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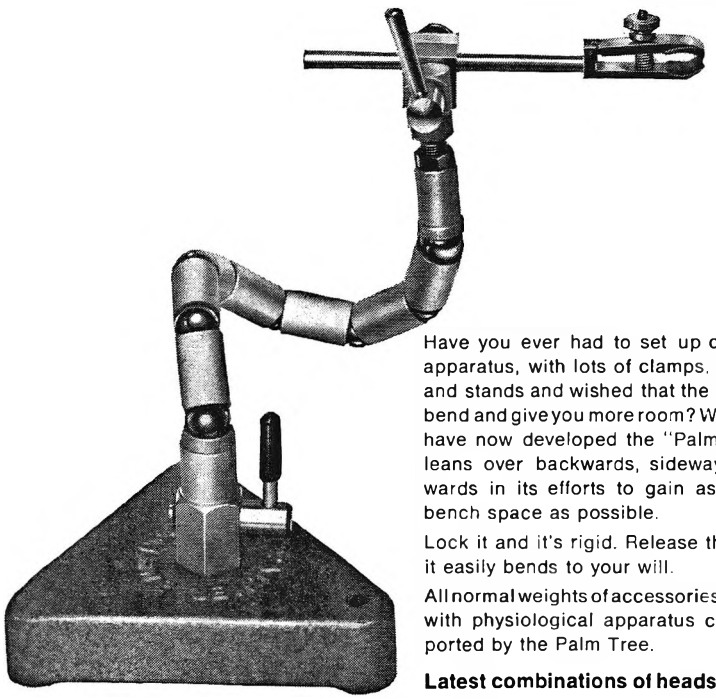

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Effects of the *NN'*-triethyl analogue of suxamethonium on neuromuscular transmission

H. DOWD, S. J. JENNINGS, I. G. MARSHALL* AND B. M. TRACY

The effects of succinyltriethylcholine on neuromuscular transmission in the cat and chick, and on the output of acetylcholine from the isolated rat hemidiaphragm, are described. Succinyltriethylcholine exhibits an initial post-junctional non-depolarizing blocking action. A secondary prejunctional inhibitory action on acetylcholine output, is due to succinyltriethylcholine rather than to the hydrolysis products. The compound also exhibits a facilitatory action which may be due to anticholinesterase activity.

THE neuromuscular blocking action of the *NN'*-triethyl analogue of suxamethonium (succinyl-TEC; I) was briefly reported by Bovet (1951, 1959). Experiments on avians indicated that it was devoid of depolarizing activity, and, in the rabbit head-drop test, it was found to possess about 1/60 of the potency of suxamethonium.

$$Et_3N^+.CH_2.CH_2.O.CO.CH_2.CH_2.CO.O.CH_2.CH_2.N^+Et_3 \ 2I^-$$

I

It might be expected that succinyl-TEC, like suxamethonium, would possess short duration of action and, since relatively weak potency need not be a disadvantage in an otherwise useful drug, it was decided to re-examine the effects of the compound in more detail. In view of the prejunctional action of many ethonium compounds on acetylcholine synthesis and output (Bowman, Hemsworth & Rand, 1967), succinyl-TEC was also examined for this effect.

Experimental

CHEMISTRY

Bis-2-ethylaminoethyl succinate was prepared by esterifying 2-diethylaminoethanol (0·75 mole) with succinic acid (0·25 mole). The mixture was first refluxed in toluene (1 ml conc. sulphuric acid catalyst) for 8 hr after which a toluene/water azeotrope was distilled off. The reaction mixture was dissolved in ether and extracted with sodium bicarbonate solution. After drying the ether solution over anhydrous sodium sulphate, removal of the solvent left a yellow oil.

The quaternary ammonium salt was prepared by heating the above ester (0·03 mole) with ethyl iodide (0·06 mole) in acetone in a sealed tube for 48 hr. The precipitated quaternary salt gave a colourless crystalline solid, bis-2-triethylaminoethyl succinate iodide (m.p. 169·5–170°) when crystallized from absolute ethanol/methyl ethyl ketone. Found; C, 38·3; H, 6·7; I, 39·5; N, 4·4%. $C_{20}H_{42}I_2N_2O_4$ requires C, 38·2; H, 6·7; I. 40·4; N, 4·5%.

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In a second synthesis bis-2-chloroethyl succinate was prepared using the method of Walker (1950) from 2-chloroethanol and succinic acid. This was converted to the iodo-ester by refluxing with 15% sodium iodide in acetone solution. When the iodo ester was refluxed with triethylamine in acetone a solid separated out which on crystallization from alcohol yielded white crystals (m.p. 168–169°). Mixed melting point determination and infrared spectra confirmed the identical nature of the compounds obtained from the two syntheses.

PHARMACOLOGY

Pharmacological tests were made on the following preparations:

1. The tibialis anterior muscles of cats anaesthetized with chloralose (80 mg/kg injected intraperitoneally). Maximal twitches of a tibialis anterior muscle were elicited by stimulation of the sciatic nerve with rectangular pulses of 50 μsec duration and of about twice the strength required to evoke a maximal twitch. In most experiments, maximal twitches of both tibialis anterior muscles were elicited simultaneously, one being excited once every sec through a 1:1 isolation transformer, and the other once every 10 sec (Bowman & Rand, 1961; Bowman, Hemsworth & Rand, 1962). Drugs were injected intravenously through a cannula in a jugular vein or close-arterially by the technique of Brown (1938).

2. Observations were made after intravenous injection into young conscious chicks (Buttle & Zaimis, 1949).

3. The effects of succinyl-TEC on the acetylcholine output from the isolated innervated rat hemidiaphragm preparation were studied using a method identical with that described by Bowman & Hemsworth (1965).

4. The isolated transmurally stimulated oesophagus preparation of the young chick mounted in Krebs-Henseleit solution of the following composition g/litre: NaCl, 6·95; KCl, 0·34; $CaCl_2$, 0·28; KH_2PO_4, 0·162; $MgSO_4$, 0·294; $NaHCO_3$, 2·1; dextrose, 2·0, which was bubbled with 5% carbon dioxide in oxygen and at 32°.

The drugs used were: choline chloride, α-chloralose, acetylcholine chloride and atropine sulphate (British Drug Houses), neostigmine methylsulphate and edrophonium chloride (Roche). The doses quoted, including those of succinyl-TEC, refer to the base or to the cation.

Results

NEUROMUSCULAR BLOCKING ACTION

Chicks. On intravenous injection into young conscious chicks succinyl-TEC (10–20 mg/kg) produced flaccid paralysis, confirming Bovet's (1951, 1959) finding that the blocking action was of a non-depolarizing nature.

