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Research Papers

Effects of tyramine on a spinal reflex in the anaesthetised chick

W. C. BOWMAN, B. A. CALLINGHAM AND G. OSUIDE

The effects of tyramine on polysynaptic spinal reflexes in anaesthetised chicks and cats have been studied. Very large intravenous doses of tyramine depressed the reflexly evoked contractions of skeletal muscles by an action exerted in the spinal cord. Since tyramine is normally present in the central nervous system, its function may be to exert some inhibitory controlling influence on muscular activity.

THE presence, in mammalian tissues, of an enzyme capable of catalysing the decarboxylation of tyrosine to tyramine was demonstrated by Lovenberg, Weissbach & Udenfriend (1962); tyramine is normally present in the urine of man, the amount excreted being about 0·2–0·5 mg/day (Levine, Oates, Vendsala & Sjoerdsma, 1962). By a combination of solvent extraction and fluorimetric assay, Spector, Melmon, Lovenberg & Sjoerdsma (1963) examined mammalian tissues for the presence of tyramine. They concluded that the amine is present in amounts of 1 to 6 μg/g in various parts of the central nervous system of rats, rabbits, dogs and cats, the highest concentrations being in the brain stem and spinal cord. Spector and others also mention some experiments in which they found that after convulsive doses of strychnine, the tyramine level in the spinal cord of rabbits was decreased.

These results suggest that tyramine may, either directly or indirectly, influence the motoneurones innervating skeletal muscles. However, in preliminary experiments on the cat, intravenously injected tyramine, in doses up to 2 mg/kg, was found to be without effect on spinal reflexes.

It was considered that the inactivity of intravenous tyramine in the mammal might be because it cannot penetrate from the blood stream to its possible site of action in the central nervous system. Studies by Zaimis (1960) and Key & Marley (1962) have suggested that the blood-brain barrier, at least with respect to some drugs, is defective in the domestic fowl during the first few days after hatching and for this reason it was decided to study the effects of tyramine on a spinal reflex evoked in young chicks.

Methods

The experiments were made on 84 male chicks (Silver Link) ranging in age from 1 to 15 days after hatching. The chicks were anaesthetised with chloralose (60 mg/kg) injected intraperitoneally. The chick was laid on its back, the trachea cannulated and artificial respiration commenced immediately. A stout pin was placed through the lower end of the femur of the right leg and firmly clamped so that the lower leg could

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swing freely. One end of a thread was tied to the ankle and the other end was attached, via a pulley-wheel, to a light spring-loaded writing lever. Extension of the limb caused the lever to write on a kymograph. Crossed extensor reflexes were elicited once every 15 or 20 sec by stimulation of the peripheral end of the central stump of the severed contralateral sciatic nerve with single rectangular shocks of 0·5–1 msec duration. Injections were made, in a volume not exceeding 0·2 ml, through a fine polythene cannula tied into a jugular vein. In many experiments, the sciatic artery of the left leg was also cannulated and arterial blood pressure was recorded by means of a Condon manometer. Twelve chicks were treated with 1 mg/kg of reserpine injected subcutaneously on each of the first and second days after hatching. Reflex contractions

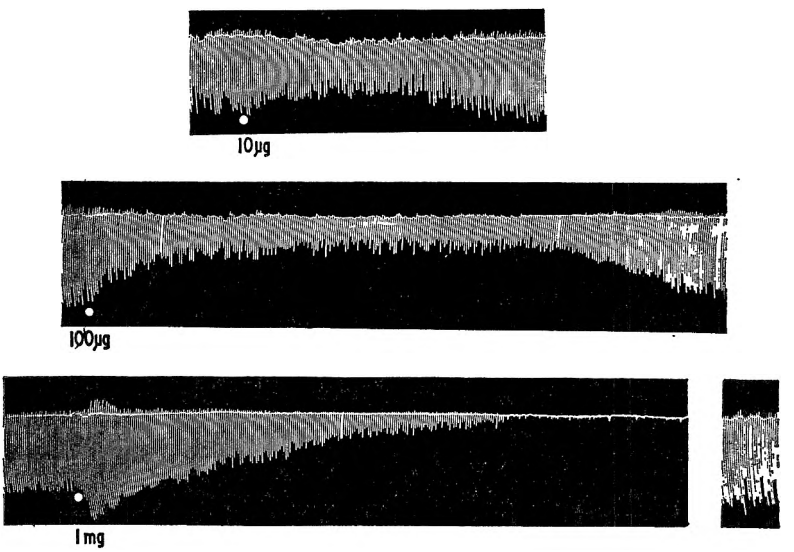

FIG. 1. Chick aged 2 days and weighing 32 g. Crossed extensor reflex elicited every 15 sec; contractions downwards. At the white dots tyramine was injected intravenously in the absolute doses indicated. The second part of the lowest record was recorded 20 min after the end of the first part.

were recorded on the third day. Twenty-four chicks, aged between 1 and 6 days, were decapitated during the experiment. The head was severed after tightly ligating the neck excluding the trachea and the cannulated jugular vein. Ten chicks were spinalised during the experiment by sectioning the cord at the level of C 10 between two ligatures tied tightly round the spinal column and adhering muscles.

In some experiments contractions of the gastrocnemius muscle were recorded in response to motor nerve stimulation. In these experiments, a pin was placed through the lower end of the femur and another through the lower end of the tibia, so that the lower limb could be clamped in a horizontal position. The tendon of the gastrocnemius muscle was attached, via a pulley wheel, to the writing lever and maximal twitches

were elicited by stimulation of the peripheral stump of the severed ipsilateral sciatic nerve with single rectangular shocks of 100 μsec duration.

Similar experiments were made on three cats under chloralose anaesthesia (80 mg/kg injected intravenously). The method was identical to that previously described (Bowman & Sanghvi, 1963). Flexor reflex contractions of a tibialis anterior muscle and crossed-extensor reflexes of a quadriceps femoris muscle were recorded. The reflex contractions of the tibialis anterior muscle were elicited by stimulation of the ipsilateral musculo-cutaneous branch of the peroneal nerve with single rectangular shocks of 0·5 msec duration. The reflex contractions of the quadriceps muscle were elicited as in chicks but with 0·5 sec bursts of repetitive stimulation at a frequency of 10/sec. Drugs were injected intravenously through a cannula in a jugular vein and arterial blood pressure was recorded from a common carotid artery.

The drugs used were tyramine hydrochloride (BDH), 3-hydroxytyramine hydrochloride (dopamine, BDH), noradrenaline bitartrate (BDH), strychnine nitrate (BDH), reserpine (Serpasil ampoules, Ciba) and nialamide (Pfizer).

Results

Intravenously injected tyramine always depressed the crossed extensor reflex in chicks but the effective doses and the duration of the depression varied in different experiments. Fig. 1 illustrates an experiment on a 2 days-old chick in which tyramine was unusually effective. A dose of 10 μg produced a definite depression of the contractions and a dose of 1 mg completely abolished them after an initial small potentiation. In this experiment the time from injection to maximal depression was longest with the largest dose possibly because, with the brain intact, the effect of large doses of tyramine was the result of both a stimulant and a depressant action in the central nervous system.

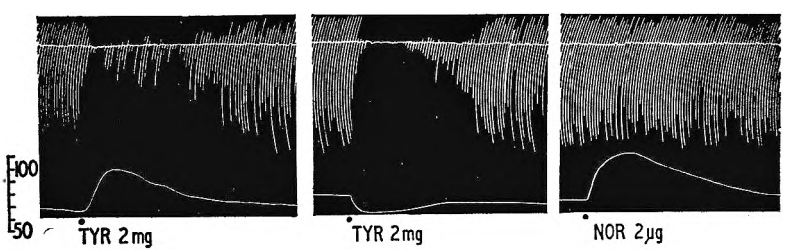

FIG. 2. Chick aged 2 days and weighing 35 g. Crossed extensor reflex (upper record) recorded as in Fig. 1. Blood pressure (lower record) recorded from the sciatic artery (calibration in mm Hg). At TYR, tyramine and at NOR, noradrenaline injected intravenously in the absolute doses indicated. The first two panels show the first and the fourth injection of the same dose of tyramine.

Fig. 2 illustrates an experiment, again on a 2 days-old chick, in which the effective doses of tyramine lay at the other extreme of the dose-range. In this experiment an intravenous dose of 2 mg of tyramine produced

an immediate but relatively short-lasting depression of the contractions. In most experiments, including all those in which chicks more than 6 days old were used, large doses of tyramine of about 0·5 mg/10 g body weight were required to depress the reflex contractions. The depression was occasionally preceded by a brief stimulant effect. Even the largest doses required were without effect on maximal twitches of the gastrocnemius muscle elicited by motor nerve stimulation.

At the start of the experiment, the blood pressure of the anaesthetised chick was usually between 70 and 100 mm Hg. During the first hour or so, the blood pressure usually fell gradually, occasionally to as little as 40 mm Hg. However, the amplitude of the reflex contractions was little effected by this fall in blood pressure. Large doses of tyramine (0·2–0·5 mg/10 g body weight) produced rises in blood pressure of some 30 to 60 mm Hg (Figs 2 and 5) which were often preceded by a small fall (Fig. 5). Tachyphylaxis of the pressor effect was usually quickly established when large doses were repeatedly injected. The first panel of Fig. 2 illustrates the effect of an initial large dose of tyramine. The second panel of Fig. 2 illustrates the effect of the fourth injection of the same dose. In contrast to the first injection the fourth injection produced a fall in blood pressure but a similar depression of the reflex contractions was produced. The third panel of Fig. 2 illustrates the absence of effect on the reflex contractions of a dose of noradrenaline which produced a rise in blood pressure greater than that produced by the first injection of tyramine.

In chicks pre-treated with reserpine, the pressor effect of tyramine was weak or absent but a depression of the reflex contractions similar to that occurring in the non-reserpinised chicks was produced.

Decapitation of the chick caused a rise in blood pressure lasting 1–2 min and this was followed by a fall to 30–40 mm Hg where it remained constant, in the absence of drugs, for the remainder of the experiment (Fig. 5). Decapitation always abolished the reflex contractions but this effect was not always immediate, suggesting that it was not simply the result of spinal shock. Fig. 5 illustrates an experiment in which the reflex contractions persisted unchanged for 5–6 min after decapitation but disappeared as the blood pressure fell to 30 mm. However, in other experiments, in which the blood pressure was already low and was not lowered further by decapitation, the reflex contractions disappeared immediately on severing the head. It, therefore, appeared that the abolition of reflex contractions was the combined result of mild spinal shock, produced by decapitation, and of the fall in blood pressure. When left alone, reflex contractions did not re-appear during the rest of the experiment (up to 3 hr after decapitation). However, contractions could always be restored at any time after decapitation by the intravenous injection of small doses of strychnine (1–2 μg/chick). After the initial large potentiation produced by strychnine, the amplitude of the contractions subsided to a lower level where they remained constant for the rest of the experiment. In the experiment of Fig. 5, the contractions remained constant, at the level illustrated at the end of the lower panel,

for a further 2 hr, after which time the experiment was terminated. Tyramine, injected at any time after decapitation, produced a depression of the restored reflex contractions similar to that occurring before decapitation.

When spinalisation was done by sectioning the cord in the cervical region, the fall in blood pressure was less severe than that following decapitation. Reflex contractions were depressed in amplitude but not

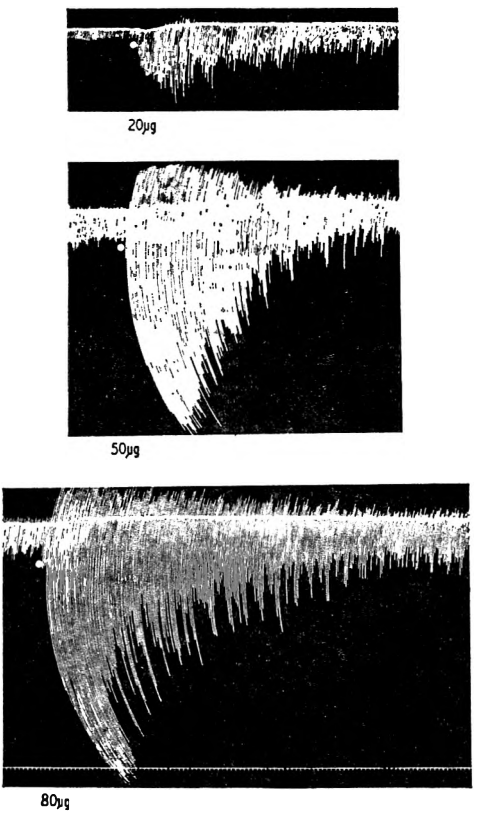

FIG. 3. Chick aged 15 days and weighing 124 g. Recording as in Fig. 1. At the white dots, strychnine injected intravenously in the absolute doses indicated. Time trace on the lowest panel, 0·5 min intervals.

abolished by this procedure, and, in the absence of drugs, they continued at a constant level for the remainder of the experiment. Contractions elicited from these preparations were always more constant in amplitude than those produced when the central nervous system was left intact. Tyramine injected after spinalisation produced a depression of the reflex contractions similar to that occurring before transection of the cord.

Tyramine was also tested for an anti-strychnine action. Fig. 3 illustrates the potentiating effect of three doses of strychnine on the reflex contractions elicited in a 15 days-old chick. On a dose per body weight basis,

strychnine was found to be more active, the younger the chick. In fact the effective doses of strychnine in μg was found to correspond approximately to the age of the chick in days. Thus in a 1 day-old chick, the smallest effective dose of strychnine was about 1–2 μg intravenously while in a 15 days-old chick, 15–20 μg was required. The gain in weight of the chicks over the first 15 days was only 300–500%.

The effect of tyramine on the strychnine potentiation depended on the dose of strychnine and on the time of injection of tyramine. Fig. 4

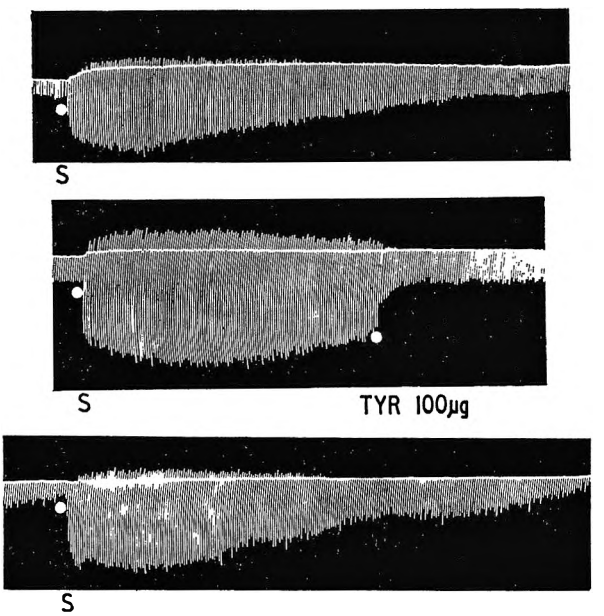

FIG. 4. Chick aged 2 days and weighing 30 g. Recording as in Fig. 1. At S, 2 μg of strychnine and at TYR, 100 μg of tyramine injected intravenously. 35 min elapsed between injections of strychnine.

illustrates an experiment on an intact chick in which 100 μg tyramine, injected towards the end of the potentiation produced by a small dose of strychnine, depressed the contractions to the pre-strychnine level. When the dose of strychnine was larger and when tyramine was injected soon after strychnine, no depressant effect was produced even by doses of tyramine which, before strychnine, were big enough to abolish the reflex contractions completely (Fig. 5, upper panels). When tyramine was injected at the height of the strychnine potentiation in an intact chick, the evoked contractions were often slightly increased rather than depressed (Fig. 5, upper panels) and sometimes rapid additional contractions of the muscle were produced. When tyramine was injected before strychnine, the potentiating effect of strychnine was only slightly depressed.

In the decapitate preparation and the preparation spinalised in the cervical region, tyramine always blocked the reflex contractions in the

presence of strychnine irrespective of the time it was injected. Under these circumstances, the depression of contractions was always preceded by rapid and powerful contractions of the muscle which were not related to the electrical stimulation. Fig. 5 illustrates an experiment in which, in the intact preparation, tyramine depressed the normal reflex contractions but did not depress potentiation produced by strychnine. However, after decapitation, the same dose of tyramine abolished the effect of strychnine after an initial short-lasting stimulant effect.

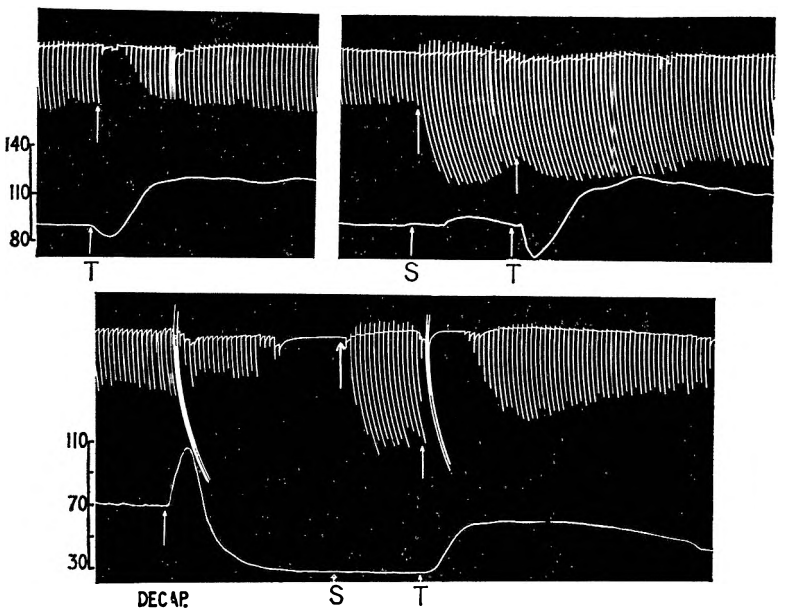

FIG. 5. Chick aged 4 days and weighing 63 g. Recording as in Fig. 2. Crossed-extensor reflexes elicited once every 20 sec. At T, 1 mg tyramine and at S, 5 μg strychnine injected intravenously. At DECAP, the head was completely severed.

In a few spinalised preparations, the effect of dopamine was tested. In similar doses, dopamine produced greater pressor effects than tyramine but it did not depress the reflex contractions either in the presence or in the absence of strychnine. When the blood pressure of the chick was very low, the pressor effect of dopamine was often accompanied by a small increase in the amplitude of the contractions.

The depressant action of tyramine, both in the presence and in the absence of strychnine, was potentiated by the previous injection of the monoamine oxidase inhibitor, nialamide (100 μg/10 g weight). After nialamide, the depressant effect of tyramine (0·25 mg/10 g weight) was occasionally permanent, no recovery of the reflex contractions occurring throughout the remainder of the experiment.

The doses of tyramine required to affect the reflex contractions in the chick were extremely large. For example, doses of 1–2 mg in a 2 days-old

chick weighing about 30 g are equivalent, on a body weight basis, to doses of about 100–200 mg in an average sized cat. Since the chick could withstand repeated doses of this magnitude, it was decided to test the effect of equivalent doses in cats.

In the cat, intravenous doses of about 50 mg/kg caused a depression of both the crossed extensor and the flexor reflex contractions from which recovery was slow and incomplete. Fig. 6 illustrates this effect on the flexor reflex and shows that the depression was preceded by a powerful but short-lasting stimulant effect. Although the evoked reflex contractions were completely abolished, contractions of the muscle, unrelated to the electrical stimulation, occurred intermittently after the injection of tyramine. At the height of the block of the flexor reflex, stimulation of the motor nerve still evoked maximal twitches of the tibialis anterior muscle. A stimulant action of large doses of tyramine was also evident in what appeared to be an analeptic effect. Immediately after injection, respiratory rate and depth were increased and side to side head movements occurred. This effect lasted for about 5 min after injection.

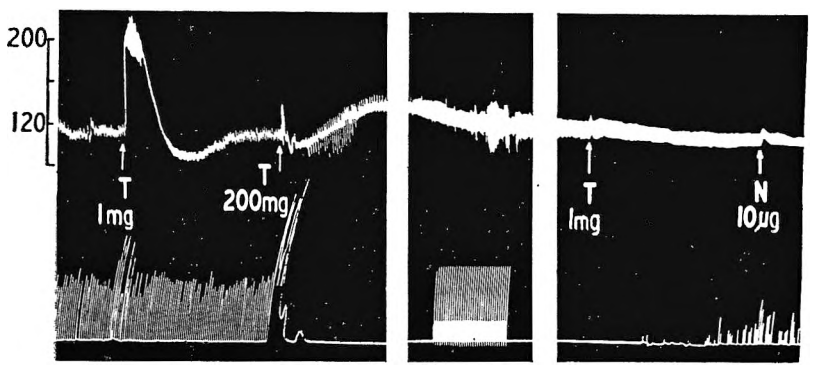

FIG. 6. Adult cat weighing 4 kg. Flexor reflex contractions of a tibialis anterior muscle elicited once every 10 sec (contractions upwards) and blood pressure from a carotid artery (calibration in mm Hg). At T, tyramine and at N, noradrenaline injected intravenously in the absolute doses shown. The middle panel shows maximal twitches of the tibialis anterior muscle elicited by stimulation of the sciatic nerve once every 10 sec at a time when the reflex contractions were completely abolished. The gaps between panels represent intervals of about 15 min. The recovery of the reflex contractions shown at the end of the third panel was the maximal degree of recovery which occurred.

