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Mechanism of amphetamine accumulation in the isolated perfused heart of the rat

H. THOENEN, A. HÜRLIMANN AND W. HAEFELY

Rat hearts were perfused with 10 to 1,000 ng/ml of (±)-[^{3}H]amphetamine. The time course of accumulation and the maximal tissue/medium ratio (T/M) were identical for all concentrations studied. The maximal T/M varied between 5 and 6 and was reached after 5 min of perfusion. The accumulation of amphetamine was not inhibited by cocaine or noradrenaline. It was not impaired by combined inhibition of aerobic and anaerobic energy metabolism and it was not dependent on the intactness of sympathetic nerve endings, indicating that the greater amount of amphetamine was located extraneuronally. The diminished accumulation of amphetamine following reduction of temperature or sodium concentration of the perfusion fluid most probably reflects impaired tissue perfusion resulting from vascular constriction. The time course of accumulation and decay of amphetamine is compatible with a rapidly reversible phase-distribution of this amine possibly related to its relatively high lipophilic properties. The possible significance of phenolic hydroxyl groups in membrane transport and diffusion of phenethylamines is discussed.

THERE is strong evidence that amphetamine, like many other phenethylamines, exerts its sympathomimetic effect mainly by liberating noradrenaline from sympathetic nerve endings (Fleckenstein & Stöckle, 1955; Burn & Rand, 1958; Trendelenburg, Muskus & others, 1962; Haefely, Hürlimann & Thoenen, 1964). In addition to this indirect sympathomimetic effect these substances inhibit the uptake of noradrenaline into sympathetically innervated organs (Hertting, Axelrod & Whitby, 1961; Iversen, 1964a). Several of them have been shown to interfere with the membrane transfer of noradrenaline by being transported themselves and possessing kinetic uptake properties very similar to those of noradrenaline (Carlsson & Waldeck, 1965; Iversen, 1966). Thus the question arises whether amphetamine inhibits noradrenaline uptake in a similar manner or whether it interferes with noradrenaline uptake without being transported itself. The accumulation of amphetamine and its uptake kinetics were therefore studied in the isolated perfused heart of the rat.

Experimental

METHODS

Charles-River male rats weighing 180 to 210 g were injected intraperitoneally with 2,000 units of heparin. 5 to 10 min later the animals were killed by cervical dislocation. The hearts were removed and perfused by the Langendorff technique at a constant rate of 10 ml/min at 37° with the following modified Krebs-Henseleit solution: NaCl 5·54 g, KCl 0·354 g, KH_2PO_4 0·163 g, $MgSO_4.7H_2O$ 0·294 g, $CaCl_2$ 0·282 g, $NaHCO_3$ 2·1 g, Na-pyruvate 0·542 g, Na-fumarate 0·474 g, Na-l-glutamate 0·416 g, dextrose 2·08 g, ethylenediamine tetra-acetic acid disodium salt 0·01 g, ascorbic acid 0·01 g in 1,000 ml. At this perfusion

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rate the perfusion pressure varied between 50 and 70 mm Hg, the heart rate between 150 and 200 beats/min. Usually the perfusion pressure was checked only at the end of the equilibration period to be certain that it was within this range. In a limited number of experiments in which the sodium concentration or the temperature of the perfusion medium was reduced, the perfusion pressure was recorded throughout the experiment. The hearts could be perfused by either one of two independent perfusion systems connected to the perfusion cannula by a three-way stopcock. One of the two systems contained (±)-amphetamine in concentrations of 10 to 1,000 ng/ml; irrespective of the total concentration of amphetamine, all solutions contained 0·15 μc/ml of (±)-[^{3}H]amphetamine.

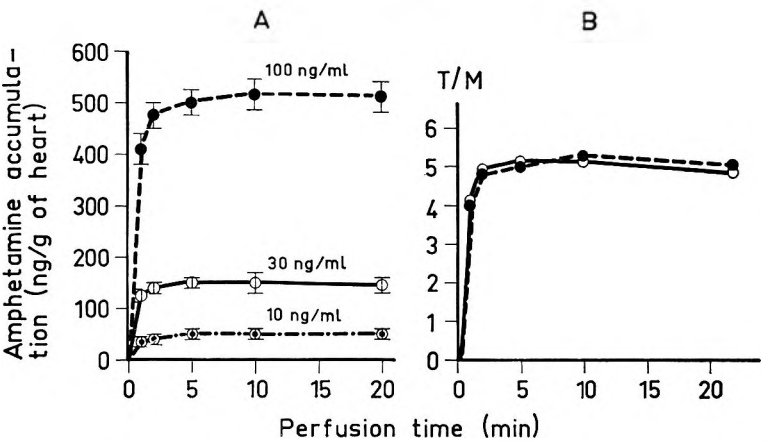

FIG. 1A and B. Time course of amphetamine accumulation in the isolated perfused rat heart. T/M = tissue/medium ratio. In Fig. 1B only the values for 30 and 100 ng/ml of amphetamine are given since those for 10 ng/ml lay between the two other concentrations. Each value in Figs 1–6 is the mean ± standard error of 4–5 experiments.

After an initial perfusion period of 2 to 3 min with amphetamine-free solution, the hearts were perfused for 1, 2, 5, 10 or 20 min with amphetamine solutions of different concentrations (10 to 1,000 ng/ml). At the end of the perfusion, the hearts were blotted, frozen in light petroleum (b.p. 90–120°) at −80°, weighed and homogenized in 5 ml of 0·4 N $HClO_4$. After centrifugation the radioactivity of the supernatant was determined in a liquid scintillation counter (Mark I, Nuclear-Chicago) using Bray's solution. The amphetamine content of the hearts was expressed in ng/g wet weight, corrected for the recovery of (±)-[^{3}H]amphetamine added to homogenates of hearts perfused without amphetamine. No corrections were made for the extracellular space.

In two series of experiments the effect of cocaine and noradrenaline on the accumulation of amphetamine was examined. The two substances were infused into a rubber tube placed immediately ahead of the perfusion cannula during the entire duration of the experiment, i.e. as early as during the initial equilibration phase with amphetamine-free solution.

For "chemical sympathectomy" rats were injected intravenously with four doses of 20 mg/kg of 6-hydroxydopamine (Tranzer & Thoenen, 1967) given over a period of 48 hr. On account of the extreme susceptibility to oxidative degradation at neutral and alkaline pH it was necessary to dilute 6-hydroxydopamine in 0·001 N hydrochloric acid.

Isolation and chromatographic identification of amphetamine and its possible metabolites formed in hearts during perfusion with (±)-[^{3}H]-amphetamine were as described previously (Thoenen, Hürlimann & others, 1966).

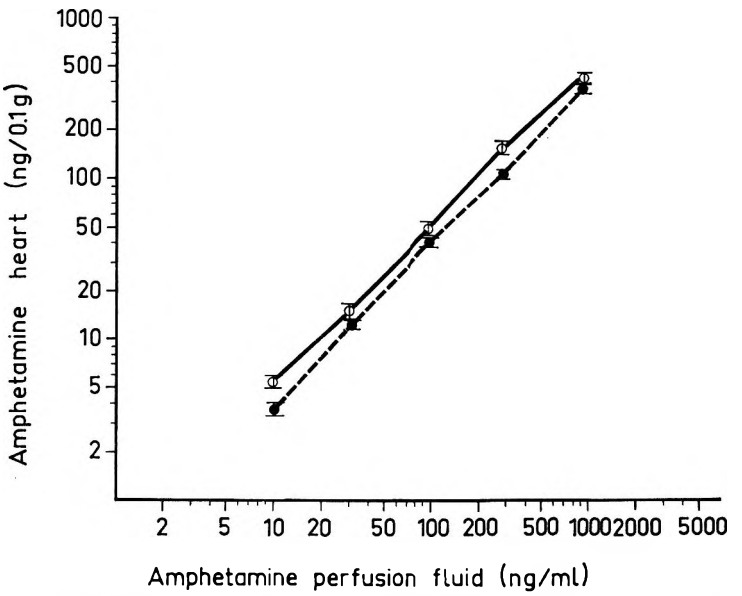

FIG. 2. Relationship between the concentration of amphetamine in the perfusion fluid (ng/ml) and its accumulation in the heart tissue (ng/0·1 g) after 1 (●— —●) and 20 (O——O) min of perfusion.

Drugs used: (−)-noradrenaline (Arterenol, Hoechst), cocaine hydrochloride, 6-hydroxydopamine hydrobromide and (±)-[^{3}H]amphetamine sulphate (tritiated in *ortho*-position, activity 15 mc/mg). 6-Hydroxydopamine was synthesized by Dr. M. Scheer, tritiated amphetamine by Dr. J. Würsch and Dr. H. Bruderer of the Chemical Research Department of Hoffmann-La Roche & Co. Ltd., Basle. All doses refer to the base.

Results

METABOLISM OF AMPHETAMINE IN THE RAT ISOLATED PERFUSED HEART

The labelled compounds present in homogenates of rat hearts perfused for 20 min with 30 ng/ml of amphetamine were separated on Dowex-50 columns into acidic and basic fractions. The entire activity was found in the latter fraction and its chromatographic analysis revealed that the total activity was confined to the position of amphetamine. In particular, no

activity could be detected at the position of norephedrine (β-hydroxylated amphetamine), *p*-hydroxyamphetamine and its β-hydroxylated derivative *p*-hydroxynorephedrine, metabolites found in cat spleen and heart after pretreatment with (±)-[^{3}H]amphetamine (Thoenen & others, 1966). The activity determined in homogenates of rat hearts will therefore be expressed in terms of amphetamine.

TIME COURSE AND DOSE DEPENDENCE OF AMPHETAMINE ACCUMULATION

As shown in Fig. 1, the time course of amphetamine accumulation in isolated perfused rat hearts was virtually identical for amphetamine concentrations of 10, 30 and 100 ng/ml. After 1 min of perfusion, the amphetamine content of the hearts (expressed in ng/g wet weight) was about 4 times higher than the concentration in the perfusion fluid (ng/ml), and there was only a small further increase in the following minutes.

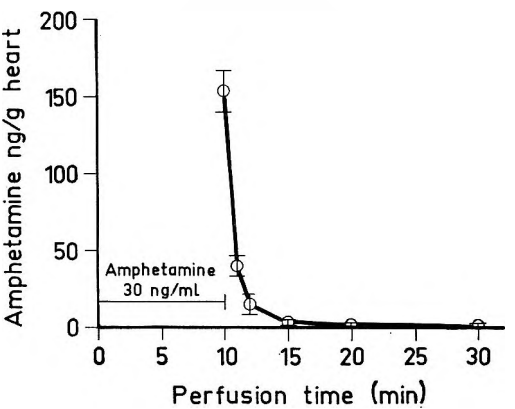

FIG. 3. Decay of the amphetamine content in rat hearts. The hearts were first perfused for 10 min with 30 ng/ml of amphetamine, followed by perfusion without amphetamine for 1, 2, 5, 10 and 20 min.

The maximal T/M was reached after 5 min and remained at this level as long as 20 min. In the dose range studied (10 to 1,000 ng/ml) the amphetamine accumulation revealed no signs of saturation which accords with observations on brain slices (Ross & Renyi, 1966b). There is a statistically significant ($P < 0.05$) linear correlation between the amphetamine concentrations in the perfusion fluid and its accumulation in heart tissue both for 1 and 20 min of perfusion (Fig. 2). The slope of the regression line is 1·01 for the 1-min values and 1·07 for the 20-min values.

ELIMINATION OF AMPHETAMINE ACCUMULATED IN RAT HEARTS

In a further series of experiments the hearts were perfused during 10 min with 30 ng/ml of amphetamine. The amphetamine content of the hearts at the end of this perfusion period was compared with that after continuation of perfusion for 1, 2, 5, 10 or 20 min without amphetamine. The time course of the decay (Fig. 3) is virtually identical to that of accumulation during perfusion with 30 ng/ml of amphetamine (Fig. 1).

EFFECT OF COCAINE AND NORADRENALINE ON AMPHETAMINE ACCUMULATION

In a series of experiments the hearts were perfused with 10 μg/ml of cocaine, a concentration which almost completely abolishes the noradrenaline uptake into the isolated perfused rat heart (Iversen, 1964b). The effect on amphetamine accumulation, however, was very small (Fig. 4A) and there was no statistically significant ($P > 0.05$) difference between the amount of amphetamine accumulating in hearts perfused with and without cocaine. These results agree with the observation on mouse heart slices published by Ross & Renyi (1966a) while our experiments were in progress.

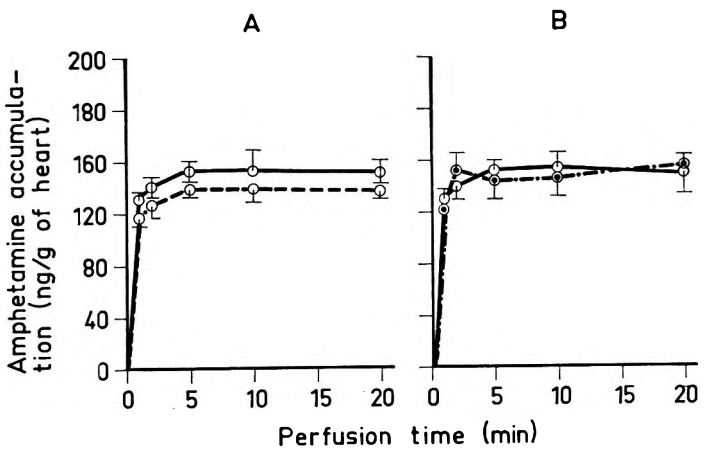

FIG. 4. The effect of (A) cocaine (10 μg/ml) and (B) noradrenaline (0·1 μg/ml) on the accumulation of amphetamine in the isolated perfused rat heart. The concentration of amphetamine was 30 ng/ml in all experiments. Control O——O; cocaine O — — O; noradrenaline O— · —O.

As amphetamine interferes with the uptake of noradrenaline into the isolated perfused rat heart, we studied the effect of noradrenaline on amphetamine accumulation to decide whether this interference is a mutual one. As shown in Fig. 4B, the presence of 100 ng/ml of (−)-noradrenaline had no statistically significant ($P > 0.05$) effect on amphetamine accumulation, although the affinities of (±)-amphetamine and (−)-noradrenaline for noradrenaline uptake sites are very similar (Iversen, 1964a).

DEPENDENCE ON TEMPERATURE, SODIUM CONCENTRATION AND ENERGY METABOLISM

The time course of amphetamine accumulation is very similar at 37, 30 and 20° (Fig. 5A). However, the maximal T/M reached after 5 min of perfusion showed a clear-cut temperature dependence and was reduced to 69% at 20° and to 84% at 30° (37° = 100%).

The transport of noradrenaline has been found to be sodium dependent (Iversen & Kravitz, 1966), the uptake being diminished in proportion to the sodium reduction in the perfusion fluid. A reduction of the sodium

concentration to 50% (correction of osmolarity by sucrose) resulted in a reduction of the heart rate from 185 ±3 to 74 ±3, whereas the perfusion pressure remained within the normal range. The accumulation of amphetamine did not differ significantly ($P > 0.05$) from that of controls (Fig. 5B). If the sodium concentration was further reduced to 25% the hearts contracted only sporadically and the perfusion pressure regularly exceeded the control range of 50 to 70 mm Hg. The accumulation of amphetamine amounted to about 70% of that of controls after 5 min of perfusion with 30 ng/ml of amphetamine.

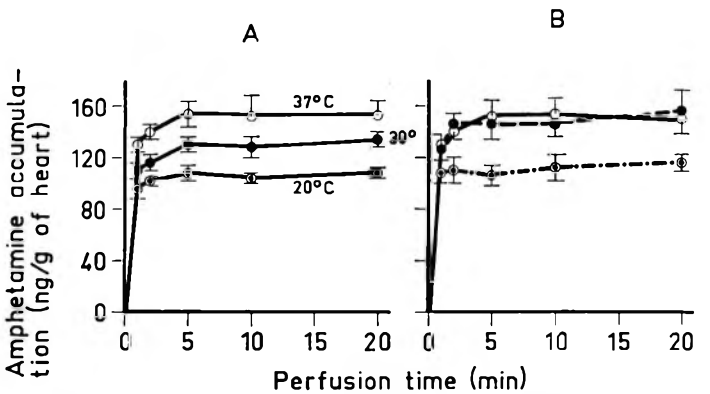

FIG. 5. Dependence of amphetamine accumulation on temperature (A) and sodium concentration (B) control O——O; 1/2 sodium ●— —●; 1/4 sodium ⊙— · —⊙.

Wakade & Furchgott (1966) have shown that both anoxia and glucose deprivation are necessary to block noradrenaline uptake into isolated atria of guinea-pigs, whereas alone neither was effective. If iodoacetate (2×10^{-4}M) was added to the perfusion fluid, saturated with nitrogen 95% and carbon dioxide 5%, the hearts stopped beating after 2 to 3 min. The perfusion pressure remained within the normal range. In these experiments the perfusion with amphetamine (30 ng/ml) was preceded by a perfusion period of 10 min with amphetamine-free medium. As shown in Fig. 6, impairment of the glycolytic and aerobic energy metabolism did not diminish the accumulation of amphetamine in heart tissue. On the contrary the accumulation was even larger than under control conditions. The difference, however, was statistically significant ($P < 0.05$) only for 2 min of perfusion with 30 ng/ml of amphetamine.

EFFECT OF CHEMICAL SYMPATHECTOMY

6-Hydroxydopamine has been shown to produce an efficient and extremely long-lasting noradrenaline depletion in various species (Porter, Totaro & Stone, 1963; Stone, Stavorski & others, 1963; Laverty, Sharman & Vogt, 1965). Recent electronmicroscopic studies revealed that this particular "noradrenaline depletion" was due to selective destruction of sympathetic nerve endings (Tranzer & Thoenen, 1967). 6-Hydroxydopamine therefore provides a unique tool for chemical sympathectomy.

To study whether the accumulation of amphetamine is linked to functionally intact sympathetic nerve endings, as is so for noradrenaline (Hertting, Axelrod & others, 1961; Hertting & Schiefthaler, 1964; Iversen, Glowinski & Axelrod, 1966), we examined the effect of pretreatment with 6-hydroxydopamine. The rats were injected intravenously with four doses of 20 mg/kg of 6-hydroxydopamine given over a period of 48 hr. The perfusion experiments were made 10 days after the last dose. At this time, the noradrenaline content was reduced to about 10% of that of controls and electronmicroscopic examination revealed alterations of sympathetic nerve endings (Tranzer, unpublished results) similar to those observed in various organs of the cat (Tranzer & Thoenen, 1967). The heart rate of these pretreated preparations was lower (133 ±7)

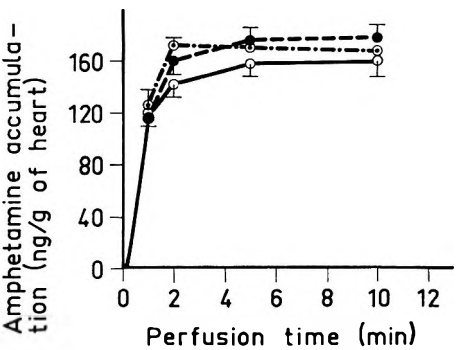

FIG. 6. Dependence of amphetamine accumulation on aerobic and anaerobic energy metabolism and intactness of sympathetic nerves. Control O——O; iodoacetate (2×10^{-4}M) and saturation of perfusion fluid by 95% N_2 and 5% CO_2 ⊙— · —⊙; hearts of rats pretreated with 4×20 mg/kg 6-hydroxydopamine (chemical sympathectomy) ●— —●.

than that of controls (185 ±3), whereas the perfusion pressure was within the normal range. As evident from Fig. 6, the amphetamine accumulation was even somewhat larger than in control hearts, indicating that amphetamine is not selectively accumulated in sympathetic nerves as in the case of noradrenaline.

RELATIONSHIP BETWEEN PERFUSION PRESSURE AND AMPHETAMINE ACCUMULATION

In the preceding experiments in which the sodium concentration or the temperature of the perfusion fluid was reduced, we came to suspect the possibility of a relationship between the increase of perfusion pressure and the diminution of amphetamine accumulation in the perfused hearts.

Since the perfusion pressure was not continuously recorded but checked only during the equilibration phase, an additional series of experiments was undertaken in which the perfusion pressure was measured during the whole duration of the experiments and in which the sodium concentration was further reduced to 12·5% and the temperature to 10°. The hearts

were equilibrated for 5 min with amphetamine-free solution and then perfused for 2 min with 30 ng/ml of amphetamine.

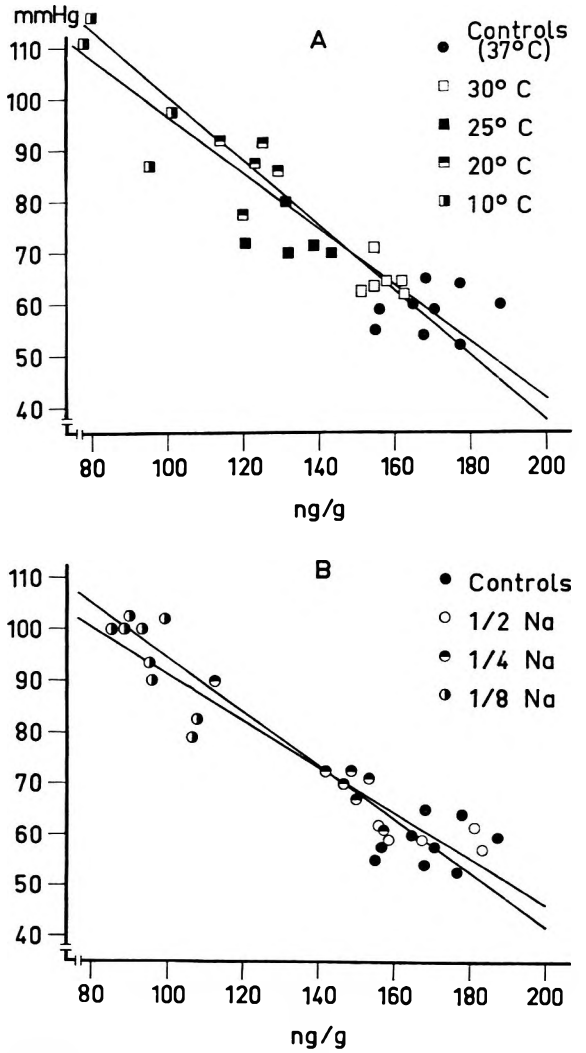

FIG. 7. Relationship between perfusion pressure and amphetamine accumulation in the isolated perfused rat heart for changes in temperature (A) and changes in sodium concentration (B). The hearts were perfused at a constant rate of 10 ml/min with 30 ng/ml of amphetamine. There is a significant correlation ($P < 0{\cdot}001$) between perfusion pressure and the amount of amphetamine accumulating in the heart both for changes in temperature (A) and sodium concentration (B). Each value is the result of a single experiment.

After 2 to 3 min of perfusion with amphetamine-free solution, the perfusion pressure equilibrated and was not changed by switching to the amphetamine solution. As evident from Fig. 7, there is a significant

($P < 0.001$) correlation between the height of perfusion pressure and the amount of amphetamine accumulating in the hearts both for changes in temperature and in sodium concentration.

Discussion

The uptake of noradrenaline into the isolated perfused rat heart has been shown to be mediated by a stereochemically specific mechanism which exhibits the properties of an active membrane transport (Iversen, 1963; Iversen & Kravitz, 1966). The complexity of the biological systems usually studied does not permit direct proof, and the assumption of an active transport is therefore based in most cases on dependence on temperature and energy metabolism, uphill transport and saturation kinetics.

The present study has shown that in the rat isolated perfused heart amphetamine, which interferes with the uptake of noradrenaline (Iversen, 1964a), is not accumulated in the same manner as noradrenaline. The accumulation of amphetamine was not inhibited by cocaine or noradrenaline. It was not impaired by combined inhibition of aerobic and anaerobic energy metabolism. It did not obey the principles of saturation kinetics. The time course of accumulation and decay after subsequent perfusion without amphetamine suggests instead a rapidly reversible phase-distribution, possibly related to the relatively high lipophilic properties of amphetamine. The distribution coefficient between benzene and Krebs-Henseleit solution (pH 7·4) is 0·212 for amphetamine and 0·004 for noradrenaline. The corresponding values for heptane and Krebs-Henseleit solution are 0·043 and <0.001 respectively (unpublished results). Both accumulation and decay curves are complex and do not fulfil the requirements of a first order reaction which might be taken as evidence that equilibration takes place between several compartments.

The dependence of amphetamine accumulation on temperature and sodium concentration could be interpreted as favouring an active membrane process. However, the fact that the quantity of amphetamine which accumulates in the rat heart is inversely correlated with the height of the perfusion pressure (Fig. 7) makes it more probable that the diminished accumulation of amphetamine reflects impaired tissue perfusion resulting from vascular constriction provoked by a reduced sodium concentration or a reduction of temperature.