Cat tibialis anterior muscle. In the cat tibialis anterior muscle preparation stimulated indirectly once every 10 sec, doses of 10–15 mg/kg of

succinyl-TEC injected intravenously produced a brief (2–3 min) period of twitch augmentation. Larger doses (20–50 mg/kg) produced neuromuscular block of a duration intermediate between that of tubocurarine and suxamethonium, taking about 10–15 min to full recovery from a 90–100% block of twitch height. A short period of twitch augmentation was occasionally observed before the blocking action of the compound, and frequently, after the block, the twitches recovered to an amplitude greater than that before the block. This post-block augmentation of twitches, when observed, usually lasted for 5–15 min, and was always preceded by a two-stage recovery from the block; an initial slow steady recovery up to about 75% of the original twitch height was followed by a secondary rapid recovery leading into the twitch augmentation (Figs 1 and 4).

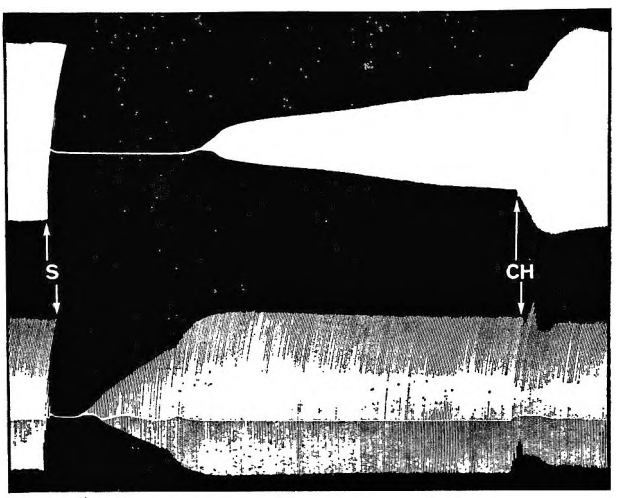

FIG. 1. Cat 2·65 kg, chloralose anaesthesia. Maximal twitches of right and left tibialis anterior muscles elicited indirectly by 1 shock/sec and 1 shock/10 sec respectively. At S, 50 mg/kg succinyl-TEC, and at CH 5 mg/kg of choline were injected intravenously.

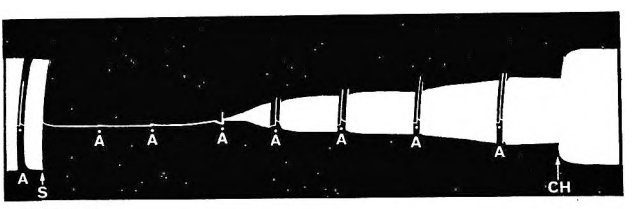

FIG. 2. Cat 2·85 kg. Maximal twitches of a tibialis anterior muscle elicited indirectly once every second. At A, electrical stimulation was temporarily stopped and 7·5 μg of acetylcholine was injected close-arterially. At S, 50 mg/kg of succinyl-TEC and at CH 5 mg/kg of choline were injected intravenously. The horizontal bar corresponds to a period of 10 min.

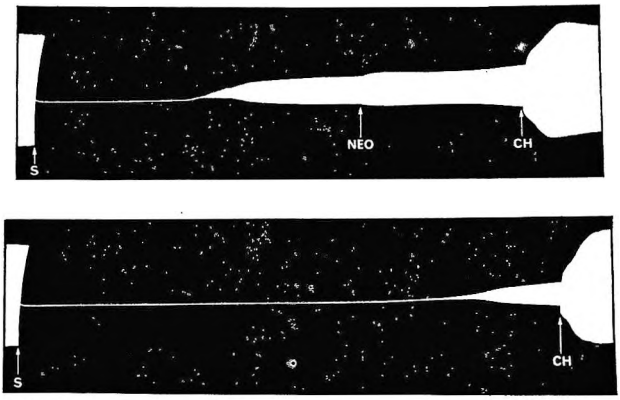

FIG. 3. Cat 2·65 kg. Maximal twitches of a tibialis anterior muscle elicited indirectly once every second. The lower trace is a continuation of the upper trace, the time between the two being 60 min. In both traces at S, 50 μg/kg of succinyl-TEC, and at CH, 5 mg/kg of choline were injected intravenously. In the upper trace at NEO, 100 μg/kg of neostigmine was injected intravenously. Note the transient effect of neostigmine on the secondary phase of block, and compare with the complete reversal produced by choline. In the lower trace 100 μg/kg of neostigmine was injected 5 min before the succinyl-TEC. Note the prolongation of the initial stage of block when the lower trace is compared with the upper. The horizontal bar corresponds to 10 min.

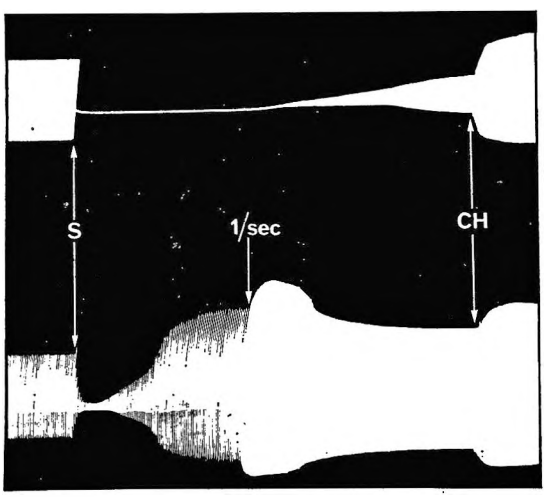

FIG. 4. Cat 2·5 kg. Maximal twitches of the right and left tibialis anterior muscles elicited indirectly by 1 shock/sec and 1 shock/10 sec respectively. At the marked arrow the stimulation rate of the right muscle was increased to 1/sec. At S, 40 mg/kg succinyl-TEC, and at CH, 5 mg/kg of choline were injected intravenously. Note the two-stage recovery from the initial blocking action of succinyl-TEC in the more slowly stimulated muscle, and the post-block twitch augmentation.

Neostigmine and edrophonium (100 μg/kg intravenously or 5 μg close-arterially) failed to hasten recovery from the blocking action of succinyl-TEC. The depth of block produced by succinyl-TEC was not reduced

by previous administration of neostigmine (100 μg/kg, i.v.), but the duration of action of the compound was prolonged. During partial block produced by succinyl-TEC, tetanic tension, elicited by stimulating the sciatic nerve at 50/sec for 5 sec, although depressed, did not wane during the period of repetitive stimulation. Immediately after the tetanus there was a reduction in the depth of block when single shock indirect testing was resumed.