The effects of large doses of tyramine on the blood pressure of the cat were surprisingly small. An intravenous injection of 50 mg/kg caused a small and transient rise after which the blood pressure quickly returned to normal. The effect of 50 mg/kg was actually smaller than the effect of 0·25 mg/kg given previously (Fig. 6). After the large dose of tyramine, subsequent doses of the same drug, and of noradrenaline, were completely without effect on the blood pressure (Fig. 6).

The effect of tyramine in the cat was therefore similar to its effect in the chick. However, the cat was less able than the chick to withstand repeated large doses of tyramine. In two of the three experiments on cats, a second large dose of tyramine caused a profound fall in blood pressure, cessation of respiration and death.

Discussion

The depressant effect of tyramine on the polysynaptic reflexes was most probably exerted in the central nervous system since the amine did not depress twitches of skeletal muscle elicited by direct motor nerve stimulation. Since the same effect was produced in the spinalised preparations, the site of action may be located in the spinal cord. The effect did not appear to be a consequence of cardiovascular changes for the following reasons: (1) it still occurred when complete tachyphylaxis to the cardiovascular effects had been established; (2) doses of noradrenaline or dopamine which produced equal or greater rises in blood pressure did not depress the reflex contractions; (3) in the reserpinised chick, tyramine produced very little change in blood pressure but still depressed the reflex contractions. Spectrophotofluorimetric assays made in this laboratory by Callingham, Cass & Osuide of the brains and hearts of chicks treated with the same doses of reserpine have shown a 50 to 75% depletion of catecholamine content. This suggests that, unlike its peripheral actions (Burn & Rand, 1958), the central actions of tyramine are not mediated through noradrenaline release.

The central effects of tyramine were produced in the adult cat by doses equivalent in size to those found effective in the chick. In both species the effective doses were very large suggesting that the particular barrier to the site of action of tyramine is as well developed in the chick as it is in the cat. This does not contradict the general statement that the blood-brain barrier in the chick is under-developed, since it is clear from the results of Zaimis (1960) and Key & Marley (1962) that sympathomimetic amines do penetrate well into some areas of the central nervous system of the chick.

Since tyramine has been found in the central nervous system, and since it exerts a depressant action on polysynaptic reflex activity, its function may be to exert an inhibitory modulating influence on synaptic activity. Although the intravenous doses of tyramine required were large, the amine might prove very active if it could be applied directly to the site of the depressant action. We have not yet made any real attempt to analyse the site and mechanism of action of tyramine. However, it did not appear to inhibit the anterior horn cells directly since, at the height of the block of the evoked contractions, tyramine occasionally initiated irregular contractions of the muscle which were unrelated to the electrical stimulation.

The depressant action of tyramine was antagonised by strychnine. The convulsive action of strychnine is due to interruption of transmission at inhibitory synapses, probably by competitive blockade of the inhibitory

transmitter (Eccles, Fatt & Koketsu, 1954; Curtis, 1959, 1962; Eccles, 1964). However, since tyramine bears little chemical relationship to strychnine, it seems unlikely that both should act on the same receptor. Furthermore, the finding of Spector & others (1963), that the tyramine content of the central nervous system is depleted by strychnine, is difficult to reconcile with the possibility that tyramine is released from the nerve endings at inhibitory synapses. It may be, however, that when inhibitory transmitter is blocked by strychnine, the unchecked excitation activates some mechanism which causes the release of tyramine. The tyramine may then be exposed to destruction by amine oxidase and its concentration in the CNS depleted. It might be that tyramine acts, not by a direct depressant action, but by stimulating inhibitory neurones. Signs of excitation followed the injection of tyramine and this may be a reflection of a predominantly stimulant action.

The depressant effect of intravenously injected tyramine on the polysynaptic flexor and crossed-extensor reflexes appeared similar to the effect of intravenously injected adrenaline (Schweitzer & Wright, 1937) and topically applied dopamine (McLennan, 1961) on the monosynaptic knee-jerk in the cat. By intravenous injection, dopamine was relatively very weakly active both in the cat (McLennan, 1961) and in the chick. However, it seems likely that the two closely related amines, tyramine and dopamine, may act at the same site.

The finding that strychnine was fully effective in the chick on the first day after hatching suggests that inhibitory mechanisms in the CNS are already well developed at this early age. The chick therefore differs from the kitten in which inhibitory processes develop slowly and in which strychnine is without effect up to 3 to 6 days after birth (Malcolm, 1955). The fact that strychnine was most potent in the youngest chicks and gradually became less so with increase in age, may reflect the gradual development of the blood-brain barrier. Even in the youngest chicks, however, strychnine was less potent on a body weight basis than it is in the adult cat probably because inhibitory mechanisms in the fowl never become as well developed as in the mammal.

The weak and transient effects of huge doses of tyramine on the blood pressure, particularly in the cat, seemed to be due to a self-blocking action since the pressor effect of subsequent doses of tyramine and noradrenaline were abolished. We did not test the effect of non-sympathomimetic pressor agents, so we cannot say whether the blocking action of large doses of tyramine was due to a specific α-receptor blocking action or to a general impairment of the reactivity of the cardiovascular system. It is possible that this self-blocking action of tyramine contributes to the development of tachyphylaxis to this amine when a series of smaller doses is injected. Spriggs (1964) has recently reached a similar conclusion from experiments on rats.

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Release of phosphorus-32-containing compounds from *Micrococcus lysodeikticus* treated with chlorhexidine

R. M. RYE AND DAVID WISEMAN

Chlorhexidine has been shown to cause the release of cellular constituents from phosphorus-32 labelled cells of *Micrococcus lysodeikticus* suspended in distilled water or in phosphate buffer. An initial rapid release is followed by a slower secondary release. This secondary release is inhibited by high concentrations of chlorhexidine. The release depends on the chlorhexidine to cell ratio and not on the absolute bactericide concentration.

CHLORHEXIDINE, 1,6-di-(4-chlorophenyldiguanido)hexane, an antibacterial agent, is the most active of a large number of related biguanides which were synthesised (Davies, Francis, Martin, Rose & Swain, 1954; Rose & Swain, 1956). It is bactericidal against a wide range of both Gram-positive and Gram-negative organisms although the Gram-positive organisms are the more sensitive (Calman & Murray, 1956; Lawrence, 1960). Its activity is highly dependent upon its chemical structure, small changes in which cause a marked decrease in activity (Davies & others, 1954).

Experimental

MATERIALS

Chlorhexidine diacetate, Imperial Chemical (Pharmaceuticals) Ltd., Wilmslow, Cheshire.

Sodium phosphate-^{32}P, the Radiochemical Centre, Amersham, Buckinghamshire.

Micrococcus lysodeikticus, (NCTC 2665).

Suspending medium, distilled water or M/15 phosphate buffer pH 7·2.

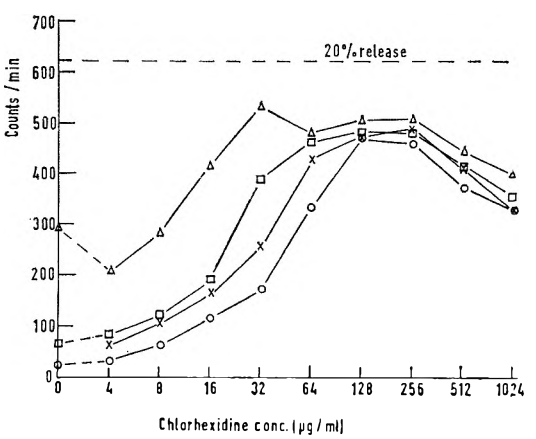

FIG. 1. Release of phosphorus-32 from *Micrococcus lysodeikticus* treated with chlorhexidine. Suspending medium distilled water. Cell concentration $1{\cdot}7 \times 10^{10}$/ml. Contact times: ○ ½ hr, × 2 hr, □ 6 hr, △ 21 hr.

From the Department of Pharmacy, Bradford Institute of Technology

METHODS

Conditions of culture and harvesting. The organisms were grown for 48 hr at 30° on Oxoid tryptone soya agar containing 0·125 μc/ml of sodium phosphate-^{32}P. The cells were harvested, washed three times with the suspending medium and the total count of the final suspension determined by opacity measurements.

Treatment of cells and measurement of released radioactivity. Reaction mixtures were prepared from samples of the bacterial suspensions and equal volumes of suitable dilutions of chlorhexidine dissolved in suspending medium. The mixtures were maintained at room temperature (18–20°).

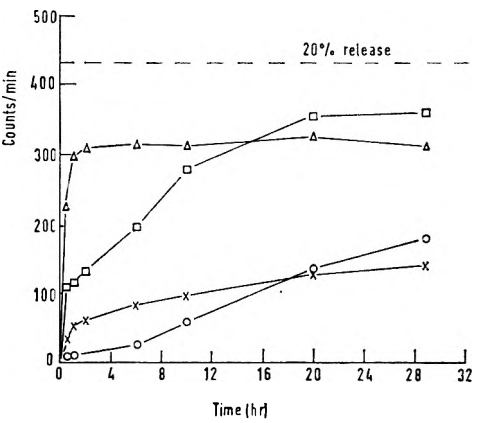

FIG. 2. Rate of release of phosphorus-32 from *Micrococcus lysodeikticus* suspended in distilled water alone and in the presence of chlorhexidine. Cell concentration 1·8 × 10^{10}/ml. ○ Distilled water alone: × 8, □ 32, △ 128 μg chlorhexidine/ml.

5 ml samples were taken from the mixtures after various time intervals, centrifuged at 4,500 rpm for 10 min and the radioactivity of the supernatant liquids measured by counting 3 ml samples using a Mullard MX 124/01 Liquid sample tube. In the experiments with "dilute" bacterial suspensions, 10 ml samples were taken and 7 ml of the supernatant liquid was counted. The total radioactivity of the bacteria was determined by counting 1 ml samples of the original suspensions. Each sample was counted for 15 min which in most cases reduced the random error (P = 68·3%) to below 2% of the observed count.

Results

Release from cells suspended in distilled water. Cells suspended in distilled water alone gradually release compounds containing ^{32}P.

The addition of chlorhexidine causes an initial rapid release during the first hr. The amount released during this period increases with increasing concentrations of chlorhexidine up to a maximum at 128 μg/ml. Concentrations in excess of 256 μg/ml cause less than this maximum release (Fig. 1).

In low concentrations of chlorhexidine, the initial release is followed by a slower secondary release extending over at least 30 hr. A secondary release was not observed in concentrations of chlorhexidine above 128 μg/ml (Fig. 2).

Release from cells suspended in M/15 *phosphate buffer.* Cells suspended in phosphate buffer alone show virtually no release over 30 hr. In the presence of chlorhexidine, the initial release is similar to that obtained with cells treated in distilled water. The subsequent secondary release however, differs from that occurring in water. This release is small with concentrations of chlorhexidine up to 8 μg/ml but with concentrations between 16 and 32 μg/ml, the secondary release is much greater and a peak in the release curve is observed (Figs 3 and 4).

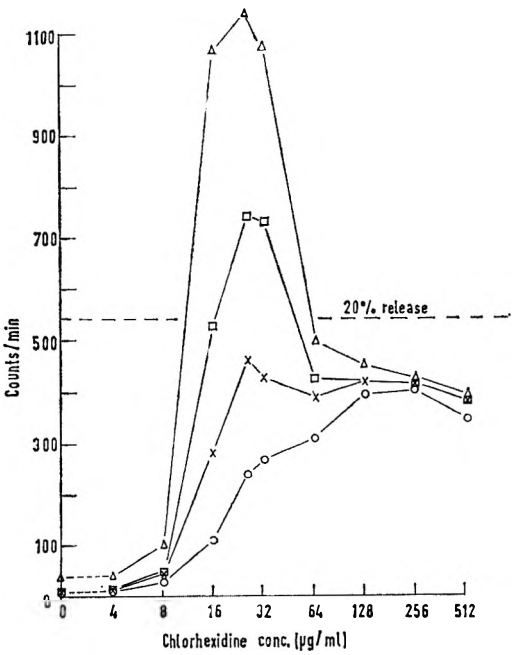

FIG. 3. Release of phosphorus-32 from *Micrococcus lysodeikticus* treated with chlorhexidine. Suspending medium M/15 phosphate buffer (pH 7·2). Cell concentration $1{\cdot}5 \times 10^{10}$/ml. Contact times: ○ ½ hr, × 2 hr, □ 6 hr, △ 21 hr.

Release from "dilute" cell suspensions. The experiments in phosphate buffer were repeated using a much lower cell concentration. The results are given in Fig. 5. A similar pattern of release was observed but occurred at proportionately lower chlorhexidine concentrations.

Discussion

Several antibacterial agents have been shown to initiate or accelerate the release of cellular constituents. These compounds fall into three main groups:

(i) *Surface-active agents.* Hotchkiss (1946), Gale & Taylor (1947), Salton (1950, 1951), Newton (1953), Stedman, Kravitz & King (1957).

(ii) *Phenolic compounds.* Hotchkiss (1946), Gale & Taylor (1947), Beckett, Patki & Robinson (1959), Joswick (1962), Judis (1962, 1963).

(iii) *Polypeptide antibiotics.* Hotchkiss (1946), Gale & Taylor (1947), Newton (1953), Few & Schulman (1953).

Chlorhexidine does not readily fit into any of the above groups. It bears closest resemblance to the quaternary ammonium compounds in its chemical structure, the concentrations required for activity and in its antibacterial spectrum. Unlike the quaternary compounds however, chlorhexidine has little effect on the surface tension of water. A concentration of 256 μg/ml chlorhexidine diacetate in distilled water gives readings between 68–70 dynes/cm using a Du Nouy Tensiometer.

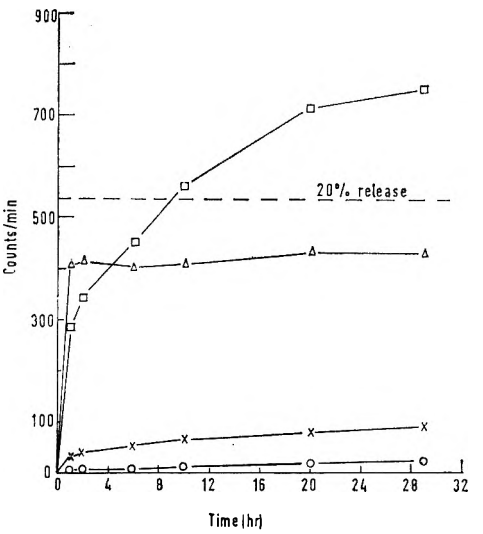

FIG. 4. Rate of release of phosphorus-32 from *Micrococcus lysodeikticus* suspended in M/15 phosphate buffer (pH 7·2) alone and in the presence of chlorhexidine. Cell concentration $1{\cdot}8 \times 10^{10}$/ml. ○ Buffer alone: × 8, □ 32, △ 128 μg chlorhexidine/ml.

The release of cellular constituents from bacteria is regarded by many workers as occurring in two stages.

Hotchkiss (1946) states that adsorption of the surface-active agent onto the bacterial surface results in irreversible damage to the cellular membrane so that the total content of soluble nitrogen and phosphorus compounds is released from the cell. The membrane injury is the signal for the beginning of a series of enzymatic processes which may lead to the virtual dissolution of the cell. The exposure of staphylococci to various bactericides for 20 min at 25° is sufficient to allow autolysis.

Salton (1951), studying the release of 260 mμ absorbing materials from *Staphylococcus aureus* treated with cetyltrimethylammonium bromide

found the primary release to be completed in 3–4 hr. The autolytic breakdown follows this and does not appear to make a significant contribution during the initial leakage.

Newton (1953), studying the release of cellular constituents from *Pseudomonas aeruginosa* treated with cetyltrimethylammonium bromide obtained results similar to those of both Salton and Hotchkiss, and showed that the release of 260 mμ absorbing material, pentose and total phosphate all occurred at the same rate.

Stedman, Kravitz & King (1957) also observed a two stage release of carbon-14 labelled compounds from *Serratia marcescens* treated with a quaternary ammonium compound.

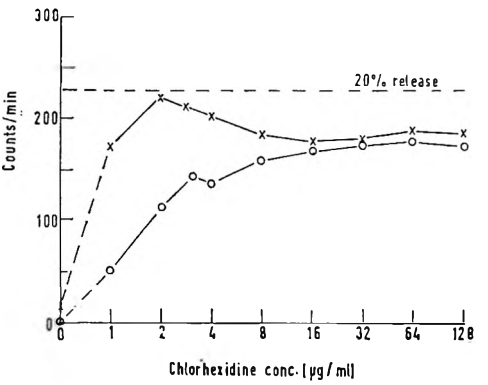

FIG. 5. Release of phosphorus-32 from "dilute" suspensions of *Micrococcus lysodeikticus* treated with chlorhexidine. Suspending medium M/15 phosphate buffer (pH 7·2). Cell concentration 9×10^8/ml. Contact times ○ 3 hr, × 20 hr.

Our results using chlorhexidine show that the release of phosphorus-containing compounds occurs in two stages. The rate and extent of release depends on the ratio of chlorhexidine to bacterial cells and not upon the absolute concentration of chlorhexidine. The initial release is rapid, being completed in 1–2 hr, and is compatible with the hypothesis that cell membrane damage has occurred. We found the maximum initial release produced by chlorhexidine to be between 12% and 22% of the total activity.

The secondary release, which is observed to occur only at low ratios of chlorhexidine to bacteria, takes place over at least 30 hr. This secondary release, which may be due to autolysis in the damaged cells, contributes little to the amount released during the first 2 hr.

Our observations that the autolysis is inhibited by high concentrations of chlorhexidine resemble the findings of Newton (1953) with cetyltrimethylammonium bromide and Joswick (1962) with hexachlorophane.

The increased rate of autolysis in phosphate buffer may result from the involvement of phosphate-requiring enzymatic processes, or it may be due simply to the effect of pH.

The apparent decreased release at concentrations of chlorhexidine in excess of 256 μg/ml may be attributable to a precipitation of labelled compounds by the chlorhexidine and to their removal during centrifuging.

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Anticholinesterase activity and charge delocalisation in "aliphatic" and "aromatic" quaternary ammonium compounds

J. THOMAS AND D. STANIFORTH

The anticholinesterase activities of a homologous series of trimethyl(phenylalkyl)-ammonium bromides have been determined and compared with anti-acetylcholinesterase activities previously reported. The pattern of results obtained with the two enzymes differs widely. The surface activities of the compounds at a constant molar concentration was determined in order to investigate the influence of the "distribution effect" on the anticholinesterase activities of the compounds. The anticholinesterase activities of a series of polycyclic aromatic quaternary compounds have also been determined and compared with their previously reported anti-acetylcholinesterase activities. With these compounds, the pattern of results obtained with the two enzymes was similar.

UNDER standardised conditions the degree of inhibition of acetylcholinesterase by quaternary ammonium compounds is dependant upon the forces of attraction between the onium ion and the active site of the enzyme. The total force of adsorption between onium ions and acetylcholinesterase is comprised of the following constituent forces (Bergmann, 1955, Bernhard, 1955): (*a*) coulombic interaction between the positive charge of the quaternary ammonium group and the anionic site of the enzyme; (*b*) Van der Waal's forces between the hydrocarbon moiety of the quaternary ammonium ion and the enzyme surface.

From a study of the inhibition of acetylcholinesterase by quaternary ammonium compounds, Thomas & Marlow (1963) have suggested that onium ions may be divided into "aromatic" and "aliphatic" types based on their anti-acetylcholinesterase activity. The characteristics of the two types of compound are: (*a*) "aromatic"; the positive charge is delocalised around a flat aromatic ring so it is considered that practically all of the charge is available for coulombic interaction with the anionic site of the enzyme. (*b*) "aliphatic"; the positive charge is delocalised among the four α-carbon atoms arranged tetrahedrally around the nitrogen atom. It is believed that such a situation leads to only a portion of the unit positive charge being available for coulombic interaction between an onium ion and the enzyme anionic site.

