Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 16 No. 6
June 1964

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

17 Bloomsbury Square, London, W.C.1. HOLborn 8967

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J. Pharm. Pharmacol., 1964, **16**, 369–374 Received December 20, 1963

Research Papers

Effects on anaesthetised animals of an oxytocic glycoside extracted from certain species of *Albizia*

A. LIPTON

The actions on anaesthetised guinea-pigs, rabbits, cats and monkeys of a glycoside, "albitocin", obtained from *Albizia gummifera*, and some other species of *Albizia*, used by East African witchdoctors to accelerate labour and induce abortion, are described. On intravenous injection there is a transient fall in systemic blook pressure and an increase in uterine activity, with the pregnant uterus exhibiting greater sensitivity. Both responses occur in the presence of atropine and antihistamines, and in monkeys after bilateral vagotomy and hexamethonium, but a prolonged small rise in blood pressure occurs on intraperitoneal injection of the drug. Electrocardiograph records in rabbits and monkeys exhibit no consistent acute or chronic changes in the electrical activity of the heart resulting from the injection of the drug even in lethal doses, almost up to the time of death.

THE isolation of an oxytocic glycoside, "albitocin", from *Albizia gummifera* (Gmel.) C. A. Smith, var *gummifera*, and some other species of *Albizia* (extracts of which are used by East African witchdoctors to accelerate labour and induce abortion), the partial chemical characterisation of the active principle, and its actions on uterus and ileum preparations *in vitro*, have been described (Lipton, 1960; Lipton, 1963). The present communication deals with its *in vivo* actions on anaesthetised mammals.

Method

Twenty guinea-pigs, 12 rabbits, 8 cats and 14 vervet monkeys (*Cercopithecus aethiops*) were used. Animals were anaesthetised with urethane (1·5 g/kg), pentobarbitone (20–60 mg/kg) or chloralose (60–100 mg/kg) and were heparinised (1,000 i.u./kg).

Pentobarbitone was also used in the electrocardiograph work, in which recordings were made repeatedly over several days and bemegride (10–12 mg/kg intravenously plus 10–12 mg/kg intraperitoneally), was administered to the monkeys after each ECG recording to avoid the poor survival due to dehydration, salt loss, and anorexia with animals almost continuously anaesthetised, and also the poikilothermia due to barbiturate which has been found to occur in monkeys (Luck, 1953). This reduced the duration of each anaesthesia from 6–8 hr to 20–90 min. These animals remained in good condition throughout the experiments, control animals each surviving up to fourteen pentobarbitone inductions over 8–9 days. 30,000–50,000 i.u. of sodium benzylpenicillin B.P. were administered daily to all surviving animals.

As in the *in vitro* work previously reported (Lipton, 1963) the assumption has been made that the glycoside albitocin is the only new active substance present in the plant extract.

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The drug was administered in solution in isotonic saline checked for neutrality with Universal indicator, and as shown previously, contained no nitrogen hence no polypeptide or alkaloid spasmogens. Free inorganic ions were removed by dialysis during purification.

Respiration was recorded by Gaddum's (1941) method. Carotid or femoral *arterial blood pressure* was recorded by a Condon (1951) modified manometer, where appropriate, otherwise an ordinary U-tube mercury manometer was used. *Venous pressure* was recorded from the right jugular vein in three monkeys using a miniature volume recorder (Luck, 1952).

UTERINE ACTIVITY

Four different methods were used for recording uterine activity.

In Method A, a 5–8 cm mid-line abdominal incision was made, the skin and body wall stitched to an elliptical brass ring, and raised slightly on threads to keep them clear of the abdominal contents. The uterine cervix was clamped through this ring and a thread was attached to the ventral surface of the uterus 4–6 cm from it, leading to a frontal lever. Alternatively (Method B), a Cushny myograph was used via a ventral abdominal incision, the ends of the lever arms stitched direct to the uterus 5–8 cm apart.

A small steady rhythm due to respiration was observed super-imposed on the large irregular spontaneous uterine contractions.

Because of the laparotomy, cooling and drying of the surface of the uterus occurred to a variable extent, with a consequent increase in activity due to irritation. The attachments of the threads and levers were also foci of irritation, and in some cases the non-specific activity set up by these causes confused the results obtained by injecting the drugs.

In method C an internal catheter was inserted via the uterine cervical os and, with or without a small fluid-filled rubber balloon at its end, was attached to a pressure transducer device connected to an ink-writing galvanometer (Smyth, 1957). This method left the uterus almost undisturbed and such preparations showed a steady base-line trace without the occasional increase in spontaneous activity which characterised methods A and B.

Method D, which could only be applied to animals in an advanced stage of gestation, was the use of a tocograph recording head (Smyth, 1957) which was attached externally to a shaved area of the abdomen, over the uterus, and recordings made with an ink-writing galvanometer.

ELECTROCARDIOGRAPHY

A portable "Sanborn Visette" electrocardiograph was used. The anaesthetised animals were insulated on a sheet of dry polythene, and the electrodes were attached to shaved areas over electrode paste.

The standard leads were recorded in the order 1, 2, 3, AVR, AVL, AVF, each for 8 to 10 beats without moving the animals or the electrodes.

This was repeated immediately after administration of the drug, and then at regular intervals subsequently during the survival time of the animals. The animals were re-anaesthetised when necessary for this purpose.

Records were taken continuously from two of the dosed rabbits during and just after injection, using Lead 1 for several min, to observe any acute effects on the heart.

Results

ORAL ROUTE

Gastric tube administration of up to 5 mg/kg albitocin in six urethane anaesthetised pregnant guinea-pigs had no effect on the uterine activity recorded by method A, or on the respiration, in up to 8 hr of recording. A dose of albitocin, 0·25 mg/kg, was then dripped onto the exposed uterus and immediately caused a series of powerful contractions, persisting for 30–120 min.

It was concluded that the guinea-pig pregnant uterus would respond *in situ* as *in vitro*, to direct application of the extract, but the oral dose was either destroyed in the digestive tract, or was absorbed from it too slowly or in inadequate amounts.

INTRAVENOUS ROUTE

Doses containing from 0·1–8·0 mg/kg albitocin produced, in all animals, falls in blood pressure proportional to the dose and lasting up to 1 min, and sometimes brief hyperpnoea (Figs 1–3).

In 8 pregnant guinea-pigs, 2 pregnant and 2 non-pregnant rabbits, 1 pregnant and 2 non-pregnant cats, 2 pregnant and 1 post-partum monkey, intravenous injection of up to 8 mg/kg albitocin gave an increase in the rate and force of spontaneous uterine contractions where present, or

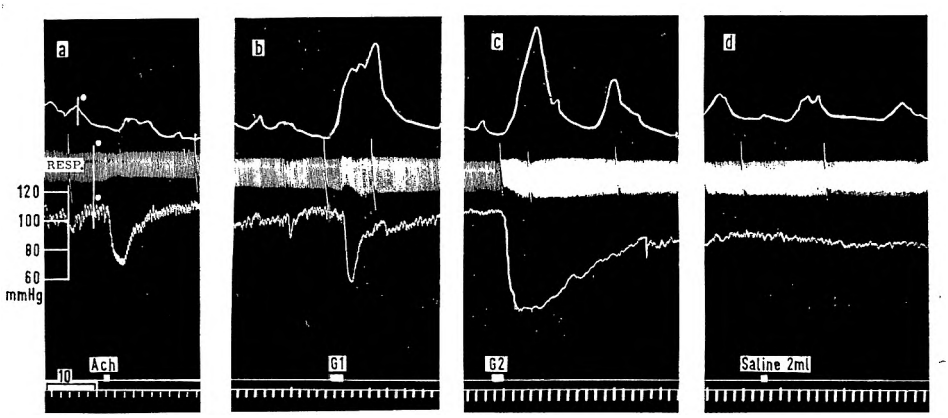

FIG. 1. The effect of albitocin on an anaesthetised rabbit, virgin, wt, 2·48 kg. pentobarbitone anaesthesia. Uterus by method B. All doses intravenous, Ach = 2 μg/kg acetylcholine chloride. G1 = 0·3 mg/kg and G2 = 0·4 mg/kg albitocin. Upper trace, uterus; middle trace, respiration; lower trace blood pressure. Time: 10 sec.

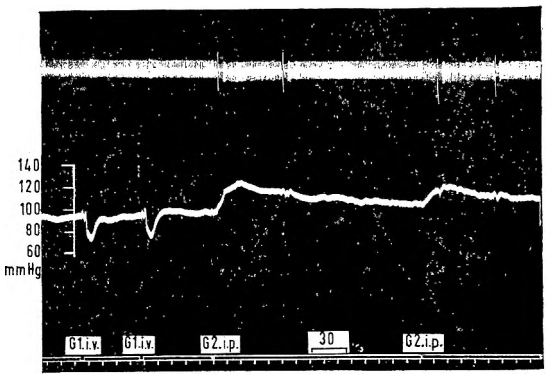

FIG. 2. Effect of route of administration on response to albitocin in an anaesthetised cat. Male, wt 2 kg, pentobarbitone anaesthesia. G1 = 1·25 mg/kg albitocin i.v. G2 = 0·25 mg/kg albitocin i.p. Upper trace, respiration; lower trace, blood pressure. Time: 30 sec.

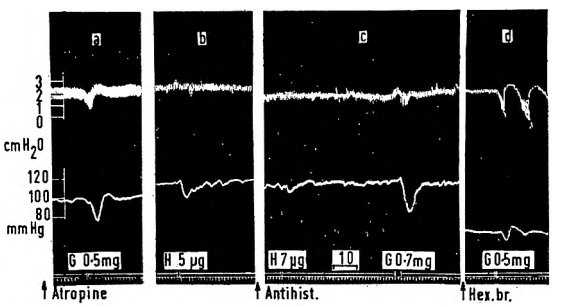

FIG. 3. Comparison of albitocin with other depressant drugs in the monkey (*Cercopithecus aethiops*), wt 3·2 kg, pentobarbitone anaesthesia. Both cervical vagi cut, atropine sulphate 2·5 mg/kg. All doses intravenous. G = albitocin; H = histamine acid phosphate. Antihist = 2 mg/kg promethazine HCl. Hex.br. = 15 mg/kg hexamethonium bromide. Upper trace, venous pressure; lower trace, blood pressure. Time: 10 sec.

produced powerful contractions in quiescent uteri, all responses were proportional to the doses (Fig. 1b, c).

The depressor response was instantaneous and usually compensated in less than 1 min, but the uterine response had a latency of up to 1 min, and sometimes continued for 3–5 min.

Control intravenous doses of saline, and of acetylcholine and histamine giving comparable depressor effects to the doses of albitocin had no effect on the uterus (Fig 1a, d), and doses of antihistamines (pyranisamine maleate and promethazine hydrochloride), and atropine, sufficient to minimise the depressor responses to histamine and acetylcholine respectively, had no effect on the depressor (Fig. 3) or uterine responses to albitocin.

DEPRESSOR ACTION

Three adult male monkeys were examined in an attempt to determine the character of the transient blood pressure fall occurring on intravenous injection.

Atropine, bilateral vagotomy, and massive doses of antihistamines had no effect on the arterial blood pressure fall resulting from up to 0·7 mg/kg of albitocin, even when all these were applied together (Fig. 3, a, b, c). The venous blood pressure showed only a corresponding small change at the time of the arterial pressure fall.

In one animal, after atropine, bilateral vagotomy, and antihistamines, 20 mg/kg of hexamethonium bromide was also injected, and after stabilisation of the arterial blood pressure at a lower level, 0·5 mg/kg albitocin gave a smaller but still quite distinct and reproducible fall (Fig. 3, d).

INTRAPERITONEAL ROUTE

In contrast to the above results, an interesting reversal of the depressor effect occurred in all the animals if administration of the drug was by the intraperitoneal route, this was a prolonged rise in arterial blood pressure from doses as low as 0·2–0·5 mg/kg albitocin (Fig. 2).

ELECTROCARDIOGRAPHY

Four adult male rabbits (doses from 2·3–2·7 mg/kg albitocin) showed no acute or chronic differences in ECG from normal before dosing up to a short time before death 1–7 days later. A control animal dosed with saline also showed no effects throughout.

In two other rabbits lead 1 was recorded continuously during drug administration, and one showed a brief T wave reversal lasting about 30 sec which then returned to normal. The other showed only a brief tachycardia.

Similar results were obtained with seven non-pregnant adult female monkeys (doses from 3·3–6·6 mg/kg albitocin). Only one, which had had 3·3 mg/kg on each of two successive days, survived, the others all died within 72 hr. In three cases the monkeys were so moribund after a few hr, that although they were conscious there was no need for anaesthesia for restraint. The only change observed was bradycardia when the animals were almost on the point of death.

One monkey received a total of 37 mg/kg albitocin over 3 hr in gradually increasing dosage, while all ECG leads were recorded. This animal died 24 hr later without any changes in ECG until the last few min before death.

Discussion

These results demonstrate that albitocin or extracts containing it can act powerfully on the uterus *in vivo*, in moderate doses, causing marked increase in activity in all the species tried, as it does *in vitro* (Lipton, 1963), and the greater sensitivity of the gravid uterus to the drug which has been demonstrated *in vitro* is also apparent *in vivo*, both in the

minimal dose required for an effect, and in the relative increase in activity with larger doses.

In all the animals, the extracts, on intravenous injection, gave a transient fall in blood pressure proportional to the dose, which persisted, like the uterus response, in the presence of atropine and antihistamines, while bilateral vagotomy and hexamethonium also failed to prevent the depressor response in the monkeys. This would appear to localise the cause to a direct action of the drug on the peripheral vessels or heart. On the other hand, intraperitoneal injection of the extracts gave a persistent rise in blood pressure, the mechanism of which is under investigation.

The drug caused no consistent changes in the electrical activity of the hearts of the rabbits or monkeys (Dr G. Shaper, personal communication) even with lethal doses, so that its action is not like that of the common cardiac glycosides.

These results provide further justification for the known use of extracts of these plants by African witchdoctors to accelerate labour and procure abortion.

Acknowledgements. The author wishes to express his appreciation of the generosity of the Makerere College Research Grants Committee and the Wellcome Foundation for grants in support of the work. Grateful acknowledgement is also due to Professor C. P. Luck for his advice and guidance, and to Mrs. T. Walker for technical assistance.

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J. Pharm. Pharmacol., 1964, **16**, 375–380 Received October 17, 1963

Some effects of altering onium substituents on the internitrogen distance in ganglionic and neuromuscular blocking agents

P. H. ELWORTHY

The conductances of butaethonium chloride and bromide, hexaethonium chloride and bromide, and hexadecaethonium iodide have been measured in water at 25°. The limiting equivalent cationic conductances have been interpreted to give a measure of the internitrogen distances, which are larger than in the corresponding methonium compounds, being 5·7–6·7 Å for butaethonium, 6·5–8·7 Å for hexaethonium, and 13·6–14·9 Å for hexadecaethonium. The changes of potency observed on changing the onium substituents are attributed to changes in internitrogen distance and of molecular geometry.

IN a previous paper (Elworthy, 1963b) the conductances of hexa-, deca-, and hexadecamethonium cations were interpreted in terms of internitrogen distance, showing that these molecules were considerably contracted in aqueous solution, due to the high interfacial energy present between the hydrocarbon chains and water. Measurements on decaethonium showed that the contraction was less pronounced than for decamethonium. As considerable changes in biological activity occur when the onium group substituents are altered, studies on additional ethonium compounds have been made in an attempt to relate changes of internitrogen distance and molecular geometry to biological activity.

Experimental

MATERIALS

The compounds were prepared by reacting the redistilled α–ω dibromo- or diiodo-alkanes with excess triethylamine in dry ethanol. The reaction mixture was kept for one week in the cold, refluxed for 4–5 hr, and the excess triethylamine and ethanol removed by distillation. The residue was recrystallised at least three times from a suitable solvent, and dried. Assays by releasing the free acid or free base on a strong cation (Zeocarb 225) or anion (Deacidite FF) exchange column, and titrating the effluent with standard alkali or acid, gave purities of 99·9% or better. Butaethonium and hexaethonium chlorides were prepared from the relevant bromides by passing their solutions down a strong anion-exchange column, which had been converted to the chloride form with Analar potassium chloride.

Water for preparing solutions was twice distilled and had specific conductance of 0·6–0·9 $\times$ 10^6 mho.

MEASUREMENTS

The conductance bridge used has been described (Elworthy, 1963b). The two cells previously used have been replaced by one flask type cell,

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k = 0·6057, calibrated with solutions of recrystallised Analar potassium chloride using the Fuoss-Onsager equation to calculate the conductances of the solutions (Lind, Zwolenik & Fuoss, 1959). The cell had lightly platinised platinum electrodes, and included a polythene coated magnetic follower. Approximately 300 g water was weighed into the cell, which was then placed in thermostated oil bath (25° ± 0·003°), allowed to stand overnight, and the resistance of the water determined on the following day. The compounds were weighed into small pyrex tubes, which were then introduced one at a time into the cell, the stirrer started, and readings of the resistance taken until constant values were obtained for each tube. A run consisted of successively introducing 4–5 samples in this manner. 2–3 runs were made on each compound. The measuring resistance was calibrated from a Cambridge Precision Decade Bridge.

Results

The conductance results are shown in Figs 1 and 2 as plots of equivalent conductance (Λ) against (normality)$^{\frac{1}{2}}$; preliminary Λ_0 values were found by extrapolating these phoreograms to zero concentration. All the salts studied appeared to be slightly associated, approaching the Onsager limiting tangent from below, and the final fitting of the results to obtain Λ_0 was by the method of Fuoss & Shedlovsky (see Fuoss & Accascina, 1959). Decaethonium iodide was found to be associated in previous work. Using 78·1 for $\lambda_0(Br^-)$, 76·8 for $\lambda_0(I^-)$, and 76·4 for $\lambda_0(Cl^-)$ the λ_0 values found for the cations were:

	λ_0 from chloride	λ_0 from bromide	λ_0 from iodide
Butaethonium	42·8	42·7	
Hexaethonium	39·4	39·4	
Hexadecaethonium			31·6

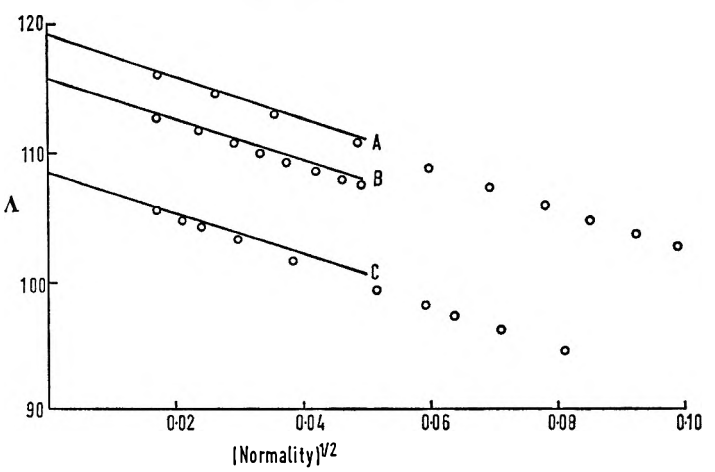


FIG. 1. Plots of equivalent conductance (Λ) against (normality)$^{\frac{1}{2}}$ for A. Butaethonium chloride. B. Hexaethonium chloride. C. Hexadecaethonium iodide.

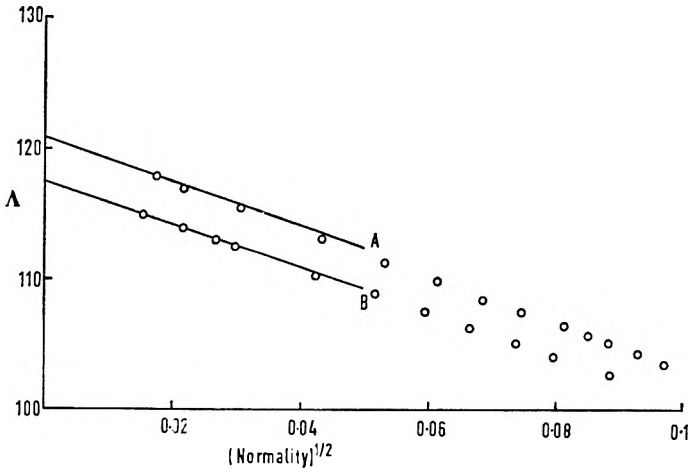


FIG. 2. Plots of equivalent conductance (Λ) against (normality)$^{\frac{1}{2}}$ for A. Butaethonium bromide. B. Hexaethonium bromide.

The limiting cationic conductances are interpreted in terms of molecular shape using the methods previously described (Elworthy, 1962, 1963a, 1963b) representing the molecules as prolate ellipsoids, and calculating the long (a) and short (b) semi-axes. From a, b, and the volume of each grouping in the molecule, values of the distance from the centre of the ellipsoid (distances, x, along the a axis) corresponding to the volume of the molecule inside and outside the nitrogen atoms were found (equation 6, Elworthy, 1963b).

The results on the ethonium compounds do not provide as clear an estimate of internitrogen distance as those on the methonium salts. This is due to the several possible arrangements of the ethyl groups on the nitrogen atoms. The three principal arrangements (see Elworthy, 1963b) are: (1). Ethyl groups lying in close contact with the main hydrocarbon chain which links the nitrogen atoms. (2). Two ethyl groups close to the main hydrocarbon chain, and one facing outwards in the x direction. (3). One ethyl group close to the chain, and two directed outwards. The internitrogen distances calculated for these alternative arrangements are given in Table 1.

TABLE 1. INTERNITROGEN DISTANCES FOR ETHONIUM COMPOUNDS (Å)

$Et_3\overset{+}{N}[CH_2]_n\overset{-}{N}Et_3$

Position of ethyl groups	n = 4	n = 6	n = 10	n = 16
1	8·3	10·4	13·2	16·7
2	6·8	8·8	11·5	15·0
3	5·7	7·5	10·2	13·6
Nordic cross	6·0	7·9	10·6	14·0

The results of Wait & Powell (1958), on the crystal structure of tetraethylammonium iodide showed that the cation had the configuration of

a nordic cross. Although in the case of the ethonium compounds, this configuration may not be maintained in solution, it falls between positions 2 and 3, which appear, from molecular models, to be the most strain free arrangements. In all it seems likely that the shaded area in Fig. 3 represents the most probable inter-onium distance for these compounds.