When tested on cat tibialis anterior muscles stimulated at 1/sec the first dose of succinyl-TEC (40–50 mg/kg) usually produced a block which, though deeper than that in the more slowly stimulated muscle, recovered at about the same rate. Other neuromuscular blocking drugs are also more effective at higher rates of stimulation (Preston & Maanen, 1953; Wislicki, 1958). Subsequent doses of succinyl-TEC produced a biphasic block of the more rapidly stimulated muscle. The twitches remained completely blocked for a period of 15–30 min. They then began to recover, either very slowly and continuously over a period of 1–2 hr, or partially, to become constant at a reduced level for about 30 min before full recovery occurred. Contractions produced by close-arterially injected acetylcholine were blocked during the initial block of the twitches. Responses to acetylcholine then recovered at a faster rate than did the twitches, so that, during the second phase of block the responses to acetylcholine returned to their original height while the twitch height remained partially depressed (Fig. 2). This result is in sharp contrast to that recorded when the block is entirely due to a post-junctional action, such as with tubocurarine (see for example, Bowman & others, 1962). When tubocurarine is the blocking agent used, twitches evoked by nerve stimulation return to control levels much more quickly than do contractions produced by injected acetylcholine. The results therefore indicate that whereas the initial phase of block produced by succinyl-TEC is post-junctional in origin, the second phase is pre-junctional.

Choline (5 mg/kg, i.v.) rapidly and permanently reversed the secondary phase of block produced by succinyl-TEC in the more rapidly stimulated muscle (Figs 1–4). When injected during the initial phase, choline prevented the onset of the secondary phase of block. Neostigmine (100 μg/kg intravenously), injected during the secondary block, produced only a small increase in the twitch tension (Fig. 3), an effect similar to that observed during block produced by hemicholinium or triethylcholine (Reitzel & Long, 1959; Bowman & Rand, 1961).

The dependence of the secondary block on the rate of stimulation was further demonstrated in an experiment in which the twitches of the slowly stimulated muscle were allowed to recover completely after a dose of 50 mg/kg of succinyl-TEC. The stimulation rate was then increased to equal that for the more rapidly stimulated muscle when there was an initial increase in twitch tension, followed, after about 3 min, by a rapid decrease in tension until a partial but constant degree of block was maintained. Choline reversed the blocks in both muscles (Fig. 4).

Biphasic blocks similar to those described above were produced by

succinyl-TEC in cats which had been pretreated with neostigmine (0·1–0·2 mg/kg), the only difference being that the duration of the complete block of the more rapidly stimulated muscle was prolonged (Fig. 3). In an experiment in which 60 mg/kg of succinyl-TEC was incubated with 10 ml cat blood for 90 min at 37°, intravenous injection of the incubation mixture produced only a slightly smaller effect than did a control dose of the compound injected 2 hr earlier. The secondary choline-reversible phase of block was noted in both cases.

RELEASE OF ACETYLCHOLINE

Succinyl-TEC reduced the amount of acetylcholine released during nerve stimulation; the results are shown in Fig. 5. Succinyl-TEC added

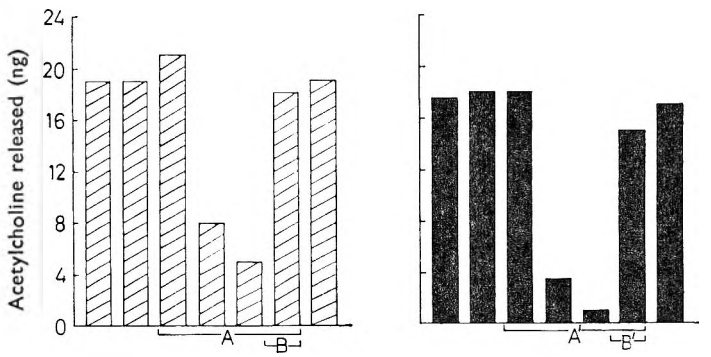

FIG. 5. Each column represents the acetylcholine (as cation) released during a 15 min period of stimulation at 1 shock/sec. The period between each 15 min collection period was 43 min. The first two columns in each histogram represent the release during control collections of acetylcholine before the addition of drugs. The third, fourth, fifth and sixth columns represent the acetylcholine released in the presence of succinyl-TEC (A, 150 μg/ml; A', 250 μg/ml) which was initially added 2 min before the collection period represented by the third column. Choline (B, 50 μg/ml; B', 75 μg/ml) was also present during the collection period represented by the sixth column, having been initially added 40 min before the collection period began. The last column represents the release after washing out both the succinyl-TEC and the choline. Each column represents the mean of results obtained on three diaphragms.

to the bath fluid 2 min before a collection period, showed no immediate depressant effect on acetylcholine release, but at the lower dose level used (150 μg/ml) slightly increased the acetylcholine output during the first collection period after its administration. During the second collection period after succinyl-TEC administration, the acetylcholine output was reduced by 58–81%, depending upon the dose used, and during the subsequent collection period, the acetylcholine output was reduced by 74–94%. When choline (50–75 μg/ml) had been present together with the succinyl-TEC for a period of 40 min, the acetylcholine output returned to over 90% of the control output. After washing out all the drugs from the bath the acetylcholine output returned to the control level. Control experiments confirmed the finding of Bowman &

Hemsworth (1965) that in the absence of succinyl-TEC the acetylcholine output remained constant over a period of 8 hr.

CHICK OESOPHAGUS

Succinyl-TEC (35–50 μg/ml) added to the fluid bathing an isolated transmurally stimulated chick oesophagus preparation produced a gradual increase in the size of the contractions elicited by electrical stimulation. When left in contact with the tissue for more than 5–10 min, succinyl-TEC produced spasm of the tissue, which was only relieved by cessation of stimulation and washing.

The response to added acetylcholine (5 μg/ml) was increased after succinyl-TEC, and the compound reversed the block of contractions produced by atropine (0·1 μg/ml).

Discussion

The results confirm Bovet's (1951, 1959) observation that succinyl-TEC is without depolarizing action, and they show that its effects on neuromuscular transmission are the result of both facilitatory and blocking actions. Because of its complexity of action, the relatively prolonged duration of its effect when compared with suxamethonium, and the inability of neostigmine to antagonize it, succinyl-TEC would not be of value as a neuromuscular blocking agent in surgical anaesthesia.

Facilitation of neuromuscular transmission produced by succinyl-TEC was evidenced by an increase in twitch tension which preceded and followed the blocking action of the compound. Two mechanisms of action probably contribute to the facilitatory action. Measurements of acetylcholine output showed that succinyl-TEC initially increased transmitter release, an action shared by other ethonium ions including tetraethylammonium (Collier & Exley, 1963) and triethylcholine (Bowman & Hemsworth, 1965). In addition succinyl-TEC probably possesses anticholinesterase activity as shown by its ability to augment responses of the isolated chick oesophagus to acetylcholine, and to produce a slowly developing spasm of the tissue. Several other ethonium ions have also been shown to possess anticholinesterase activity (Blaschko, Bülbring & Chou, 1949; Karczmar, 1957; Koelle, 1957; Arnold, Soria & Kirchner, 1954). These facilitatory components of action of succinyl-TEC may account for the maintenance of tetanic tension during partial neuromuscular block. An anticholinesterase action of the compound may explain the inability of neostigmine or edrophonium to antagonize the initial postjunctional blocking action of the compound. The non-depolarizing drug, benzoquinonium, also possesses anticholinesterase activity and its blocking action is similarly resistant to antagonism by anticholinesterase drugs (Hoppe, 1951; Bowman, 1958, 1966).