The basic difference between the two types of compound is their interaction with the anionic site of acetylcholinesterase. Since there is conflicting evidence about the presence and nature of an anionic site in cholinesterase (Adams & Whittaker, 1950; Bergmann, 1955; Bergmann & Segal, 1963) it was thought of interest to compare the anticholinesterase activities of a series of quaternary ammonium compounds, in which both "aromatic" and "aliphatic" types were represented, with their anti-acetylcholinesterase activities. A homologous series of trimethyl-(phenylalkyl)ammonium salts has therefore been examined. Also, the anticholinesterase activities of quinolizinium bromide and of some of its

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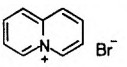
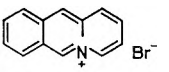
benzo-homologues have been determined and compared with the results obtained with the same compounds using erythrocyte acetylcholinesterase (Thomas, 1963). These results give an indication of the effect of the size of the condensed ring system on the anticholinesterase activity of "aromatic" quaternary compounds.

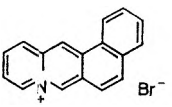
N-Methylpyridinium iodide



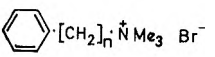
Quinolizinium bromide



Benzo[*b*]quinolizinium bromide



Naphtho[2,1-*b*]quinolizinium bromide



Trimethyl(phenylalkyl)ammonium bromide

Experimental

CHEMICAL

With the exceptions of trimethylphenylammonium bromide and trimethylbenzylammonium bromide, all compounds used have been previously prepared by Thomas & Marlow (1963) and Thomas (1963). The two compounds have now been synthesised by condensing the appropriate dimethylamine with methyl bromide.

Trimethylphenylammonium bromide, m.p. 214·5° (McDowell & Kraus, 1951, report 215°). Found: C, 50·2; H, 6·9. Calc. for $C_9H_{14}BrN$: C, 50·0; H, 6·5.

Trimethylbenzylammonium bromide, m.p. 236·5–237·5° (Kharasch, Williams & Nudenberg, 1955, report 235°). Found: C, 52·0; H, 7·1. Calc. for $C_{10}H_{16}BrN$: C, 52·2; H, 6·96.

MEASUREMENT OF ANTICHOLINESTERASE ACTIVITY

An electrically heated, thermostatically controlled water-bath adjusted to 37° was used. A beaker (100 ml) was supported in the water-bath and into it was placed a glass electrode, a glass stirrer, one arm of an agar bridge and the tip of a microburette. The other end of the agar bridge dipped into a saturated potassium chloride solution into which also dipped a calomel electrode. Both glass and calomel electrodes were connected to a pH meter, the temperature compensator of which was set at 37°.

A solution of the inhibitor (x ml) of selected concentration, was pipetted into the beaker followed by distilled water (43·5 — x ml). The enzyme preparation (1·5 ml) was added and the mixture incubated at 37° for 30 min. Acetylcholine perchlorate (5 ml of 0·12M solution) was added and the pH of the solution quickly adjusted to 7·4 with CO_2-free 0·02N sodium hydroxide solution. The constantly stirred mixture was then maintained

at pH 7·4 for 15 min by addition of further sodium hydroxide solution, the volume used being recorded. The determination was repeated using different concentrations of inhibitor until inhibition values from 30 to 70% were obtained. The pI50 values were obtained from graphs drawn with the ordinates representing the –ve log of the concentration of inhibitor and the abscissae representing % inhibition. To check that the apparatus was working correctly (particularly the response of the glass electrode), a determination was made without an inhibitor before and after a series of experiments on a particular compound. The results were corrected for non-enzymic hydrolysis of the acetylcholine, the rate of which was determined by replacing the enzyme preparation by a buffer solution (pH 7·4, 1·5 ml) and noting the volume of 0·02N sodium hydroxide added every 5 min for 45 min to maintain the pH at 7·4. All conditions were those used in inhibition experiments. A plot of the volume of alkali added against time was a straight line and the volume of the sodium hydroxide solution consumed in 15 min was obtained from the graph.

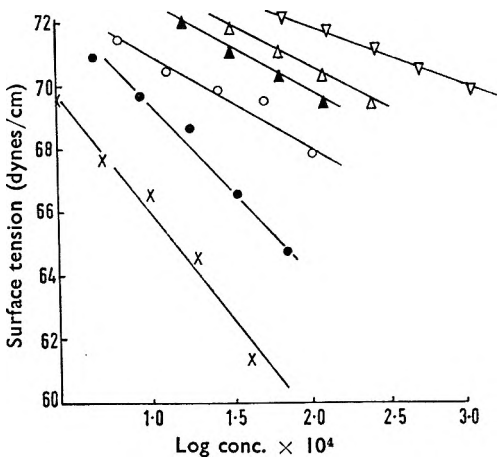


FIG. 1. Surface tension/concentration curves for trimethyl(phenylalkyl)ammonium bromides.

▽ Trimethylphenylammonium bromide.
△ Trimethylbenzylammonium bromide.
▲ Trimethylphenethylammonium bromide.
○ Trimethyl(3-phenylpropyl)ammonium bromide.
● Trimethyl(4-phenylbutyl)ammonium bromide.
× Trimethyl(5-phenylpentyl)ammonium bromide.

SOURCE OF CHOLINESTERASE

Horse serum obtained from commercial sources and preserved with chloroform was used as it has been shown that it has a high cholinesterase activity and that the chloroform has no effect on the activity (Stedman, Stedman & White, 1933). Also, even though horse serum contains an ali-esterase this has no action on acetylcholine (Richter & Croft, 1942).

MEASUREMENT OF SURFACE TENSION OF SOLUTIONS OF TRIMETHYL(PHENYLALKYL)AMMONIUM COMPOUNDS

The surface tension of solutions of the trimethyl(phenylalkyl)ammonium bromides was determined as described by Thomas & Clough (1963) and the results shown in Fig. 1. From this, surface tensions of the solutions of constant molar concentration were obtained.

Results and Discussion

TRIMETHYLPHENYLALKYLAMMONIUM COMPOUNDS

The results of the anticholinesterase determinations are compared in Fig. 2, with a curve similarly obtained by Thomas & Marlow (1963) on the same compounds using red blood cell acetylcholinesterase. Since different enzyme preparations were used to determine the activities, the pI50 values are not comparable, but as it is the pattern of change *within* each series which is compared, rather than absolute pI50 values, differences in the enzyme preparations used are of little consequence to the interpretation of the results.

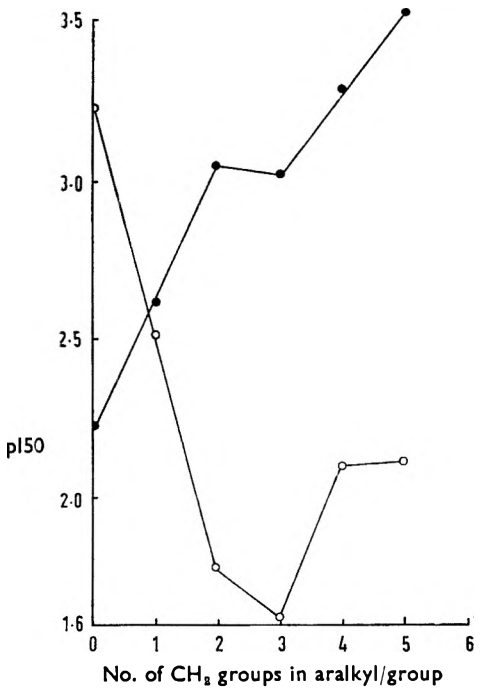


FIG 2. Anticholinesterase and antiacetylcholinesterase activities of trimethyl-(phenylalkyl)ammonium compounds. Anticholinesterase activities —●—: anti-acetylcholinesterase activities —○— (values taken from Thomas & Marlow, 1963).

It can be seen from Fig. 2 that the change in activity towards the two enzymes differs widely in the homologous series. The pattern of results

obtained with red blood cell acetylcholinesterase has been explained by Thomas & Marlow (1963). The essential point in their explanation of a fall in activity followed by a rise as the series is ascended is that the compounds change from being "aromatic" in type to "aliphatic." Consequently, there is a decrease in coulombic interaction between the positive charge of the quaternary ammonium group and the anionic site of the enzyme. However, the Van der Waal's component of the total adsorption force increases as the series is ascended and so two factors are operating to change the activities of successive homologues; the first tending to decrease activity and the second tending to increase it as the series is ascended.

The pattern of results now obtained with the horse serum cholinesterase is quite different. The activity rises progressively as the the number of methylene groups in the aralkyl group increases, except with phenethyl and 3-phenylpropyl compounds, in which the number changes from 2 to 3. This pattern poses two questions: (i) why are the results so different wth the two enzymes and (ii) why have trimethylphenethylammonium bromide and trimethyl(3-phenylpropyl)ammonium bromide the same activity? Since an increase in the number of methylene groups in the aralkyl group should cause a regular increase in Van der Waal's forces of attraction with both enzymes, and this has been shown to be so with the trimethylalkylammonium series (Bergmann, 1955), it appears that the differences between the behaviour with the two esterases must be ascribed to the coulombic component of the total adsorption force. The most reasonable assumption is that the difference is a reflection of variations in the anionic sites in the two enzymes but precisely what these are is obscure.

The "plateau" in the regular rise in activity as the number of methylene groups in the aralkyl group changes from 2 to 3 is difficult to explain. Even though only two sets of pI50 values are reported, these two compounds have been examined four times. Different salts of the two compounds have been synthesised and examined to exclude the possibility of an error, but in all instances the two compounds have virtually the same activity. It is noticeable that the "plateau" occurs in the same part of the series as did the change in activity with acetylcholinesterase. Also in Fig. 2 the points representing the anticholinesterase activity of compounds with 0, 3, 4, 5 methylene groups in the aralkyl group lie on a straight line, suggesting that the compounds with 1 and 2 such groups have abnormally high anticholinesterase activity.

It has been suggested that the "distribution effect" will have an influence on the anticholinesterase activities of quaternary ammonium ions (Thomas & Marlow, 1963). The "distribution effect" was described by Thomas & Marlow (1963) as the increased concentration of quaternary ammonium ions at the interface of the water and enzyme surfaces, compared with the concentration in the bulk of the solution, due to the effect of water on amphipathic ions. To examine whether the "plateau" was due to the "distribution effect," the surface tension of the trimethyl(phenylalkyl)-ammonium compounds at constant molar concentration was determined. The results, given in Fig. 3, show that the surface tension values fall in a

regular manner as the number of methylene groups in the aralkyl group increases. Hence the "distribution effect" will rise regularly as the series is ascended and is not, therefore, the cause of the "plateau."

The results indicate that the adsorption of quaternary ammonium ions onto cholinesterase is not as sensitive to changes in the nature of the quaternary ammonium ion as is the case with acetylcholinesterase.

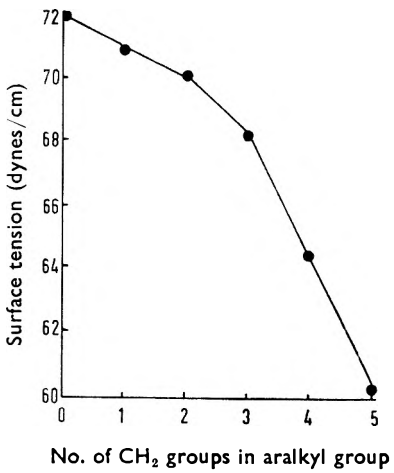


FIG. 3. Surface tension of solutions of trimethyl(phenylalkyl)ammonium bromides at constant molar concentration (log molar conc. $\times$ 10^4 = 1·9) plotted against the number of methylene groups in aralkyl groups.

QUINOLIZINIUM COMPOUNDS

The pI50 values for this series of compounds are given in Table 1, together with the values obtained by Thomas (1963) for the antiacetylcholinesterase activities of the same compounds. It can be seen that unlike the results for the trimethyl(phenylalkyl)ammonium compounds,

TABLE 1. ANTICHOLINESTERASE ACTIVITIES OF QUINOLIZINIUM COMPOUNDS. pI50 VALUES GIVEN IN g/m/l. HORSE SERUM USED AS SOURCE OF ENZYME. TEMP. 37°. SUBSTRATE CONCENTRATION 0·012M ACETYLCHOLINE PERCHLORATE. (Anti-acetylcholinesterase values taken from Thomas, 1963)

	Anticholinesterase		Antiacetylcholinesterase	
	pI50	Relative activities	pI50	Relative activities
N-Methylpyridinium iodide	1·655	0·00000084	2·485	0·00877
Quinolizinium bromide	3·023	0·000195	2·95	0·0296
Benzo[*b*]quinolizinium bromide	6·730	1·0	4·575	1·0
Naptho[2,1-*b*]quinolizirium bromide	5·712	0·0959	3·945	0·294

the pattern of results obtained with the two enzymes is similar. Activity increases as the number of rings in the compound increases up to three and then decreases. Also the increased activity of quinolizinium bromide compared with that of *N*-methylpyridinium iodide (223 times) is much less than the difference observed between benzo[*b*]quinolizinium bromide

and quinolizinium bromide (5,120 times). A similar result was obtained with acetylcholinesterase, the respective values being 3·4 times and 33 times (Thomas, 1963). The results suggest that the benzo[*b*]quinolizinium ion has optimal characteristics for adsorption onto both cholinesterase and acetylcholinesterase.

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Gamma-irradiation of spores of *Bacillus subtilis*

A. M. COOK AND T. A. ROBERTS*

Freeze-drying from a 5% aqueous solution of glucose produced a marked protection of spores of *Bacillus subtilis* subjected to spent fuel gamma-irradiation over the range $2{\cdot}5$–$12{\cdot}5 \times 10^5$ rads. Freeze-drying from aqueous suspension produced no protection. Irradiation in 5% aqueous glucose and in aqueous suspension gave log % survivor/dose regressions with similar slopes.

BACTERIAL spores have been used in studies of radiation resistance, but only recently has evidence been forthcoming that conditions during (Tallentire, 1958; Powers & Kaleta, 1960) and after (Powers, Webb & Ehret, 1960; Tallentire & Davies, 1961) irradiation have any marked effect on the recovery after irradiation.

We have examined the effect of irradiation on spores freeze-dried from water and glucose and lactose solutions and compared their viability with that of suspensions in water and glucose after irradiation.

Experimental

MATERIALS AND METHODS

Spore suspension. *Bacillus subtilis* NCTC 8236 was grown on a Lemco agar containing 0·0001% manganous sulphate at 37°. After 14 days spores washed from the surface were washed five times with sterile water, heated at 78–80° for 20 min to kill vegetative cells, and stored at 0–4°. Suspensions were made in water or 5% glucose or lactose as required.

Counting. Decimal dilutions were made in sterile distilled water. Ten replicates of 0·5 ml were spread on overdried peptone agar (Oxoid) plates, and incubated at 37° for at least 36 hr. Colonies were counted at 18 and at 36–40 hr.

Freeze-drying. 0·1 ml samples of spore suspension in water or 5% glucose or lactose solutions were snap-frozen in ampoules and dried over phosphorus pentoxide for 5–6 hr (Model L.T.5, Edwards and Co. Ltd.). The phosphorus pentoxide was then replenished and drying continued overnight, maintaining a pressure of 0·01 mm Hg by continuous pumping. Ampoules were sealed under air. Freeze-dried spores were recovered by adding 1 ml sterile water to the ampoule, transferring the reconstituted suspension to 9 ml sterile water, and rinsing the ampoule at least 5 times with the bulk dilution.

Storage of aqueous spore suspension. No change in microscopical, colonial or biochemical characteristics was detected in 2 years of storage in water or 5% glucose solution, and the resistance of the spores to wet heat (100°), dry heat (100°) and phenol, chlorocresol, phenylmercuric nitrate, chloramine-T, crystal violet, cetrimide, aminacrine HCl, chlorhexidine diacetate, benzylpenicillin and streptomycin remained the same.

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Irradiation. Irradiation at the Spent Fuel Gamma Irradiation Unit of the Atomic Energy Research Establishment, Harwell, was at approximately 20° and over the dose range of 2·5–12·5 × 10^5 rads. Storage before and after irradiation was at 16–17°. At least 3 ampoules were irradiated at each dose level. Spores were irradiated in the presence of air.

Storage of irradiated spores in aqueous suspension. At 0–4° the viable count of the stock spore suspension fell from 6 × 10^8 spores/ml to 3 × 10^8 spores/ml after 1 year. Serial dilutions of irradiated spore samples were stored at 0–4°, and plated at intervals up to 215 days. Although a fall in count occurred in irradiated and unirradiated samples, the slope of the regression of log % survivors against dose did not change significantly over this period.

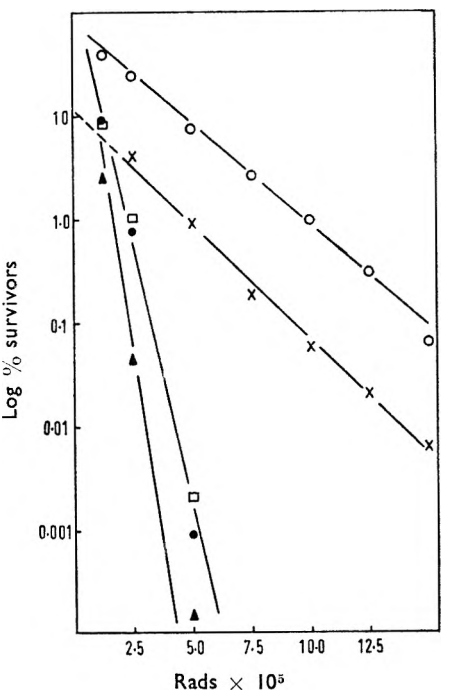

FIG. 1. Gamma irradiation of *Bacillus subtilis* spores. Regressions of log% survivors against radiation dose for: □ aqueous suspension of spores. ● suspension of spores in 5% aqueous glucose. ▲ spores freeze-dried from aqueous suspension. ○ spores freeze-dried from 5% aqueous solution of glucose. × spores freeze-dried from 5% aqueous solution of lactose.

Statistical analysis. Results from irradiation experiments were expressed as % survivors, using counts from unirradiated ampoules as representative of 100%. Log % survivor/dose regressions were calculated, including the 100% value except in the case of freeze-dried lactose. Correlation coefficients indicated that the regressions could be considered linear.

Groups of regression coefficients were compared for parallelism using an analysis of variance (Yousef, 1954). If a significant difference between regression coefficients was thus established, the slopes of pairs of lines were compared by a modified '*t*' test (Bailey, 1959). In some cases a total regression was calculated assuming that all the points were scattered along one regression line and a common slope established.

Results

Exponential survivor/dose curves were obtained upon gamma-irradiation of *B. subtilis* spores, whether in aqueous suspension or freeze-dried and also in the presence of glucose but not lactose.

No significant difference was established in slopes of the curves of spores in aqueous suspension, spores suspended in 5% aqueous glucose, or spores freeze-dried from water (slope (b) = $-0{\cdot}9331$, $-1{\cdot}0112$, and $-1{\cdot}1663 \times 10^{-5}$ rads respectively).

Freeze-drying from 5% aqueous glucose resulted in a marked protective effect ($b = -0{\cdot}1949$ rads $\times 10^{-5}$) (Fig. 1).

Addition of glucose to spores freeze-dried from water was without effect on the radiation resistance.

Freeze-drying from 5% aqueous lactose produced an initial rapid fall in viability to 5–10% of the original, whereupon the slope of the regression changed to almost that of freeze-dried glucose ($-0{\cdot}2226$ rads $\times 10^{-5}$).

Discussion

Linear log survivor/dose curves have previously been reported for the radiation inactivation of *B. subtilis* spores by Donnellan & Morowitz (1957) and Weese (1958).

Moos (1952) interpreted a statistically significant protective effect on freeze-drying *Pasteurella pestis* and *Escherichia coli* from distilled water as a reduction of the water content of the cells preventing formation of certain toxic radicals on subsequent irradiation. Experiments with rigidly controlled water content show sensitisation to X- and gamma-radiation of spores irradiated in the presence of, and stored in the presence of, oxygen. Sensitivity is a function of water content, decreasing sensitivity occurring with increasing water content (Tallentire & Davies, 1961; Tallentire & Powers, 1963). Intracellular dehydration is therefore unlikely to be the reason for the increased radiation resistance demonstrated.

Freeze-drying from 5% aqueous glucose under the conditions described produced a glass. This may be regarded as a supercooled syrup, amorphous in nature, and containing some water. A true glass is impermeable to gases, but if crystallisation occurs, pores form and permit passage of gases. Since the glass forms during freeze-drying, and is impermeable, spores encased in it may exhibit typical anoxic radiation resistance, which is greater than resistance in the presence of air (Proctor, Goldblith, Oberle & Miller, 1955). If the glass is incompletely formed, or unstable, as with lactose, only some of the spores will remain protected by the residual entire glass.

