, hence particles did not appear to be retained in preference to liquid or vice versa.

The exception to the above general conclusion arises in the case of suspensions applied to hairy leaves with a "closed" pattern of trichomes, where spores were retained despite difficulties in wetting the surface and particles were probably trapped by the trichomes. In addition to variations in roughness attributed to different trichome patterns and densities, surface roughness of individual trichomes often occurs, and together with natural water repellent chemicals could influence wetting of leaves.

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OBSERVATIONS ON THE USE OF A MOUSE BIOASSAY METHOD FOR INVESTIGATING PURGATIVE ACTIVITY

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Some essentially practical observations have been made on the use of a simple purgative assay in mice. Modifications of the method have improved both the accuracy and precision of the assay. Senna extracts have been assayed in terms of a sennoside A standard.

THE mouse is well suited for investigating the purgative activity of some anthraquinone drugs (Geiger, 1940; Collier, Fieller and Paris, 1948; Lou, 1949; Fairbairn, 1959). Moreover, McClure Browne, Edmunds, Fairbairn and Reid (1957) have shown that the clinical results obtained with senna preparations are in good agreement with the purgative activity predicted from the mouse assay method described by Lou (1949). In this present work, the latter method of assay has been systematically examined to improve its inherent accuracy and precision. Furthermore, an attempt has been made to use the mouse method of assay for routine standardisation of senna extracts in terms of sennoside A as a reference standard.

MATERIALS AND METHODS

Materials

Throughout this work two powdered extracts of Alexandrian senna pod (S.1, S.2) were used; immediately before use these extracts were suspended in distilled water. A sample of sennoside A (prepared in the Pharmaceutical Research Department, Allen & Hanburys Limited) was used as the laboratory standard. Solutions of this material were prepared in distilled water to which trace amounts of sodium bicarbonate were added.

Methods

The basic method of assay used is that described by Lou (1949), although subsequent modifications have been made. At the commencement of this work, it was thought that the following procedure would be suitable for assaying senna extracts with a reasonable degree of accuracy and precision.

Male albino mice of body weight 18 to 22 g. are conditioned by being subjected at weekly intervals to a "dummy" assay procedure, involving starving, dosing with a purgative and placing in cages, since D'Arcy, Grimshaw and Fairbairn (1960) and D'Arcy (1962) have shown that the initial training of the animals improves the precision of the assay. After this initial training period, which may take from 3 to 4 weeks, the mice are used routinely at weekly or fortnightly intervals. Food, but not water, is removed 2 hr. before the assay and during this time the mice are isolated

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in perspex cages placed on wire grids over blotting paper; if any animal should show evidence of diarrhoea it is excluded from the test.

The animals are then randomised in groups of 10, and solutions or suspensions of standard and test materials are prepared immediately before use and administered orally in a dose volume of 0·5 ml./mouse, regardless of body weight. Depending upon the material being assayed, either 2 or 3 dose groups are used for the standard and each test preparation. After dosage the mice are returned to the perspex cages, two mice per compartment. The compartments are $5 \times 3\frac{1}{2} \times 7$ in. high, open top and bottom and are built of perspex in blocks of 10. They stand on $\frac{1}{4}$ in. mesh wire grids raised $\frac{1}{2}$ in. above sheets of blotting paper, the cage tops are also covered with grids. A small container, filled with a mixture of 10 parts crushed cubes (Diet 41) to 7 parts water, is placed in each compartment 6 hr. after the start of the assay.

At 3, 6 and 22 hr. from the start of the assay the grids and blotting papers are changed, and the number of unformed faeces counted for each pair of mice. Unformed faeces are wet, shapeless, relatively large in size and stain the blotting paper. The total unformed faeces excreted in 22 hr. by pairs of mice within each dose group is used as the response metameter. The potency ratio and fiducial limits of the assay are calculated by standard statistical methods.

RESULTS

Dose Response Relationships

Response-time. Purging of mice can be induced by oral doses of 0·5 to 2·5 mg. sennoside A/mouse and 5 to 30 mg. of the senna extracts/mouse. Initial experiments indicated that the total number of wet faeces excreted by pairs of mice, during 22 hr. after drug administration, provided a satisfactory response metameter. To test whether the purgative activity was complete by 22 hr., three experiments were made in which senna extract (S.1) was assayed against sennoside A, the total number of unformed faeces being counted at 3, 6, 22, 25 and 28 hr. Table I summarises the cumulative responses for each dose; responses from the three separate assays have been summed.

TABLE I

SUMMED CUMULATIVE RESPONSES FOR THREE ASSAYS OF SENNA EXTRACT (S.1) AGAINST SENNOSIDE A

Sample	Dose mg./mouse orally	No. of unformed faeces* excreted; time after dosage				
		3 hr.	6 hr.	22 hr.	25 hr.	28 hr.
Senna extract S.1	10	10	36	43	44	44
	20	26	65	88	89	89
Sennoside A	0·75	12	32	47	47	47
	1·5	15	60	84	86	87

* Each figure is the cumulative total, for 5 pairs of mice, summed over three assays.

The results show that, for both sennoside A and the senna extract (S.1), the responses were substantially complete by 22 hr., thus confirming that this was a suitable time at which to terminate an assay.

Dose-response curves. Using the cumulative responses at 22 hr., a log dose-response curve was established for sennoside A. The responses produced by doses of 0·5 to 2·5 mg. sennoside A/mouse lay on the rectilinear portion of the curve. In some experiments, sennoside A was administered at higher dosage, i.e., 3·0 mg./mouse; however, this dose was not suitable for assay use since it produced severe purging and prostration.

Dose-response curves were similarly established for the two senna extracts (S.1, S.2) and were linear within the range 7·5 to 30 mg. extract/mouse. The dose-response curve for each extract did not deviate significantly in parallelism (P > 0·05) from that of sennoside A.

Housing of Animals During the Assay Procedure

Two dose-response curves were established for sennoside A using 10 mice per dose group. In the first series of experiments the animals were housed separately and their unformed faeces counted over a 22 hr. period. Secondly, mice were housed in pairs and the responses measured as previously described. The results of these experiments (Table II) show that there is no gain in precision to be obtained by housing the mice singly; in subsequent experiments the mice were therefore caged in pairs.

TABLE II

EFFECT OF HOUSING MICE EITHER SINGLY OR IN PAIRS ON THE PRECISION OF THE DOSE-RESPONSE CURVE FOR SENNOSIDE A

Statistic	Animals housed singly	Animals housed in pairs
b	6·5	11·5
s.e.$_b$	0·47	0·77
b/s.e.$_b$	13·83	14·93

Effects of Dose Volume and Adjustment for Body Weight

Effect of dose volume. The effect of variation in dose volume on the precision of the assay was studied. Three groups of 24 mice were used and they were dosed with volumes of either 0·25, 0·5 or 1·0 ml./mouse. For each of the three groups a dose-response curve was established using 8 mice per dosage group at levels of 0·5, 1·0 and 2·0 mg./mouse of sennoside A. The results of this experiment and their analysis are presented in Table III. It is evident that, in all three dose-volume groups, the response to sennoside A was linear over the dose range studied, since the sums of squares attributed to both "combined curvature" and "opposed curvature", were not significant. In addition, there was no significant difference in the level of responses to a specific dose of sennoside within each dose-volume group, since the "between dose-volume" term was not significant. Furthermore, as the "parallelism" term was also not significant, there was no difference between the slopes of the dose-response curves within the three dose-volume groups. However, the "within dose error" for the 0·5 ml. dose volume group was less than that of the other two groups.

TABLE III

THE EFFECT OF DOSE VOLUME ON ASSAY PRECISION

Assay protocols

Dose Volume ml./mouse orally	Dose of sennoside A mg./mouse	No. of unformed faeces				Total response
		Cumulative 22 hr. response per pair of mice				
0·25	0·5	3,	0,	1,	4	8
	1·0	6,	7,	3,	5	21
	2·0	10,	9,	9,	9	37
0·5	0·5	1,	1,	3,	2	7
	1·0	6,	2,	7,	6	21
	2·0	10,	10,	10,	10	40
1·0	0·5	1,	4,	2,	4	11
	1·0	5,	9,	3,	5	22
	2·0	8,	9,	8,	11	36

Analysis of Variance

Source	S.S.	d.f.	M.S.	F.
Between dose volumes	0·389	2	0·195	<1
Linear regression	315·375	1	315·375	123·87*
Parallelism	4·000	2	2·000	<1
Combined curvature	1·681	1	1·681	<1
Opposed curvature	0·111	2	0·056	<1
Total between doses	321·556	8		
Within dose error (Volume 0·25 ml.) ..	19·500 ⎫	9	⎫	
Within dose error (Volume 0·5 ml.) ..	17·500 ⎬ 68·750	9	⎬ 2·546	
Within dose error (Volume 1·0 ml.) ..	31·750 ⎭	9	⎭	
Total	390·306	35		

* Very highly significant.

Adjustment of dosage for body weight. In order to examine the effect of dosing per body weight or per mouse, it was necessary to house the animals individually. Solutions of 2 or 4 mg. sennoside A/ml. were administered to groups of 10 mice of body weight 27 to 34 g. In dosing, a volume of 0·5 ml./30 g. was used; in dosing irrespective of body weight, a volume of 0·5 ml./mouse was given. Results are shown in Table IV; there was no difference in level of response which ever method of dosage was used, nor was any gain in precision obtained by dosing on a bodyweight basis.

Effects of Training on Accuracy and Precision

The effect of the training procedure on the accuracy and precision of the assay method has been correlated with age and weight of the animals. The purgative activity of senna extract (S.2) was assayed against sennoside A using a 2 × 2 design, at weekly intervals for 4 weeks and subsequently at fortnightly intervals. For each assay the mean body weight of the animals was determined; the dose volume administered was 0·5 ml./mouse regardless of body weight; animals were housed in pairs, 5 pairs per dose group. The results of these experiments (Table V) show that the best accuracy and precision was obtained in the fifth week of training. However, since the mice increased in weight during the training period it was deemed necessary to investigate the effect of weight alone. Therefore,

the purgative activity of senna extract (S.2) was again assayed against sennoside A using, for each separate assay, groups of mice within a narrow body weight range. In five assays (Table VI), the mean weight of the mice varied from 18·8 to 36·8 g., all mice being untrained.

<div align="center">TABLE IV</div>

<div align="center">THE EFFECT OF ADJUSTMENT OF DOSE VOLUME FOR BODY WEIGHT ON ASSAY PRECISION</div>

<div align="center">Assay protocols</div>

Mean weight g. ± s.d.*	Dose Sennoside A		No. of unformed faeces		Total response
	mg./ml.	Dosage	Cumulative 22 hr. response per mouse		
30·3 ± 1·70	4·0	0·5 ml./30 g.	5, 5, 8, 4, 3, 6, 5, 5, 5, 4		50
29·5 ± 1·51	4·0	0·5 ml./mouse	5, 4, 3, 4, 2, 4, 6, 4, 6, 6		44
30·8 ± 2·15	2·0	0·5 ml./30 g.	5, 0, 3, 4, 1, 1, 5, 4, 5, 2		30
29·8 ± 2·30	2·0	0·5 ml./mouse	2, 6, 4, 2, 3, 1, 2, 4, 3, 4		31

* Standard deviation (s.d.) has been tabulated since it provides the best measure of scatter between the body weights.

<div align="center">Analysis of variance</div>

Source	S.S.	d.f.	M.S.	F.
Between methods of dosage	0·625	1	0·625	<1·0
Linear regression	27·225	1	27·225	11·77†
Parallelism	1·225	1	1·225	<1·0
Total between doses	29·075	3		
Error 1 (0·5 ml./30 g.)	48·0	18	}2·313	
Error 2 (0·5 ml./mouse)	35·333	18		
Total	112·375	39		

<div align="center">† Highly significant.</div>

The results indicate that accuracy and precision in the untrained mice is satisfactory when their mean body weight lies between 27 to 31 g. Although accuracy and precision are improved to some extent by using heavier animals, comparison with the previous assay data (Table V) indicates that the degree by which these indices are improved is greater with trained heavier mice. In current assays, the use of mice, initially weighing 25 to 30 g. and subsequently conditioned for 2 weeks, gives good results.

<div align="center">DISCUSSION</div>

The method of determining the purgative activity of senna extracts using mice is relatively simple and easy to perform, and in our experience and in the hands of others (Fairbairn, 1958) it has proved reliable and repeatable. Although the method we have described has been successful for the examination of senna, senna extracts and senna preparations, it is not so well suited to the evaluation of the activity of other anthraquinone purgatives, for example, cascara and rhubarb. The method does not demonstrate the purgative activity of aloin and phenolphthalein (D'Arcy, Grimshaw and Fairbairn, 1960).

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The selection of sennoside A as a reference standard has provided a single chemical compound, in terms of which, the purgative activity of senna extracts can be evaluated, since in our experience dose-response curves for senna extracts are parallel to that of the standard sennoside. Although we have observed that the rates of onset of activity of the extracts and sennoside A differ, purgative activity is complete after 22 hr. for both extracts and sennoside A. Calculation of assay results over this period therefore provides a valid basis for assay.

TABLE V

THE EFFECT OF TRAINING ON THE ACCURACY AND PRECISION OF THE ASSAY OF SENNA EXTRACT (S.2) AGAINST SENNOSIDE A

Week of training	Mean weight g. ± s.d.*	Known relative potency†	Estimated relative potency	95 per cent fiducial limits (per cent)	λ = s/b
0	20·1 ± 1·2	0·25	Assay	invalid‡	
1	24·9 ± 1·8	0·25	0·23	62–161	0·21
2	28·2 ± 2·3	0·25	0·19	61–164	0·19
3	30·1 ± 2·8	0·25	Assay	invalid‡	
5	31·8 ± 4·0	0·25	0·24	76–131	0·12
7	34·8 ± 2·8	0·25	0·26	74–136	0·14
9	36·4 ± 6·0	0·25	0·24	65–153	0·19
11	38·0 ± 4·3	0·25	0·20	68–146	0·20

* As for Table IV.
† Mean of 10 assays using conditioned mice. 95 per cent fiducial limits of the mean 95–105 per cent.
‡ Dose response lines of test and standard deviated significantly from parallelism.

The potency of senna extract relative to sennoside A is the same whether mice are housed singly or in pairs during the assay. When low doses of purgative are administered to animals housed singly, the percentage of zero responses is large and their subsequent statistical treatment is questionable. However, by housing animals in pairs, and taking the sum of responses from the two animals, zero responses can be almost eliminated.

TABLE VI

THE EFFECT OF BODY WEIGHT ON THE ACCURACY AND PRECISION OF THE ASSAY OF SENNA EXTRACT (S.2) AGAINST SENNOSIDE A

Mean weight g. ± s.d.*	Known relative potency†	Estimated relative potency	95 per cent fiducial limits (per cent)	λ = s/b
18·8 ± 1·1	0·25	0·26	62–162	0·22
25·3 ± 1·9	0·25	0·16	68–147	0·15
27·5 ± 1·7	0·25	0·23	66–151	0·17
31·4 ± 3·7	0·25	0·25	70–143	0·16
36·8 ± 4·0	0·25	0·17	50–200	0·28

* As for Table IV.
† As for Table V.

In considering the effects of dose volume and adjustment for body weight on the accuracy and precision of the assay, the optimal dosage was found to be 0·5 ml./mouse regardless of body weight. Since, in practice, it is quicker and far more convenient to dose irrespective of body weight, this procedure is entirely suitable for routine work.