Discussion

Elworthy (1963b) has pointed out how an interfacial energy effect led to greater molecular extensions in the ethonium series, compared to the methonium series. This result is not predictable from Gill's (1959) calculations. At this stage it seems profitable to discuss some of the results in relation to biological activity.

The principal conclusions are:

(1) The molecules are much contracted in solution.

(2) Introduction of ethyl for methyl on the head groups increases the internitrogen distance.

It is usual to suggest a two point attachment theory for neuromuscular block, and in many cases for ganglionic block. It is also common to consider "holes" in receptor surfaces into which head groups drop neatly. The implication of conclusion (1) is that, due to contraction, the molecule resembles an ellipse from a side view; the diameter of the head group may thus be smaller than the diameter (2b) at the centre of the molecule in the methonium series. The implication of conclusion (2) is that the more extended configuration produced by substituting ethyl for methyl groups, which causes the chain to lengthen, also decreases the diameter at the centre of the molecule; the diameter at the position of the head groups is increased by the substitution. Some pharmacological results are examined in the light of these considerations.

For neuromuscular block, decamethonium had peak activity, having an internitrogen distance of 9·5 Å. Decaethonium, however, has an internitrogen distance of 10·8 Å (mean value from shaded area in Fig. 3), and its potency is 10 to 25% that of decamethonium (Barlow, Roberts & Reid, 1953; Thesleff & Unna, 1954). Wein, Mason, Edge & Langston (1952) showed that converting the methonium into an ethonium head group for compounds with $n = 6$ and 7 enhanced their neuromuscular blocking power. For $n = 6$ the change in internitrogen distance is from 6·3 to 8·0 Å, for $n = 7$ from 7·2 to 8·8 Å; the ethonium compounds come closer to the 9·5 Å distance of decamethonium, which is presumably necessary for peak activity. Thesleff & Unna give results showing the same general trend. However, the potency (or type of action) of a molecule expanded by introduction of ethyl groups cannot be expected to be the same as that of a longer chain methonium compound, because the geometry of the molecules is different, as is the fit on the receptor site, and the distances from the centre of the onium group to the receptor atoms. Nevertheless, the change of internitrogen distance on altering

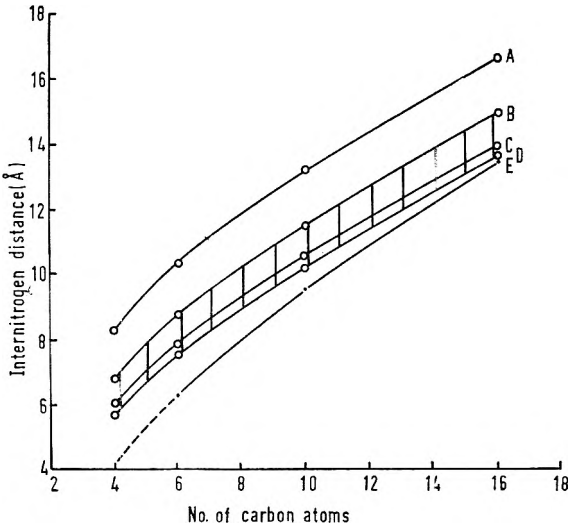
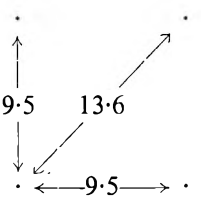


FIG. 3. Internitrogen distance against number of carbon atoms separating the nitrogen atoms. Ethonium Compounds: A. Position 1. B. Position 2. C. Nordic cross. D. Position 3. E. Methonium Compounds.

the head groups does give a guide to explaining some of the potency changes.

It is interesting to speculate on the implications of a geometrical arrangement of receptor points for neuromuscular block. Using 9·5 Å as the critical distance between receptor points, and letting them be present on the corners of a square, we have a hypotenuse of 13·6 Å.



This distance should provide a second set of receptor points for attaching molecules to the surface. Molecules having the required internitrogen distance are n = 16–17 for methonium and n = 14–15 for ethonium derivatives. Barlow & Ing (1948) showed that the most potent member of the ethonium series tested was n = 13, no tests of longer chain compounds being made; n = 13 has an internitrogen distance of 12·6 Å. Examination of Paton & Zaimis's (1949) results shows that in most of the species of animal used, the n = 18 methonium derivatives was more potent than n = 11 or n = 12 methonium derivatives. The n = 18 compounds give an approximate fit on the second site. It was realised that the n = 16 methonium compound should also fit the longer site; a preliminary test by courtesy of Mr. J. J. Lewis indicated a comparable

potency of the n = 16 and n = 18 methonium compounds (comparison of n = 16 methonium and tubocurarine, and using Paton & Zaimis' n = 18 methonium and tubocurarine figures).

Turning now to ganglionic block, the n = 4 methonium compound shows an increase of potency on introducing ethyl groups, the increase in internitrogen distance being from 4·2 Å to 6·4 Å. This second distance is almost exactly that of hexamethonium, but the ganglionic blocking potency of butaethonium is 6% that of hexamethonium. The indication is that other geometrical factors in the molecule are as important as internitrogen distance. The shorter chain molecules, like hexamethonium, show a smaller percentage contraction in solution than the longer ones; their equatorial diameter (2b) is not so great. Thus the bulkiness of head groups should have a more significant effect on the fit of short chain molecules to receptor sites than on that of long chain ones. Of particular interest are the potencies of n = 5 and n = 6 compounds; with Me_2EtN^+ and $BuMe_2N^+$ head groups these compounds have greater potencies than the corresponding methonium compounds. Although the hydrocarbon chains will be more expanded in these derivatives, some small groups are still present, suitable orientation of which could still bring the onium nitrogens atoms close to the receptor surface.

Acknowledgement. I thank Miss M. Buchanan for technical assistance.

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J. Pharm. Pharmacol., 1964, **16**, 381–384 Received December 12, 1963

Response of standardised suspensions of *Escherichia coli* to iodine

J. M. NEWTON AND J. A. VICKERS

Suspensions of *Escherichia coli* prepared from agar slopes by a standardised method were compared with washed suspensions, prepared by a centrifuging process, the dry weight of which was less than that of the unwashed suspensions for the same number of viable cells. In a series of experiments the dry weight of a given number of washed cells proved to be more constant than the dry weight of the same number of unwashed cells. The washed bacteria were more susceptible to iodine than the unwashed bacteria.

METHODS used to evaluate antibacterial agents vary in form and complexity, but most involve an assessment of the bactericidal or bacteriostatic activity of the test substance. In an attempt to provide bacteria which give a constant response to bactericides, specific directions for preparing the organisms are often stated, for instance, by Berry & Bean (1954) and in the Rideal Walker test. The final form in which the bacteria are used differs with the test and includes broth cultures (Rideal Walker test), dried films (Hoy & Clegg, 1953) and suspensions in sterilised quarter strength Ringer's solution (Berry & Bean, 1954).

When assessing the bactericidal activity of iodine, using aqueous suspensions and a counting technique, both Chang & Morris (1953) and Carroll (1955) found a great variation in response. Carroll (1955) attributed this to the presence of traces of organic matter in the suspension. Newton (1962) had found that suspensions of *Staphylococcus aureus*, giving similar viable counts, did not always have the same dry weight, and the same was true for *Escherichia coli*. Hugo & Newton (1964a) found that variations in response of aqueous suspensions of *Escherichia coli* and *Staphylococcus aureus* to iodine could be related to the dry weight of the suspension. An attempt has now been made to prepare bacterial suspensions of a more reproducible nature.

Experimental

MATERIALS

The materials of the culture media were of Oxoid bacteriological grade. The nutrient broth had the following formula (%); Lab Lemco 0·5, peptone 1·0, sodium chloride 0·5, distilled water to 100·0. Nutrient agar was prepared by solidifying the above medium with 1·8% Agar No. 3. Both media had a pH of 7·2 after sterilisation. The other chemicals were of Analar reagent grade. Iodine solutions were prepared by dissolving iodine crystals in sterile distilled water, and adjusted to known concentrations after estimating against standard sodium thiosulphate, with an amperometric determination of the end-point.

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PREPARATION OF SUSPENSIONS

The bacterium was *E. coli* (NCTC 5933). An unwashed suspension of the organism was prepared from a 24 hr culture which had been grown on nutrient agar, centrifuged for 1 min at 1500 rpm to remove debris and large clumps, and shaken with glass beads for 5 min to break up remaining clumps. The bacteria were washed by centrifuging the unwashed suspension at 2400 rpm for 5 min, they were then resuspended in an equal volume of sterile distilled water, and re-centrifuged and re-suspended. If a volume of water equal to the original volume was taken for resuspension and the clumps of sedimented organisms thoroughly dispersed, a single washing with water produced suspensions with a consistent dry weight: number relationship.

Viable counts were made by serial dilution in quarter strength Ringer's solution, adding 0·5 ml of the final dilutions to 4 ml of nutrient agar in a roll tube. To obtain the dry weight of the suspensions, they were heated to constant weight under an infra-red lamp.

The interaction of iodine and bacteria was at room temperature (20°) by mixing bacteria and iodine solutions in a fixed volume of 5 ml. After 2 min, 1 ml of the mixture was removed, the iodine inactivated with sterile 0·01N sodium thiosulphate, and a count made.

Results and discussion

Analysis of the initial counts according to the method of Fisher (1958) gave values for $\sqrt{2\chi^2} - \sqrt{2n-1}$ of 0·118 for the unwashed suspension, and 1·035 for the washed suspension. As these values did not exceed 1·645 (representing the 5% level of probability), we may assume that the counting technique was satisfactory. The variance ratio (F) of the counts of unwashed and washed suspensions was found to be below 95% probability level. Hence when the dry weight of 10^8 bacteria is calculated, for the unwashed and washed suspensions, any large difference will not be caused by the errors of counting. The results in Table 1

TABLE 1. RESULTS FOR WEIGHT OF 10^8 BACTERIA, WASHED AND UNWASHED

	Unwashed bacteria	Washed bacteria
Number of results	11	11
Mean weight of 10^8 bacteria in μg/ml	120·5	36·55
Range	88·1–164·0	32·9–41·5
Standard deviation	24·47	3·00
Coefficient of variation	20·3	8·2

show that the dry weights of washed suspensions are more reproducible than the dry weights of unwashed suspensions when a comparison is made with count. A reduction of about 70% in dry weight occurs after washing in most experiments.

Known dry weights of the two kinds of suspensions were treated with 10 μg/ml of iodine, and the log % survivors after 2 min recorded (Fig. 1). Treating suspensions of approximately the same dry weight gave results

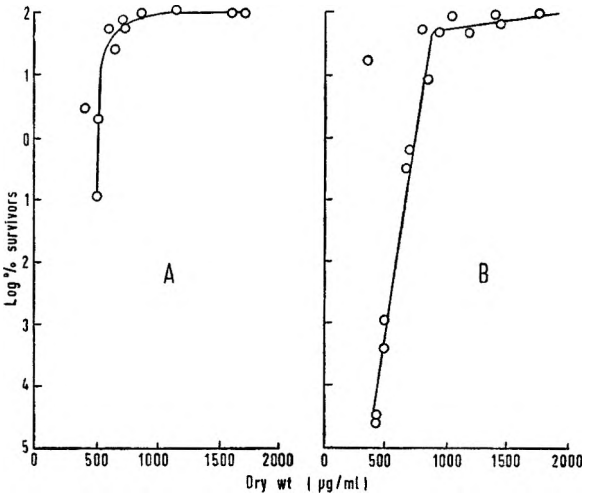

FIG. 1. The effect of iodine (10 μg/ml) on washed and unwashed bacteria of different dry weights. Graph of log % survivors after 2 min at 20° plotted against dry weight. (A) Unwashed bacteria. (B) Washed bacteria.

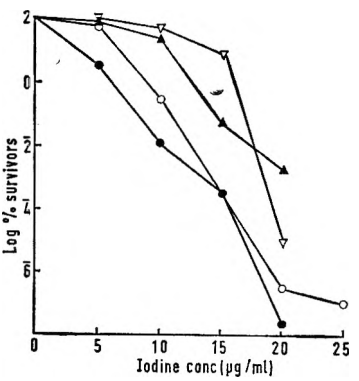

FIG. 2. The effect of varying concentrations of iodine on washed and unwashed bacteria of similar dry weights after 2 min at 20°. ○ Washed bacteria, 680 μg/ml. ● Washed bacteria, 490 μg/ml. ▽ Unwashed bacteria, 740 μg/ml. ▲ Unwashed bacteria, 660 μg/ml.

shown in Fig. 2. The washing process appears to make the bacteria more susceptible to iodine. The following explanations are offered.

(1) Washing removes a protective substance from the cell surface. In a washed suspension of a given dry weight all the available iodine can attack the cell.

(2) Material removed by the washing process has a greater affinity for iodine than the bacterial cell. In an unwashed suspension this material takes up iodine and does not allow an adequate amount to reach the surface of the cell for killing. Evidence supporting this is that, when a bacterial suspension and the washings from the preparation of

such a suspension, both of the same dry weight, were treated for 15 min with a high concentration of iodine, more iodine remained in the presence of the bacteria than in the presence of the washings. Hugo & Newton (1964b) also showed that the adsorption of iodine from solutions by micro-organisms and serum differed.

Said, Lambin, German & Bernard (1963) found that when bacterial suspensions were washed by centrifugation after treatment with various bactericides, in most instances the surviving organisms were killed by lower concentrations of the bactericides. Washing, before and after treatment with bactericides, could therefore affect recovery of the bacteria by removing materials essential to growth.

Whatever the cause of the difference in response of the washed and unwashed suspensions, it appears that when assessing the bactericidal activity of iodine, it is essential to have a closely controlled method of preparing the suspensions. For a more predictable suspension, a washed suspension would appear preferable, and whilst this conclusion can only be drawn for iodine, the behaviour of other bactericides in similar circumstances would seem worthy of investigation.

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J. Pharm. Pharmacol., 1964, **16**, 385–393 Received October 25, 1963

The role of histamine and 5-hydroxytryptamine in inflammatory processes

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During aseptic inflammation there is a rise in the histamine and 5-hydroxytryptamine (5-HT) contents of the skin and several internal organs in mice and rats. The time course of this rise has been worked out. Depletion of histamine or 5-HT, adversely affects the exudative or reparative phases of inflammation in the rat. Reserpine, the agent used to deplete 5-HT, produces an effect which resembles the action of anti-inflammatory steroids like prednisolone.

THE occurrence of high levels of histamine at sites where the organism meets the external environment, led to the suggestion that histamine might be involved in a defence mechanism (Paton, 1955).

The close association of histamine with 5-hydroxytryptamine (5-HT) and the mast cell, and the demonstration that characteristic mast cell changes occur during aseptic inflammation (Sanyal, 1959), has strengthened the hypothesis of a defensive function for histamine and suggested a similar role for 5-hydroxytryptamine (5-HT).

The changes in histamine and 5-HT contents after aseptic inflammation have been studied and the effect of their depletion has been separately studied both for the exudative and reparative phases of inflammation. A preliminary communication was published earlier (Bhatt & Sanyal, 1963).

Materials and methods

Animals. Albino rats, 80–120 g, and albino mice 20–25 g, of either sex were obtained from local dealers and fed with Anidiet A, the composition of which has been previously described (Dhar & Sanyal, 1962). Animals were maintained in air conditioned rooms and water was allowed *ad lib.*

Production of aseptic inflammation. Rats or mice were anaesthetised with ether, and on the shaved dorsal skin an aseptic incision was made 2–3 cm long in mice and 3–5 cm in rats. The skin flaps were mobilised and then the wound was closed with suture clips. Similar animals anaesthetised and then allowed to recover served as controls. All were then transferred to sterilised cages. Animals, from control and test groups were killed at varying intervals, and tissues from 6 or more similarly-treated animals were collected and pooled for extraction and assay of histamine and 5-HT as described by Parratt & West (1957a). Mast cell examinations were made at the same time.

Production of granuloma pouch. The granuloma pouch technique of Selye, as described by Finney & Somers (1958) was used to examine the exudative phase of inflammation in rats (150–200 g, either sex). A pouch in the subcutaneous tissue of the dorsum was formed in anaesthetised animals by injecting 25 ml of air, 0·5 ml of a 2% solution of croton

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oil in arachis oil was subsequently injected into the pouch. The intense irritation thus set up leads to inflammatory changes in the pouch which can be assessed qualitatively while the amount of exudate can be measured.

Cotton wool pellet test. To study the phenomenon of fibrosis, the cotton wool pellet method of Meier, Schuler & De Soulles, as described by Finney & Somers (1958) was adopted. Sterilised cotton wool pellets (8/animal) each weighing 10 mg were implanted aseptically in the subcutaneous tissue of the back. They were removed after 7 days, cleared of extraneous tissues, and dehydrated in an oven to constant weight. The rise in weight denote the amount of fibrous tissue formed.

Depletion of tissue histamine and 5-HT. Tissue histamine or 5-HT was preferentially depleted by repeated injections of polymyxin B, or reserpine respectively, as described by Parratt & West (1957a).

Results

CHANGES IN MAST CELLS, TISSUE HISTAMINE AND 5-HT CONTENT DURING ASEPTIC INFLAMMATION

Changes in rats. Throughout the experiment, the operated animals were in apparent good health, their behaviour being indistinguishable from that of control animals. Animals were killed 1, 2, 3, 4, 6, 8, 12, 16 and 26 days after operation. Controls were killed at the same time.

A slight swelling and accumulation of serosanguineous fluid was noticed during the first 2–3 days, but this subsided later. The inflammatory changes were seen to be subsiding and strands of fibrous tissue had made their appearance 6 days after operation. The incised edges were united on day 12, and the union was quite firm after 26 days.

The mast cells in an adjacent site were swollen and degranulated after 24 hr, many had disappeared. The changes were more marked after 48 hr when very few cells were detected. In 4 days a large number of small mast cells made their appearance along with vascular strands, which were seen to invade the operated area. Some of these cells migrated away from blood vessels, and rapidly divided to form cell clumps.

After 8 days, mast cells were normal in appearance.

The changes in histamine and 5-HT contents of skin from operated animals expressed as % of values obtained in control animals are shown in Figs. 1, 2 and 3 for the site of injury, an adjacent site and a distal area respectively. There was an initial lowering of histamine values at the injured site which was later followed by a rise. In other areas, there was a rise from the beginning, which, as in the injured site, was maximal in 8–12 days. The values in the injured area were subnormal in the 16–26 day period, but in other areas, by this time the contents were comparable with values obtained in control animals.

5-HT values from all the areas showed a rise, which was maximal in 6–8 days, and returned to basal levels after 16–26 days.

The changes in histamine and 5-HT contents of other tissues are shown in Table 1. The histamine content of the intestine was slightly raised, and returned to normal in 12 days; that of the spleen showed minor or no

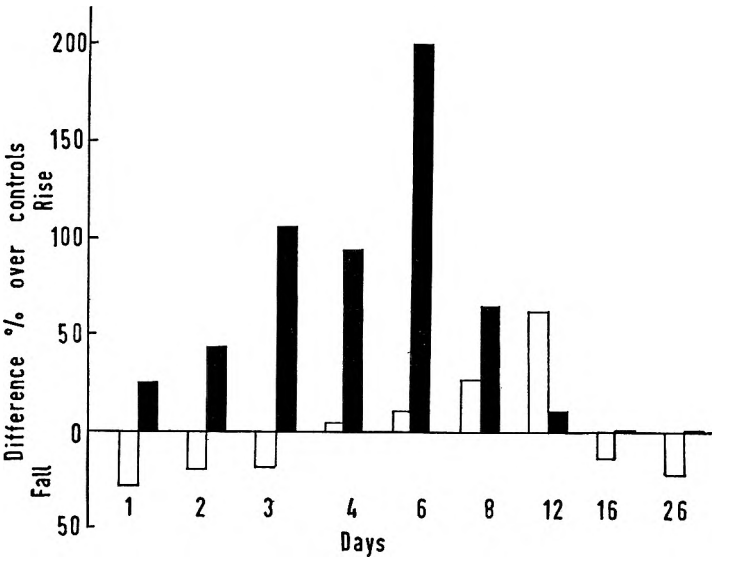

FIG. 1. Changes in histamine and 5-HT content of rat skin at the site of injury after production of aseptic inflammation. Results expressed as percentage differences over control values. Open columns = histamine; solid columns = 5-HT.

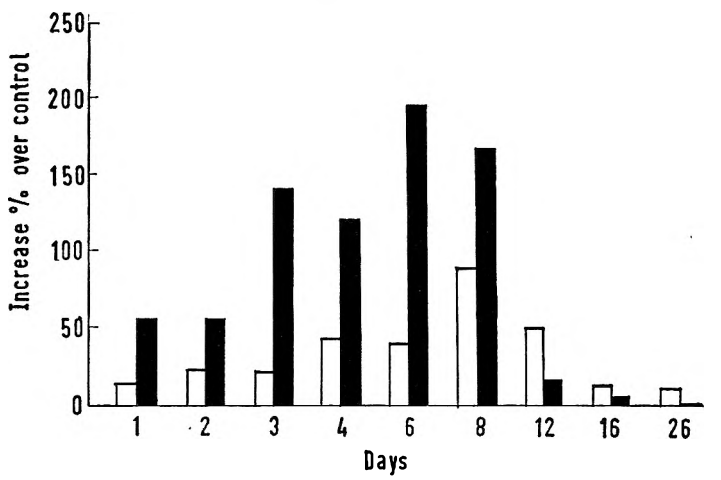

FIG. 2. Changes in histamine and 5-HT content of rat skin in an area adjacent to the site of injury after production of aseptic inflammation, expressed as percentage increase over control values. Open columns = histamine; solid columns = 5 HT.

changes; that of the lung showed erratic changes. Experiments were repeated several times, but no definite pattern could be established. The 5-HT values of tissues other than skin showed insignificant alterations.