Alternative explanations should also be considered. As the bacterial spore is permeated by glucose (Black & Gerhardt, 1961) a glass may form within the spore. Stabilisation of toxic free-radicals similar to that reported (Cloutier, 1961) in a boric acid glass may then occur.

There is also evidence that the state of molecular aggregation has a marked effect on the radiation decomposition of α-D-glucose (Phillips & Baugh, 1963). It may be possible for the most resistant aggregation to protect spores encased in it against radiation damage.

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Bactericidal activity of chloroxylenol in aqueous solutions of cetomacrogol

A. G. MITCHELL*

The bactericidal activity of chloroxylenol in water and in solutions of the non-ionic surface-active agent cetomacrogol is shown to be related to the degree of saturation of the system, expressed as a Saturation Ratio, which is the ratio of the amount of chloroxylenol present to its solubility. A saturated solution of chloroxylenol in water is shown to have the same bactericidal activity as saturated surfactant solutions containing up to 100 times as much chloroxylenol. It is apparent that bactericidal activity depends on the amount of chloroxylenol in the true aqueous "phase" and not the total amount present. The bactericidal activity of undersaturated solutions of chloroxylenol in cetomacrogol falls markedly as the Saturation Ratio is reduced. This is attributed to a change in the distribution of chloroxylenol in favour of the micelles where it is apparently without bactericidal activity.

THE effects of surface-active agents on the bactericidal properties of phenols and other compounds have been much investigated. Bean & Berry (1951, 1953) using benzylchlorophenol (5-chloro-2-hydroxydiphenylmethane) and chloroxylenol (4-chloro-3,5-xylenol) solubilised in potassium laurate solutions claimed that bactericidal activity depends on the concentration of phenol in the micelles and that the amount of phenol in water alone makes no significant contribution to the total bactericidal activity even though the water is saturated with phenol. In contrast, Allawala & Riegelman (1953a, b) concluded that the bactericidal action of iodine solubilised in Antarox A-400 (a polyethylene glycol nonyl phenol derivative) is related to the degree of saturation of the water with iodine and not the total concentration of iodine present, i.e., in the water and micelles combined. Further work by the same authors (1954) showed, in accordance with the proposals of Ferguson (1939), that the bactericidal activity of many phenols in water depends on the thermodynamic activity of the solution. Equitoxic solutions were shown to be those in which the thermodynamic activity or degree of saturation and not the actual concentration was the same. The thermodynamic activity of the phenol solution was expressed in terms of the percentage saturation by setting the saturated solution as the standard state of reference.

The bactericidal activity of solutions of chloroxylenol in water and in Cetomacrogol 1,000 B.P.C. (polyethylene glycol 1,000 monocetyl ether) against *Escherichia coli* is now reported. In agreement with the findings of Allawala & Riegelman, bactericidal activity is shown to be related to the degree of saturation of the solution. The degree of saturation is expressed as a Saturation Ratio R, in which

$$R = \frac{c}{c_s} \qquad (1)$$

where c is the concentration and c_s the solubility of chloroxylenol. The degree of saturation is expressed as a ratio rather than as a percentage

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since equation (1) has been used previously to show the dependence of rates of oxidation (Carless & Mitchell, 1962) and rates of hydrolysis (Mitchell, 1963, 1964) of solubilised and emulsified systems on the degree of saturation.

Method

Determination of the solubilities of chloroxylenol in water and in aqueous solutions of cetomacrogol 1,000. The solubilities of chloroxylenol in water and in solutions of cetomacrogol at 20° were determined by the methods described by Mulley & Metcalf (1956).

Determination of the mean death-time of E. coli *in chloroxylenol solutions.* The death-times of *E. coli* in solutions of chloroxylenol in water and in cetomacrogol were estimated by an extinction time method similar to that described by Berry & Bean (1954). The method of cultivating the test organism and sampling the reaction mixture was modified as follows:

(i) *E. coli* from a freeze dried culture was transferred to a slope of peptone agar. Subcultures were made every 24 hr for 4 weeks. From the fourth day slopes were used to prepare suspensions containing $2{,}000 \times 10^6$ organisms per ml in quarter strength Ringer's solution.

(ii) 0·2 ml of bacterial suspension was inoculated into 5 ml of chloroxylenol solution at 20°. At pre-determined time intervals, corresponding to 1/6th to 1/10th of the anticipated death-time, samples were taken from the reaction mixture with a platinum loop and transferred to 5 ml of nutrient broth at 37°. At the end of the experiment the tubes were transferred to an incubator at 37° and examined for evidence of growth after 3 days. Each experiment was made in replicate and the mean death-time calculated according to the method of Berry & Bean (1954).

Results and discussion

The solubility of chloroxylenol in water at 20° was 0·031 g/100 ml. The solubilities of chloroxylenol in cetomacrogol solution at 20° agreed with the values of Mulley & Metcalf (1956). The results in Tables 1–3 show that the death-time of *E. coli* in solutions of chloroxylenol in cetomacrogol

TABLE 1. DEPENDENCE OF DEATH-TIME OF *E. coli* IN CHLOROXYLENOL-CETOMACROGOL SOLUTIONS AT 20° ON THE SATURATION RATIO

Saturation Ratio R	Chloroxylenol % c	Chloroxylenol % c_s	Cetomacrogol moles/litre	Number of replicates	Mean death-time (min)	Standard deviation	Coefficient of variation
1·00	0·1	0·1	0·0026	16	52	3·596	6·92
1·00	0·3	0·3	0·0098	24	53	4·667	8·81
1·00	1·5	1·5	0·0490	16	49	3·124	6·38
1·00	3·0	3·0	0·0960	19	49	7·431	15·17
0·90	0·1	0·111	0·0030	16	108	7·528	6·84
0·90	0·3	0·333	0·0110	16	104	11·58	11·13
0·90	1·5	1·670	0·0540	16	104	10·20	9·81
0·90	3·0	3·333	0·1070	16	98	11·98	12·48

$$R = c/c_s \quad .. \quad .. \quad .. \quad (1)$$

depends on the Saturation Ratio (R) as defined in equation (1). For any given value of R the death-times agree closely. When R = 1·0 the death-times in chloroxylenol-cetomacrogol solutions are approximately the same as in a saturated solution of chloroxylenol in water (Table 4). Thus a saturated solution of chloroxylenol in water has the same bactericidal activity against *E. coli* as a saturated solution of chloroxylenol in 0·096 M cetomacrogol containing 100 times as much chloroxylenol.

TABLE 2. DEPENDENCE OF DEATH-TIME OF *E. coli* IN CHLOROXYLENOL-CETOMACROGOL SOLUTIONS AT 20° ON CHLOROXYLENOL CONCENTRATION. (Cetomacrogol concentration 0·096 moles/litre)

Chloroxylenol %	Saturation ratio R	Number of replicates	Mean death-time (min)	Standard deviation	Coefficient of variation
3·00	1·00	19	49	7·431	15·17
2·85	0·95	16	84	5·007	5·96
2·70	0·90	16	96	9·037	9·51
2·55	0·85	5	75–79 hr	—	—

When R = 1·0 both the micelles and the true aqueous "phase" are fully saturated with chloroxylenol. Since the death-times in these solutions are the same as in a saturated solution of chloroxylenol in water it is apparent that the bactericidal activity is due to the fraction of chloroxylenol present in the true aqueous "phase" and not to the amount of chloroxylenol in the micelles nor to the total amount present in the system. These findings are in agreement with the Ferguson principle (Ferguson, 1939) as developed by Allawala & Riegelman (1953a, 1954) who showed that equitoxic solutions, both in water and in surface-active agents, are those having the same thermodynamic activity or degree of saturation and not the same concentration. (The reader is referred to the original papers for a full discussion of these concepts).

TABLE 3. DEPENDENCE OF DEATH-TIME OF *E. coli* IN CHLOROXYLENOL-CETOMACROGOL SOLUTIONS AT 20° ON CETOMACROGOL CONCENTRATION. (Chloroxylenol concentration 1·5%)

Cetomacrogol (moles/litre)	Saturation ratio R	Number of replicates	Mean death-time (min)	Standard deviation	Coefficient of variation
0·049	1·00	16	49	3·124	6·38
0·051	0·95	16	88	7·192	8·08
0·054	0·90	16	104	10·20	9·81
0·057	0·85	5	75–79 hr	—	—

In solutions of chloroxylenol in water the bactericidal activity decreases as the concentration of chloroxylenol, and thereby the Saturation Ratio is reduced (Table 4). In chloroxylenol-cetomacrogol solutions the Saturation Ratio can be reduced either by decreasing the concentration of chloroxylenol (Table 2) or by increasing the concentration of cetomacrogol (Table 3). The resulting increase in death-time is more marked than in corresponding chloroxylenol-water solutions and when R = 0·85 the death-time becomes nearly the same as in water or cetomacrogol solutions

without chloroxylenol (Table 4). Such results accord with the numerous reports that non-ionic surface-active agents inactivate preservatives (Beckett & Robinson, 1958). The difference in death-time between solutions of chloroxylenol in water and in cetomacrogol when $R < 1{\cdot}0$ may be attributed to the change in distribution of chloroxylenol in favour of the micelles in a solution of surface-active agent on reducing the Saturation Ratio. Presumably when $R = 0{\cdot}85$ there is insufficient chloroxylenol in the true aqueous "phase" to exert a bactericidal action. From solubility studies and the evidence of ultra-violet absorption spectra Mulley & Metcalf (1956) suggested that incorporation of chloroxylenol into micelles of cetomacrogol is governed by the hydrogen bonding which occurs between the phenolic hydroxyl group of chloroxylenol and the ether chain of the non-ionic surface-active agent. This hydrogen-bonded complex is presumably inactive.

TABLE 4. DEATH-TIME OF *E. coli* IN SOLUTIONS OF CHLOROXYLENOL IN WATER AND IN SOLUTIONS OF CETOMACROGOL AT 20°

Chloroxylenol %	Cetomacrogol (moles/litre)	Saturation ratio	Replicates	Mean death-time (min)
0·031	0·000	1·00	16	47
0·029	0·000	0·95	16	74
0·028	0·000	0·90	16	85
0·026	0·000	0·85	16	121
0·000	0·000	—	5	79–84 hr
0·000	0·096	—	5	79–84 hr
0·000	0·120	—	5	79–84 hr

In simple solutions of chloroxylenol in water, changes in concentration are related directly to the thermodynamic activity of phenol and so to its bacterial activity. The bactericidal activity of chloroxylenol in the solubilised state, however, is due to its concentration in the true aqueous "phase" and not the total concentration. Hence a better index of bactericidal activity would be the degree of saturation of the true aqueous "phase" rather than the total solution as defined in equation (1). This would require a knowledge of the distribution of chloroxylenol between the micelles and the true aqueous "phase". Nevertheless the concept of Saturation Ratio provides a simple and convenient means of defining the degree of saturation of the solution as a whole. Moreover, the death-times in undersaturated solutions in cetomacrogol at a given value of R are in close agreement indicating that the degree of saturation of the true aqueous "phase" with chloroxylenol is the same. Hence the degree of saturation as expressed by the Saturation Ratio, can be used as an index of the bactericidal activity of chloroxylenol-cetomacrogol solutions.

It is of interest to note that in solutions of surface-active agents, phenomena as different as bactericidal activity, rates of oxidation (Carless & Mitchell, 1962) and rates of hydrolysis (Mitchell, 1963, 1964) are dependent on the degree of saturation of the system. This is to be expected since the degree of saturation of a given solvent system is a measure of the thermodynamic activity of the system and therefore of its chemical potential. Solutions of surface-active agents differ from simple solvents

in that rates of reaction in the former are apparently controlled by the distribution of reactant between the micelles and the true aqueous "phase". With bactericidal activity and hydrolysis the rate of reaction appears to depend mainly on the amount of reactant in the true aqueous "phase", whereas in oxidation it is controlled mainly by the amount in the micelles.

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Hypoglycaemic agents*

D. F. HAYMAN, V. PETROW AND O. STEPHENSON

Various 3-substituted derivatives of 1-(*p*-vinylbenzenesulphonyl)urea, 1-[*p*-(2-chloroethyl)benzenesulphonyl]urea and 1-[*p*-(2-bromoethyl)benzenesulphonyl]urea are described. Several of the compounds possess noteworthy hypoglycaemic activity on oral administration in rabbits. In contrast, several related 5-substituted derivatives of 2-[*p*-(2-chloroethyl)benzenesulphonamido]-1,3,4-thiadiazoles and -1,3,4-oxadiazoles were virtually inactive.

REPLACEMENT of the methyl group of tolbutamide (I; R = Me, R′ = Bu) by one of the lower alkyl groups leads to sulphonylureas which still possess significant hypoglycaemic activity (Gryglewski, 1957; Gourley, 1958). A new structural type in which the methyl group is replaced by an alkenyl group, and specifically by a vinyl group (I; R = $CH:CH_2$, R′ = Bu), is reported herein. As this compound showed significant biological activity the work was extended to the preparation of the *p*-vinylbenzenesulphonylureas listed in Table 2. The most potent compounds in the series proved to be the n-butyl, cyclopentyl, cyclohexyl and cycloheptyl derivatives (I; R = $CH:CH_2$, R′ = Bu^n, cyclopentyl, cyclohexyl or cycloheptyl).

New intermediate isocyanates were prepared by standard methods, viz—reaction of the amine hydrochloride with excess of phosgene in an appropriate solvent at or near the boiling-point. Several of the isocyanates (Table 1) were characterised by conversion into the phenylureas. *p*-Vinylbenzenesulphonamide, used in early preparative work, was obtained from 4-(2-bromoethyl)benzenesulphonamide (II; X = Br, Y = NH_2) by an improved process based upon the earlier work of Inskeep & Deanin (1947) and of Wiley & Ketterer (1953). It was later found to be more convenient to prepare the *p*-vinylbenzene derivatives by the action of aqueous ethanolic alkali hydroxide upon the appropriate l-substituted 3-[*p*-(2-bromo- or chloro-ethyl)benzenesulphonyl]ureas (Table 3). Later biological data revealed that several of these compounds themselves (I; R = $CH_2{\cdot}CH_2{\cdot}Br$ or $CH_2{\cdot}CH_2{\cdot}Cl$, R′ = Bu^n, cyclohexyl or, cycloheptyl) were at least equal to the derived *p*-vinylbenzene compounds in hypoglycaemic activity.

p-(2-Chloroethyl)benzenesulphonyl chloride (II; X = Y, Y = Cl), a compound not previously described in the literature, was obtained by direct chlorosulphonation of phenethyl chloride at 15–20°, and converted into the required sulphonamide (II; X = Cl, Y = NH_2) by reaction with ammonia in a two-phase chloroform–water medium.

Three sulphonylthioureas (III; R = $CH:CH_2$, R′ = Pr^n, Bu^n or allyl), were prepared by reaction of *p*-vinylbenzenesulphonamide with the appropriate isothiocyanate. Reaction of *p*-(2-chloroethyl)benzenesulphonamide with butyl isothiocyanate yielded the sulphonylthiourea (III; R =

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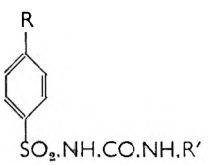
* The fourth paper in this series.

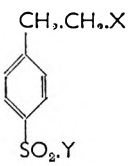
$CH_2{\cdot}CH_2{\cdot}Cl$, R′ = Bu^n), which was smoothly oxidised to the sulphonylurea (I; R = $CH_2{\cdot}CH_2{\cdot}Cl$, R′ = Bu^n) by the action of hydrogen peroxide in alkaline solution (compare Shah, Mhasalkar, Patki & Deliwala, 1959). Some of the 2-chloroethyl and 2-bromoethyl compounds (Table 3) were also prepared by routes other than that involving reaction of the sulphonamide with an isocyanate in aqueous alkaline acetone (see Experimental), but these routes invariably gave inferior yields of products.

Finally, a series of 2-[*p*-(2-chloroethyl)benzenesulphonamido]-1,3,4-thiadiazoles (IV; R = $CH_2{\cdot}CH_2{\cdot}Cl$, R′ = alkyl, X = S) [compare Janbon, Chaptal, Vedel & Schaap (1942) and Loubatières (1944, 1955)], and of 2-[*p*-(2-chloroethyl)benzenesulphonamide]-1,3,4-oxadiazoles (IV; R = $CH_2{\cdot}CH_2{\cdot}Cl$, R′ = alkyl, X = O) [compare O'Neal, Rosen, Russell & Blumenthal (1962)] were synthesised. Two of the thiadiazoles were converted into the corresponding 2-(*p*-vinylbenzenesulphonamido)-1,3,4-thiadiazoles (IV; R = $CH{:}CH_2$, R′ = Pr^i or Bu^i, X = S) by treatment with ethanolic alkaline hydroxide solution. Surprisingly, these latter heterocyclic derivatives were all less active than the aromatic types (I).

We are indebted to Dr. A. David and his colleagues for biological data.



(I)



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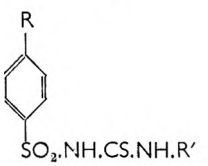

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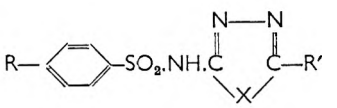


(IV)

Experimental

Most of the examples given typify the methods used for the preparation of the compounds listed in Tables 1 to 3, which contain relevant analytical data.

trans-4-*Methylcyclohexyl isocyanate.* A suspension of *trans*-4-methylcyclohexylamine hydrochloride in chloronaphthalene (200 ml, "mixed isomers") was heated to 140° and treated with a fairly rapid stream of phosgene gas for 3 hr. The phosgene was stopped and nitrogen passed into the mixture whilst the temperature was raised to 180–200° for 3 hr. The residual oil was distilled at 5 mm to yield crude material (50·8 g), b.p. 60–100° at 5 mm. This was refractionated to give the pure *product* (35·5 g), b.p. 60 to 60·5° at 6 mm.

1-(trans-4-*Methylcyclohexyl*)-3-*phenylurea.* The foregoing isocyanate

TABLE 1. ISOCYANATES (R.NCO) AND DERIVED PHENYLUREAS (R.NH.CO.NH.Ph)

R	Reaction solvent for R.NCO	b.p. °C	mm	n_D	°C	m.p. °C of phenylurea	Formula	Found C	Found H	Found N	Required C	Required H	Required N
t-Pentyl	c	110–114	A.P.	1·4320	28	127–129	$C_6H_{11}NO$ $C_{12}H_{18}N_2O$	70·1	8·4	13·8	69·85	8·8	13·6
t-Pentyl													
Heptyl	e	184	A.P.	1·4295	26	65–66	$C_8H_{15}NO$ $C_{14}H_{28}N_2O$	68·4 71·3	11·1 9·2	9·6 11·9	68·1 71·7	10·7 9·5	9·9 12·0
Heptyl													
Octyl	e	196	A.P.	1·4460	22	72–73	$C_9H_{17}NO$ $C_{15}H_{24}N_2O$	70·2 72·6	10·7 9·9	8·8 11·0	69·6 72·5	11·0 9·7	9·0 11·3
Octyl													
Cyclopentyl	d	145–146	A.P.	1·4470	26	204–206	C_6H_9NO $C_{12}H_{16}N_2O$	64·5 70·8	8·3 8·0	12·7 13·6	64·9 70·55	8·2 7·9	12·6 13·7
Cyclopentyl													
trans-2-Methylcyclohexyl	e	58–60	6	1·4550	23	157–159*	$C_8H_{13}NO$ $C_{14}H_{20}N_2O$	69·6	9·4	9·9	69·0	9·4	10·1
trans-2-Methylcyclohexyl													
trans-3-Methylcyclohexyl	e	66	10	1·4522	22	165–167*	$C_8H_{13}NO$ $C_{14}H_{20}N_2O$	69·7	9·6	10·0	69·0	9·4	10·1
trans-3-Methylcyclohexyl													
trans-4-Methylcyclohexyl	e	60	6	1·4500	23	214–216*	$C_8H_{13}NO$ $C_{14}H_{20}N_2O$	68·6	9·2	10·2	69·0	9·4	10·1
trans-4-Methylcyclohexyl													
Cycloheptyl	e	69–70	16	1·4670	27	185–187	$C_9H_{15}NO$ $C_{15}H_{22}N_2O$	72·4	8·7	11·8	72·4	8·7	12·1
Cycloheptyl													
Cyclo-octyl	d	85–87	7	1·4814	25	154–156	$C_9H_{15}NO$ $C_{15}H_{22}N_2O$	73·3	8·9	11·3	73·2	9·0	11·4
Cyclo-octyl										10·1			10·1
Cyclohexylmethyl	e	194–196	A.P.	1·4751	22	128–130	$C_9H_{15}NO$ $C_{14}H_{20}N_2O$	72·9	8·4	11·9	72·4	8·7	12·1
Cyclohexylmethyl													
2-Cyclohexylethyl	e	215–217	A.P.			106–108	$C_{10}H_{17}NO$ $C_{16}H_{22}N_2O$	73·2	8·7	11·4	73·2	9·0	11·4
2-Cyclohexylethyl													
2-Phenoxyethyl	b	122	7·5			134–136	$C_9H_9NO_2$ $C_{15}H_{16}N_2O_2$	66·5 70·0	5·6 6·4	8·4 10·9	66·2 70·35	5·6 6·3	8·6 10·9
2-Phenoxyethyl													
2-Ethylthioethyl	a	188–190	A.P.				C_9H_9NOS			12·5			12·5
2-Ethylthioethyl						75–76	$C_{11}H_{16}N_2OS$	59·2	6·9	14·0′	58·9	7·2	14·3′

(*a*) toluene.
(*b*) chlorobenzene.
(*c*) nitrobenzene.
(*d*) *o*-dichlorobenzene.
(*e*) α-chloronaphthalene (usually mixed isomers).