The secondary blocking action of succinyl-TEC exhibited the characteristics of prejunctional block arising from inhibition of acetylcholine synthesis. Thus, like that produced by hemicholinium, triethylcholine and related compounds (Schueler, 1960; Bowman & others, 1967), the

secondary block produced by succinyl-TEC occurred selectively in the more rapidly stimulated muscle and was reversed by choline. Its prejunctional nature was indicated by the fact that, during the block, contractions produced by acetylcholine were similar to the controls. Experiments on the rat diaphragm preparation confirmed that succinyl-TEC decreases the output of acetylcholine from the stimulated nerve and that this effect is reversed by choline.

Since succinyl-TEC hydrolyses to produce succinic acid and triethylcholine, it was considered that the secondary prejunctional block may have been due to triethylcholine, as was the case in a series of *NNN*-trisonium esters tested by Marshall (1968). However, destruction of succinyl-TEC on incubation with blood was very slow, as would be expected of a compound showing the properties of an inhibitor rather than of a substrate for cholinesterase. Furthermore, pretreatment with neostigmine did not change the type of block produced by succinyl-TEC and these results indicate that the effects are mainly due to the parent compound rather than to the products of hydrolysis.

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The uptake and retention of [^{3}H]noradrenaline in rat sciatic nerves after ligation

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The uptake and retention of [^{3}H]noradrenaline (^{3}H-NA) was examined in sciatic nerves of albino rats. In the 1 cm part of nerve proximal to a 12 hr ligation the uptake and retention of exogenous noradrenaline was about 4 times the uptake in 1 cm of normal unligated nerves. Treatment with reserpine 10 hr before killing caused a marked decrease in the estimated amount of ^{3}H-NA, while injection of nialamide 15 min before ^{3}H-NA administration in ligated, reserpine-treated animals caused a somewhat larger uptake and retention of ^{3}H-NA in the nerve part above the ligation. Protriptyline, a blocker of the "membrane pump", was approximately 3 times less effective in 12 hr-ligated nerves than in unligated nerves, indicating a reduced efficiency of the "membrane pump" in the distended axons above a ligation.

THE accumulation of noradrenaline proximal to a ligation in peripheral adrenergic nerves has been noted previously (Dahlström & Fuxe, 1964; Dahlström, 1965; Dahlström & Häggendal, 1966; 1967; Kapeller & Mayor, 1966). Reserpine, which is known to empty the monoamine stores in central and peripheral tissues by blocking the storage mechanism of the amine storage granules (*cf.* Carlsson, Hillarp & Waldeck, 1963; Carlsson, 1965; Dahlström, Fuxe & Hillarp, 1965; Malmfors, 1965), depletes the noradrenaline accumulated in sciatic nerves above a ligation (Dahlström, 1965; 1967a). If the monoamine oxidase inhibitor nialamide is given before reserpine to nerve ligated rats the noradrenaline fluorescence is unchanged (Dahlström, 1967a). For these reasons it has been assumed that the accumulation of noradrenaline occurring proximal to a ligation in adrenergic axons is due to a piling up of noradrenaline storage granules transported proximo-distally in the axons (Dahlström, 1966; 1967a; b; Dahlström & Häggendal, 1966; 1967).

The uptake and retention of [^{3}H]noradrenaline (^{3}H-NA) in adrenergic nerves of normal animals has been shown to be dependent on the efficiency of the amine transport mechanism at the level of the cell membrane (the so called "membrane pump") (Hillarp & Malmfors, 1964; Carlsson & Waldeck, 1965a; Lindmar & Muscholl, 1964). This transport mechanism can be blocked by protriptyline (Carlsson & Waldeck, 1965b; Malmfors, 1965). The uptake and retention of ^{3}H-NA is also dependent on the presence of functioning amine storage granules (cf. Lundborg & Stitzel, 1967). Therefore it was thought of interest to examine the uptake of ^{3}H-NA in the sciatic nerve of the rat under different conditions. In the present study the influence of ligation, reserpine, nialamide and protriptyline was investigated.

Experimental

MATERIAL AND METHODS

Male albino rats of the Sprague–Dawley strain (180–200 g) were given a single intravenous injection of ^{3}H-NA (specific activity 10 c/mmole)

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5 μg/kg 30 min before death. The animals with bilateral ligation of the sciatic nerve were operated under light ether anaesthesia 12 hr before death. Reserpine (Serpasil ampoules 2·5 mg/ml, CIBA) was given intraperitoneally in one single dose of 10 mg/kg 10 hr before killing. Nialamide was administered intraperitoneally to reserpine-pretreated animals with ligations either 2 hr (10 mg/kg) or 15 min before ^{3}H-NA injection (100 mg/kg). Protriptyline was given intravenously (10 mg/kg) to normal rats or to rats with 12 hr bilateral ligations 10 min before the injection of ^{3}H-NA. Before the intravenous administration of ^{3}H-NA, the rats were anaesthetized with pentobarbitone (35 mg/kg).

The animals were killed by decapitation and the 1 cm part of the nerve just above the ligation was dissected. Nerves from 2 rats were always pooled, so too were the nerves from unligated rats where 3 cm of nerve on each side was dissected. The nerve parts were put into 10 ml of ice-cooled perchloric acid (0·4N), homogenized and centrifuged. Noradrenaline was separated from the supernatant by chromatography on columns of a strong cation-exchange resin (Dowex 50W X4), and the radioactive noradrenaline was then estimated by liquid scintillation counting essentially as described by Carlsson & Waldeck (1963).

Results

The uptake and retention of ^{3}H-NA in 1 cm of normal unligated rat sciatic nerve was found to be 2·6 pg as a mean. Twelve hr after ligation, the amount had increased to 9·3 pg in the 1 cm just proximal to the ligation. Reserpine treatment 10 hr before death reduced the amount of recovered ^{3}H-NA in ligated nerves to about 0·7 pg/cm. In reserpine-treated animals, nialamide caused an increase in the amount of ^{3}H-NA, particularly in the group given a high dose 15 min before the ^{3}H-NA administration. In normal rats, given protriptyline 10 min before ^{3}H-NA, the amount of ^{3}H-NA recovered was reduced to about a quarter. In ligated rats, however, the effect of protriptyline was much less pronounced, the amount of ^{3}H-NA recovered was reduced only by a factor of 1·4.