Changes in mice. Groups of animals were killed 1, 3, 7 and 15 days after operation for mast cell study and extraction and assay of histamine

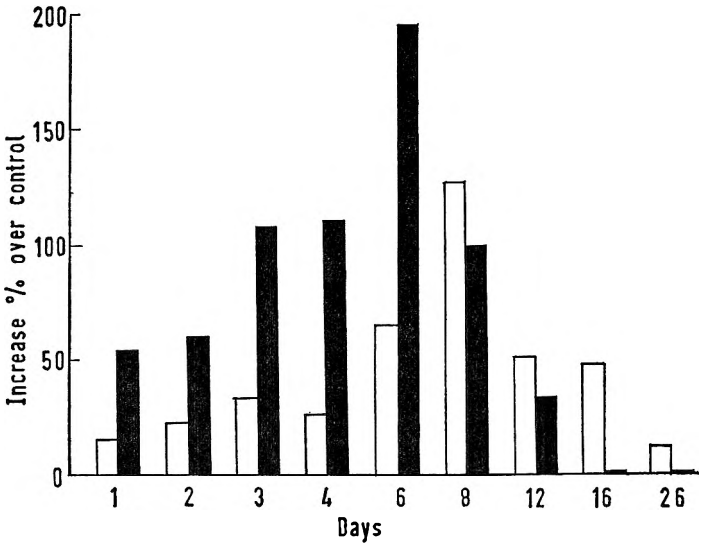

FIG. 3. Changes in histamine and 5-HT content of rat skin in an area distal to the site of injury after production of aseptic inflammation, expressed as percentage increase over control values. Open columns = histamine; solid columns = 5-HT.

TABLE 1. HISTAMINE AND 5-HT CONTENT OF SPLEEN AND INTESTINE OF RAT, AFTER PRODUCTION OF ASEPTIC INJURY ON THE SKIN EXPRESSED AS % OF CONTROL

	Spleen		Intestine	
Days after injury	Histamine	5-HT	Histamine	5-HT
1	105	112	146	82
2	105	85	135	76
3	105	102	108	82
4	110	80	116	100
6	97	100	114	100
8	91	120	136	100
12	92	89	108	100
16	100	100	108	100
26	100	100	100	87

and 5-HT from various tissues. Control animals were killed at similar times.

No animals developed obvious sepsis, and a minimal amount of serosanguineous discharge was seen only in the first 24 hr. By day 7 inflammation had subsided, and the wound edges were uniting. The union was firm by day 15.

Mast cell changes were similar to those seen in the rats, a later near-normal state being obtained by day 15.

The changes in the histamine and 5-HT contents are shown in Table 2. In an area adjacent to the operated site, there was an initial lowering, followed by a rise of histamine content which returned to near normal values in 15 days. In distal areas, high values were seen from the beginning and these returned to normal in 15 days. 5-HT values were raised from

TABLE 2. CHANGES IN THE HISTAMINE AND 5-HT CONTENT OF THE AREA ADJACENT AND DISTAL TO THE SITE OF INJURY OF MOUSE SKIN AND INTESTINE AFTER PRODUCTION OF ASEPTIC INFLAMMATION ON THE SKIN EXPRESSED AS % OF CONTROL VALUES

Days after production of injury	Area adjacent to site of injury		Area distal to site of injury		Intestine	
	Histamine	5-HT	Histamine	5-HT	Histamine	5-HT
1	73·00	116·66	150·00	125·00	333	320
3	131·62	140·62	154·28	150·00	100	90
7	156·66	200·00	200·00	275·00	100	100
15	100·00	100·00	115·00	112·50	106	104

the beginning and were maximal in 7 days, normal values were obtained in 15 days.

CHANGES IN HISTAMINE CONTENTS OF SKIN DURING SEPTIC INFECTION

As studies were in progress, rats received in a batch from a local dealer were seen to have multiple abscesses in the skin, and it was thought that it might afford an opportunity of studying the histamine content during natural infections. A smear of the pus, on examination, showed clumps of organisms resembling staphylococci. The clinically non-infected animals were separated and served as control. There was a 50% rise in the histamine content of the skin of infected animals as compared with controls.

EFFECT OF DEPLETIONS OF HISTAMINE OR 5-HT ON GRANULOMA POUCH PRODUCTION IN RATS

Granuloma pouch in control animals. Granuloma pouches were produced in a number of rats, and animals were killed after 3, 5, 8, 13 and 18 days. Intense inflammation and serosanguineous discharge was seen in 3 days, between 5–8 days, the pouch became adherent to adjacent structures with much thickening of the wall, and the discharge also became seropurulent. Later, there was greater thickening of the wall and the pus became thick and viscid. Changes were most marked between 5 and 8 days and in all further experiments pouches were examined after 6 days.

Granuloma pouch in histamine-depleted animals. Groups of animals received repeated injections of polymyxin B beginning with 0·5 mg/kg and increasing to 5·0 mg/kg over 4 days. Control animals received injections of normal saline at similar times. One group each of polymyxin B-treated, and control animals were killed for extraction and assay of tissue histamine and 5-HT. The histamine content of the skin and lung was much reduced, the residual amount being approximately 10% of control values. Spleen values were not significantly altered, and the intestinal contents were depleted by about 50%. Changes in 5-HT values were insignificant.

In another set of polymyxin B-treated and control animals, granuloma pouches were induced. On the sixth day, a mild to moderate degree of

inflammation was seen in 50% of animals and in the rest, inflammation was patchy and mild; the amount of exudate was small (Fig. 4).

Granuloma pouch in 5-HT *depleted rats.* Injections of reserpine, 10 mg/kg, were given on two consecutive days. This resulted in a 75–95% depletion of 5-HT from most organs, leaving the histamine values unaffected. The granuloma pouches in this group, in contrast to the control animals, were not warm to the touch. The skin was thin and parchment-like. Mild inflammation was present in only 25% of animals. In the rest there was no sign of inflammation. The amount of exudate was insignificant (Fig. 4).

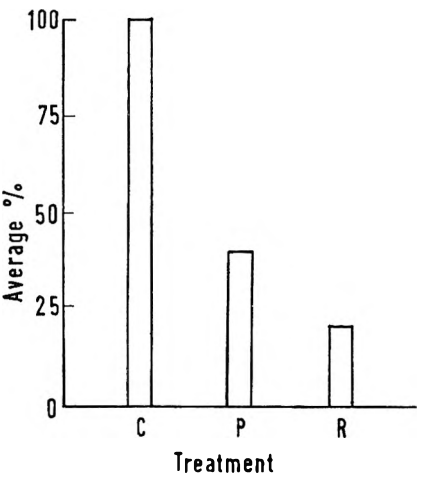

FIG. 4. Amount of exudate in granuloma pouch after various treatments expressed as average % (control value = 100%). C = control; P = polymixin; R = reserpine.

Granuloma pouch in prednisolone-treated animals. Groups of rats were given intraperitoneal injections of prednisolone hemisuccinate, 20 mg/kg, for 3–4 days. There was a 80% reduction in the 5-HT content of skin and lungs, whereas the amounts in the spleen and intestine were approximately halved.

Histamine content of skin and intestine was reduced by approximately 30%, the lung and spleen values being unaffected. The changes in the granuloma pouch after prednisolone treatment were similar to those seen in reserpine-treated animals. The exudate was insignificant, and inflammation was minimal compared with control animals.

GRANULATION TISSUE FORMATION AFTER DEPLETION OF TISSUE AMINES

One group of 6 rats received injections of polymyxin B, as in the previous experiment, another group of 6 received injections of reserpine, a further 6, the control group, received injections of normal saline. Cotton wool pellets were then inserted in all the animals as described. The amount of fibrous tissue developed in 7 days is shown in Fig. 5.

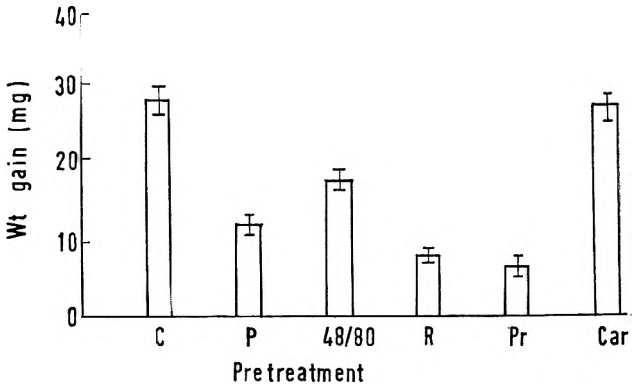

FIG. 5. Illustrating the gain in weight of implanted cotton wool pellets in rats after various pretreatments. Data based on average of 48 observations for each. C = control; P = polymixin; 48/80 = compound 48/80; R = reserpine; Pr = Prednisolone; Car = carbachol.

Development of granulation tissue was minimal in reserpine-treated animals; the amount in polymyxin B-treated animals was also lower than that of the controls. The differences were statistically significant.

The prevention of granulation tissue formation after reserpine treatment could be due to depletion of 5-HT, or depletion of catecholamines (Muscholl & Vogt, 1957) or to its own pharmacological actions. To test these possibilities the experiments were repeated after the following pretreatments.

(i) Compound 48/80, graded doses 1 to 5 mg/kg over 4 days, a substance depleting both histamine and 5-HT (Parratt & West, 1957b).

(ii) Carbachol, 0·5 mg/kg twice daily for two days, a substance depleting catecholamine stores (Butterworth & Mann, 1958).

(iii) Prednisolone, a substance with known anti-inflammatory actions, and known to deplete 5-HT from several tissues.

The results are in Fig. 5. Prednisolone prevented development of grannulation tissue to a marked extent; carbachol had no action. Compound 48/80 pretreatment reduced the amount of fibrosis around the implanted pellets.

The differences were statistically significant for prednisolone and compound 48/80.

Discussion

During aseptic inflammation in rats and mice there is a rise in tissue histamine and 5-HT, the process being most marked in the skin. Depletions of histamine or 5-HT adversely affected the exudative as well as the reparative phases of the inflammation. A rise also occurred in septic infections. Depletion of histamine or 5-HT has been shown previously by Mishra & Sanyal (1959) to increase the invasiveness of pathogenic bacteria in rats, to which in natural state they are relatively immune.

There is some parallelism between the changes in histamine and 5-HT levels with those occuring in mast cells, but the association is not very close. An interesting feature of the rise is that it is not localised but is found over all the skin in sites distal to injury and in some internal organs. This suggests the possibility of some general mechanism which it is hoped to consider at a later date.

The anti-inflammatory action of reserpine is most likely to be related to its 5-HT depleting properties, as this is shared with other substances having similar actions.

The reserpine action resembles that of prednisolone. Both substances affect the actions of 5-HT more than they do those of histamine, though there are qualitative and quantitative differences. It is debatable whether reserpine action is mediated through the cortical hormones, or that both substances have a similar site of action.

Much evidence has now accumulated to suggest that histamine and more particularly 5-HT may be involved in defensive functions in response to injury, infection, inflammation and repair.

This may be summarised as follows:

(i) There is a rise in skin histamine after aseptic injury as well as in naturally infected states (see also Geiringer & Hardwick, 1953).

(ii) The inflammatory response in the granuloma pouch method, and development of fibrous tissue round implanted cotton wool pellets are reduced after histamine depletion, and more particularly after depletion of 5-HT. Stern & Nikulin (1960) also noted that histological changes in the granuloma pouch were less marked after histamine or 5-HT depletion.

(iii) The resistance of rats to pathogenic organisms is reduced after histamine or 5-HT depletion (Mishra & Sanyal, 1959).

(iv) Inflammatory exudates contain both histamine and 5-HT and each substance is capable of increasing capillary permeability (Dale & Richards, 1918; Spector & Willoughby, 1957).

(v) Depletion of histamine or the use of antihistamine substances reduces the amount of pleural exudate produced by injection of turpentine (Spector & Willoughby, 1958).

(vi) Histamine and 5-HT stimulate phagocytosis (Kato & Gozsy, 1956; Buttle & Northover, cit. Mishra & Sanyal, 1959).

(vii) Tensile strength of healing skin incisions is increased when the formation of histamine is accelerated and decreased when it is inhibited (Kahlson, 1960).

Spector and Willoughby (1958) did not find any reduction in exudate of turpentine pleurisy in reserpine-treated animals or in animals pretreated with 2-bromolysergic acid diethylamide, an antagonist of 5-HT. However, the dosage of reserpine used was only 20% of the amount we gave.

The exact mechanism of action of histamine or 5-HT in these processes is not very clear. Recently it has been suggested that histamine liberated from mast cells may prepare many more connective tissue cells than are

normally available, to receive heparin or heparin-containing granules, and these may be used in preparing ground substances; 5-HT may possibly, particularly in the rat and mouse, act similarly (Riley, 1962; West, 1962).

Most of the experiments reported were in rats and mice, and it may be a fair conclusion that at least in these two species, histamine as well as 5-HT participate in tissue defence and repair during inflammation.

Acknowledgements. We thank Burroughs Wellcome (India), who supplied the Polymyxin B used, the Wellcome Research Laboratories (U.S.A.) for the Compound 48/80 and Sandoz (Basle) for the 5-HT.

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J. Pharm. Pharmacol., 1964, **16**, 394–399 Received January 3, 1964

Preparation and surface-active properties of glycyrrhizic acid and its salts

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Methods are given for the preparation in a pure form of glycyrrhizic acid and its salts. From conductivity and surface tension measurements and unsuccessful solubilisation studies it would appear that micelle formation does not occur in pure solutions of these substances.

JAMES & Stanford (1962) have found from conductance and surface tension measurements that liquorice extracts contain at least two amphipathic substances which form micelles, and which, they suggested were probably glycyrrhizin and gum. Solubilisation by the extracts of benzene, chloroform and hexane was also demonstrated. Glycyrrhizin was isolated from liquorice by Tschirch & Cederberg (1907), but their method gives a poor yield of a coloured product, heavily contaminated with gum, and difficult to purify. No other method of isolation has been reported, but all subsequent workers who have described glycyrrhizin agree that it is a complex salt of glycyrrhizic acid. The major matter of controversy is the identity of the cations; Tschirch & Cederberg (1907) said that glycyrrhizin is a mixed calcium and potassium salt, but Paris (1956) and Pointet-Guillot (1958) have described it as the calcium and ammonium salt, while Tocco-Tocco (1924) and Orsi (1956) claim that it is a mixed potassium and ammonium salt.

In view of the indeterminate nature of the cations of glycyrrhizin and the fact that the surface-active properties would be due to the anion, whose identity is agreed, we have preferred to examine the surface activity of glycyrrhizic acid and some of its salts to determine if this molecule is in fact one of the solubilising constituents of liquorice.

Experimental

Analyses for C, H and N were made by the Department of Chemistry, Welsh College of Advanced Technology, Cardiff, and the Department of Pharmaceutical Chemistry, School of Pharmacy, London.

Potassium was determined volumetrically after incineration, and ammonia distillations were carried out in a Kjeldahl apparatus using octanol as an anti-foam agent. Water contents were determined by drying to constant weight over calcium chloride *in vacuo* at 90°. Spectral absorption between 200–300 mμ was measured with a Uvispek quartz spectrophotometer.

PREPARATION OF MATERIALS

Monoammonium glycyrrhizate was prepared from ammoniated glycyrrhizin (Stafford Allen Ltd.) by a method described by Marsh & Levvy (1956). In preference to their lead salt method, the residue was

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purified by recrystallising four times from 80% ethanol, using decolourising charcoal each time, and twice from 85% ethanol. Yield 2·1%. M.p. 211–217° (decomp.) after turning yellow at 169–170°. $[\alpha]_D^{20}$ + 45·1° (*c* 3·1 in 40% EtOH). Found: C, 54·8; H, 8·3; N, 1·6; NH_3, 1·9; H_2O, 7·8. $C_{42}H_{65}O_{16}N,4H_2O$ requires C, 55·3; H, 8·1; N, 1·5; NH_3, 1·9; H_2O, 7·9. λ_{max} (water) 256 mμ (log ε 4·05), λ_{max} (EtOH) 248 mμ (log ε 4·05). Marsh & Levvy (1956) gave m.p. 212–217° (decomp.), $[\alpha]_D^{20}$ + 46·9° (*c* 1·5 in 40% EtOH), λ_{max} (EtOH) 248 mμ (log ε 4·06). Water content equivalent to $5H_2O$. Onrust, Jansen & Wöstmann (1955) quote a water content equivalent to $4H_2O$.

Tripotassium glycyrrhizate was prepared from monoammonium glycyrrhizate according to the method of Voss, Klein & Sauer (1937). Yield 90% $[\alpha]_D^{20}$ + 41·5° (*c* 4·3 in 40% EtOH), $[\alpha]_D^{20}$ + 46·3° (*c* 4·0 in water). Found: C, 49·4; H, 6·9; K, 11·9; H_2O, 7·15. $C_{42}H_{59}O_{16}K_3$, $4H_2O$ requires C, 50·0; H, 6·7; K, 11·6; H_2O, 7·1 λ_{max} (water) 257 mμ (log ε 4·04). Voss, Klein & Sauer (1937) gave $[\alpha]_D^{19}$ + 44·8° (in water) and water content equivalent to $2H_2O$.

Monopotassium glycyrrhizate was prepared by dissolving tripotassium tetrahydrate (1·3 g) in a mixture of glacial acetic acid (6 ml) and ethanol (4 ml) under reflux. After filtration the solution was allowed to stand for 24 hr. Separation and washing with ethanol gave 0·8 g (62%) of monopotassium glycyrrhizate tetrahydrate as white crystals. $[\alpha]_D^{20}$ + 43·8° (*c* 3·0 in 40% EtOH). Found: C, 54·6; H, 7·3; K, 4·0; H_2O, 7·7. $C_{42}H_{61}O_{16}K$, $4H_2O$ requires C, 54·1; H, 7·45; K, 4·2; H_2O, 7·7. λ_{max} (water) 256–257 mμ (log ε 4·05). Voss, Klein & Sauer (1937) quote a water content equivalent to $2H_2O$.

Glycyrrhizic acid was prepared by suspending monoammonium glycyrrhizate (1 g) in 1% sulphuric acid (10 ml) for 30 min on a hot water-bath. It was important to avoid excessive heating to prevent conversion into glycyrrhetic acid. On cooling, the turbid solution gave a white precipitate which was filtered off and washed with purified water until free of sulphuric acid. Further washing with chloroform gave on drying 0·58 g (58%) of glycyrrhizic acid tetrahydrate. M.p. 203–206°. $[\alpha]_D^{20}$ + 56·4° (*c* 3·1 in EtOH). Found: C, 56·1; H, 7·7; H_2O, 8·0. $C_{42}H_{62}O_{16}$, $4H_2O$ requires C, 56·4; H, 7·9; H_2O, 8·05. λ_{max} (water) 256 mμ (log ε 4·05).

Monosodium glycyrrhizate was supplied by W. H. Ransom Ltd. This material (5·6 g) was recrystallised twice from 80% ethanol, using decolourising charcoal each time, and twice from 85% ethanol to give 2·5 g (45%) of monosodium glycyrrhizate tetrahydrate as white crystals. $[\alpha]_D^{20}$ + 45·0 (*c* 3·1 in 40% EtOH). Found: C, 55·25; H, 7·9; H_2O, 7·9. $C_{42}H_{61}O_{16}Na$, $4H_2O$ requires C, 55·0; H, 7·6; H_2O, 7·9. λ_{max} (water) 256–257 mμ (log ε 4·05).

CONDUCTANCE MEASUREMENTS

The water used for the preparation of solutions and for conductance measurements was prepared by passing distilled water through a bed of

mixed ion-exchange resins and then allowing it to equilibrate with the atmosphere for 24 hr. Its specific conductance, at 25°, varied between $1{\cdot}21$–$1{\cdot}36 \times 10^{-6}$ mhos cm^{-1}.

The conductances of aqueous dilutions of glycyrrhizic acid and its salts were measured at $25 \pm 0{\cdot}1°$, using a Pye conductance bridge. Plots of equivalent conductance against the square root of molarity for glycyrrhizic acid and its monoammonium and tripotassium salts, are shown in Fig. 1. Since the conductance curves for the mono-substituted salts were nearly identical only one of these has been included.

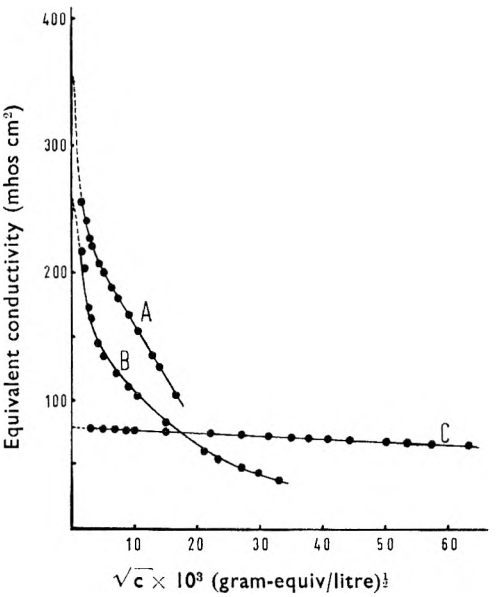

FIG. 1. Variation of equivalent conductivity of glycyrrhizic acid and its salts with concentration. A, Glycyrrhizic acid. B, Monoammonium glycyrrhizate. C, Tripotassium glycyrrhizate.

SURFACE TENSION MEASUREMENTS

A static method (Wilhelmy plate) was used for the measurement of surface tensions. The apparatus employed has been described by Robins & Thomas (1963). Measurements were made on aqueous dilutions of glycyrrhizic acid and its salts and surface tensions plotted against molarity: the graphs are shown in Fig. 2.

The surface tensions of solutions of glycyrrhizic acid and its salts did not change with time.

SOLUBILISATION EXPERIMENTS

Aqueous solutions of various concentrations of glycyrrhizic acid and its salts were shaken for 6 hr at 25° with dimethyl yellow. Insoluble material was then filtered off and the extinction of the filtrate determined using a Spekker absorptiometer. No significant increase in absorption

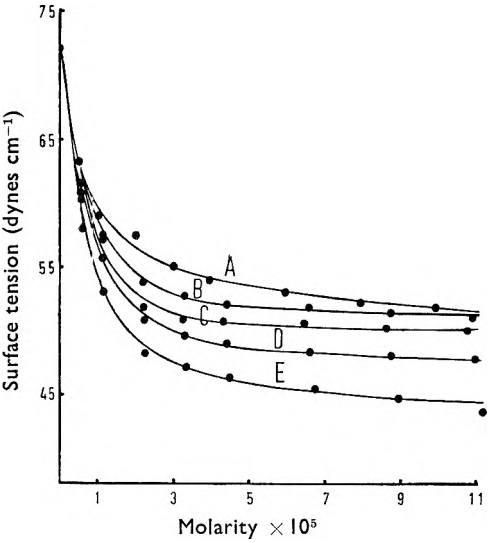

FIG. 2. Variation of surface tension of glycyrrhizic acid and its salts with concentration. A, Tripotassium glycyrrhizate. B, Monosodium glycyrrhizate. C, Monopotassium glycyrrhizate. D, Monoammonium glycyrrhizate. E, Glycyrrhizic acid.

was observed over that of the control with water only. To confirm this result, chloroform, which has been shown to be solubilised by liquorice extracts (James & Stanford, 1962) was examined. Various quantities were shaken with 0·01M tripotassium glycyrrhizate, and optical densities measured as before. The extinction rose rapidly as soon as the aqueous solubility of chloroform was exceeded, indicating that emulsification and not solubilisation, had taken place.