′ = sulphur.
* = identical with compounds synthesised from amine, $R.NH_2$ and phenyl isocyanate.

(1 g) was added to a solution of aniline (1 ml) in dry benzene (10 ml) and the mixture was heated on a steam-bath for 10 min and then diluted with light petroleum (b.p. 60–80°). The *product* had m.p. 214–216° (from ethanol) and was identical with the material obtained by reaction of phenyl isocyanate with *trans*-4-methylcyclohexylamine.

Styrene-4-sulphonamide (i) A solution of *p*-(2-bromoethyl)benzenesulphonamide (80 g) [compare Inskeep & Deanin (1947) and Wiley & Ketterer (1953)] in ethanol (800 ml) was treated with a solution of potassium hydroxide (60 g) in ethanol (800 ml) and the mixture heated under reflux for 8 hr after which time the bulk of the ethanol was boiled off. The residual solid was dissolved in water (550 ml), and the solution was heated to 90° and filtered after the addition of decolorising charcoal. The cooled filtrate was acidified with hydrochloric acid to yield the *product* (52·5 g), m.p. 138–139°. This material was pure enough for use. A sample crystallised from water had m.p. 140–141°. Found: C, 52·3; H, 4·8; N, 7·3; S, 17·0. Calc. for $C_8H_9NO_2S$: C, 52·4; H, 5·0; N, 7·6; S, 17·5%.

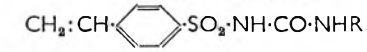
(ii) (*a*) p-(2-*Chloroethyl*)*benzenesulphonyl chloride.* 2-Phenethyl chloride (216 g) was added dropwise with stirring to chlorosulphonic acid (537 g) at 15–20°, and the mixture was stirred for a further hr and then poured onto crushed ice (5 litres). The oil was extracted with chloroform and the extract washed successively with water, 5% sodium bicarbonate solution and water. The chloroform was boiled off from the extract and the residual oil distilled at 0·6 mm to give the *product* (55% yield), b.p. 125–130°, m.p. 54–56° [from ether–light petroleum (b.p. 40–60°)]. Found: C, 40·6; H, 3·4; S, 13·6. $C_8H_8Cl_2O_2S$ requires C, 40·2; H, 3·4; S, 13·4%. A small quantity of a lower-boiling fraction (110–125° at 0·6 mm) presumably contained the *ortho*-sulphonyl chloride.

(*b*) p-(2-*Chloroethyl*)*benzenesulphonamide.* A solution of the foregoing sulphonyl chloride (131 g) in chloroform (400 ml) was added slowly with stirring to aqueous ammonia (800 ml, d = 0·880). After 1 hr the *product* (92·6 g) was collected; it had m.p. 179–181° (from water). Found: C, 43·7; H, 4·6; Cl, 16·1; N, 6·4; S, 14·7. $C_8H_{10}ClNO_2S$ requires C, 43·7; H, 4·6; Cl, 16·1; N, 6·4; S, 14·6%.

(*c*) A solution of the foregoing compound (11·0 g) in ethanol (50 ml) was treated with a solution of potassium hydroxide (8·4 g) in ethanol (75 ml) and the mixture heated under reflux for 3 hr. The solvent was distilled off at reduced pressure, and water (200 ml) added to dissolve the residual solid. The solution was filtered and the filtrate acidified with 5N hydrochloric acid to yield the *product* (7·8 g), m.p. 141–143° (from water containing a little hydroquinone).

1-*Phenyl*-3-(p-*vinylbenzenesulphonyl*)*urea.* A solution of styrene-4-sulphonamide (7·3 g) in acetone (90 ml) was treated with a solution of sodium hydroxide (1·8 g) in a minimum volume of water. The mixture was cooled to 0° and treated with phenyl isocyanate (3·13 g). Stirring was continued for 1 hr at 0°, then for 3 hr at 25°; the mixture was then poured onto crushed ice (800 ml) and filtered. The filtrate was acidified with dilute hydrochloric acid to yield the *product* (11·4 g), m.p. 162–164°.

TABLE 2. 3-SUBSTITUTED 1-*p*-VINYLBENZENESULPHONYL-UREAS AND -THIOUREAS



R	m.p. °C	Formula	Found C	Found H	Found N	Found S	Required C	Required H	Required N	Required S
Ethyl	100–102	$C_{11}H_{14}N_2O_3S$	51·7	5·5	10·8	12·5	52·0	5·5	11·0	12·6
Propyl	105–106	$C_{12}H_{16}N_2O_3S$	53·4	6·0	10·6	12·0	53·7	6·0	10·4	12·0
Butyl	116–117	$C_{13}H_{18}N_2O_3S$	55·3	6·0	10·0	11·3	55·3	6·4	9·9	11·4
Pentyl	86–88	$C_{14}H_{20}N_2O_3S$	56·5	6·9	9·5	10·7	56·7	6·8	9·5	10·8
Hexyl	139–140	$C_{15}H_{22}N_2O_3S$	58·0	7·2	9·0	10·4	58·0	7·1	9·0	10·3
Heptyl	118–119	$C_{16}H_{24}N_2O_3S$	59·3	7·4	8·8	10·0	59·3	7·5	8·6	9·9
Octyl	92–94	$C_{17}H_{26}N_2O_3S$	60·5	7·5	8·2	9·8	60·3	7·7	8·3	9·5
Isopropyl	112–113	$C_{12}H_{16}N_2O_3S$	53·7	5·8	10·3	11·7	53·7	6·0	10·4	12·0
Isobutyl	150–151	$C_{13}H_{18}N_2O_3S$	55·6	6·3	10·0	11·5	55·3	6·4	9·9	11·4
s-Butyl	133–134	$C_{13}H_{18}N_2O_3S$	54·9	6·3	9·7	11·2	55·3	6·4	9·9	11·4
t-Butyl	134–135	$C_{13}H_{18}N_2O_3S$	44·4	6·6	9·7	11·3	55·3	6·4	9·9	11·4
Isopentyl	113–114	$C_{14}H_{20}N_2O_3S$	56·8	6·8	9·4	10·8	56·7	6·8	9·5	10·8
t-Pentyl	118–119	$C_{14}H_{20}N_2O_3S$	56·5	6·9	9·4	10·7	56·7	6·8	9·5	10·8
1-Ethylpropyl	134–135	$C_{14}H_{20}N_2O_3S$	56·7	6·6	9·7	11·0	56·7	6·8	9·5	10·8
Isohexyl	113–114	$C_{15}H_{22}N_2O_3S$	58·2	6·7	9·0	10·5	58·0	7·1	9·0	10·3
1-Propylbutyl	158	$C_{16}H_{24}N_2O_3S$	59·4	7·6	8·5	9·5	59·3	7·5	8·6	9·9
Allyl	131–132	$C_{11}H_{14}N_2O_3S$	54·0	5·3	10·7	12·1	54·1	5·3	10·5	12·0
3-Propoxypropyl	120–122	$C_{15}H_{24}N_2O_4S$	55·6	6·8	8·6	9·9	55·2	6·8	8·6	9·8
3-Butoxypropyl	70–72	$C_{16}H_{26}N_2O_4S$	56·1	6·9	8·5	9·4	56·4	7·1	8·2	9·4
3-Isobutoxypropyl	96–97	$C_{16}H_{26}N_2O_4S$	56·3	7·0	8·1	9·2	56·4	7·1	8·2	9·4
3-Cyclohexyloxypropyl	97–99	$C_{18}H_{28}N_2O_4S$	59·0	7·2	7·7	8·7	59·0	7·2	7·6	8·7
Cyclopentyl	161·5–162*	$C_{14}H_{18}N_2O_3S$	57·0	6·0	9·5	10·8	57·1	6·2	9·5	10·9
Cyclohexyl	169·5–170*	$C_{15}H_{20}N_2O_3S$	58·7	6·6	9·0	10·2	58·4	6·5	9·1	10·4
Cycloheptyl	146·5–147*	$C_{16}H_{22}N_2O_3S$	59·3	6·6	8·7	9·8	59·6	6·9	8·7	9·9
Cyclo-octyl	140–142	$C_{17}H_{24}N_2O_3S$	60·3	7·1	8·2	9·9	60·7	7·2	8·3	9·5
trans-2-Methylcyclohexyl	157	$C_{16}H_{22}N_2O_3S$	59·6	6·9	8·7	9·9	59·6	6·9	8·7	9·9
trans-3-Methylcyclohexyl	155–156	$C_{16}H_{22}N_2O_3S$	59·5	6·8	8·5	9·6	59·6	6·9	8·7	9·9
trans-4-Methylcyclohexyl	183–184	$C_{16}H_{22}N_2O_3S$	59·7	6·9	8·7	10·1	59·6	6·9	8·7	9·9
Cyclohexylmethyl	170	$C_{16}H_{22}N_2O_3S$	59·2	7·0	8·4	9·8	59·6	6·9	8·7	9·9
2-Cyclohexylethyl	101–102	$C_{17}H_{24}N_2O_3S$	60·5	7·2	8·5	9·6	60·7	7·2	8·3	9·5
Benzyl	158	$C_{16}H_{14}N_2O_3S$	61·0	5·2	8·9	10·0	60·7	5·1	8·9	10·1
Phenethyl	137–138	$C_{17}H_{16}N_2O_3S$	62·1	5·7	8·6	9·4	61·8	5·5	8·5	9·7
2-Phenoxyethyl	168–169	$C_{17}H_{16}N_2O_4S$	59·0	5·4	8·3	9·0	58·9	5·2	8·1	9·3
Phenyl	168–169	$C_{16}H_{14}N_2O_3S$	59·6	4·9	9·2	10·5	59·6	4·7	9·3	10·6
p-Tolyl	169–170	$C_{17}H_{16}N_2O_3S$	61·1	5·3	8·8	10·3	60·7	5·1	8·9	10 1
p-Methoxyphenyl	138–139	$C_{16}H_{16}N_2O_4S$	57·9	4·8	8·3	9·6	57·8	4·9	8·4	9·6
p-Ethoxyphenyl	166–167	$C_{17}H_{18}N_2O_4S$	59·1	5·4	7·9	9·1	58·9	5·2	8·1	9·3
p-Chlorophenyl	178	$C_{15}H_{13}ClN_2O_3S$	53·3	3·8	8·1	9·1 10·1′ }	53·5	3·9	8·3	9·5 10·5′ }

TABLE 2—*continued*

R	m.p. °C	Formula	Found C	Found H	Found N	Found S	Required C	Required H	Required N	Required S
p-Bromophenyl	178–180	$C_{15}H_{12}BrN_3O_3S$	47·5	3·6	7·3	8·1 20·8+	47·3	3·4	7·4	8·4 21·0+
1-Naphthyl	161	$C_{19}H_{15}N_3O_3S$	65·0	4·7	7·9	9·1	64·8	4·6	8·0	9·1
2-Ethylthioethyl	109–110	$C_{13}H_{19}N_3O_3S_2$	49·6	5·7	8·8	20·0	49·70	5·8	8·9	20·4
		$CH_2{:}CH\cdot C_6H_4\cdot SO_2\cdot NH\cdot CS\cdot NHR$								
Propyl	90–91	$C_{12}H_{16}N_2O_3S_2$	50·9	5·3	9·7	22·5	50·7	5·7	9·8	22·6
Butyl	94–95	$C_{13}H_{18}N_2O_3S_2$	52·5	6·1	9·8	21·2	52·3	6·1	9·4	21·5
Allyl	89–91	$C_{12}H_{14}N_2O_3S_2$	50·5	4·6	9·5	—	51·0	5.0	9·9	—

* Corrected m.p. ' = chlorine. + = bromine.

TABLE 3. 3-SUBSTITUTED 1-(*p*-2-HALOGENOETHYL)BENZENESULPHONYLUREAS

$X\cdot CH_2\cdot CH_2\cdot C_6H_4\cdot SO_2\cdot NH\cdot CO\cdot NHR$

R	X	m.p. °C	Formula	Found C	Found H	Found Hal	Found N	Found S	Required C	Required H	Required Hal	Required N	Required S
Propyl	Cl	142–144	$C_{12}H_{17}ClN_2O_3S$	47·4	5·8	11·7	9·1	10·2	47·3	5·6	11·6	9·2	10·5
Butyl	Cl	122–123	$C_{13}H_{19}ClN_2O_3S$	49·2	6·2	10·9	8·9	10·0	49·0	6·0	11·1	8·8	10·1
t-Butyl	Cl	142–143	$C_{13}H_{19}ClN_2O_3S$	49·4	6·1	10·8	8·8	9·8	49·0	6·0	11·1	8·8	10·1
Cyclopentyl	Cl	111–112	$C_{14}H_{19}ClN_2O_3S$	51·2	5·9	10·5	8·2	9·6	50·8	5·8	10·7	8·4	9·7
Cyclohexyl	Cl	129–130	$C_{15}H_{21}ClN_2O_3S$	52·3	6·0	10·4	7·9	8·9	52·2	6·1	10·3	8·1	9·3
Cycloheptyl	Cl	127–129	$C_{16}H_{23}ClN_2O_3S$	53·4	6·2	10·2	8·0	9·3	53·5	6·5	9·9	7·8	8·9
Butyl	Br	132–134	$C_{13}H_{19}BrN_2O_3S$	43·2	5·1	22·2	8·1	8·9	43·0	5·3	22·0	7·7	8·8
t-Butyl	Br	145–146	$C_{13}H_{19}BrN_2O_3S$	42·7	5·5	22·2	7·5	9·2	43·0	5·3	22·0	7·7	8·8
Cyclopentyl	Br	150–151	$C_{14}H_{19}BrN_2O_3S$	44·6	5·0	21·3	7·2	8·2	44·8	5·1	21·3	7·5	8·6
Cyclohexyl	Br	165–166	$C_{15}H_{21}BrN_2O_3S$	46·4	5·3	21·0	7·4	8·4	46·3	5·4	20·5	7·2	8·2
Cycloheptyl	Br	152–155	$C_{16}H_{23}BrN_2O_3S$	48·0	5·5	19·6	6·9	8·2	47·6	5·7	19·8	6·9	7·9

Crystallisation from acetone–light petroleum (b.p. 60–80°) raised the m.p. to 168–169°.

1-[p-(2-*Bromoethyl*)*benzenesulphonyl*]-3-*cyclohexylurea*. Cyclohexyl isocyanate (68·8 g) was added with stirring to a mixture of *p*-(2-bromoethyl)benzenesulphonamide (132 g) in acetone (1200 ml) with a solution of sodium hydroxide (20 g) in water (20 ml) at 8–12° during 20 min. Stirring was continued at room temperature for 1 hr and then at 35–45° for 2 hr. The mixture was cooled and poured onto crushed ice (*ca* 4 litres). The resultant solution was filtered and the filtrate acidified with dilute hydrochloric acid to yield the *product* (187 g), m.p. 165–166° (from aqueous ethanol).

1-*Cyclohexyl*-3-(p-*vinylbenzenesulphonyl*)*urea*. A refluxing suspension of the foregoing compound (38·9 g) in ethanol (150 ml) was treated with a solution of sodium hydroxide (8·8 g) in water (10 ml) and heating continued for 3 hr. It was then evaporated to dryness at reduced pressure and the residue dissolved in water (500 ml), heated to 80° with decolorising charcoal and filtered. The filtrate was cooled and acidified with dilute hydrochloric acid to yield the *product* (18·2 g), m.p. 169·5–170° (decomp.) (corr.) after crystallisation from aqueous ethanol.

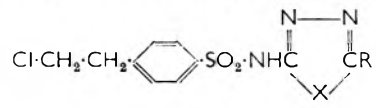
3-[p-(2-*Chloroethyl*)*benzenesulphonyl*]-1-*t-butylurea* was prepared in 80% yield by reaction of t-butyl isocyanate with *p*-(2-chloroethyl)benzenesulphonamide in aqueous acetone containing an equivalent of sodium hydroxide as described earlier. It had m.p. 142–143° (from aqueous ethanol). 3-(p-*Vinylbenzenesulphonyl*)-1-*t-butylurea* was obtained in 80% yield when a solution of the foregoing compound (8 g) in ethanol (75 ml) was treated with a solution of sodium hydroxide (2·5 g) in water (4 ml) and the mixture refluxed for 3 hr. It had m.p. 134–135° (from aqueous ethanol). 1-*Propyl*-3-(p-*vinylbenzenesulphonyl*)*thiourea*. Propyl isothiocyanate (2·4 g) was added with stirring to a solution of styrene-4-sulphonamide (3·7 g) in acetone (45 ml) containing 10N sodium hydroxide solution (2 ml) at 0–5°. The mixture was stirred at 20–25° for 4 hr, and then poured onto crushed ice. After filtration the filtrate was acidified to yield the *product* (4·4 g), m.p. 90–91° [from benzene-light petroleum b.p. 60–80°)].

The following examples illustrate other methods used for the preparation of some of the compounds listed in the Tables:

1-[p-(2-*Chloroethyl*)*benzenesulphonyl*]-3-*cyclohexylurea*. (*a*) *Ethyl* p-(2-*chloroethyl*)*benzenesulphonylcarbamate* (see Marshall & Segal, 1958). A mixture of *p*-(2-chloroethyl)benzenesulphonamide (11 g) and anhydrous potassium carbonate (20 g) in acetone (100 ml) was heated under reflux with stirring, and ethyl chloroformate (6 g) added during 1 hr. Heating was continued for a further 3 hr and the solid collected after cooling. This was dissolved in water (80 ml) and acidified with hydrochloric acid to yield the *product* (10·1 g), m.p. 107–109° (from dilute ethanol). Found: C, 45·5; H, 5·0; Cl, 12·2; N, 5·1; S, 10·9. $C_{11}H_{14}ClNO_4S$ requires C, 45·3; H, 4·8; Cl, 12·2; N, 4·8; S, 11·0%.