Discussion

In earlier reports, the proximo-distal transport of noradrenaline in the adrenergic axons has been described. In the rat the rate of this transport has been calculated to be about 5 mm/hr (Dahlström & Häggendal, 1966). There are several reasons to believe that the measured noradrenaline content in normal and ligated nerves is stored within granules and that these granules are formed in the nerve cell body and transported down to the nerve terminals via some kind of active transport mechanism in the axons (see Discussions in Dahlström, 1965; 1966; Dahlström & Häggendal, 1966). One piece of evidence for this view is the disappearance of noradrenaline fluorescence on reserpine treatment; and that this disappearance can be inhibited by pretreatment with nialamide (Dahlström, 1966; 1967). Such behaviour of the noradrenaline is consistent with the idea that the amine is stored within granules (Carlsson, 1965; Malmfors, 1965).

In the present experiments it was found that 12 hr after ligation the uptake and retention of labelled noradrenaline was much larger compared to that in unligated nerves. Reserpine treatment caused a great decrease in the amount of ^{3}H-NA recovered, probably due to the granule-blocking effect of this drug. Inhibition of monoamine oxidase in reserpine-pretreated animals caused some increase in the ^{3}H-NA recovered particularly when a relatively large dose of nialamide was given at a short interval before ^{3}H-NA administration. There exists a very subtle balance between enhancing and antagonizing effects of monoamine oxidase inhibitors on the uptake and retention of ^{3}H-NA in reserpine-treated animals (Carlsson & Waldeck, 1967). In view of this it is not surprising that the time and dosage schedules for nialamide are critical (compare group E and F in Fig. 1). As seen from the Results, protriptyline (known

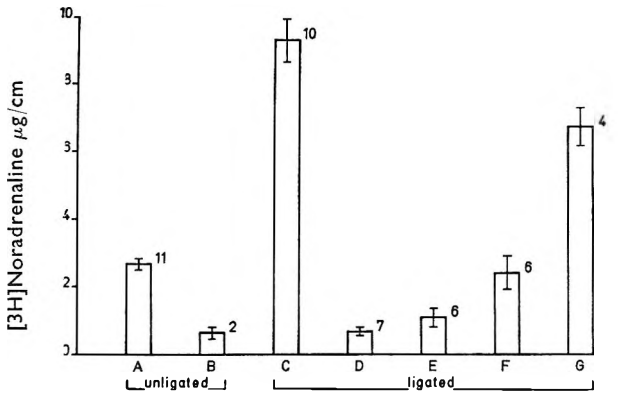

FIG. 1. Effect of drugs on the uptake and retention of [^{3}H]noradrenaline (^{3}H-NA) in the rat sciatic nerve. Rats, normal (unligated) and rats whose sciatic nerves had been ligated 12 hr beforehand (see text) received ^{3}H-NA 1 µg/kg i.v. 30 min before being killed. The amount of ^{3}H-NA/cm sciatic nerve (in ligated nerves the 1 cm just above lig.) was estimated. A and C, no drug, B and G, protriptyline 10 mg/kg i.v. 10 min before ^{3}H-NA. D, reserpine 10 mg/kg i.p. 10 hr before ^{3}H-NA. E, reserpine 10 mg/kg i.p. 10 hr and nialamide 10 mg/kg i.p. 2 hr before ^{3}H-NA. F, reserpine 10 mg/kg i.p. 10 hr and nialamide 100 mg/kg i.p. 15 min before ^{3}H-NA. Also shown are the mean ± s.e. Figures above bars indicate the number of observations.

to block the uptake of catecholamines across the nerve membrane, see above) caused a marked decrease in the recovery of ^{3}H-NA in unligated nerves, suggesting that the non-terminal adrenergic axons in the sciatic nerve have mainly the same properties as the adrenergic nerve terminals. In ligated nerves, however, protriptyline had little effect (see below).

In earlier experiments on endogenous noradrenaline content in ligated sciatic nerves, the ratio between 1 cm of unligated nerve and the proximal 1 cm just above a 12 hr-ligation was found to be about 1 : 7. The present experiments reveal a difference in uptake and retention of exogenous noradrenaline in normal and ligated nerves of about 1 : 3·6. This discrepancy may be due to, for instance, the following possibilities. The procedure of ligation may reduce the blood supply to this nerve part.

This is unlikely since the ligation is made by a short-lasting compression of the nerve without any sideways tearing, and since peripheral nerves are supplied with blood by a mesoneurium sending vessels to the nerve perpendicularly. Also, experiments with tandem ligations and tetrabenazine [a short-lasting blocker of granular storage mechanism (Carlsson, Hillarp & Waldeck, 1963; Carlsson & Lindqvist, 1966; Häggendal, 1968)] have shown that granules in a nerve part separated by two ligations behave normally in respect of storing and synthesizing noradrenaline (Dahlström, 1967), indicating an unimpaired blood supply to the nerves. A second possibility which has to be discussed, is that a large part of the endogenous noradrenaline that accumulated above a 12 hr ligation (Dahlström & Häggendal, 1966) might be situated extragranularly in the cytoplasm, and that the real intragranular part was demonstrated by the uptake of exogenous noradrenaline. This can in all probability be ruled out, since with the sensitive histochemical fluorescence method used in earlier work very little or no noradrenaline fluorescence could be observed after treatment with reserpine. A third possibility is that the ratio of membrane-surface to volume is greatly decreased in the axons above a 12 hr-ligation compared to normal axons and nerve terminals, as pointed out earlier (Dahlström, 1965). Exogenous noradrenaline would theoretically have more difficulty in penetrating to the centrally located granules in these enlarged, bulky axons than in normal thin nerves and terminals. Finally, the explanation which seems to be most reasonable is that the amine uptake mechanism at the level of the cell membrane, the so called "membrane pump" (Hillarp & Malmfors, 1964; Lindmar & Muscholl, 1964; Carlsson & Waldeck, 1965a) is impaired by the distention of the axonal membrane during the process of accumulation. This last mentioned alternative is strongly supported by the results obtained with protriptyline which is known to block the membrane pump. In animals ligated 12 hr beforehand, this drug was approximately three times less effective than in unligated animals.

The results in the present experiments thus support the view that above a ligation of sympathetic adrenergic nerves, noradrenaline storage granules formed in the nerve cell body accumulate in large numbers. They also suggest an impairment of the uptake mechanism across the nerve membrane, possibly as a result of mechanical distention of the membrane.

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The effects of drugs on circular muscle strips from the isolated ileum of the rabbit

M. G. TWEEDDALE

Circular muscle strip preparations were contracted by high concentrations of acetylcholine, methacholine or carbachol, but not by other smooth muscle stimulants. The responses to acetylcholine were antagonized by muscarinic blocking agents but not by ganglion-blocking drugs or local anaesthetics. Anticholinesterases induced violent and prolonged activity which was unaffected by repeated washing, by atropine or by local anaesthetics. The insensitivity to acetylcholine and the anomalous responses to anticholinesterases and to the stimulant drugs are discussed. It is suggested that there is some basic difference between the pharmacological responses of the two layers of the mammalian small intestine.