Discussion

The many methods that have been described for the preparation of glycyrrhizic acid and its salts have been reviewed recently (Muravev & Ponomarev, 1962; Gilbert, 1963). After critically examining most of these, we are in agreement with Brieskorn & Mahran (1960) that much of the work is difficult to reproduce. The methods of preparation we have used were those we considered to be the most satisfactory. Our main difficulty was to establish the purity of our product, the usual criterion, namely melting-point, was of little value, since only that of the acid and that of the monoammonium salt were sufficiently low, and these are known to extend over several degrees. Other reported physical properties are both few and variable and when quoted, the purity of the material has not been proved. For this reason we quote a full analysis for each compound even though none of them is new. To prepare solutions of known molarity, it was necessary to know the quantity of water of crystallisation and moisture determinations were made because of the lack of agreement amongst the published figures, $4H_2O$ (Onrust,

Jansen & Wöstmann, 1955) and $5H_2O$ (Marsh & Levvy, 1956) for monoammonium glycyrrhizate, and $2H_2O$ (Voss, Klein & Sauer, 1937) for the monopotassium and tripotassium salts. The presence of $4H_2O$ was confirmed by the elemental analysis, and was the same for all the compounds; this was anticipated since the water would be expected to be bound to the anion. A report (Tschirch & Cederberg, 1907) that glycyrrhizic acid salts form yellow solutions in aqueous alkali was tested by observing the effect of alkali on the electronic spectra. Not even 5N sodium hydroxide could produce a bathochromic shift and so it was assumed that this early observation was due to impurities in the sample.

Fig. 2 shows that the surface tension is greater when the acid is neutralised with strong basic cations. The curves are typical of amphipathic substances, and their shape could suggest micelle formation. This was considered unlikely however since the surface tension continued to decrease after the initial sharp fall. Similar curves were obtained with the saponins by Ruyssen & Loos (1947) who confirmed by several other methods that the systems they studied did not form micelles.

The conductance curve for glycyrrhizic acid is typical of that for a weak electrolyte and that for tripotassium glycyrrhizate typical of a strong electrolyte. For the mono-substituted salts, the unneutralised carbonyl groups behave as weak electrolytes, while the carboxyl group involved in salt formation is completely ionised, and, as expected, the curves for these salts lay between those for glycyrrhizic acid and the tripotassium salt (Fig. 1). Extrapolation of the straight line for tripotassium glycyrrhizate gave a limiting equivalent conductance of 79·5 mhos cm^2, equivalent to a mobility of 18 mhos cm^2 $equiv^{-1}$ for the glycyrrhizate ion. This agrees favourably with the values of 15 and 16 mhos cm^2 $equiv^{-1}$ respectively quoted for the similar sapoalbin and senegin ions by Ruyssen & Loos (1947). Accurate extrapolation of the conductance curves of the other compounds was not feasible, but the approximate limiting conductances obtained by this means agreed with those calculated assuming an anionic mobility of 18 mhos cm^2 $equiv^{-1}$. There is therefore no sharp decrease in the slopes of these curves, and these compounds, like the tripotassium salt, are therefore molecularly dispersed electrolytes. The solubilisation experiments support this conclusion.

The glycyrrhizin content of liquorice root differs from plant to plant but is usually within the range 7·0–10·0% (Nieman, 1957) and an aqueous extract containing 7·5% has been prepared by James & Stanford (1962). In this study, aqueous solutions of tripotassium glycyrrhizate could be prepared up to a concentration of 4% but heating was necessary to aid solution. For glycyrrhizic acid and monoammonium glycyrrhizate 0·03 and 0·5% aqueous solutions, respectively, could be prepared only by vigorous mixing at 80°, and even much weaker solutions required a little heat to aid solution. Our observations suggest that glycyrrhizin behaves differently in liquorice extracts than in aqueous solution. It is possible that other constituents in liquorice may either promote micelle formation, or combine with glycyrrhizin to form compound micelles.

Acknowledgements. We are grateful to W. H. Ransom Ltd., for a gift of monosodium glycyrrhizate, and to Dr Hans Schmidt of S. B. Penick Ltd., New York, for his advice on the preparation of glycyrrhizic acid.

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J. Pharm. Pharmacol., 1964, **16**, 400–407 Received December 16, 1963

Antifungal activity of some imidazole derivatives

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A series of 4(5)-arylimidazoles and their *N*-alkyl and other derivatives has been synthesised. Some of these compounds possessed good *in vitro* antifungal action, especially against strains of *Trichophyton* and less activity against *Microsporum*, *Aspergillus* and *Candida* strains; their activity against all mycological pathogens was reduced in the presence of serum. The three most active compounds are 1-alkyl-4-(3,4-dichlorophenyl)imidazoles in which the alkyl group is an n-propyl, n-butyl or isobutyl group. 4(5)-(2,3,4-Trichlorophenyl)imidazole is also almost as potent as an antifungal compound. Structure-antifungal activity relations for these compounds are discussed.

THE imidazole ring occurs in several physiologically important compounds such as histamine, histidine and the purines. 4(5)-Aminoimidazole-5(4)-carboxyamide promotes the growth of some microorganisms and may be converted into purines by biological or chemical methods. In view of this and of the widespread chemical attention which has been paid to imidazole derivatives (Hofmann, 1953), it is surprising that few therapeutically useful compounds containing the simple imidazole ring exist. The antibiotic azomycin is 2-nitroimidazole (Nakamura, 1955); imidazole-4,5-dicarboxyamide and its 1-alkyl derivatives have been claimed to be effective against coccidiosis in poultry (Merck and Co., 1959); some imidazole-2-thiols have bacteriostatic activity (Simon & Kovtunovskaya-Levshina, 1957) and the use of metronidazole has been described (Cosar & Jolou, 1959). We have explored and now report the pharmacological and microbiological properties of a range of imidazole derivatives.

The compounds described consist of imidazoles containing a substituted benzene ring in position 4 or 5 and optionally an alkyl, cyanoalkyl, carboxyalkyl, hydroxyalkyl or pyridylethyl group attached to the ring nitrogen atom. Substituents in the benzene ring included one or more halogen atoms, methyl, cyano, nitro or carboxy groups. A few other related compounds are also included. The synthesis of some typical compounds is described in the experimental section, and the melting- or boiling-point and analysis of the compounds are listed in Table 1, except for those compounds whose analysis is given in the text.

Substituted 4(5)-arylimidazoles were generally prepared by the method of Bredereck & Theilig (1953) in which a phenacyl halide is heated under reflux with formamide. The phenacyl halides were synthesised by a Friedel-Crafts reaction between the aromatic compound and chloroacetyl chloride.

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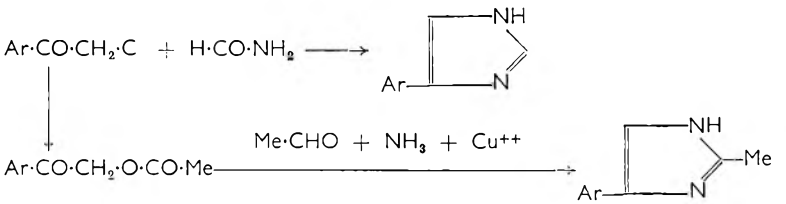
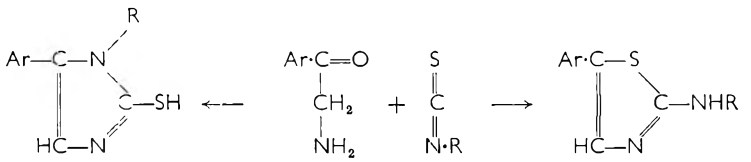


4(5)-*p*-Chlorophenyl-2-methylimidazole was prepared by heating the phenacyl acetate, acetaldehyde, aqueous ammonia and cupric acetate as described by Weidenhagen & Herrmann (1935) for related compounds. The ring nitrogen atom was alkylated by treatment first with sodium in ethanol and then with an alkyl halide in an autoclave at 100°. Since the formation of two isomeric products (the 4- and 5-arylimidazoles) is possible in this reaction, it was necessary to identify the isomer formed. When a phenacylamine is reacted with an alkyl isothiocyanate two products are formed, a 2-alkylamino-5-arylthiazole and a 1-alkyl-5-aryl-imidazole-2-thiol:



The thiazole, being less soluble in alkali, can be separated from the imidazole-2-thiol, which is then oxidised to the imidazole. When this sequence of reactions was carried out using 3,4-dichlorophenacylamine and methyl isothiocyanate, the dichlorophenylmethylimidazole eventually isolated was not identical with the isomer obtained by the methylation of 4(5)-(3,4-dichlorophenyl)imidazole. Direct alkylation of a 4(5)-aryl-imidazole therefore yields a 4-arylimidazole. A few 4(5)-arylimidazoles in which the imide nitrogen atom carries a 2-(pyrid-2-or-4-yl)ethyl group were prepared by reacting the imidazole with 2- or 4-vinylpyridine respectively. Although there is no direct proof of structure, it is assumed that the products of this reaction are also 4-arylimidazoles. A few nitro-phenyl compounds were obtained as by-products of the nitration of the imidazoles and their preparation will be described in a later paper.

Experimental

4-(3,4-*Dichlorophenyl*)-1-*methylimidazole.* 4(5)-(3,4-Dichlorophenyl)-imidazole (21·4 g) (prepared from 3,4-dichlorophenacyl chloride and formamide) in ethanol (50 ml) was treated with a solution of sodium (2·3 g) in ethanol (50 ml), followed by methyl iodide (14·2 g). The mixture was heated in an autoclave at 100° for 16 hr, then cooled and filtered. The filtrate was diluted with water and extracted with chloroform. After the extracts had been washed and dried (Na_2SO_4), the solvent was removed. The remaining oil distilled at 170–180° at 0·02 mm, and gave the *imidazole* (1·8 g) as crystals, m.p. 84–85° (from ether and light petroleum).

5-(3,4-*Dichlorophenyl*)-1-*methylimidazole*. To a stirred solution of sodium (7·7 g) in ice-cold ethanol (200 ml) was added 3,4-dichloroacetophenone (63 g), followed by the slow addition of butyl nitrite (34 g) in ethanol (50 ml) and ether (50 ml). The mixture was kept in a refrigerator overnight and the sodium salt was collected on a filter, washed with ether and dissolved in water (4 litres). The aqueous solution was filtered and acidified with 33% acetic acid (220 ml), whereupon 3,4-dichloro-α-isonitroso-acetophenone (15 g), m.p. 145°, was precipitated. A solution of this (8·8 g) in ethanol (250 ml) containing concentrated hydrochloric acid (4 ml) was shaken with 10% platinum on charcoal (0·9 g) in an atmosphere of hydrogen until the uptake of hydrogen ceased. Removal of the catalyst and solvent gave 3,4-dichlorophenacylamine hydrochloride (6·4 g), m.p. 210–220°. This hydrochloride (3·6 g), methyl isothiocyanate (1·1 g) and dry pyridine (30 ml) were heated under reflux for 2 hr, then poured into water and left to stand overnight. The solid was collected and stirred with hot 10% sodium hydroxide solution; the solution was filtered, and the filtrate was cooled and acidified to produce 5-(3,4-dichlorophenyl)-1-methyl-imidazole-2-thiol, m.p. 185–188° (decomp.). This was dissolved in ethanol and refluxed for 3 hr with an excess of Raney nickel. Removal of the catalyst and distillation of the solvent left an oil, b.p. 160° at 0·5 mm which solidified to give 5-(3,4-*dichlorophenyl*)-1-*methylimidazole* (0·6 g), m.p. 80–82°. An admixture of this with 4-(3,4-dichlorophenyl)-1-methylimidazole described above melted at 50°.

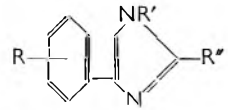
4(5)-(2,4,5-*Trichlorophenyl*)*imidazole*. (*a*) 4(5)-(2-Amino-4,5-dichlorophenyl)imidazole dihydrochloride (1·7 g) was diazotised and converted via a Sandmeyer reaction with cuprous chloride to 4(5)-(2,4,5-*trichlorophenyl*)*imidazole* (0·7 g), m.p. 228–229° (from aqueous methanol).

(*b*) 1,2,4-Trichlorobenzene (19 g), acetyl chloride (15·6 g) and aluminium chloride (25·4 g) were reacted together, first in refluxing carbon disulphide and then, after removal of the solvent, at 130° for 2 hr. 2,4,5-Trichloroacetophenone (4·2 g), b.p. 136–146° at 15 mm, was obtained and was reacted with bromine (3 g) in chloroform to form the phenacyl bromide. The chloroform was distilled off and the residue was heated under reflux for 2 hr with formamide (45 ml). After acidifying, filtering and basifying with ammonia, crystals of 4(5)-(2,4,5-*trichlorophenyl*)-*imidazole* (1·5 g), m.p. 226–227°, were obtained.

4(5)-(4,5-*Dichloro*-2-*cyanophenyl*)*imidazole*. 4(5)-(2-Amino-4,5-dichlorophenyl)imidazole dihydrochloride (7·7 g) dissolved in N hydrochloric acid (60 ml) was diazotised and treated with cuprous cyanide (3·8 g) and sodium cyanide (7 g). The *nitrile* (1·2 g) formed colourless leaflets, m.p. 245–246° (from aqueous ethanol).

1-(3,4-*Dichlorophenyl*)*imidazole*-2-*thiol*. 2,2-Diethoxyethyl isothiocyanate (Easson & Pyman, 1932) (6·6 g) and 3,4-dichloroaniline (6·2 g) were heated together on a steam-bath for 1 hr. The product was then heated under reflux with 5N sulphuric acid (140 ml) for 2 hr, cooled and filtered. The solid was recrystallised from ethanol to obtain the *thiol* (6·1 g),

TABLE 1. ARYLIMIDAZOLE DERIVATIVES



R″ = H except for compound No. 5, where R″ = Me

Compound No.	Position of aryl ring	R	R′	m.p. (or b.p.) °C	Formula	Analysis							
						Found				Required			
						C	H	Cl	N	C	H	Cl	N
1	4 (5)	*p*-NO_2	H	225	—	Grant & Pyman, 1921							
2	4 (5)	*p*-Br	H	140–142	—	Theilig, 1953							
3	4 (5)	*p*-Cl	H	146–147	—	Weidenhagen, Herrmann & Wegner, 1937							
4	4 (5)	*p*-MeO	H	139–140	—	Weidenhagen & Herrmann, 1935							
5	4 (5)	*p*-Cl	H (R″ = Me)	172–174	$C_{10}H_9ClN_2$	61·9	5·0	18·1	14·6	62·3	4·7	18·4	14·5
6	4 (5)	*p*-CN	H	168–169	$C_{10}H_7N_3$	70·7	4·3	—	25·2	71·0	4·2	—	24·8
7	4 (5)	*p*-Ph	H	222–223	$C_{15}H_{12}N_2$	82·3	5·3	—	12·6	81·8	5·5	—	12·7
8	4 (5)	4-Cl-2-NO_2	H	201	$C_9H_6ClN_3O_2$	48·3	2·9	15·9	18·7	48·4	2·7	15·9	18·8
9	4 (5)	4-Cl-3-NO_2	H	245	$C_9H_6ClN_3O_2$	48·1	2·8	16·0	18·6	48·4	2·7	15·9	18·8
10	4 (5)	4-Cl-3-Me	H	140	$C_{10}H_9ClN_2$	—	—	—	17·9	—	—	—	18·4
11	4 (5)	4-Cl-2-NH_2	H	274–275	$C_9H_{10}Cl_2N_3$	—	—	26·4[a]	—	—	—	26·4[a]	—
12	4 (5)	4-Cl-2-CO_2H	H	202–204	$C_{10}H_7ClN_2O_2$	53·4	3·3	16·1	13·1	53·9	3·2	16·0	12·6
13	4	*p*-NO_2	2-(pyrid-2-yl)ethyl	225–227	$C_{16}H_{15}Cl_3N_4O_2$	—	—	18·9	—	—	—	19·4	—
14	4 (5)	3,4-Cl_2	H	181	$C_9H_6Cl_2N_2$	50·8	2·9	33·2	13·4	50·7	2·8	33·3	13·1
15	4 (5)	2,4-Cl_2	H	143–144	$C_9H_6Cl_2N_2$	50·6	3·0	33·3	13·1	50·7	2·8	33·3	13·1
16	4 (5)	2,5-Cl_2	H	237–238	$C_9H_6Cl_2N_2$	50·8	2·7	33·1	12·8	50·7	2·8	33·3	13·1
17	4 or 5	3,4-Cl_2	D-Glucosyl	243–245	$C_{14}H_{16}Cl_2NO_5$	—	—	—	7·2	—	—	—	7·2
18	4	3,4-Cl_2	Me	84–85	$C_{10}H_8Cl_2N_2$	52·8	4·0	31·2	12·1	52·8	3·5	31·3	12·3
19	4	3,4-Cl_2	Et	(158–162/0·03 mm)									
				Picrate	$C_{17}H_{13}Cl_2N_5O_7$	43·3	2·9	—	14·7	43·4	2·8	—	14·9
20	4	3,4-Cl_2	Pr^n	249–250 (179/0·2 mm)									
				Picrate	$C_{18}H_{15}Cl_2N_5O_7$	44·5	2·9	—	14·7	44·6	3·1	—	14·5
21	4	3,4-Cl_2	Pr^i	203–204 (206–210/0·06 mm)									
				Picrate	$C_{18}H_{15}Cl_2N_5O_7$	44·5	3·3	—	14·3	44·6	3·1	—	14·5
22	4	3,4-Cl_2	Allyl	252 (196–200/0·05 mm)									
				Picrate	$C_{18}H_{13}Cl_2N_5O_7$	43·5	2·8	—	14·4	44·8	2·7	—	14·5
23	4	3,4-Cl_2	Bu^n	203 (170–174/0·02 mm)									
				Picrate	$C_{19}H_{17}Cl_2N_5O_7$	45·5	3·7	—	13·8	45·7	3·4	—	14·0
24	4	3,4-Cl_2	Bu^i	206 (210–220/0·06 mm)									
				Picrate	$C_{19}H_{17}Cl_2N_5O_7$	45·5	3·4	—	13·7	45·7	3·4	—	14·0

[a] Ionic chlorine.

TABLE 1—*continued*

Compound No.	Position of aryl ring	R	R′	m.p. (or b.p.) °C	Formula	Analysis							
						Found				Required			
						C	H	Cl	N	C	H	Cl	N
25	4	3,4-Cl_2	Bu^s	(210–215/0·05 mm)									
			Picrate	217	$C_{19}H_{17}Cl_2N_5O_7$	45·9	3·4	—	14·1	45·7	3·4	—	14·0
26	4	3,4-Cl_2	n-C_6H_{11}	(188–192/0·05 mm)									
			Picrate	177	$C_{20}H_{19}Cl_2N_5O_7$	46·6	3·9	—	13·8	46·8	3·7	—	13·7
27	4	3,4-Cl_2	n-C_6H_{13}	(192–196/0·05 mm)									
			Picrate	168	$C_{21}H_{21}Cl_2N_5O_7$	47·6	4·1	—	13·1	47·9	4·0	—	13·3
28	4	3,4-Cl_2	n-C_7H_{15}	(200–205/0·05 mm)									
			Picrate	163	$C_{22}H_{23}Cl_2N_5O_7$	48·5	4·4	—	13·0	49·0	4·3	—	13·0
29	4	3,4-Cl_2	n-$C_{16}H_{33}$	51–52	$C_{25}H_{38}Cl_2N_2$	67·8	8·8	—	6·5	68·7	8·7	—	6·4
30	4	3,4-Cl_2	$CH_2{\cdot}CH_2{\cdot}CN$	113	$C_{12}H_{12}Cl_2N_2$	53·9	3·5	—	15·6	54·1	3·4	—	15·8
31	4	3,4-Cl_2	$CH_2{\cdot}CH_2{\cdot}CO_2H$	187–188	$C_{12}H_{12}Cl_2N_2O_2$	50·3	3·6	—	9·4	50·6	3·5	—	9·8
32	4	3,4-Cl_2	$CH_2{\cdot}CF_2OH$	132–133	$C_{21}H_{16}Cl_2N_2O$	51·2	3·4	27·6	10·7	51·3	3·9	27·6	10·9
33	4	3,4-Cl_2	2-(pyrid-2-yl)ethyl	126	$C_{13}H_{12}Cl_2N_2$	—	—	22·1	—	—	—	22·3	—
34	4	3,4-Cl_2	2-(pyrid-2-yl)ethyl	218–219	$C_{13}H_{13}Cl_4N_2$	48·7	4·0	17·7[a]	—	49·1	3·8	18·1[a]	—
35	4	3,4-Cl_2	2-(pyrid-4-yl)ethyl	235	$C_{13}H_{13}Cl_4N_2$	48·8	3·9	17·9[a]	—	49·1	3·8	18·1[a]	—
36	4 (5)	4,5-Cl_2-2-NO_2	H	225–226	$C_9H_6Cl_2N_2O_2$	42·2	1·9	27·7	16·1	41·9	1·9	27·5	16·3
37	4	4,5-Cl_2-2-NO_2	Me	139–140	$C_{10}H_8Cl_2N_2O_2$	44·3	2·6	25·9	15·5	44·1	2·6	26·1	15·4
38	4	4,5-Cl_2-2-NO_2	2-(pyrid-2-yl)ethyl	220–224	$C_{19}H_{14}Cl_4N_4O_2$	—	—	16·0[a]	—	—	—	16·3[a]	—
39	4	4,5-Cl_2-2-NH_2	2-(pyrid-2-yl)ethyl	240–243	$C_{19}H_{16}Cl_4N_4$	—	—	17·2[a]	—	—	—	17·5[a]	—
40	4 (5)	4,5-Cl_2-2-NH_2	H	171–173	$C_9H_7Cl_2N_3$	46·8	3·2	31·4	17·9	47·3	3·1	31·1	18·4
41	4 (5)	4,5-Cl_2-2-Me	H	214–215	$C_{10}H_9Cl_2N_3$	52·6	4·0	—	12·0	52·8	3·5	—	12·3
42	4 (5)	4,5-Cl_2-2-CN	H	245–246	$C_{10}H_6Cl_2N_4$	50·9	2·5	29·6	16·7	50·4	2·1	29·8	17·6
43	4 (5)	4,5-Cl_2-2-CO_2H	H	283–284	$C_{10}H_8Cl_2N_2O_2$	46·3	2·4	27·4	11·0	46·7	2·3	27·6	10·9
44	4 (5)	2,3,4-Cl_3	H	195–196	$C_9H_6Cl_3N_3$	43·2	1·8	43·2	11·3	43·7	2·0	43·0	11·3
45	4 (5)	2,4,5-Cl_3	H	229–230	$C_9H_6Cl_3N_3$	43·7	2·0	43·2	11·1	43·7	2·0	43·0	11·3
46	4 (5)	2,4-Me_2	H	82–83	$C_{11}H_{12}N_2$	76·6	7·1	—	16·0	76·8	7·0	—	16·2

[a] Ionic chlorine.

m.p. 247–249°; found C, 45·0; H, 2·7; Cl, 28·1; N, 11·4; S, 12·8. $C_9H_6Cl_2N_2S$ requires C, 44·1; H, 2·5; Cl, 28·9; N, 11·4; S, 13·0%.