(*b*) A solution of the foregoing carbamate (2·9 g) in boiling toluene (25 ml) was treated with cyclohexylamine (1 g) and the mixture heated

TABLE 4. 5 SUBSTITUTED 2-[*p*-(2-CHLOROETHYL)BENZENESULPHONAMIDO]-1,3,4-THIADIAZOLES AND -1,3,4-OXADIAZOLES



R	X	m.p. °C	Formula	Found					Required				
				C	H	Cl	N	S	C	H	Cl	N	S
Propyl	S	126–127	$C_{13}H_{16}ClN_3O_2S_2$	45·1	4·8	10·4	12·0	18·7	45·1	4·7	10·25	12·1	18·55
Isopropyl	S	133–134	$C_{13}H_{16}ClN_3O_2S_2$	45·3	4·5	10·3	12·2	18·6	45·1	4·7	10·25	12·1	18·55
Cyclopropyl	S	166–168	$C_{13}H_{14}ClN_3O_2S_2$	45·5	3·7	10·4	12·1	18·5	45·4	4·1	10·3	12·2	18·65
Isobutyl	S	134–135	$C_{14}H_{18}ClN_3O_2S_2$	46·5	4·8	9·9	11·6	17·9	46·7	5·0	9·85	11·7	17·8
s-Butyl	S	121–122	$C_{14}H_{18}ClN_3O_2S_2$	47·0	5·1	10·1	11·4	18·2	46·7	5·0	9·85	11·7	17·8
t-Butyl	S	204–206	$C_{14}H_{18}ClN_3O_2S_2$	46·7	5·0	9·9	11·6	17·8	46·7	5·0	9·85	11·7	17·8
Cyclopentyl	S	151–153	$C_{15}H_{18}ClN_3O_2S_2$	48·9	5·2	9·6	11·0	17·3	48·5	4·9	9·5	11·3	17·25
Cyclohexyl	S	180–183	$C_{16}H_{20}ClN_3O_2S_2$	49·5	5·1	9·4	10·8	16·7	49·8	5·2	9·2	10·9	16·6
2-Cyclopentylethyl	S	204–206	$C_{17}H_{22}ClN_3O_2S_2$	51·1	5·9	9·2	10·5	16·4	51·0	5·5	8·9	10·5	16·6
Ethyl	O	139–141	$C_{12}H_{14}ClN_3O_3S$	45·9	4·4	11·1	13·2	10·0	45·6	4·5	11·2	13·3	10·15
Propyl	O	110–112	$C_{13}H_{16}ClN_3O_3S$	47·7	4·7	11·0	12·9	9·9	47·3	4·9	10·75	12·7	9·7
Isopropyl	O	118–120	$C_{13}H_{16}ClN_3O_3S$	47·6	5·0	11·0	12·6	10·0	47·3	4·9	10·75	12·7	9·7
Isobutyl	O	100–102	$C_{14}H_{18}ClN_3O_3S$	49·0	5·0	10·7	12·4	9·6	48·9	5·3	10·3	12·2	9·3
t-Butyl	O	161–163	$C_{14}H_{18}ClN_3O_3S$	48·6	5·5	10·3	12·2	8·9	48·9	5·3	10·3	12·2	9·3
Cyclohexyl	O	102–103	$C_{16}H_{20}ClN_3O_3S$	52·1	5·4	10·0	11·7	9·1	51·95	5·45	9·6	11·4	8·7

under reflux for 4 hr, after which time volatile material was distilled off at reduced pressure. The residue was extracted with 1% aqueous ammonia solution, filtered, and the filtrate acidified with dilute hydrochloric acid. The *product* (1·2 g) had m.p. 129–130° (from aqueous ethanol).

1-*Butyl*-3-[p-(2-*chloroethyl*)*benzenesulphonyl*]*urea* [see Georgiev (1960) and Das Gupta (1961)]. (*a*) p-(2-*Chloroethyl*)*benzenesulphonylurea.* A mixture of p-(2-chloroethyl)benzenesulphonamide (22 g) and potassium cyanate (10·1 g) in ethanol (200 ml) and heated under reflux for 2 hr, and then ethanol (100 ml) was distilled off. The potassium salt was collected, dissolved in water, filtered, and the filtrate acidified with dilute hydrochloric acid to yield the *product* (18 g), m.p. 178–180° (from ethanol). Found: C, 41·2; H, 4·3; Cl, 13·2; N, 10·7; S, 11·9. $C_9H_{11}ClN_2O_3S$ requires C, 41·2; H, 4·2; Cl, 13·5; N, 10·7; S, 12·2%.

(*b*) A mixture of the foregoing sulphonylurea (6·6 g), isobutyl methyl ketone (20 ml) and acetone (2 ml) was treated with butylamine (2 g), added during 20 min with occasional shaking. It was then heated under reflux for 2 hr, cooled, treated with 4% aqueous sodium hydroxide solution (25 ml), and the organic layer separated and washed with water. The combined aqueous extracts were acidified with dilute sulphuric acid and the *product* (6·55 g) collected. It had m.p. 122–123° (from aqueous ethanol).

2 (*a*) 1-*Butyl*-3-[p-(2-*chloroethyl*)*benzenesulphonyl*]*thiourea.* Butyl isothiocyanate (12·7 g) was added with stirring to a mixture of p-(2-chloroethyl)benzenesulphonamide (22·0 g) in acetone (225 ml) containing sodium hydroxide (4 g) in water (4 ml) at 0–5°. It was then warmed to 50° for 3 hr, cooled and poured onto crushed ice (2 litres). The solution was filtered and the filtrate acidified with hydrochloric acid to yield the *product* (26·5 g), m.p. 110–112° (from aqueous ethanol). Found: C, 46·9; H, 5·7; Cl, 10·5; N, 8·6; S, 18·7. $C_{13}H_{19}ClN_2O_2S$ requires C, 46·6; H, 5·7; Cl, 10·6; N, 8·4; S, 19·2%.

(*b*) Hydrogen peroxide (10 ml of 10% solution) was added to a stirred solution of the foregoing sulphonylthiourea (4 g) in water (60 ml) containing sodium hydroxide (2·0 g) and the mixture was warmed to 40° for 1 hr. The solution was cooled, acidified with dilute hydrochloric acid and extracted with chloroform. The chloroform extract was washed with water and the chloroform distilled off to yield the *product* (1·6 g), m.p. 122–123° (from aqueous ethanol).

1-[p-(2-*Bromoethyl*)*benzenesulphonyl*]-3-*cyclohexylurea* [see Nantka-Namirski & Betzecki (1959)]. (*a*) O-*Methylcyclohexylurea.* A mixture of cyclohexylurea (25·6 g) and dimethyl sulphate (25·2 g) was heated carefully to 100°, kept at that temperature for 10 min, then it was cooled, poured onto crushed ice and basified with 30% aqueous sodium hydroxide solution. The mixture was extracted with benzene, the benzene was distilled off and the residual oil distilled at 8 mm to yield the *product*, b.p. 115–118°. Found: N, 17·8. $C_8H_{16}N_2O$ requires N, 17·9%.

(*b*) Solutions of the foregoing urea (3·9 g) in acetone (10 ml) and of p-(2-bromoethyl)benzenesulphonyl chloride (7·1 g) in acetone (10 ml) were added simultaneously to a stirred mixture of potassium carbonate

(3·4 g) in acetone (15 ml) and water (10 ml) at 10° and stirring was continued for 1 hr further. The mixture was poured onto crushed ice (150 ml), extracted with benzene and the extract washed once with water and concentrated. The residual material was hydrolysed by heating with concentrated hydrochloric acid on the steam-bath for 10 min. The crude material (7 g) was collected, washed with water, dissolved in 2% aqueous ammonia solution and filtered to remove insoluble material. Acidification of the filtrate with dilute hydrochloric acid yielded the *product* (3·3 g), m.p. 165–166° (from aqueous ethanol).

2-*Amino*-5-*cyclopentyl*-1,3,4-*thiadiazole*. Cyclopentylcarbonyl chloride (48·5 g) was added to a mixture of thiosemicarbazide (30·5 g) and phosphorus trichloride (40 ml), which was then heated at 60–70° for 5 hr. The mixture was then cooled, diluted with water (300 ml) and the solution basified with 20% aqueous sodium hydroxide solution. The solids were collected, washed with cold water, dissolved in warm dilute hydrochloric acid, filtered and the filtrate basified with aqueous sodium hydroxide solution. The *product* (26·2 g) had m.p. 234–236° (decomp) (from aqueous ethanol). Found: C, 49·6; H, 6·3; N, 24·9; S, 19·0. Calc. for $C_7H_{11}N_3S$: C, 49·7; H, 6·55; N, 24·8; S, 18·9%.

2-*Amino*-5-(2-*cyclopentylethyl*)-1,3,4-*thiadiazole* had m.p. 234–236° (from ethanol). Found: C, 54·7; H, 7·7; N, 21·1; S, 16·6. $C_9H_{15}N_3S$ requires C, 54·8; H, 7·7; N, 21·3; S, 16·25%.

2-[p-(2-*Chloroethyl*)*benzenesulphonamido*]-5-(2-*cyclopentylethyl*)-1,3,4-*thiadiazole*. A solution of the foregoing thiadiazole (9·85 g) in pyridine (35 ml) was cooled slightly and treated with a solution of *p*-(2-chloroethyl)-benzenesulphonyl chloride (12 g) in pyridine (35 ml) and the mixture allowed to stand at room temperature overnight. It was then poured into water (250 ml) and the solution acidified with hydrochloric acid. The solids (15·8 g) were collected, washed with water, dissolved in 2% aqueous ammonia solution and the solution heated to 60° and filtered after the addition of decolorising charcoal. The filtrate was acidified with dilute hydrochloric acid to yield the *product*, m.p. 204–206° (from ethanol).

5-*Isobutyl*-2-(p-*vinylbenzenesulphonamido*)-1,3,4-*thiadiazole*. A solution of 2-[*p*-(2-chloroethyl)benzenesulphonamido]-5-isobutyl-1,3,4-thiadiazole (18·05 g) in ethanol (200 ml) was treated with a solution of sodium hydroxide (5 g) in water (5 ml) and the mixture was heated under reflux for 4 hr. Solvent was distilled off at reduced pressure, the residual solid was dissolved in water (200 ml) and the solution was acidified with hydrochloric acid. The *product* (12·2 g) had m.p. 135–136° (from aqueous ethanol). Found: C, 51·6; H, 5·7; N, 12·8; S, 19·6. $C_{14}H_{17}N_3O_2S_2$ requires C, 52·0; H, 5·3; N, 13·0; S, 19·8%.

5-*Isopropyl*-2-(*p-vinylbenzenesulphonamido*)-1,3,4-*thiadiazole*, prepared by the foregoing method, had m.p. 122–123° (from aqueous ethanol). Found: C, 50·5; H, 5·1; N, 13·4; S, 20·5. $C_{13}H_{15}N_3O_2S_2$ requires C, 50·4; H, 4·9; N, 13·6; S, 20·7%.

2-*Amino*-5-*isopropyl*-1,3,4-*oxadiazole* (compare Swain, U.S. Patent 2,883,391). A solution of isobutyrohydrazide (36·8 g) in methanol

(70 ml) was added dropwise with stirring to a solution of cyanogen bromide (38·6) in methanol (70 ml), with cooling to 20–25°. After the addition was complete the mixture was refluxed for 2 hr, and then concentrated to remove most of the methanol. The residual oil was dissolved in boiling water (70 ml) and the solution brought to pH 8–9 by the addition of ammonia solution. The solids (17·8 g) were dissolved in acetone and the solution was filtered to remove insoluble material. Dilution of the filtrate with light petroleum (b.p. 60–80°) furnished the *product*, m.p. 180–182° (from acetone). Found: C, 47·5; H, 7·5; N, 33·4. $C_5H_9N_3O$ requires C, 47·2; H, 7·1; N, 33·05%.

2-*Amino*-5-*isobutyl*-1,3,4-*oxadiazole* had m.p. 167–169° after crystallisation from acetone-light petroleum (b.p. 40–60°). Found: C, 51·4; H, 8·3; N, 29·7. $C_6H_{11}N_3O$ requires C, 51·0; H, 7·85; N, 29·8%.

2-*Amino*-5-*t-butyl*-1,3,4-*oxadiazole* had m.p. 222–224° (from aqueous ethanol). Found: C, 50·9; H, 7·8; N, 30·2. $C_6H_{11}N_3O$ requires C, 51·0; H, 7·85; N, 29·8%.

2-[p-(2-*Chloroethyl*)*benzenesulphonamido*]-5-*propyl*-1,3,4-*oxadiazole*. A solution of 2-amino-5-propyl-1,3,4-oxadiazole (12·7 g) in pyridine (60 ml) was cooled below 20° and treated with a solution of *p*-(2-chloroethyl)-benzenesulphonyl chloride (23·9 g) in pyridine (60 ml). The mixture was allowed to stand overnight and then diluted with water (250 ml) and acidified with concentrated hydrochloric acid with cooling. The solids were collected, washed with water and dissolved in dilute ammonia solution. The solution was heated to 50° and filtered after the addition of decolorising charcoal. Acidification of the filtrate with dilute hydrochloric acid furnished the *product* (13·1 g), m.p. 110–112° (from aqueous ethanol).

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The influence of hydrochloric acid on the chromatographic behaviour of sympathomimetic catecholamines

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The behaviour of three catecholamines on paper chromatograms is shown to be markedly influenced by hydrochloric acid. Double spot formation exhibited by noradrenaline acid tartrate, for example, results from the use of hydrochloric acid in the developing solvent, but the formation of the second spot is prevented when the amine is applied to the paper from solution in dilute hydrochloric acid. Retention of amine at the application area and additional multiple spot phenomena are exhibited by all of the three amines investigated when chromatographed from concentrated hydrochloric acid. The significance of these and related observations is discussed in relation to the use of hydrochloric acid during the separation of catecholamines from biological tissues and fluids.

IN 1952 Shepherd & West demonstrated that adrenaline can form multiple spots when chromatographed on paper in the presence of trichloroacetic and certain other acids and they attributed their results to the formation of a complex between the phenolic amines and the acids as described by Kendall (1916). Tissue amines other than catecholamines can in certain circumstances also produce more than one spot (West, 1959). The phenomenon was further investigated and elucidated by Beckett, Beaven & Robinson (1960a,b) who warned against the use of trichloroacetic acid as a protein precipitant during the preparation of biological extracts for paper chromatography.

Double spot formation by noradrenaline acid tartrate in the presence of hydrochloric acid has also been reported (Roberts, 1963a) and because hydrochloric acid is used during both the extraction and the paper chromatographic separation of sympathomimetic catecholamines (Vogt, 1952; Lockett, 1954; Roberts, 1963b) it was decided to investigate the ways in which this acid might influence the chromatographic behaviour of these amines.

Methods

The apparatus, materials and techniques used have been previously described (Roberts, 1963a). Noradrenaline, adrenaline and isoprenaline were chromatographed on Whatman No. 1 papers (washed or sprayed with 0·01 N hydrochloric acid or untreated) from distilled water or hydrochloric acid (0·01, 0·1, 1·0 and 10 N) solution. The developing solvent was phenol containing 15% v/v 0·1 N hydrochloric acid and the amines were located by spraying the developed chromatograms with potassium ferricyanide (0·44 g) in sodium hydroxide solution (100 ml, 0·5 N).

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Two dimensional chromatograms were first developed as in the single dimensional experiments and the papers were washed free of the phenolic solvent with benzene (without locating the amines). When dry the papers were refashioned into new cylinders at right angles to the original direction of flow, and development was continued in this second direction. The positions of the amines after the first development were arrived at by comparison with single dimensional chromatograms developed simultaneously.

Drugs. (−)-Noradrenaline acid tartrate (L. Light & Co. Ltd.), (−)-adrenaline acid tartrate (Burroughs Wellcome & Co.) and (±)-isoprenaline sulphate (Burroughs Wellcome & Co.) were obtained commercially. The doses quoted in the text refer to the quantity of amine calculated as its salt.

Results

Each amine (25μg) chromatographed from 0·01 ml distilled water or hydrochloric acid (0·01, 0·1, 1·0 and 10 N) behaved typically as shown in Fig. 1. When chromatographed from distilled water, isoprenaline and

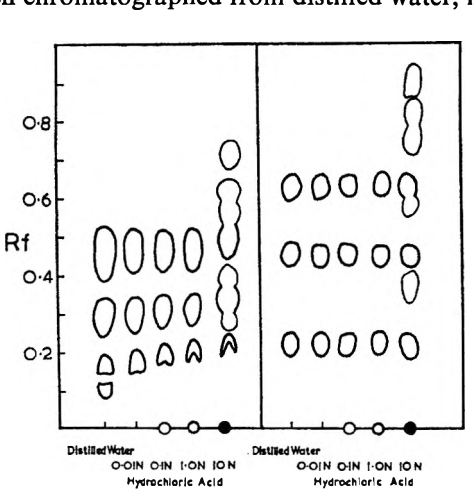

FIG. 1. The influence of application from hydrochloric acid on the chromatographic behaviour of noradrenaline (lower spots), adrenaline (middle spots) and isoprenaline (upper spots) on untreated (left hand side) and acid-treated (right hand side) papers. Circles on the base line represent retention of catecholamine. Developing solvent, phenol containing 15% v/v 0·1 N HCl.

adrenaline showed tailing, and noradrenaline complete separation into two spots, on untreated paper; on acid-washed or acid-sprayed papers all three amines gave compact spots with higher Rf values. On untreated papers increasing the concentration of acid was without effect on the Rf values of isoprenaline and adrenaline and did not prevent the tailing phenomenon; noradrenaline produced a variety of results ranging from double spot formation (distilled water), through a single spot with the higher Rf value (0·01 N HCl), to a crescent-shaped spot showing even

greater movement up the paper (0·1 and 1·0 N HCl). On acid-treated papers compact spots were obtained from all three amines and although the Rf values were higher than on the untreated papers, application from hydrochloric acid was without effect on these values (Fig. 1). When the conditions were such that noradrenaline applied from hydrochloric acid had similar Rf values to those of adrenaline applied from distilled water (untreated paper, less than 25 cm solvent development), the adrenaline and isoprenaline Rf values obtained from the acid solutions were also increased.

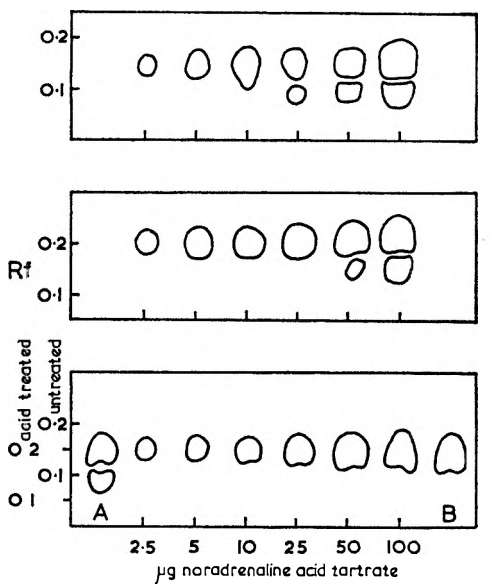

FIG. 2. The influence of hydrochloric acid, in the developing solvent (upper diagram, untreated paper), on the paper (middle diagram, acid-treated paper) and as a solvent for the amine (lower diagram, untreated or acid-treated paper), on the chromatographic behaviour of noradrenaline acid tartrate. Developing solvent, phenol containing 15% v/v 0.1 N HCl.

The double spot formation exhibited by noradrenaline was further examined by chromatographing 2·5, 5, 10, 25, 50 and 100 μg of the amine (1·0 mg/ml) from distilled water and hydrochloric acid (0·01 N). Typical results are shown in Fig. 2. Application from distilled water on untreated papers resulted in single spots of the higher Rf value when 10 μg or less were chromatographed; double spot formation was evident when 25 μg or more were used (Fig. 2, upper diagram). When the amines were applied from hydrochloric acid, however, the faster running spot only was obtained from all concentrations chromatographed (Fig. 2, lower diagram) On acid treated papers, 2·5 to 25 μg gave single spots of high Rf value, 50 μg gave a similar spot plus a smaller one of lower Rf value and 100 μg gave two equally sized spots, when applied from distilled water (Fig. 2, middle diagram). Application from hydrochloric acid again resulted

in compact spots of the higher Rf value for all concentrations of noradrenaline applied. However, when 100 μg amounts of noradrenaline were chromatographed from more concentrated solutions (10 mg/ml, distilled water or hydrochloric acid), double spot formation was obtained from distilled water and hydrochloric acid (0·01 N), but from hydrochloric acid (0·1 N) only a single spot of the higher Rf value was obtained (Fig. 2, lower diagram A and B). These results were obtained on both untreated and acid-treated papers.

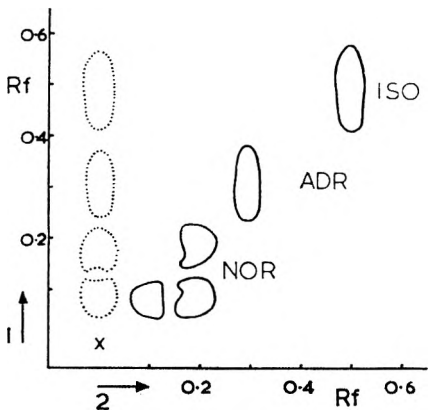

FIG. 3. Combined two-dimensional chromatograms of noradrenaline acid tartrate (NOR), adrenaline acid tartrate (ADR) and isoprenaline sulphate (ISO) developed in phenol containing 15% v/v 0·1 N HCl on untreated papers. Continuous outline indicates the positions of the spots after development in the second dimension. Dotted outline indicates the positions of the spots after the first development.

Further information was obtained by developing individual two dimensional chromatograms of noradrenaline, adrenaline and isoprenaline (100 μg) applied from distilled water to untreated papers. The single dimensional chromatogram of noradrenaline showed two spots but on re-chromatographing in the second direction the lower spot again separated into two while the upper spot persisted (Fig. 3). The shape and relative location of the two new spots were characteristic of the shape and location of the two spots obtained in the single dimensional chromatogram. One way development of adrenaline and isoprenaline resulted in elongated spots, but on re-chromatographing in the second direction no further tailing was observed and there was no indication of any multiple spot phenomena (Fig. 3).