Perhaps the most important difference between the accumulation of noradrenaline and amphetamine is the lack of dependence of the latter on the intactness of sympathetic nerves. Indeed no difference was found between the content of amphetamine in hearts with functionally intact sympathetic nerves and those which were chemically denervated by treatment with 6-hydroxydopamine. Thus the bulk of amphetamine accumulating in the heart must be located extraneuronally. It cannot be determined from the present results whether cardiac sympathetic nerves are capable of concentrating amphetamine to a greater degree than the extraneuronal tissue. However, an accumulation of amphetamine in

sympathetic nerves of the same order of magnitude as that found after perfusion with noradrenaline can be excluded. After 30 min of perfusion with 10 ng/ml of (±)-[^{3}H]noradrenaline the tissue/medium ratio (T/M) is about 40 (Iversen, 1963). Since noradrenaline is accumulated almost exclusively in adrenergic nerves (Hertting & others, 1961; Hertting & Schiefthaler, 1964; Iversen & others, 1966) and since the volume of nerve terminals represents only a minute part of the total heart tissue the ratio nerve terminal/perfusion medium for noradrenaline must be even much higher. If amphetamine were accumulated in adrenergic nerve terminals to a similar extent the T/M should be much higher in innervated than in chemically denervated hearts despite additional extraneuronal accumulation.

The lack of significant accumulation of amphetamine in adrenergic nerve terminals, however, does not permit the definite conclusion that it is not transported by the noradrenaline transfer mechanism. A marked uptake of amphetamine could be masked by a rapid outward diffusion along the concentration gradient. In this context it is interesting to recall that α-methyltyramine which differs from amphetamine only by a phenolic hydroxyl group in the *para*-position is very efficiently accumulated by the isolated perfused rat heart (Iversen, 1966) the T/M being 10 after 5 min perfusion with 1 ng/ml. In contrast to amphetamine this amine is concentrated selectively in sympathetic nerves (Iversen & others, 1966) and since it is not stored in the granular vesicles (Kopin, 1966), it must be present to a large extent in free diffusible form in the axoplasm of the sympathetic nerve endings.

The differences between the accumulation of amphetamine and α-methyltyramine are open to the interpretation that at least one phenolic hydroxyl group is a prerequisite for the transportation of phenethylamines by the noradrenaline transfer system and that amphetamine, though able to block this mechanism, is not itself transported. In consequence, the interference of cocaine with the sympathomimetic action of amphetamine (Fleckenstein & Stöckle, 1955; Burn & Rand, 1958) would not be due to inhibition of its active membrane transfer but possibly to interference with its effect on intraneuronal storage sites from which noradrenaline is liberated. However, it may equally well be assumed that the phenolic hydroxyl group of phenethylamines is not essential for their membrane transport into the sympathetic nerves but that this group changes their physico-chemical properties in such a way that their diffusion through the lipid membrane along the concentration gradient is impaired. Thus, in spite of an active transport an accumulation of amphetamine in sympathetic nerve endings would not occur because of its rapid passive outward diffusion.

Acknowledgement. The authors wish to thank Mr. W. J. Ziegler for the statistical evaluation.

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The metabolism of 5,5′-methylenedisalicylic acid in various species

CLARKE DAVISON* AND R. T. WILLIAMS

Commercial methylenedisalicylic acid has been shown to be grossly impure. Pure 5,5′-methylenedisalicylic acid (4,4′-dihydroxydiphenylmethane-3,3′-dicarboxylic acid) has been prepared and labelled with ^{14}C. The fate of the pure compound in the rat, mouse, hamster, rhesus monkey, rabbit, guinea-pig and chicken has been investigated. The compound is excreted entirely unchanged in the urine and faeces in all the above species and no metabolites have been found. The biliary excretion of the injected compound is high (50–60%) in the rat and dog and low (5%) in the guinea-pig and rabbit. In the monkey, rabbit and guinea-pig, the compound is excreted almost exclusively in the urine. In the rat about 50% of the dose is excreted in the faeces. In the mouse and hamster, the main route of excretion is the urine, about 10% appearing in the faeces.

THE compound 5,5′-methylenedisalicylic acid (4,4′-dihydroxydiphenylmethane-3,3′-dicarboxylic acid) has been used in the synthesis of triarylmethane dyes and corrosion protectives. It forms complexes with drugs such as phenacetin, theophylline and prednisolone (Higuchi & Pisano, 1964) and salts with organic bases. A salt of considerable interest is bacitracin methylenedisalicylate (Siminoff, Price & Bywater, 1953) which is widely used as an animal feed supplement designed to enhance growth and prevent disease in poultry, swine and mink. Whilst this salt and the acid itself have received toxicological testing (Radomski, Hagan & others, 1954), essentially no studies have been made of the metabolism of the acid. Simon (1962) using a non-specific colorimetric assay reported that 30% of an oral dose of piperidine methylenedisalicylate was excreted in the urine in three days by rabbits.

We find commercial samples of methylenedisalicylic acid and samples synthesized by standard methods to be grossly impure. Purified samples of [^{14}C]methylenedisalicylic acid are not metabolized but excreted unchanged in the urine and faeces of a number of animal species. The species differences in biliary excretion of the acid are similar to those reported by Abou-El-Makarem, Millburn & others (1967a,b) for other compounds.

Experimental

4,4′-Dihydroxydiphenylmethane-3,3′-dicarboxylic acid. Samples of this acid (methylenedisalicylic acid) of m.p. varying from 225–240° (decomp.) were obtained commercially (S. B. Penick, New York; Hopkin & Williams Ltd., Essex) and by preparation from salicylic acid and formaldehyde or paraformaldehyde (e.g. Clemmensen & Heitman, 1911; Kahl, 1898). All these samples were grossly impure and on paper chromatography at least 8 spots, one of which was salicylic acid, were seen by their blue

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fluorescence in ultraviolet light or by the colour given with 1% aqueous ferric chloride. Recrystallization of this material from various solvents or column chromatography on a variety of packings failed to give a product of the desired purity.

Pure samples of methylenedisalicylic acid were eventually obtained by chromatographing 100 mg samples of the crude acid as a band on Whatman No. 3MM paper or on a thin-layer (1/16 in) of Silica Gel G (Merck) without binder, using isopropanol–5N ammonia solution (10:3 v/v) as solvent. The band of Rf 0·6 was extracted from the paper or silica with N ammonia solution. The extract was acidified with 2N hydrochloric acid and the methylenedisalicylic acid extracted with ether. It was purified by adding water to a hot acetone solution from which it formed colourless needles (yield 45–70% of crude material). The 4,4′-dihydroxydiphenylmethane-3,3′-dicarboxylic acid had m.p. 255–257° (decomp.) [Smith, Sager & Siewers (1949) give m.p. 247° (decomp.).] [Found: C, 62·6; H, 4·2%; *M* 266 (Rast), 305 (isothermal dist.); equiv.; 147 (titration). Calc. for $C_{15}H_{12}O_6$; C, 62·5; H, 4·2%; *M* 288; equiv.; 144.] The apparent pK_a by titration was about 3·5. On acetylation, the diacetyl derivative, m.p. 146–149°, was obtained (Clemmensen & Heitman (1911) give m.p. 142°).

4,4′-Diaminodiphenylmethane (Koch-Light & Co. Ltd., Colnbrook, Bucks) was converted into 4,4′-dihydroxydiphenylmethane, m.p. 158–160° according to Haase & Moyat (1894). This phenol showed on Whatman No. 1 paper, Rf values of 0·65 with 2% aqueous sodium carbonate, 0·80 with 20% aqueous acetic acid and 0·71 with benzene–acetic acid–water (100:32:0·5 by vol.) as solvents. On fusing a little pure methylenedisalicylic acid with potassium hydroxide according to Clemmensen & Heitman (1911), a small amount of material was obtained which was chromatographically identical in the above three solvents with 4,4′-dihydroxydiphenylmethane. 5,5′-Methylenedisalicylic acid was further characterized by conversion to aurin tricarboxylic acid with salicylic acid and nitrous acid according to Smith & others (1949). The aurin tricarboxylic acid behaved chromatographically on paper identically with an authentic sample (British Drug Houses Ltd., Poole, Dorset).

TABLE 1. PARTITION OF RADIOACTIVITY IN CRUDE [^{14}C]METHYLENEDISALICYLIC ACID BY THIN-LAYER CHROMATOGRAPHY. See text for details of solvent and supporting medium. The values given are the mean for four plates.

Rf	% of ^{14}C	Specific activity μc/mg
Above 0·6	0·1	—
0·6*	42·9	0·57
0·45†	34·0	0·75
0·1–0·2 (2 bands)	17·7	0·71
0·0–0·1 (3 bands)	5·6	0·64

* 5,5′-methylenedisalicylic acid.
† Trimeric form, see text.

The major contaminant (Rf 0·45; see Table 1) occurred to the extent of up to 35% of the crude methylenedisalicylic acid. This was isolated as above and crystallized from acetone–water mixtures as needles which

softened at 264° and decomposed at 272°. This was probably a trimeric form derived from three salicylic acid molecules. [Found: C, 62·2; H, 4·3%; *M* 509 (isothermal dist.). Calc. for $C_{23}H_{18}O_9$; C, 63·0; H, 4·1%; *M* 438.] Several other impurities at lower Rf values amounted to 25%, whereas free salicylic acid was only 3% of the crude material.

4,4'-*Dihydroxydiphenyl*[^{14}*C*]*methane*-3,3'-*dicarboxylic acid.* Salicylic acid (1·05 g; 0·076 mole) and [^{14}C]paraformaldehyde (108·9 mg; 0·036 mole; 500 μc; Radiochemical Centre, Amersham) were dissolved in glacial acetic acid (1·4 ml) by warming at 95° for 5 min in a filtration tube (20 ml) fitted with a separatory funnel. The side arm of the filtration tube was connected to successive dinitrophenylhydrazine and silver nitrate solution traps to collect any [^{14}C]formaldehyde. A mixture of conc. sulphuric acid and glacial acetic acid (0·4 ml, 1 : 5 by vol.) was introduced and the mixture left in a boiling water bath for 3 hr. Air was sucked through the traps and hot distilled water (100 ml) then added to the reaction tube. After cooling, the crude [^{14}C]methylenedisalicylic acid was filtered, washed with water three times and dried (yield 0·76 g, 73%). The crude [^{14}C]acid in quantities of 78–80 mg was chromatographed on thin-layer silica gel plates and the pure [^{14}C]acid (Rf 0·6) separated as before. The average recoveries from four plates of methylenedisalicylic acid and other products are shown in Table 1.

ANIMALS

The animals used were Wistar albino rats, rhesus monkeys, European hamsters, albino mice (I.C.I. strain), New Zealand White rabbits, Abyssinian guinea-pigs, mongrel dogs and Light Sussex hens. These animals had free access to water and suitable food.

The methylenedisalicylic acid was administered as the sodium salt in water. Biliary cannulation of the animals was performed by Dr. M. M. Abou-El-Makarem (St. Mary's Hospital Medical School).

DETERMINATION OF ^{14}C

A Packard Tri-Carb scintillation counter (Model 3204) and a Packard radiochromatogram scanner (Model 7200) were used. For the assay of urine, bile and other clear solutions, the scintillator used was that of Bray (1960) (POPOP/PPO/dioxan). For plasma and homogenates of tissues and faeces in dioxan-methanol (1 : 1 by vol.), a thixotropic gel (Cab-O-Sil, Packard Instrument Co.) was added to the above scintillator solution.

CHROMATOGRAPHY

Urine and various extracts were chromatographed on Whatman No. 3MM paper or thin-layer silica gel as referred to earlier. The solvent systems used were isopropanol–5N ammonia solution (10 : 3 by vol.) or phenol–water (4 : 1 by vol.). For detecting possible metabolites on chromatograms, 1% aqueous ferric chloride and Gibbs reagent (dichloroquinonechloroimide) was used for phenols, naphthresorcinol for glucuronides, and *p*-dimethylaminobenzaldehyde in acetic anhydride for glycine conjugates. Since no metabolite of methylenedisalicylic acid

is formed *in vivo* further details are omitted. Methylenedisalicylic acid itself is readily detected on chromatograms by its blue fluorescence in ultraviolet light and Rf value.

DETERMINATION OF METHYLENEDISALICYLIC ACID BY ISOTOPE DILUTION

To a sample of radioactive urine (enough to contain 0·05–0·2 μc) was added 0·8 g of pure methylenedisalicylic acid. The mixture was made alkaline with sodium hydroxide and filtered. The methylenedisalicylic acid was precipitated by acidification and this process was repeated eight times on each sample. Dissolution in acetone and precipitation with water was also used as an alternative method of purification. Constant specific activity was usually attained after four precipitations by either method.

DETERMINATION OF THE PLASMA HALF-LIFE OF METHYLENEDISALICYLIC ACID

The [^{14}C]acid (10 mg/kg) was injected intravenously into rats, dogs, guinea-pigs or rabbits. Plasma samples were prepared every 5–10 min for the first hr and subsequently at longer intervals. The ^{14}C of each sample was determined by scintillation counting. No radioactive compound other than methylenedisalicylic acid was found in the plasma.

TABLE 2. THE EXCRETION OF [^{14}C]METHYLENEDISALICYLIC ACID IN RATS. The dose of methylenedisalicylic acid was 10 mg/kg and of ^{14}C 5·8 μc/kg. The compound was administered as the sodium salt in 0·21 ml of water. The excreta of the animals in each group were pooled for analysis.

	Males	Females	Males
Number of rats used	9	3	6
Weight, mean, g	280	235	295
Route of administration	oral	oral	i.p.
	% of dose of ^{14}C excreted* in		
Urine, day 1	25·3	29·0	55·4
2	7·3	10·3	2·4
3	1·6	0·0	0·1
Total	34·2†	39·3	57·9
Faeces, day 1 and 2	50·2	48·6	30·3
Total excreted	84·4	87·9	88·2

* In a separate experiment it was shown that in female rats receiving the above dose of [^{14}C]methylenedisalicylic acid, the amount of radioactive CO_2 in the expired air collected for 6 hr after dosing was <0·03% of the dose.

† By isotope dilution 98% of this material was shown to be 4,4′-dihydroxydiphenylmethane-3,3′-dicarboxylic acid.

Results and discussion

Preliminary studies in rats injected with non-radioactive methylenedisalicylic acid (10–200 mg/kg) indicated that the drug was not metabolized and only the original substance appeared in the urine and faeces. Chromatograms of the urine before and after acid hydrolysis showed one and the same spot characterized as methylenedisalicylic acid. No glycine or glucuronic acid conjugates were detected. This was confirmed, using the [^{14}C]acid, by isotope dilution and by use of the radiochromatogram scanner.

The fate of the compound in rats is shown in Table 2. There is no sex difference in its excretion, for in both male and female rats 30–40% of an oral dose is excreted in the urine in 3 days and about 50% in the faeces in 2 days. These figures may suggest that the acid is not completely absorbed, but as shown in Table 4 there is a considerable biliary excretion of the acid in the rat after intravenous injection. When given intraperitoneally, nearly 60% is excreted in the urine and 30% in the faeces. Thus, both incomplete absorption and biliary excretion could account for the faecal excretion after oral administration. The faecal excretion after intraperitoneal injection is largely the result of biliary excretion.

TABLE 3. ELIMINATION OF ^{14}C IN VARIOUS SPECIES RECEIVING [^{14}C]METHYLENEDISALICYLIC ACID. The dose of [^{14}C]methylenedisalicylic acid was 10 mg/kg. except in mice which received 20 mg/kg. The monkeys and chickens were females, whilst the guinea-pigs, hamsters, mice and rabbits were males.

Animal (No.)	Monkey (2)*	Rabbit (3)†	Guinea-pig (3)†	Hamster (3)‡	Mouse (8)‡	Chicken (3)‡§
Dose of ^{14}C, μc/kg	0·58	0·58	0·58	5·8	11·6	1·16
Body wt, mean, kg	3	3·8	0·72	0·1	0·025	2·9
Route of administration	s.c.	i.v.	i.p.	i.p.	i.p.	i.p.
			% of the dose of ^{14}C excreted in			
Urine, day 1	89, 92	43 (0–93)	82 (78–89)	69	87	68
2	1, 1	34 (2–92)	0·7 (0·4–1)	6	7	15
3	—	19 (1–50)	—	—	—	—
Total	90, 93	96 (79–90)	83 (79–90)	75	94	83
Faeces, day 1 and 2	0·4, 0·7	0	0	15	8	—
Expired air day 1	—	—	—	< 0·1	< 0·1	—
Total excreted	90·4, 93·7	96	83	90	102	83
			% of ^{14}C excreted in urine			
% of urinary ^{14}C present as methylenedisalicylic acid‖	97	97	102	98	97	90

* For monkeys individual values are given.
† Mean values are given with ranges in parentheses.
‡ Excreta were pooled.
§ The chickens were fasted for one day to reduce faeces; values are for total excreta.
‖ Determined by isotope dilution.

The fate of the compound after injection in six other species is shown in Table 3. In the monkey, rabbit and guinea-pig, methylenedisalicylic acid is almost entirely excreted in the urine, whereas in the hamster and mouse, although the urine is the major channel of excretion, some 15 and 8%, respectively, is excreted in the faeces. With the hen, the urine and faeces are not readily separated, and although the birds were starved for a day the urine collected did contain a small amount of faeces. The figure of 82% excretion therefore does not clearly distinguish between urinary and faecal excretion. By isotope dilution it was shown that the material excreted in the urine of the monkey, hamster, mouse, rabbit and guinea-pig was unchanged methylenedisalicylic acid, the value found 97–102% (Table 3) being within the experimental error of the method. The value for hens was 90% but there was no evidence of any other material than methylenedisalicylic acid in the excreta. The expired air of the rats (Table 2), mice and hamsters (Table 3) was also examined for

radioactivity. None was found and it was concluded that methylenedisalicylic acid is not degraded to carbon dioxide.

Table 4 summarizes the findings on the biliary excretion of methylenedisalicylic acid in four species. Excretion is high in the rat (54% of the dose) and dog (50–70%), and low in the rabbit and guinea-pig (4–5%); at the dose level used (10 mg/kg) biliary excretion is practically complete in all these species in 6 hr. These observations on species differences

TABLE 4. BILIARY EXCRETION OF [^{14}C]METHYLENEDISALICYLIC ACID IN VARIOUS SPECIES. The acid (10 mg/kg) as the sodium salt in water was injected intravenously into biliary cannulated animals. The rats and rabbits were females, the guinea-pigs males and one dog was male and the other female. Bile and urine were collected for 6 hr after dosing.

Animal (No.)	Rat (4)	Dog (2)	Guinea-pig (2)	Rabbit (3)
Body wt, mean, kg	0·265	8 (m), 7·2 (f)	0·88	3·8
Bile vol. ml /hr	1·2	5	4	12·0
Plasma half life, hr	2·0	1	3	3·0
	% of ^{14}C* administered excreted in			
Bile 0–0·5 hr	17 (8–26)	39, 32	1·2, 2·6	1·2 (0·9–1·8)
0·5–1·0	14 (12–16)	14, 13	1·5, 0·5	1·6 (1·3–1·7)
1–2	11 (9–13)	9, 10	1·2, 0·6	1·2 (1·1–1·4)
2–3	6 (4– 8)	4, 3	0·4, 0·6	0·6 (0·6–0·5)
3–4	3 (1– 5)	2, 2	0·0, 0·2	0·3 (0·2–0·5)
4–5	2 (0·4–3)	1, 1	—	—
5–6	1 (0·2–2)	—	—	—
Total	54 (46–62)	69, 61	4·3, 4·5	4·9 (3·8–5·3)
Urine 0–6 hr	15, 23†	1, 11	59, 73	44 (37–52)

* The values for the rat and rabbit are given as mean values with ranges in parentheses; individual values are given for the dog and guinea-pig.
† Individual values for two of the rats.

agree with others found in this laboratory (Abou-El-Makarem & others, 1966, 1967a,b). According to Millburn, Smith & Williams (1967), for extensive biliary excretion of a compound to take place in the rat the compound should have a molecular weight of not less than 325 ± 50, and a strongly polar anionic group, or it must be converted into such a compound by metabolism. 5,5′-Methylenedisalicylic acid has a molecular weight of 288 and a pK_a of about 3·5 and appears to fit these criteria without undergoing conjugation.

No metabolite of methylenedisalicylic acid was found in the bile or in the urine. Since it is closely related to salicylic acid, there was the possibility that, like salicylic acid, it might have formed a glycine or glucuronic acid conjugate or an oxidation product by further hydroxylation (see Williams, 1959). However, none of these were found in any of the species examined.

METABOLISM OF CRUDE METHYLENEDISALICYLIC ACID

Methylenedisalicylic acid as used commercially, contains large amounts of impurities probably from the variety of ways in which salicylic acid and formaldehyde can be condensed. The major impurity (Rf 0·45; see Table 1) is probably a product containing three salicylic acid residues and two methylene groups and is referred to below as "trimeric product." In the experiments from which Table 1 was constructed, it can be calculated that the specific activity of a product containing three salicylic acid

residues and two methylene groups should be about 0·75 which agrees with the value found (Table 1). In some experiments using crude [^{14}C]methylenedisalicylic acid, only 26% of the ^{14}C of an injected dose of this material (10 mg/kg) was excreted in the urine in 6 hr by the rat, whilst in a parallel experiment with purified [^{14}C]methylenedisalicylic acid, 46% of the ^{14}C was excreted in the same time. Furthermore, the urinary material from the crude compound contained appreciably less of the contaminants than the injected compound. Since the "trimeric product" has a molecular weight (calc. 438) higher than methylenedisalicylic acid it might be expected to have a higher biliary excretion and a greater faecal excretion than the latter. A solution of the ^{14}C-labelled "trimeric product" (10 mg/kg) as the sodium salt in water, was injected intraperitoneally into three biliary cannulated female rats and the bile and urine collected for 3 hr. In this time an average of 74% of the injected ^{14}C appeared in the bile and 1% in the urine. From Table 4 it can be seen that the biliary excretion of methylenedisalicylic acid is about 47% in 3 hr. In another experiment the ^{14}C-labelled "trimeric product" (10 mg/kg) was injected intraperitoneally into three normal male rats with anal cups and the urine and faeces collected for 48 hr. In this time an average of 92% of the injected ^{14}C appeared in the faeces and 4% in the urine and these figures should be compared with those for purified methylenedisalicylic acid (Table 2) which are 50% in the faeces and 34% in urine. It appears, therefore, that the contaminants may have a markedly different pattern of excretion from that of 5,5′-methylenedisalicylic acid. However, no further work was done on these contaminants due to the difficulty of separating and characterizing them in sufficient quantities.

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The response of an isolated artery to sympathomimetic amines

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The rabbit ear central artery preparation responded with contractions to noradrenaline, dopamine, tyramine, octopamine, phenylethylamine, β-phenylethanolamine and periarterial sympathetic nerve stimulation. Noradrenaline, dopamine and nervous stimulation gave monophasic responses. Tyramine and octopamine gave biphasic responses consisting of an initial fast contraction followed by a second contraction of slow onset and long duration. Phenylethylamine and phenylethanolamine also gave biphasic responses, but the second contraction was of quick onset and short duration of action and often merged with the first contraction. Cocaine, superior cervical ganglionectomy and reserpinization reduced the second phase of the contraction to tyramine, octopamine and phenylethylamine. The second phase of the response to phenylethanolamine was reduced by reserpine but not by cocaine or denervation.

A BIPHASIC response of the isolated central artery of the rabbit ear to tyramine has been described by Farmer (1966). It was concluded that this biphasic response consists of a primary phase probably due to a direct α-receptor stimulation and a secondary phase due to release of catecholamines from stores within the sympathetic neurone. The aim of the present investigation was to examine, qualitatively and quantitatively, a number of chemically related sympathomimetic amines on the isolated artery preparation. The responses of the artery to noradrenaline, dopamine, tyramine, octopamine, β-phenylethylamine, β-phenylethanolamine and periarterial nerve stimulation and their modification by sympathetic denervation, reserpine, cocaine are described. Although the responses of this tissue to noradrenaline and tyramine have previously been described they are included in these experiments to enable a direct comparison of all these amines on one tissue to be made.

Experimental

METHODS

Lop-eared or semi-lop-eared rabbits weighing 2·0–5·0 kg were anaesthetized with pentobarbitone 30 mg/kg injected intravenously. The central artery of the ear was cannulated and removed according to de la Lande & Rand (1965). The arterial segment, usually 4–5 cm long, was perfused with McEwen solution delivered from a constant output pump (Watson-Marlow). The artery was also immersed in McEwen solution, both solutions being maintained at 37° and gassed with oxygen 95%, carbon dioxide 5%. Perfusion pressure was measured with a Devices blood pressure transducer recording on a Devices multi-channel recorder. Periarterial nerve stimulation was by means of bipolar platinum electrodes. Trains of impulses of supramaximal voltage and 1·0 msec pulse width were delivered for 5 sec from a Palmer electronic square-wave stimulator.

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Injections of drugs dissolved in McEwen solution were given into a rubber connection close to the artery.