THERE have been many accounts of the effects of drugs on the longitudinal smooth muscle of the mammalian small intestine (see, for example, Kosterlitz & Lees, 1964). By comparison, the pharmacology of the circular muscle layer of the small intestine has attracted little attention. Isolated preparations from only three species have so far been investigated. These are the cat (Gasser, 1926; Evans & Schild, 1953), the guinea-pig (Harry, 1963; Brownlee & Harry, 1963) and man (Fishlock & Parks, 1963; Fishlock, 1964). In addition, there have been some experiments on circular muscle strips from the large intestine. In the present work, the pharmacological investigation of the intestinal circular muscle has been extended to a fourth species, the rabbit.

Experimental

METHODS

Rabbits of either sex weighing between 1·25 and 3·0 kg were killed by a blow on the neck and bled. The abdomen was opened and a marking thread was sewn into the wall of the small bowel close to the ileo-caecal junction. The gut was transected distal to this thread and then freed from its mesenteric attachments. Approximately one third of the distal small bowel was so mobilized, removed from the animal and placed in Krebs solution chilled to 10°.

Thirty min later, when the gut was fully relaxed, it was measured and any length in excess of 95 cm from the ileo-caecal junction was discarded. Circular muscle strips were made from the proximal 10 cm of the remaining length of small gut, i.e., from 85–95 cm proximal to the ileo-caecal junction.

A small segment 2·5 cm long was cut from the chosen portion of the small bowel (Fig. 1a), freed from mesenteric debris and opened by a cut along the mesenteric border (Fig. 1b). The resulting sheet of intestinal wall was pinned out, lightly stretched, under chilled Krebs solution with the mucosal surface uppermost. Threads were tied into the margins of the sheet of intestinal wall, opposite one another and in the line of the circular muscle fibres (Fig. 1c). A strip 4 mm wide was cut between the

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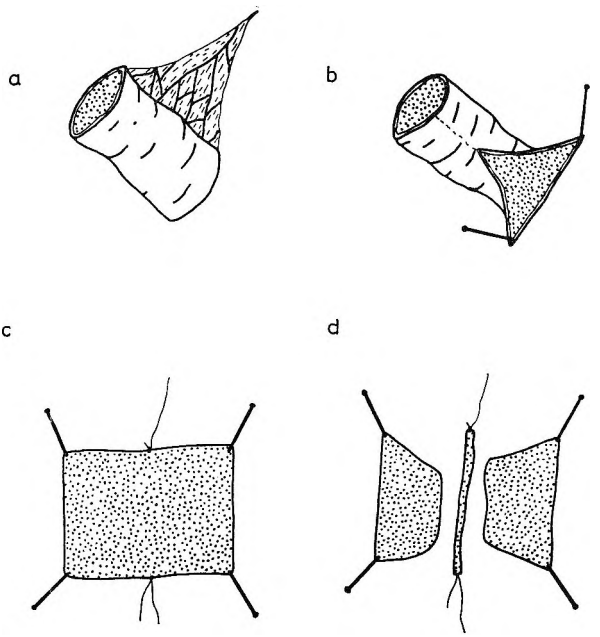

FIG. 1. The preparation of a circular muscle strip. (a) A segment 2·5 cm long is removed from the ileum about 90 cm proximal to the ileo-caecal junction (see text). (b) Cleaned of mesenteric attachments, the segment is opened along the mesenteric border. (c) The sheet of intestinal wall is pinned out, mucosa uppermost, under chilled Krebs solution. Threads are tied into the margins of the sheet in the direction of the circular muscle fibres. (d) The strip, 4 mm wide, is cut between the threads.

threads (Fig. 1d) using two parallel scalpel blades attached to a handle. Usually two such strips were cut from each 2·5 cm segment of ileum.

Longitudinal muscle strips were prepared in a similar manner except that the threads were tied and the cuts were made at right-angles to the line of the circular muscle fibres.

Each circular muscle strip prepared by the method described was anchored at one end to a glass tissue holder and mounted vertically in 15 ml of Krebs solution maintained at 37° (±0·5°) and gassed with 5% carbon dioxide in oxygen. The upper end of the strip was attached to a light, isotonic, balsa-wood lever fitted with a glass side-writing point (Foster, 1963) recording on a lightly smoked drum. The movements of the tissue were magnified 10 times and the load on the tissue was 300 mg. All preparations were left for 1 hr before the experiments were begun.

Immediately before the addition of a drug to the bath fluid, 1 ml of bath fluid was withdrawn. Each dose of a drug was added to the bath fluid in this volume (1 ml) of Krebs solution. A drug was left in contact with the preparation for 60 sec in a 10 min cycle. The bath fluid was changed at least three times at ½ min intervals after each dose of a drug. A resting period of 45–60 min was required between each series of six 10 min cycles if the preparation was to survive.

When the dose response relations were being investigated the first dose of each series was repeated because the sensitivity of the preparations increased transiently after each rest period. Successive doses were quadrupled to reduce the number of cycles required to cover a wide range of drug concentrations.

Antagonist drugs were made up to the required concentration in the Krebs solution used to replace the bath fluid. All antagonists were in contact with the tissue for the duration of a rest period (i.e., 45–60 min) before responses to the agonist were again tested.

Each experiment was made with at least four preparations.

DRUGS AND SOLUTIONS

Drugs used were: acetylcholine chloride, acetyl-β-methylcholine chloride, carbachol, atropine sulphate, hyoscine hydrobromide, hexamethonium bromide, dimethylphenylpiperazinium iodide, cocaine hydrochloride, procaine hydrochloride, neostigmine methylsulphate, eserine sulphate, di-isopropyl-phosphodiamidic fluoride (Mipafox), histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, nicotine acid tartrate, crude substance P, potassium chloride, barium chloride, angiotensin II and sucrose. All concentrations are in mg/ml or μg/ml of base.

The Krebs solution had the following composition (in g/litre of distilled water): NaCl 6·92; KCl 0·354; $CaCl_2$ 0·282; $NaHCO_3$ 2·10; NaH_2PO_4 0·162; $MgSO_4.7H_2O$ 0·294; and glucose 2·00.

Results

Circular muscle strips from the rabbit ileum were never spontaneously active. Most preparations survived for about 5 hr after the start of the experiment. This was sufficient to allow three dose response series, each consisting of six cycles, to be completed.

ESTERS OF CHOLINE

Typical responses of a circular muscle strip to increasing concentrations of acetylcholine, methacholine and carbachol are shown in Fig. 2. The responses to acetylcholine or to methacholine increased with increasing concentrations of the agonist over a wide range (50 μg/ml–12·8 mg/ml for acetylcholine; 2·5–640 μg/ml for methacholine). Most preparations were more sensitive to carbachol than to the other two choline esters. Usually two phases were seen; the first an increase in response with increasing concentrations followed by the second, a subsequent decrease with increasing concentrations. In some preparations, the response to the second and subsequent concentrations of carbachol was delayed for a variable latent period. Because of these inconsistencies, the responses of circular muscle strips to carbachol were not further investigated.