Conditioning of the mice before routine purgative testing greatly improves the assay. Initial studies, using mice 18 to 22 g. body weight,

showed that the best accuracy and precision was attained after a 5-week training period.   In addition, later work using untrained animals, showed that when heavier mice were used, results were optimal with a weight range of 27 to 31 g.   Thus it would seem that both the actual conditioning of the mouse to the test procedure, and the gain in weight during this training period influence the accuracy and precision of the assay.   Current work using trained heavier mice has amply confirmed this observation.

When mice are fully conditioned, the assay procedure using 5 pairs of mice per dose group routinely gives 95 per cent fiducial limits of 75 to 130 per cent.   This is satisfactory for routine estimations of purgative activity of senna extracts.   Greater precision can be achieved by either using larger numbers of animals per dose group in individual assays or alternatively combining the results of two or more separate assays.   The simple modifications that have been described have resulted in an increase in both the accuracy and the precision of the assay method.

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THE DISTRIBUTION AND EXCRETION BY CATS OF A NEW HYPOTENSIVE DRUG, N-BENZYL-N'N"-DIMETHYLGUANIDINE

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The distribution and excretion of ^{14}C-labelled N-benzyl-N'N"-dimethylguanidine (BW 467C60) have been studied in cats. The drug, like bretylium, had a selective affinity for adrenergic neurones. At 3 mg./kg., about half the dose was excreted unchanged in urine within 24 hr. and no metabolites were found. Excretion continued for more than 7 days.

N-BENZYL-N'N"-DIMETHYLGUANIDINE (BW 467C60) is a new hypotensive drug with pharmacological properties intermediate between those of bretylium and guanethidine (Boura and Green, 1962). Bretylium localises in the adrenergic neurones of cats (Boura, Copp, Duncombe, Green and McCoubrey, 1960), a property shared by two analogues of the drug (Boura, Duncombe and McCoubrey, 1961). We wished to know whether BW 467C60 also shared this property. For the study of distribution the drug was labelled with ^{14}C at the benzyl carbon atom. The same material was used to measure the urinary excretion by cats and to search for metabolites.

MATERIALS

Unlabelled drugs were supplied by Dr. E. Walton who also kindly supplied details of the preparation of BW 467C60 from benzylamine.

[*Carboxy*-^{14}*C*]*benzoic acid.* Phenyl magnesium bromide absorbed [^{14}C]carbon dioxide to give benzoic acid in 95·5 per cent yield with a specific activity of 5·1 mc/mmole.

[*Carbonyl*-^{14}*C*]*benzamide.* The above benzoic acid (60 mg.; 2·5 mc) was diluted with inactive benzoic acid (40 mg.) and refluxed for 3 hr. under dry nitrogen with thionyl chloride (0·3 ml.) and dry benzene (0·3 ml.). Additional dry benzene (1 ml.) was added and the mixture was evaporated at about 50 mm. pressure. The residue was transferred in dry benzene (2 ml.) to a tube connected to a vacuum manifold. It was cooled to about −40° and the air pumped out. The tube was disconnected from the manifold by stopcock and the manifold was then filled with dry ammonia. The gas was admitted to the tube in small quantities with shaking and cooling as required, until no more was absorbed. The semi-solid mass was treated with water (2 ml.) and extracted with benzene (3 × 5 ml.). Inactive benzamide (23 mg.) was dissolved in the aqueous residue by warming and the extraction repeated. The mixed benzene extracts were evaporated to give a white residue of benzamide (121 mg.; 99 per cent yield).

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[α-^{14}C]*Benzylamine.* The above benzamide was refluxed with lithium aluminium hydride (135 mg.) in dry ether (5 ml.) under dry nitrogen for 5 hr. Excess hydride was destroyed by moist ether followed by 5N sodium hydroxide (5 ml.). Benzylamine was extracted by ether (4 × 3 ml.), the extract dried over sodium sulphate, and distilled. Yield 82·5 mg.; 77 per cent.

N-[α-^{14}C]*Benzyl*-N′N″-*dimethylguanidine hydriodide.* The above benzylamine was refluxed with methyl isocyanate (56 mg.) in ether (5 ml.) for 10 min. and the solvent evaporated. The residue was refluxed with methyl iodide (1 ml.) in ethanol (4 ml.) for 20 min. and the ethanol and excess iodide evaporated. The residue was refluxed with ethanolic methylamine (33 per cent; 7 ml.) with addition of more methylamine (1 ml.) every 30 min. during 3·5 hr. Ethanol was distilled and the residue diluted with ether. After standing for 2 hr. the product was centrifuged down (204 mg.). It was crystallised from ethanol-ether as yellowish prisms, m.p. 190–194° (181 mg.). Radioassay indicated 2·36 mc/mmole, equivalent to 56 per cent overall yield from benzoic acid based on ^{14}C used.

Autoradiography of chromatograms developed in s-butanol: acetic acid: water (11:5:3) revealed a single compact spot R_F 0·78, the same as authentic BW 467C60 visualised by Dragendorff's reagent.

METHODS

Doses of BW 467C60 were given to cats by stomach tube under light ether anaesthesia.

Tissue samples were taken at 16 hr. after the dose as described for bretylium (Boura, Copp, Duncombe, Green and McCoubrey, 1960). The radioactivity of small samples (<30 mg. dry weight) was measured by Schöniger combustion and liquid scintillation counting (Kelly, Peets, Gordon and Buyske, 1961) using ethanolamine as CO_2-trapping reagent (Jeffay and Alvarez, 1961). Larger samples were dried over P_2O_5 under moderate vacuum for several days. They were powdered and plated in polythene planchettes for counting at infinite thickness.

Urines were diluted with an equal volume of ethanol, cooled to 0° and filtered. Aliquots (40 μl.) were plated on lens paper in planchettes for counting under a mica end window counter. Standards were prepared for each sample by adding labelled BW 467C60 to an aliquot sufficient roughly to double the counting rate.

RESULTS

Tissue Distribution

Table I shows the concentrations of BW 467C60, calculated from the radioactivity present, in the larger organs of two cats at 16 hr. after a subcutaneous dose (3 mg./kg.) of the drug. The bile of one cat (1·3 ml.) contained radioactivity equivalent to 3·1 μg. of the drug.

Table II shows the concentrations of BW 467C60 found in the nerves and ganglia of these cats. The nictitating membranes of both were almost fully relaxed when they were killed.

Urinary Excretion

The urine of one cat used to study distribution contained radioactivity equivalent to 5·9 mg. of the 9 mg. dose given. Autoradiographs of chromatograms developed in s-butanol:acetic acid:water (11:5:3) or propanol:10 per cent ammonia (6:4) revealed one spot only at R_F 0·78 and 0·84 respectively, the same positions occupied by BW 467C60. In butanol:pyridine:water (1:1:1) two spots were revealed, one well marked at R_F 0·82, corresponding to unchanged drug, and one weak spot at R_F 0·70. This last result could not be repeated subsequently.

TABLE I

CONCENTRATIONS OF *N*-BENZYL-*N′N″*-DIMETHYLGUANIDINE (BW 467C60) IN CAT TISSUES AT 16 HR. AFTER A SUBCUTANEOUS DOSE

Dose: 3 mg./kg. of *N′*-[α-^{14}C]benzyl labelled drug
Concentrations are expressed as mμmoles/g. wet tissue
Values from two cats

Adrenal	..	..	..	17.5, 53.2	Kidney ..	..	..	1·3, 1·4
Lung ..	..	..	..	6·7, 16·4	Cerebral cortex ..	..	..	0·3, 0·0
Spleen	..	..	..	14·5, 14·0	Cerebellar cortex	..	..	0·4, 0·0
Left ventricle ..	..	..	..	6·9, 11·8	Hypothalamus ..	..	..	Trace, 0·0
Liver ..	..	..	..	4·5, 6·8	Spinal cord	..	..	2·0, 0·0
Diaphragm	..	..	..	5·4, 5·4	Blood ..	..	..	1·3, 1·7
Thyroid	..	..	..	2·8, 2·2	Area postrema ..	..	..	1·9, –
Pancreas	..	..	..	2·3, 2·2	Pituitary ..	..	..	52·0 –
Parotid	..	..	..	– 6·6	Lymph gland ..	..	..	– 0·8
Renal fat	..	..	..	– Trace	Cerebrospinal fluid	..	..	– 0·0

In further experiments the urine was collected at intervals of 24 hr. after subcutaneous or oral doses. The samples were assayed for radioactivity and radioautographs prepared if sufficient activity was present. Table III shows the results. In all instances save one, the urines gave single spots that travelled slightly more slowly than marker spots prepared from aqueous solutions of the drug. Since the lower R_F value corresponded to that of *N*-benzyl-*N′*-methylguanidine (BW 783C60), a search was made for this possible metabolite of the drug. The 24, 48 and 72 hr. specimens from orally dosed cats were pooled, evaporated to about 50 ml. and extracted with ethanol (100 ml.). The process was repeated using acetone. The extract was evaporated and passed down a column of Zeo-Karb 226 (H) (25 × 2 cm.) and bases were eluted by 2N hydrochloric acid. The radioactive fractions were evaporated to dryness *in vacuo* and the residue was chromatographed on Whatman 3MM paper in s-butanol:acetic acid:water (11:5:3). The single radioactive zone was eluted from the paper and upon evaporation gave an oily residue (92 mg.) with radioactivity corresponding to 5·0 mg. of BW 467C60. This resisted further efforts at purification.

The picrates of BW 467C60 and BW 783C60 were prepared by diluting ethanolic solutions of picric acid plus the drugs with an equal volume of water. BW 467C60 picrate had m.p. 146–148° (Found: N, 20·4. $C_{16}H_{18}N_6O_7$ requires N, 20·7 per cent). BW 783C60 picrate had m.p. 128–129° (Found: N, 21·2. $C_{15}H_{16}N_6O_7$ requires N, 21·4 per cent).

The above oily residue (70 mg.) was dissolved in acetone (3 ml.) and two portions (1 ml.) were pipetted onto the above picrates (0·25 g.)

respectively. Specific activities of the picrates, estimated by plating 40 μl. of the above solutions onto lens paper, were 1275 $\pm$ 59 (BW 467C60) and 1284 $\pm$ 14 counts/min./mg. (BW 783C60). After three crystallisations from 50 per cent ethanol the specific activities were respectively 1147 $\pm$ 32 and 72 $\pm$ 7 counts/min./mg. The bulk of the radioactivity in the urine extract can be accounted for as BW 467C60 though the

TABLE II

CONCENTRATIONS OF N-BENZYL-$N'N''$-DIMETHYLGUANIDINE (BW 467C60) IN NERVES AND GANGLIA OF CATS AT 16 HR. AFTER A SUBCUTANEOUS DOSE

Dose: 3 mg./kg. of the N-[α-^{14}C]benzyl labelled drug
Concentrations are expressed as mμmoles/g. wet tissue. Values from two cats

Sympathetic ganglia				Adrenergic nerves			
Superior cervical	..	..	43·0, 28·4	Postganglionic superior cervical		33·0, 21·6
Stellate ..	..	..	31·0, 59·3	Hypogastric	..	..	46·0, 33·8
Coeliac ..	..	..	31·0, 11·7	Inferior cardiac ..	..	..	40·0, 67·2
Inferior mesenteric	..	..	14·5, 43·5	Splenic ..	..	..	30·0, 75·4
				Gastric ..	..	..	– 49·6
				Colonic ..	..	..	– 16·4
Other ganglia						
Nodose ..	..	..	– 2·3			
Ciliary ..	..	..	7·1, 8·6	Other nerves		
Dorsal root	..	..	1·4, 3·7	Vagus ..	..	..	5·2, 2·6
Semilunar	..	..	3·8, –	Preganglionic superior cervical		2·1, 5·7
				Sciatic ..	..	..	2·0, 3·1
				Greater splanchnic	..	..	9·3, 10·8

possibility exists that about 10 per cent may be the monodemethylated product. Comparison of chromatograms of normal cat urine containing authentic BW 467C60 with those of aqueous drug showed that the urinary constituents slightly retarded the drug (R_F 0·81 and 0·77 respectively). Addition of the oily extract to the same specimen of normal urine gave a spot at R_F 0·77.

Radioassay of respired carbon dioxide from a rat kept in a metabolism apparatus after receiving the labelled drug (1 mg.) intraperitoneally showed that 0·08 μg. was completely oxidised during 24 hr. and 0·81 μg. in the subsequent 24 hr. (total 0·89 μg. = 0·09 per cent of the dose).

TABLE III

EXCRETION OF N-BENZYL-$N'N''$-DIMETHYLGUANIDINE (BW 467C60) IN CAT URINE
Figures are mg. drug as hydriodide calculated on radioactivity present

Dose	Time after dose							Total excreted	Per cent of dose
	24 hr.	48 hr.	72 hr.	96 hr.	120 hr.	144 hr.	168 hr.		
9·3 mg. s.c.	5·25	1·18	0·284	0·128	0·095	0·024	–	6·961	75
8·4 mg., s.c.	4·15	0·80	0·95	–	–	–	–	5·90	70
9 mg., oral after feeding	1·39	0·90	0·12	0·203	0·040	0·050	0·010	2·713	30
9 mg., oral after fasting	3·38	0·87	0·42	0·108	0·046	0·028	0·017	4·869	54

DISCUSSION

The distribution of BW 467C60 in cats at a time when there is a maximum effect on the nictitating membranes resembles that of bretylium (Boura, Copp, Duncombe, Green and McCoubrey, 1960) and its analogues (Boura, Duncombe and McCoubrey, 1961). The amounts in adrenergic nerves and ganglia were considerably higher than in other nerves and

ganglia and, on the whole, were higher than in other tissues. Assuming that the drug was present solely in the aqueous phase of an adrenergic ganglion the concentration there varied from about 1·3 to 6·6 × 10^{-5}M. The comparable figures for bretylium and BW 172C58 are 2·87 to 9·9 × 10^{-4}M and 3·8 to 6·0 × 10^{-6}M respectively. It seems that anti-adrenergic activity of these three drugs in the cat is associated with their specific affinity for adrenergic nerve tissue though, as concluded previously (Boura, Duncombe and McCoubrey, 1961), there is no simple quantitative relationship. It is possible that an adequate concentration of the drug is needed in the aqueous phase of the adrenergic tissue to maintain a loose chemical combination of the drugs with some unknown tissue component by mass action. Other hypothetical explanations are possible but information is needed on the detailed disposition within a ganglion to reach any firm conclusion. It is interesting that those tissues with high noradrenaline content, liver, spleen, and heart, had relatively high concentrations of these drugs, though there was little affinity for the adrenal.

BW 467C60 appears to escape metabolic modification and its urinary excretion showed no peculiar characteristics.

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BIOCHEMICAL PROPERTIES OF BRETYLIUM

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The effect of bretylium (10^{-3} M) on the respiration of brain slices and certain enzyme systems has been investigated. This concentration of drug is similar to that present in cat sympathetic ganglia after doses of bretylium sufficient to impair adrenergic nerve function. It was a weak inhibitor of monoamine oxidase, but had no effect on the other enzymes investigated. Prolonged treatment with bretylium (10 mg./kg.) did not affect the catecholamine levels of cat adrenals and sympathetic ganglia; larger doses depleted the amine from the ganglia. The noradrenaline content of rabbit spleen tended to rise after chronic dosage with the drug and this treatment appeared to render the amine refractory to the depleting action of reserpine.

Bretylium (N-o-bromobenzyl-N-ethyl-NN-dimethylammonium) can impair the function of the peripheral adrenergic nervous system without affecting cholinergic nerves (Boura and Green, 1959). It accumulates in the adrenergic neurones of cats (Boura, Copp, Duncombe, Green and McCoubrey, 1960) and at the time when the adrenergic blocking action is at a peak, judged by the degree of relaxation of the nictitating membranes, the sympathetic ganglia have considerably higher concentrations of bretylium than the other tissues examined. If the bretylium in the sympathetic ganglia at the time of peak effect were uniformly distributed in the tissue fluids, the concentration there would be of the order 10^{-3}M. This high concentration could be inhibitory to neural biochemical reactions if the possibility is envisaged that bretylium is a drug with relatively non-specific biochemical properties but whose pharmacological effects are rendered specific by selective localisation. To test this hypothesis the effect of bretylium at about 10^{-3}M concentration was examined on a range of biochemical systems.