The maximum dose volume used was 0·1 ml. The total doses of the drugs expressed in terms of their salts is given in all figures. Dose-response curves to the sympathomimetic amines and frequency response curves for periarterial nerve stimulation (2, 5 and 10 cycles/sec) were determined on each artery. In experiments using denervated arteries the superior cervical ganglia of the rabbits were removed 7 to 10 days before the animal was used. Reserpine, 2·5 mg/kg was administered intraperitoneally, dissolved in 20% ascorbic acid in distilled water, 19 hr before the arteries were used. In experiments using cocaine the drug was added to the perfusion fluid to give a concentration of 10 μg/ml.

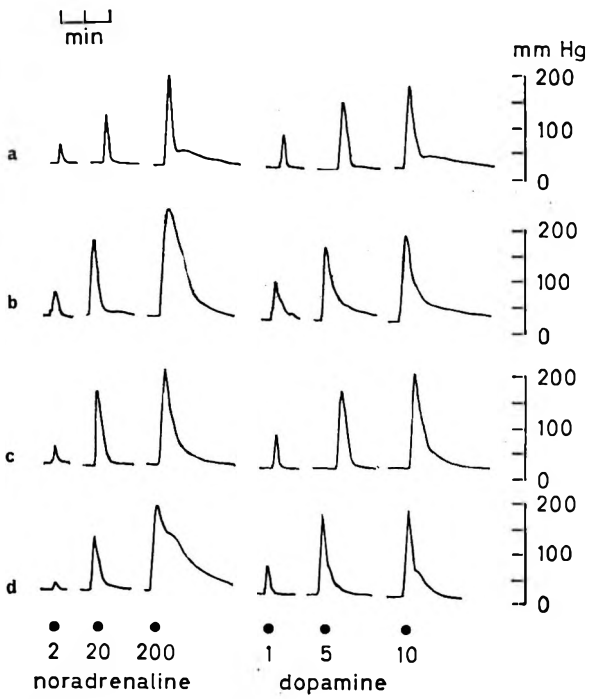

FIG. 1. (a) The response of an isolated central ear artery of the rabbit to noradrenaline (2, 20 and 200 ng) and dopamine (1, 5 and 10 μg). (b) The effect of superior cervical ganglionectomy on responses of an isolated ear artery to the same doses of noradrenaline and dopamine as used in the control experiments. (c) The effect of reserpine pretreatment (2·5 mg/kg 19 hr before experiment) on responses of an isolated ear artery to the same doses of noradrenaline and dopamine as used in the control experiments. (d) The effect of cocaine (10 μg/ml in perfusion fluid) on responses of an isolated ear artery to the same doses of noradrenaline and dopamine as used in the control experiments.

DRUGS

(−)-Noradrenaline bitartrate, dopamine hydrochloride, tyramine hydrochloride, (±)-octopamine hydrochloride, β-phenylethylamine hydrochloride, (±)-β-phenylethanolamine hydrochloride, cocaine hydrochloride

and reserpine. Solutions of all sympathomimetic amines contained 1 μg/ml ascorbic acid as an antioxidant.

RESPONSES OF THE ISOLATED ARTERY TO SYMPATHOMIMETIC AMINES AND SYMPATHETIC NERVE STIMULATION

In 10 arteries, dose-response curves to noradrenaline, dopamine, phenylethylamine, phenylethanolamine, tyramine, octopamine and perivascular sympathetic nerve stimulation at 2, 5 and 10 cycles/sec were obtained. Dopamine produced a monophasic response, like noradrenaline, but was some 50 times less potent. The slope of the dose response curve for dopamine was the same as for noradrenaline (Fig. 1a). Octopamine gave a monophasic response at low doses (5 and 10 μg) and in this respect was approximately 1000 times less potent than noradrenaline.

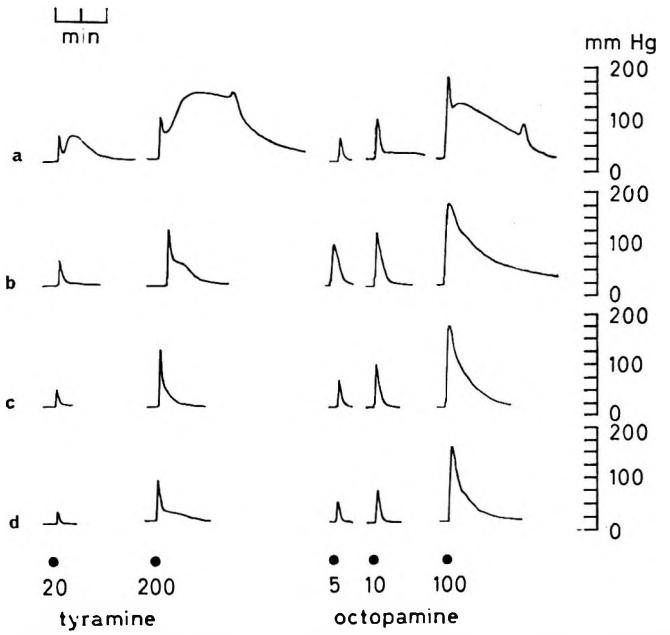

FIG. 2. (a) The responses of an isolated central artery of the rabbit ear to tyramine (20 and 200 μg) and octopamine (5, 10, 100 μg). (b) The effect of superior cervical ganglionectomy on responses of an isolated ear artery to the same doses of tyramine and octopamine as used in the control experiments. (c) The effect of reserpine pretreatment (2·5 mg/kg, 19 hr before experiment) on responses of an isolated ear artery to the same doses of tyramine and octopamine as used in the control experiments. (d) The effect of cocaine (10 μg/ml in perfusion fluid) on responses of an isolated ear artery to the same doses of tyramine and octopamine as used in the control experiments.

At higher dose levels (100 μg) octopamine elicited a biphasic response, the second phase was prolonged but less intense than the first phase (Fig. 2a). Phenylethylamine and phenylethanolamine produced monophasic responses at low dose levels (10 μg) and were of a similar potency to octopamine; however, a biphasic response at higher dose-levels was observed

with phenylethylamine and phenylethanolamine but the response was not clearly differentiated into two phases (Fig. 3a). The second phase was of similar magnitude to the first phase at any given dose-level, but was of shorter duration than that observed with either tyramine or octopamine. The maximum response that could be obtained with phenylethylamine was only 50% of that obtainable with noradrenaline.

THE EFFECT OF SUPERIOR CERVICAL GANGLIONECTOMY ON RESPONSES OF THE ISOLATED ARTERY TO SYMPATHOMIMETIC AMINES AND SYMPATHETIC NERVE STIMULATION

The superior cervical ganglia of four rabbits were removed one week to 10 days before the animals were used for experiments. On the eight arteries, dose-response curves to noradrenaline, dopamine, β-phenylethylamine, phenylethanolamine, tyramine and octopamine were obtained. No responses were obtained to periarterial nerve stimulation at 2, 5 or

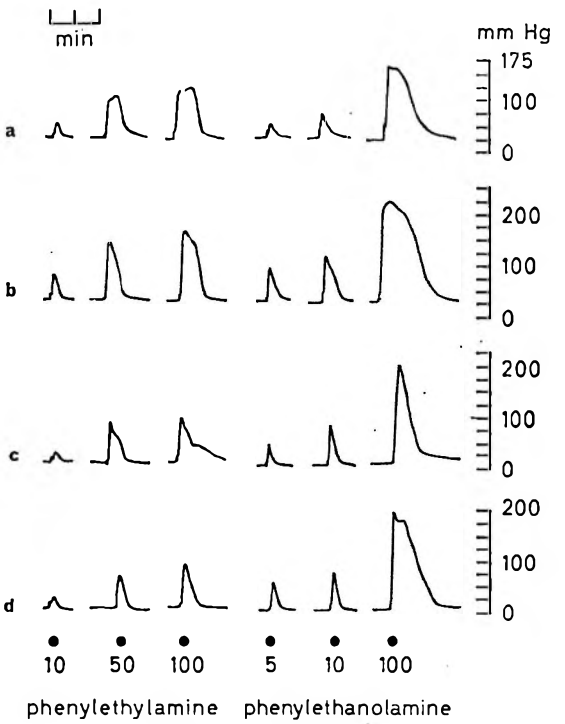

FIG. 3. (a) The response of an isolated central artery of the rabbit ear to phenylethylamine (10, 50 and 100 μg) and phenylethanolamine (5, 10, 100 μg). (b) The effect of superior cervical ganglionectomy on responses of an isolated ear artery to the same doses of phenylethylamine and phenylethanolamine as used in the control experiments. (c) The effect of reserpine pretreatment (2·5 mg/kg, 19 hr before experiment) on responses of an isolated ear artery to the same doses of phenylethylamine and phenylethanolamine as used in the control experiments. (d) The effect of cocaine (10 μg/ml in the perfusion fluid) on responses of an isolated ear artery to the same doses of phenylethylamine and phenylethanolamine as used in the control experiments.

10 cycles/sec (Fig. 4b). Responses to high doses of noradrenaline and dopamine were enhanced by denervation, but in the case of dopamine this potentiation was not significant (Fig. 1b). As described previously, tyramine, octopamine, phenylethylamine and phenylethanolamine elicited biphasic constrictions of the artery preparation. Sympathetic denervation enhanced the first phase of the response to these amines and reduced the second phase, although the reduction in the second phase was not marked with phenylethanolamine (Figs 2b and 3b).

THE EFFECT OF RESERPINE PRETREATMENT ON RESPONSES OF THE ISOLATED ARTERY TO SYMPATHOMIMETIC AMINES AND SYMPATHETIC NERVE STIMULATION

Four rabbits were pretreated with reserpine (2·5 mg/kg) 19 hr before the experiments were made. Dose-response curves were obtained on eight arteries to noradrenaline, dopamine, phenylethylamine, phenylethanolamine, tyramine and octopamine. The responses to noradrenaline, dopamine, and the primary phase of the responses to tyramine, octopamine, phenylethylamine and phenylethanolamine was not significantly altered by pretreatment with reserpine (Figs 1c, 2c, 3c). The second phase of the responses to the above amines were much reduced or

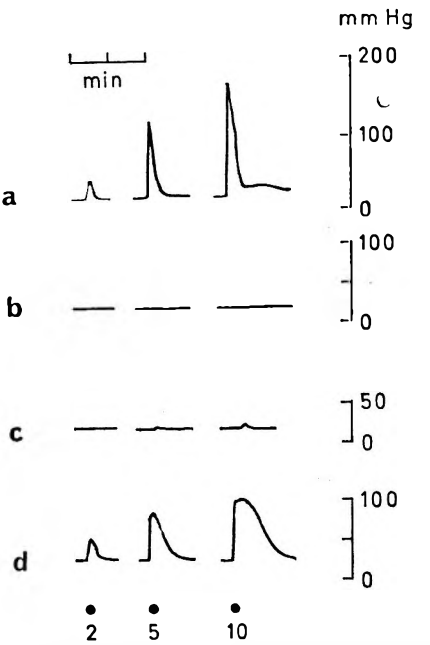

FIG. 4. (a) The response of an isolated central ear artery of the rabbit to periarterial nerve stimulation (2, 5, 10 cycles/sec). (b) The effect of superior cervical ganglionectomy on responses of an isolated central ear artery to the same frequencies of nervous stimulation as used in control experiments. (c) The effect of reserpine pretreatment (2·5 mg/kg, 19 hr before experiment) on responses of an isolated central ear artery to the same frequencies of nervous stimulation as used in control experiments. (d) The effect of cocaine (10 μg/ml) in perfusion fluid on responses of an isolated central ear artery to the same frequencies of nervous stimulation as used in control experiments.

absent (Figs 2c, 3c). The response to sympathetic nerve stimulation was also absent (Fig. 4c).

THE EFFECT OF COCAINE ON RESPONSES OF AN ISOLATED ARTERY TO SYMPATHOMIMETIC AMINES AND SYMPATHETIC NERVE STIMULATION

Eight arteries were perfused with McEwens solution containing 10 μg/ml cocaine hydrochloride, and dose-response curves to noradrenaline, dopamine, phenylethylamine, phenylethanolamine, tyramine and octopamine were determined after perfusion for 2–3 hr. The responses to periarterial nerve stimulation were significantly reduced by cocaine, the effect being greater at higher rates of stimulation (Fig. 4d). Responses to noradrenaline were unaffected in height by cocaine, but increased in duration (Fig. 1d). Responses to dopamine were not altered by cocaine (Fig. 1d). The primary phase of the response to the four other amines was not altered, however the second phase of the response to tyramine, octopamine, and phenylethylamine were much reduced (Figs 2d, 3d). The second phase of the response to phenylethanolamine was not altered by cocaine (Fig. 3d).

Discussion

Three types of response to sympathomimetic amines have been observed on the isolated central artery ear preparation of the rabbit. Firstly, a monophasic response was produced by noradrenaline and dopamine. Secondly, a well differentiated biphasic response was produced by tyramine and octopamine. Thirdly, a poorly differentiated biphasic response in which the second phase of contraction was of rapid onset and short duration, produced by β-phenylethylamine and phenylethanolamine. Phenylethylamine was also of interest because the maximum response obtainable was considerably smaller than that obtainable with noradrenaline.

Superior cervical ganglionectomy, reserpinization and the addition of cocaine to the perfusion fluid all produced significant reductions in the second phase of the response to tyramine, octopamine and phenylethylamine. These results suggest that the second phase of the response to tyramine, octopamine and phenylethylamine is produced by a release of noradrenaline from tissue stores. The evidence for such an action of phenylethanolamine was by no means as clear cut since only reserpine pretreatment affected the response to this amine; denervation and cocaine having little effect. However the decreased response of the artery after reserpine pretreatment confirms the observation of Burn & Rand (1958) who observed that this amine was without action on the perfused hindleg of the reserpine pretreated dog.

The primary phase of the response to all amines used was enhanced by denervation. This may be expected in the absence of an adrenergic innervation. Reserpine potentiated the action of all amines with the exception of β-phenylethylamine and β-phenylethanolamine. The preferential inhibition of uptake of amines into adrenergic nerves by reserpine

can possibly explain these results. No potentiation of the primary phase of the response to any of the sympathomimetic amines was observed in the presence of cocaine. These results are surprising since cocaine is known to inhibit neuronal uptake of sympathomimetic amines. However the method of administration, by the injection into perfusion fluid of the sympathomimetic amines, is not an ideal way to show marked changes in sensitivity since equilibrium conditions cannot occur.

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Acetylcarbocholine and acetylsilicocholine: directly or indirectly acting cholinergic spasmogens?

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The cholinergic and anticholinergic actions of nitrogen-free isosteres of acetylcholine and benzilylcholine are described. Esters of two kinds of choline analogues, carbocholine and silicocholine, were used. The spasmogenic activity of acetylcarbocholine and acetylsilicocholine on the guinea-pig ileum was identified as an indirect cholinergic action, in contrast to the direct cholinergic action of furtrethonium and the mainly noncholinergic action of barium ions. In addition to this indirect cholinergic action, both esters show a weak anticholinergic and a weak noncompetitive "papaverine-like" spasmolytic activity. The corresponding benzilyl esters, although without an onium group, are relatively potent anticholinergic compounds.

GRADUAL elimination of the ester group-bearing side-chain of the acetylcholine molecule results in a gradual increase in the dose required to induce a response, such as contraction of the isolated gut of the rat. However, a maximum response equal to that of acetylcholine can still be obtained as long as the quaternary ammonium group, tetramethyl ammonium, is maintained. Elimination of the onium group, giving for example ethyl acetate, results in a loss of the cholinergic action. Ethylation of the onium group in cholinergic compounds as a rule results in a decrease or loss of the cholinergic intrinsic activity. These observations emphasize the significance of the trimethylammonium group in acetylcholine for its cholinergic action (Ariëns, 1964, 1965, 1966a, b; Ariëns & Simonis, 1960, 1964).

For anticholinergic compounds, where only an affinity to and no intrinsic activity on the cholinergic receptors is required, the quaternary ammonium group is less critical and ethylation or a change to a tertiary amino-group does not abolish the anticholinergic action. Even elimination of the onium group does not abolish anticholinergic action, as was demonstrated in the investigation of various nitrogen-free analogues of anticholinergic drugs of the benzilylcholine type (see Table 1 in which the anticholinergic activity is expressed as pA_2 values). The *N*-free anticholinergic compounds, although less active than their choline analogues, can nevertheless still be regarded as potent anticholinergics (Funcke, Rekker & others, 1959, 1960; Ellenbroek, 1964; Ariëns, 1965, 1966a, b, c).

The blocking activity of the benzilylcholine type of anticholinergic compounds is based mainly on the interaction of the ring-bearing acyl moiety to the accessory receptor areas. These are areas with which large acyl moieties will bind and they are located in the immediate vicinity of, but are not a part of, the cholinergic receptors in a strict sense. There is good evidence for identifying the mechanism of action of the *N*-free anticholinergic compounds and their analogues as an interaction with the same accessory receptor areas (Ariëns, 1965, 1966a, b, c). The elimination

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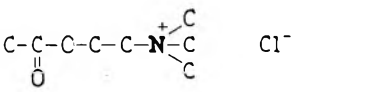
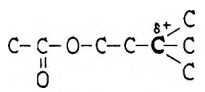
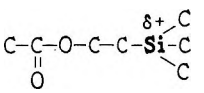
of the rings from the acyl moiety results in a strong decrease in the affinity (the pA_2 values) of these anticholinergic compounds for their receptors (see Table 1). As reported earlier (Ariëns, 1965, 1966a, b, c; Burgen, 1965) an unexpected finding was that 3,3-dimethylbutyl acetate, an isostere of acetylcholine called acetylcarbocholine, was spasmogenic on the isolated gut of the rat or guinea-pig, which might indicate a cholinergic action for this compound. In addition the same is found to hold true for acetylsilicocholine (trimethylsilylethylacetate*). The maximal contraction obtained with these compounds in cumulative concentration-response curves is smaller than that obtained with acetylcholine itself and cholinergic



acetylcholine



acetyl"carbocholine"



acetyl"silicocholine"

compounds such as furtrethonium. If tested on the isolated gut of the guinea-pig the maximal contractions for acetylcarbocholine are 40–100% and the maximal contractions for acetylsilicocholine are 20–80% of those obtained with furtrethonium. If tested on the isolated gut of the rat these figures are 50–100% and 50–100% respectively. This may imply that acetylcarbocholine and acetylsilicocholine have to be classified as partial agonists (Table 1). Acetylcarbocholine was studied extensively by Whittaker (1954) for its action on acetylcholinesterase. In a series of related esters this isostere of acetylcholine appeared to be hydrolysed most quickly by the esterase. The spasmogenic action of acetylcarbocholine was not mentioned.

The question arises whether the acetylcholine-like action of these onium group-free isosteres of acetylcholine results from a direct cholinergic action as suggested by Burgen (1965) or whether it results from an indirect cholinergic action, either by the liberation of endogenous acetylcholine or by the protection of endogenous acetylcholine against inactivation by the inhibition of acetylcholinesterase, which would make these compounds comparable in their action to drugs like physostigmine and neostigmine.

Spasmogens acting on smooth muscle tissues such as the isolated gut can be differentiated into (*a*) directly acting cholinergic spasmogens which have an acetylcholine- or muscarine-like action based on the interaction between the drug concerned and the cholinergic receptors on the smooth muscle tissue, (*b*) indirectly acting cholinergic spasmogens, the action of

* Synthesized according to Limburg & Post (1962).

TABLE 1. INTRINSIC ACTIVITIES AND AFFINITIES OF SOME ESTERS OF CHOLINE, CARBOCHOLINE, BUTANOL AND SILICOCHOLINE

R	C-C-R (O)			Ph, OH, C-C-R (O), Ph			C_6H_{11}, OH, C-C-R (O), Ph (−)		
Rat intestine	i.a.*	pD_2	pA_2 E/O	i.a.	pA_2	E/O	i.a.	pA_2	E/O
$-O-C-C-N^+(C)_3$	1·0	6·6 ± 0·12	27/8	0	8·6 ± 0·18	26/5	0	9·6 ± 0·26	19/18
$-O-C-C-C(C)_3$	0·65 ± 0·07	3·9 ± 0·17		0	7·5 ± 0·07	19/14	0	7·3 ± 0·17	22/21
$-O-C-C-C-C$	0		3·1 ± 0·15 11/8	0	7·4 ± 0·10	15/15	0	6·8 ± 0·15	23/19
$-O-C-C-Si(C)_3$	0·74	3·4 ± 0·11	5/2		not tested			not available	
	0		3·9 1/1						
Guinea-pig intestine	i.a.*	pD_2	pA_2 E/O	i.a.	pA_2	E/O	i.a.	pA_2	E/O
$-O-C-C-C(C)_3$	0·58 ± 0·18	3·9 ± 0·14	9/7 14/9		not tested			not tested	
	0		3·7 ± 0·15 18/9						
$-O-C-C-C-C$	0		3·3 ± 0·09 14/8	0	7·3	5/5		not tested	
$-O-C-C-Si(C)_3$	0·54 ± 0·10	4·0 ± 0·28	11/11 15/5	0	7·1 ± 0·13	20/5		not available	
	0		3·9 ± 0·43 8/6						

* i.a. expressed as the ratio: maximal effect of the compound/maximal effect of furtrethonium, the ± values represent S.x.
E/O: number of experiments/number of organs used in those experiments.

which is dependent on the presence of endogenous acetylcholine, probably liberated at the nerve terminals at the level of the effector system (smooth muscle). This endogenous acetylcholine then acts on the acetylcholine (muscarinic) receptors on the effector tissue, or (*c*) non-cholinergic spasmogens, in the action of which neither acetylcholine nor its receptors are involved.

These groups of spasmogens can be differentiated by testing them in the presence of different inhibitors.

Experimental

(1) Anticholinergic drugs such as atropine and lachesine in concentrations of 10^{-9}–10^{-6}M cause a parallel shift over a wide dose range of the log concentration-response curves for spasmogens acting directly on cholinergic receptors (Ariëns, 1964, 1966a, b, c; Ariëns & Simonis, 1960). For the indirectly acting cholinergic spasmogens the doses of the anticholinergic drugs mentioned cause a decline in the concentration-response curves which is occasionally preceded by a slight parallel shift. The log concentration-response curves for non-cholinergic spasmogens are not affected by the dose of 10^{-9}–10^{-6}M of atropine or lachesine. Cumulative log concentration-response curves of the spasmogens are made in the presence of different concentrations (10^{-9}–10^{-6}M) of lachesine. In the presence of a relatively high concentration of lachesine (10^{-4}M) the responses to directly-acting cholinergic drugs, in doses producing a submaximal response, and the response obtained with indirectly acting cholinergic spasmogens, are abolished. The responses to non-cholinergic spasmogens such as barium ions are unaffected.

(2) Hemicholinium (HC-3)* inhibits the synthesis of acetylcholine possibly by an inhibition of the transport of choline into the nervous elements (MacIntosh, Birks & Sastry, 1956; Long, 1961; MacIntosh, 1961). Treatment of the isolated organ with hemicholinium $1{\cdot}75 \times 10^{-3}$M, causes, after an adequate period of incubation, a large reduction in the response to the indirectly-acting cholinergic spasmogens. At $1{\cdot}75 \times 10^{-3}$M, hemicholinium has practically no influence on the response to directly-acting cholinergic and non-cholinergic spasmogens. The spasmogens are tested after incubation of the organ for 15 min with hemicholinium and with it still in the organ bath.

The inhibitive effect of hemicholinium can be counteracted to some extent by choline. If sufficient choline is available, the acetylcholine synthesis goes on in the presence of hemicholinium. Choline, 5×10^{-2}M, is applied simultaneously with hemicholinium and the spasmogens are tested after an incubation of the organ for 15 min and with the hemicholinium and choline still in the bathing fluid.

The inhibition of the response to a spasmogen by hemicholinium and the counteraction thereof by choline are strong arguments for the involvement of endogenous acetylcholine in the action of the spasmogen and therefore its classification as an indirectly-acting cholinergic spasmogen.

* Synthesized according to Long & Schueler (1954).

(3) The action of procaine on isolated organs results in the reduction of the output of acetylcholine at the nerve terminals (Feldberg & Lin, 1949; Harry, 1962). It blocks the propagation of action-potentials along the nerve fibres. In a concentration of 3×10^{-3}M, procaine blocks the responses to the indirectly-acting cholinergic spasmogens. In the concentration mentioned it leaves the responses to directly-acting cholinergic and noncholinergic spasmogens practically unaffected. The spasmogens are tested after incubation of the organ for 40 min with procaine, 3×10^{-3}M, and subsequent washing of the organ 3 times in succession with fresh Tyrode solution, which takes about 2 min.

(4) Ganglionic blocking agents competitively block the response to compounds with a ganglionic stimulant, nicotine-like action. The responses to drugs acting on receptors different from those for nicotine are practically unaffected. The spasmogens are tested in the presence of the ganglionic blocking drug hexamethonium in a concentration of 3×10^{-4}M without any pre-incubation.