1-(3,4-*Dichlorophenyl*)*imidazole.* 1-(3,4-Dichlorophenyl)imidazole-2-thiol (5·1 g) was added in portions to hot 10% nitric acid (100 ml). When all the reaction had ceased the liquid was filtered, cooled and basified with ammonia. The precipitate gave crystals of the *imidazole* (3·6 g), m.p. 83–84°, from aqueous ethanol. Found C, 49·3; H, 3·0; Cl, 31·9; N, 12·6. $C_9H_6Cl_2N_2.\frac{1}{2}H_2O$ requires C, 48·6; H, 3·1; Cl, 31·9; N, 12·6%.

Biological methods

In vitro ASSAY OF FUNGISTATIC ACTIVITY

The following pathogenic fungi and yeast were obtained from the National Collection of Type Cultures, Colindale, London, and were used as test organisms: *Trichophyton rubrum* (T 88), *T. mentagrophytes* (T 95), *T. interdigitale* (T 72), *T. verrucosum* (T 97), *Candida albicans* (T 71), *Epidermophyton floccosum* (T 99), *Aspergillus fumigatus* (T 98), *Microsporum canis* (T 90) and *M. audouini* (T 100). The two culture media used were:

(*a*) for growth inhibition, Sabouraud's broth composed of 0·5% w/v pancreatic digest of casein, 0·5% w/v peptic digest of fresh meat and 2% w/v glucose in water at pH 5·7; this was sterilised for 15 min at 15 lb/in².

(*b*) to provide a potential biological antagonist of the growth inhibition provided by the test compound, a supplement of 10% v/v of heat-inactivated horse serum (Oxo) was added to the medium described in (*a*) and was provided in 9 ml and 5 ml quantities in 1 oz bottles.

A concentrated stock solution of the test compound at 1% w/v was prepared in either liquid macrogol (Carbowax 300) or glycerol formal. Neither of these solvents alone at 2% (drug concentrations equivalent to 200 μg/ml) exerted inhibitory activity but the neat solvents served as sterilising agents.

The fungistatic activity of the compounds was determined by a procedure similar to that of Collier, Potter & Taylor (1955), involving two-fold serial dilutions, with test compound concentrations over the ranges of 150–4·5, 100–3 and 10–0·3 μg/ml.

Inocula. (*a*) In tests against *C. albicans*, a heavily grown one-day-old culture of the yeast in Sabouraud's broth was diluted one hundred-fold and 0·1 ml of this suspension was introduced into bottles containing 5 ml of medicated broth as well as a bottle containing drug-free broth as growth control.

(*b*) In tests for fungistatic activity, 0·2 ml suspensions of mycelium and spores were taken from 7–21-days-old broth cultures of the filamentous moulds and added to 10 ml Sabouraud's broth in a screw-capped bottle, shaken well and then allowed to stand at room temperature for 10 min to allow any large fragments to settle. The slightly opalescent supernatants were diluted ten-fold and then 0·1 ml of the respective inoculum

was used to inoculate 5 ml volumes of the serially diluted test compound in Sabouraud's broth.

After inoculation, the bottles were incubated at 28° for 7 days and the degree of inhibition was read by eye, recording after 3 and 7 days the maximum dilution of the drug to give partial growth as compared with the growth control. In addition, the dilution required to give complete visual inhibition of the fungi, that is, the minimum inhibitory concentration in μg/ml was observed.

TABLE 2. ANTIFUNGAL ACTIVITY OF IMIDAZOLE DERIVATIVES

Compound No.	Minimum inhibitory concentration after 7 days at 28° (μg/ml)			
	Trichophyton	*Microsporum*	*Aspergillus*	*Candida*
7	20			
14	25			
18	25			
19	25			
20	4 (15)	15 (45)	20 (40)	25 (50)
21	25			
22	20			
23	5 (12)	12 (25)	18 (35)	25 (50)
24	5	20	—	50
25	25			
26	10	25	—	50
27	7·5	100	—	50
28	20			
29	20			
30	25			
33	8 (22)	60 (100)	50 (75)	100
41	25			
42	12	50	—	50
43	25			
44	6 (15)	12 (25)	—	—
45	25			
Hedaquinium chloride	1 (3)	1 (1)	5 (5)	0·5 (1)
Nystatin	2 (5)	2 (5)	5 (10)	2 (5)
N-Butyl-4-chloro-2-hydroxybenzamide (Jadit)	25 (100)	25 (100)	25 (150)	50 (200)

The figures in parentheses were determined in the presence of serum.

Results

Of the compounds described in this paper, those listed in Table 2 exhibited activity at a concentration of 25 μg/ml or less against at least one of the species mentioned the previous section. The minimum inhibitory concentrations given were determined in medium without serum supplement, except for the values shown in brackets which were determined in the presence of 10% inactivated horse serum.

Discussion

CHEMICAL STRUCTURE—BIOLOGICAL ACTIVITY CONSIDERATIONS

The *in vitro* tests show a distinct relationship between the structure and the antifungal activity of the imidazoles studied. The presence of only one halogen atom in the benzene ring (as in compounds 2 and 3) does not confer fungicidal activity and neither does a pair of chlorine atoms in positions 2 and 4 or 2 and 5 (compounds 15 and 16), but the 3,4-dichlorophenyl derivative does show moderate activity. It is of interest to mention that a clinically used antifungal drug, Dybenal, is

2,4-dichlorobenzyl alcohol (Taylor & D'Arcy, 1961). Alkylation of the ring nitrogen atom had no effect on potency if the alkyl group contained up to three carbon atoms, although the allyl compound (22) showed a marginal improvement. Longer alkyl chains, however, increased the potency until a maximum was reached at the n-hexyl derivative, after which activity rapidly diminished. A similar variation of activity with length of alkyl chain has been demonstrated for a number of 2-alkoxybenzamides (Coates, Drain, Macrae & Tattersall, 1959). It cannot be stated that branching of the alkyl chain has a uniform effect on antifungal activity (compare compounds 20, 21 and 23, 24, 25), neither has increasing the number of halogen atoms in the aryl ring as it depends on their positions, the 2,3,4- orientation (compound 44) giving a higher activity than the 2,4,5- arrangement (compound 45). Difficulties in synthesis prevented further exploration of these trichlorophenyl compounds.

The presence in the aryl ring of a nitro, amino, carboxy or methyl group in addition to the halogens had no appreciable effect on potency, but a nitrile group (compound 42) appeared to double the activity. A nitrile group without the halogen atoms (compound 6) however, had no merit. In the only active compound (7) not containing a halogen atom or nitrile group, the aryl substituent consisted of a *p*-phenyl substituent.

Although the potency of a few of the compounds was high, especially against *T. interdigitale*, the activity was adversely affected by the presence of serum.

Acknowledgements. The authors wish to thank Mr. K. F. Clarke and Mr. P. B. Johnson for experimental assistance and Mr. C. Campbell, B.Sc., F.R.I.C., for analytical results.

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J. Pharm. Pharmacol., 1964, **16**, 408–421 Received September 19, 1963

Catechu (Gambier): its microscopical characters*

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24 different commercial samples of catechu have been examined microscopically for the presence of vegetable fragments, starch and crystalline material. The components of the vegetable débris have been identified as being derived from the leaves and young shoots of *Uncaria gambier* Roxb. used in the preparation of the extract. The main microscopical characters of value in the identification of catechu are enumerated.

CATECHU is the dried aqueous extract prepared from the leaves and young shoots of *Uncaria gambier*, Roxb. family Rubiaceæ, a climbing shrub which is indigenous to Malaya, Borneo, Sumatra and Java. An excellent account of its preparation is given by Roebuck (1936) and descriptions of the various processes involved have also been summarised by Flückiger and Hanbury (1900), Burkill (1935), Howes (1953), Brumwell (1911) and others (Trease, 1960; Wallis, 1960). Although the methods used vary from one territory to another, the basic principles underlying the preparation are essentially the same; the process consists of boiling the young shoots and leaves of *Uncaria gambier* in water, straining, concentrating the resulting aqueous extract to a thick syrup, pouring it into a mould, allowing it to set, cutting the solidified extract into pieces of desirable shapes or forms and finally drying them in the sun.

In view of the method of preparation and the fact that frequently somewhat primitive filtering media have been employed for straining the extract (Brumwell, 1911; Howes, 1953), it is to be expected that the final product will contain varying amounts of vegetable material. Descriptions of the microscopical structures found in catechu are included in certain textbooks of pharmacognosy (Trease, 1960; Wallis, 1960) and some of the standard books on the microscopy of crude drugs (Barclay, 1900; Greenish, 1910; Parry, 1911; Schneider, 1921; Kay, 1938). Little attempt has been made, however, to relate these fragments to the plant, *Uncaria gambier* from which they presumably have been derived. It was decided, therefore, to carry out a full investigation of all the microscopical characters of commercial catechu and to compare their structure with that of the shoots of *Uncaria gambier* previously described (Leong & Jackson, 1964); in this way the origin of the fragments could be established.

Materials

Catechu occurs in commerce in more or less regular cubes, sometimes in blocks or angular masses or occasionally in the form of tablets or discs. It is usually light and friable. The colour of the external surface varies from light brown to almost black but the freshly broken surface

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* The subject-matter of this communication forms part of a thesis by one of us (M.S.L.) accepted by the University of London for the degree of Master of Pharmacy.

is usually a light cinnamon brown colour, although it may be somewhat streaky. It has no odour and a bitter, astringent taste.

24 different commercial samples have been used in this investigation, including one (number 18, Table 1) obtained direct from one of the few remaining gambier factories in Malaya. 15 of these samples (Nos. 4, 6, 8–10, 13–17, 20–24, Table 1) had the characteristic form of most commercial catechu, that is, they occurred as more or less regular cubes. Of these, 8 (Nos. 15–17, 20–24, Table 1) were in the form of relatively large

TABLE 1. MICROSCOPICAL CHARACTERS OF COMMERCIAL CATECHU

Sample No.	Detached covering trichomes	Leaf and perianth fragments	Pollen grains	Starch	Extraneous crystalline material	Other identified microscopical structures
1	+++	+++	++	absent	absent	fragments of cork
2	+	+	absent	absent	absent	absent
3	+	+	absent	absent	absent	absent
4	+++	++++	++++	absent	absent	absent
5	+	+	+	present (rice)	absent	rice bran and rice husk
6	+	+	++	absent	hexagonal plates and silica	absent
7	+++	+++	++	absent	absent	absent
8	+	+	absent	absent	hexagonal plates	absent
9	+++	+++	++++	absent	absent	fragments of cork
10	++++	+++	++++	absent	absent	absent
11	+	+	++	absent	silica crystals	absent
12	+	+	++	absent	silica crystals	absent
13	+	+	++	absent	hexagonal plates and silica	absent
14	+++	+	++	present (rice)	absent	rice bran but no husk
15	+++	+++	++++	absent	common salt	fragments of cork
16	++++	+++	++++	absent	absent	fragments of cork
17	++++	+++	++++	absent	common salt	fragments of cork
18	++++	+++	+++	present (rice)	absent	rice bran and rice husk
19	+++	++	++	absent	absent	absent
20	++++	+++	++	absent	absent	absent
21	++++	+++	++++	absent	absent	absent
22	++++	+−+	+++	absent	absent	fragments of cork
23	++++	+−+	++	present (sago)	absent	absent
24	++++	+−+	+++	absent	absent	fragments of cork

Plus signs indicate the relative abundance of the particles.

irregular cubes exceeding 2 cm side and externally dark brown to almost black in colour; they were purchased from wholesale drug houses between 1953 and 1962, and seem to be typical of present-day material. The other cubes were all pre-1939 samples; they were reddish-brown in colour, about 2 cm side and were very regular in shape, having apparently been prepared more carefully than the preceding type; 3 of these samples (Nos. 6, 8, 13, Table 1) had a characteristic trade-mark symbol impressed on one of the sides of each cube.

The remaining 9 samples varied considerably in size and shape from flat circular plates to slender sticks, and from balls to irregular masses. All but two of these were Museum specimens manufactured before 1885 and it is unlikely that these forms of catechu are available on the present-day market; the other two samples (Nos. 18, 19) occurred in irregular masses and small angular pieces and were probably broken from large blocks, an alternative form in which catechu is occasionally manufactured at the present time.

Experimental methods

For the microscopical examination of all the post-1945 samples of catechu, in which the abundance of vegetable débris made its detection relatively easy, a small amount of the crushed material was mounted directly in solution of chloral hydrate (B.P.) and heated to clear. Most of the pre-1939 samples, however, contained only very small amounts of vegetable material and this was concentrated by boiling some of the powdered material in water and allowing to stand; mounts of the deposit were then made in solution of chloral hydrate as above.

Iodine (0·02N) was used to detect the presence of starch in the samples, and mounts were also made in solution of lactophenol (B.P.) to detect catechins and other crystalline material.

Microscopical characters

VEGETABLE FRAGMENTS

The following are the more important fragments found in the vegetable débris from the catechu samples; they were identified as being derived from the shoots of *Uncaria gambier* used in the preparation of the extract.

Covering trichomes. These are abundant and occur singly or in groups of up to 50 or more. They all are unicellular, but vary considerably in size, shape and degree of lignification and on the basis of these characters 4 types could be recognised:

(i) Large, stout, about 253–540 μ long and 21 to 43 μ wide; walls fairly thick and lignified; lumen nearly always filled with yellowish brown contents and often containing 1 or 2 thin, transverse, lignified septa; pitted at the base and only slightly enlarged; limb somewhat sinuous, narrowed near the base and gradually enlarging towards the centre, then remaining the same width to near the apex where it rapidly tapers to a point (Fig. 1, i).

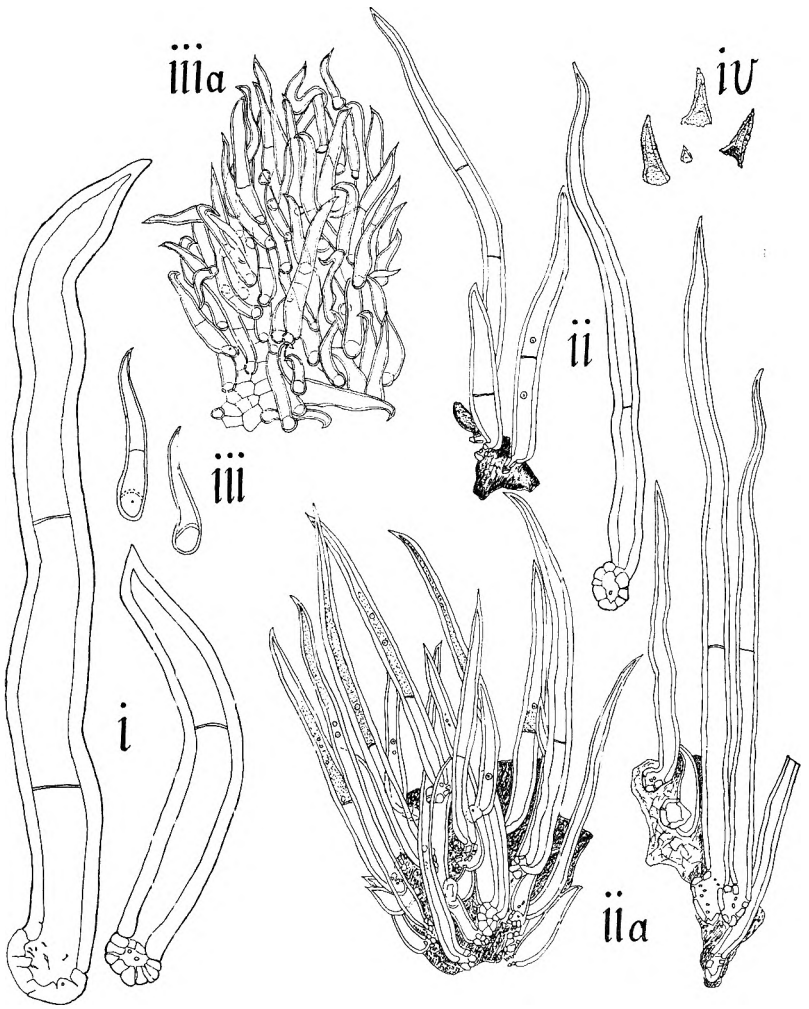

FIG. 1. Vegetable débris from pale catechu. Different types of covering trichomes: (*i*) from the inner epidermis of the petals; (*ii*) from the outer surface of the petals and the basal region of the calyx; (*iii*) from the outer epidermis of the lobes of the sepals; (*iv*) from the epidermis of the leaf midrib, stem or hook. (*iia*) and (*iiia*) show groups of trichomes of types (*ii*) and (*iii*) respectively massed together giving a flame-like appearance. All ×200.

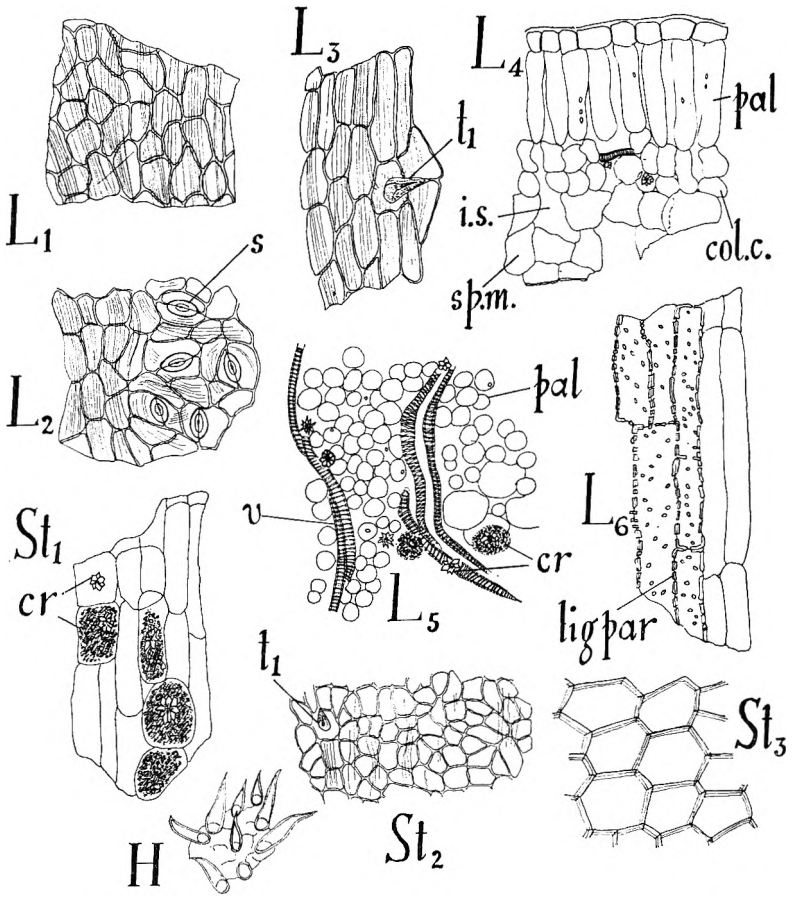

FIG. 2. Vegetable débris from pale catechu. Fragments from the leaves, stems and hooks. L_1 to L_6, fragments from the leaves; L_1, upper epidermis; L_2, lower epidermis; L_3, lower epidermis of the midrib; L_4, transverse section of the lamina; L_5, mesophyll seen in surface view; L_6, portion of the midrib medulla in longitudinal section; St_1 to St_3, fragments from the stems; St_1, portion of the phloem in longitudinal section; St_2, epidermis in surface view; St_3, cork; *H*, fragment of the epidermis of the hooks in surface view; *col.c.*, collecting cells; *cr*, crystals of calcium oxalate; *i.s.*, intercellular space; *lig.par*, lignified parenchyma; *pal*, palisade; *s*, stoma; *sp.m.*, spongy mesophyll; t_1, short covering trichomes; *v*, vessels. All ×200.

The trichomes of this type are probably derived from the *inner surface* of the *petals.*

(ii) Usually shorter and more slender than type (i), about 234 to 522 μ long and 10 to 15 μ wide at the midpart of the limb; walls fairly thick and lignified; lumen frequently filled with granular substances or a yellowish brown fluid and often containing 1 to 3 (usually 2) lignified septa; bases sometimes slightly enlarged, densely pitted and rather irregular in outline; limb often bent at a right angle to the base and somewhat sinuous, slightly constricted near the base, then enlarging rapidly and remaining approximately the same width to near the apex, where it gradually tapers to a point (Fig. 1, ii).

These trichomes are derived from the *outer surface* of the *petals* and the *calyx tube.*

(iii) These are smaller than either of the preceding types, measuring approximately 151 to 270 μ long and 10 to 15 μ wide at the base; walls thin and only slightly lignified; lumen usually empty and may be divided by a single, slightly lignified septum; base rounded with few pits; limb somewhat sinuous and gradually tapering towards the pointed apex (Fig. 1, iii).

These trichomes are derived from the *outer surface* of the *calyx lobes.*

(iv) Small, conical, with a warty cuticle; about 25 to 43 μ long and 15 to 36 μ at the base, walls variable in thickness but unlignified; base usually very much enlarged with no pits; lumen with no septa, wide at the base but sometimes becoming occluded near the apex (Fig. 1, iv).