EXPERIMENTAL

Bretylium bromide and iodide were supplied by Dr. F. C. Copp.

Respiration of brain slices. Slices of guinea-pig cerebral cortex about 0·35 mm. thick were cut manually by template and razor blade. They were trimmed in cold oxygenated salines to about 70 mg. weight, drained on glass and weighed on a torsion balance. They were incubated under oxygen in salines (2 ml.) at 37° and the oxygen uptake followed manometrically for periods up to 2·5 hr. Drugs were added to the saline just before gassing the vessels with oxygen.

Salines. These were basically either a glucose-phosphate saline or a supplemented saline low in bicarbonate (medium II of Krebs, 1950). The potassium content of the latter was sometimes raised to 21 mM by

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using the calculated proportion of potassium bicarbonate to replace some of the sodium bicarbonate needed to neutralise the acidic additives. In some experiments additional potassium chloride equivalent to 20 or 30 mM, or ammonium chloride to 10 mM was added to the glucose phosphate saline. In a few experiments glucose was replaced by sodium L-glutamate, succinate, pyruvate or oxoglutarate (0·02M).

Assay of bretylium in brain slices. ^{14}C-*N*-Methyl labelled bretylium iodide was used in some experiments to facilitate determination of the amount of drug present in brain slices after completion of manometry. Slices from 5 flasks (about 400 mg. wet tissue) were pooled after rinsing for 2 sec. in saline containing unlabelled bretylium at the concentration used during manometry. They were drained on glass, dried at 100°, powdered and plated on polythene planchettes for counting to ±10 per cent error at infinite thickness under an end window counter. The results were referred to known standards by combustion of a few samples in oxygen and counting the carbon dioxide as gas. The author is indebted to Dr. W. G. Duncombe for these standards and for counting the solid samples. The concentrations in the slices were calculated relative to the initial wet weight of the tissue and were not corrected for swelling. An increase in weight by one third due to swelling (Stern, Eggleston, Hems and Krebs, 1949) would reduce the stated concentrations by about one quarter.

Ammonia production by brain slices. The saline medium after incubation of brain slices (about 100 mg. in 2 ml.) was removed, rapidly cooled to 0° and centrifuged. An aliquot (1·5 ml.) was taken for determination of ammonia by the microdiffusion method of Conway (1947) using 0·002N HCl for the titration. The standard deviation in determination of ammonia from 20 μg. amounts of ammonium chloride was ±3·7 per cent in 5 experiments.

Assay of catecholamines. Tissues from cats or rabbits were dissected, cooled in ice, weighed and homogenised in 6 per cent trichloroacetic acid. They were assayed the same day by the fluorimetric method of Euler and Floding (1956). The cats were exsanguinated from the aorta under ether anaesthesia. The author is indebted to Mr. A. L. A. Boura for supplying the tissue specimens and for dosing the animals. Recovery of 2·7 μg. amounts of noradrenaline added to rat liver homogenates averaged 52 per cent in 4 trials (range 41–65). Bretylium did not fluoresce under the conditions of assay and there was no evidence for the presence of fluorescent metabolites in the tissues or urines from treated animals (Duncombe and McCoubrey, 1960).

Enzymes

Adenosine triphosphatase. An acetone dried powder of rat liver was prepared and assayed as described by Lardy and Wellman (1953). The adenosine triphosphate was 75 per cent pure and contained no inorganic phosphate, using the analytical methods of Eggleston and Hems (1952). The inorganic phosphate liberated by the enzyme was determined by the method of Weil-Malherbe and Green (1951).

Carbonic anhydrase. The enzyme was provided by cat erythrocytes, prepared by the method of Roughton and Booth (1956) for assay by the veronal buffer method of Miller, Dessert and Roblin (1950).

Choline dehydrogenase. The enzyme was provided by acetone-dried rat liver mitochondria, solubilised by sodium desoxycholate and assayed as described by Williams and Sreenivasan (1953).

Dopa decarboxylase. The enzyme was obtained from rabbit kidney cortex and assayed manometrically (Bertler and Rosengren, 1959).

Glucose-6-phosphate dehydrogenase. The enzyme was provided by an extract of rat adrenals and was assayed spectrophotometrically (Glock and Maclean, 1954).

Glutamic decarboxylase. The enzyme was obtained from a homogenate of guinea-pig brain in 0·05M phosphate buffer, pH 5·9, fortified with pyridoxal phosphate (1 mg. for two brains in 15 ml. buffer). Activity was assayed manometrically (Roberts and Frankel, 1951).

Monoamine oxidase. The enzyme was provided by an acetone dried preparation of guinea-pig liver mitochondria and was assayed manometrically with oxygen gas phase at 37° in phosphate buffer pH 7·4. Substrates, all at 0·01M final concentration were tryptamine, tyramine, 5-hydroxytryptamine and 3-hydroxytyramine. A short pre-incubation period of 10 min. with the drugs preceded addition of substrate from the side arm.

Thiaminase. Fresh bracken was extracted and assayed as described by Kenten (1958) using piperidine as the acceptor amine.

Trypsin. A 1 per cent solution of commercial trypsin (1 ml.) was incubated at 37° with bovine albumen (10 mg.) in 0·05M phosphate buffer pH 7·5 (3 ml.) for 1 hr. Protein was assayed as described by Robinson and Hogben (1943).

RESULTS

Respiration of brain slices. Table I shows that bretylium at 5×10^{-4}M inhibited the resting respiration of guinea-pig cerebral cortex slices in glucose phosphate saline containing 6·7 mM potassium ion by 21 per

TABLE I

INHIBITION OF RESPIRATION OF BRAIN SLICES BY BRETYLIUM

Guinea-pig cerebral cortex slices were incubated in glucose
phosphate saline containing 5×10^{-4} M bretylium
Figures express μmoles O_2/g. tissue/hr. with S.D.

	6·7 mM K⁺	26·7 mM K⁺	36·7 mM K⁺
No bretylium	56 ± 8	70 ± 12	82 ± 11
With bretylium	44 ± 6	60 ± 8	80 ± 9

cent. It almost suppressed the 25 per cent increase in respiratory rate due to additional 20 mM potassium ion but had no effect on the larger increment in rate due to 30 mM potassium ion. Bretylium was inactive at 10^{-4}M in similar experiments. The drug (10^{-3}M) had no influence on

the rate of respiration of rat liver slices in glucose phosphate saline. The rate of respiration of brain slices in the potassium enriched saline with added bretylium sometimes slackened progressively after 1 hr. incubation. The effect was not consistent even with slices from the same animal and the degree of slackening varied considerably. Of the results from 113 slices there was no slackening observed in those without added bretylium (56) whether the saline was potassium enriched or not, or in those with bretylium but without additional potassium (14). Slackening however occurred in 20 of 36 experiments with both bretylium and additional potassium. Attempts to make this effect reproducible for further study by replacing glucose with other substrates, L-glutamate, pyruvate, succinate or oxoglutarate (0·02M), or by reducing endogenous substrate by incubation of the slices for 30 min. before adding glucose, were all unsuccessful. In a short series of experiments, histamine phosphate 10^{-4}M, a component of adrenergic nerves (Rexed and Euler, 1951), also prevented the increment in respiratory rate of brain slices in glucose phosphate saline due to added potassium salts but there was no slackening of rate as observed for bretylium. Histamine did not enhance the inhibitory effect of bretylium on respiration.

TABLE II

INHIBITION OF RESPIRATION OF BRAIN SLICES BY BRETYLIUM

Guinea-pig cerebral cortex slices were incubated in the supplemented medium II of Krebs (1950) with the stated K^+ concentration. The average rates of oxygen uptake during the first and second hours are expressed as μmoles O_2/g. tissue/hr. $\pm$ S.D.

			5·9 mM K^+		21·1 mM K^+	
			A	B	A	B
1st hr.	..	..	89 $\pm$ 9	92 $\pm$ 7	105 $\pm$ 16	93 $\pm$ 18
2nd hr.	..	..	66 $\pm$ 15	59 $\pm$ 12	80 $\pm$ 14	53 $\pm$ 14

A. No bretylium. B. With bretylium (10^{-3} M)

Brain slices incubated in the supplemented medium low in bicarbonate (Krebs, 1950) respired at higher rates compared to those in glucose phosphate saline and these progressively slackened during 2·5 hr. During the second hr. the average rate was 80 per cent of that during the first hr. The initial respiratory rate was slightly increased by addition of potassium salts to a final concentration of 21 mM and fell by an average of 24 per cent during the second hr. In this saline without additional potassium salts the fall in respiratory rate during the second hr. was slightly increased by bretylium (10^{-3}M) to 36 per cent. With additional potassium salts the rate fell by 43 per cent. Bretylium had no influence on the initial rate of respiration in this saline without additional potassium. Bromide ion (10^{-3}M) introduced by the bretylium, had no influence when added as sodium bromide. Results are summarised in Table II.

Brain slices respiring in glucose phosphate saline with added ammonium chloride (10 mM) showed a slightly enhanced rate of oxygen uptake (60 $\pm$ 5 μmoles/g. tissue/hr. which was reduced to 45 $\pm$ 9 μmoles/

g./hr. by addition of bretylium (10^{-3}M). The flasks without bretylium showed a 15 per cent reduction in rate during the second hr. of incubation whereas those with bretylium showed a 50 per cent reduction.

Ammonia production by brain slices. Bretylium (10^{-3}M) had no influence on the amount of ammonia released into glucose phosphate saline by brain slices incubated anaerobically for 1 hr. ($3\cdot3 \pm 1\cdot0$ μmoles/g./hr.). There was a 30 per cent fall in ammonia output due to added bretylium by slices incubated anaerobically in the Krebs supplemented saline during 2·5 hr. but the difference was not significant in 6 paired results ($1\cdot52 \pm 0\cdot68$ compared to $1\cdot07 \pm 0\cdot59$ μmoles/g./hr.). Table III shows that the ammonia liberated into glucose-free phosphate saline under aerobic conditions was reduced by bretylium at 1 hr. but there was no difference at 3 hr. The drug did not inhibit tryptic digestion of albumen.

TABLE III

PARTIAL INHIBITION OF AMMONIA PRODUCTION IN BRAIN SLICES BY BRETYLIUM
Guinea-pig cerebral cortex slices were incubated aerobically with
bretylium (10^{-3} M) in a glucose-free phosphate saline
Figures expressed in μmoles NH_3/g. tissue are means of 4 results

	0·5 hr.	1 hr.	2 hr.	3 hr.
Control	5·5 ± 0·5	8·6 ± 1·3	11·6 ± 1·8	13·4 ± 2·0
Bretylium	—	4·7 ± 0·7	8·6 ± 2·1	11·8 ± 1·3

Uptake of bretylium by brain slices. The mean concentration of bretylium in brain slices after respiring aerobically for 1 hr. in glucose phosphate saline containing 0·5 μmoles/ml. drug was $1\cdot44 \pm 0\cdot71$ μmoles/g./wet tissue. In saline with additional potassium ion (20 mM) this concentration was reduced to $0\cdot67 \pm 0\cdot33$ μmoles/g. ($P = 0\cdot1$). The bretylium was washed out of the slices fairly readily, one wash in saline reducing the concentration to about one quarter. After washing the respiratory rate returned to normal control value.

Influence of bretylium on enzymes. Of the nine enzymes tested, bretylium (10^{-3}M) inhibited only monoamine oxidase. Using tryptamine or 3-hydroxytyramine as substrate, inhibitions were 39 ± 4 and 38 ± 2 per cent respectively (means of 4 results). With tyramine or 5-hydroxytryptamine as substrate the inhibitions were 73 ± 8 and 69 ± 11 per cent respectively. The inhibition was readily reversed by washing. These results are comparable in so far as they were obtained with one batch of enzyme. Bretylium was almost inactive at 10^{-4}M.

Influence of bretylium on the catecholamine content of tissues. Table IV shows that the levels of noradrenaline in representative cat tissues after one dose of saline were reasonably constant. Cats receiving bretylium (10 mg./kg. s.c.) showed more variation in tissue amine levels with a tendency to increased values in the sympathetic ganglia. The levels in spleen and heart were little affected though there may have been some reduction after two weeks of daily dosage. The high values found in sympathetic ganglia could not be accounted for by an increased proportion of adrenaline which, in the assay used, gives a higher fluorescence intensity

compared to noradrenaline. The levels of adrenaline remained barely detectable. A larger dose of bretylium (30 mg./kg.) daily for 14 days caused about 50 per cent fall in the noradrenaline content of the superior cervical and stellate ganglia (P = 0·01). A similar fall was found in spleen and heart, but the adrenal amine levels remained unaffected. The loss of noradrenaline in sympathetic ganglia, spleen and heart due to the larger dose of bretylium was not enhanced by reserpine (1 mg./kg.) given with the last dose of bretylium about 12 hr. before killing the animal but the adrenals showed a marked depletion of their catecholamines. By contrast the spleens of 4 rabbits given bretylium (30 mg./kg.) daily for one month contained 0·62 ± 0·43 μg./g. noradrenaline compared to 0·27 ± 0·11 μg./g. in 4 controls. The adrenals were not affected.

TABLE IV

THE CATECHOLAMINE CONTENT OF CAT TISSUES AFTER PROLONGED DOSAGE WITH BRETYLIUM

Bretylium bromide given daily to cats, s.c. The figures are μg./g. wet tissue by fluorometric assay. Control values are the means from 3–5 cats receiving one dose of saline.

Figures given for the 30 mg./kg. dose are the mean of 3 results

Duration of dosage (days)	10 mg./kg. dose							Adrenaline
	Noradrenaline							Adrenaline
	Superior cervical ganglion	Stellate ganglion	Coeliac ganglion	Splenic and gastric nerves	Spleen	Heart	Adrenal	Adrenal
Controls	5·5 ± 0·7	5·7 ± 0·5	9·4 ± 4·9	5·7 ± 2·7	0·56 ± 0·15	0·50 ± 0·07	355 ± 171	342 ± 54
1	4·9	2·7	10·5	12·5	—	—	436	598
3	10·7	17·1	1·9	8·8	0·6	0·6	—	—
7	5·0	14·6	33·2	20·2	—	0·3	—	—
10	7·4	6·4	18·7	4·2	0·8	0·4	173	358
15	—	11·6	21·7	0·7	0·2	0·1	340	149
22	—	11·0	—	0·8	0·6	0·6	427	366
31	3·0	4·5	11·9	—	0·3	0·1	414	1005
	30 mg./kg. dose							
14	2·2 ± 1·1	1·8 ± 1·6	—	—	0·28 ± 0·23	0·07 ± 0·04	709 ± 286	509 ± 128
14*	2·6 ± 1·2	2·2 ± 1·3	—	—	0·11 ± 0·00	0·20 ± 0·20	102, 231	149, 270

* Reserpine (1 mg./kg.) given on the 13th day.

When reserpine (1 mg./kg.) was given to rabbits followed at daily intervals by either saline or bretylium (30 mg./kg.), the noradrenaline content of the spleen fell more slowly in the bretylium group (Table V). The long delay in the return of noradrenaline content in the spleens of these rabbits. is notable. They were sensitive to reserpine and 2 mg./kg. caused delayed fatalities.