Results

The anticholinergic drug lachesine effects a parallel shift in the log concentration-response curves of the directly-acting cholinergic spasmogen furtrethonium, but a depression of the curves for acetylsilicocholine as shown in Fig. 1a and b. The same is found for acetylcarbocholine. The

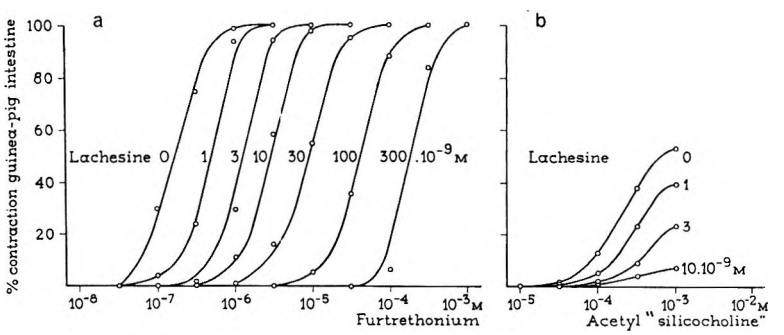


FIG. 1a. Cumulative log concentration-response curve for furtrethonium in the presence of increasing concentrations of the anticholinergic lachesine (benzilyl-*N*-dimethyl-*N*-ethylethanolamine). Note the parallel displacement of the curves indicating the competitive relation between furtrethonium and lachesine. b. Cumulative log concentration-response curve for acetylsilicocholine in the presence of increasing concentrations of lachesine (benzilyl-*N*-dimethyl-*N*-ethyl-ethanolamine). Note the depression in the curves, caused by the anticholinergic indicating an indirect cholinergic spasmogenic action for acetylsilicocholine.

depression of the log concentration-response curves for these spasmogens with relatively low concentrations of the anticholinergic indicates an indirect cholinergic action.

The contractions obtained with the various spasmogens applied as a single dose in combination with the various inhibitors are presented in

TABLE 2. DIFFERENTIATION OF VARIOUS TYPES OF SPASMOGENS

	Furtrethonium		Acetyl-"carbocholine"		Acetyl-"silicocholine"		"Acetylglycol"		Nicotine		$BaCl_2$	
In the presence of lachesine	0	(6)	0	(8)	1	(6)	2	(12)	1	(7)	128	(7)
After hemicholinium incubation	76	(9)	2	(8)	1	(9)	0	(8)	2	(9)	68	(10)
After procaine treatment	95	(9)	7	(7)	3	(12)	0	(12)	6	(6)	116	(9)
In the presence of hexamethonium	98	(6)	106	(15)	73	(20)	77	(12)	2	(19)	101	(8)

The contractions of the various spasmogens are expressed as mean percentage of the contractions under control conditions. The number of experiments is given in brackets. For further details see text.

Table 2. The contractions are expressed as percentages of the corresponding contractions in the control conditions, i.e. in the absence of blocking agents.

The doses of the spasmogens were respectively: furtrethonium 3×10^{-5}M, acetylcarbocholine 10^{-3}M, acetylsilicocholine 10^{-3}M, acetylglycol 10^{-2}M, nicotine 3×10^{-5}M, $BaCl_2$ 10^{-3}M. These doses just cause a maximal contraction of the guinea-pig ileum. Because of the desensitization phenomenon observed after furtrethonium, the application of

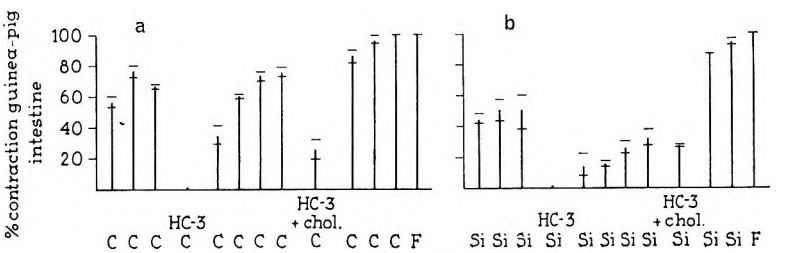


FIG. 2. The responses of the guinea-pig intestine to a single dose of acetylcarbocholine (C) (1×10^{-3}M) (a) and of acetylsilicocholine (Si) (1×10^{-3}M) (b) expressed in % of the responses to a single dose of furtrethonium (F) (3×10^{-5}M) and the inhibition thereof by incubation of the organ (15 min) with hemicholinium (HC-3) ($1{\cdot}75 \times 10^{-3}$M), as well as the antagonistic action of choline (5×10^{-2}M) with respect to the inhibitive action of hemicholinium. Note that the effects of acetylcarbocholine and acetylsilicocholine are abolished during the incubation with hemicholinium. This is in contrast to the effect of furtrethonium (see Table 2). During incubation with hemicholinium and choline the response to acetylcarbocholine and acetylsilicocholine are partially maintained. After removal of the hemicholinium the response is even enhanced. The interval between the various doses of spasmogen is 5 min, with the exception of the interval (15 min) preceding the application of the spasmogen in the presence of hemicholinium.

other spasmogens shortly after furtrethonium was avoided. When tested in the presence of lachesine (10^{-4}M), the responses of the ileum to all spasmogens except barium ions were abolished. This implies that barium chloride has to be considered mainly as a non-cholinergic directly acting spasmogen.

After 15 min incubation with hemicholinium ($1{\cdot}75 \times 10^{-3}$M) the actions of all spasmogens were inhibited with the exception of the directly-acting

cholinergic furtrethonium and the non-cholinergic spasmogen barium chloride. The partial reduction in the response to barium ions may, however, be ascribed to an indirect cholinergic component in its action. The inhibiting action of hemicholinium on acetylcarbocholine and acetylsilicocholine could be counteracted by choline (5×10^{-2}M) (Fig. 2). These experiments with hemicholinium and with the combination of hemicholinium and choline are a strong argument in favour of the involvement of acetylcholine in the spasmogenic actions of acetylcarbocholine, acetylsilicocholine and nicotine.

The influence of 40 min incubation of the ileum with procaine (3×10^{-3}M) is seen as a depression of the effect of all spasmogens used except furtrethonium and Ba^{++}.

The influence of hexamethonium (3×10^{-4}M) on the effect of the various spasmogens is seen as an inhibition of the effect of nicotine; the effects of the other spasmogens remained practically unchanged.

Discussion

These results strongly indicate that acetylcarbocholine and acetylsilicocholine are to be considered as indirectly acting cholinergic, non-nicotine-like compounds, their action being based on the stimulation of the release of acetylcholine from the presynaptic nerve terminals, probably induced at the level of these nerve terminals. Although nicotine-like compounds also act by way of the liberation of acetylcholine, this liberation is not induced at the level of the nerve terminals but at the level of the ganglionic synapse and therefore can be antagonized by hexamethonium. Besides the onium-free drugs mentioned, other compounds have been reported to have an indirect, non-nicotine-like cholinergic action (Levy & Michel-Ber, 1956; Takagi, Takayanagi & others, 1960; Carlyle, 1963; Koelle, 1963; Kosterlitz & Lees, 1963; Takagi & Takayanagi, 1966a, b). Of special importance in this respect are (*a*) esters of phenol with acids such as acetic or propionic acid, and (*b*) various acetic acid esters of alcohol, e.g. propanol, butanol, pentanol and 3-methylbutanol, which Takagi & Takayanagi (1966a) reported to act as indirect cholinergic spasmogens. In our experiments on the intestine of the rat the acetic acid esters of butanol and 3-methylbutanol had little or no spasmogenic action but mainly acted as weak anticholinergics (Ariëns, 1965). The question arises whether cholinergic receptors—other than muscarinic or nicotinic—are involved in the non-nicotinic indirect cholinergic action of the onium-free compounds studied. The fact that the central carbon and silicon atom have a certain positive charge (δ^+) might suggest a chemical relationship with acetylcholine and therefore a relation to acetylcholine receptors (Burgen, 1965). It was found, however, that the ester in which the onium group of acetylcholine was substituted by an OH-group, i.e. acetyl glycol half-ester ($MeCOOCH_2CH_2OH$) also acted as a spasmogen. On the isolated gut of the guinea-pig a pD_2 value of 2·2 was found while the maximal response obtained in a cumulative concentration-response curve was 60–100% of that of furtrethonium. In the present investigation

acetylglycol behaved in the same way as acetylcarbocholine and acetylsilicocholine (Table 2). Consequently it too may be considered as a non-nicotinic indirect cholinergic spasmogen. The hydroxy group in the ester, however, has no positive charge but has some polarity. This implies that a positive charge as present in acetylcarbocholine and acetylsilicocholine is not essential for their action. The indirect cholinergic spasmogenic action of esters such as n-pentyl acetate and n-propyl acetate reported by Takagi & Takayanagi (1966a) can also be regarded as evidence in this direction.

Acetylcarbocholine and acetylsilicocholine are related to the nitrogen free anticholinergic compounds in Table 1. Elimination of the rings from the acyl moiety in the more potent nitrogen-free anticholinergic compounds, as expected, results in a strong decrease in their activity, seen as a decrease in the pA_2 values. The question arises whether besides having an indirect cholinergic action, these nitrogen-free compounds still have some anticholinergic action An anticholinergic activity if present in acetylcarbocholine and acetylsilicocholine may manifest itself if tested against a directly-acting cholinergic drug such as furtrethonium on organs kept in the ice box at 2° for 24 hr, as these are practically irresponsive to the indirectly-acting spasmogens. As demonstrated in Figure 3a,

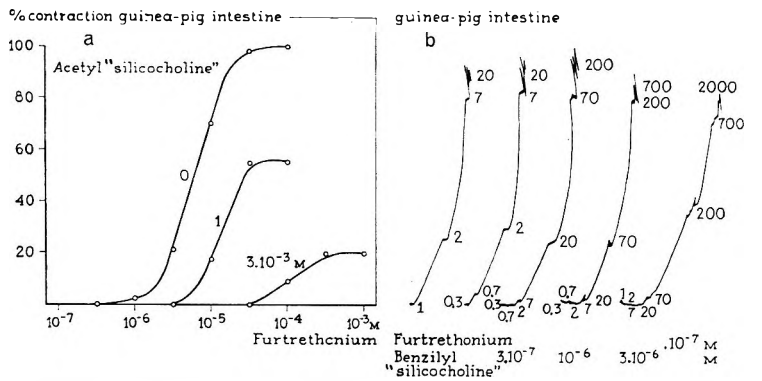


FIG. 3.a. Cumulative log concentration-response curve for furtrethonium, tested in the presence of increasing concentrations of acetylsilicocholine obtained on a piece of isolated guinea-pig ileum (stored in the ice-box for 24 hr). Note that on the gut, so pretreated, acetylsilicocholine has no spasmogenic action of its own, but behaves as a non-competitive (the depression in the curves) and a competitive (the parallel displacement of the curves) antagonist of furtrethonium. b. Registrogram for cumulative dose-response curves, obtained with furtrethonium in the presence of various concentrations of benzilylsilicocholine. Note that the parallel shift in the curves indicates an anticholinergic action of benzilylsilicocholine. The depression in the curves, which becomes more manifest if higher doses of benzilysilicocholine are used, indicates a non-competitive spasmolytic action for this compound.

acetylsilicocholine antagonizes furtrethonium. There is a decline in the log concentration-response curves indicating a non-competitive, papaverine-like spasmolytic action which is combined with a parallel shift in the curves. This indicates the anticholinergic component in the spasmolytic action of acetylsilicocholine. Analogous results were obtained with

acetylcarbocholine in concentrations of 10^{-3}M and other acetyl esters of simple alcohols like 3-methylbutanol and butanol. A schematic representation of the mode of action of acetylcarbocholine and acetylsilicocholine is given in Fig. 4. On the receptor system R_{II}, acetylcarbocholine and acetylsilicocholine, because of their anticholinergic action, have an action as competitive antagonists of the acetylcholine liberated. On the receptor system R'_{II}, a noncompetitive, "papaverine-like" spasmolytic action is induced by acetylcarbocholine and acetylsilicocholine. One of the consequences of this multiplicity in actions is that the spasmogenic action induced on the receptor system R_I, by virtue of the liberation of endogenous acetylcholine acting on the receptor system R_{II}, is inhibited because of the inhibitory actions on the receptor systems R_{II} and R'_{II}. The result is that in the cumulative log concentration-response curves of acetylcarbocholine and acetylsilicocholine an auto-inhibition becomes manifest. The hemiacetate of glycol, "acetyl-glycol" is devoid of anticholinergic properties in concentrations up to $3 \cdot 10^{-2}$M.

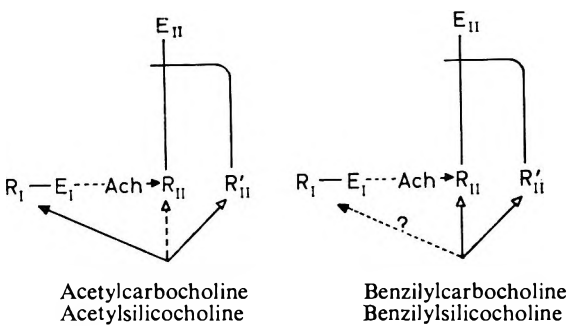


Fig. 4. Probable sites of action of carbocholine and silicocholine esters. (Solid arrows stimulant action; open arrows inhibiting action). R_I are the receptors located at the nerve terminal, E_I is the effector system on which the liberation of endogenous acetylcholine is induced. R_{II} are the cholinergic (muscarinic) receptors on which the acetylcholine liberated induces its spasmogenic effect. The smooth muscle fibres represented by E_{II} serve as the final effector system. R'_{II} represents the receptors on which a non-competitive spasmolytic action is induced.

The elimination of the aromatic rings and the hydroxy-group from the acidic moiety in benzilylcarbocholine and related compounds results in a strong decrease in the anticholinergic activity (Ariëns, 1965, 1966a, b) (Table 1). As shown in Fig. 3a, acetylsilicocholine has weak anticholinergic properties. Introduction of aromatic rings and an hydroxy-group in the acidic moiety, leading to benzilylsilicocholine, results in an increase in the anticholinergic activity again (Fig. 3b). Benzilylsilicocholine*, tested on the isolated gut of the guinea-pig, has a relatively high anticholinergic activity (pA_2 7·1). On the isolated heart of the frog both benzilylcarbocholine and benzilylsilicocholine showed a clear-cut anticholinergic

* Obtained by coupling trimethylsilylethanol (silicocholine) with diphenylchloroacetylchloride followed by hydrolysis to eliminate the chloro-groups.

action. A schematic representation of the mode of action of benzilylcarbocholine and benzilylsilicocholine is given in Fig. 4. These compounds act primarily as competitive antagonists of acetylcholine on the cholinergic (muscarinic) receptors R_{II}. In higher concentrations they also have a non-competitive, papaverine-like spasmolytic action induced on the receptors R'_{II}. Whether an indirect cholinergic action is also present remains unanswered. The blockade of the receptor system R_{II} at relatively low concentrations of these esters may interfere with the effectuation of such an action.

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The effects of physiological concentrations of noradrenaline on the coronary resistance of isolated perfused hearts of the cat, dog and monkey

E. PROCTOR

The effects of "physiological" concentrations of noradrenaline on the coronary resistance of isolated, perfused hearts of the cat, dog and monkey were determined. At the lowest active concentrations of noradrenaline (2–4 ng/ml), the preparations always responded with an increase in coronary resistance associated with an unrecordable or minimal myocardial response. As the concentration increased, the effect of the amine on the coronary resistance became biphasic. There was an initial transient increase followed by a more prolonged decrease in resistance associated with a significant myocardial response. These results support other evidence that the direct action of noradrenaline on the coronary vessels is one of vasoconstriction, and that the decrease in resistance associated with larger concentrations of noradrenaline is secondary to the myocardial stimulation, perhaps by way of vasodilating metabolites. Lack of agreement of results may well arise due to the use of concentrations of noradrenaline which also affect the myocardium.

NORADRENALINE is used as a pressor agent in cardiogenic shock due to myocardial ischaemia, although the nature of its actions on the coronary vessels is still in dispute. In man, it has been reported to cause a decrease in coronary resistance (Feinberg & Katz, 1958), an increase in coronary resistance (Yurchak, Rollett & others, 1964), or a biphasic change (Berne, 1958). Apparent disparity of this kind is found in the intact animal, including man, and in the isolated heart.

Recent investigations with the isolated mammalian heart, using a sensitive coronary resistance recorder (Proctor, 1964), suggest that the variation in results may arise, in part, from the difficulties of measuring small changes in coronary resistance, and the consequent use of concentrations of catecholamines far in excess of those found physiologically.

Experimental

Isolated hearts of the cat, dog and monkey were perfused by the Langendorff technique with Krebs solution at a perfusion pressure of 50 cm H_2O and a temperature of 37°. The Krebs solution contained in g/100 ml: NaCl 0·692, KCl 0·0354, $CaCl_2$ 0·0282, $NaHCO_3$ 0·21, KH_2PO_4 0·0162, $MgSO_4.7H_2O$ 0·0294, glucose 0·2. The solution was gassed with oxygen 95% and carbon dioxide 5%, and its pH immediately before it entered the coronary arteries was in the range 7·34–7·44. Number and weight range of animals in each species were: cats (10) (0·8–1·2 kg); dogs (8) (0·9–1·3 kg); monkeys (2) (*Cynomolgus* 1·8–1·9 kg). The perfusion apparatus was based on that of Baker (1951).

Since the coronary resistance is a function of vascular and myocardial support, kymograph recordings were also made of the amplitude of contraction (spring-loaded lever with a tension of 9 g), and heart-rate (Thorp impulse counter). The sensitivity of the coronary resistance recorder

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was such that a change in coronary resistance of 1·5% gave a deflection of 1·0 cm on the drum. Noradrenaline was injected in a volume of 0·05 ml of Krebs solution into the aortic cannula, which had a volume of 1·0 ml. At least 45 min were allowed for the heart to become steady in amplitude, rate, and coronary resistance before any injections were made.

DRUGS

Acetylcholine chloride (Hopkins and Williams). (−)-Noradrenaline bitartrate (Bayer). Doses given are in terms of the base.

Results

Early in perfusion, the coronary vessels of the isolated heart have a low tone and show an exaggerated vasoconstrictor response. However, if perfusion is maintained for 45–60 min much of the smooth muscle tone returns and the preparation will respond with a decrease in coronary resistance to doses of acetylcholine which do not significantly affect the

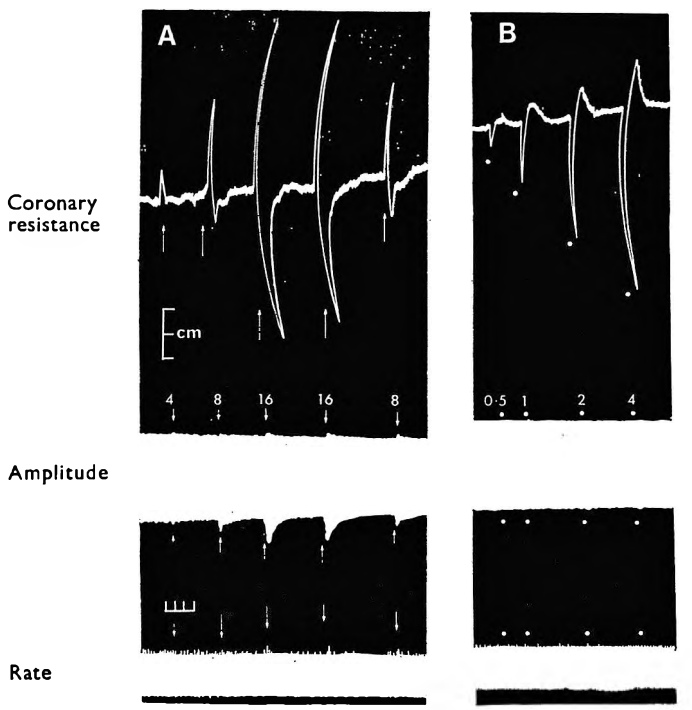

FIG. 1. Isolated perfused cat heart. Krebs solution. 37°. 1 cm≡1·5% change in coronary resistance. A. A simple increase in coronary resistance with the smallest effective dose of noradrenaline (4 ng), changes to a biphasic response as the dose is increased to 16 ng, and myocardial amplitude and rate are more affected. B. Indicating the sensitivity of the recording system, and the effect on the coronary resistance of small doses of acetylcholine which have a minimal or insignificant effect on the myocardium. Time scale: min.

myocardium (Fig. 1B). Noradrenaline is not used until this point is reached.

Cats. Fig. 1A shows the effect of very small doses of noradrenaline on the coronary resistance, heart rate and amplitude of contraction in the cat isolated perfused heart. The smallest dose of noradrenaline seen to affect the coronary resistance (4 ng) caused a transient increase in coronary resistance. At 8 ng, a biphasic response was seen in which there was first a brief increase in resistance, followed by a prolonged decrease. At 16 ng, the decrease in resistance became the larger component. The rate was unaffected until the dose reached 16 ng, and the amplitude of contraction was increased minimally at 4 ng and moderately at 16 ng.

Dog. Fig. 2A shows a similar response in the dog isolated heart, with the difference that the effect on the coronary resistance was recordable at 2 ng, and the biphasic response was dominated by the decrease in resistance at a lower dose of noradrenaline than the cat heart. The effect on the myocardium was similar to that of the cat preparation except that the heart rate was affected at a lower dose of noradrenaline.

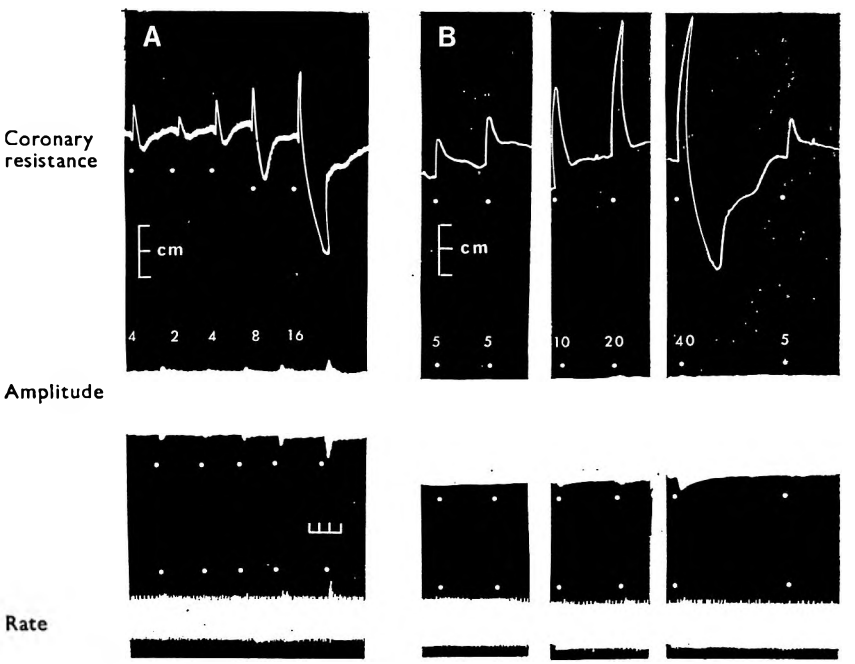

FIG. 2. A. Isolated perfused dog heart. Krebs solution. 37°. 1 cm=1·5% change in coronary resistance. A simple increase in coronary resistance with the smallest effective dose of noradrenaline (2 ng), changes to a biphasic response as the dose is increased to (8 ng), and myocardial amplitude and rate are more affected. B. Isolated perfused monkey heart. Krebs solution. 37°. 1cm=1·5% change in coronary resistance. A simple increase in coronary resistance with the smallest effective dose of noradrenaline (5 ng), changes to a biphasic response as the dose is increased to (40 ng), and myocardial amplitude and rate are more affected. Time scale: min.

Monkey. Fig. 2B shows qualitatively that the monkey isolated heart gave a response similar to the cat and dog preparations, but differed in that the increase in coronary resistance with the smallest dose (5 ng) was more prolonged than that seen with the cat and dog hearts, and the biphasic response did not begin until the dose reached 40 ng. The effect on the myocardium was less marked than with the other species. There was no increase in rate until 20 ng. At 5 ng it is doubtful if the slight increase in amplitude of contraction could be called significant, but a clear, though small, increase did occur at 10 ng.

In all three species, as the dose was increased towards the levels generally used (0·1–1·0 μg), the initial increase in resistance became proportionately smaller and its duration became shorter until the decrease in resistance became the predominant effect. This response would not be apparent with a summated type of record of greater than a few seconds duration, which would indicate a decrease in resistance.

Discussion

The concentration of noradrenaline in the plasma of the rabbit is about 1 ng/ml (Muscholl & Vogt, 1957), in the rat and cat it is 15 ng/ml and 27 ng/ml respectively (Farrant, Harvey & Pennefather, 1964), in the dog 2 ng/ml (Goott, Rosenberg & others, 1960), and in the whole blood of the "non-resting" man it is 5 ng/ml (Weil-Malherbe & Bone, 1953). Most recent work on the effect of catecholamines on the coronary resistance has been with dog and man. The concentrations of noradrenaline used were some fifty to two hundred times those found physiologically and caused marked stimulation of the myocardium. With a dose of noradrenaline which did not apparently affect the myocardium (Fig. 2B), or only minimally so (Figs 1A and 2A), the effect on the coronary resistance was one of transient increase. As the dose was increased the myocardium became increasingly affected and this was associated with the development of the biphasic response in the coronary resistance.