In contrast to the results described above, chromatography of 25–50 μg of each amine from 0·01 ml hydrochloric acid (10 N) resulted in the formation of many extra spots, but as in the previous experiments higher Rf values were obtained on acid-treated papers (Fig. 1). The pink colour of these additional spots, produced on oxidation with alkaline potassium ferricyanide, was not very intense and the overall impression was one of considerable tailing and streaking between and around the three main spots of noradrenaline, adrenaline and isoprenaline. More definite

boundaries to the additional spots were observed when 100 μg quantities of each amine were chromatographed individually on acid-washed papers. The use of 10 N hydrochloric acid resulted in each amine producing three spots (Fig. 4). The more intense of these spots were obtained at the expected Rf values of the amines chromatographed. The remaining spots had Rf values higher than the corresponding amine from which they were formed, and in each case the colour intensity of the spot nearest the parent catecholamine was greater than that of the spot which had travelled farthest up the paper.

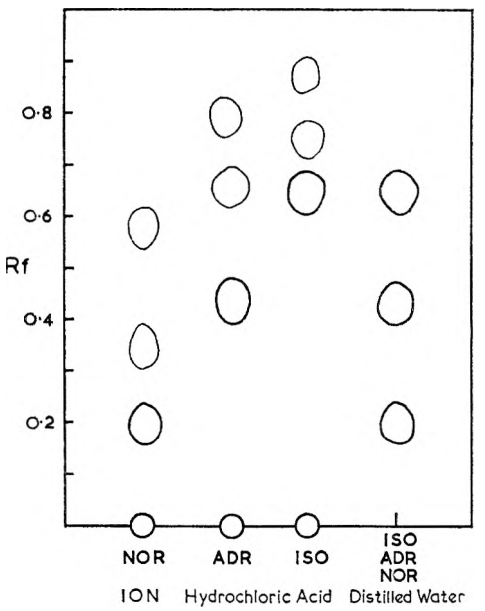

FIG. 4. Multiple spot phenomena exhibited by 100 μg of noradrenaline acid tartrate (NOR), adrenaline acid tartrate (ADR) and isoprenaline sulphate (ISO) when chromatographed from hydrochloric acid (ION) in phenol containing 15% v/v 0.1 N HCl on acid-treated papers. Circles on the base line represent retention of amine. Extreme right, Rf values of the amines obtained from distilled water, for comparison. Some streaking was observed when noradrenaline was chromatographed from 10 N HCl but in the interests of clarity this is not shown.

An additional phenomenon was observed when the stronger acid solutions were used. In all instances, application from 0·1, 1·0 and 10 N hydrochloric acid resulted in considerable quantities of amine(s) being left at the starting point (Figs. 1 and 4). When individual amines (25 μg) were chromatographed from hydrochloric acid (0·01 ml) it became apparent, using the density of colour produced on oxidation as an index of concentration, that the stronger the acid then the greater was the retention of the amines. However, when 25 μg of any one amine was chromatographed from 0·1 ml of hydrochloric acid (0·01 and 0·1 N) even the dilute acid now caused some amine to be left at the application point. Furthermore, the amount of retention obtained with 0·1 N hydrochloric

acid was equivalent to that obtained previously (i.e. 25 μg of amine from 0·01 ml of acid) with 1·0 N hydrochloric acid. This phenomenon was still evident after continuously washing the papers with acid for three days before use.

Discussion

The multiple spot phenomenon observed with noradrenaline agrees with the arguments presented by Beckett, Beaven & Robinson (1960a, b). On papers untreated with acid the hydrochloric acid present in the developing solvent converts some, but not all, of the 25 μg of noradrenaline acid tartrate applied from distilled water to noradrenaline hydrochloride. This partial conversion results in the formation of two distinct spots, the lower one being noradrenaline base associated with tartaric acid and the upper one being noradrenaline base associated with hydrochloric acid. The results expressed in Fig. 2 (upper diagram) confirm this and show that there is sufficient concentration of acid in the solvent to totally convert 10 μg of noradrenaline acid tartrate to the hydrochloride salt thereby resulting in only a single spot of the higher Rf value. After treatment of the papers with hydrochloric acid (0·01 N), however, the combined concentrations of acid on the paper and in the solvent is sufficient to convert 25 μg of noradrenaline acid tartrate to noradrenaline hydrochloride (Fig. 2, middle diagram). The small size of the lower spot (indicative of incomplete conversion to hydrochloride) obtained when 50 μg of the acid tartrate was chromatographed, suggests that, even when this amount was applied, only a small proportion of the base remained unassociated with hydrochloric acid.

The results obtained when noradrenaline acid tartrate was chromatographed from hydrochloric acid solution confirm that the upper spot is associated with hydrochloric acid rather than tartaric acid and are in agreement with expected results when considered on a molar basis. A solution of 1 mg noradrenaline acid tartrate (i.e. 0·5 mg noradrenaline base) in 1·0 ml hydrochloric acid (0·01 N) represents more than a three-fold molar excess of acid and all of the noradrenaline must be present as the hydrochloride salt. Chromatography of any amount of noradrenaline from this solution is thus expected to result in only a single spot of the upper Rf value and this was found to be so (Fig. 2, lower diagram). A solution of 10 mg noradrenaline acid tartrate (i.e. 5 mg noradrenaline base) in 1·0 ml of hydrochloric acid (0·01 N), however, represents a three-fold molar excess of base and as this will result in a large proportion of noradrenaline remaining associated with tartaric acid, two spots are to be expected and are in fact found (Fig. 2, lower diagram, A). Since this was observed on both untreated and acid-treated papers it is inferred that the combined acid concentration of the developing solvent and that present on the paper is insufficient to complete the conversion of noradrenaline acid tartrate (100 μg) to the hydrochloride salt. Similarly, a concentration of 10 mg noradrenaline acid tartrate (i.e. 5 mg noradrenaline base) in 1·0 ml hydrochloric acid (0·1 N) again represents more than

a threefold molar excess of acid and the upper spot only is observed (Fig. 2, lower diagram, B).

The final proof that the double spot phenomenon exhibited by noradrenaline is due to partial conversion of the acid tartrate salt to the hydrochloride salt is obtained from the results of the two dimensional chromatograms. The single dimensional chromatography of 100 μg noradrenaline acid tartrate from distilled water on untreated papers resulted in a fast running spot associated with hydrochloric acid and a slower running spot associated with tartaric acid. If the arguments presented above are correct then re-development of the chromatogram in the second direction, at right angles to the first direction, should result in the formation of three spots. The spot which consists of noradrenaline associated with hydrochloric acid in the first dimension will still run as a discrete spot in the second dimension. The noradrenaline-tartaric acid spot of the first dimension, however, will again separate into two spots in the second dimension as more of the acid tartrate is converted to hydrochloride by the developing solvent; one of these spots, and the single noradrenaline-hydrochloric acid spot, will be equi-distant from the solvent front of the second development. The experimental observations (Fig. 3) comply with these requirements exactly. The fact that adrenaline acid tartrate and isoprenaline sulphate do not exhibit any multiple spot phenomena under similar conditions is interpreted as being the result of the Rf values of these amines differing sufficiently from those of tartaric hydrochloric and sulphuric acids to be uninfluenced by them. The increases in Rf values of these amines occasionally seen on increasing the concentration of acid used to dissolve them are the result of simple displacement caused by the large upward shift of the noradrenaline spots. The crescent-shaped spots exhibited by noradrenaline when the acid concentration was increased (Fig. 1) are presumably due to concentration of amine at the apex of an elongated acid spot (Beckett, Beaven & Robinson, 1960a, b).

The higher Rf values and absence of tailing by adrenaline and isoprenaline observed on acid treated papers have already been discussed (Roberts, 1963a).

The retention of amine at the application point when applied from strong hydrochloric acid is possibly the same as that noted before with adrenaline by Shepherd & West (1952), although no explanation was offered and drying at an elevated temperature was a prerequisite for this phenomenon in their experiments. In the present case the amount of retention is dependent on the concentration of hydrochloric acid at the application area, for the amount of amine retained from 0·1 ml hydrochloric acid (0·1 N) was the same as that from 0·01 ml hydrochloric acid (1·0 N). A possible cause of the phenomenon is the formation of some insoluble complex between the amines and inorganic ions (not removed from the paper by washing with acid), either with or under the influence of the hydrochloric acid. Alternatively the acid might be causing some change in the molecular and/or ionic structures of the amines causing them to be held at the starting line. Substitution of hydrogen atoms by

chlorine atoms, and the existence of the amines as quaternary ammonium salts in the presence of strong hydrochloric acid, are suggested examples of such changes. The faster moving multiple spots observed when the amines were chromatographed from 10 N hydrochloric acid (Fig. 4) may be due to similar changes in structure giving compounds of differing Rf values.

Double spot formation of the type exhibited by noradrenaline acid tartrate is unlikely to interfere with the chromatography of tissue extracts providing sufficient hydrochloric acid is present in both test and control solutions to ensure that all the catecholamines are in the form of their hydrochloride salts. It is, however, further evidence that the use of different acids in the salt (i.e. during the process of preparing an extract) and solvent system can result in the formation of more than one spot in the chromatography of individual catecholamines. It is significant that when the amines are chromatographed from 10 N hydrochloric acid using the phenol-HCl solvent system, the same acid is being used in the salt and the developing solvent and the multiple spots formed under these conditions must be of different origin to those reported previously in the literature. Furthermore, it may be that the practice of evaporating hydrochloric acid-ethanol extracts to small volume (Vogt, 1952; Lockett, 1954; Roberts, 1963b) is resulting in sufficient concentration of the acid to cause the formation of the substances of higher Rf values discovered in my experiments by the use of 10 N hydrochloric acid. The amounts of these substances formed from the naturally occurring catecholamines present in biological fluid and tissue extracts may be insufficient to show up as spots on the paper, but if they are pharmacologically active they might interfere with the biological assays of the eluates. In this context it is of interest that one of the additional spots formed from adrenaline has an Rf value similar to that of isoprenaline.

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The effect of some ganglionic stimulants and blocking drugs on acetylcholine release from the mammalian neuromuscular junction

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The effect of nicotine, dimethylphenylpiperazinium (DMPP), hexamethonium and pempidine on release of acetylcholine from the guinea-pig phrenic nerve diaphragm preparation has been investigated. Neither nicotine nor DMPP, 2×10^{-5}, modified acetylcholine release from the hemidiaphragms at rest or indirectly stimulated at 50/sec: therefore their neuromuscular blocking action has only a postjunctional origin. Hexamethonium, 4×10^{-4}, significantly reduced the output of transmitter from preparations stimulated at 50/sec at 38°. It did not affect the release of acetylcholine from hemidiaphragms at rest or the acetylcholine content of the muscle. The presynaptic effect of hexamethonium is probably related to its linkage with "receptors" present on the surface of the nerve endings. Pempidine, 1×10^{-4}, diminished the release of acetylcholine from the preparations at rest or stimulated either at 50 or at 6/sec. The effect was related to the frequency and to the temperature. Moreover, the drug reduced the acetylcholine content of the muscle. This effect may be the result of non-specific metabolic inhibition or of an impairment of choline transport system.

ACCORDING to Paton & Zaimis (1949, 1951) and Hesleff & Unna (1954), hexamethonium interrupts ganglionic and neuromuscular transmission solely by acting on the postjunctional membrane as a competitive blocking agent. However, this statement is not fully accepted by Riker & Szreniawsky (1959) who suggest that the nerve endings are an additional site of action of the drug. It has also been suggested that the tertiary amine, pempidine, blocks ganglionic transmission by acting at both pre- and post-synaptic sites (Corne & Edge, 1958). Dimethylphenylpiperazinium (DMPP), like nicotine, possesses both stimulant and blocking actions on autonomic ganglion cells and at the neuromuscular junction (Ling, 1959) and there is evidence that these two drugs also may exert an action on nerve endings, either facilitating (Lee & Shideman, 1959) or inhibiting (Wilson, 1962) transmitter release.

In the experiments now described we have studied the pre-junctional action of hexamethonium, pempidine, nicotine and DMPP directly, by measuring their effect on acetylcholine release from motor nerve endings and on tissue stores of acetylcholine. These experiments have been briefly reported elsewhere (Beani, Bianchi, Bieber & Ledda, 1962).

Experimental

The experiments were made on phrenic nerve-hemidiaphragm preparations from guinea-pigs weighing 250–350 g. Each preparation was suspended in 3 ml of oxygenated Tyrode solution. Both hemidiaphragms from each animal were prepared; one was treated with the chosen drug and the other served as a control.

The methods for (i) estimation of transmitter release, and (ii) detection of acetylcholine stores, have been previously described (Beani & Bianchi, 1961; Beani, Bianchi & Ledda, 1962) and may be summarised as follows:

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(i) The preparations, pre-incubated with 500 μg/ml dyflos (DFP) for 150 min, were indirectly stimulated by rectangular pulses (0·1 msec duration, at a voltage twice the threshold) for five 10 min periods of high frequency stimulation, interspersed with usually 20 min periods of rest. Immediately before every period of high frequency stimulation, the height of contraction at 15/min was briefly tested. The contractions of both preparations were recorded on smoked paper with an isotonic lever, amplification 1:7, load 2 g. The acetylcholine released during stimulation or rest was estimated on the guinea-pig ileum without any special sensitising procedure. The effect of drug on the transmitter output was examined at 33° and 38° and at stimulation rates of 6 and 50/sec.

The drug was added to one preparation of each pair after the end of the second 20 min period of rest, left in contact for 30 min before starting the third period, and maintained during the third and fourth period of stimulation and rest. After repeated washing, the fifth period of stimulation was carried out. Every experimental point was determined on six pairs of preparations.

(ii) The tissue stores of acetylcholine were estimated at the end of the fourth stimulation period (50/sec, 33°) by the method of Bentley & Shaw (1952) as modified by Beani, Bianchi & Ledda (1962). The estimations were made on the hemidiaphragms treated with drugs found to modify the release of acetylcholine and in the contralateral preparations of the same animals, which acted as controls. Every experimental point was determined on ten pairs of hemidiaphragms. Amounts of acetylcholine are given as chloride; the final concentration of the drugs is (w/v) as base.

TABLE 1. EFFECT OF GANGLIONIC STIMULANT AND BLOCKING DRUGS ON NEUROMUSCULAR TRANSMISSION AND ACETYLCHOLINE RELEASE AND TISSUE STORES

Drug	Indirect contraction at 15/min before the 3rd and 4th period, as % of the control		Stimulation rate/sec	Acetylcholine release in 3rd and 4th period of stimulation from treated preparations as % of expected values*		Tissue ACh/hemidiaphragms (ng±s.d.) after the 4th period of stimulation, 50/sec, 33°	
	33°	38°		33°	38°	Controls	Treated
Nicotine 2×10^{-5}	0	0	50	98·2±19·6	102·1±21·2	not estimated	not estimated
DMPP 2×10^{-4}	0	0	50	104·1±26·5	105·0±32·9	not estimated	not estimated
Hexamethonium 1×10^{-4}	42	37	6	108·4±17·6	83·1±16·9	117·4±15	122·8±33·7
			50	89·4±13·9	57·9±14·6		
Pempidine 1×10^{-4}	26	14	6	70·0±24·7	55·8± 6·3	113·5±22	83·1±29·9●
			50	50·7±18·9	44·7±17·1		

● = Statistically different ($0{\cdot}02 > P > 0{\cdot}01$) from the control group.
* = Results are means of 6 experiments.

Results

The evaluation of the drug effect on the transmitter release was made by comparing the absolute values of acetylcholine released in the third and fourth stimulation and rest periods, from treated and untreated

preparations. The drug effect was also assessed by expressing the mean of the acetylcholine released during the third and fourth periods from every treated preparation, as a percentage of the "expected" value. The "expected" value was calculated from the average releases from both test and control preparations in the first and second period, and from the average release from the controls in the third and fourth periods.

The reliability of these evaluations is based upon the observation that the absolute release from the untreated right and left hemidiaphragms of the same animal is nearly equal in any given period.

Nicotine and DMPP, 2×10^{-5}, did not modify the release of acetylcholine either at 33 or at 38°, from preparations stimulated at 50/sec (Table 1) or at rest (values not given).

Lower stimulation frequencies were not used because we have found that, in general, the effect of a drug able to inhibit the release of acetylcholine, is directly related to the frequency of stimulation (Beani & Bianchi, 1961; Beani, Bianchi & Ledda, 1963).

Hexamethonium, 4×10^{-4}, significantly ($P < 0.001$) reduced the output of acetylcholine only in the fourth period from the hemidiaphragms at 38° and stimulated at 50/sec (Table 1). Acetylcholine output was still depressed during the fifth period of stimulation, although the drug had been washed out and the contractions elicited by stimulation at 15 min had fully recovered. At 33° and 50/sec, inhibition of acetylcholine output was evident ($P < 0.01$) only after washing, although a slight reduction was present in the fourth period. When the lower stimulation rate is employed (6/sec) the effect of hexamethonium was not significant either at 33 or at 38°. The resting release was never reduced by the drug. The reduction of acetylcholine release was brought about by hexamethonium at a concentration insufficient to block neuromuscular transmission completely and the drug had no effect on acetylcholine stored in the tissue, at the end of the fourth stimulation period at 50/sec, 33° (Table 1).

Pempidine, 1×10^{-4}, strongly reduced the output of acetylcholine from the stimulated preparations. The effect was greater when higher stimulation rate and temperature were employed (Table 1). After washing out the drug, the difference between the acetylcholine released from the control and the treated groups remained significant in the fifth period at 50/sec, either at 33 or at 38°. Pempidine also decreased the acetylcholine released from the preparations at rest (values not given), the effect being most pronounced at 38°: in the group stimulated at 6/sec, the amount released in 10 min of rest was 5.8 ± 1.8 ng in the controls, and 3.3 ± 0.7 ng in the treated group ($P < 0.01$).

A noteworthy difference between hexamethonium and pempidine was that the latter, even in a concentration insufficient to block neuromuscular transmission completely, significantly reduced the tissue stores of acetylcholine (Table 1).

Discussion

The drugs were added to the preparations 30 min before starting the third period of stimulation, to allow their uniform distribution in the

tissue. Consequently, no information was obtained about their initial effect on acetylcholine release: the short acetylcholine-like activity of nicotine and DMPP on the guinea-pig diaphragm, for instance, may result not only from stimulation of the motor end-plate, but also from stimulation of the motor nerve endings. This last effect, if present, was not detected in our experimental procedure. However, it is clear that neither nicotine nor DMPP reduces acetylcholine output during the long-lasting neuromuscular block, the mechanism of which is "competitive" (Beani, Bianchi & Conti, 1960) and limited to the post-junctional membrane.

The inhibition of acetylcholine release by hexamethonium was only evident at 38° and at the higher stimulation rate. Hexamethonium was without effect on acetylcholine release at rest, or on tissue stores.

This effect may suggest that the drug slowly combines with (and dissociates from) "receptors" present on the surface of the axon. The consequence may be a reduced ability of the motor nerve endings to follow the high frequency impulses. Therefore the mechanism of the presynaptic effect of hexamethonium may be similar to that proposed for tubocurarine (Beani & Bianchi, 1961) although the inhibition brought about by the former increases at higher instead of at the lower temperatures. The effect of pempidine has a different pattern. It reduced the output of acetylcholine even at 33° and at the lower stimulation rate. Moreover, it diminished the release from the unstimulated preparations and the acetylcholine stores. Mitchell & Silver (1963) have shown that a great part of the transmitter released from the muscle at rest does not have a nervous origin. Pempidine does not inhibit choline-acetyltransferase (Parkinson, 1959) and its effect on the acetylcholine release and storage may therefore be a consequence of a non-specific metabolic effect or of an impairment of choline transport mechanism (Birks & McIntosh, 1961). Pempidine appears to reduce the safety factor of neuromuscular transmission, chiefly through its presynaptic effect; this may be the reason for the observed sensitisation towards curarising agents (Beani & others, 1960).

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Spectrophotometric method for the estimation of guanethidine

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A spectrophotometric method for the estimation of guanethidine sulphate, based on the development of blue colour in alkaline medium after reaction with lithium and sodium molybdophosphotungstate B.P. reagent, is described. The method is more sensitive and rapid than the existing British Pharmacopoeia methods. Both powder and tablets can be estimated by this method.

IN the British Pharmacopoeia 1963, two methods of estimation for guanethidine sulphate are described: the cation-exchange resin method, which requires 2·5 g of powder, and the colorimetric method for tablets, for which acid extraction and development of colour requires more than 1 hr.

We describe a rapid colorimetric method, requiring only a small sample of either powder or tablets. The method is a modification of the observation that guanethidine, in weakly acid or neutral solution, produces molybdenum blue from phosphomolybdic acid in alkaline medium. As molybdenum blue is unstable, the reaction can be utilised only qualitatively. By using lithium and sodium molybdophosphotungstate B.P. reagent solution, a stable colour is produced and there is also a ten-fold increase in the sensitivity.