DISCUSSION

Since a pharmacologically effective dose of bretylium (10 mg./kg.) given daily for as long as 31 days did not deplete the catecholamine content of sympathetic ganglia or adrenals, it seems reasonable to exclude the inhibition of biosynthesis of noradrenaline as a mode of action of the drug, unless the biosynthesis is linked firmly to the processes of release. The tendency to higher values of noradrenaline in sympathetic ganglia after prolonged dosage at this level suggests rather that the catecholamine

release mechanism is impaired and accumulation of the amine occurs by continued, though possibly slower, biosynthesis. Conversely, the depletion of the amine from the ganglia by larger doses suggests that the noradrenaline storage mechanism becomes deranged, indicating an affinity of bretylium with guanethidine or reserpine, both of which deplete tissue noradrenaline stores. The noradrenaline content of the spleen of rabbits increased, though the wide scatter of the results suggest that the effect is inconsistent under the experimental conditions described. The result however is consistent with the finding that the release of noradrenaline from rabbit spleen by reserpine appeared to be slower when bretylium was given subsequent to the dose. The results obtained with

TABLE V

NORADRENALINE CONTENT OF RABBIT SPLEEN AFTER RESERPINE FOLLOWED
BY DAILY DOSES OF BRETYLIUM

Reserpine (1 mg./kg.) was followed after 12 hr. by bretylium (30 mg./kg.) or saline at daily intervals. Figures are μg./g. noradrenaline of single rabbits.
Normal controls 0.27 ± 0.11 μg./g.

	Days after reserpine									
	1	4	8	9	11	15	17	19	22	26
Saline	0.084	0.057	0.063	0.015	0.044	—	0.050	0.061	0.027	0.166
Bretylium ..	—	0.037	0.185	0.136	0.133	0.114	0.050	0.160	0.074	0.058

rabbits suggest that bretylium may limit the egress of noradrenaline from its site of biosynthesis or its binding site, possibly by affecting membrane permeability. These alternative hypotheses seem preferable to the hypothesis that bretylium has non-specific biochemical properties rendered pharmacologically specific by localisation at selected sites. On the contrary, the drug has no influence on a number of enzymes that attack substrates with formal resemblance to bretylium. The evidence presented here suggests that bretylium may produce its effects by specifically affecting catechol amine storage and release mechanisms.

The partial inhibition of ammonia output by brain slices deprived of glucose derives interest from observations (Larrabee, Horwicz, Stekiel and Dolivo, 1957) that stimulated sympathetic ganglia can oxidise nitrogenous substrates (see, however, Borowicz and Larrabee, 1962), but further work on this topic was deferred since major sources of ammonia in nervous tissue are conjectural and probably composite. Unlike a related drug, benzethonium (Beck, Pinter and McKenna, 1960), bretylium did not inhibit trypsin.

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A NOTE ON THE INFLUENCE OF CHLORPROMAZINE AND DIETHAZINE ON THE STORES OF CATECHOLAMINE IN THE ADRENAL GLANDS AND AORTIC WALLS OF RATS

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Stores of catecholamines accumulated in the adrenal glands and in the walls of the aortae of rats during treatment with diethazine or chlorpromazine. Diethazine failed to antagonise the release by reserpine of tissue stores of catecholamines and increased tissue sensitivity to the pressor effects of tyramine, probably because release of catecholamines from tissue stores by tyramine is enhanced. Chlorpromazine antagonised the release of adrenal medullary amines by reserpine and delayed the restoration of these stores after depletion.

THE factors controlling the magnitude of the stores of catecholamine in the walls of blood vessels (Schmiterlow, 1948) and the part these stores may play in postganglionic sympathetic activity are little understood. For this reason we were interested to compare the effect of two amino-derivatives of phenothiazine on the catecholamine stores in the adrenal glands and aortae of rats. The two compounds, chlorpromazine and diethazine, were selected because of known differences in their actions within the autonomic nervous system. Chlorpromazine depresses vaso-motor reflexes operative through the medulla and hypothalamus (Das-gupta and Werner, 1954) and has peripheral anti-adrenaline action (Courvoisier, Fournel, Ducrot, Kolsky and Koetschet, 1953) but exerts no blocking action in sympathetic ganglia (Reuse, 1954; Holzbauer and Vogt, 1954). By contrast, diethazine does not block cardiovascular reflexes in the medulla and has no anti-adrenaline action: it causes peripheral vasodilation by depression of ganglionic transmission and sup-presses cardiac inhibitory reflexes by means of an atropine-like effect (Heymans, 1949).

METHODS

Female wistar rats, 150 to 200 g. in weight, fed diet 41 b of Stein with water, were divided at random into groups which were separately housed under similar conditions of heating, lighting and humidity. Different daily treatments were assigned to each group of rats. Thus: saline only; chlorpromazine 5 mg./kg. or diethazine 10 mg./kg. once daily or mec-amylamine 7·25 mg./kg. twice daily, each in 0·2 ml. 0·9 per cent NaCl w/v, by stomach tube. Treatments were given on four consecutive days; experiments were made on the fifth day.

Preparation of Extracts

Adrenal glands and aortae, removed under deep ether anaesthesia, were rapidly weighed, sliced and ground in ice cold 0·1N HCl with a knife point of silver sand. Each extract was transferred with washings (total volume, adrenals 5 ml.; aorta, 2·5 ml.) to a centrifuge tube, which was heated in a boiling water bath for 2·5 min., rapidly cooled and spun.

Supernatants were stored for 3 to 7 hr. at $-7°$ and were thawed and brought to pH 6·7 with 0·5N NaOH and to a known volume just before bioassay. Assays of total catecholamine in extracts of adrenal glands were made either on the rat colon (Gaddum, Peart and Vogt, 1949) or on the mean arterial pressure of the spinal cat, using 4×4 Latin Square designs and $(-)$-adrenaline as standard. The adrenaline present in extracts of aortae was assayed on the rat uterus (Gaddum, Peart and Vogt, 1949) against $(-)$-adrenaline; since the quantities of amine in these extracts were small it was rarely possible to complete more than three lines of a 4×4 block design. Assays of tyramine in terms of $(-)$-noradrenaline were made on the mean arterial pressure of treated and control rats under urethane anaesthesia.

TABLE I

THE EFFECT OF DRUGS ON THE TOTAL CATECHOLAMINE IN THE ADRENAL GLANDS OF RATS. CHLORPROMAZINE 5 MG./KG., DIETHAZINE 10 MG./KG. ONCE DAILY, MECAMYL-AMINE 7·5 MG./KG. TWICE DAILY, ORALLY, FOR 4 DAYS. RESERPINE 5 MG./KG., I.P. ONCE ON THE FOURTH DAY

Drug used	Body weight, g.	Assay method	Total catecholamine in adrenal glands as $(-)$-adrenaline, μg.
Chlorpromazine	166 ± 5·2 (5)	Cat B.P.	76·4 ± 6·7 (5)**
Diethazine	166 ± 6·8 (5)		99·0 ± 9·2 (5)**
Mecamylamine	160 ± 5·3 (5)		55·0 ± 6·7 (5)*
None	172 ± 4·7 (5)		42·6 ± 4·0 (5)
Chlorpromazine	178 ± 4·4 (8)	Rat colon	88·9 ± 13·4 (8)**
Diethazine	181 ± 3·6 (8)		82·4 ± 7·8 (8)**
None	183 ± 6·3 (8)		36·6 ± 5·6 (8)
Chlorpromazine	170 ± 5·5 (6)	Rat colon	66·6 ± 7·4 (6)*
Reserpine	189 ± 5·8 (6)		23·7 ± 4·2 (6)*
Chlorpromazine, Reserpine	168 ± 5·3 (6)		59·7 ± 8·1 (6)
Diethazine	173 ± 6·2 (6)		83·3 ± 6·7 (6)*
Diethazine, Reserpine	175 ± 5·7 (6)		41·6 ± 5·9 (6)††
None	191 ± 6·4 (6)		46·8 ± 5·7 (6)

The significance of drug effects has been examined by '*t*' test and is indicated by asterisks for the effect of a single drug and by † for the action of a second drug: one, $P = <0·05$; two, $P = <0·01$.
The values shown are means ± standard errors of the means followed by the numbers of rats within brackets.

RESULTS

Daily oral treatment of rats with either chlorpromazine, 5 mg./kg., or diethazine 10 mg./kg. for 4 days had, by the fifth day increased the total pressor catecholamine in the adrenal glands (Table I) and the adrenaline extractable from the aortic walls (Table II). Whereas reserpine

TABLE II

THE EFFECT OF DRUGS ON THE STORES OF ADRENALINE IN THE WALLS OF RAT AORTAE. TREATMENTS AS IN TABLE I

Drug used	Body weight, g.	Assay method	ng. $(-)$-adrenaline/100 mg. aorta
Chlorpromazine	174 ± 7·2 (5)	Rat uterus	36 ± 5·2 (5)**
Diethazine	181 ± 6·2 (6)		52 ± 9·0 (6)**
None	177 ± 5·8 (6)		14 ± 2·3 (6)
Chlorpromazine	170 ± 5·5 (6)	Rat uterus	28 ± 4·7 (6)*
Reserpine	189 ± 5·8 (6)		10 ± 4·3 (6)
Chlorpromazine, reserpine	168 ± 5·3 (6)		26 ± 4·9 (6)
Diethazine	173 ± 6·2 (6)		49 ± 5·4 (6)*
Diethazine, reserpine	175 ± 5·7 (6)		27 ± 4·3 (6)†
None	191 ± 6·4 (6)		15 ± 3·4 (6)

Tests for the significance of drug effects as for Table I.

5 mg./kg. i.p., given on the fourth day, depleted greatly or reduced the catecholamine stored in the adrenal glands of normal rats and of rats under treatment with diethazine, reserpine was ineffective in this respect in the rats pretreated with chlorpromazine (Table I). Significant reduction in the adrenaline extractable from the aortic walls was demonstrable, after reserpine only, in those rats which had been pretreated with diethazine (Table II). Mecamylamine, 7·5 mg./kg. orally twice daily for 4 days, also increased the total catecholamine in the adrenal glands (Table I).

TABLE III

THE EFFECT OF DRUGS ON THE RESTORATION OF CATECHOLAMINE IN RAT ADRENAL GLANDS AFTER DEPLETION BY RESERPINE. RESERPINE 5 MG./KG. I.M. TO ALL ANIMALS AT ZERO TIME. THEREAFTER, CHLORPROMAZINE 5 MG./KG. OR DIETHAZINE 10 MG./KG. ORALLY AT 6 HR., THEN 12 HRLY. EXPERIMENTS AT 40 TO 46 HR.

Drugs used	Body weight, g.	Assay method	Total catecholamine as µg. (−)-adrenaline
Reserpine alone	193 ± 6·9 (6)	Rat colon	35·6 ± 6·8 (6)
Reserpine, chlorpromazine ..	190 ± 6·5 (6)		20·7 ± 4·2 (6)*
Reserpine, diethazine	189 ± 7·8 (6)		51·3 ± 5·4 (6)*
Reserpine alone	175 ± 4·7 (6)	Rat colon	44·6 ± 8·1 (6)
Reserpine, chlorpromazine ..	178 ± 6·2 (6)		33·3 ± 6·4 (6)
Reserpine, diethazine	177 ± 5·9 (6)		53·8 ± 7·4 (6)

Test for significance as in Table I.

Daily oral treatment of rats for 4 days with diethazine 10 mg./kg. increased the pressor effect of tyramine relative to (−)-noradrenaline. Chlorpromazine was ineffective in this respect (Table IV).

TABLE IV

THE EFFECT OF DRUGS ON RELATIVE SENSITIVITY OF RATS UNDER URETHANE ANAESTHESIA TO THE PRESSOR EFFECTS ACTIONS OF (−)-NORADRENALINE BITARTRATE AND TYRAMINE HYDROCHLORIDE. TREATMENTS AS IN TABLE I

Drug used	µg. tyramine HCl equivalent to 1 µg. (−)-noradrenaline bitartrate
None	141 ± 21·6 (6)
Chlorpromazine ..	125 ± 23·4 (6)
Diethazine	73 ± 12·4 (6)*

Tests for significance as in Table I.

DISCUSSION

The results of experiments summarised in Tables I and II clearly demonstrate the accumulation of stores of catecholamine in the adrenal glands and in the walls of the aortae of rats during treatment with either diethazine or chlorpromazine. Similar accumulation of adrenal medullary stores results from the prolonged administration of small quantities of the ganglion blocking agents hexamethonium, tetraethylammonium or pentolinium ions (Mawji and Lockett, 1962) and from treatment with mecamylamine (Table I). Diethazine resembles the ganglion blocking drugs (Mawji and Lockett, 1962) in failing to antagonise the action of reserpine on tissue stores (Tables I and II) but differs from the ganglion

blocking drugs in increasing sensitivity to the pressor effects of tyramine (Table IV). This potentiation of the pressor effects of tyramine is almost certainly attributable to enhancement of the release of catecholamine from tissue stores by tyramine (Burn and Rand, 1958, 1959; Lockett and Eakins, 1960; Euler and Lishajko, 1960) since the action of reserpine on tissue stores appears to be increased by treatment with diethazine (Table II). Chlorpromazine, like cocaine (Mawji and Lockett, 1962) antagonised the release of adrenal medullary amines by reserpine (Table I) and delayed the restoration of these stores after their depletion (Table III) probably by reduction in the rate of resynthesis of catecholamine.

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THE EFFECTS OF HYDROCORTISONE ON THE CHANGES IN LIPID METABOLISM INDUCED IN GUINEA-PIG LUNG TISSUE BY ANAPHYLAXIS *IN VIVO*

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Exposure of sensitised guinea-pigs to aerosolised antigen causes alterations in the lipid metabolism of their lung tissue. The responses of control animals exposed to aerosolised distilled water has suggested that these metabolic disturbances are probably a manifestation of a "stress reaction". Pretreatment of sensitised animals with anti-anaphylactic dosage of sodium hydrocortisone hemisuccinate abolished this reaction.

CERTAIN forms of stress influence the anaphylactic reaction in man; thus localised infections, traumatic shock, surgical shock and pregnancy all temporarily relieve asthmatic symptoms. But attempts by Samter and Kofoed (1952) and Feinberg, Malkiel and McIntire (1953) to demonstrate a similar protection in sensitised guinea-pigs using sterile abscesses and treatment with piromen were unsuccessful.

The hypothesis that the beneficial effects of stress were mediated by the adrenal-hypophyseal axis received some confirmation when good clinical results were obtained in asthmatic patients treated with cortisone or ACTH (Bordley, Carey, Harvey, Howard, Kattus, Newman and Winkwerder, 1949; Carryer, Koelsche, Prickman and Maytum, 1950; M.R.C., 1956). In addition, Eriksson-Lihr (1951), Rose, Fyles and Venning (1955) and Siegel, Ely, Birnberg and Kelley (1956) found diminished 17-ketosteroid excretion in asthmatics and this seemed to be related to the severity of the disease. Furthermore, Kenipow (1922) found that partially adrenalectomised guinea-pigs showed increased sensitivity to anaphylactic reactions.

But it is usually impossible in a sensitised guinea-pig to prevent death from anaphylactic shock with cortisone or ACTH (Freidlander and Friedlander, 1950; Leger, Leith and Rose, 1948; Dworetsky, Code and Higgins, 1950; Malkiel, 1951). Herxheimer and Rosa (1952) also showed that a single injection of cortisone given a short time before exposure to aerosolised antigen did not influence the time for production of dyspnoea and cough in actively sensitised guinea-pigs. In 1953, Feinberg, Malkiel and McIntire reported cortisone to be capable of prolonging the "preconvulsion time" in passively sensitised animals, provided that the drug was administered 18 hr. before exposure to aerosolised antigen.