It is tempting to assume that in the lower "physiological" range of doses, in which the myocardial effect and therefore the support component of the coronary resistance was minimal, the effect on the coronary resistance arose directly from the action of noradrenaline on the coronary vessels, i.e. vasoconstriction. However, these results do not constitute proof of a direct vasoconstrictor action by noradrenaline on the coronary vessels but, taken with other reports (see below) they add to the evidence for such an action. Also, in the biphasic response seen with larger doses of noradrenaline and associated with moderate stimulation of the myocardium, it may well be that the prolonged decrease in resistance seen after the initial increase arises from the release of vasodilating metabolites associated with myocardial stimulation rather than as a direct action on the vessels, as suggested by Folkow, Frost & Uvnäs (1949).

Support for this delayed indirect action has been growing, and Krasnow, Hood & others (1964) have shown that although noradrenaline and isoprenaline stimulate the myocardium and increase myocardial oxygen

consumption, isoprenaline causes the coronary flow to rise out of proportion to demand and the coronary venous oxygen content to rise, while noradrenaline causes an increase in the oxygen extraction and a decrease in the coronary venous oxygen content. These responses were interpreted as indicating that isoprenaline has a direct vasodilating action in addition to the metabolic response it induces, and that noradrenaline must be primarily vasoconstrictor in spite of an increase in coronary flow. Further support may be inferred from the work of Szakacs & Cannon (1958) with animals treated with noradrenaline. These authors described pathological changes in the myocardium resembling sub-endocardial infarction which occurs from excessive coronary vasoconstriction.

The present results show that if the method of recording the coronary resistance is sufficiently sensitive and rapid in response, it is possible to use concentrations of noradrenaline approximating to those found physiologically, and to reduce the chance of artifact caused by excessive concentrations at which the myocardial stimulation becomes the dominant feature and two components of the coronary resistance—metabolic vasodilation and myocardial compression—are exaggerated.

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Subcellular distribution of [^{3}H]ε-aminocaproic acid and its effects on amine storage mechanisms

ROBERT STITZEL, PER LUNDBORG AND HOPÉ OBIANWU

ε-Aminocaproic acid (EACA) is an amino-acid reported to cause almost complete depletion of cardiac noradrenaline stores. The present report indicates that this compound can be found in both the particulate and supernatant fractions derived from heart homogenates. The [^{3}H]EACA in the supernatant probably represents a mixture of intra- and extraneuronally located drug. Protriptyline pretreatment decreases the uptake of [^{3}H]EACA suggesting that the amino-acid probably utilizes the amine membrane transport system. EACA, like reserpine, can both impair the retention of exogenously administered amines by adrenergic storage particles and cause the release of amines previously stored in such particles.

THE synthetic amino-acid ε-aminocaproic acid (EACA) is a potent inhibitor of plasminogen activation and has been demonstrated to be an effective therapeutic agent for the control of disordered fibrinolytic states in man (Alkjaersig, Fletcher & Sherry, 1959; Ablondi, Hagan & others, 1959; Nilsson, Sjoerdsma & Waldenström, 1960; Nilsson, Andersson & Björkman, 1966).

Recently, EACA has been reported to inhibit the dual amine uptake-concentration mechanisms of the adrenergic neurons (Obianwu, 1967) and to cause almost complete depletion of cardiac noradrenaline stores (Lippmann, Wishnick & Buyske, 1965; Andén, Henning & Obianwu, in preparation). The EACA-induced depletion of tissue noradrenaline is accompanied by a loss of adrenergic nerve function (Andén, Henning & Obianwu, in preparation). In an attempt to elucidate the mechanisms by which EACA exerts some of its amine-depleting effects, we have examined both the subcellular distribution of [^{3}H]EACA and its effect on amine storage mechanisms.

Experimental

METHODS

In vivo *experiments*. Mice, divided at random into groups of six, were given [^{3}H]α-methylnoradrenaline, 100 μg/kg (30 mc/mM); [^{3}H]metaraminol, 40 μg/kg (100 mc/mM) or [^{3}H]EACA, 200 μg/kg (60 mc/mg) intravenously. The animals were either pretreated or subsequently given various drugs and were killed at appropriate time intervals. A more detailed presentation of the injections schedules can be found in the Results section.

All animals were killed by decapitation and hearts removed and homogenized in the cold. A coarse fraction was obtained by centrifugation of the homogenate in the cold at 2000 *g* for 10 min. The supernatant obtained was then centrifuged at 100,000 *g* for 60 min in a Spinco Model L Ultracentrifuge, providing two more fractions, particulate (sediment) and high speed supernatant. Details of the subcellular fractionation and isolation of [^{3}H]α-methylnoradrenaline and [^{3}H]metaraminol have been

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described previously (Carlsson & Waldeck, 1963; Lundborg & Stitzel, 1967; Stitzel & Lundborg, 1967).

[^{3}H]EACA was isolated by ion-exchange chromatography. Tissue extracts were neutralized to pH 3·5 with 5N potassium carbonate solution and placed on a column of Dowex 50-X4 resin (50 × 4·2 mm). The column was washed with 40 ml of redistilled water and [^{3}H]EACA eluted with 18 ml of 0·4N hydrochloric acid. The first 3 ml of the eluate was discarded. Eluates were freeze-dried and the radioactivity was estimated by liquid scintillation counting. The identity of the isolated [^{3}H]EACA was established by subjecting the freeze-dried eluates, dissolved in a few drops of distilled water, to paper chromatography (butanol–acetic acid–water; 4:1:5, 18–20 hr). All the radioactivity was localized in an area with an Rf value (0·56–0·60) which was similar to that of authentic EACA. The recovery of known amounts of [^{3}H]EACA added to tissue homogenates or extracts ranged from 80–90%. No corrections have been made for recovery.

In vitro *granule experiment*. Bovine adrenal medullae were homogenized in 0·3M sucrose. Unbroken cells and nuclei were removed by centrifugation at 800 *g* for 5 min. The supernatant thus obtained was decanted and centrifuged at 26,000 *g* for 20 min. The granules were then suspended in 0·3M sucrose. The preparation and incubation of the granules were essentially the same as described by Hillarp (1958) and Carlsson, Hillarp & Waldeck (1963). Incubations were made without shaking at 0° and 31° for 30 min.

MATERIALS

[^{3}H]α-methylnoradrenaline and [^{3}H]metaraminol were prepared by the research laboratory of Hässle Ltd. in co-operation with this department. [^{3}H]EACA was kindly donated by Dr. K.-F. Benitz of Lederle Laboratories, Pearl River, New York. [^{14}C]Adrenaline was obtained from New England Nuclear Corp.

Results

Subcellular distribution of [^{3}H]EACA *in the mouse heart*. The subcellular distribution of [^{3}H]EACA ½, 1, 2 and 4 hr after its intravenous administration (400 μg/kg) is shown in Table 1. After ½ hr only about 6% of the [^{3}H]EACA retained by the heart was localized in the particulate fraction, while after 4 hr 28% of the remaining tritiated compound was associated

TABLE 1. SUBCELLULAR DISTRIBUTION OF [^{3}H]EACA IN THE MOUSE HEART

Time (hr)	[^{3}H]EACA — ng/g ± s.e.m.*		$\frac{p}{p+s} \times 100$
	Particulate	Supernatant	
0·5	1·91 ± 0·25	32·83 ± 4·42	6·1 ± 1·6
1·0	2·05 ± 0·23	29·54 ± 0·72	6·5 ± 0·7
2·0	4·56 ± 1·10	19·09 ± 1·76	18·3 ± 2·9
4·0	4·39 ± 0·22	11·68 ± 0·93	27·5 ± 1·5

[^{3}H]EACA (400 ng/kg, i.v.) was given and the animals killed at various time intervals thereafter. Each experiment used six pooled hearts.
* Mean of four experiments.

with this fraction. During the 4 hr following its injection, there was an increase in the amount of EACA found in the particulate fraction with a concomitant decrease in the supernatant content.

Effects of blockade of the neuronal membrane pump on the uptake and subcellular distribution of [^{3}H]EACA. Substances capable of releasing tissue amines have been separated into two groups depending on their ability to utilize the amine transport mechanism located in or on the adrenergic axonal membrane. Protriptyline, a compound which markedly inhibits this uptake mechanism, was found to decrease the accumulation of [^{3}H]EACA in both subcellular fractions by about 32% (Table 2).

TABLE 2. EFFECT OF PROTRIPTYLINE ON THE UPTAKE AND SUBCELLULAR DISTRIBUTION OF [^{3}H]EACA

Treatment	No. of experiments	[^{3}H]EACA – ng/g ± s.e.m.		$\frac{P}{P+S} \times 100$
		Particulate	Supernatant	
Control	6	1·86 ± 0·19	28·13 ± 1·20	6·2 ± 0·5
Protriptyline	3	1·23 ± 0·18	19·15 ± 0·33	6·0 ± 0·7

Protriptyline-treated animals were given the drug (10 mg/kg, i.p.) 1 hr before the administration of [^{3}H]EACA (400 ng/kg, i.v.). Each experiment used six pooled hearts.

Effect of EACA *on the total uptake of* [^{3}H]α*-methylnoradrenaline and its metabolic conversion to* [^{3}H]α*-methylnormetanephrine.* EACA exerted a moderate inhibitory effect on the total accumulation of [^{3}H]α-methylnoradrenaline (Fig. 1). The impairment of [^{3}H]α-methylnoradrenaline uptake was most pronounced ½ hr after an intraperitoneal injection of EACA (1 g/kg) and then gradually declined over the next 12 hr. The early stages of uptake blockade were accompanied by a slight increase in the amount of *O*-methylated metabolite, [^{3}H]α-methylnormetanephrine, which could be recovered from the heart (Table 3). A decrease in amine uptake impairment was accompanied by a gradual decrease in the amount of [^{3}H]α-methylnormetanephrine formed.

TABLE 3. EFFECT OF EACA PRETREATMENT ON THE FORMATION AND RETENTION OF [^{3}H]α-METHYLNORMETANEPHRINE ([^{3}H]α-MeNM)

	Time after EACA administration	No. of experiments	[^{3}H]α-MeNM ng/g
Control ..	0	6	19·85 ± 1·10
EACA	(½ hr)	4	26·04 ± 1·65
EACA	(1 hr)	4	27·76 ± 2·23
EACA	(2 hr)	4	20·34 ± 1·86
EACA	(4 hr)	4	19·26 ± 0·74

At varying intervals after the administration of EACA (1 g/kg i.p.) [^{3}H]α-methylnoradrenaline was given (100 μg/kg i.v.) and the [^{3}H]α-methylnormetanephrine content determined 15 min later. Values are means ± s.e.m. and each experiment used six pooled hearts.

Effect of EACA *pretreatment on the uptake of* [^{3}H]α*-methylnoradrenaline into subcellular fractions of the mouse heart.* EACA pretreatment (1 g/kg) greatly impaired the retention of [^{3}H]α-methylnoradrenaline in the particulate fraction derived from heart homogenates (Table 4). This impairment was accompanied by a moderate increase in the amount of tritiated compound found in the supernatant fraction. The inhibitory

effect of EACA on amine uptake appeared to be maximal 1–2 hr after its administration while 12 hr after EACA administration there was a significant recovery of the granular storage function.

TABLE 4. EFFECT OF EACA PRETREATMENT ON THE UPTAKE OF [^{3}H]α-METHYLNORADRENALINE ([^{3}H]α-MeNA) INTO SUBCELLULAR FRACTIONS OF THE MOUSE HEART

Treatment	Time (hr)	No. of experiments	[^{3}H]α-MeNA — ng/g ± s.e.m.		$\frac{P}{P+S} \times 100$
			Particulate	Supernatant	
Control	0	6	23·44 ± 2·12	32·55 ± 2·31	41·9
EACA	0·5	4	10·59 ± 2·32	37·72 ± 4·20	21·9
EACA	1·0	4	9·69 ± 1·11	45·56 ± 1·91	17·5
EACA	2·0	4	8·87 ± 0·91	40·53 ± 1·53	21·8
EACA	4·0	4	11·69 ± 1·20	35·24 ± 2·07	24·9
EACA	12·0	4	19·30 ± 1·38	27·95 ± 2·79	40·8

Animals were pretreated with EACA (1 g/kg, i.p.) and at various intervals thereafter were given [^{3}H]α-methylnoradrenaline (100 μg/kg, i.v.) and killed 15 min later. Each experiment used six pooled hearts.

Effect of EACA *on the release of* [^{3}H]*metaraminol from subcellular fractions of the mouse heart.* Mice were pretreated with [^{3}H]metaraminol (40 μg/kg) intravenously either 15 min or 23¼ hr before EACA (1 g/kg, i.p.). All animals were killed 45 min after EACA administration. EACA caused a loss of [^{3}H]metaraminol from both the particulate and the supernatant fractions (Table 5) at both the shorter and longer time intervals after [^{3}H]metaraminol administration. However, the loss appeared to be greater from the particulate than from the supernatant fractions as evidenced by the decline in the percentage of labelled amine recovered in the particulate fraction (relative to the P + S fractions) (Table 5). EACA appeared to cause a more pronounced loss from the particulate fraction of animals which had been given [^{3}H]metaraminol 23¼ hr previously than those given the amine 45 min before.

TABLE 5. EFFECT OF EACA ON THE RELEASE OF [^{3}H]METARAMINOL ([^{3}H]MA) FROM SUBCELLULAR FRACTIONS OF THE MOUSE HEART

Treatment	No. of experiments	[^{3}H]MA — ng/g ± s.e.m.		$\frac{P}{P+S} \times 100$
		Particulate	Superantant	
[^{3}H]MA (1 hr)	4	6·40 ± 0·86	29·76 ± 3·46	18·8 ± 1·19
[^{3}H]MA (1 hr) + EACA ..	3	3·81 ± 0·46	22·04 ± 1·96	14·7 ± 0·69
[^{3}H]MA (24 hr) ..	4	6·77 ± 0·40	20·06 ± 1·12	25·2 ± 0·54
[^{3}H]MA (24 hr) + EACA	6	1·17 ± 0·09	12·12 ± 0·92	9·9 ± 0·30

[^{3}H]MA (40 μg/kg, i.v.) was given either 15 min or 23¼ hr before the injection of EACA (1 g/kg, i.p.) Animals were killed 45 min after EACA administration. The figures in parenthesis indicate the time elapsed between [^{3}H]MA injection and decapitation. Each experiment used six pooled hearts.

Influence of EACA *on the* in vitro *uptake of* [^{14}C]*adrenaline by bovine adrenal medullary granules.* EACA, in a concentration of $1{\cdot}5 \times 10^{-5}$M, caused a slight inhibition of the uptake of [^{14}C]adrenaline by bovine adrenal granules. Higher concentrations did not markedly increase the inhibitory effect. EACA $2{\cdot}6 \times 10^{-4}$M caused a 35·8 ± 1·42% inhibition of [^{14}C]adrenaline (4 exp.) while at $5{\cdot}2 \times 10^{-4}$M inhibition was 41·8 ± 3·5% (2 exp.). The concentration of adrenaline (labelled and unlabelled) was 3×10^{4}M. The inhibitory effect of EACA could not be attributed to a

lysis of the granules since an estimation of the adrenaline content in the incubation media showed no appreciable difference between the control flasks and those to which EACA had been added.

Discussion

The uptake mechanisms present in adrenergic nerves consist of two major components, transport through the nerve cell membrane and incorporation into an amine storage granule complex (Carlsson & others, 1963). Both of these mechanisms can be selectively blocked by drugs. Reserpine is known to impair the storage mechanism of the adrenergic granules (Green & Sawyer, 1960; Campos & Shideman, 1962), thereby greatly inhibiting their ability to retain a variety of substances such as [^{3}H]noradrenaline (Stitzel & Lundborg, 1967), [^{3}H]α-methylnoradrenaline (Carlsson, Lundborg & others, 1967) and [^{3}H]guanethidine (Chang, Costa & Brodie, 1965). Antidepressant agents such as protriptyline and desmethylimipramine can block the uptake of circulating (Carlsson & Waldeck, 1965) or neurally released (Malmfors, 1964) amines at the level of the cell membrane. The above agents are, therefore, useful tools in assessing the role played by the two uptake mechanisms.

The data presented in Table 1 indicate that intravenously administered [^{3}H]EACA is initially bound to the amine storage particles to only a small extent, most of the drug being recovered from the supernatant fraction. The amount of [^{3}H]EACA found in the supernatant fraction then declined while there was some increase in the particulate content. The amount found in the supernatant fraction probably represents a mixture of intra- and extra-neuronally located drug. The relatively rapid loss of [^{3}H]EACA observed from this fraction may be indicative of a rapid elimination from the blood with a concomitant decrease in extraneuronal concentrations. However, some of the labelled substance is probably associated with adrenergic nerves since it was found in a subcellular fraction usually associated with adrenergic granules and protriptyline pretreatment reduced the amount recovered from subcellular fractions by about 32%. The latter observations indicates that EACA probably utilizes the amine membrane transport system. Thus it is likely that EACA can be taken up into adrenergic nerves by an active process, and once accumulated is bound to some extent within the storage particles.

A quantitative estimation of the inhibition of uptake caused by protriptyline is difficult since EACA is generally distributed through body tissues, and therefore would tend to accumulate extraneuronally after membrane pump blockade. However, since protriptyline can reduce the amount of [^{3}H]EACA taken up, it is probable that at least 32% of that taken up by the mouse heart is associated with adrenergic nerves. This view is further supported by the observation of Obianwu (to be published) that denervation reduces the amount of EACA retained by sympathetically innervated tissues.

Inhibition of the storage mechanism in adrenergic granules leads to a depletion of the endogenous catecholamines (Bertler, Hillarp & Rosengren, 1961; Carlsson, Hillarp & Waldeck, 1962; Kirshner, 1962) and to

depletion of exogenous amines previously administered (Hertting, Axelrod & Whitby, 1961). The released amines appear to be preferentially metabolized by monoamine oxidase (Kopin & Gordon, 1962). However, if this enzyme is inhibited or an amine resistant to its action is administered, such as α-methylnoradrenaline or metaraminol the released amine accumulates in the axoplasm (Stitzel & Lundborg, 1967; Carlsson & others, 1967). The data presented in Table 3 and Fig. 1 indicate that EACA

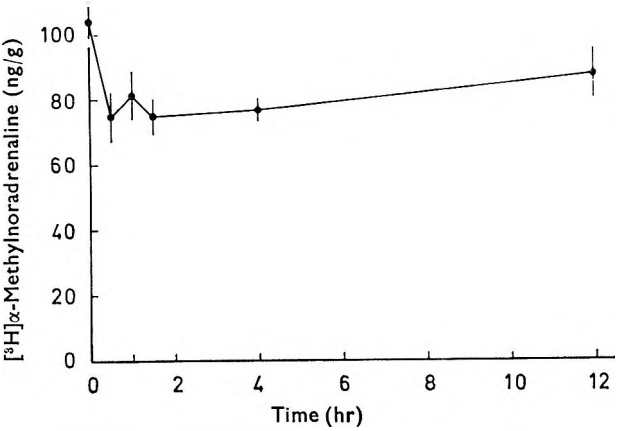

FIG. 1. Effect of EACA on total uptake of [^{3}H]α-methylnoradrenaline in the mouse heart. [^{3}H]α-methylnoradrenaline (100 mg/kg, i.v.) was given at various intervals after EACA (1 g/kg, i.p.) and animals were killed 15 min after injection of the labelled amine. Vertical lines are standard errors of the mean.

greatly inhibits the retention of [^{3}H]α-methylnoradrenaline by the particulate fraction and that this reduction was accompanied by an increase in the level of labelled amine found in the supernatant fraction. Apparently EACA, like reserpine, can impair the retention of exogenously administered amines by adrenergic storage particles. The ability of EACA to impair uptake processes present in adrenergic granules is further supported by the finding that it can cause a moderate inhibition of [^{14}C]adrenaline uptake into bovine adrenal medullary granules.

After administration of metaraminol this amine, like noradrenaline, accumulates and is retained in sympathetically innervated tissues (Andén, 1964: Shore, Busfield & Alpers, 1964; Gram & Wright, 1966). Previous studies have indicated that there is a gradual transfer of this amine from a labile to a more stable pool with time (Crout, Alpers & others, 1964; Carlsson & Waldeck, 1966) and that reserpine more effectively depletes metaraminol given 24 hr rather than 1 hr previously (Stitzel & Lundborg, 1967). It was of interest, therefore, to see if EACA also could displace metaraminol from subcellular pools. The distribution studies indicate that EACA, like reserpine, was more effective as a depleting agent when given 24 hr after [^{3}H]metaraminol than 1 hr after its administration. The primary site of action of EACA was the particulate fraction since depletion was accompanied by a decrease in the P/(P + S) ratio. The EACA-induced depletion of tissue monoamine (Lippmann & others, 1965;

Lippmann & Wishnick, 1965) probably results from this action of EACA on the granular storage mechanism.

The adrenergic nerve blockade caused by EACA does not set in until severe depletion of tissue noradrenaline has occurred. However, function returns when tissue amine levels are still low (Andén, Henning & Obianwu, in preparation). In both the rat and the cat this occurs 12–18 hr after EACA administration. The present finding that EACA-induced blockade of amine storage mechanisms has partially recovered 12 hr after its administration appears to coincide with recovery of adrenergic nerve function. Reports by Andén, Magnusson & Waldeck (1964), Lundborg (1963) and Andén & Henning (1966) also point to the importance of the recovery of uptake rather than the restoration of tissue amine levels for the return of nerve function. In this respect EACA appears to exert similar actions to reserpine on amine storage and nerve function.

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Estimation of the stability of dry horse serum cholinesterase by means of an accelerated storage test

B. R. COLE AND L. LEADBEATER

The thermal inactivation of dry horse serum cholinesterase was found to obey first order kinetics over the temperature range 38–140°. Rogers' (1963) accelerated storage test was applied to this reaction and gave estimates of the rate coefficients and energy of activation of the inactivation reaction which agreed with the values determined in isothermal experiments. The stability of the enzyme preparation at 38° predicted by means of the accelerated storage data coincided with that found by isothermal storage of the enzyme at 38°.

MANY biological and pharmaceutical preparations are stored in the solid state. Hitherto the stabilities of these solid preparations have been estimated empirically by relating the rate of inactivation at some arbitrary temperature, higher than that of storage, to the established stabilities of similar preparations at the elevated and storage temperatures. This type of experiment can take several weeks or months to perform and a more rapid means of estimating the stability of solid preparations would be advantageous.

In a previous report (Cole & Leadbeater, 1966) a critical assessment of the accelerated storage test, described by Rogers (1963), was made and it was confirmed that the technique could be applied successfully to reactions in solution. The object of the present work was to establish that Rogers' technique can be used to estimate the stability of solid preparations of horse serum cholinesterase [EC 3.1.1.8].

Experimental

CHOLINESTERASE

Two preparations of partially purified horse serum cholinesterase were investigated. One was a preparation made in these laboratories by a method involving chromatography on diethylaminoethyl cellulose (DEAE-cholinesterase) which hydrolyzed 0·1 μmole acetylcholine/mg enzyme/min at 25°: the other, supplied by Organon Laboratories Ltd., was obtained by ammonium sulphate fractionation of horse serum (Organon-cholinesterase) and was about 40 times more active. The enzyme preparations were stored over phosphorus pentoxide.

TECHNIQUE

Cholinesterase (10 mg) was pressed onto the bottom of a tube of 10 mm internal diameter giving a layer of enzyme about 0·1 mm thick, assuming it to be uniformly distributed over the hemispherical surface of the base of the tube. The atmosphere above the sample was replaced with nitrogen. The tubes were fitted with guard-tubes containing silica gel

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and were heated in an oil bath. At appropriate times during the experiments tubes were withdrawn from the bath and cooled in an ice-water bath. The enzyme was dissolved in water, to a final activity of 0·2 units/ml, and the cholinesterase activity determined from the rate of hydrolysis of acetylcholine, using an automatic titrimeter (Cole & Leadbeater, 1966).

TEMPERATURE CONTROL

The temperature of the oil bath was controlled by means of an Ether Programme Temperature Controller, Type 994. For the accelerated storage experiments the temperature was within $\pm 0{\cdot}5°$ of that required by the programme and in the isothermal experiments the temperature control was better than $\pm 0{\cdot}1°$.