ATROPINE AND HYOSCINE

Fig. 3 shows the results of four experiments in which circular muscle strips were treated with atropine (0·01 μg/ml) before the repetition of the

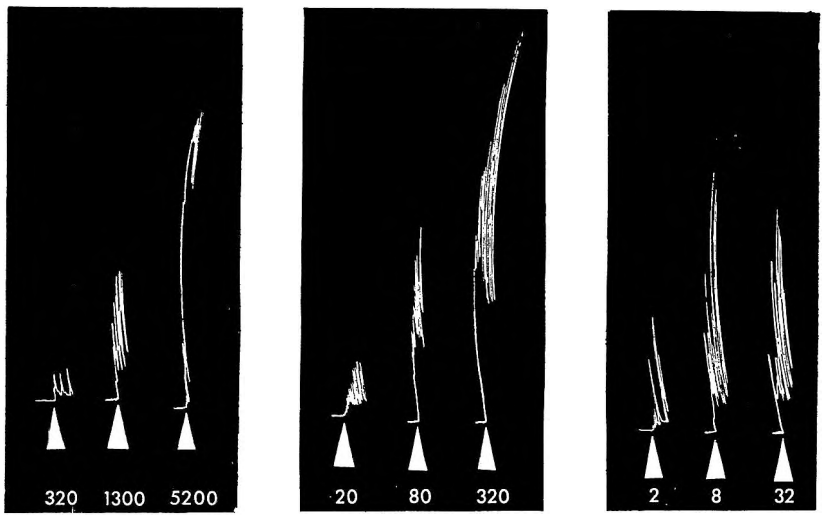

FIG. 2. The responses of a circular muscle strip from the rabbit ileum to acetylcholine, methacholine and carbachol. The first panel shows the responses to increasing concentrations of acetylcholine and the second and third panel to methacholine and carbachol. All concentrations are in μg/ml. Methaboline was 20 times more active than acetylcholine, but 10 times less active than carbachol, The responses to carbachol did not increase with increasing concentrations of the agonist.

dose response curve to methacholine. Atropine (0·01 μg/ml) or hyoscine (0·005 μg/ml) always depressed the responses of preparations stimulated by either acetylcholine or methacholine.

HEXAMETHONIUM

Four preparations were treated with the competitive ganglion-blocking agent hexamethonium (100 μg/ml) after a control dose response curve to acetylcholine had been completed. The responses of two preparations to acetylcholine were unaffected by hexamethonium, and two additional preparations showed a slight potentiation of the responses to the lower concentrations of acetylcholine.

Three out of four preparations stimulated by methacholine were unaffected by hexamethonium (100 μg/ml) and one preparation showed potentiation of the responses to the lower concentrations of methacholine.

DIMETHYLPHENYLPIPERAZINIUM

The compound, 1,1-dimethyl-4-phenylpiperazinium (DMPP) has been shown to cause ganglion blockade by depolarization (Leach, 1957; Ling, 1959). Brownlee & Johnson (1963) presented evidence that depolarizing blockade by DMPP differed from that produced by nicotine in not reverting to a competitive type of blockade after a short time. The dose response curves to acetylcholine and methacholine of circular muscle strips from the rabbit ileum were unaffected by the presence of DMPP (100 μg/ml).

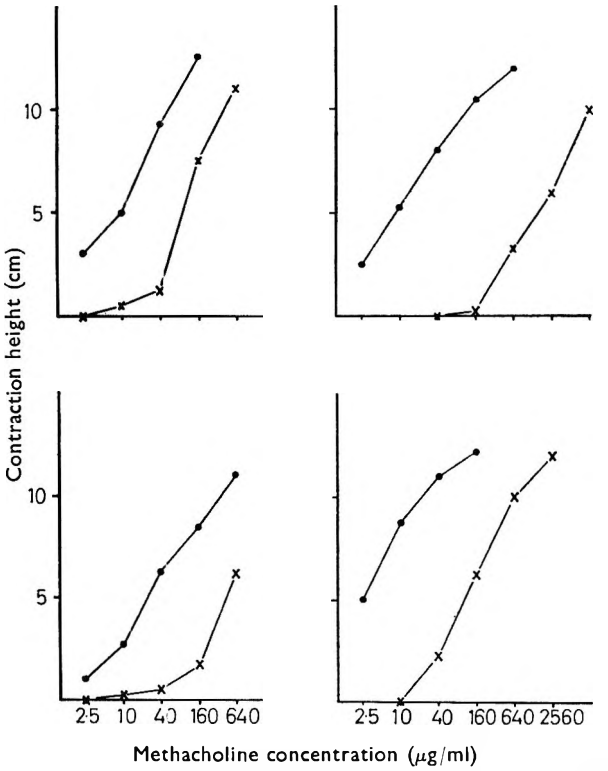

FIG. 3. The depressant effect of atropine on the responses to methacholine. The dose response curves from four circular muscle strip preparations are shown. The contractions of each preparation (cm) are plotted against the concentration of methacholine (μg/ml). The closed circles represent the control responses; the crosses represent the responses obtained 1 hr later in the presence of atropine (0·01 μg/ml). Atropine antagonized the responses to methacholine, moving the dose response curve to the right.

COCAINE AND PROCAINE

The responses of two acetylcholine-treated circular muscle strips were unchanged in the presence of cocaine (50 μg/ml). Two other preparations showed a slight potentiation of the responses to acetylcholine after cocaine.

Three preparations stimulated by methacholine showed potentiation of the responses after cocaine (50 μg/ml) and another was unaffected. A further methacholine-treated preparation went into spasm on the addition of cocaine (50 μg/ml) to the bath fluid and this experiment was abandoned.

Procaine (100 μg/ml) failed to modify the responses of circular muscle strips stimulated by acetylcholine or methacholine.

ANTICHOLINESTERASES

Neostigmine (1·0–2·6 μg/ml) always stimulated circular muscle strips from the rabbit ileum. Three types of activity were recorded. Firstly,

large, irregular contractions which continued for 60–90 min and which were followed by spasmodic activity for a further 60–90 min. During this latter period, bursts of activity were triggered by any interference with the preparation (e.g., changing the bath fluid). Following the period of intermittent activity, the preparation became quiescent and unresponsive to electrical, mechanical or pharmacological stimuli. Secondly, a slow, well-maintained contracture which persisted for several hours, and thirdly, occasional preparations contracted intensely and then relaxed slowly. Such contractions occurred singly, or in groups of two or three, at irregular intervals for many hours. Eserine (1·0–5·0 μg/ml) produced similar effects on the preparations, as did the organophosphorus anti-cholinesterase di-isopropylphosphodiamidic fluoride (Mipafox, 50 μg/ml).