Recent investigations by Smith (1962) using an isolated, sensitised guinea-pig lung have demonstrated marked changes in lipid content during anaphylactic shock. Alterations in the lipid metabolism of several organs as a result of cortisone administration have been reported

in man (Adlersberg, Schaefer and Dritch, 1950), in rabbits (Adlersberg, Schaefer and Wang, 1951) and in rats and guinea-pigs (Hausberger, 1958).

The purpose of this investigation was to examine any changes occurring in the lung lipid content of intact sensitised guinea-pigs when exposed to aerosolised antigen and to determine possible modifications induced by corticosteroid therapy.

METHODS

Pharmacological

Guinea-pigs, 250 to 350 g., fed on Diet 18 (Oxo) and receiving 50 mg. of ascorbic acid every morning in solution in drinking water contained in amber glass bottles were sensitised to egg albumin (BDH) by the intra-peritoneal injection of 100 mg. (5 per cent) in water.

Three weeks after sensitisation, eight animals were killed by a blow on the head, their lungs were excised and perfused for 10 min. at 1 ml./min. through the pulmonary artery with Tyrode's solution at 37° to remove blood, and then chopped into small pieces and freeze dried. This was the control group.

After sensitisation a second group of eight animals was injected with 50 mg./animal (about 100 mg./kg.) of sodium hydrocortisone hemi-succinate intramuscularly, killed, and their excised lungs treated similarly. This was the "treatment control group".

Three weeks after sensitisation six groups of eight animals were exposed to an aerosol of 1 per cent egg albumin. Thereafter they were exposed to antigen at weekly intervals (Herxheimer, 1951). After exposures to determine their "normal collapse time" (Smith, 1961), each group was divided into two subgroups of four animals of approximately equal sensitivity to antigen. One week later one subgroup was exposed to antigen as before and the other subgroup received aerosolised distilled water for a period of time equivalent for each animal to its normal collapse time.

The animals in Groups I, II, III received no premedication but the animals in Groups IV, V, VI received 50 mg. per animal of sodium hydrocortisone hemisuccinate intramuscularly 18 hr. before exposure to aerosol. Group I and IV were killed 15 min. after exposure to aerosol. Groups II and V were killed 30 min. and Groups III and VI 1 hr. after exposure to aerosol. All the lungs were excised, chopped and freeze dried.

Biochemical

Each freeze dried lung was powdered and then extracted for 24 hr. with 200 times its weight of chloroform : methanol (12 : 1). After filtration, the extract was dried *in vacuo*, redissolved in the original volume of chloroform and stirred for 5 min. with 5 g. of silicic acid (Malinkrodt). The filtrate was examined for cholesterol by the method of Hanel and Dam (1955) and glyceride by the method of Van Handel and Zilversmit (1957). The silicic acid was allowed to dry and transferred to a volume of methanol equivalent to the original volume of extract. After 5 min.

the methanol was filtered and examined for lipid phosphorus (Bartlett, 1959). The fatty acids of the lipids present in the chloroform and methanol were methylated. An aliquot (5 ml.) of the methanol (phospholipid) solution was dried *in vacuo*, dissolved in 1 ml. of 1·7N methanolic hydrochloric acid and kept overnight at 37°. Water (2 ml.) was added and the solution extracted with 4 ml. of light petroleum (60–80°). The separated light petroleum was dehydrated with anhydrous sodium sulphate and then dried. The separated fatty acid methyl esters were dissolved in 0·2 ml. of light petroleum (80–100°). A larger aliquot (20 ml.) of the chloroform (neutral lipid) solution was first dried, and dissolved in 0·5 ml. of 0·1N ethanolic potassium hydroxide. After 30 min. at 37°, 0·5 ml. of 0·4N sulphuric acid was added, and the solution extracted with 4 ml. of light petroleum (60–80°). After separating and dehydrating the light petroleum layer, it was dried *in vacuo*. The fatty acids were then methylated with 1·7N methanolic hydrochloric acid.

The methyl esters of both neutral and phospholipids were examined by gas-liquid chromatography (Pye Argon). The stationary phases were Apiezon L grease at 190° and polyethylene glycol adipate at 175°. Identification of individual fatty acids was carried out by measuring the log relative retention times on these two columns (James, 1959).

Reagents

Hydrogen peroxide (100 vol.) (phosphorus free) was kindly supplied by Laporte Chemicals, Luton. A range of pure fatty acid standards for gas chromatography was kindly supplied by Prices (Bromborough) Ltd., Bromborough Pool, Near Birkenhead.

Other reagents and solvents were Analar grade, except zinc chloride, which was reagent grade.

TABLE I

LIPID CONTENT OF LUNGS FROM SENSITISED GUINEA-PIGS EXPOSED TO AN AEROSOL OF DISTILLED WATER OR AEROSOLISED ANTIGEN (EGG ALBUMIN). RESULTS AS MEAN ± STANDARD ERROR

Lipid fraction	Controls	Time after aerosol (hr.)		
		0·25	0·5	1·0
		Distilled water		
Cholesterol.. ..	19·45 ± 0·78	29·06 ± 2·17	19·14 ± 1·02	22·75 ± 2·84
Glyceride	19·10 ± 1·25	21·83 ± 2·28	81·53 ± 5·73	21·64 ± 3·76
Phospholipid ..	121·00 ± 4·50	130·97 ± 9·24	100·79 ± 9·59	77·04 ± 4·89
		Egg albumin		
Cholesterol.. ..	19·45 ± 0·78	21·98 ± 3·90	17·34 ± 0·39	17·39 ± 1·43
Glyceride	19·10 ± 1·25	30·08 ± 2·85	66·31 ± 14·58	21·16 ± 5·47
Phospholipid ..	121·00 ± 4·50	117·16 ± 15·25	101·81 ± 4·08	85·93 ± 4·88

RESULTS

The changes in the lung lipids seen in animals exposed to aerosolised distilled water are shown in Table I, together with corresponding changes in animals exposed to aerosolised antigen. The most prominent changes are a fall in phospholipid content, and an increase in the glyceride content which is pronounced 30 min. after exposure to aerosol. One hr. after

exposure, the glyceride content had returned to normal but the phospholipid level appeared to be still falling.

In Table II the lipid fractions of the "control group" and the "treatment control group" are compared. It can be observed that hydrocortisone treatment did not appreciably alter the lipid content of the lungs.

TABLE II

LIPID CONTENT OF LUNGS FROM GUINEA-PIGS IN THE CONTROL GROUP COMPARED WITH LUNGS FROM GUINEA-PIGS PRETREATED WITH HYDROCORTISONE. RESULTS AS MEAN ± STANDARD ERROR

Lipid fraction	Treatment Controls	Hydrocortisone treated
Cholesterol	19·45 ± 0·78	20·09 ± 0·55
Glyceride	19·10 ± 1·25	17·15 ± 1·68
Phospholipid ..	121·00 ± 4·50	125·29 ± 2·91

The results obtained in animals pretreated with hydrocortisone are given in Table III. In animals exposed to either antigen or distilled water there are only small changes of the lipid fractions.

TABLE III

LIPID CONTENT OF LUNGS FROM SENSITISED GUINEA-PIGS EXPOSED TO AN AEROSOL OF DISTILLED WATER OR TO AEROSOLISED ANTIGEN, 18 HR. AFTER PRETREATMENT WITH HYDROCORTISONE. RESULTS AS MEAN ± STANDARD ERROR

Lipid fraction	Treatment Controls	Time after aerosol (hr.)		
		0·25	0·5	1·0
		Distilled water		
Cholesterol	20·09 ± 0·55	19·78 ± 0·87	19·63 ± 0·88	19·45 ± 0·01
Glyceride	17·15 ± 1·68	22·28 ± 1·23	25·10 ± 2·57	16·95 ± 1·43
Phospholipid ..	125·29 ± 2·91	140·10 ± 3·43	126·60 ± 0·86	119·75 ± 9·91
		Antigen		
Cholesterol ..	20·09 ± 0·55	18·77 ± 0·44	17·55 ± 0·44	19·45 ± 0·02
Glyceride	17·15 ± 1·68	23·02 ± 3·05	25·15 ± 1·57	23·20 ± 4·31
Phospholipid ..	125·29 ± 2·91	131·25 ± 6·65	126·50 ± 3·02	116·95 ± 6·23

The results of the gas chromatographic analysis of the neutral and phospholipid fractions of some groups of animals are given in Table IV. These examinations were confined to the controls and lungs obtained 30 min. after exposure to aerosol.

From Table IV it can be concluded that the constituent fatty acids of both the neutral lipid and phospholipid fractions were substantially the same in both the "controls" and "treatment controls". By comparing the data in Table IV (A and B) the effects of exposing animals to either distilled water or antigen can be deduced. The constituent fatty acids of the neutral lipids of animals exposed to distilled water have higher proportions of 16:1 and 18:1 acids and less 18:0 acid than the controls. The neutral lipids of animals exposed to antigen are also different in proportional fatty acid content from their controls. They have less 18:0 and more 18:1 and 18:3 acids. The phospholipid fractions also show differences. Animals exposed to distilled water have less 14:0 and more 16:1 and 18:1 acids than their controls. Animals exposed to

TABLE IV

The proportional fatty acid analysis of the neutral lipid and phospholipid fractions of sensitised guinea-pig lungs (A) from control groups, (B) obtained 30 min. after exposure to aerosols of distilled water or antigen, (C) obtained 30 min. after exposure to aerosolised distilled water or antigen 18 hr. after pretreatment with hydrocortisone. The results are expressed as the mean per cent and standard error.

Fatty* acid	A Neutral lipids Controls	A Neutral lipids Treatment controls	A Phospholipids Controls	A Phospholipids Treatment controls	B Neutral lipids Distilled water	B Neutral lipids Antigen	B Phospholipids Distilled water	B Phospholipids Antigen	C Neutral lipids Distilled water	C Neutral lipids Antigen	C Phospholipids Distilled water	C Phospholipids Antigen
C14·0	2·77 S.E. 0·33	2·63 S.E. 0·12	3·30 S.E. 0·24	3·13 S.E. 0·20	2·46 S.E. 0·17	2·11 S.E. 0·20	2·47 S.E. 0·18	2·30 S.E. 0·37	3·37 S.E. 0·34	3·34 S.E. 0·38	3·51 S.E. 0·35	3·78 S.E. 0·27
U$_1$	Nil	0·12 S.E. 0·03	0·10 S.E. 0·03	0·14 S.E. 0·09	0·22 S.E. 0·04	0·19 S.E. 0·04	0·39 S.E. 0·08	0·46 S.E. 0·12	0·50 S.E. 0·17	0·44 S.E. 0·70	0·32 S.E. 0·19	0·63 S.E. 0·22
U$_2$	0·54 S.E. 0·09	0·43 S.E. 0·06	0·66 S.E. 0·07	0·67 S.E. 0·10	Nil	Nil	Nil	Nil	Nil	Nil	0·79 S.E. 0·30	0·25 S.E. 0·15
U$_3$	0·38 S.E. 0·38	0·15 S.E. 0·04	2·87 S.E. 0·09	2·31 S.E. 0·15	Nil	Nil	1·88 S.E. 0·43	1·67 S.E. 0·11	Nil	Nil	1·84 S.E. 0·68	1·93 S.E. 0·21
C16·0	43·37 S.E. 1·13	44·83 S.E. 1·02	51·37 S.E. 1·17	51·23 S.E. 1·07	49·63 S.E. 5·24	39·54 S.E. 5·13	47·21 S.E. 4·94	49·62 S.E. 1·19	42·47 S.E. 1·48	49·98 S.E. 1·53	46·29 S.E. 3·49	50·85 S.E. 1·54
C16·1	2·74 S.E. 0·28	3·92 S.E. 0·35	3·97 S.E. 0·37	4·65 S.E. 0·26	4·00 S.E. 0·12	3·61 S.E. 1·24	7·80 S.E. 1·53	6·46 S.E. 0·74	4·59 S.E. 0·89	3·49 S.E. 0·25	3·43 S.E. 0·68	3·61 S.E. 0·65
C18·0	9·25 S.E. 1·30	9·52 S.E. 0·34	7·89 S.E. 0·05	8·00 S.E. 0·28	4·40 S.E. 0·60	4·47 S.E. 0·14	7·04 S.E. 0·79	6·37 S.E. 0·30	7·58 S.E. 1·25	7·20 S.E. 1·04	9·05 S.E. 0·66	7·53 S.E. 0·30
C18·1	23·36 S.E. 0·63	21·16 S.E. 1·19	14·04 S.E. 0·26	13·64 S.E. 0·39	26·18 S.E. 1·00	31·11 S.E. 1·53	16·36 S.E. 0·41	15·54 S.E. 0·56	24·80 S.E. 0·65	22·09 S.E. 1·57	17·10 S.E. 0·96	15·60 S.E. 0·93
C18·2	15·43 S.E. 1·95	12·77 S.E. 0·76	11·89 S.E. 0·64	10·53 S.E. 0·48	11·58 S.E. 1·10	15·89 S.E. 1·95	12·67 S.E. 2·31	12·76 S.E. 0·80	15·13 S.E. 0·78	12·18 S.E. 0·86	13·64 S.E. 0·77	10·92 S.E. 1·06
C18·3	2·15 S.E. 0·19	1·79 S.E. 0·19	0·14 S.E. 0·14	0·55 S.E. 0·17	2·26 S.E. 0·25	4·82 S.E. 0·33	0·23 S.E. 0·23	0·50 S.E. 0·69	1·50 S.E. 0·13	1·26 S.E. 0·27	0·43 S.E. 0·16	0·37 S.E. 0·21
C20·4	Nil	Nil	3·79 S.E. 0·22	5·14 S.E. 0·30	Nil	Nil	3·17 S.E. 1·10	4·33 S.E. 0·51	Nil	Nil	3·16 S.E. 0·21	4·53 S.E. 0·30

* Figures before decimal point = number of carbon atoms; those after the decimal point, the number of double bonds.

antigen have less 18:0 acids and more 16:1 and 18:1 acids than their controls. One general change detected throughout is an increase in unsaturated acids at the expense of saturated ones.

A parallel comparison of the constituent fatty acids of the neutral and phospholipid fractions of animals pretreated with hydrocortisone can be made by comparing the data in Table IV (A and C). The neutral lipids of the animals exposed to aerosolised distilled water showed an increase in 16·1 fatty acids and a decrease in 18·0 acids when compared with the hydrocortisone treated controls. The animals exposed to antigen showed an increase in 16·0 acids. The phospholipid fraction of the lungs of the animals exposed to distilled water showed an increase in 18·0 and 18·1 acids whilst the 20·4 acids showed a decrease. The phospholipid fraction of the lungs of animals exposed to antigen showed no changes.

DISCUSSION

There is evidence that exposing guinea-pigs to aerosolised antigen altered the lipid content of their lungs. Lung phospholipid fell after exposure to aerosol and was still continuing to fall 1 hr. afterwards. During the same period of time, there was a substantial rise in glyceride content 30 min. after exposure, but this had returned to normal at 1 hr. Changes in the component fatty acids of both neutral and phospholipid fractions indicated that these fractions were in a state of metabolic turnover.

These changes are of sufficient magnitude to influence, at least transiently, the whole of the intermediary metabolism of lung tissue. Whilst the experiments were designed with the expectation that this might be so in animals exposed to antigen, it is surprising to observe changes which were almost identical in animals exposed to distilled water. There are two possible explanations.

The changes were determined in animals receiving antigen in the form of an aerosol for the fourth time. Earlier exposures were made to measure their sensitivity and express it in the form of a "collapse time". This was done to enable the subgroups to be matched in terms of antigen sensitivity. It is thus possible that the changes in animals exposed to distilled water were a conditioned response in the sense that the animals were expecting to be exposed to antigen with its resultant severe symptoms. Alternatively, it can be reasoned that the actual procedure of aerosol exposure (involving transfer to a noisy and uncomfortable aerosol chamber) constituted a form of stress followed by a "stress reaction" on the part of the lung metabolism.