Results

ISOTHERMAL EXPERIMENTS

The thermal inactivation of both cholinesterase preparations followed first order kinetics. Typical curves are shown in Fig. 1, in which exponential curves for first order inactivation were fitted to the experimental data

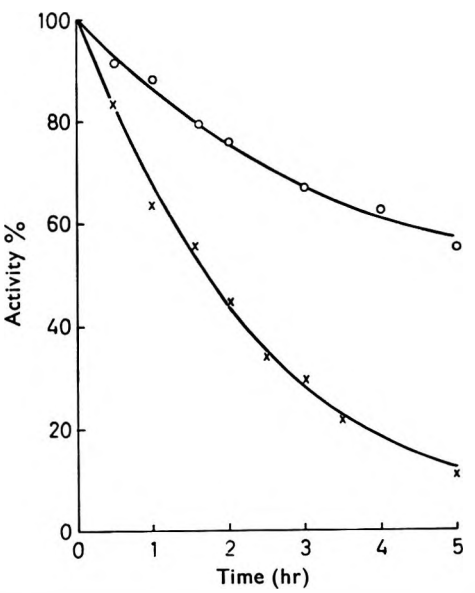

FIG. 1. The isothermal inactivation of dry cholinesterase. The points represent the experimental data for the inactivation of DEAE-cholinesterase at 120° (○) and Organon-cholinesterase at 140° (X) and the lines are the exponential curves fitted to the data.

by the method of least squares using a Ferranti Mercury computer. The exponential curves had the general form

$$\frac{a - x}{a} \cdot 100 = e^{-kt}$$

where a is the enzymic activity at zero time; x is the enzymic activity at time t; k is the rate coefficient. The average standard deviation of the points from the curves for all the isothermal data was $\pm$ 2·9.

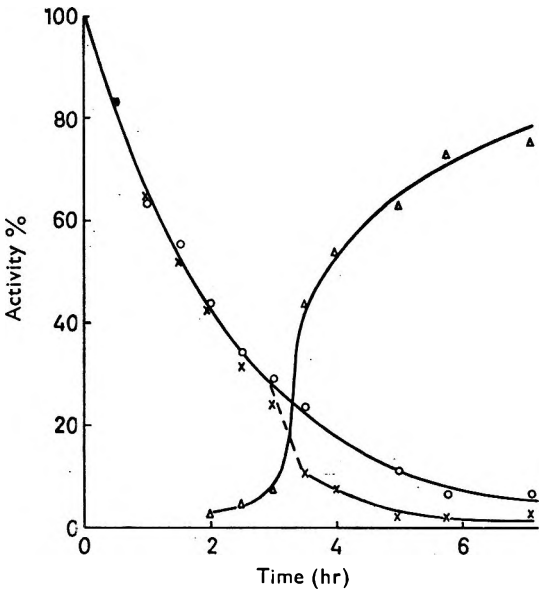

FIG. 2. The isothermal inactivation and denaturation of dry cholinesterase at 135°. The activities of the whole suspension of enzyme (○) and of the soluble fraction of the enzyme (×) are shown. The denaturation of the enzyme (△) was measured by the decrease in material absorbing at 280 mμ in the soluble fraction.

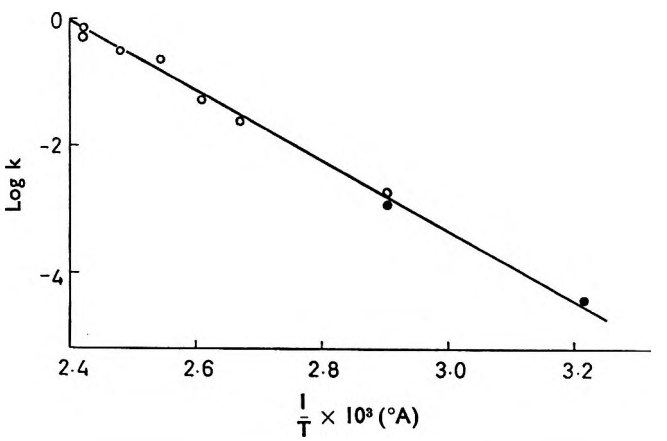

FIG. 3. The Arrhenius plot for the thermal inactivation of dry cholinesterase. The circles represent the rate coefficients determined isothermally and the line was calculated from the accelerated storage data. The solid circles represent data for the inactivation of the enzyme at 71° and 38° obtained by Mr. C. Stratford of this laboratory.

In most of the experiments the enzyme was readily soluble, but when it was more than 80% inactivated some of the protein would not dissolve. In these cases the suspension was homogenized for 10 sec using the microhead of a Silverson laboratory mixer (the enzyme was not inactivated under these conditions). The homogenate was used in the assay and smooth activity - time curves were obtained (Fig. 2). If the insoluble material was removed by centrifuging there was a break in the curve which corresponded with the point of rapid denaturation of the enzyme. Denaturation was measured by the decrease in the material absorbing at 280 mμ in the supernatent solution after centrifuging.

The effect of temperature on the rate of inactivation of DEAE-cholinesterase is shown in Fig. 3. The inactivation reaction obeyed the Arrhenius equation over the temperature range (140–71°) used in this series of experiments. Data obtained at lower temperatures (71 and 38°) by other workers in this laboratory were in excellent agreement with the present data (Fig. 3).

ACCELERATED STORAGE EXPERIMENTS

The enzyme was equilibrated at 110° and then the temperature was raised to 150° over a period of 6 hr according to the following program:

$$\frac{1}{T_o} - \frac{1}{T_t} = 2{\cdot}303.\ B.\log(1 + t)$$

Where $T_o = 383{\cdot}2°\ A$: $B = 2{\cdot}92 \times 10^{-4}$.
The Rogers' equation for the inactivation of cholinesterase, assuming first order kinetics, is shown below.

$$\log\left[2{\cdot}303.\log\left(\frac{a}{a-x}\right)\right] = \log k_{110} - \log\left(1 + \frac{EB}{R}\right) + \left(1 + \frac{EB}{R}\right)\log(1 + t) + \log\left[1 - \left(\frac{k_{110}}{k_t}\right)^{1 + \frac{R}{EB}}\right]$$

where E is the energy of activation; k_{110} is the rate coefficient at 110°; k_t is the rate coefficient at time t.

The data obtained by applying the accelerated storage technique to the thermal inactivation of both DEAE-cholinesterase and Organon-cholinesterase are shown in Table 1, together with the isothermal data for DEAE-cholinesterase. There was good agreement between the data for the two

TABLE 1. THE THERMAL STABILITY OF DRY CHOLINESTERASE

Preparation	Method of determination	No. expts.	E (kcal mole^{-1})	$k_{110} \times 10^5$ (sec^{-1})	Half-life at 38° C (years)
DEAE-cholinesterase	Accelerated storage	6	25·1 ± 2·6	1·77 ± 0·50	2·5 ± 0·7
DEAE-cholinesterase	Isothermal	1	25·9	1·42	2·2
Organon-cholinesterase ..	Accelerated storage	4	24·7 ± 1·4	1·28 ± 0·11	3·9 ± 0·3

preparations of cholinesterase, indicating that the different methods of isolation of the enzyme from horse serum yielded products with similar stabilities.

The agreement between the accelerated storage data and the isothermal data for the inactivation of DEAE-cholinesterase was very good (Table 1). This agreement is emphasized in Fig. 3 where the line was calculated from the accelerated storage data and the points represent the individual isothermal experiments.

Discussion

The data demonstrate that, over the range 38–140°, the thermal inactivation of cholinesterase follows first order kinetics. However, the data give no indication of the nature of the reaction involved in the inactivation and it is not possible to establish whether the inactivation process is in fact a true first order reaction or a complex change in the tertiary or secondary structure of the protein which yields apparent first order kinetics.

Inactivation of the enzyme preceded its denaturation since little or no insoluble protein was detected until the enzyme was about 80% inactivated. Inactivation probably involves a relatively minor change in the configuration of the peptide chain in the vicinity of the active centre of the enzyme whereas denaturation is the result of larger changes in the secondary or tertiary structures of the molecule rendering the protein insoluble. These experiments did not demonstrate whether the insoluble protein was enzymically active. The data in Fig. 2 show that the removal of the insoluble material from the enzyme suspension resulted in a loss of enzymic activity. This could have been due to the insoluble protein having enzymic activity or to molecules of the active native enzyme being carried out of solution with the insoluble material.

The present data clearly demonstrate that the accelerated storage test can be applied to the thermal inactivation of dry cholinesterase since the data obtained by this technique were reproducible (Table 1) and in good agreement with isothermal data (Table 1, Fig. 3). Moreover, the Rogers' experiments were completed within 8 hr whereas the isothermal studies at 71° and 38° were made over periods of eleven weeks and two years respectively. This technique eliminates the necessity of maintaining a standard method of assay (both chemicals and equipment must be standardized) for a long period. In addition to the great saving in time the accelerated storage procedure provides an estimate of the energy of activation of the inactivation process. This parameter enables the Arrhenius equation to be used to calculate the stability of the enzyme at any temperature in the range 38–140°, within which the inactivation was shown to follow first order kinetics. The energy of activation may also be used as a second parameter for comparing the stability characteristics of different samples of the same preparation.

Little work on the kinetics of the thermal inactivation of dry enzymes has been reported in the literature. In their review, Eyring & Stearn

(1939) quote papers by Tamman (1895) and Nicloux (1905) who showed respectively that the inactivation of dry preparations of emulsin and lipase followed first order kinetics. More recently, Mullaney (1966) demonstrated that the thermal inactivation of both trypsin and ribonuclease were first order processes. This work suggests that Rogers' accelerated storage test may have a general application in the estimation of the thermal stabilities of dry preparations of enzymes although, so far, it has only been applied to the inactivation of cholinesterase.

Acknowledgements. The computer programme for fitting the exponential curves to the experimental data was written by Dr. E. O. Powell of the Microbiological Research Establishment, Porton Down. The sample of DEAE-cholinesterase was supplied by Dr. J. J. Gordon of the Chemical Defence Experimental Establishment, Porton Down.

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A note on the determination of particle size by dielectric measurement

R. C. KAYE AND H. SEAGER

An incremental method for the determination of particle size distribution by dielectric constant measurement is described. The results obtained for griseofulvin and phenothiazine agree well with those obtained by Andreasen's method. The technique is suitable for the size-grading of low-conducting powder suspensions.

RECENTLY, Kaye & Seager (1967) described an incremental method for the determination of the droplet size distribution of emulsions by dielectric constant measurement. In this method, the proportion of dispersed phase in a narrow zone of emulsion was determined at suitable

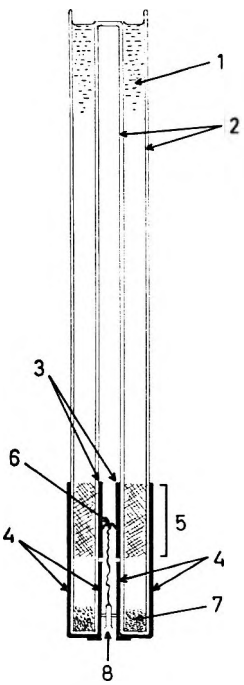

FIG. 1. Capacitance cell for the measurement of dielectric constant. 1. Annular space containing settling suspension. 2. Thin glass walls. 3. Capacitor plate at high R-F. potential. 4. Capacitor plates at earth potential. 5. Capacitance zone. 6. Copper contact. 7. Settled powder. 8. Coaxial socket.

time intervals, and by the application of Stokes' law, the droplet size distribution was calculated.

Using the same concept, this method has now been extended to the size grading of low conducting powder suspensions.

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DETERMINATION OF PARTICLE SIZE BY DIELECTRIC MEASUREMENT

METHOD

Suspensions of powdered solid (2%) (phenothiazine and coarse graded griseofulvin) were prepared using 1% Brij 98 as the wetting agent. The components were accurately weighed and the systems were carefully dispersed, the powder being gently triturated in a mortar with the aqueous phase. The dielectric constants of the settling suspensions were then measured as follows.

A cell, provided with a pair of capacitor plates at its lower end (see Fig. 1) was filled with suspension. As the suspension gradually settled and the concentration of solid in the narrow zone decreased, the dielectric constant of the suspension in this region increased. The change in dielectric value resulted in an increase of cell capacitance and this was measured by the heterodyne beat method (see Kaye & Seager, 1965, 1966, 1967; Seager, 1966).

The readings of capacitance were converted into dielectric values using the equation

$$\Sigma = \frac{Cg + C\,(Cg/Co + 1)}{Cg - C\,(Co/Cg + 1)} \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where Co = capacitance due to the air in the cell; Cg = capacitance due to the dielectric properties of the container walls; C = capacitance due to the dielectric properties of the suspension; Σ = dielectric constant of the suspension.

The composition of the suspension lying between the capacitor plates was calculated from the dielectric values employing the empirically derived equation

$$\%\ \text{Weight} = \frac{\Sigma - \Sigma_1}{d\Sigma/dC} \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

where Σ = the dielectric constant of the suspension; Σ_1 = the dielectric

TABLE 1. RESULTS FOR A SUSPENSION OF PHENOTHIAZINE IN WATER

Time		Zone capacitance in scale divisions	Dielectric constant of sample in zone	Composition of sample in zone		Diameter of particles (μ) leaving zone
min	sec			Weight %	Weight undersize %	
1	13	5,307	75·06	1·950	100	140
1	39	5,307·5	75·10	1·910	97·9	120
2	23	5,308·25	75·16	1·848	94·8	100
3	43	5,309·5	75·26	1·746	89·5	80
4	51	5,310·5	75·34	1·661	85·15	70
6	37	5,311·5	75·425	1·574	80·7	60
7	52	5,312	75·46	1·535	78·7	55
9	32	5,312·75	75·52	1·472	75·5	50
11	46	5,313·5	75·59	1·408	72·2	45
14	53	5,314·75	75·69	1·305	66·9	40
19	27	5,316	75·79	1·196	61·3	35
26	28	5,317·75	75·93	1·051	53·9	30
38	7	5,320	76·12	0·855	43·8	25
59	34	5,322·75	76·35	0·622	31·9	20
105	54	5,326	76·62	0·340	17·4	15
238	17	5,328	76·79	0·17	8·7	10

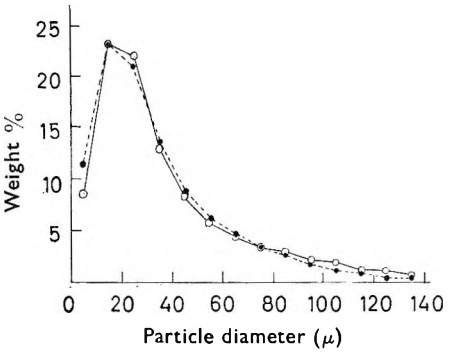

FIG. 2. Weight distribution polygons for phenothiazine suspended in water. ○—○, Dielectric methods; ●----●, Andreasen's method.

TABLE 2. RESULTS FOR A SUSPENSION OF GRISEOFULVIN (COARSE GRADE) IN WATER

Time		Zone capacitance in scale divisions	Dielectric constant of sample in zone	Composition of sample in zone		Diameter of particles (μ) leaving zone
min	sec			Weight %	Weight undersize %	
3	57	5,308·25	75·6	1·993	100	70
5	23	5,308·5	75·18	1·967	98·7	60
7	44	5,309·5	75·26	1·878	94·3	50
9	33	5,310·5	75·34	1·782	89·4	45
12	6	5,312	75·46	1·641	82·3	40
15	48	5,315·5	75·75	1·312	65·8	35
16	50	5,317	75·87	1·170	58·7	32·5
20	8	5,312·25	76·20	0·782	39·2	31
32	16	5,328·75	76·85	0·359	18	30
48	23	5,329	76·88	0	0	20
193	33	5,329	76·88	0	0	10

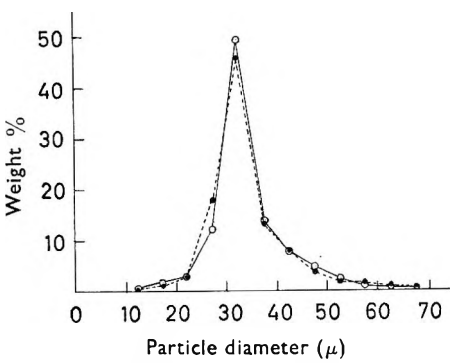

FIG. 3. Weight distribution polygons for griseofulvin suspended in water. ○—○, Dielectric method; ●—●, Andreasen's method.

constant of the continuous phase; $d\Sigma/dC$ = the rate of change of dielectric constant with suspension concentration.

The value of $d\Sigma/dC$ had been previously determined experimentally. The results of plotting dielectric constant against weight % for the suspensions of griseofulvin and phenothiazine gave straight lines of slopes 0·864 and 0·97 respectively.

The particle size distributions were then calculated from composition measurements using the modified form of Stokes' equation where d the diameter of the particles replaces r the radius of the particles.

Cumulative weight undersize curves were constructed from which weight distributions of particle size were obtained. Results for the cumulative weight undersize data for the two powdered suspensions are given in Tables 1 and 2. The weight distribution curves obtained for phenothiazine and griseofulvin suspensions, plotted alongside the size distributions obtained by the Andreasen's pipette method, are shown in Figs 2 and 3 respectively.

The agreement between the results obtained by the two methods is excellent, showing that the dielectric method offers a simple and rapid alternative method for the size grading of low conducting suspensions.

Acknowledgements. We are indebted to Glaxo Laboratories Limited for the generous supply of griseofulvin, and to the Imperial Chemical Industries for their sample of phenothiazine.

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The distribution of salicylate in mouse tissues after intraperitoneal injection

J. A. STURMAN, P. D. DAWKINS, N. McARTHUR AND M. J. H. SMITH

The concentrations of salicylate and its principal metabolites were measured in blood, liver, brain, kidney, heart, spleen, diaphragm and skeletal muscle after the intraperitoneal injection of a fixed amount of radioactive salicylate and varying doses of unlabelled salicylate. The patterns of distribution of salicylate in the various organs with time were similar, the peak level being attained in 30–60 min after injection. Salicylate was eliminated from the blood after 8 hr but persisted in liver in measurable amounts up to 24 hr.

SALICYLATE inhibits a number of enzyme systems *in vitro*, including aminotransferases, dehydrogenases and oxidative phosphorylation reactions (Smith & Smith, 1966). The degree of inhibition of any particular enzyme depends on several factors, one of the most important being the concentration of salicylate in the reaction mixture. Similar considerations apply to the possible *in vivo* actions of the drug on tissue enzymes. A knowledge of the tissue concentrations of salicylate occurring after the administration of the drug is therefore a necessary preliminary to any studies designed to investigate the *in vivo* interactions of salicylate and enzyme systems. The present work describes the measurement of the concentrations of salicylate in mouse tissues at several time intervals after the injection of varying amounts of the drug.

Experimental

ANIMAL PREPARATION

Male mice, 25–30 g, of the albino strain maintained at King's College Hospital on M.R.C. modified cube diet No. 41B were divided into five groups, each of 45 animals. Each mouse received an intraperitoneal injection (0·2 ml) containing 2 μC of [^{14}C]salicylic acid (specific activity 15·5 mc per mmole, obtained from the Radiochemical Centre, Amersham, Bucks) plus sodium salicylate at a dose level of either 50, 100, 200, 400 or 800 mg/kg body weight. The injections were adjusted by adding sufficient sodium chloride to contain the same final concentration of sodium. Subgroups, each of five mice, were killed by cervical fracture at 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 18 hr and 24 hr after injection. Food was withdrawn during the experiment but the animals were allowed to drink freely. Blood samples were collected, after decapitation, into lithium heparin tubes and the liver, brain, kidneys, spleen, heart, diaphragm muscle and both quadriceps femoralis muscles were rapidly removed, weighed on a torsion balance and stored in 80% (v/v) ethanol at −20°.

DETERMINATION OF TOTAL RADIOACTIVE MATERIAL IN THE TISSUE

The excised tissues were homogenized in 5 ml of 80% (v/v) ethanol using a Potter all-glass homogenizer. The homogenate was transferred to a centrifuge tube and the homogenizer washed out with a further 5 ml

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of the ethanol. The combined homogenate and washings were heated to 80° with frequent stirring and centrifuged at 2000 *g* for 30 min. The supernatant was decanted and the residue was resuspended in 5 ml of 80% (v/v) ethanol and the extraction repeated a further eight times with liver and five times with the other tissues. The solvent from the combined supernatants of each tissue was removed in a rotary evaporator at 55° under reduced pressure and the final residue dissolved in 5 ml of 80% (v/v) ethanol. Aliquots (0·5 ml) were used for the determination of total radioactivity in a Beckman liquid scintillation counter using as phosphor 5 ml of 0·4% 2,5-diphenyloxazole, 0·02% 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene and 60% naphthalene in 1,4-dioxan. The counting efficiency, determined by using [^{14}C]toluene as an internal standard, was 92 $\pm$ 2% for all the samples. Each sample was counted in duplicate until at least 10,000 counts were recorded.

DETECTION AND MEASUREMENT OF METABOLITES OF SALICYLATE

The radioactive substances present in the ethanolic solution of the final residue (see above) were separated by ascending chromatography, using Whatman No. 4 chromatography paper and benzene–acetic acid–water (4:2:2) and located by radioautography, using Kodak Blue Brand X-ray film and an exposure of 3 weeks. Radioactive salicylic acid occurred at the solvent front and its identity, and that of salicyluric acid (Rf = 0·35), were confirmed by co-chromatography with authentic material in a variety of solvent systems. Most of the chromatograms also showed a radioactive spot at the origin and this metabolite behaved as a single substance in a number of solvents. It was presumptively identified by its chromatographic position as a mixture of salicylglucuronides and this was confirmed by acid hydrolysis which gave a mixture of salicylic and glucuronic acids (see Quilley & Smith, 1952). The radioactivity in the separated metabolites was measured directly on the chromatography paper by a Packard Tri-Carb liquid scintillation counter using as phosphor 10 ml of 0·4% 2,5-diphenyloxazole and 0·01% 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene in toluene. The salicylate was completely extracted by the phosphor but salicylurate and the salicylglucuronides were insoluble and remained on the paper. Appropriate corrections were therefore made for the differing counting efficiencies.

PROTEIN BINDING OF SALICYLATE BY MOUSE PLASMA

The binding curve of salicylate and the proteins of human and mouse plasma were determined by the equilibrium dialysis method of Davison & Smith (1961) using [^{14}C]salicylic acid and measuring the radioactivity outside the dialysis sac before and after equilibration for 3 hr at 37° by the Beckman scintillation counter.

Results

Salicylate concentrations in mouse tissues between 5 min and 24 hr after the injection of doses ranging from 50 to 800 mg/kg are given in Table 1. The values for the highest dose are incomplete since the mice

TABLE 1. DISTRIBUTION OF SALICYLATE IN MOUSE TISSUES. The values are the means of five animals and are expressed as mg/litre for blood and mg/kg wet weight for the other tissues.

Tissue	Dose injected (mg/kg)	Time after injection								
		5 min	15 min	30 min	1 hr	2 hr	4 hr	8 hr	18 hr	24 hr
Blood	50	32·8	41·7	51·1	26·9	8·1	1·3	0	0	0
	100	53·2	84·6	102·1	63·3	30·1	6·1	0	0	0
	200	122·6	167·9	174·6	121·9	98·9	11·7	1·9	0	0
	400	291·6	291·4	387·7	233·1	162·7	37·4	22·9	0	0
	800	675·9	564·4	604·7	491·7	205·0	—	—	—	—
Liver	50	8·5	10·6	22·5	21·5	13·9	4·4	0·5	0	0
	100	24·0	44·0	62·9	46·5	41·6	14·6	1·3	0·1	0
	200	92·0	120·5	143·6	111·4	85·0	42·4	4·8	2·9	1·3
	400	173·3	198·6	270·4	227·6	178·4	96·8	23·3	13·0	6·4
	800	282·8	303·1	325·8	258·5	208·4	—	—	—	—
Brain	50	4·5	9·4	9·1	5·3	1·6	0·3	0	0	0
	100	6·8	23·4	24·4	15·9	7·3	0·8	0	0	0
	200	17·5	48·0	53·0	40·0	32·9	4·1	1·4	0·2	0
	400	47·3	67·5	123·9	95·9	91·3	32·0	6·3	1·0	0
	800	104·0	177·0	226·0	238·7	224·0	—	—	—	—
Kidney	50	23·8	28·4	37·7	33·0	12·8	2·2	0·4	0	0
	100	40·0	51·9	62·9	48·2	32·5	5·9	0·8	0	0
	200	75·1	90·5	100·9	92·8	74·4	21·0	11·3	0·2	0
	400	130·4	160·6	255·9	174·0	160·4	66·0	36·4	0·9	0·5
	800	263·6	281·0	412·5	331·1	269·1	—	—	—	—
Heart	50	12·8	15·9	16·2	9·0	3·7	0·6	0·4	0	0
	100	27·0	39·5	37·9	30·4	17·1	1·8	0·9	0	0
	200	66·8	79·9	78·2	67·5	57·1	8·0	3·0	0	0
	400	122·3	149·0	192·5	187·8	139·8	48·0	18·7	0	0
	800	176·3	235·2	274·4	245·4	162·8	—	—	—	—
Spleen	50	8·1	10·7	12·6	8·5	4·0	0·6	0	0	0
	100	19·7	27·9	31·9	26·4	17·7	2·0	0	0	0
	200	49·3	63·5	71·8	61·6	47·4	5·9	0·1	0	0
	400	105·6	133·8	173·8	150·5	123·9	21·5	1·3	0	0
	800	189·1	232·2	256·7	278·1	141·4	—	—	—	—
Diaphragm	50	10·8	12·0	21·6	10·2	8·0	1·9	1·4	0	0
	100	28·7	33·7	45·7	34·3	30·6	4·6	2·9	0	0
	200	75·0	75·2	79·5	91·7	75·4	12·1	8·9	1·5	0
	400	473·1	222·0	204·5	221·9	196·9	33·4	32·7	5·5	0
	800	904·4	336·2	248·2	227·4	175·1	—	—	—	—
Muscle	50	5·6	7·4	9·5	5·8	2·7	1·0	0·3	0	0
	100	7·2	12·4	17·7	16·2	14·5	2·1	0·8	0	0
	200	22·2	37·8	44·8	39·2	38·2	26·6	2·5	0	0
	400	74·7	93·1	123·4	106·3	88·0	74·6	8·5	0	0
	800	205·7	238·5	271·3	317·0	157·5	—	—	—	—

died 2–4 hr later. No deaths occurred in the other groups. The blood salicylate reached a peak at 30 min, except for the 800 mg/kg dose when it was reached at 5 min. Salicylate disappeared from the circulation between 4 and 8 hr with the two lowest doses and between 8 and 18 hr with the 200 and 400 mg/kg doses. The distribution of the salicylate with time in the other tissues followed a similar pattern except that very high concentrations of salicylate were observed in the diaphragm muscle at 5 min after the injection of the high doses (400 and 800 mg/kg) and that the salicylate persisted in some of the tissues, particularly the liver, up to 24 hr. One possible explanation for these initial high levels of salicylate in the diaphragm is that the drug entered the tissue directly from the peritoneal cavity and not via the circulation during a time interval when the salicylate concentration in the peritoneal fluid was at its maximum.