These trichomes are probably derived from the *epidermis* of the *leaves, hook* or *stem.*

Leaf fragments. These are most readily recognised when occurring as portions of the epidermis seen in surface view (Fig. 2, L_1 and L_2) when the diagnostic characters are: the polygonal epidermal cells with a finely striated cuticle, the cells of the upper surface with straight anticlinal walls and those of the lower surface with somewhat curved or slightly sinuous anticlinal walls; paracytic stomata present on the lower epidermis only and covering trichomes usually absent from both surfaces.

Occasionally the leafy fragments are seen in transverse section (Fig. 2, L_4), when they may be recognised by the following characters: the cells of the upper epidermis with a fairly thick, striated cuticle; the row of collecting cells beneath the single layer of palisade cells, and the occasional idioblasts in the spongy mesophyll each containing a single cluster crystal of calcium oxalate. The lower epidermis is generally absent from these fragments.

Fragments of epidermis from the veins of the leaf are also occasionally found; these consist of subrectangular or polygonal, thin-walled cells with a striated cuticle; some of the cells bear short conical trichomes of type (iv) (Fig. 2, L_3).

Other leafy fragments which occasionally occur are portions of the mesophyll with vascular strands and crystals of calcium oxalate (Fig. 2, L_5)

and, more rarely, small groups of lignified parenchyma probably derived from the medulla of the midrib (Fig. 2, L_6).

Stem fragments. (i) Groups of parenchyma from the phloem, some containing sandy crystals and cluster crystals of calcium oxalate (Fig. 2, St_1).

(ii) Portions of the epidermis with striated cuticle and occasional trichomes of type (iv). These are rather similar to the fragments of the epidermis of the midrib of the leaf, but can be distinguished from them by the presence of stomata, and sometimes by the shape and size of the epidermal cells (Fig. 2, St_2).

(iii) Fragments of cork, reddish-brown in colour, composed of 2 or 3 layers of thin-walled cells, seen in surface view (Fig. 2, St_3).

Hook fragments. Very occasionally, fragments of epidermis are found bearing short, conical trichomes in greater numbers than occur on the stem or leaf midrib; also some of these trichomes may have a smooth cuticle. Such fragments are probably small portions of the epidermis of the hook near the apex (Fig. 2, H).

Stipule fragments. These also occur only very rarely in the débris; they are recognised by the appearance and distribution of the trichomes and stomata on the epidermis (Fig. 3, Stp).

Perianth fragments. These are commonly found in most samples of catechu and, like the leaf fragments, are most readily recognised when occurring as portions of the epidermis seen in surface view; they are distinguished from the leaf fragments by the presence of numerous epidermal trichomes, also the petal fragments are reddish-brown in colour.

(i) The outer epidermis of the *corolla lobes* is more readily recognised than the inner; it consists of polygonal cells with straight or slightly sinuous anticlinal walls which are very thin and not always clearly visible, being frequently obliterated by a layer of covering trichomes of type (ii) which lie with their apices pointing in the same direction, thus giving the fragments a "flame-like" appearance; frequently some of the trichomes are broken off leaving a characteristic cicatrix which is somewhat irregular in outline, finely pitted, lignified and usually surrounded by 4 to 6 epidermal cells; stomata infrequent (Fig. 3, P_1).

(ii) Fragments of *corolla tubes* occur less frequently than those from the corolla lobes; the outer epidermis is composed of very thin-walled, elongated cells, the length of which is about three to four times greater than the width; numerous covering trichomes are present, lying parallel to the epidermal cells with their apices pointing in the same direction; they are of type (ii) but measure only up to 234 μ in length and usually contain a single lignified septum situated in the lower half of the limb; the slightly sinuous anticlinal walls of the underlying mesophyll cells may also be visible as faint, disjointed lines giving a somewhat "beaded" appearance (Fig. 3, P_2).

(iii) The *calyx lobes* are the only recognisable fragments from the sepals; the outer epidermis consists of thin-walled, polygonal or sub-rectangular cells with very slightly sinuous anticlinal walls; paracytic

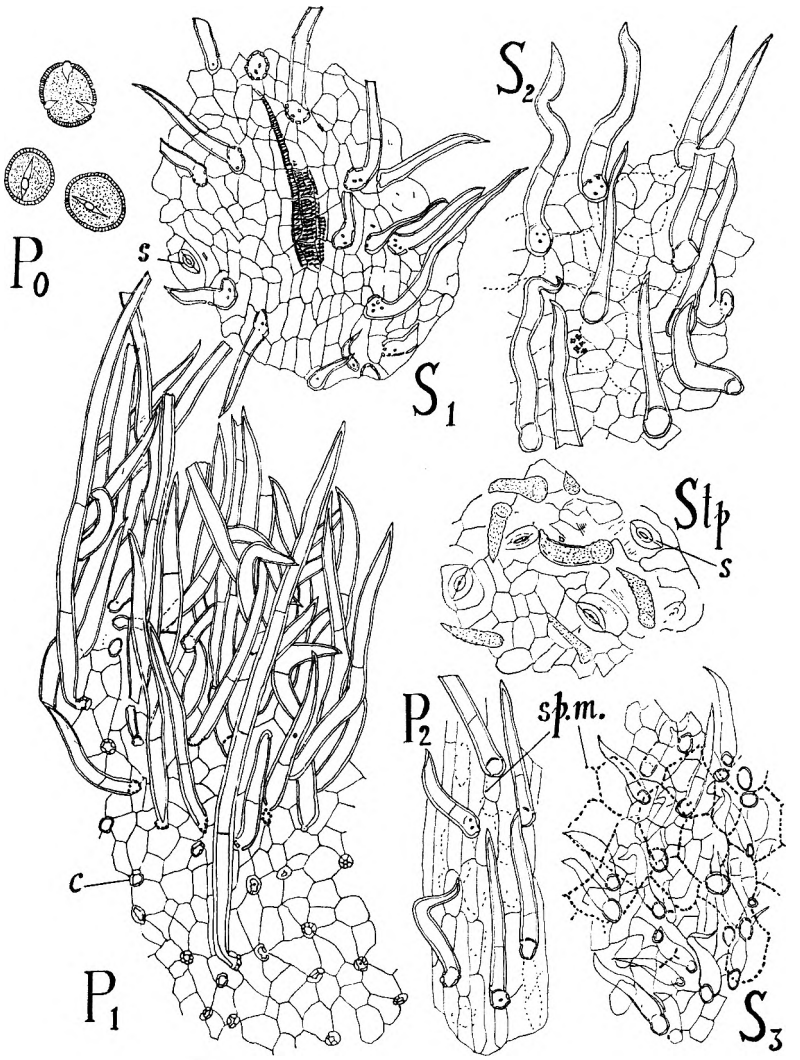

FIG. 3. Vegetable débris from pale catechu. Fragments from the flowers and stipules as seen in surface view. P_0, pollen grains; P_1, outer epidermis of the lobe of the petals and P_2, outer epidermis of the corolla tube; S_1, S_2 and S_3, fragments from the outer epidermis of the central region of the lobes of the sepals; S_1 and S_2 show the underlying vascular tissue and mesophyll respectively; S_3 shows the mesophyll lying uppermost and the epidermis underneath; *Stp*, lower epidermis of the stipule; *c*, cicatrix; *s*, stoma; *sp.m.*, spongy mesophyll. P_0, $\times 450$, others $\times 200$.

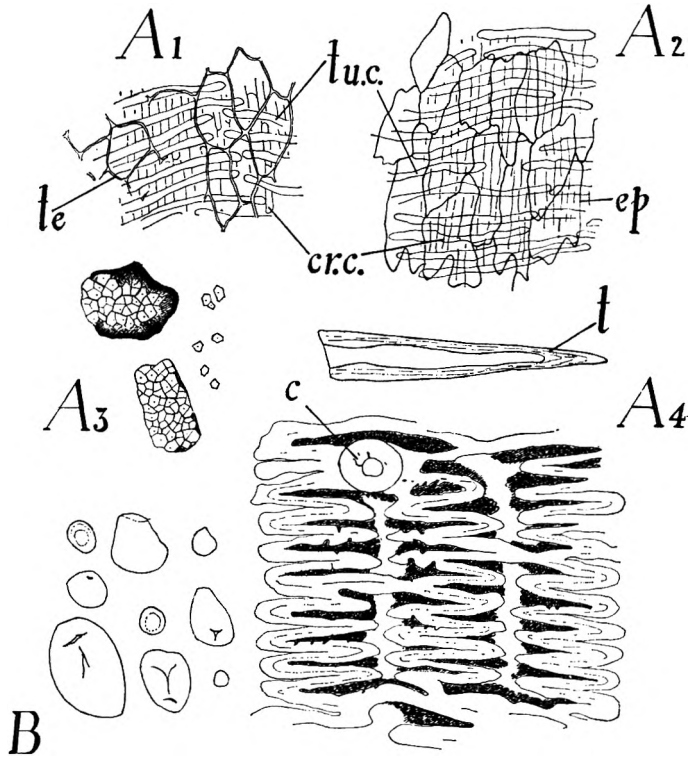

FIG. 4. Starch and other foreign material found in pale catechu. *A*, rice, *Oryza sativa* L.; A_1, surface view of the inner layers of the pericarp and adherent testa; A_2, surface view of the outer layers of the pericarp; A_3, starch granules; A_4, outer epidermis of the palea in surface view; *B*, starch granules from sago. All ×230. *c*, cicatrix; *cr.c.*, cross cells; *ep*, epicarp; *t*, covering trichome from the palea; *te*, testa or seed coat; *tu.c.*, tubular cells.

stomata fairly numerous but absent over the veins; covering trichomes of type (iii) fairly abundant, also scattered rounded or ovoid cicatrices which are lignified and frequently pitted; the underlying spongy mesophyll cells with "beaded" anticlinal walls and occasional groups of slender annular and spiral vessels are frequently visible; the inner epidermis is thin-walled, with very few trichomes (Fig. 3, S_1, S_2, S_3).

Pollen grains. These were present in most of the samples and although an occasional extraneous pollen grain was seen, the majority were similar to those of *Uncaria gambier* previously described (Leong & Jackson, 1964) and were obviously derived from the flowers present in the material used for making the extract (Fig. 3, P_0).

EXTRANEOUS VEGETABLE FRAGMENTS

In addition to the fragments derived from *Uncaria gambier* described above, 3 of the samples (Nos. 5, 14, 18, Table 1) also contained *Rice bran*; this was identified by reference to Winton (1945) and Wallis (1957) and confirmed by comparison with the bran and husks removed from whole fruits of *Oriza sativa.* The fruit wall, portions of which constitute rice bran, is composed of a number of layers of thin-walled cells and several of these adhere together; the epidermis, or epicarp, is a single layer of elongated, somewhat wavy-walled cells and beneath this a layer of very thin-walled elongated transverse cells is faintly discernible, followed by a layer of tubular cells with intercellular spaces; these cells are orientated with their long axes at right angles to those of the cells of the other layers, thus giving a very characteristic appearance to the fragments (Fig. 4, A_2). Frequently the testa is also adherent to the layers of the fruit wall; this consists of a layer of rectangular cells with straight, slightly thickened anticlinal walls, and in the fragments in which this layer is lying uppermost the underlying tubular cells of the fruit wall are clearly seen with the very faint striations of the transverse cells lying underneath (Fig. 4, A_1).

In two of these samples, in addition to the rice bran described above, fragments of the *Rice husks*, or paleae, were also present; these were recognised by the structure of the outer epidermis which is very characteristic (Fig. 4, A4), being composed of a layer of cells with very deeply sinuous, thickened and partially lignified walls and occasional covering trichomes which are unicellular, elongated and sharply pointed at the apex and have thickened, partially lignified walls; these trichomes frequently become detached from the epidermis, leaving a characteristic cicatrix (Fig. 4, A_4, c), and occur scattered in the vegetable débris (Fig. 4, A_4, t).

Starch. The 3 samples (5, 14, 18) which contained rice bran also contained starch and, as is to be expected, this was typical rice starch; it consisted of small, polygonal granules, 3 to 8 μ in diameter, occurring singly or more frequently in large groups closely packed together to form angular masses, some granules with a faintly discernible central hilum (Fig. 4, A_3).

Starch was also found in one other sample (23); the granules were much larger, measuring 7 to 45 to 69 μ in diameter, some ovoid to subspherical

but the majority muller-shaped indicating that they were components of original compound granules; hilum a split, usually 1/3 to 1/5 eccentric; striations not visible, but many granules with deep cracks or fissures (Fig. 4, B). Reference to the literature (Wallis, 1957) indicated that the starch was commercial sago, and this was confirmed by comparison with authentic material.

CRYSTALLINE MATERIAL

Of all the microscopical structures found in commercial catechu the most characteristic and diagnostic are the acicular crystals of *catechin* which constitute the main bulk of the solid extract. They occur in compact, yellowish-brown masses, and are birefringent; the size of the individual crystals varies in different samples and appears to be larger in those containing only a small amount of vegetable débris (Fig. 5, A).

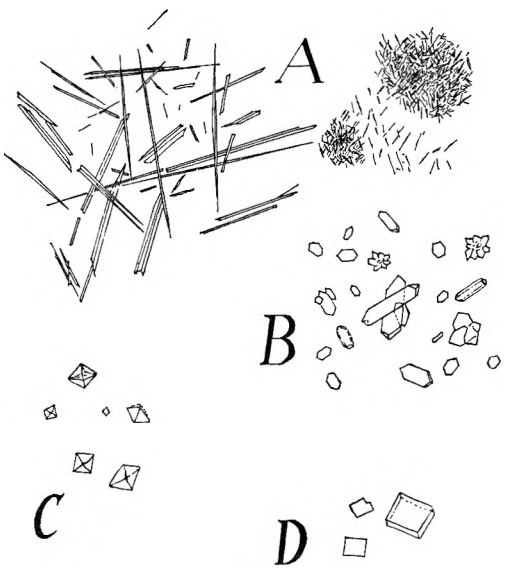

FIG. 5. Crystalline material from pale catechu. *A*, acicular crystals of catechin from two different samples, showing the variation in size; *B*, crystals of unknown composition in single hexagonal plates and in clusters; *C*, silica crystals; *D*, common salt crystals. All ×330.

Varying amounts of other crystalline materials, in characteristic forms, were also found in a number of the samples; these included common salt crystals, silica crystals and some crystalline substance of unknown composition. *Common salt crystals* were found in two samples, and they were identified chemically and also microscopically by comparison with pure sodium chloride; they occurred in the form of very thin, relatively square plates (Fig. 5, D).

Silica crystals were occasionally seen in nearly all the samples but the amount present was usually very small; they occurred in the form of small, birefringent hexahedrons or double pyramids (Fig. 5, C).

In three of the pre-1939 samples other crystals were found which were birefringent and in the form of thin, relatively regular hexagonal plates, usually lying singly with their flat faces uppermost but occasionally clustered together in small groups. They occurred only in relatively small amounts and were not identified (Fig. 5, B).

Discussion

Table 1 summarises the composition of the débris in the 24 samples examined. As the *hook* and *stipule* fragments occurred relatively infrequently they are not included under a separate heading but they were usually present in all samples which had an abundance of leaf and perianth segments. Portions of parenchyma and epidermis of the *stem* also occurred in most samples but cork fragments, indicating the use of older stems in the preparation of the extract, were only found in 7 of the samples and these are indicated in the last column of the Table.

Reference to the Table shows that *covering trichomes* occurred in varying amounts in all samples; they were particularly numerous in those manufactured since 1945 (i.e., Nos 15 to 24). Portions of the *leaves* and *perianth segments* were also found in all samples, but generally they were slightly less abundant than the covering trichomes. *Pollen grains* were not detected in all the samples but they were present in considerable quantities in those which contained abundant trichomes and other vegetable fragments. Of the four samples which contained *starch*, in only one was vegetable débris relatively scarce, suggesting that the reported practice (Brumwell, 1911; Howes, 1953) of the addition of starch to the extract is not associated with a particular manufacturing process. It is interesting to note that such addition of starch is still practised, as indicated by the abundance of rice starch in sample 18, recently obtained from Malaya. The two samples which contained *common salt crystals* also contained abundant vegetable débris, including fragments of cork; as salt is an unusual adulterant of catechu it is possible that these 2 samples, although they were obtained at different times, originally came from the same source. The other extraneous crystalline materials found—namely *silica* and the *unidentified hexagonal crystals*—were associated with only a small amount of vegetable débris, and were found only in samples manufactured before 1939.

Catechin crystals have not been included in Table 1 as they were found in very large quantities in all the samples examined.

Of the fragments of the leaves and shoots of *Uncaria gambier* present in commercial catechu, it is interesting to note that most of them are derived from superficial tissues, e.g. covering trichomes, epidermises of leaves and perianth segments and pollen grains, which would readily become detached from the plant when the marc is stirred during the extraction process. The almost complete absence of any internal structures from the core of the plant, e.g., vessels and fibres, indicates that the harvested leafy twigs are not subjected to comminution before the extraction process; this is in accordance with the normal manufacturing procedure as reported.

DIAGNOSTIC CHARACTERS

The diagnostic microscopical characters which are of most value in the identification of commercial and pharmaceutical catechu are as follows:

1. The abundant crystalline masses consisting of numerous *acicular crystals of catechin.*

2. A very large number of unicellular *covering trichomes*, either whole or fragmented, occurring singly or in groups still attached to pieces of epidermises, and showing different characters depending on the part of the plant from which they have been derived.

Isolated, whole covering trichomes are of four types, as described on page 410 (Fig. 1).

3. Fragments from the *lobes of the petals* showing the outer epidermis and, occasionally, the underlying spongy mesophyll, as described on page 414 (Fig. 3).

4. Fragments from the *central region of the lobe of the sepals* showing the outer epidermis and frequently also the underlying spongy tissues, the mesophyll being occasionally traversed by slender vascular strands; cells of the epidermis with slightly sinuous anticlinal walls; stomata numerous and paracytic; covering trichomes of type (iii) fairly numerous; cicatrices, scattered and usually rounded or ovoid in outline.

5. A large number of *pollen grains* of *Uncaria gambier*, subspherical in shape, about 11 to 18 μ in diameter, with three pores and three germinal furrows and a warty exine.

6. Fragments of *leaf* showing polygonal epidermal cells with striated cuticle. The details are described on page 413 (Fig. 2).

7. Fragments of *cork*.

All the above are derived from the source material, young twigs of *Uncaria gambier.*

If *rice bran* or *rice husks* are present in the extract, the additional diagnostic microscopical characters are:

(a) *Rice bran:* fragments of the pericarp in surface view, showing the epicarp, very frequently with underlying layers of *transverse* and *tubular* cells; described on page 417; see also Fig. 4.

(b) *Rice husk:* fragments of the outer epidermis of the paleæ (Fig. 4) described on page 417.

(c) The abundant angular masses of *starch granules* typical of rice.

Acknowledgements. The authors wish to express their thanks to Dr. T. E. Wallis, Mr. D. C. Harrod, Mr. H. K. Leong and Dr. D. E. Hathway for their supply of samples of catechu used in this investigation.

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J. Pharm. Pharmacol., 1964, **16**, 422–426 Received December 20, 1963

Influence of adrenaline on the diabetogenic effect of alloxan in the rat

H. ABD-EL-WAHED, H. A. GHALEB AND M. R. HEGAZY

The effect of alloxan and adrenaline on blood glucose was investigated in 84 rats. The diabetogenic action of alloxan, 200 mg/kg, was prevented by mixing it for 10 min with adrenaline, 50 μg/kg, before injection. No histological changes in the islets were observed after the injection of this mixture. Similar doses of adrenaline and alloxan, injected intramuscularly at the same time but separately produced a diabetogenic action greater than that produced by alloxan alone.

SINCE the discovery of alloxan-induced diabetes by Dunn, Sheehan & McLetchie (1943), drugs which can protect the animal against the diabetogenic effects of alloxan have been sought.

Kass & Waisbren (1945) found that the intraperitoneal administration of adrenaline along with the subcutaneous injection of alloxan, diminished the diabetogenic effect of the latter compound. The diabetogenic effect of alloxan has been reported to be antagonised by glutathione acting by virtue of its -SH group (Patterson, Lazarow & Levey, 1949), by nicotinamide, benzamide, propylene glycol propanol and isopropanol (Janes & Schueler, 1955).

Drugs which antagonise the diabetogenic effect of alloxan are of particular interest since alloxan has been used in the treatment of certain malignant neoplasms (Gilman, Hathorn & Lamont, 1957).

The present study, which deals with the effect of adrenaline on the diabetogenic action of alloxan, involved the intramuscular injection in rats of both drugs either separately or as a pre-incubated mixture at one site.

Material and methods

Eighty-four male adult albino Wistar rats of from 150–200 g were used. All were kept under the same conditions.

The rats were divided into 5 groups as follows:

Group I (alloxan group), comprising 23 rats, were injected intramuscularly with alloxan 200 mg/kg weight.

Group II (alloxan + adrenaline group); 18 rats were injected intramuscularly with an incubated mixture of alloxan, 200 mg, and adrenaline hydrochloride, 50 μg, for each kg weight. Incubation of this mixture was at room temperature (25°) for 10 min.

Group III (separate alloxan and adrenaline group); 13 rats were injected intramuscularly at the same time with alloxan, 200 mg/kg, in one thigh and adrenaline hydrochloride, 50 μg/kg, in the other thigh.

Group IV (adrenaline group); 14 rats were injected intramuscularly with adrenaline hydrochloride 50 μg/kg weight.

Group V (control group); this comprised 16 rats each injected intramuscularly with 0·8 ml of physiological saline.

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Drugs. Alloxan monohydrate (Merck) 5% aqueous solution and adrenaline hydrochloride (May & Baker Ltd.) 0·00125% aqueous solutions were used. Solutions were prepared and kept at room temperature, 25°, for 10 min before injection.

Collection of blood samples. All rats were fasted overnight before the collection of blood for estimation of blood glucose. Samples of 0·15 ml blood were taken before treatment, and 36 hr, 72 hr, 1, 2 and 3 weeks after the drug by cardiac puncture using needle No. 20. Lithium oxalate, 1 mg/ml blood, was used as an anticoagulant.

Blood glucose estimation. Blood samples, 0·1 ml, were measured accurately using dry micropipettes; the glucose concentration in these samples was determined using Somogyi's technique (1945), utilising Nelson's (1944) arsenomolybdate colour reagent. The optical densities were measured using a Zeiss Spectrophotometer at a wavelength of 490 mμ.