Irrespective of the reasons for the metabolic changes which followed exposure to distilled water, it is significant that animals pretreated with hydrocortisone did not show a response to aerosols of distilled water or of antigen. The "stress reaction" or "conditioned response" did not occur.

This particular experimental study is thus of interest since it offers evidence from which it may be concluded that pretreatment of animals with hydrocortisone at a dose which imparts anti-anaphylactic activity

(Feinberg and others, 1953; Goadby and Smith, unpublished) in some way protects the lung tissue of these animals from disturbances of their lipid metabolism. It implies that the anti-anaphylactic activity of hydrocortisone may have a metabolic basis.

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A NOTE ON THE USE OF CELLULOSE PHOSPHATE CATION-EXCHANGE PAPER FOR THE SEPARATION OF CATECHOLAMINES, AND SOME OTHER BIOGENIC AMINES

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Cellulose phosphate cation-exchange paper has been investigated for its ability to separate biogenic amines. Descending chromatography with an ammonium acetate:isopropanol solvent mixture gave good separation of a number of these amines, including the principal catecholamines. The technique has the advantage of eliminating neutral and acidic compounds which may interfere in conventional partition methods. It has been applied to tissue extracts containing catecholamines.

THE separation of noradrenaline, adrenaline and other catecholamines by filter paper chromatography was first described by James (1948). Subsequent extensive application of the technique to tissue extracts has, however, revealed some limitations and certain pitfalls for the unwary. As Vogt (1959) explains in a recent symposium, one is liable to get contamination of catecholamines, both with amino-acid precursors such as dihydroxyphenylalanine, and with other biologically active amines such as histamine, in the commonly employed phenol:hydrochloric acid solvent system. Some of the acid metabolites, for example dihydroxyphenylacetic acid, are also not too well separated in this system. There are ways, of course, of circumventing these difficulties by the use of specific chemical reactions, or of specific pharmacological antagonists in a biological type of assay. But it would be an advantage to have available other means of paper chromatographic separation for these amines, if only to increase the certainty of identification of doubtful compounds in tissue extracts.

An obvious approach would be to utilise some of the recently developed cation-exchange papers, which should adsorb basic compounds much more strongly than neutral or acidic precursors and metabolites. Two types are now available: (*a*) cellulose impregnated with resins, and (*b*) modified celluloses such as cellulose phosphate, cellulose citrate and carboxymethyl cellulose. Group (*a*) appears to adsorb catecholamines too powerfully to achieve much individual separation, but cellulose phosphate paper in the second group has many properties that should make it a useful additional tool in this increasingly complex field. It is as easy to use as ordinary filter paper, and separations can be obtained in aqueous salt solutions. However, the rate of irrigation with purely aqueous solvents is rather rapid, and slowing the rate by addition of water-miscible organic solvents gives sharper separations and more compact spots. The pH of the solutions used, and the nature and proportions of the organic solvent mixed with them, influence the R_F value of a compound. The increased flexibility implied by this wide possible

range of combinations of different pH, organic solvent, salt concentration, etc., promises to give the method advantages over ordinary filter paper chromatography. In this preliminary note, results with one solvent system only (ammonium acetate : isopropanol) are reported.

EXPERIMENTAL

Cation-exchange paper: cellulose phosphate paper in the ammonium form (Whatman P20, Reeve Angel and Co. Ltd., London, E.C.4) was cut into 3 × 45 cm. or 3 × 55 cm. strips, and used without pre-treatment for descending irrigation in a standard cylindrical chromatography tank (Aimer Products Ltd., London). The manufacturers point out that P20 is an experimental product, and one should therefore be on the watch for batch differences in adsorption properties. None has so far been found.

Solvent system. 0·2M ammonium acetate (Analar) was adjusted to pH 6·0 with 1·0N acetic acid, and 2 parts mixed with 1 part (v/v) of iso-propanol (Analar). A small beaker of isopropanol was placed in the bottom of the chromatography tank.

Compounds. 20–50 μg. of the compounds listed in Table I were spotted by micropipette from solutions containing 2–5 mg./ml. These included some of the amino-acid precursors of the amines studied, and a number of methylated and acidic metabolites of the catecholamines. Histamine was used as the acid phosphate, 5-hydroxytryptamine as the creatinine phosphate, noradrenaline as the acid tartrate, and most of the other bases and amino-acids as hydrochlorides or the free compound.

Development of chromatograms. When the 3 × 45 cm. strips were irrigated for 6–9 hr., the solvent front moved 28–38 cm., and this was convenient for determination of R_F values (Table I). Longer runs with the 3 × 55 cm.

TABLE I

R_F AND R_{ISO} VALUES IN 0·2M AMMONIUM ACETATE + ISOPROPANOL (2:1 v/v), WITH CELLULOSE PHOSPHATE PAPER; (R_{ISO} VALUES IN BRACKETS)

Amines—			Amino-acids—	
Histamine	0·02	(0·04)	L-Histidine	0·12 (0·23)
(−)-Noradrenaline	0·18	(0·39)	L-Dihydroxyphenylalanine	0·58
5-Hydroxytryptamine	0·24	(0·53)	DL-Tryptophan	0·60
Dopamine	0·26	(0·54)	DL-Tyrosine	0·61
(−)-Adrenaline	0·32	(0·63)	DL-β-Phenylalanine	0·69
Normetanephrine	0·35	(0·76)	*Acids—*	
Tryptamine	0·43	(0·92)	±-Dihydroxymandelic	0·86
Tyramine	0·40–0·46	(0·90–0·93)	Dihydroxyphenylacetic	0·91
Metanephrine	0·45	(1·00)	±-3-Methoxy-4-hydroxy-mandelic	0·96
(±)-Isopropylnor-adrenaline	0·46	(1·00)	Homovanillic	0·96

strips (16–24 hr.), allowing the solvent to run off the paper, were done in the presence of added isopropylnoradrenaline (20 μg. of isoprenaline sulphate B.P.). This compound was chosen as a "marker," because it travelled the farthest of the catecholamines tried and is probably not a naturally-occurring substance. An "R_{ISO}" value of the compound under test was measured (ratio of the distance travelled by the compound to the

distance travelled by the isopropylnoradrenaline: Table I, figures in brackets).

Detection of compounds on paper. (1) acetone-dip techniques, using ninhydrin and Ehrlich's Reagent (Smith, 1960, pp. 95, 96), and a diazotised *p*-nitraniline spray (Smith, 1960, p. 297: "Nitraniline Reagent II") were applied to the appropriate compounds. (2) A convenient and simple acetone-dip technique, exploiting the fluorescent ethylenediamine reaction, was devised for the detection of the catechols. The cellulose phosphate
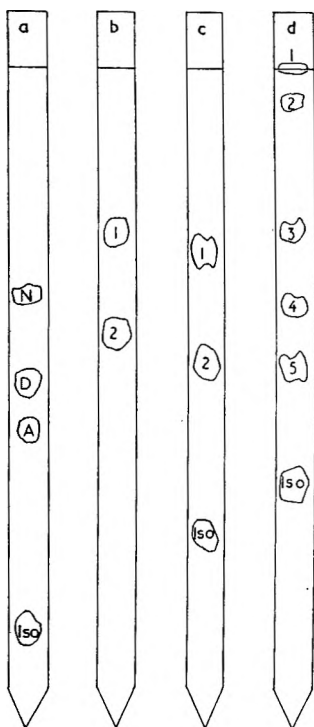

FIG. 1. Cellulose phosphate strips, 3 × 55 cm., run for 18–20 hr. in ammonium acetate-isopropanol; spots located with ethylene diamine and ammonia (for details, see text).

a, Separation of noradrenaline (N, R_{ISO} 0·40), dopamine (D, R_{ISO} 0·56) and adrenaline (A, R_{ISO} 0·64).

b, Ox adrenal extract, showing 2 spots (1 and 2).

c, Ox adrenal extract run with isopropylnoradrenaline; R_{ISO} of spot 1 is 0·38 (consistent with noradrenaline), and of spot 2 0·63 (consistent with adrenaline).

d, Banana peel extract run with isoprenaline. Spots 1, 2 and 5 have R_{ISO} 0·0, 0·08 and 0·72, not consistent with any compounds tried. Spots 3 and 4 have R_{ISO} 0·40 and 0·56, consistent with noradrenaline and dopamine respectively.

strips, allowed to dry at room temperature (15–20°), were dipped in a mixture of 1 part by volume of ethylenediamine (M. & B. Laboratory Chemicals) and 9 parts by volume of Analar acetone. After evaporation of the acetone, the strips were hung overnight in a large glass tank containing a beaker of 0·880 ammonia solution. The papers were then examined under ultra-violet light (Hanovia Chromatolite). Quantities of adrenaline and noradrenaline as low as 0·2 μg. can be detected by this method, even after chromatography. 5-Hydroxytryptamine also gave a strong green fluorescence with ethylenediamine, but the 3-methoxy derivatives reacted only very slowly (48–96 hr.).

RESULTS

R_F and R_{ISO} values. R_F values recorded in Table I show clearly that the compounds separated into 3 main groups: (1) the acids, running

well towards the solvent front (R_F 0·86–0·96); (2) the neutral amino-acids (R_F 0·58–0·69), and (3) the bases. The imidazole derivatives histamine and histidine were strongly adsorbed near the origin. The other, more weakly basic, compounds ran between R_F 0·18 and 0·46. 5-Hydroxytryptamine and dopamine were not well separated in this solvent system. Tyramine, alone among the compounds tried, gave somewhat variable R_F and R_{ISO} values, and therefore a range is quoted in the Table. The 16–24 hr. irrigation, using isopropylnoradrenaline as marker, increased the separation of these bases, and eliminated the neutral amino-acids and acidic metabolites which ran off the paper. An example of the separation of noradrenaline, dopamine and adrenaline is shown in Fig. 1a.

Application to tissue extracts. Some tissues, known to contain catecholamines, were homogenised with 0·4N perchloric acid, and the extracts, after removal of perchlorate as the potassium salt, run on cellulose phosphate paper. Ox adrenal medulla (containing principally adrenaline and noradrenaline) and banana peel (noradrenaline and dopamine, with some 5-hydroxytryptamine) were used as a test of the technique, and typical chromatograms are illustrated in Fig. 1, b, c and d. 5-Hydroxytryptamine was not detected by Ehrlich's reagent in the banana peel extract; it is known to occur in much smaller quantities than noradrenaline and dopamine (Waalkes, Sjoerdsma, Creveling, Weissbach, and Udenfriend, 1958).

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THE INFLUENCE OF ENVIRONMENTAL CHANGES ON THE CARDIOTOXICITY OF ISOPRENALINE IN RATS

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The influence of environment on the cardiotoxicity of isoprenaline has been examined in rats. Animals kept for 3 months in individual cages exhibited an increased sensitivity compared to their community caged controls when either mortality or the severity of the heart lesion was considered. Animals exposed to cold for one week showed a 1,000–10,000 times increased sensitivity to isoprenaline compared to their controls. A probable mechanism of sensitisation is discussed.

It has been shown by Chappel, Rona, Balazs, and Gaudry (1959) that the infarct-like myocardial necrosis produced by isoprenaline (1-(3,4-di-hydroxyphenyl)-2-isopropylaminoethanol hydrochloride) is more severe than that produced by other catecholamines. Several factors that influence the cardiotoxicity of isoprenaline have been investigated in laboratory animals. A relation between body weight, thyroid function and the severity of isoprenaline-induced myocardial necrosis in rats has been demonstrated by Rona, Chappel, Balazs, and Gaudry (1958) and Chappel, Rona, and Gaudry (1959a). The cardiotoxicity of this compound is aggravated by mineralocorticoids (Chappel, Rona and Gaudry, 1959b), low potassium diet (Rona, Chappel and Gaudry, 1961) and excess body fat (Balazs, Sahasrabudhe, and Grice, 1962).

In the course of investigations on the influence of various diets on the cardiotoxicity of isoprenaline in rats, it appeared that those animals which were housed in individual cages showed increased sensitivity when compared with community caged animals. It is known that long-term individual housing of laboratory animals has an effect on behaviour and physiological responses to stress (Stern, Winokur, Eisenstein, Taylor, and Sly, 1960) but the effects of such housing on toxicological responses have not been explored.

The first part of this study was undertaken to compare the cardiotoxicity of isoprenaline between individually and group-caged rats.

EXPERIMENTAL

Rats of the Wistar strain bred and raised in this laboratory were kept in an air-conditioned room at a temperature of $24 \pm 1°$ and a relative humidity of 45 ± 5 per cent. They had a commercial diet,* and water *ad libitum*, and were weighed weekly but were not handled otherwise.

In one experiment 80 female rats, 5–6 weeks of age and weighing 50–60 g. were divided into two comparable groups. Forty rats were placed in large community cages (50 × 50 × 25 cm.), each housing 10 rats, and

* Fox Chow, Toronto Elevators Ltd., Toronto, Canada.

forty were in individual cages (28 × 20 × 20 cm.) with metal sides and screened rear wall, floor and door. The rats were housed for 3 months in the manner described. At the end of 3 months, housing was exchanged between 10 community caged animals and 10 individually caged animals for 1 week. This was done to determine whether a short term exchange in the housing of the animals would modify the cardiotoxicity of iso-prenaline. The mean terminal body weights of the rats were 199 ± 3 g. for the individually caged and 199 ± 4 g. for the community caged groups.

In a second experiment 100 rats 5–6 weeks of age and weighing 50–70 g. were divided into two groups of 25 females and 25 males each. One group was placed in community cages, the other in individual cages as described above for 3 months. At the end of the 3 months housing the mean body weights for the individual and community groups respectively were as follows: females, 188 ± 3 g. and 199 ± 3 g.; males, 336 ± 6 g. and 322 ± 7 g.

Injections of isoprenaline were given after the 3-month preparatory period. A single injection of an aqueous solution of isoprenaline hydro-chloride was given subcutaneously at two dose levels in the first experi-ment. In the second experiment four dose levels were used and those animals that survived for 24 hr. were given a second injection. The doses were the same as that given for the first injection. The number of rats injected and the doses in mg./kg. are shown in Tables I and II. Control rats were injected with saline. Mortality was recorded up to 24 hr. The myocardial lesions in the survivors and in those dying after the second injection were graded as described by Rona and others (1959b). The grading was done under single blind conditions. Differential white cell counts and the weights of adrenals were taken. Adrenal ascorbic acid content was determined using the method of Bessey (1938) on the saline injected controls.

RESULTS

The manner of housing had a pronounced effect on the rat's behaviour. The individually caged animals in this experiment were excitable and intractable whereas the community caged rats were docile and easily handled. Such disturbances in behaviour of experimental animals kept in solitary confinement have been observed by Barnes (1960).

The results of the isoprenaline treatment are summarised in Tables I and II. In the first experiment (Table I) a single dose of either 100 or 50 mg./kg. of isoprenaline caused severe dyspnoea, prostration and convulsions in the individually caged rats. Nine out of ten rats in this group died at both dose levels. A temporary dyspnoea without mortality was observed in the animals kept in community cages and receiving 100 or 50 mg./kg. doses. The ten community caged rats, transferred into individual cages for a one-week period before isoprenaline administration, were not killed by a 100 mg./kg. dose. However, individually caged animals placed in community cages for 1 week died from this dose.

In the second experiment (Table II) the mortality of the females, housed individually, was comparable to that obtained in the first experiment.