Gentisic acid and other hydroxylated metabolites of salicylate were not detected in any tissue. Salicylurate was found only in kidney and appeared

in samples obtained 30 min after injection to the extent of 3 to 7% of the dose of salicylate but was not detected in animals killed 2 hr or more after injection. Salicylglucuronides were present in all the tissues except brain. The concentrations of glucuronide present varied with time similarly in all tissues. The glucuronides accumulated for the first 15 min, then the concentrations fell sharply and slowly recovered over the next 2 hr, finally decreasing to zero between 2 and 24 hr. This effect was most pronounced in the liver and is illustrated in Fig. 1.

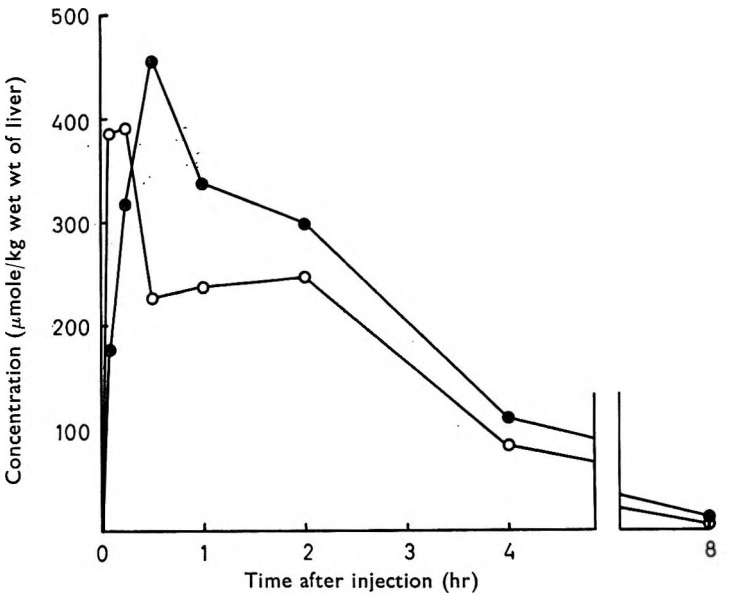

FIG. 1. Concentration of salicylate and salicylglucuronide in mouse liver after the intraperitoneal injection of 100 mg/kg body weight of sodium salicylate. ●, Salicylate; ○, salicylglucuronides.

Discussion

Many workers have studied the plasma salicylate concentrations in man and animals after single or repeated doses of the drug. However, apart from blood and urine, little information is available about the distribution of salicylate and its metabolites in tissues. Fragmentary data have been published about the levels of salicylate in human organs in cases of suicide, accidental poisoning and death during salicylate therapy and isolated reports have appeared giving values for a few animal tissues (Gross & Greenberg, 1948). The most comprehensive experiments have been done with the rat. Smith, Gleason & others (1946) determined the salicylate levels in liver, muscle, kidney, brain and lung after the oral administration of the drug. They found that the salicylates in the tissue fluid of the liver, kidneys and lung was approximately the same as that in the serum while the corresponding levels in muscle and brain were much lower. The distribution of radioactive salicylate and its metabolites in many rat organs

at 0·5, 1, 2 and 4 hr after the intraperitoneal injection of [^{14}C]salicylate was investigated by Wolff & Austen (1958). The principal findings were that the peak level of salicylate occurred 1 hr after the injection and 90–95% of the radioactivity in the tissues was salicylate itself. Traces of salicylglucuronides and salicylurate were detected in all tissues, except brain and thyroid gland, and the liver and kidney contained 2–6% of the radioactivity as glucuronides and 1–2% as gentisic acid.

The present results (Table 1) give a more complete picture of the distribution of salicylate in mouse tissues at several time intervals after the intraperitoneal injection of varying doses of salicylate. The patterns of distribution of salicylate in the various organs with time were similar in all the tissues examined, the peak level being attained in 30–60 min after the injection. Salicylate was eliminated from the blood after 8 hr but persisted in the liver, in measurable amounts, up to 24 hr.

The plasma salicylate concentrations found in the mouse were much lower than would have been predicted on the basis of similar experiments in man. Thus a single dose of 2 g of sodium salicylate (equivalent to 30 mg/kg body weight) in man gives plasma salicylate concentrations varying between 75 and 200 mg/litre (Smith & others, 1946). The 50 mg/kg dose in the mouse only produced a concentration of 50 mg/litre at the peak level and the values at the other time intervals were much lower (Table 1). The main factor which determines circulating salicylate levels is the extent to which salicylate is bound to the serum proteins, particularly albumin (Reynolds & Cluff, 1960). In man, the relative extent of the binding decreases with increasing salicylate concentration (Davison & Smith, 1961) and the present work shows that this is also true for the mouse except that the amount of salicylate bound at a given salicylate concentration is much less in the latter species (Fig. 2).

This finding could explain the relatively low circulating total salicylate concentrations observed in the mouse plasma samples. It also implies that the elimination of salicylate from the blood, i.e. the half-life of circulating salicylate, should be lower in the mouse than in man. Done (1960) has shown that the regression curve for plasma salicylate levels in man quickly assumes the characteristics of a first-order reaction and that the levels declined at a reasonably constant fractional rate. It is possible to derive a value for the mean slope of this regression curve and hence to calculate the average half-life for circulating salicylate in man to be 20 hr (Done, 1960). A similar calculation for the mouse, using the present results, gives a value of 1 hr for the half-life of circulating salicylate.

Salicylglucuronides were detected in all the mouse organs except the brain. It has been shown by Schachter, Kass & Lannon (1959) that the liver, kidney, lung, spleen and urinary bladder of many animal species are able to conjugate salicylate to salicylglucuronides *in vitro* but that other tissues, including the brain, were unable to effect the biosynthesis of the salicylglucuronides. These results suggest that the occurrence of salicylglucuronides in mouse skeletal muscle, diaphragm and heart is due to entry of the conjugates from the circulation after their biosynthesis in the other mouse tissues. The present work also shows that the circulating

glucuronides do not enter the mouse brain and that the blood-brain barrier must be impermeable to salicylglucuronides.

The concentrations of circulating salicylglucucuronides fluctuated with time in a different manner to the concentrations of circulating salicylate (Fig. 1). One possible explanation is that during the initial phase of the experiments (0–15 min after injection) the biosynthesis of the

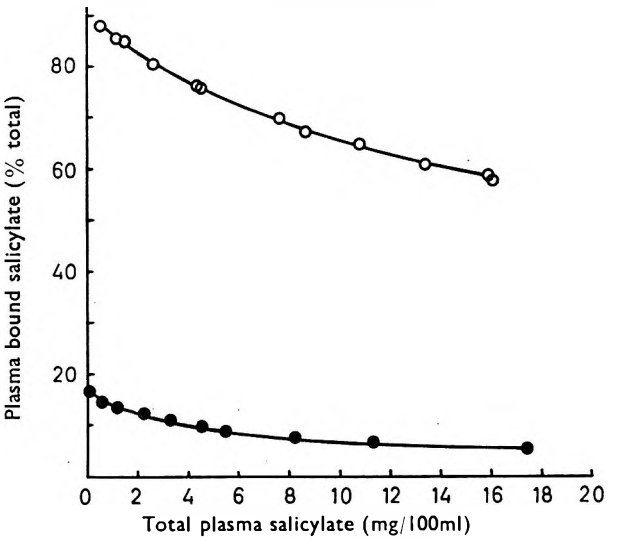

FIG. 2. Protein binding curves of salicylate in human and mouse plasma. ○, human plasma; ●, mouse plasma.

glucuronides in the liver and other tissues proceeded normally. However, when the liver salicylate concentration increased to the peak level (15–60 min) the biosynthesis of the glucuronides was partially inhibited due to the uncoupling action of salicylate on oxidative phosphorylation processes interfering with energy-dependent synthetic reactions. The concentrations of salicylglucuronide in the liver were therefore decreased during this period and their levels only started to rise again when the salicylate concentrations decreased after the peak level.

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The relation between primidone and phenobarbitone blood levels

JAMES BOGAN AND HAMILTON SMITH

A dose of primidone or one fifth of the same weight of phenobarbitone produced equal levels of phenobarbitone in the blood.

IN 1965, Bogan, Rentoul & Smith described the findings in a fatal poisoning by primidone. It appeared from this case and from the others summarized in that paper that most, if not all, of the effects of primidone are due to its conversion in the body to phenobarbitone. This paper describes work designed to clarify this point.

Experimental and results

ULTRAVIOLET SPECTROMETRY

The quantitative estimation of barbiturates is based on the method of Broughton (1956) as modified by Bogan & Smith (1967).

GAS-LIQUID CHROMATOGRAPHY

The method for the estimation of primidone and phenobarbitone by gas-liquid chromatography is summarized below.

A sample of 5 ml of blood or urine (acidified to pH 3) is shaken with chloroform (50 ml). The chloroform layer is filtered, washed with phosphate buffer (5 ml, pH 7·4) and extracted with N sodium hydroxide

TABLE 1. PHENOBARBITONE IN BLOOD

Subject	Body weight (kg)	Dose (mg)	Dose/kg (mg)	Phenobarbitone in blood (mg/100 ml)	*Corrected phenobarbitone in blood (mg/100 ml)
1	61·0	195	3·2	1·7	0·51
2	60·5	133	2·2	1·9	0·86
3	57·5	98	1·7	0·8	0·47
4	65·2	98	1·5	0·7	0·47
5	65·0	195	3·0	1·4	0·47
6	54·5	98	1·8	0·9	0·50
7	56·0	185	3·3	2·1	0·64
8	98·0	88	0·9	0·4	0·44
9	68·0	88	1·3	0·5	0·38
10	55·0	88	1·6	1·1	0·69
11	50·0	185	3·7	0·9	0·24
Average	62·8	132	2·2	1·1	0·52

* Corrected to a dose of 1 mg phenobarbitone/kg body weight.

solution (5 ml). The aqueous layer is adjusted to pH 3 with hydrochloric acid and re-extracted with chloroform (50 ml). The chloroform is evaporated to a suitable small volume and an aliquot used for gas-liquid chromatography.

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Column: stainless steel, 6 ft × $\frac{1}{8}$ in O.D. packed with 5% SE30 on aeropak 30 (100–120 mesh), maintained at 180°; injection port, 225°, flame ionization detector at 250°. Nitrogen carrier gas and hydrogen supply for the flame flow rate 60 ml/min. Under these conditions the retention times of phenobarbitone and primidone are 1·6 and 3·4 min respectively. A disc integrator is used to measure peak areas. Primidone concentrations are calculated by comparison with the phenobarbitone peak obtained spectrophotometrically. The relationship of phenobarbitone and primidone peak areas is found in the usual manner using internal standards.

THIN-LAYER CHROMATOGRAPHY

The method used is that reported by Bogan, Rentoul & Smith (1964).

SAMPLES

Arrangements were made to collect blood from patients on long term treatment with phenobarbitone, primidone or a mixture of both. Samples were taken 3 hr after a dose. The samples were extracted without delay, and the phenobarbitone levels measured quantitatively spectrophotometrically. As the patients all received differing weights of drugs, the values obtained were adjusted for dose and body weight so that a valid comparison could be made. The values for phenobarbitone are given in Table 1 and for primidone in Table 2. Table 3 gives the blood levels of phenobarbitone and primidone in patients treated with a mixture of these drugs.

TABLE 2. PRIMIDONE-PRODUCED PHENOBARBITONE IN BLOOD

Subject	Body weight (kg)	Dose (mg)	Dose/kg (mg)	Phenobarbitone in blood (mg/100 ml)	* Corrected phenobarbitone in blood (mg/100 ml)
12	61·0	750	12·3	1·40	0·114
13	73·0	750	10·3	0·55	0·053
14	65·5	1,500	23·0	2·85	0·124
15	63·8	750	11·8	1·60	0·135
16	91·0	2,000	22·0	3·10	0·141
17	71·0	1,500	21·0	2·90	0·138
18	58·0	1,500	26·0	1·75	0·067
19	80·0	2,000	25·0	2·20	0·088
20	52·0	750	14·5	2·00	0·138
Average	68·4	1,280	18·4	2·04	0·111

* Corrected to a dose of 1 mg primidone/kg body weight.

RESULTS

If the average blood phenobarbitone level [mg/mg (dose)/kg (body weight)] is calculated, the values found for administered phenobarbitone and primidone are 0·520 and 0·111 respectively. When the value of 0·520 is used to calculate the contribution of the administered phenobarbitone to the phenobarbitone blood level resulting from mixed primidone-phenobarbitone treatment the resulting average contribution by the primidone works out to 0·118 mg/mg (primidone administered)/kg (body

weight). Comparison of this value with that of 0·111 found in pure primidone therapy shows surprisingly good agreement considering the small number of samples.

METABOLISM STUDIES

When examining the above samples using thin-layer chromatography, both phenobarbitone and hydroxyphenobarbitone were produced during primidone metabolism. The metabolism was investigated further by the *in vitro* metabolism method of Smith, Waddell & Butler (1963) using rat liver microsomes and soluble fraction. The method uses a solution of nutrients made up as follows: (mg) nicotinamide, 123; nicotinamide adenine dinucleotide phosphate, 11·7; glucose 6-phosphoric acid, 37·2; semicarbazide, 75·6; magnesium chloride, 100; disodium hydrogen phosphate, 492; sodium dihydrogen phosphate, 54·2; water to 25 ml. Metabolism was in 25 ml flasks, flushed with oxygen in which 2 mg primidone (or phenobarbitone) was placed together with 2·5 ml of a nutrient solution

TABLE 3. PHENOBARBITONE IN BLOOD FROM MIXED PHENOBARBITONE AND PRIMIDONE

Subject	Body weight (kg)	Drug	Dose (mg)	Dose/kg (mg)	Phenobarbitone in blood (mg/100 ml)	Calculated contribution of phenobarbitone (mg/100 ml)	Phenobarbitone due to primidone (mg/100 ml)	*Corrected primidone contribution (mg/100 ml)
21	64·7	Primidone	1,500	23·2	4·20	1·57	2·63	0·118
		Phenobarbitone	195	3·02				
22	56·4	Primidone	750	13·3	2·90	1·76	1·14	0·082
		Phenobarbitone	195	3·45				
23	75·5	Primidone	1,000	13·2	2·10	0·89	1·21	0·092
		Phenobarbitone	130	1·72				
24	65·5	Primidone	1,500	23·0	4·40	1·03	3·37	0·146
		Phenobarbitone	150	1·99				
25	57·5	Primidone	750	13·0	2·25	0·26	1·99	0·154
		Phenobarbitone	30	0·52				

* Corrected to a dose of 1 mg primidone/kg body weight.

and 2·5 ml of rat liver homogenate. [This was prepared by homogenizing liver in cold 0·2N potassium phosphate buffer of pH 7·4 (1 g liver/4 ml buffer). The homogenate was centrifuged at 9000 *g* for 30 min in a refrigerated centrifuge. The supernatant containing microsomes and the soluble fraction was refrigerated and used within a few hr.] The flasks were again flushed with oxygen and the resulting mixture shaken for 3 hr at 37°. Protein was then precipitated by adding 0·1 ml sodium hydroxide solution (10% w/v), 1·0 ml sodium tungstate solution (10% w/v) and 0·35 ml of sulphuric acid (10% v/v) to the flask which was then shaken and heated on a boiling water bath for 10 min. The precipitated protein was filtered off and the filtrate shaken directly with 100 ml of chloroform to extract the barbiturate. The chloroform layer was filtered, dried with anhydrous sodium sulphate and evaporated to dryness. The residue was ready for thin-layer chromatography.

RESULTS

Analysis of the solutions from metabolism studies containing primidone showed the presence of phenobarbitone, hydroxyphenobarbitone and primidone, while those containing phenobarbitone showed phenobarbitone and hydroxyphenobarbitone. Though the thin-layer chromatographic method allowed only semiquantitative measurement of barbiturates in blood, it appeared that the concentrations of phenobarbitone and hydroxyphenobarbitone were almost equal in the system starting from primidone but phenobarbitone was in excess in the system starting from phenobarbitone. This may indicate either the direct production of hydroxyphenobarbitone from primidone or that the phenobarbitone is being metabolized almost as rapidly as it is produced. This would certainly be in keeping with the fact that much larger amounts of primidone are required to give the same effect as phenobarbitone.

Discussion

From the Tables it can be seen that for primidone an average dose of 18·4 mg/kg body weight produces an average blood level of phenobarbitone of 2·04 mg/100 ml. For phenobarbitone the corresponding values are 2·2 mg/kg and 1·1 mg/100 ml, i.e. for an equivalent blood level of phenobarbitone the average dose of primidone should be 10 mg/kg. Therefore, if the total effect of primidone is due to its conversion to phenobarbitone we would expect that about five times the dose of phenobarbitone would be required. It has been reported by Gruber, Moisier & Grant (1957) that the dose of primidone required to replace phenobarbitone for similar clinical effects is five times the phenobarbitone dose.

This fivefold increase may be due to the fact, shown by the comparison of blood and urine levels of primidone and phenobarbitone, that there is a large discrimination by the kidneys against primidone leading to rapid excretion. It would appear that the action of primidone is due to its metabolite phenobarbitone.

Acknowledgements. The authors wish to thank the many people who have contributed and collected samples for this study.

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An inverse relationship between the pressor response to noradrenaline and the resting blood pressure

SIR,—In rats under pentobarbitone anaesthesia (45 mg/kg, i.p.), in which initial and final mean arterial pressures have differed by less than 10 mm Hg; the pressor response to noradrenaline is inversely related to the resting blood pressure. Fig. 1 makes a comparison of responses to 20 and 200 ng noradrenaline, intravenously in two groups of rats, A and B, weighing 180–220 g, in which group A had a resting mean arterial pressure of 161·0 ± 2·7 and group B 143·0 ± 1·1 mm Hg. As can be seen from Fig. 1, the pressor response elicited by noradrenaline was greater for the group of rats with the lower resting arterial blood pressure at both the low ($P < 0.01$) and high ($P < 0.001$) dose levels. In these experiments the mean of three responses from each rat to each dose has been used and order of dose administration has been determined by randomization.

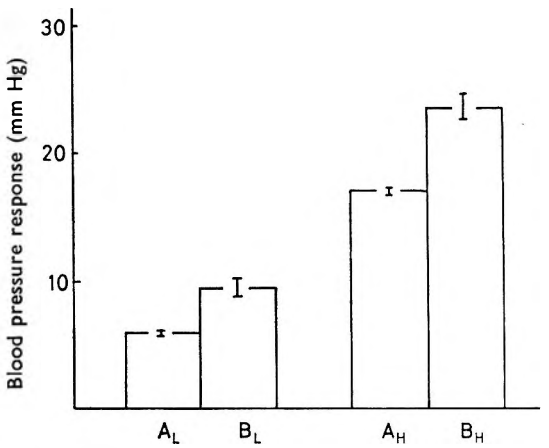

FIG. 1. The mean ± s.e. of the pressor response of two groups of rats (A and B) to a low dose (L=20 ng) and a high dose (H=200 ng) of noradrenaline i.v. The mean resting arterial blood pressure of group A (17 rats) was 161·0 ± 2·7 (mean ± s.e.) while that of group B (31 rats) was 143·0 ± 1·1 mm.

The maximum response to noradrenaline obtainable from these preparations is at least 60 mm Hg, and linearity of the log-dose effect curve theoretically extends from responses of 12 to 48 mm Hg. Since the rise in arterial pressure caused by the low dose of noradrenaline in group A was only 5·98 ± 0·13 mm Hg, this effect fell outside the linear range. Hence the apparent difference between the slopes of the log-dose effect curves for group A and B (Fig. 1) is an artifact.

There is also a similar relationship between the pressor effect of a fixed dose of angiotensin and the resting mean arterial pressure. Therefore this inverse relationship may hold for all pressor agents. Caution should then be exercised when comparing pressor responses of groups of anaesthetized animals with different resting blood pressures.

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Reciprocal potentiation of neuromuscular blocking agents

SIR,—When the rat phrenic nerve diaphragm preparation is used for quantitative work, the washout period, after the application of a neuromuscular blocking agent, is adjusted so that the subsequent addition of an equal concentration of the same agent will cause a similar response. We have already noted that a rat phrenic nerve diaphragm preparation, previously treated with tubocurarine and washed out as described, was unexpectedly sensitive to diallylnortoxiferine (Pleuvry & Hunter, 1967). Some further investigations have now been made.

Approximately equipotent concentrations of two neuromuscular blocking agents were applied, for 3 min to a rat phrenic nerve diaphragm preparation. Blocking agent A was applied first and the percentage reduction in lever movement measured. The preparation was then washed in the appropriate manner and then blocking agent B was added for 3 min. After a washout period appropriate for blocking agent B, blocking agent A was repeated and the percentage increase in response over the initial response was measured.

TABLE 1. RECIPROCAL POTENTIATION OF NEUROMUSCULAR BLOCKING AGENTS

Drug A	Drug B	% Potentiation of Drug A (± s.e.)
Diallylnortoxiferine	Tubocurarine	119 (±21·2) (6)*
Tubocurarine	Diallylnortoxiferine	22·3 (±3·70) (6)*
Tubocurarine	Toxiferine	67·0 (±8·66) (5)*
Diallylnortoxiferine	Toxiferine	80·7 (±10·4) (6)*
Gallamine	Toxiferine	1·2 (±5·59) (6)*

* Number of experiments from which the standard errors were calculated.

TABLE 2. EFFECT OF ANTICHOLINESTERASES AGENTS ON RECIPROCAL POTENTIATION

Anticholinesterase agents	Increase (%) in response to diallylnortoxiferine on a tissue sensitized by tubocurarine (± s.e.)	No. of experiments
Neostigmine	132·4 (±15·64)	6
Physostigmine	13·1 (±5·94)	7
Edrophonium	67·1 (±16·04)	6
Amberonium	71·6 (±19·54)	5
Dyflos	26·9 (±19·54)	7

The neuromuscular blocking agents used were gallamine, diallylnortoxiferine, toxiferine and (+)-tubocurarine. The results (Table 1) show that treatment of the preparation with toxiferine and tubocurarine potentiated diallylnortoxiferine. Toxiferine also potentiated tubocurarine and to a lesser, but still significant, extent diallylnortoxiferine potentiated tubocurarine. Only gallamine was not significantly potentiated by toxiferine.

The effect of anticholinesterases was investigated only on the potentiation of diallylnortoxiferine by tubocurarine. Edrophonium, ambenonium, physostigmine and neostigmine were used in approximately equipotent concentrations, and each agent was added to the preparation 1 min before each administration of neuromuscular blocking agent. The results (Table 2) showed that only physostigmine reduced the potentiation to insignificant levels. Complete

inhibition of cholinesterase with di-isopropylfluorophosphonate (dyflos, DFP, 20 μg/ml for 1 hr) also inhibited the potentiation. It is interesting to note that physostigmine and dyflos have by far the highest lipid solubility and this may be related to their ability to inhibit this potentiation.

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The influence of hypoxia upon toxicity of a nucleotoxic agent, mustine hydrochloride

SIR,—Although many radioprotective substances also provide protection against nucleotoxic agents (Scarborough & Thomas, 1962), this is not so for 5-hydroxytryptamine (5-HT) which is a powerful chemical radioprotector (Uroić, Rabadjija & Supek, 1964) but enhances the toxicity of a typical representative of radiomimetic poisons—mustine hydrochloride (nitrogen mustard).