Histological studies. Three rats from each group were killed at different intervals and their blood glucose levels estimated. The results are not presented in the tables to avoid interference with the mortality rates. Histological sections of the pancreas from these animals were prepared and stained with haematoxylin and eosin to see if there was evidence of histological changes in the islet cells after drug administration.

Statistical analysis of the results. The results of the blood glucose estimations were subjected to statistical analysis. The significance of differences between means of the various groups was tested by their *t* values and the level of P (Burn, Finney & Goodwin, 1952).

Results

BLOOD GLUCOSE LEVEL

The blood glucose concentrations in the different groups of animals at different intervals after drug administration are shown in Table 1. The statistical significance of the difference between means of blood glucose in the different groups are presented in Table 2.

From these results, there is no significant difference in the mean blood glucose levels of the animals in the control group and those injected with adrenaline alone or those injected with the incubated mixture of adrenaline and alloxan. The animals of the remaining two groups developed hyperglycaemia. In the rats that received alloxan only, the hyperglycaemia was noticed after 36 hr and was maintained during the first two weeks. On the third week, some animals showed marked hypoglycaemia.

The group of animals receiving alloxan and adrenaline separately at the same time, developed more hyperglycaemia than the group on alloxan. The difference is highly significant (Table 2).

MORTALITY RATE

The cumulative percentage mortality in each group at different intervals after drug administration is in Table 3. The highest mortality rate was in group III whereas the rate in group II was much lower than either that of group I or III.

TABLE 1. ACTION OF ADRENALINE ON THE DIABETOGENIC EFFECT OF ALLOXAN IN RATS

Drugs (i.m.)		Fasting blood glucose mg/100 ml blood					
		Initial	36 hr	72 hr	1 week	2 weeks	3 weeks
Group I, 20 animals Alloxan	Mean	60·85	151·50	144·05	110·12	89·30	82·60
	s.d.	±17·93	±75·17	±90·13	±50·95	±32·37	±34·34
	s.e.	±4·01	±16·80	±20·16	±12·36	±10·24	±10·86
		(20)	(20)	(20)	(17)	(10)	(10)
Group II, 15 animals Adrenaline and alloxan incubated mixture	Mean	66·00	80·14	82·46	84·67	73·10	66·42
	s.d.	±8·31	±15·34	±18·82	±14·49	±15·95	±2[illegible]·21
	s.e.	±2·15	±4·10	±5·22	±3·74	±4·60	±6·12
		(15)	(14)	(13)	(15)	(12)	(12)
Group III, 10 animals Adrenaline and alloxan in separate sites	Mean	64·00	285·57	515·50	205·33	104·00	82·00
	s.d.	±6·68	±90·68	±185·60	±94·56		
	s.e.	±2·11	±34·27	±75·77	±54·66		
		(10)	(7)	(6)	(3)	(2)	(1)
Group IV, 11 animals Adrenaline	Mean	58·45	84·27	64·50	83·27	83·10	60·09
	s.d.	±12·65	±16·19	±14·52	±14·36	±16·98	±8·18
	s.e.	±3·81	±4·88	±4·59	±4·33	±5·37	±2·47
		(11)	(11)	(10)	(11)	(10)	(11)
Group V, 13 animals .. Control	Mean	62·90	76·38	74·77	87·27	81·23	65·15
	s.d.	±7·77	±13·70	±11·10	±8·31	±10·92	±11·12
	s.e.	±2·46	±3·80	±3·08	±2·50	±3·03	±3·08
		(10)	(13)	(13)	(11)	(13)	(13)

s.d. = Standard deviation.
s.e. = Standard error of the mean.
( ) = Number of samples.
Variations were due to mortalities or to loss of samples.

TABLE 2. STATISTICAL SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS OF BLOOD GLUCOSE IN VARIOUS GROUPS AS TESTED BY THEIR *t* VALUE AND THE LEVEL OF *P*

Groups compared		Initial	Time after drug administration				
			36 hr	72 hr	1 week	2 weeks	3 weeks
Alloxan group .. .. Alloxan + adrenaline group	*t*	1·13	4·125	2·478	1·972	1·443	1·298
	P	<0·15	<0·0005	=0·01	<0·05	<0·15	<0·15
Alloxan + adrenaline group Separate alloxan and adrenaline group ..	*t*	0·664	5·938	5·702	2·205		
	P	=0·25	<0·0005	<0·0005	<0·025		
Alloxan group .. .. Control group	*t*	0·436	4·359	3·398	1·812	0·756	1·546
	P	<0·35	<0·0005	=0·0005	=0·05	<0·25	<0·1
Alloxan + adrenaline group Control group	*t*	0·95	0·673	1·269	0·577	1·475	0·185
	P	<0·20	=0·25	<0·1	=0·3	<0·1	<0·4
Adrenaline group .. Control group	*t*	0·98	1·275	1·854	0·80	0·303	1·281
	P	<0·2	<0·1	<0·05	=0·2	<0·35	<0·1

TABLE 3. THE CUMULATIVE PERCENTAGE MORTALITY IN THE VARIOUS GROUPS OF RATS

Group	Time after drug administration				
	36 hr	72 hr	1 week	2 weeks	3 weeks
Alloxan group	0	0	10%	40%	50%
Alloxan + adrenaline mix group	0	0	0	20%	20%
Separate alloxan and adrenaline group ..	30%	40%	70%	80%	90%
Adrenaline group	0	0	0	0	0
Control group	0	0	0	0	0

MICROSCOPICAL CHANGES

Twelve hr after administration of alloxan in group I, the nuclei of the beta cells in the islets of Langerhans showed marked pycnosis (Fig. 1).

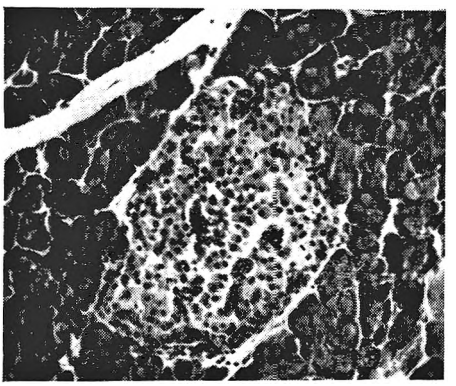

FIG. 1. Islets of Langerhans in a rat 12 hr after i.m. injection of 200 mg alloxan/kg. The nuclei of the beta cells show marked pycnosis. The fasting blood glucose level was 112 mg/100 ml.

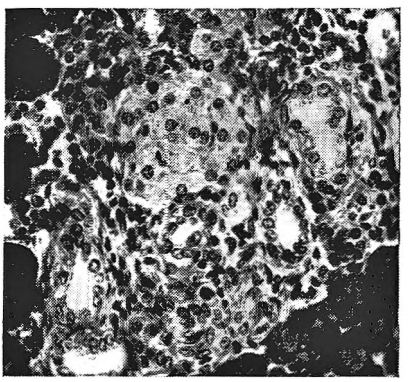

FIG. 2. Islets of Langerhans in a rat 10 days after i.m. injection of 200 mg alloxan/kg. Regeneration in the pancreas is apparent; complex small tubules arranged focally in small islands have begun to appear. The fasting blood glucose level was 57 mg/100 ml.

In the animals that received alloxan only and in those injected with alloxan and adrenaline at the same time by separate injections, there were marked histological changes in the islet cells 36 hr after the drug. Some beta cells were vacuolated and appeared to retain shadows of nuclei which showed various stages of pycnosis, karyorrhexis or karyolysis. The blood sinusoids were dilated and occasionally the islets were infiltrated with leucocytes. In these animals marked hyperglycaemia was observed.

The pancreatic tissue of rats which were injected with alloxan only and were killed 10 days after the drug, was characterised by the appearance of small tubules indistinguishable in all respects from small pancreatic ductules present in normal pancreas (Fig. 2). These were arranged focally in little islands. The tubules were lined by cubical epithelium and the lumen was usually empty. In the animals which recovered from the diabetic condition particularly those which exhibited hypoglycaemia, the pancreatic islets looked almost normal, although many small islets were seen.

Microscopical examination of the pancreatic tissues of the animals of the control group, those injected with adrenaline only and those injected with the mixture of adrenaline and alloxan, showed that there were no relevant microscopical changes in the islet cells.

Discussion and conclusions

The results show that the groups of animals injected intramuscularly with a mixture of alloxan and adrenaline neither developed hyperglycaemia nor any microscopical changes in the pancreas. However, those animals injected intramuscularly at the same time with alloxan and adrenaline

at different sites, showed hyperglycaemia even more severe than that occurring in the alloxan group and degenerative changes in the beta cells of the islets were observed.

It appears that the process of mixing adrenaline with alloxan abolishes the diabetogenic property of the latter. But, adrenaline injected intramuscularly at the same time as alloxan but at a different site did not protect against the diabetogenic action of alloxan, on the contrary it enhanced the action.

The reduction of the diabetogenic effect of alloxan, 200 mg/kg, after mixing it with adrenaline, 50 μg/kg, for 10 min at 25° before administration might result from a chemical interaction. Lazarow (1954) pointed out that loss of the diabetogenic action of alloxan results either from an attack on the ketone group or the =NH group. Since the amount of adrenaline is so small compared to the amount of alloxan, it may be that oxidation products of adrenaline resulting from interaction with alloxan might also be responsible for the antagonism of the diabetogenic action. A recent report of the activity of adrenochrome on oxidative phosphorylation showed that at micromolar levels, it decreased both oxygen uptake and phosphorylation (Krall, Siegel & Gozansky, 1962).

The action of alloxan when it was simultaneously administered along with adrenaline at separate sites could be the result of the very rapid destruction of alloxan (Goodman & Gillman, 1957) *in vivo* and also the very rapid disappearance of adrenaline from the blood stream (Lund, 1951) which would not allow much time for any chemical interaction. However, the possibility that the vasodilator action of adrenaline on the blood vessels of the skeletal muscles might enhance the rate of absorption of alloxan from its site of injection in the thigh and hence accentuate the diabetogenic effect must not be overlooked.

In the report of Kass & others (1945), the protection against the diabetogenic action of alloxan administered subcutaneously, by intraperitoneal injection of adrenaline, could be due to delayed absorption of alloxan from the subcutaneous tissue with consequent low blood levels.

Acknowledgement. The authors wish to thank Professor A. A. Tarkhan and Professor M. A. Khayyal for their interest and valuable suggestions.

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J. Pharm. Pharmacol., 1964, **16**, 427–428 Received December 16, 1963

A preparation of the spinal cat by an anterior approach

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A method for preparation of the spinal cat through an anterior approach is described. It has certain advantages over the classical posterior approach and is particularly useful in preparations for studying the superior cervical ganglion or nictitating membrane since the entire procedure may be carried out through the same exposure.

DESPITE the frequent use of the spinal cat-nictitating membrane preparation for the study of autonomic drugs, the operational details have been infrequently described. Most investigators use Dale's method as described by Burn (1952) for preparation of the spinal cat. This method is somewhat inconvenient because one must first do a tracheotomy and temporarily clamp the carotids, then turn the cat over and carry out the posterior approach to the spinal cord.

The removal of the larynx and oesophagus as suggested by Trendelenberg (1957) offers a clearer operative field for the necessary manipulations of the superior cervical ganglion and the post ganglionic fibres to the nictitating membrane. When this was done, it was found that a simple retraction from the midline of the anterior spinal muscles exposed a natural space between the skull and the first cervical vertebra, especially on hyperextension of the head. It was covered only by a dural membrane and offered a simple approach for the transection of the spinal cord.

Not only was the posterior dissection eliminated, but other advantages accrued. By avoiding the thick and vascular spinal muscles, less trauma and haemorrhage was inflicted. No bones or cartilage had to be resected, and the approach avoided the vertebral arteries.

Method

The cat was anaesthetised with diethyl ether in an ether box. Anaesthesia was maintained with ether by the open drop technique using a nose cone. A tracheotomy was performed just above the sternal notch, anaesthesia now being maintained with an ether vapouriser attached to the tracheotomy tube. The trachea and oesophagus were severed between a double ligature placed immediately cephaled to the tracheotomy tube. These two structures were then easily peeled away from the anterior spinal muscles. A second ligature was placed around these structures above the larynx. The larynx, trachea, and oseophagus were now removed. Careful ligature of the branches of the carotid, such as the thyroid artery, virtually eliminated all haemorrhage.

The anterior spinal muscles were then retracted from the midline between the base of the skull and the first cervical vertebra. For better exposure of the superior cervical ganglion, the anterior spinal muscles on the desired side might be pulled away or even resected (Fig. 1).

Transection of the spinal cord was best performed with the head hyperextended. A small incision was made in the dural membrane of

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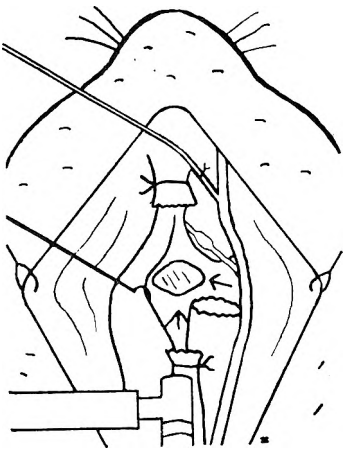

FIG. 1. Anterior view of the cervical region of the cat. A tracheal tube is in place and connected to the respirator tube. A ligature is around the base of the oropharynx at the cephalic end. The larynx, trachea and oesophagus are removed. The left anterior spinal muscles are severed, the right is retracted. The left carotid artery is outlined and a catheter is in the cut end of the lingual branch. The left superior cervical ganglion is seen against the tympanic bullae, as the post ganglionic trunk enters the skull. An arrow points to the natural opening between the base of the skull and the first cervical vertebra, through which the procedure is carried out.

the now exposed space between the skull and the first cervical vertebra. The spinal cord was severed with a suitable instrument such as a small, curved, blunt spatula. Then the instrument was quickly passed through the foramen magnum and the brain destroyed. Obviously, destruction of the brain may be omitted in preparations where it is unnecessary or undesired. Ether anaesthesia was now stopped and artificial respiration instituted. Pledgets of cotton were held ready to be packed into the spinal canal immediately after the instrument was removed. When the canal was tightly packed, pressure was maintained over the opening with a gauze sponge for a few min until all bleeding stopped. The opening may be covered with beeswax.

During the transection, bleeding could be avoided by applying bulldog clamps to both carotids. Egress of spinal fluid was minimised by first aspirating through the dura with a wide bore needle and syringe before it was cut. In over 20 cats prepared in this fashion, the described operative procedure proved to be eminently satisfactory in all respects.

Acknowledgement. We wish to thank Mr. Robert McMichael for valuable technical assistance. This work was supported in part by a Public Health Service Fellowship No. 1-F2-HE-20, 148-01 from the National Institutes of Health, National Heart Institute, U.S. Public Health Service.

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J. Pharm. Pharmacol., 1964, **16**, 429–432 Received December 23, 1963

A note on the stability of actinomycin D

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Visible absorption spectra and an agar-diffusion microbiological assay with *Staphylococcus aureus* were used to examine the effects of temperature, time, and pH on the stability of actinomycin D in distilled water, Clark and Lubs buffer solutions, and plasma. The most stable storage pH range for actinomycin D in buffer solution is 5 to 7. Actinomycin D in distilled water and stored 5° C (refrigeration) preserved its stability for at least 150 days. Except in buffer at pH 5, the antibiotic is appreciably degraded by autoclaving.

THE antibiotic actinomycin D, a dipeptide derivative of the chromophore 2-amino-4,5-dimethylphenoxazin-3-one-1,8-carboxylic acid (Bullock & Johnson, 1957; Johnson, 1960) has a broad spectrum of biological activity as evidenced by bacteriostatic action against gram-positive bacteria and some fungi (Brockman, 1954; Foley, 1955; Pugh, Kutz & Waksman, 1956; Slotnick, 1957), cytotoxicity produced in many mammalian lines in cell culture (Eagle & Foley, 1956; Goldstein, Slotnick, Hillman & Gallagher, 1959), and antineoplastic activity against experimental tumours in rodents (see, for example, Garatti, Costa, Murelli, Palma & Vegeto, 1956; Sugiura, Stock, Reilly & Schmid, 1958).

Although chemical and microbiological changes of actinomycin D have been described (Bullock & Johnson, 1957; Brockman, 1960; Johnson, 1960; Katz, 1960), to date there is no information on the stability of this agent under usual laboratory storage conditions. The purpose of this study was to investigate the effect of varied pH and temperature levels over extended periods of time on the stability of actinomycin D in aqueous solution.

Experimental

Actinomycin D concentrated stock solutions, 300 μg/ml, were prepared from dried powder‡ and refrigerated sterile distilled water. Subsequent dilutions were prepared at pH 1, 3, 5, 7, 9, 11, such that the final dilution contained 30 μg/ml. Clark and Lubs buffers (Remington, 1961) were used except for the solutions at pH 1·0 and 11·0, for which 0·1N hydrochloric acid and 0·1N sodium hydroxide were used respectively.

Solutions were stored at −10°, 5° (refrigeration), 25° (room temperature) and 37° and were assayed at zero, 6, 14, 29, 55 and 150 days, by spectrophotometric and microbiological methods. In addition, solutions of actinomycin D at each pH level were autoclaved for 20 min at 15 pounds pressure and assayed.

SPECTROPHOTOMETRIC ASSAY

The spectral range observed was over 500–360 mμ, curves at zero time for each solution of actinomycin D were used as a point of reference.

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Supported by USPHS Grants CA 06660–01 and AI 05170–01 and American Lebanese Syrian Associated Charities (ALSAC).

‡ Courtesy of Merck Sharp & Dohme, West Point, Pennyslvania.

MICROBIOLOGICAL ASSAY

A standard agar diffusion paper-disc assay using *Staphylococcus aureus* was made on each solution. An actinomycin D solution of 30 μg/ml in sterile distilled water (pH 7·0) was used as control for each assay. The percentage inhibition was based on the comparison of zones of inhibition of the test samples to the standard solution.

STABILITY OF ACTINOMYCIN D IN PLASMA

Plasma solutions of actinomycin D (30 μg/ml) were prepared aseptically from fresh sterile human plasma. These solutions were held at 37° and assayed at zero time, 7 and 14 days, microbiologically and spectrophotometrically.

Tables 1 and 2 list the result of the spectrophotometric and microbiological assays.

TABLE 1. SPECTROPHOTOMETRIC ASSAY OF SOLUTIONS OF ACTINOMYCIN D

pH	Temp. °C	6 days	14 days	29 days	55 days	150 days
1	−10	*0·89	0·87	0·95	S	S
	5	S	S	S	S	S
	25	S	S	S	S	S
	37	S	S	S	S	—
3	−10	0·95	—	0·95	0·85	0·85
	5	0·97	0·95	0·93	0·85	S
	25	0·87	0·83	S	S	S
	37	0·83	S	S	S	—
5	−10	0·98	0·95	0·96	0·88	0·88
	5	0·98	0·98	0·96	0·96	0·91
	25	0·98	0·98	0·96	0·91	0·77
	37	0·98	0·97	0·93	0·91	—
7	−10	0·92	0·92	0·92	0·80	0·82
	5	1·0	1·0	0·97	0·95	0·92
	25	0·97	0·97	0·95	0·95	0·85
	37	0·95	0·92	0·90	0·87	—
9	−10	0·95	0·90	—	0·70	0·82
	5	0·78	0·70	0·61	0·42	S
	25	0·20	S	S	S	S
	37	S	S	S	S	S
11	−10	0·71	S	S	S	S
	5	S	S	S	S	S
	25	S	S	S	S	S
	37	S	S	S	S	S
Distilled water	5	1·0	1·0	1·0	1·0	0·95

* Values in table are fractional representations of the optical density maximum of the sample as compared to the zero time standard which was taken as unity.
S indicates shifts in spectral curves which did not allow comparison with standard maximum absorbance readings.
— Indicates data could not be recorded.

EFFECT OF pH

The most stable storage pH range between 5 and 7. As pH level was increased above 7 there was a more rapid alteration in spectra and more rapid decrease in microbiological activity than when pH level was decreased below 5.

Solutions of actinomycin D changed from a dark golden brown colour at pH 1 to colourless at pH 11.

TABLE 2. MICROBIOLOGICAL ASSAY OF SOLUTIONS OF ACTINOMYCIN D

pH	Temp. °C	6 days	14 days	29 days	55 days	150 days
1	−10	*1·0	1·0	0·95	0·93	0·72
	5	1·0	0·82	0·76	0·65	—
	25	0·79	—	—	—	—
	37	—	—	—	—	—
3	−10	1·0	1·0	0·95	0·95	0·94
	5	1·0	1·0	0·93	0·98	0·69
	25	0·95	0·85	0·65	—	—
	37	0·87	0·70	—	—	—
5	−10	1·0	0·95	0·98	1·0	0·96
	5	1·0	1·0	0·98	0·93	0·84
	25	1·0	1·0	0·95	0·88	0·81
	37	1·0	1·0	0·91	0·88	—
7	−10	1·0	0·98	1·0	1·0	0·98
	5	1·0	1·0	0·98	0·94	0·88
	25	1·0	1·0	0·95	0·88	0·83
	37	1·0	1·0	0·93	0·88	—
9	−10	1·0	1·0	0·98	0·95	0·94
	5	1·0	0·95	0·88	0·81	—
	25	0·87	0·70	—	—	—
	37	—	—	—	—	—
11	−10	0·67	0·67	—	0·67	—
	5	—	—	—	—	—
	25	—	—	—	—	—
	37	—	—	—	—	—
Distilled water	5	1·0	1·0	1·0	1·0	1·0

* Zones of inhibition, measured in mm, have been converted to a decimal basis with inhibition at zero time an arbitrary 1·0. All other readings are compared to fractional inhibition of control.
— Indicates data could not be recorded.

EFFECT OF TEMPERATURE

With temperature rise, solutions buffered at pH 7 and stored at −10° showed a significant spectral alteration after 7 days while buffered solutions stored at 5° were stable for the same period of time. On the other hand, this was not observed with unbuffered aqueous solutions of actinomycin D treated in the same manner. We have no explanation for this phenomenon.

Solutions of actinomycin D which were autoclaved, degraded significantly except at pH 5·0. At this pH, spectra and microbiological activity remained similar to the standard (Table 3).

TABLE 3. SPECTROPHOTOMETRIC AND MICROBIOLOGICAL ASSAY OF SOLUTIONS OF ACTINOMYCIN D AFTER AUTOCLAVING

Buffer solution pH	After autoclaving	
	Spectrophotometric	Microbiological
1	0·89	—
3	0·83	0·89
5	0·95	1·00
7	0·73	0·80
9	0·00	—
11	0·00	—
In water about pH 7	0·75	0·76

All solutions before autoclaving were assayed microbiologically and spectrophotometrically and given values of 1·0. Cf. Tables 1 and 2.