Furthermore, the pathological findings indicated an increased sensitivity of the isolated females. Twenty mg./kg. of isoprenaline induced more severe cardiac lesion in these rats (grade 3·5) than did a 200 mg./kg. dose in community caged females (grade 1·5). The cardiotoxicity of isoprenaline in males was also influenced by isolation. This was shown by mortality at the 100 and 200 mg./kg. doses (5/5 vs. 1/5) and also by the grade of myocardial damage at the 20 and 50 mg./kg. dose levels (3·5 vs. 1; 4 vs. 2 respectively). However, the community caged male rats were more sensitive to the cardiotoxic effect of the amine than were the community caged females. This is evident when both mortality and the severity of the myocardial lesion at the 100 and 200 mg./kg. dose levels are considered.

TABLE I

COMPARISON OF THE EFFECT OF ISOPRENALINE ON MORTALITY OF INDIVIDUALLY AND COMMUNITY-CAGED FEMALE RATS

Experiment I

Group	Dose mg./kg.	Mortality
Individual*	100	9/10
	50	9/10
	0	0/10
Community*	100	0/10
	50	0/10
	0	0/10
Individual* Exchanged‡ to Community	100	10/10
Community* Exchanged‡ to Individual	100	0/10

* Caged for 3 months.   ‡ Exchanged for 1 week.

Differential white cell counts and adrenal ascorbic acid content were the same in the individual and community caged groups. There was also no significant difference between the adrenal weights of the individually and community caged rats. The hearts in the saline treated groups appeared normal on gross and histologic examination. No evidence of intercurrent disease was observed in any groups of this study.

The results of this study indicate that rats housed for 3 months in individual cages develop an increased sensitivity to the cardiotoxic effect of isoprenaline. A single dose of 50 mg./kg., which caused mortality in the individually caged female rats represents approximately 6 per cent of the LD50 of 815 ± 70 mg./kg. as determined in this laboratory for community caged 200 g. female rats (Balazs, Sahasrabudhe and Grice, 1962). The community caged males showed an increased sensitivity as compared with the community caged females. It is known that a positive relationship exists between body weight and isoprenaline toxicity (Rona and others, 1958) and the increased susceptibility to this compound in the heavier males may be explained on this basis. Because of the effect of this weight factor the sensitivity difference between individually and

community caged rats was not as pronounced for the males as for the females in this study.

The sensitivity to the cardiotoxic effect of isoprenaline was not altered by a period of 1 week of exchanged housing, thus the underlying mechanism of the sensitisation is not of an acute character.

TABLE II

COMPARISON OF CARDIOTOXICITY OF ISOPRENALINE IN INDIVIDUALLY AND COMMUNITY-CAGED RATS

Experiment II

Group			Dose* mg./kg.	Mortality	Average grade of myocardial lesions
Females—					
Individual	..	..	200	5/5	—
Community	..	..	200	0/5	1·5
Individual	..	..	100	4/5	3·0
Community	..	..	100	0/5	1·5
Individual	..	..	50	4/5	3·0
Community	..	..	50	0/5	1·5
Individual	..	..	20	0/5	3·5
Community	..	..	20	0/5	1·0
Individual	..	..	0	0/5	0
Community	..	..	0	0/5	0
Males—					
Individual	..	..	200	5/5	—
Community	..	..	200	1/5	4·0
Individual	..	..	100	5/5	—
Community	..	..	100	1/5	4·0
Individual	..	..	50	1/5	4·0
Community	..	..	50	0/5	2·0
Individual	..	..	20	1/5	3·5
Community	..	..	20	0/5	1·0
Individual	..	..	0	0/5	0
Community	..	..	0	0/5	0

* 24 hr. survivors received a second similar dose.

EXPOSURE TO COLD STRESS

Since a minor environmental change was found to have an effect on the cardiotoxicity of isoprenaline further experiments were made to examine the effect of a more drastic change of the environment. Exposure of rats to cold was chosen for this purpose. The physiological changes accompanying cold exposure in rats have been reviewed by Smith and Hoijer (1962). It was thought that if a major environmental change having a known physiological effect influenced more profoundly the cardiotoxicity of this compound, it might shed some light on the mechanism of sensitivity due to such changes.

EXPERIMENTAL

Seventy-two male rats of the Wistar strain, bred and raised in our laboratory, were divided into two groups of 36. The rats were assigned to each group so that the weights were comparable; cold exposed:

360 $\pm$ 17 g.; control: 378 $\pm$ 20 g. The first group were placed in individual cages in a cold room having a mean temperature of 4 $\pm$ 1°. The second group were kept at room temperature (24 $\pm$ 1°) also in individual cages. All rats were fed water and food *ad libitum*.

After seven days the rats in each environment were randomly subdivided into 6 groups of 6 and injected with various doses of isoprenaline subcutaneously. The cold exposed rats were taken from the cold room, injected immediately and were then placed in individual cages in the same room as the controls. Doses and mortality are presented in Table III.

In the second experiment 48 female rats weighing 260–280 g. were divided into two groups of 24 each. The rest of the experiment was conducted as described above, except that the rats from both environments were divided into 4 groups of 6. Doses and mortality are presented in Table III.

Animals were necropsied and the adrenal weights were recorded. Hearts from both groups were examined histologically.

RESULTS AND DISCUSSION

The results of the experiments are summarised in Table III. Severe dyspnoea was observed in both groups, followed by a shock-like stage. It is apparent that a remarkable sensitivity to isoprenaline is induced

TABLE III

COMPARISON OF THE EFFECT OF ISOPRENALINE ON MORTALITY IN RATS EXPOSED FOR ONE WEEK TO COLD AND ROOM TEMPERATURE

Group	Dose mg./kg.	Mortality
Control males	220	2/6
	180	1/6
	160	2/6
	140	3/6
	120	1/6
	100	0/6
Cold exposed males	20	6/6
	10	5/6
	5	6/6
	1	6/6
	0·75	5/6
	0·25	4/6
Control females	800	2/6
	700	2/6
	600	3/6
	500	1/6
Cold exposed females	10	6/6
	5	6/6
	0·1	4/6
	0·01	0/6

in rats that have been exposed to a cold environment for 1 week. In the male rats the difference in sensitivity compared to control rats appears to be about 1000. The cold exposed female rats in the second experiment appear to be even more sensitive to the drug; the difference being about 10,000.

The adrenal weights of both male and female rats exposed to cold increased as compared with those of the control rats. However, this was significant statistically only in the females (P < 0·05). It is interesting to note that the cold exposed females developed a proportionately higher sensitivity when compared with their controls than did the cold exposed males. However, the control males were more sensitive to the amine than the control females because of their heavier body weight.

Histological examination of the heart revealed separation of muscle fibre with oedema and haemorrhage. No evidence of intercurrent disease was observed in any groups of this study.

That thyroid and adrenal weights increase during cold exposure is well documented (Smith and Hoijer, 1962). Chappel and others (1959a, 1959b) have shown that thyroid hormone and mineralocorticoids increase the cardiotoxicity of isoprenaline in rats. Raab and others (1960), demonstrated, in rats exposed to certain stressful situations, that these hormones sensitised the myocardium to the cardiotoxic properties of catecholamines. There is thus reason to believe that these hormones played a role in the sensitisation of the myocardium to isoprenaline in our rats.

A drastic environmental change, like cold exposure, which is known to alter homeostasis, had a potent effect in sensitising rats to isoprenaline while a minor environmental change, like long-term isolation, induced a minor increase in sensitivity to the amine. It is possible that both environmental changes produced the increased sensitivity in rats by a common mechanism of action.

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POTENTIAL RESERPINE ANALOGUES

Part III* Derivatives of 4-ethoxycarbonyloxy-3,5-dimethoxy-benzoic acid, 3-dimethylaminobenzoic acid, p-hydroxybenzanilide and N-(3,4-dimethoxyphenethyl)-3(or 4)-hydroxycyclohexane carboxyamide

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4-Ethoxycarbonyloxy-3,5-dimethoxybenzanilide (I), 3-dimethylamino-benzanilide (II), N-(3,4-dimethoxyphenethyl)-4-ethoxycarbonyloxy-3,5-dimethoxybenzamide (III), N-(3,4-dimethoxyphenethyl)-3-di-methylaminobenzamide (IV), 4-(3,4,5-trimethoxybenzoyloxy)-benzanilide (V), 4-(4-ethoxycarbonyloxy-3,5-dimethoxybenzoyloxy)-benzanilide (VI), 3-acetoxy-N-(3,4-dimethoxyphenethyl)cyclohexane-carboxyamide (VII), 3-anisoyloxy-N-(3,4-dimethoxyphenethyl)cyclo-hexanecarboxyamide (VIII), N-(3,4-dimethoxyphenethyl)-3-veratroyl-oxycyclohexanecarboxyamide (IX), N-(3,4-dimethoxyphenethyl)-3-trimethoxybenzoyloxycyclohexanecarboxyamide (X), and N-(3,4-di-methoxyphenethyl)-4-trimethoxybenzoyloxycyclohexanecarboxyamide (XI) have been prepared. Eight of the compounds, I-VII and X, were compared with reserpine for their ability to potentiate barbiturate hypnosis in mice and to deplete the 5-hydroxytryptamine content of rat brain. None of them lowered the brain 5-hydroxytryptamine content but several showed barbiturate potentiation. Compound V was the most potent of the series in producing potentiation of barbiturate hypnosis, being about one tenth as action as reserpine.

Following the elucidation of the structure of resperine a number of analogues have been synthesised but so far there has been no analogue of reserpine (methyl O-3,4,5-trimethoxybenzoylreserpate) prepared that has shown a similar or higher activity on the blood pressure or sedation. Lucas and others (1959) reported two most promising analogues; methyl O-3-dimethylaminobenzoylreserpate and syrosingopine [methyl-O-(4-ethoxycarbonyloxy-3,5-dimethoxybenzoyl)reserpate; carbethoxy syringoylmethyl reserpate] respectively. Karim, Sharp and Linnell (1960) reported that comparatively simple amides of 3,4,5-trimethoxy-benzoic acid appeared to possess promising pharmacological activity and among the compounds reported, 3,4,5-trimethoxybenzanilide showed about one-eighth the activity of reserpine.

It seemed possible, therefore, that new compounds containing a combination of the favoured molecular fragments of the compounds already prepared might reveal increased pharmacological activity. As a result compounds I-XI were prepared. The present work describes the preparation of these compounds.

* Parts I and II, *J. Pharm. Pharmacol.*, 1960, **12**, 74-86.

Pharmacological

The compounds I–VII and X were dissolved or suspended in solvents such as glycerol formal, or dilutions of ethanol in water, and then compared with reserpine for their ability to potentiate the hypnosis in mice produced by an intravenous dose of hexobarbitone (50 mg./kg.), and to deplete the 5-hydroxytryptamine content of rat brain. The standard intraperitoneal dose of reserpine in the first test was 2 mg./kg., and in the second, 2·5 mg./kg.

Evaluation of these compounds was difficult, because of the lack of suitable solvents. However, none of the compounds reduced the brain 5-hydroxytryptamine content but several potentiated barbiturate hypnosis. Compound V was the most potent in this respect, although other compounds (e.g. VII–X) showed closer structural resemblances to reserpine.

We should like to express our thanks to Mr. L. I. L. Ndika and Dr. G. B. West of the Department of Pharmacology of this School for carrying out the pharmacological tests.

TABLE I

COMPARISON OF THE PHARMACOLOGICAL ACTIVITIES OF VARIOUS COMPOUNDS

Compound No.	Potentiation of barbiturate hypnosis in mice		Depletion of 5-hydroxytryptamine in rat brain	
	Maximum dose (mg./kg.)	Relative activity	Maximum dose (mg./kg.)	Relative activity
I	80	0·5	—	—
II	80	0·8	—	—
III	8	2·5	20	0·5
IV	20	2·0	20	0·5
V	10	10	10	0·1
VI	40	0·4	10	1·0
VII	—	—	40	0
X	10	8·0	20	0·5

EXPERIMENTAL

4-*Ethoxycarbonyloxy*-3,5-*dimethoxybenzoic acid.* Ethyl choroformate (16 g.) was added gradually to an ice-cold mechanically stirred solution of syringic acid (20 g.) in N aqueous sodium hydroxide (240 ml.). When the odour of the ester was no longer perceptible (left overnight), the solution was acidified with dilute hydrochloric acid, the precipitate collected, washed with water and dried in a vacuum desiccator. The crude acid was recrystallised twice from 50 per cent aqueous acetone. M.p. 178–181°. Yield 26 g. (95 per cent). Found C, 53·4; H, 4·8 per cent. $C_{12}H_{14}O_7$ requires C, 53·3; H, 5·1 per cent.

4-*Ethoxycarbonyloxy*-3,5-*dimethoxybenzanilide* (*I*). Freshly prepared 4-ethoxycarbonyloxy-3,5-dimethoxybenzoyl chloride (6 g.) (obtained from 4-ethoxycarbonyloxy-3,5-dimethoxybenzoic acid and thionyl chloride) was dissolved in ether (50 ml.) and added to an ice-cold mechanically

stirred solution of redistilled aniline (4 g.) in ether (50 ml.). The ethereal layer, after treatment with dilute hydrochloric acid and water, was dried with anhydrous magnesium sulphate. The solvent was removed and the solid residue was dissolved in benzene, treated with animal charcoal and crystallised by the addition of light petroleum (60–80°); after leaving in the refrigerator pale yellow needle-shaped crystals were obtained. Yield 4·5 g. (30 per cent), m.p. 165–166°. Found C, 62·6; H, 5·4, N, 3·9 per cent $C_{18}H_{19}NO_6$ requires C, 62·6; H, 5·5; N, 4·0 per cent.

N-(3,4-*Dimethoxyphenethyl*)-4-*ethoxycarbonyloxy*-3,5-*dimethoxybenzamide* (III). To 4-ethoxycarbonyloxy-3,5-dimethoxybenzoyl chloride (2·25 g.) dissolved in tetrahydrofuran (50 ml.) was added redistilled homoveratrylamine (1·81 g.) dissolved in tetrahydrofuran (50 ml.) over 1 hr. The reaction mixture was mechanically stirred for 1 hr. and the precipitated hydrochloride was removed by filtration. The solvent was removed under reduced pressure to leave gum, which was crystallised from tetrahydrofuran and ether, after treatment with animal charcoal and leaving overnight in the refrigerator. Further recrystallisation from 50 per cent ethanol gave colourless, long, needle-shaped crystals. Yield 1·7 g. (39 per cent), m.p. 93–94°. Found C, 59·3; H, 6·2; N, 3·2 per cent. $C_{22}H_{27}NO_8$ requires C, 60·4; H, 6·2; N, 3·3 per cent.

3-*Dimethylaminobenzanilide* (II). 3-Dimethylaminobenzoic acid was esterified with diazomethane and the ester (4·5 g.) was heated with aniline (3 ml.) on a metal bath at 200–210° for 7 hr. The reaction mixture was dissolved in benzene and refluxed with decolorising charcoal for 30 min. and filtered. To the filtrate was added light petroleum (60–80°). After one day silvery, shining clusters of crystals were obtained. Yield 2·5 g. (62 per cent); m.p. 133–134°. Found C, 75·0; H, 6·4; N, 11·5 per cent. $C_{15}H_{16}N_2O$ requires C, 75·0; H, 6·6; N, 11·7 per cent.

N-(3,4-*Dimethoxyphenethyl*)-3-*dimethylaminobenzamide* (*IV*). Redistilled homoveratrylamine (1·71 g.) and methyl *m*-dimethylaminobenzoate (1·64 g.) were heated together on a metal bath at 160–180° for 3 hr. The brown-coloured transparent reaction product was treated as in the previous experiment and recrystallised from benzene and light petroleum (60–80°) to give needle-shaped crystals, m.p. 100–101°, Yield 2·6 g. (84 per cent). Found C, 69·8; H, 7·2; N, 8·8 per cent. $C_{19}H_{24}N_2O_3$ requires C, 69·5; H, 7·3; N, 8·5 per cent.