Experimental

Freshly prepared aqueous solution of guanethidine sulphate, on treatment with lithium and sodium molybdophosphotungstate reagent, produces a yellow precipitate, changing to deep blue on making alkaline with dilute solutions of ammonia, sodium hydroxide and carbonate.

TABLE 1. GUANETHIDINE CONTENT OF COMMERCIAL TABLETS

Sample	Content as labelled mg	mg recovered	Deviation %
1	10	10·1	+1
2	10	10·0	—
3	10	10·0	—
4	10	10·2	+2
5	10	9·9	−1

The colour that develops with the first two alkalies is not stable enough for quantitative estimation but is satisfactory with the 10% sodium carbonate, except that solutions become turbid at 20 μg/ml. This can be rectified by diluting the reagent 1:10. The extinction of the solution is then measured in a Beckman spectrophotometer model DU, having 1 cm standard Corex cells.

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ESTIMATION IN POWDER

To known concentrations of the compound in 1 ml of water, add the molybdophosphotungstate reagent, 1 ml of 1 : 10, and mix. Add sodium carbonate solution, 8 ml 10%, to each strength of solution and develop the colour for 15 min. Measure the extinction at 650 mμ. There is agreement with Beer's law up to 20 μg/ml. A blank prepared in the same manner, omitting the compound, is run simultaneously. The extinction shows a linear relation, for example:

Concentrated μg/ml	2	4	6	8	10	12	14	16	18	20
Extinction	0·046	0·086	0·137	0·181	0·229	0·268	0·319	0·357	0·409	0·456

ESTIMATION IN TABLETS

Weigh and powder a known quantity of tablets and extract with water in a mortar. Adjust the solution after filtration to a final strength of 20 μg/ml. The results of estimations are shown in Table 1. The binding material did not interfere with the assay.

COMPARISON WITH THE BRITISH PHARMACOPOEIA COLORIMETRIC METHOD

The samples of powder and tablets were then compared using the proposed and the B.P. method (Table 2).

TABLE 2. RECOVERY % OF THE KNOWN QUANTITY OF GUANETHIDINE BY B.P. AND SPECTROPHOTOMETRIC METHODS

Sample	mg taken	B.P. method		Spectrophotometric method	
		mg recovered	Deviation %	mg recovered	Deviation %
Powder I	100	99·5	−0·5	99·8	−0·2
Powder II	100	99·6	−0·4	99·4	−0·6
Tablets I	10	9·9	−1	9·9	−1·0
Tablets II	10	10·2	+2	10·0	—
Tablets III	10	9·8	−2	10·1	+1·0

It is evident that though the % recovery of the compound is similar with both the methods, that proposed is the more rapid and sensitive. The estimation can be made with an accuracy of $\pm 2\%$.

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Letters to the Editor

Effect of acetylsalicylic acid on foetal rats

SIR,—During the course of testing drugs for those most likely to exhibit teratogenic activity in man, a study was made of the action of orally administered acetylsalicylic acid on foetal development in rats. In the preliminary experiments, doses about ten times the maximum human therapeutic dose were used and, as other workers (e.g. Obbink & Dalderup, 1964) have reported, not one live birth was obtained. In later experiments, however, lower doses similar to those used clinically (3 × 5 grain tablets four times a day = 50 mg/kg daily) were used, and a relatively large number of dead foetuses and resorption sites were still obtained.

Acetylsalicylic acid powder was mixed with each of two diets, one which had a high sucrose content (sucrose 65%, casein 24%) and one with a high casein content (casein 89%). The remainder of each diet consisted of corn oil (5%) and the vitamin and salt mixture (6%), as used by Colby & Frye (1951). Drinking water was allowed *ad lib.* Each hooded Lister rat of approximately 200 g body-weight consumed 14–16 g food per day when this was made up into a thick paste with water. Males were left in with the females for three days, after which they were removed and the females were given the test diet in place of the standard diet (No. 41 B, London Flour Millers). With this mating regimen, 70–80% of the females were successfully mated. Animals were killed on the 20th day of gestation, the number of live and dead foetuses and resorption sites were counted, and foetal mortality was thus calculated for each dose of acetylsalicylic acid. Results are shown in Table 1 and Fig. 1.

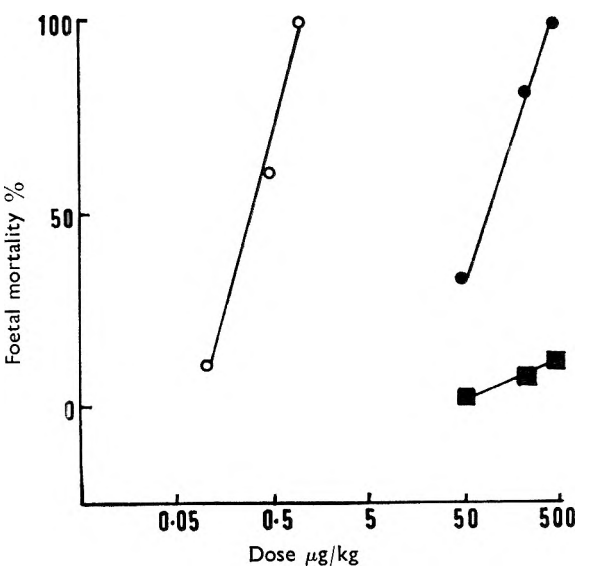

FIG. 1. The relation between foetal mortality and log dose of acetylsalicylic acid (●), thalidomide (■) and reserpine (○) in rats fed on the high sucrose diet.

TABLE 1. THE EFFECT OF ACETYLSALICYLIC ACID ON FOETAL DEVELOPMENT IN RATS FED ON DIFFERENT DIETS

Diet	Dose of acetyl-salicylic acid (mg/kg)	No. of rats	Mean weight gain (g) Day 6–Day 16	No. of implants (and per litter)	No. of foetuses			No. of resorption sites	Deaths (%)
					Total	Live	Dead		
High Sucrose	0	11	+45·3	95 (8·6)	94	94	0	1	1
	50	6	+41·9	74 (12·3)	64	49	15	10	34
	250	4	+31·0	46 (11·5)	23	10	13	23	80
	500	10	−10·0	120 (12·0)	6	0	6	114	100
High Casein	0	10	+16·6	102 (10·2)	100	100	0	2	2
	500	9	+10·0	96 (10·7)	32	0	32	64	100

The following points should be made.

1. The steep slope of the dose-response curve for acetylsalicylic acid is similar to that of reserpine but unlike that of thalidomide (West, 1963); in consequence, acetylsalicylic acid appears to be less likely to produce congenital malformations in the young since a 10-fold increase in dose produced a large increase in lethal action.

2. The smallest dose of acetylsalicylic acid used in the present experiments was equivalent to the maximal B.P. dose used clinically; as it produced a relatively large number of dead foetuses and resorption sites, the question is raised of the possibility of acetylsalicylic acid producing foetal death in humans.

3. Rats fed on the high casein diet containing the largest dose of acetylsalicylic acid continued to increase in weight during gestation whereas those on the high sucrose diet lost weight; there were also relatively more whole foetuses, suggesting that the high protein diet was beneficial and tended to reduce the toxic effects of acetylsalicylic acid, despite the fact that in the absence of acetylsalicylic acid foetuses on the high casein diet were usually smaller than those of the high sucrose diet.

4. The incidence of gastric ulceration was greater in the rats fed on the high sucrose diet (45%) than in those on the high casein diet (22%), again illustrating the beneficial effect of the high protein diet; however, rats which later proved to be non-pregnant showed no toxic effects of acetylsalicylic acid when fed on either of the diets.

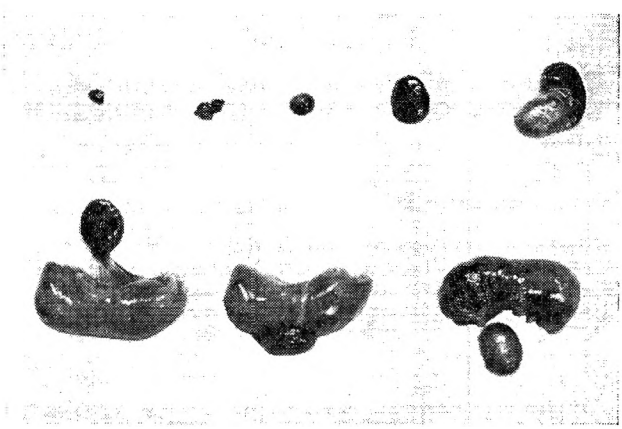

FIG. 2. Some of the contents of the uterus of a pregnant rat after receiving daily doses of acetylsalicylic acid (50 ml/kg) mixed with the high sucrose diet. Animal killed on 20th day of gestation. Note the different stages of disintegration of the foetuses.

5. Acetylsalicylic acid affects the course of pregnancy in rats but it has not been established whether the foetuses are directly damaged or whether the drug acts on the placenta; as foetuses of many sizes were found in some of the rats (Fig. 2), it is probable that the haemorrhagic effect of the drug on the foetuses predominates.

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Mechanism of action of monoamine oxidase inhibitors in enhancing amphetamine toxicity

SIR,—The occurrence of toxic effects in man due to the simultaneous or successive administration of monoamine oxidase (MAO) inhibitors and either amphetamine or methylamphetamine has been reported by several workers (Mason, 1962; Dally, 1962; Hay, 1962). Since these reports Brownlee & Williams (1963a, b) have shown that there is a marked potentiation of amphetamine toxicity in mice after pretreatment with the MAO inhibitor phenelzine.

Amphetamine is known to possess central and peripheral sympathomimetic activities typified by its central nervous system stimulant activity and pressor activity on the cardiovascular system respectively. Fencamfamin (*N*-ethyl-3-phenylbicyclo[2,2,1]hept-2-ylamine hydrochloride,) is also a central sympathomimetic drug which is a chemical analogue of amphetamine and, so far as can be determined, acts on the central nervous system by a similar mechanism to that of amphetamine (Hotovy & others, 1961). By contrast, however, fencamfamin does not possess peripheral sympathomimetic activity. For example, it has no pressor effect even when given in high intravenous doses to anaesthetised cats.

The reason for the increased toxicity of amphetamine in animals previously treated with MAO inhibitors is not known with certainty, but it may be due to an enhancement of the drug's central or peripheral effects or both. If the main effect was central, the previous administration of MAO inhibitors would also be expected to increase the toxicity of fencamfamin, but for a peripheral effect, the toxicity of fencamfamin should not be greatly affected by previous administration of MAO inhibitors. Accordingly it seemed to us that it was desirable to determine:

1) the relative central stimulant activities of amphetamine and fencamfamin, and

2) the acute toxicities of these compounds in normal animals and in animals treated with effective MAO inhibitors.

We report here our findings.

Reserpine-reversal activities of amphetamine and fencamfamin. A severe depressive state, characterised by ptosis, locomotor inactivity, piloerection and hypothermia, was produced in mice by the intravenous injection of reserpine, 1·0 mg/kg. Four hr later, amphetamine (0·5 or 5·0 mg/kg) or fencamfamin (2 or 20 mg/kg) was administered orally to reverse this depression. This reversal was measured quantitatively by determining the rise in body temperature 1 hr

later (maximum antihypothermic effect) using an oesophageal thermocouple and electric thermometer (Brittain & Spencer, 1964) to determine body temperatures. The mean residual hypothermia was calculated for each group by comparison with a group of untreated control mice (Table 1). By plotting the mean residual hypothermia against log dose, it was found that fencamfamin had 34% of the activity of amphetamine.

TABLE 1. ANTIHYPOTHERMIC ACTIVITIES OF AMPHETAMINE AND FENCAMFAMIN IN RESERPINE-TREATED MICE

Treatment	Body temp., °C, 5 hr after reserpine mean ± s.e.*	Hypothermia °C	Relative antihypothermic activity
Untreated control mice	36·86 ± 0·37	—	—
Reserpine only, 1·0 mg/kg i.v.	31·64 ± 0·75	5·22	—
Reserpine i.v. followed 4 hr later with amphetamine orally			
0·5 mg/kg	34·10 ± 0·41	2·76	1·0
or			
5·0 mg/kg	35·48 ± 0·55	1·38	
Reserpine i.v. followed 4 hr later with fencamfamin orally			
2·0 mg/kg	34·34 ± 0·52	2·52	0·34
or			
20 mg/kg	35·62 ± 0·38	1·24	

* Male albino mice, 18–22 g, were used in groups of 5 for each determination.

2. *Effect of* MAO *inhibition on the acute toxicities of amphetamine and fencamfamin.* The oral LD50 values of amphetamine and fencamfamin were determined under non-crowded conditions in untreated mice and in mice pretreated orally 4 or 16 hr previously with an MAO inhibitor. Two dose levels of both a hydrazine (phenelzine) and a non-hydrazine (tranylcypromine) MAO inhibitor were investigated. The results are set out in Table 2. In untreated

TABLE 2. ACUTE ORAL TOXICITIES OF AMPHETAMINE AND FENCAMFAMIN IN MICE PRETREATED WITH MAO INHIBITORS

MAO inhibitor	Pretreatment time hr	Oral dose mg/kg	Oral LD50* amphetamine (95% limits) mg/kg	Oral LD50* fencamfamin (95% limits) mg/kg
Phenelzine	4	none	126 (97·7–163)	55·0 (45·8–66·0)
		20	7·52 (4·50–12·6)	47·5 (39·7–56·8)
		50	5·00 (3·09–8·10)	29·2 (22·5–37·9)
	16	none	89·0 (61·3–129)	58·3 (48·6–70·0)
		20	7·00 (5·47–8·96)	54·8 (43·8–68·6)
		50	9·88 (7·22–13·5)	45·4 (36·6–56·3)
Tranylcypromine	4	none	95·0 (76·6–118)	71·0 (55·9–88·9)
		2	13·7 (10·1–18·6)	68·0 (48·6–95·2)
		5	5·52 (4·18–7·29)	38·2 (30·3–48·1)
	16	none	145 (120–176)	63·5 (49·2–81·9)
		2	20·7 (14·6–29·4)	65·3 (48·3–88·2)
		5	19·3 (14·4–25·9)	56·0 (47·1–66·7)

* Male albino mice, 18–22 g were used in totals of 40–50 for each determination. LD50's (95% fiducial limits) were calculated by the method of Litchfield & Wilcoxon (1949).

mice, fencamfamin is between 1½ and 2 times as toxic as amphetamine. However, the toxicity of amphetamine is markedly increased by both phenelzine and tranylcypromine. In contrast, the toxicity of fencamfamin is unaffected except 4 hr after the larger dose of phenelzine or tranylcypromine, when an approximately 2 fold increase was produced. Under identical conditions, the toxicity of amphetamine was increased some 15 to 25 times. Simultaneously

with these LD50 determinations, mice from the same source and of similar weight range were used in biochemical determinations of brain and liver MAO activity. Both phenelzine and tranylcypromine, at each dose level and after 4 and 16 hr pretreatment produced 85 to 100% inhibition of liver MAO activity, and 90 to 100% inhibition of brain MAO activity.

Conclusions. Both fencamfamin and amphetamine antagonise reserpine-induced depression in mice, fencamfamin having about one-third the potency of amphetamine in this test. However, the toxicity of fencamfamin, unlike that of amphetamine, is not markedly enhanced by previous administration of MAO inhibitors. It seems probable, therefore, that the main cause of the increased toxicity of amphetamine in animals previously treated with MAO inhibitors is due to an enhancement of its peripheral sympathomimetic effects. The toxicity of fencamfamin was increased only by high doses of phenelzine or tranylcypromine. It is difficult to attribute this increase to a specific drug effect and it is probably due to summation of the central and peripheral toxic effects of the two drugs.

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Effect of adrenalectomy on the response of rat skin to an intradermal injection of histamine and 5-hydroxytryptamine

SIR,—The immediate oedema produced in the ears of haematoporphyrine-treated rats by illumination with a small dose of visible light is greater in adrenalectomised rats maintained on saline than in intact animals and is mediated by 5-hydroxytryptamine (5-HT) to a greater extent than by histamine (Ashford, 1963). This fact prompted an investigation into the effect of histamine and 5-HT on capillary permeability in the skin of adrenalectomised and intact rats.

Female albino Wistar rats (140 and 180 g) were bilaterally adrenalectomised through a dorsal incision under ether anaesthesia. Sham-operated and non-operated control animals were included. The room temperature was 27° and adrenalectomised animals drank saline instead of tap water. Histamine acid phosphate and 5-hydroxytryptamine creatinine sulphate were made up in isotonic saline and were injected intradermally into the dorsal, and in some instances abdominal, skin in a volume of 0·1 ml immediately after an intravenous injection of pontamine sky blue (0·1 ml 2%/100 g). Intradermal injections were made on either side, and well clear of the mid-line. The rats were killed 15 min later, and the diameter of the wheals was measured on the underside of the skin by means of calipers. The results (Table 1) show that neither saline nor histamine were more effective whealing agents in adrenalectomised than in intact rats with

TABLE 1. THE MEAN DIAMETER OF WHEALS INDUCED BY AN INTRADERMAL INJECTION OF SALINE, HISTAMINE AND 5HT IN INTACT AND ADRENALECTOMISED RATS

Treatment	No. days after surgery	No. rats/ group	Mean diameter of wheals ± s.e.				
			Saline 0·1 ml	Histamine 0·5 μg	Histamine 1·0 μg	5-HT 0·05 μg	5-HT 0·01 μg
Dorsal skin							
Adrenalectomised	7	8	—	9·0±0·4	11·1±1·1	11·1±0·6	13·1±0·7
Sham-operated	7	8	—	9·3±0·5	11·6±1·1	9·8±0·7	12·1±0·5
Adrenalectomised	8	3	8·3±1·0	—	—	9·7±1·2	15·3±0·4
Sham-operated	8	3	8·8±0·7		—	10·3±0·4	13·0±0·8
Adrenalectomised	10	8	9·4±1·0†	10·8±0·4	12·4±1·0	11·6±0·5	13·3±0·7
Sham-operated	10	8	9·4±1·1†	10·9±0·6	11·4±0·5	10·5±0·5	13·8±0·3
Non-operated	10	8	9·3±0·8†	11·1±0·5	11·3±0·6	10·5±0·9	13·1±0·6
Adrenalectomised	13	9	8·6±0·8	—	—	9·1±0·6	15·3±0·5***
Sham-operated	13	11	8·6±0·7	—	—	8·8±0·4	12·5±0·6
Adrenalectomised	13	7	10·1±1·2†	10·9±0·4	11·7±0·8	11·4±0·6	13·3±0·4
Sham-operated	13	7	9·8±0·3†	11·5±0·5	11·6±0·2	12·6±0·5	13·9±0·8
Non-operated	13	7	9·4±0·6†	11·4±0·4	11·8±0·4	12·6±0·4	13·9±0·2
Abdominal skin							
Adrenalectomised	3	10	—	10·7±0·4	15·9±0·5*	—	—
Sham-operated	3	10	—	10·0±0·5	13·9±0·7	—	—
Adrenalectomised	13	7	5·1±1·9	10·4±0·8	11·2±0·4	11·9±0·6	14·9±0·4**
Sham-operated	13	7	3·5±1·7	12·2±0·5	11·9±0·6	11·8±0·2	14·1±0·4
Non-operated	13	7	2·4±1·5	9·6±1·0	10·1±0·6	10·8±0·3	13·1±0·6

* $P < 0.05$
** $P < 0.05$—compared with non-operated controls.
*** $P < 0.01$.
†These groups contained 1 rat less than the stated number.

the exception of one experiment in abdominal skin in which 1·0 μg of histamine induced a larger wheal in the adrenalectomised group ($P < 0.05$). However, the low dose of histamine was not more active in these animals. A slight increase in the effectiveness of 5-HT was seen 13 days after adrenalectomy in dorsal skin ($P < 0.01$) in one experiment and in abdominal skin in another ($P < 0.05$). However, the potentiation in dorsal skin was not confirmed and in abdominal skin it was apparent only in relation to non-operated controls there being no difference between adrenalectomised and sham-operated animals.

The increased susceptibility of photosensitised rats to light after adrenalectomy may be due to, either, the release of an increased amount of histamine and 5-HT from storage sites in the skin, or, a lowered ability of skin to inactivate and remove the released amines, or, an increase in sensitivity of the skin capillaries to histamine and 5-HT (Spencer & West, 1962).

From the present results increased sensitivity to light is unlikely to be wholly due to a greater effect of histamine and 5-HT on skin capillaries and may be mainly due to an increase in the amount of histamine and 5-HT available for release and possibly to a reduction in the rate of inactivation of 5-HT in skin.

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