STORAGE IN AQUEOUS SYSTEM

Solutions of actinomycin D (30 μg/ml) in distilled water at pH 7·0 at 5° exhibited the greatest storage stability (Tables 1 and 2).

Plasma solutions of the antibiotic held at 37° were stable for 7 days. After this period of time, the plasma became cloudy and further spectral analysis was not feasible.

Conclusion

For the preservation of stability of actinomycin D in solution, it is suggested that actinomycin D be solubilised in refrigerated sterile distilled water and stored at refrigeration temperature. The suggested time of storage for optimal stability would be not more than 120 days.

Solutions of actinomycin D should not be sterilised by autoclaving.

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J. Pharm. Pharmacol., 1964, **16**, 433

Letters to the Editor

The influence of spectral slit width on the absorption of visible or ultra-violet light by Pharmacopoeial substances

SIR,—For accurate results in spectrophotometric assays, the spectral slit width of the spectrophotometer must be small in comparison with the half width of the absorption band, and for a number of substances official in the B.P. 1958 particular care is needed to avoid spuriously low results (Rogers, 1959). Andersen (1964) has measured the slit-width effect at wavelengths of maximum

TABLE 1. EFFECT OF CHANGE OF SPECTRAL SLIT WIDTH ON THE SPECTROPHOTOMETRIC DETERMINATION OF EXTINCTION

Substance	λmax (mμ)	Max *h* (mμ) for extinction error of < 0·2 %	1 %	2
Acetazolamide	265	1·9		
Apomorphine hydrochloride	273	0·8	1·6	
Benztropine methanesulphonate	258	0·5	0·8	1·1
Carbimazole	291	4·0		
Chloramphenicol	278	2·3		
Chlorothiazide	292	1·1	1·6	2·3
Chlorpheniramine	262*	0·5	0·9	1·3
	265†	1·1	1·6	
Chlorpromazine hydrochloride	254	1·2		
Colchicine	350	1·7	4·0	
Cyanocobalamin	361	1·5	1·8	2·3
Cyclomethycaine sulphate	261	1·4		
Cycloserine	219	0·8		
Deoxycortone trimethylacetate	240	1·3		
Dichlorphenamide	285	0·7	1·1	1·4
Dimethisterone	240	1·3		
Diphenhydramine hydrochloride	258	0·5	0·9	1·1
Ethinyloestradiol	280	1·1	1·8	2·4
Fluoxymesterone	240	1·3		
Griseofulvin	291	0·9	1·6	2·3
Hydrochlorothiazide	273·5	1·5		
Hydrocortisone esters	240	1·4		
Hydroxocobalamin	351	1·3	1·7	2·2
Levorphanol tartrate	279	0·7	1·5	2·1
Mepyramine maleate	316	0·9	2·2	
Methandienone	245	1·4		
Methyltestosterone	240	1·3		
Nandrolone phenylpropionate	240	1·3		
Nitrofurantoin	367	1·7		
Norethandrolone	240	1·3		
Norethisterone	240	1·3		
Oxytetracycline dihydrate and hydrochloride	353	3·0		
Paracetamol	249	1·5		
Perphenazine	254	1·0		
Phenindione	278	0·9	2·3	
Phenoxybenzamine hydrochloride	272	0·8	1·2	2·0
Phenoxymethylpenicillin and salts	268	0·7	0·9	1·1
Phytomenadione	249	0·7	0·9	1·1
Probenecid	248	1·5		
Prochlorperazine salts	258	1·2		
Progesterone	240	1·2		
Promazine hydrochloride	251	1·3		
Promethazine hydrochloride	249	1·2		
Pyridostigmine bromide	269·5	0·9		
Riboflavine	444	0·9		
Sodium anoxynaphthonate	570	1·0		
Testosterone and esters	240	1·2		
Thioridazine hydrochloride	310	1·4	2·5	
Trifluoperazine hydrochloride	256	1·1	1·7	
Tripelennamine hydrochloride	245	1·3		
Tubocurarine chloride	280	1·1	1·7	2·3
Warfarin sodium	308	1·0	2·1	

* Solvent water.
† Solvent 0·5 N sulphuric acid.
Solutions were prepared as directed by the B.P. 1963.

and minimum ultra-violet absorption of potassium phenoxymethylpenicillin, prednisolone and yohimbine hydrochloride, and confirmed that the effect is greater, the narrower the absorption maximum.

The earlier survey has now been extended to those substances that are subject to spectrophotometric assay in the B.P. 1963. The same experimental procedure has been used as before (Rogers, 1959), except that the spectrophotometers on this occasion were a Hilger and Watts Uvispek H.700 Mark VII and a Unicam SP.500. Table 1 lists the drugs examined and shows in the third column the widest half-intensity spectral slit width h that may safely be used. For convenience, substances that are subject to spectrophotometric assay in both the 1958 and 1963 editions of the B.P. have been included in the Table, as well as the newer substances, and the opportunity has been taken to give revised, more accurate values for one or two drugs.

Examination of the Table shows that the greatest care should be taken with those substances that show the vibrational structure of the benzenoid absorption near 255 mμ, namely apomorphine hydrochloride, benztropine methanesulphonate, chlorpheniramine maleate, dichlorphenamide, diphenhydramine hydrochloride, levorphanol tartrate, phenindione, phenoxybenzamine hydrochloride, phenoxymethylpenicillin and its salts, and phytomenadione.

The chlorpheniramine assays show a feature of some interest. With the tablets, the final solution is acid; there is little vibrational structure in the spectrum, and the slits can be opened quite widely before low results will be obtained. With the injection, however, the solution is near neutrality, and because the vibrational structure is marked in the spectrum, the slit-width setting of the spectrophotometer is critical. It would seem desirable to use an acid solvent for this assay also.

The avoidance of slit-width errors in assays may be secured (*a*) by specification of a maximum permitted half-intensity spectral slit width h (Rogers, 1959), (*b*) by requiring that "the instrumental slit width used should always be such that a further reduction does not result in an increased extinction reading," as in Appendix IV.H of the B.P. 1963, or (*c*) by adoption of a procedure in which the extinction of the sample is compared with that of a reference substance under the same conditions (Anderson, 1964). A combination of (*b*) and (*c*) would seem to be the most reliable.

Acknowledgements. I thank the Chemistry Department of this College for use of a Unicam spectrophotometer, and the manufacturers of the drugs listed for supplying samples for examination.

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April 21, 1964

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Inhibition of noradrenaline uptake by sympathomimetic amines

SIR,—The uptake of noradrenaline into tissues innervated by the sympathetic nervous system can be inhibited by a wide variety of drugs (Whitby, Hertting & Axelrod, 1960; Axelrod, Whitby & Hertting, 1961; Hertting, Axelrod & Whitby, 1961; Dengler, Spiegel & Titus, 1961; Axelrod, Hertting & Potter, 1962). Some of these inhibitors such as the sympathomimetic amines tyramine, amphetamine and ephedrine are closely related chemically to noradrenaline, and the present study was undertaken as a systematic and quantitative investigation of the inhibition of noradrenaline uptake by these and other chemically related amines to elucidate the structural specificity of the noradrenaline uptake site.

A simple test procedure has been designed for this purpose. Rat hearts were perfused by the Langendorff technique with a medium containing DL-β-^{14}C-noradrenaline (specific activity = 130 μc/mg) at a concentration of 10 ng/ml. The uptake of noradrenaline was measured by assaying the tissue content of ^{14}C-noradrenaline at the end of a 10 min perfusion. The methods used for measuring the uptake of radioactively-labelled noradrenaline in this preparation have been described in detail elsewhere (Iversen, 1963). Drugs were added

TABLE 1. INHIBITION OF NORADRENALINE UPTAKE BY SYMPATHOMIMETIC AMINES IN THE ISOLATED RAT HEART

Drug	ID50 (Drug concentration (M) producing 50% inhibition of noradrenaline uptake)	Relative affinity for uptake site (β-phenylethylamine = 100)
(−)-Metaraminol	7.6×10^{-8}	1440
Dopamine	1.7×10^{-7}	650
(±)-α-Methyldopamine	1.8×10^{-7}	610
(±)-Amphetamine	1.8×10^{-7}	610
(±)-Hydroxyamphetamine	1.8×10^{-7}	610
(−)-Nordefrin (Corbasil)	2.0×10^{-7}	550
(−)-Noradrenaline	3.3×10^{-7}	330
(±)-Nordefrin	4.2×10^{-7}	260
Tyramine	4.5×10^{-7}	245
(±)-Amphetamine	4.6×10^{-7}	240
m-Tyramine	5.1×10^{-7}	215
(+)-Methamphetamine	6.7×10^{-7}	165
(±)-Noradrenaline	7.3×10^{-7}	150
N-Methyldopamine (Epinine)	7.6×10^{-7}	145
(±)-Buphenine	8.5×10^{-7}	130
(±)-Propylhexedrine	8.5×10^{-7}	130
(−)-Adrenaline	1.0×10^{-6}	110
Mephentermine	1.0×10^{-6}	110
β-Phenylethylamine	1.1×10^{-6}	100
(+)-Noradrenaline	1.3×10^{-6}	85
(±)-Octopamine	1.3×10^{-6}	85
(±)-Cyclopentamine	1.4×10^{-6}	78
Noradrenalone	1.5×10^{-6}	75
(±)-Adrenaline	1.6×10^{-6}	70
(±)-Phenylpropanolamine	2.0×10^{-6}	55
(−)-Ephedrine	2.2×10^{-6}	50
Hordenine	2.5×10^{-6}	45
(−)-Amphetamine	3.7×10^{-6}	30
(±)-Phenylethanolamine	4.8×10^{-6}	23
(−)-Phenylephrine	5.6×10^{-6}	20
1-Methylhexylamine (Tuamine)	5.6×10^{-6}	20
(±)-*N*-Ethylnoradrenaline	9.2×10^{-6}	12
p-Methoxyphenylethylamine	1.0×10^{-5}	11
(±)-Methoxyphenamine	1.1×10^{-5}	10
(±)-Oxedrine	1.2×10^{-5}	9
(±)-Isoprenaline	2.5×10^{-6}	4·5
(±)-*N*-n-Butylnoradrenaline	3.5×10^{-5}	3·2
(±)-*N*-Isobutylnoradrenaline	4.0×10^{-5}	2·6
(−)-DOPA	6.0×10^{-5}	1·2
(±)-3-Methoxynoradrenaline	2.0×10^{-4}	0·55
3,4-Dimethoxyphenylethylamine	2.0×10^{-4}	0·55
(±)-Methoxamine	1.0×10^{-3}	0·11
Mescaline	1.5×10^{-2}	0·007

to the perfusing medium at various concentrations to determine the drug concentration which produced a 50% inhibition of the control noradrenaline uptake. This drug concentration (ID50) was taken as a reciprocal measure of the affinity of the drug molecule for the noradrenaline uptake site, and relative affinities were calculated and expressed on an arbitrary scale on which the wholly unsubstituted parent molecule β-phenylethylamine was assigned a value of 100. Two to four drug concentrations were used, each concentration was tested on a group of four hearts and the mean inhibition for the group was used in the calculation of the ID50. It should be emphasised that this test procedure does not distinguish between substances that inhibit noradrenaline uptake because they are themselves transported, and non-transported inhibitors, it should therefore be regarded as a screening test.

The results obtained with 43 drugs are presented in Table 1. All the substances tested showed some activity as inhibitors of noradrenaline uptake. From these results the following preliminary conclusions have been drawn concerning the structural specificity of the noradrenaline uptake site in the rat heart with β-phenylethylamine as the reference molecule.

1. *N*-Substitution decreases affinity; the optimal chemical structure seems to be a primary amine group.

2. β-Hydroxylation decreases affinity; in compounds with a β-hydroxyl group the (−)-isomer has a higher affinity than the (+)-isomer.

3. α-Methylation increases affinity; in compounds with an α-methyl group the (+)-isomer has a much higher affinity than the (−)-isomer.

4. Phenolic hydroxyl groups increase affinity.

5. *O*-Methylation of phenolic hydroxyl groups leads to a very striking decrease in affinity.

6. The aromatic ring is not an essential structural feature and can be replaced by saturated ring structures or even by a branched aliphatic chain without great change in activity.

The finding that many sympathomimetic amines are extremely potent inhibitors of noradrenaline uptake suggests that at least part of their pharmacological activity may be related to this property. For example, the ability of tyramine to prevent the normal inactivation of noradrenaline by tissue uptake could explain the reported sensitisation to noradrenaline produced by this compound (Lindmar & Muscholl, 1961; Furchgott, Kirpekar, Rieker & Schwab, 1963).

Acknowledgements. I am grateful to the Medical Research Council for a Scholarship and for a grant for animals and materials, to Professor A. S. V. Burgen for much helpful advice and discussion, and to the many firms who have contributed drug samples so generously.

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April 20, 1964

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Pharmacological properties and mechanism of action of atractyloside

SIR,—During the past eight years, we have investigated the biochemical and pharmacological properties of a natural glycoside, atractyloside, extracted from the rhizome of a Mediterranean member of the Compositae, *Atractylis gummifera* L. and much information has been obtained.

The toxic and therapeutic effects of this natural drug were well known in classical times, and are referred to in Dioscorides' *De Materia Medica* under the name of "Chamaeleon". Caccialanza & Landi (1960–1961) have confirmed that the use of this drug in skin diseases, as practiced in antiquity, was fully justified.

The pharmacological action of the drug can be attributed to a glycoside formed by an aglycone which has the structure of a perhydrophenanthrene, one molecule of glucose, two of potassium sulphate, and one of isovaleric acid (Ajello, Piozzi, Quilico & Sprio, 1963). The relation between chemical structure and pharmacological action of the drug is of interest (Santi, Bruni, Contessa & Luciani, 1962). We now summarise the results of our *in vivo* and *in vitro* investigations.

In rats, dogs, mice and rabbits atractyloside induces hypoglycaemia, usually preceded by a phase of hyperglycaemia, depletion of muscular and hepatic glycogen reserves (myocardial glycogen remains unchanged or is even slightly increased), and inhibition of glycogen synthesis. An increase in the blood lactic acid level and a decrease in oxygen consumption have also been observed (see Santi, 1958).

The effect elicited *in vivo* can be explained by the finding that the drug is a powerful inhibitor of oxidative phosphorylation in isolated mitochondria and in rat liver homogenate (see Santi, 1958). On this basis it may be presumed that the hypoglycaemic effect is connected with an inhibition *in vivo* of the Pasteur effect. The inhibition of glycogen synthesis as well as the inhibition of oxygen uptake and the increase in blood lactic acid are the consequence of the marked reduction in cellular oxidative and phosphorylation reactions. Subsequently, Bruni, Contessa & Luciani (1962) and Bruni & Luciani (1962) have shown that the atractyloside-induced inhibition of mitochondrial oxygen uptake is reversed by the uncoupling effect of 2,4-dinitrophenol, and in general by conditions in which the respiratory processes are not controlled by phosphorylation. Among the partial reactions of the coupling system, 2-4-dinitrophenol-stimulated adenosine-triphosphatase (ATPase), ATP-inorganic phosphate, and ATP-ADP exchanges are markedly inhibited by atractyloside, whereas Mg^{2+}-stimulated ATPase is influenced only if the activity is elicited by a partial damage of mitochondria; no effect whatever is induced if mitochondrial structure is severely disorganised.

These results have led us to conclude that atractyloside inhibits oxidative phosphorylation by a process of specific interference with energy transfer reactions.

Recently, the analysis of the mechanism of action of atractyloside on mitochondrial energy transfer has been brought a step forward by the finding

that a competitive inhibition can be shown to exist between atractyloside and ADP. These studies have led to the conclusion that atractyloside acts on the terminal phase of energy transfer, the phase in which a high-energy phosphorylated intermediate, originating from inorganic phosphate and the energy-conserving reaction of the respiratory chain, forms ATP from ADP. This assumption is supported by the following observations (Bruni, Luciani & Contessa, 1964; Bruni, Luciani, Contessa & Azzone, 1964): first, atractyloside specifically inhibits the binding of ADP and ATP to liver mitochondria; second, in contrast to the inhibition induced in respiration stimulated by inorganic phosphate (P_i) and P_i-acceptor, atractyloside does not affect arsenate-stimulated respiration, which must be ascribed to the splitting of the unstable arsenilated compound formed instead of a stable phosphorylated analogue; thus, atractyloside does not interfere with the entry of As (or of P_i) into energy transfer reactions. Third, atractyloside inhibits the substrate level phosphorylation linked to the oxidation of α-ketoglutarate. It is interesting to note that the energy transfer inhibitor oligomicin (Lardy, Johnson & McMurray, 1958), which acts on the interaction of P_i with the coupling system, inhibits arsenate-induced respiration but does not affect substrate level phosphorylation.

The lack of sensitivity to atractyloside of Mg^{2+}-stimulated ATPase in severely damaged mitochondria does not invalidate this hypothesis, since these mitochondrial preparations have lost the capacity to bind atractyloside. Analysis of atractyloside effect on oxidative phosphorylation, ATPase stimulated by 2,4-dinitrophenol or by low concentrations of sodium deoxycholate shows a competitive removal of inhibition by increasing the amounts of adenine nucleotides in the reactions involving both ADP and ATP. From these results it was concluded that the mechanism of the inhibitory effect on the terminal step of the energy transfer process, may be explained by admitting a competitive inhibition between adenine nucleotides and atractyloside on the same mitochondrial receptor site.

It seems reasonable to expect that atractyloside will acquire more importance as a biochemical tool because the most prominent feature of its action is selective inhibition of phosphorylation.

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Influence of drugs upon $^{47}Ca^{2+}$ uptake in depolarised intestinal smooth muscle

SIR,—Acetylcholine (10 μg/ml) increases $^{45}Ca^{2+}$ uptake by depolarised smooth muscle (Robertson, 1960), and carbachol (0·3 μg/ml) acts similarly in the depolarised guinea-pig taenia coli (Durbin & Jenkinson, 1961a). In frog skeletal muscle, contracture-producing drugs increase both uptake and release of $^{47}Ca^{2+}$ (Ahmad & Lewis, 1962) and in non-depolarised smooth muscle acetylcholine, carbachol, 5-hydroxytryptamine and barium chloride increase $^{47}Ca^{2+}$ uptake, but papaverine, adrenaline and histamine do not alter the inward flow of $^{47}Ca^{2+}$ although papaverine decreases $^{42}K^{+}$ influx (Banerjee & Lewis, 1963, 1964).

We have studied a group of drugs which stimulate or relax smooth muscle, using the longitudinal muscle of the guinea-pig ileum depolarised in potassium-rich Krebs solution (Durbin & Jenkinson, 1961a,b). In this preparation it is possible to observe effects upon $^{47}Ca^{2+}$ uptake in conditions in which the marked mechanical changes, associated electrical changes and alterations in ion flux seen in normal Krebs solution are absent.

The technique used was based on that of Durbin & Jenkinson (1961a). 10 cm long sections of terminal ileum were removed from freshly killed guinea-pigs. Extraneous tissues were removed, the ileum gently pulled on to a glass rod and an incision made along the attachment of the mesentery. The longitudinal muscle layer was gently removed from the circular layer, the process being carried out under the surface of Krebs solution. For each series of experiments, 4 pieces each 2 cm long were taken from the same length of longitudinal muscle. Adjacent pieces served respectively as test and control preparations. Each strip was stretched with an 0·2 g weight and incubated for 30 min at 37° in non-radioactive Krebs solution. It was then kept for not less than 2 hr in potassium-rich Krebs solution at 37°. Each strip was now exposed for 2–3 min to radioactive potassium-rich Krebs solution. Drug or control solution was added and exposure continued for a further 7–8 min. The tissues were then washed for 3 periods of 30 sec with a stream of non-radioactive, potassium-rich Krebs solution and counted for 100 sec using a thallium-activated sodium iodide crystal scintillation counter (EKCO type N.597). 22 to 24 pairs of strips were used for each drug. The number of counts taken up per mg of tissue during the period of exposure was calculated and the difference between drug and control tested for significance by Student's "*t*" test. The results are shown in Table 1.

TABLE 1. UPTAKE OF $^{47}Ca^{2+}$ BY ISOLATED DEPOLARISED STRIPS OF THE LONGITUDINAL MUSCLE OF THE GUINEA-PIG ILEUM IN RESPONSE TO DRUGS

Drug	Dose (μg/ml)	No. of pairs	Mechanical Response	Effect on $^{47}Ca^{2+}$ uptake
Acetylcholine chloride	10	22	Contraction	Increased $0{\cdot}05 < P < 0{\cdot}10$
Adrenaline hydrogen tartrate	10	23	No change	No change $0{\cdot}60 < P < 0{\cdot}70$
Carbachol	0·3	24	Contraction	Increased $0{\cdot}01 < P < 0{\cdot}02$
Histamine acid phosphate	2	24	Small contraction	No change $0{\cdot}10 < P < 0{\cdot}20$
5-Hydroxytryptamine creatinine sulphate	50	24	Small contraction	Increased $0{\cdot}05 < P < 0{\cdot}10$
Papaverine sulphate	10	24	Relaxation	Decreased $P < 0{\cdot}001$

Carbachol and acetylcholine significantly increased $^{47}Ca^{2+}$ uptake, thus confirming the results of Durbin & Jenkinson (1961a,b) and Robertson (1960) respectively. Histamine and adrenaline caused no significant change, but 5-hydroxytryptamine significantly increased $^{47}Ca^{2+}$ uptake. The highly significant decrease in uptake induced by papaverine is of considerable interest because, at the dose level used, this drug relaxes depolarised smooth muscle. Papaverine does not, however, depress $^{47}Ca^{2+}$ uptake in non-depolarised ileum (Banerjee & Lewis, 1963). Failure of adrenaline to alter $^{47}Ca^{2+}$ uptake significantly may be linked with its failure to relax the depolarised tissue and points to adrenaline and papaverine exerting their effects by different mechanisms. Calcium is probably essential in greater amounts for maintained contraction than is required for relaxation and during relaxation calcium permeability is much reduced. This effect underlines the importance of calcium ions for the contraction of smooth muscle.

We thank Dr J. F. Lamb for many helpful discussions.

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April 11, 1964

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PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.

Journal of Pharmacy and Pharmacology

JUNE 1964 VOL. 16. No. 6

Research Papers

Letters to the Editor