4-(3,4,5-*Trimethoxybenzoyloxy*)*benzanilide* (*V*). *p*-Hydroxybenzanilide (0·24 g.) was dissolved in dry pyridine (5 ml.) at room temperature. 3,4,5-Trimethoxybenzoyl chloride (0·32 g.) (obtained by inter-reaction of 3,4,5-trimethoxybenzoic acid and thionyl chloride) was slowly added with shaking. The mixture was left for 1 hr., poured into ice-cold water (50 ml.), and vigorously stirred when a precipitate was deposited. The precipitate was removed by filtration washed first with 1 per cent Na_2CO_3 solution and then with cold water, dried and recrystallised thrice from benzene. Yield 0·357 g. (77 per cent); m.p. 200–201°. Found C, 68·1; H, 5·3; N, 4·0 per cent. $C_{23}H_{21}NO_6$ requires C, 67·8; H, 5·1; N, 3·6 per cent.

4-(4-*Ethoxycarbonyloxy*-3,4-*dimethoxybenzoyloxy*)*benzanilide*   (*VI*). This was similarly prepared from *p*-hydroxybenzanilide (0·21 g.) and 4-ethoxycarbonyloxy-3,5-dimethoxybenzoyl chloride (0·32 g.) in pyridine (6 ml.). The crude product was crystallised from benzene and light petroleum (60–80°). Yield 0·34 g. (74 per cent); m.p. 162–163°. Found C, 64·9; H, 5·0; N, 3·0 per cent. $C_{25}H_{23}NO_8$ requires C, 64·5; H, 4·9; N, 3·0 per cent.

N-(3,4-*Dimethoxyphenethyl*)-3-*hydroxycyclohexanecarboxyamide* (*XII*). *Method* (*a*). 3-Hydroxycyclohexanecarboxylic acid (as prepared by Noyce and Denney (1952) (0·49 g.) and homoveratrylamine (0·615 g.) were heated together on a metal bath at 200–210° for 1½ hr. The reaction product (a gum) was dissolved in warm benzene (100 ml.) and refluxed with decolorising charcoal for 2 hr., filtered and the volume reduced to about 10 ml. On addition of light petroleum (40–60°) and leaving aside for three days a solid substance was obtained. After three crystallisations from benzene a constant melting-point was obtained. Yield 0·35 g. (17 per cent); m.p. 117–118°. Found C, 65·9; H, 8·2; N, 4·8 per cent. $C_{17}H_{25}NO_4$ requires C, 66·4; H, 8·1; N, 4·6 per cent.

Method (*b*). Ethyl 3-hydroxycyclohexanecarboxylate (as prepared by Ungnade and Morriss, 1948) (1·14 g.) and homoveratrylamine (1·15 g.) were similarly treated for 3 hr. and the reaction product was recrystallised four times from benzene to yield the crystalline amide, m.p. 117–118°. Yield 0·98 g. (50 per cent). Mixed m.p. with the product of Method (a) 117–118·5°.

3-*Acetoxy*-N-(3,4-*dimethoxyphenethyl*)*cyclohexanecarboxyamide*   (*VII*). N-(3,4-Dimethoxyphenethyl)-3-hydroxycyclohexanecarboxyamide   (XII) (0·195 g.) and acetyl chloride (1 ml.) were warmed for 5 min. on a very low flame and on cooling the reaction mixture was poured into water (10 ml.). The white precipitate obtained was washed with water, dried and recrystallised from benzene and light petroleum (40–60°) to yield needle-shaped crystals. Yield 0·23 g. (82 per cent), m.p. 108–109°. Found C, 65·4; H, 8·1; N, 3·9 per cent. $C_{19}H_{27}NO_5$ requires C, 65·3; H, 7·7; N, 4·0 per cent.

3-*Anisoyloxy*-N-(3,4-*dimethoxyphenethyl*)*cyclohexanecarboxyamide* (*VIII*). The amide (XII) (0·175 g.) was dissolved in dry pyridine (3 ml.). To this was added anisoyl chloride (0·5 g.). The mixture was refluxed for 30 min. and left aside for three days. It was then poured into ice-cold water, vigorously stirred and extracted with benzene. The benzene extracts were refluxed with decolorising charcoal, filtered, dried over anhydrous magnesium sulphate, and the solvent removed under reduced pressure on a water-bath leaving a pale yellow viscous substance. This crystallised from ether and light petroleum and two crystallisations yielded white granular crystals. Yield 0·15 g. (38 per cent); m.p. 128–130° (softens at 125°). Found C, 68·6; H, 7·0 per cent. $C_{25}H_{31}NO_6$ requires C, 68·0; H, 7·0 per cent.

N-(3,4-*Dimethoxyphenethyl*)-3-*veratroyloxycyclohexanecarboxyamide* (*IX*). On treating similarly the amide (XII) (0·31 g.) with veratroyl chloride (0·395 g.) in pyridine (4 ml.) a white flocculent precipitate was

obtained. Two recrystallisations from benzene and light petroleum (60–80°) gave a substance melting at 97–98°. Yield 0·285 g. (60 per cent). Found C, 66·4; H, 7·1; N, 3·0 per cent. $C_{26}H_{33}NO_7$ requires C, 66·2; H, 7·0; N, 3·0 per cent.

N-(3,4-*Dimethoxyphenethyl*)-3-*trimethoxybenzoyloxycyclohexane-carboxyamide* (*X*). 3,4,5-Trimethoxybenzoyl chloride (0·275 g.) was treated similarly with the amide (XII) (0·305 g.) in pyridine (5 ml.) to give a white precipitate. After two recrystallisations from benzene and light petroleum (40–60°) granular crystals melted at 140–142°. Yield 0·16 g. (32 per cent.) Found C, 65·0; H, 7·1; N, 2·6 per cent. $C_{27}H_{35}NO_8$ requires C, 64·7; H, 7·0; N, 2·8 per cent.

N-(3,4-*Dimethoxyphenethyl*)-4-*hydroxycyclohexanecarboxyamide* (*XIII*). Homoveratrylamine (1·3 g.) and ethyl 4-hydroxycyclohexanecarboxylate (as prepared by Ungnade and Morriss, 1948) (1·295 g.) were heated together on a metal bath at 200–220° for 3 hr. After working up the reaction product as in the preparation of the amide (XII), four recrystallisations from benzene gave needle-shaped crystals. Yield 0·81 g. (37 per cent), m.p. 98–99°. Found C, 66·8; H, 8·1; N, 4·3 per cent. $C_{17}H_{25}NO_4$ requires C, 66·4; H, 8·1; N, 4·6 per cent.

N-(3,4-*Dimethoxyphenethyl*)-4-*trimethoxybenzoyloxycyclohexane-carboxyamide* (*XI*). The amide (XIII) (0·100 g.) was treated exactly in the same way as for the amide (XII) with 3,4,5-trimethoxybenzoyl chloride (0·145 g.) in pyridine (1 ml.) to give a substance melting at 144–145°. Yield, 0·080 g. (49 per cent). Found C, 64·5; H, 7·0 per cent. $C_{27}H_{35}NO_8$ requires C, 64·7; H, 7·0 per cent.

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A NEW COMPLEXOMETRIC METHOD FOR THE DETERMINATION OF SOME SULPHONAMIDES

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A complexometric method for the determination of some sulphon-
amides has been evaluated. The sulphonamides are precipitated as
cupric salt, filtered and the excess copper is titrated with EDTA using
PAN as indicator.

THE different methods for the determination of some sulphonamides
were reviewed by Abdine and Abdel Sayed (1962). They include
diazotisation, electrometric, bromometric, neutralisation and non-aqueous
titration methods. Sulphonamides can also be determined by precipi-
tating with metallic ions, example silver, mercuric or copper ions and
completing the determination gravimetrically or volumetrically in aqueous
or non-aqueous media, see, for example, Lapiere (1946, 1947, 1948).

The application of a complexometric method seemed to hold possi-
bilities, the sulphonamides being quantitatively precipitated by a measured
excess of metallic ions and the excess (in the filtrate) titrated complexo-
metrically. Copper was chosen, because silver ions cannot be directly
titrated complexometrically (Flaschka, 1953) and mercury ions necessitate
special precautions lest basic salts of variable composition precipitate
(Partington, 1950).

EXPERIMENTAL

The sulpha drugs are practically insoluble in water. Sulphadiazine
was chosen initially and was dissolved in dilute sodium hydroxide solu-
tion,* and treated with a known excess of standard copper solution. The
excess copper in the filtrate was determined complexometrically. The
results obtained were high compared with the official (B.P. 1958) electro-
metric method.

The effect of changing the time of standing of the precipitate before
filtration and of changing the pH of the precipitation was investigated.

Effect of Time of Standing

Sulphadiazine (0·4 g.), accurately weighed, was dissolved in the minimum
amount of 0·1N sodium hydroxide (pH 9) then 0·1M copper sulphate
solution (30 ml.) was added slowly with constant stirring. The solution
was filtered immediately or after standing for 15 to 30 min. The precipi-
tate was washed with distilled water and the filtrate and washings com-
pleted to 250 ml. An aliquot of 50 ml. was taken, 60 ml. ethanol (to
improve the end-point) (Cheng and Bray, 1955), 10 ml. buffer solution

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* Dissolving the sulphadiazine in dilute acids or dilute ammonia prevented its
precipitation with copper.

pH 6, drops of PAN indicator were added and the solution was titrated with 0·02M EDTA.

The results obtained were calculated on the basis of a 2:1 ratio of sulphonamide to copper according to Lott and Bergeim (1939), Lapiere (1948) and Billman, Jonetos and Cheinin (1960).

In the determination, filtering immediately gave a per cent recovery (mean of three determinations) of 104·03 (+1·83 − 1·91), after standing 15 min. this was 107·43 (+1·35 − 1·04), and after standing 30 min. this was 116·04 (+1·09 − 0·92). The B.P. 1958 per cent recovery was (average of three determinations) 99·71 (+0·32 − 0·34).

Effect of pH

The high results, even when filtration was immediate, may be due to co-precipitation of copper ions at the relatively high pH of the precipitation (pH 9). Trials were therefore made to adjust the solution to lower pH values with buffer solutions at pH 8, 7, 6·5, 6, 5·5 (borax buffers) and 4 (acetate buffer) respectively, added before the solution was filtered.

In the determination at pH 8 the per cent recovery (mean of three determinations) was 102·62 (+0·75 − 0·5), at pH 7 this was 102·28 (+1·69 − 1·52), at pH 6·5, 101·02 (+0·33 − 0·42), at pH 6, 99·18 (+0·14 − 0·07), at pH 5·5, 97·62 (+0·5 − 0·54), and at pH 4 this was 86·89 (+1·31 − 1·02). The B.P. 1958 mean result = 99·71 per cent. The above results show that pH 6 is the most suitable for the precipitate. The solution is stable at pH 6 for about 15 min.

The Recommended Procedure

Weigh accurately 0·4 g. of sulpha drug and dissolve in the minimum amount of NaOH (0·1N), (faint blue to thymol blue). Add 0·1M copper sulphate (30 ml.) and borax buffer solution pH 6 (20 ml.). After filtration wash the precipitate with distilled water to a total volume of filtrate and washings of 250 ml. Take an aliquot (50 ml.) add absolute ethanol (60 ml.) and 3 to 4 drops of PAN indicator and titrate the solution with 0·02M EDTA until green.

Some comparative results are given in Table I.

Sulphaguanidine and sulphanilamide do not give a precipitate with a silver or copper ions.

Reagents

Copper sulphate: 0·1M; 24·971 g. of $CuSO_4·5H_2O$, per litre, was standardised with 0·1M EDTA by the method of Flaschka and Abdine (1956) using PAN.

Edta disodium salt (of the B.P. 1958): 0·1M, standardised with 0·1M zinc solution using Eriochrome black T.

Zinc solution: 0·1M, 6·538 g. AR metallic zinc dissolved in the minimum amount of hydrochloric acid AR and diluted to 1 litre.

Pan: 1-(2-pyridyl azo-2-naphthol) 0·1 per cent in methanol.

Acetate buffer solution pH 4: 27·22 g. sodium acetate + 40 ml. N HCl per litre.

TABLE I

RESULTS OF ASSAY OF SOME SULPHA DRUGS BY DIFFERENT METHODS

Sulpha drug	B.P. 1958 method	Argentimetric titration*	Complexometric method
	per cent found	per cent found	per cent found
Sulphadiazine	99·71 (+0·32 − 0·34)	98·19 (+0·38 − 0·22)	99·65 (+0·36 − 0·33)
Sulphathiazole (Non-official) ..	99·35 (+0·27 − 0·29)	98·85 (+0·23 − 0·27)	98·36 (+0·19 − 0·34)
Sulphadimidine	99·26 (+0·38 − 0·36)	98·87 (+0·25 − 0·30)	99·11 (+0·46 − 0·43)
Sulphamerazine	99·66 (+0·27 − 0·30)	99·56 (+0·25 − 0·43)	99·81 (+0·56 − 0·49)
Sulphapyridine (Non-official) ..	99·65 (+0·36 − 0·22)	99·30 (+0·42 − 0·37)	99·56 (+0·25 − 0·34)
Sulphacetamide sodium	99·45 (+0·32 − 0·36)	Soluble silver salt	77·82 (+3·22 − 3·34)

* Lee Kum-Tatt, 1957.

Borax buffers; solution A: 31 g. boric acid + 8·75 g. sodium chloride per litre.   Solution B: 2·86 g. sodium carbonate per litre.

Buffer solution pH 5·5: 300 ml. solution A + 6 ml. solution B.

Buffer solution pH 6: 300 ml. solution A + 12·5 ml. solution B.

Buffer solution pH 6·5: 300 ml. solution A + 50 ml. solution B.

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LETTER TO THE EDITOR

The Concentration of Catecholamines in the Turtle Heart and Vagal Escape

SIR,—A report by Hough (1895) indicated that stimulation at supramaximal voltages of the peripheral vagus nerve of the turtle produces an asystole which persists for the period of stimulation. Recent work on adrenergic factors of the heart in the phenomenon of vagal escape (Friedman and Campos, 1960; Campos and Friedman, to be published) suggested that the sustained vagally-induced cardiac arrest in the turtle might be explained on the basis of the catecholamine content of the heart. The varied ability of different species to resist vagal stimulation has been pointed out by Hough (1895).

The startling finding that vagal stimulation in the lamprey (*Lampetra fluviatilis*) resulted in cardioacceleration rather than the expected bradycardia has been reported by Augustinsson and others (1956). The myocardial catecholamine concentration of this species is the highest of any yet known. On the basis of these studies it was predicted that the catecholamine content of the turtle heart would be relatively low when compared to that of more common species such as the dog. This prediction has been verified. The supporting data is to be found in Table I.

TABLE I

CATECHOLAMINE CONTENT OF TURTLE AND DOG HEART

Tissue	Turtle		Dog[1]	
	Noradrenaline[2]	Adrenaline	Noradrenaline	Adrenaline
Atria	1·08 ± 0·08 (6)[3]	0·17 ± 0·01 (6)	2·98 ± 0·17 (8)	0·24 ± 0·08 (8)
Ventricle	0·44 ± 0·07 (6)	0·07 ± 0·01 (6)	1·09 ± 0·02 (13)	0·14 ± 0·03 (13)

[1] Data of Campos, H. A. and Shideman, F. E., *Internat. J. Neuropharmacol.* In press.
[2] Mean concentration in μg./g. fresh tissue ± S.E.
[3] Numbers in parentheses indicate number of animals used.

Catecholamines were determined by the trihydroxyindole fluormetric assay of Shore and Olin (1958).

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