Tissue hypoxia provides protection against ionizing radiation (Brues & Patt, 1953). According to van den Brenk & Haas (1961) 5-HT exerts its radioprotective effect in terms of pharmacologically-induced hypoxia. If this is true then hypoxia alone should not protect against mustine hydrochloride. To test this assumption we have examined the influence of acute hypoxia upon the chronic toxicity of mustine hydrochloride in rats. Hypoxia was produced by administering 55 mg/kg of hydroxylamine hydrochloride intraperitoneally. This dose does not change the toxicity of mustine, but exerts a strong methaemoglobinaemia (40–50%) with consequent anaemic hypoxia.

Two groups of 34 albino rats were injected intravenously with mustine hydrochloride (0·8 mg/kg). The first group received saline and the second group hydroxylamine hydrochloride (55 mg/kg) intraperitoneally 30 min before mustine. The survival of rats was observed every 12 hr during 30 days. The mortality (%) and the mean survival time (days $\pm$s.e.m.) were: 64·7 and 13·7 $\pm$ 2·09 for the saline group and 30·3 and 22·4 $\pm$ 2·04 for the group treated with hydroxylamine hydrochloride.

A decrease in mortality rate (χ^2-test; $P < 0.01$) and increase in mean survival itme (t-test; $P < 0.01$) was observed. It is evident that hypoxia in our experimental conditions significantly reduces the toxicity of mustine hydrochloride.

The present finding does not seem to be consistent with the hypothesis that 5-HT exerts its radioprotective action by means of cellular hypoxia.

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Antihistamine constriction in mouse skin microcirculation

SIR,—Evidence has recently accrued, using direct *in vivo* microscopy of various regional circulations, that some structurally unrelated antihistimines, e.g., ethanolamines (diphenhydramine), ethylenediamines (pyrilamine), phenothazines (promethazine) and dimethylpropylamines (chlorpheniramine), have a dose dependent constrictor action on the muscular micro-vessels of mesenteries and omentums of rats, rabbits and guinea-pigs which resembles the profile of vascular reactivity for adrenaline and noradrenaline in the microcirculation (Altura, 1964; Altura & Zweifach, 1965a, Haley & Harris, 1949; Haley & Andem, 1950). Further, these various antihistamines sensitized various muscular micro-vessels (precapillaries, metarterioles, arterioles, venules, small arteries, small veins) to the constrictor actions of adrenaline and noradrenaline and *vice-versa* (Altura & Zweifach, 1964, 1965a). In addition, we have shown that the contractions induced by these antihistamines cannot be ascribed to circulating noradrenaline, adrenaline or 5-hydroxytryptamine (5-HT) and are

TABLE 1. EFFECTS OF TOPICAL APPLICATION OF ANTIHISTAMINES ON MICRO-VESSELS IN MOUSE SKIN MICROVASCULATURE. Antihistamines applied to surface of exposed skin microvasculature in volumes of 0·05 ml. Symbols (+) represent degree of microvascular constriction (+ = 10–15% decrease in vessel diameter size). Groups of 5 animals were used for each dose

Antihistamine (dose in μg)		Precapillary sphincter	Arteriole	Venule
Chlorpheniramine maleate	0·1	++	+	0
	1·0	+++	++	0
	10·0	++++*	+++	0
	15·0	++++*	++++*	++**
Diphenhydramine hydrochloride	0·5	++	+	0
	5·0	+++	++	0

*++++—Represents complete closure of these vessels.
** Represents stasis, petechial formations and leucocytic sticking at these vessel sites in addition to the contractile response.

not dependent upon anticholinergic or local anaesthetic actions (Altura & Zweifach, 1965b). We have also shown that these antihistamines cannot be exerting their microvascular effects through a release of catecholamines from peripheral sympathetic stores on to the vascular smooth muscle effector unit, which may be inaccessible to adrenergic blocking agents (Altura & Zweifach, 1966).

In view of the implications of these findings, it has been thought advisable to determine the effects of some of these antihistamines on the skin microvasculature.

Male C57 BL/6J mice, weighing 20–25 g, were anaesthetized with pentobarbitone sodium, 6 mg/100 g body wt intramuscularly. Hair on the dorsal surface was shaved and depilated. A transparent lucite skin chamber was then implanted in each mouse (Zarem, Zweifach & McGehee, 1967), areolar tissue removed, and the exposed blood vessels of panniculus carnosus and skin observed. One dose of each antihistamine (made up fresh daily in isotonic saline) was then topically applied, in 0·05 ml amounts to the exposed vascular tissue of the skin chamber preparation and examined at a magnification of 65–500 × with a Leitz Ortholux microscope equipped with Ultropak lenses. The results are shown in Table 1.

Both chlorpheniramine maleate and diphenhydramine hydrochloride exerted a dose-dependent constrictor action on the skin micro-vessels, which progressed from precapillaries to arterioles to venules in terms of vessel sensitivity.

Isotonic saline (vehicle), on the other hand, exerted no vascular effects. The doses, of antihistamines, which elicited threshold precapillary contractile responses were extremely low (0·1–1·0 μg), while arteriolar-venular (A–V) shunts seemed to remain unaffected until much higher doses were applied (10–15 μg). However, these latter high antihistamine doses, approximately 100–150 $\times$ threshold, also produced irreversible microvascular injury in most of the preparations tested, in the form of postcapillary venular stasis, petechial formations and sticking of leucocytes to endothelial walls. The microcirculatory actions of the antihistamines (0·1 to 5·0 μg) lasted for protracted periods (3 to 60 min) depending on the dose; the lower the dose the shorter was the duration of constrictor action.

These experiments suggest that certain antihistamines–chlorpheniramine maleate and diphenhydramine hydrochloride, among others—may exert anti-inflammatory actions, in very low doses, at least in mouse skin microcirculation, by virtue of their ability to induce direct microvascular contractile responses possibly similar in action to the effects of the local anti-inflammatory hormones-adrenaline and noradrenaline (Willoughby & Spector, 1964). The actions of the antihistamines on the small blood vessels of the skin, like the catecholamines, result primarily in precapillary vasoconstriction (i.e., no flow into true capillaries) and would inhibit the increased capillary permeability seen in certain local inflammatory conditions, thus preventing the escape of intravascular fluids and other plasma constituents into the perivascular connective tissue. As shown by Landis (1927), vasoconstriction (closure) of metarterioles and precapillaries induces a return of extravascular fluid and other plasma constituents from the extravascular compartment into the true capillary circulation as originally suggested by Starling. In this context, Spector & Willoughby (1963), among many others found that various antihistamines inhibited a variety of permeability inducing factors, in addition to histamine, in rat skin, as measured by the trypan blue dye labelling technique.

These results on small blood vessels in mouse skin may help to explain why antihistamines are effective in ointments as anti-inflammatory agents.

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Restraint-induced gastric ulcers in the golden hamster

SIR,—Physical restraint has been reported to cause a high incidence of gastric ulcers in rat, mouse, and guinea-pig. The low incidence of spontaneous ulcers in the golden hamster has discouraged its use in experiments on ulcer formation (Brodie & Hanson, 1960).

We have found that 40 hr of fasting followed by 4 or 7 hr of restraint (Bonfils, Dubresquet & Lambling, 1960) regularly caused the formation of gastric ulcers in male golden hamsters (*Mesocricetus auratus*) weighing 100–200 g.

The areas of mucosal ulceration with or without haemorrhage occurred in the glandular portion of the stomach and were similar to those seen by Bonfils, Richir & others (1959) in the rat. The incidence and the mean number of ulcers were high in all our experiments. However, many animals were used because of the variability in response.

TABLE 1. INCIDENCE OF EXPERIMENTAL GASTRIC ULCERS IN THE GOLDEN HAMSTER: INFLUENCE OF BILATERAL VAGOTOMY AND ADRENALECTOMY

Operative procedure	Animals with ulcers	Ulcers	
		Incidence %	Mean ± s.e.
Normal*	1/26	3·8	1 —
Fasted†	19/55	34·5	2·15 ± 0·22
Fasted + restrained 4 hr	20/26	77	3·75 ± 0·58
Fasted + restrained 7 hr	65/73	89	7·23 ± 0·82
Vagotomized‡	6/24	25	1·83 ± 0·40
Sham-operated	23/31	74	4·65 ± 0·48
Adrenalectomized§	24/32	75	4·33 ± 0·58
Sham-operated	14/21	66·6	4·00 ± 0·85

* Animals fed normally and kept in usual conditions.
† 40 hr.
‡ Fasted for 40 hr and vagotomized before 4 hr restraint.
§ Adrenalectomized 2 days before fasting for 40 hr and restraint for 7 hr.

Vagotomy reduced the incidence and mean number of restraint-induced ulcers for each animal. It would seem therefore that, as in the rat (Watanabe, 1966), parasympathetic activity plays an important role in ulcer formation in this animal species. In contrast, adrenalectomy did not significantly influence the ulcerogenic effects of restraint, the incidence and mean number of ulcers being similar in the two comparable groups of sham-operated and adrenalectomized animals. Because accessory adrenals are absent in hamsters (Schroeder & Bohle, 1957), it may be concluded, that secretion of adrenal hormones is of little importance in the production of so-called stress ulcers induced by restraint.

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The detection of vocal responses in small laboratory animals

SIR,—The mechanical, thermal, electrical or chemical stimulation of nociceptors results in a reflex pain response which includes a vocal response (Woodworth & Sherrington, 1904; Guzman, Braun & Lim, 1962). The detection of this audible reaction is widely used as an index of pain in tests designed to assess the potency of analgesic drugs (Green, Young & Godfrey, 1951; Grewal, 1952; Carroll & Lim, 1960; Collier, Warner & Skerry, 1961; Nilsen, 1961; McKenzie & Beechey, 1962; Collins, Weeks & MacGregor, 1964; Dickerson, Engle & others, 1965; Winter & Flataker, 1965). However, although the response is clearly manifest in the dog, cat and guinea-pig, difficulties are encountered with its detection in small laboratory animals such as the rat or mouse. This problem is overcome with a simple and inexpensive amplification system, using readily available components.

The amplification system is placed in close proximity to the test animal, and consists of a miniature dynamic microphone, a microminiature amplifier module with a gain of 42 dbs and with a frequency response substantially flat up to 8 kc/sec and a balanced armature miniature ear piece. This innovation has been found to be of practical value in making analgesic tests, and may have additional applications in experiments in which a vocal response is taken as an end-point; such examples include the coughing reflex or a vocal response resulting from a conditioned fear response.

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The self-inhibiting effect of acetanilide on the formation of methaemoglobin

SIR,—It has been reported that as the homologous series of anilides (acetanilide to dodecylanilide) was ascended, the ability of the compounds (orally, 1 mmole/kg) to induce the formation of methaemoglobin in cats was at a maximum at propionanilide and n-butyranilide (McLean, Murphy & others, 1967a) (see Fig. 1). If, however, the same series of compounds was administered at a dose of 0·5 mmole/kg and all other experimental procedures used were as described by McLean & others (1967a) a different pattern of results was obtained (Fig. 1A). In the second case the ability of the compounds to induce the formation of methaemoglobin fell regularly as the homologous series was ascended. The problem arises as to why this difference in response should occur.

If acetanilide is considered, it may be seen from Fig. 1 that a dose of 1·0 mmlole/kg produces less methaemoglobin than 0·5 mmole/kg and it is the only anilide which does this. A log dose response curve of acetanilide (oral) and aniline (i.v.) is shown in Fig. 2. From this it can be seen that at lower doses there is a linear relationship between log dose and the mean percent methaemoglobin formed with acetanilide, but at higher doses this relationship does not hold. Aniline given intravenously also shows a linear log dose response curve over the same range of mean percent methaemoglobin formed.

When aniline was administered to cats which had previously been anaesthetized, the amount of methaemoglobin produced was less than that in control animals given the same dose of aniline (McLean, Robinson & others, 1967b). This reduction in the response was shown to be independent of the nature of the anaesthetic used or the route by which it was administered. Further, it was also established that this "anaesthetic effect" was due to the metabolism of aniline to phenylhydroxylamine (the proximal methaemoglobin forming compound) being modified rather than the effect of phenylhydroxylamine on the enzyme systems of the red blood cell being affected.

It is well known that the structurally non-specific biological depressants such

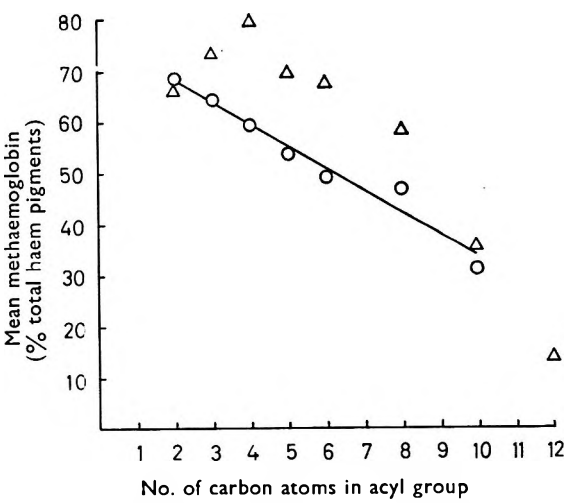

FIG. 1. Methaemoglobin formed by anilides. Each point is the mean value from either 4 or 6 cats. The value for each cat is the mean of the hourly readings of % methaemoglobin for 6 hr following the administration of each compound. The anilides were administered orally at dose levels 1·0 mmole/kg (6 cats each) △, and 0·5 mmole/kg (4 cats each) ○—○.

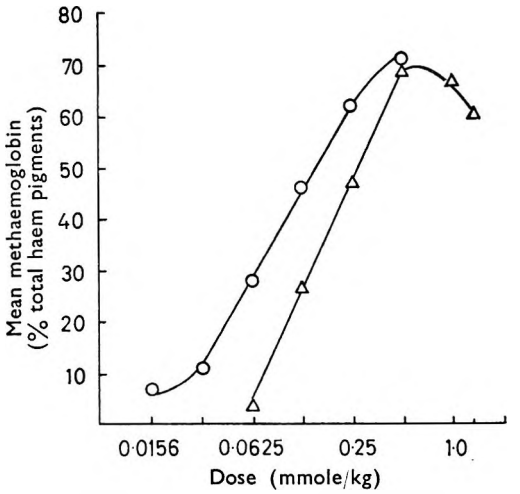

FIG. 2. Log dose response curves for aniline (i.v.) ○—○ and acetanilide (oral) △—△. Each point is the mean value from 5 cats. The value for each cat is the mean of the hourly readings of % methaemoglobin for 5 hr after the administration of aniline and 6 hr after acetanilide.

as the general anaesthetics depress the activity of many biological activities other than central nervous activity and it is not surprising, therefore, that the rate of *N*-hydroxylation was reduced by such a compound. Acetanilide in high doses has a central depressant effect and appears to act as a structurally non-specific depressant. A possible explanation of why the pattern of results obtained at 1 mmole/kg was different from the pattern obtained at 0·5 mmole/kg with the series of anilides (Fig. 1) is that acetanilide inhibited its own metabolism to phenylhydroxylamine by its structurally non-specific depressant action. However, it has been reported that acetanilide at a dose of 0·75 mmole/kg was a potent inhibitor of the metabolism of methanol in mice (Hassan, Elghamry & Abdel-Hamid, 1967). The mechanism of this action was said to be unknown but since the metabolism of ethanol was not inhibited by acetanilide the authors suggested that this action of acetanilide was due to a specific mechanism. This was thought to be due to acetanilide causing a depletion of the peroxide pool of the body. If this is so then it offers another explanation why acetanilide has a self inhibiting effect on its ability to induce the formation of methaemoglobin.

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Endotoxin shock in dogs pretreated with cellulose sulphate, an agent causing partial plasma kininogen depletion

SIR,—Cellulose sulphate, a polyanionic macromolecule previously described by Astrup, Galsmar & Volkert (1944) as a semi-synthetic anticoagulant, has been recently shown (Rothschild & Gascon, 1966; Rothschild, 1967) to be a potent activator of the kinin generating system of rat, guinea-pig and human plasma. On intravenous administration to the pentobarbitone-anaesthetized dog, cellulose sulphate causes intense, transient hypotension probably mediated by released bardykinin since a rapid fall of kinin precursor (kininogen) in plasma accompanies the hypotensive response. After return to the normotensive condition, the animal becomes refractory towards the effects of a second injection of cellulose sulphate both in so far as plasma kininogen disappearance and fall of blood pressure are concerned.

Recent results (Scharnagel, Greeff & others, 1965; Diniz, Carvalho & others, 1967) have indicated that endotoxin shock in the dog is accompanied by a pronounced fall of plasma bradykininogen levels; for these reasons we have investigated the effect of *Escherichia coli* endotoxin in dogs partly depleted of plasma kininogen by previous cellulose sulphate treatment.

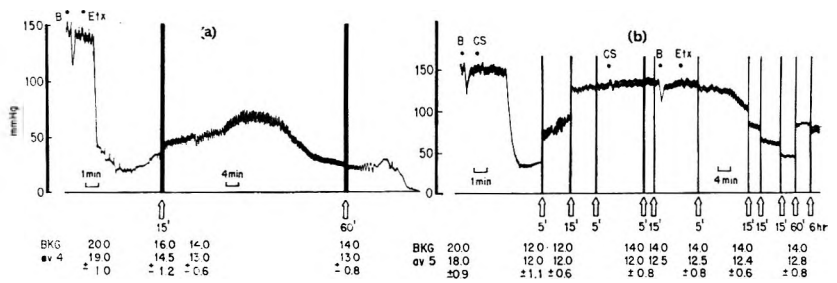

FIG. 1 (a). Effect of intravenous injection of *E. coli* endotoxin on arterial blood pressure and plasma kininogen (BKG) levels of the dog. At B, bradykinin (Sandoz), 1 μg; Etx, 5 mg/kg endotoxin. BKG = kininogen units/ml of plasma of samples withdrawn from femoral artery at times indicated: values shown in the upper line correspond to the experiment depicted; those in the lower line, to averages of four identical experiments. One unit of BKG yields 0·44 μg of synthetic bradykinin.

(b) Effect of intravenous injection of cellulose sulphate and subsequently of endotoxin, on dog arterial blood pressure and plasma kininogen content. At B, bradykinin, 1 μg; CS, cellulose sulphate, 20 mg/kg; Etx, endotoxin, 5 mg/kg. Other details as in Fig. 1 (a). Note partial recovery of blood pressure 2 hr after injection of endotoxin and increased survival time of the animal.

Fig. 1 (a) shows the typical, rapid hypotension leading to irreversible shock which is observed in dogs receiving a lethal dose of endotoxin (*E. coli* lipopolysaccharide, B26 : O26, Difco). This effect is no longer observed in dogs pretreated with cellulose sulphate; as shown in Fig. 1 (b), such animals respond to the toxin with a delayed, slowly progressing hypotension, starting approximately 15 min after the injection and reaching its lowest level 60 min afterwards. Partial recovery and a tendency towards longer survival times are noted in this group of animals. Kininogen levels, determined by the method of Diniz & others (1961), were reduced to about the same extent by either endotoxin or by cellulose sulphate; the bacterial polysaccharide, however, was unable to cause a further reduction of kinin precursor in the plasma of dogs which had first received an injection of cellulose sulphate.

These results, while lending further weight to the hypothesis of a participation of kinins in the early hypotensive response of the dog to endotoxin suggest that in addition, the toxin can induce a delayed hypotensive response appearing after a latency period of at least 15 min, and apparently not involving plasma kininogen breakdown. This response may be due to the direct impairing effect of *E. coli* endotoxin on ventricular performance (Solis & Downing, 1966), which has a similar latency period.

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A new tracheal strip preparation for the evaluation of β-adrenergic activity

SIR,—The preparation we have used in our laboratory for the evaluation of β-adrenergic activity has been the tracheal muscle of the calf, described by Ariëns & Simonis (1960). However, dependence on an abattoir supply is a disadvantage as is the lack of the animal's history, while the tracheal muscles of the usual laboratory animals are too small to be used in the same way.

The preparations described in the literature are either too tedious to prepare, such as the guinea-pig tracheal chain* according to Castillo & De Beer (1947) and its modifications by Akcasu (1952) and Foster (1960), or otherwise inconvenient, like the spirally cut guinea-pig trachea of Constantine (1965) which has a torsion strength of its own.

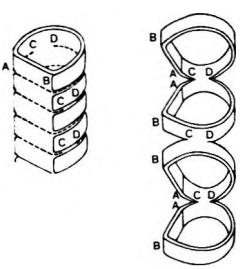

FIG. 1. Diagram of a new way of cutting the guinea-pig trachea. A-B: tracheal muscle; C-D: part which is cut open (along the dotted line).

We now wish to report that a satisfactory preparation can be obtained by cutting—with scissors—the trachea of a guinea-pig (400–600 g), after having removed all extraneous tissue, as indicated in Fig. 1. Between the cuts we left one cartilage ring and the total number of cuts was 10–15, depending on the size of the trachea.

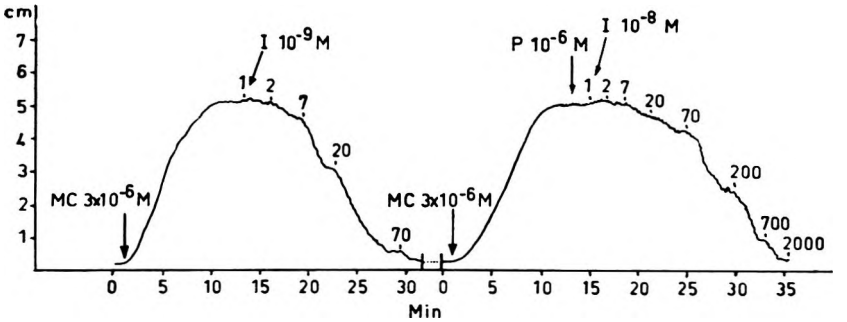

FIG. 2. Record of the spasmolytic action of isoprenaline (I) on the guinea-pig isolated trachea, with and without 10^{-6}M propranolol (P), respectively. The indicated concentrations of I are added cumulatively making the bath concentration 10^{-9}, 3×10^{-9}, 10^{-8} etc. The contractions, induced by 3×10^{-6}M methacholine (MC) are fully developed after about 30 min.

The preparation obtained is mounted and used as described by Ariëns & Simonis (1960) for the tracheal muscle of the calf. Drug-induced contractions of the preparation were recorded isotonically on a smoke drum, using a load of about 1 g and a magnification of 20 times. The contractions obtained were smaller than those from the calf tracheal muscle; the base line is stable and the contractile response to 3×10^{-6}M methacholine is constant. We assessed the β-adrenergic blocking activity of propranolol on both preparations with isoprenaline as agonist (Fig. 2). The pA_2 value found, with 95% confidence limits, for calf tracheal muscle was (9 exp.) 7·79 (7·59–7·99), and for the proposed guinea-pig tracheal preparation (32 exp.) it was 8·19 (8·03–8·35).

The preparation also proved very sensitive to histamine: the pA_2 value of diphenhydramine hydrochloride as a histamine antagonist, assessed on our preparation was about 7·3; on the guinea-pig ileum it was 7·5.

The preparation we have described would seem very suitable for the investigation of β-adrenergic activities of drugs.

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* Foster (1966) has characterized the adrenergic receptors of the guinea-pig isolated trachea as β-receptors by established criteria.

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Activation of *Bacillus stearothermophilus* spores and release of dipicolinic acid after hydrochloric acid treatment

SIR,—Without prior heat activation less than 10% of spores of *Bacillus stearothermophilus* germinate and produce colonies on a nutrient agar medium (Cook & Brown, 1964, 1965). Optimum heat activation results in about 50% of the spores germinating and forming colonies.

We have found that in the presence of 0·5 N hydrochloric acid at 25° the colony count increases to the value of the total (chamber) count and that this increase is accompanied by progressive release of dipicolinic acid (DPA), a characteristic component of bacterial spores (Powell, 1953). Spore suspensions of *B. stearothermophilus* (NCIB 8919) were prepared according to Cook & Brown (1964). The DPA released was measured polarographically using method 2 of Porter, Brown & Brown (1967). Initially about 2% of the spores germinated and formed colonies after plating. After 30 min this rose to about 97%, by which time about half the DPA had been released (Fig. 1).

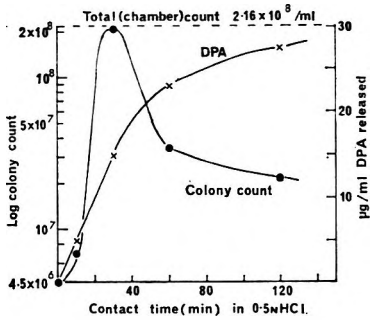

FIG. 1. Effect of 0·5 N hydrochloric acid on increase in colony count and release of DPA from *B. stearothermophilus* spores.

Keynan, Issahary-Brand & Evenchik (1965) showed that lowering the pH imitated the effects of heat activation as measured by germination rates, but they did not correlate the pH effect with release of DPA. Lewis, Snell & Alderton (1965) reported that treatment of *B. stearothermophilus* spores at pH 1·5 for 80 min at 25° increased the colony count but did not remove DPA.

Although a causal relation between pH and DPA release has not been established by the present work, it might well be that low pH imitates the effect of heat activation by releasing DPA but without the noxious effects of the relatively high temperatures which are needed for heat activation and which may kill a proportion of heat-activated spores.

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Letters to the Editor

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