The activity produced by these anticholinesterases persisted in spite of repeated washings. In other experiments, the anticholinesterase agent was added to the bath fluid together with atropine (0·01 μg/ml), cocaine (50 μg/ml) or procaine (100 μg/ml). None of these drugs prevented the anticholinesterase-induced activity.

OTHER SMOOTH MUSCLE STIMULANTS

Circular muscle strips from the rabbit ileum failed to respond to histamine, 5-hydroxytryptamine (5-HT), nicotine, DMPP, substance P, barium ions or potassium ions (Fig. 4). Each of these substances was added to the bath fluid in a concentration of 1 mg/ml, the metallic ions

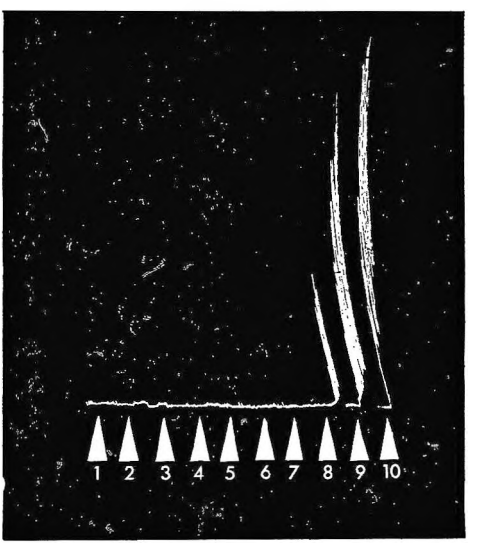

FIG. 4. Spasmogenic drugs on the circular muscle strip preparation. The record shows the effect of 1, 5-hydroxytryptamine, 2, barium chloride, 3, crude substance P, 4, nicotine, 5, histamine, 6, potassium chloride and 7, dimethylphenylpiperazinium. Each of these substances was applied to the preparation in a concentration of 1 mg/ml. The preparation did not respond to any of these drugs but graded responses were obtained to methacholine 8, 10 μg/ml, 9, 40 μg/ml and 10, 160 μg/ml.

being added as the chloride. The four circular muscle strips used for these experiments responded normally to methacholine (see Fig. 4). No

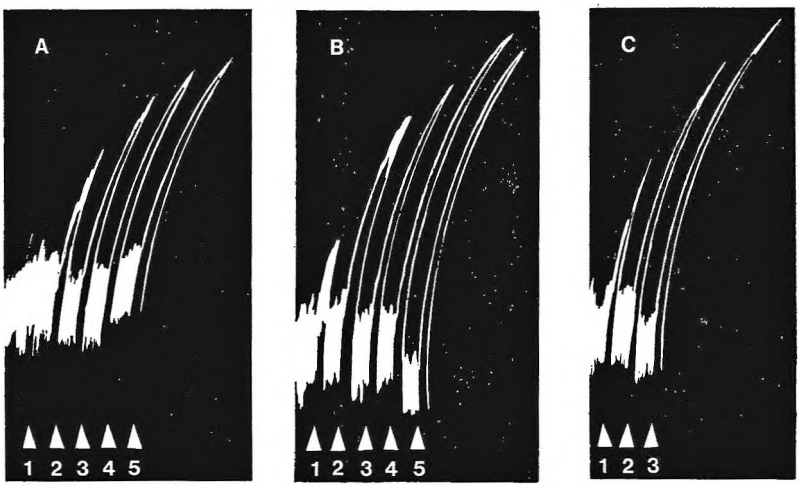

FIG. 5. The effect of acetylcholine, methacholine and carbachol on a longitudinal muscle strip from the rabbit ileum. The first panel shows the responses of the preparation to increasing concentrations of acetylcholine. The other two panels show, respectively, the responses to methacholine and carbachol. All concentrations are in μg/ml. The longitudinal muscle strip is spontaneously active and very sensitive to the stimulant drugs (compare with Fig. 2). The responses to all three agonists increase with increasing concentrations. A. Acetylcholine; 1, 0·015; 2, 0·06; 3, 0·25; 4, 1·0; 5, 4·0. B. Methacholine; 1, 0·015; 2, 0·06; 3, 0·25; 4, 1·0; 5, 4·0. C. Carbachol; 1, 0·004; 2, 0·016; 3, 0·064.

response was obtained from two preparations treated with angiotensin II (50 μg/ml).

SUCROSE

Sucrose in a concentration (330 mg/ml) osmotically equivalent to the highest concentration of acetylcholine used in these experiments produced only negligible activity compared with the large and rapid response to acetylcholine.

LONGITUDINAL MUSCLE STRIPS

Unlike circular muscle strips, preparations cut in the direction of the longitudinal muscle coat were spontaneously active. Further, a high level of inherent tone was demonstrable by the use of relaxant drugs. Longitudinal muscle strips responded to choline esters, histamine, nicotine or substance P in concentrations similar to those required on the Magnus preparation of the rabbit ileum. The responses of one longitudinal muscle strip to acetylcholine, methacholine and carbachol are shown in Fig. 5.

Discussion

Circular muscle strips from the rabbit ileum responded with concentration-dependent contractures when treated with acetylcholine or methacholine. Preparations were more sensitive to carbachol than to the other two esters of choline, but the responses to carbachol showed a mixed action and a variable latency. This pattern of responses to drugs differs from that previously reported (Tweeddale, 1963), the difference being attributable to the use of different recording systems on the two occasions. The reasons for these differences are fully discussed elsewhere (Tweeddale, 1965).

The circular muscle strip of the rabbit ileum is remarkable for its insensitivity to drugs. Very high concentrations of acetylcholine (up to 12·8 mg/ml) were needed to produce adequate contractions, and concentrations in excess of this did not produce a maximal response. The need for such high concentrations of acetylcholine leads one first to consider indirect mechanisms. The responses to acetylcholine or to methacholine were not reduced by the competitive ganglion-blocking agent hexamethonium, nor by its depolarizing counterpart, dimethylphenylpiperazinium. The possibility of a site of action of acetylcholine distal to the ganglion-cell body was excluded by the failure of local anaesthetic agents to reduce the responses. In fact, both hexamethonium and cocaine increased some of the responses to the choline esters. For hexamethonium this observation is attributed to the weak anticholinesterase activity of this drug in the concentration used of 100 μg/ml of base (Paton & Zaimis, 1949) and for cocaine to a stimulant action of the type reported by Feldberg & Lin (1949a). Because drugs which abolish neural activity failed to modify the responses to acetylcholine, or to methacholine, the responses to these agonists must occur wholly by activation of receptors on the smooth muscle cells.

The muscarinic blocking agents atropine and hyoscine in low concentrations antagonized the actions of acetylcholine or methacholine. This antagonism seemed to be competitive since the dose response curves in the presence of the antagonist were parallel to and to the right of the control curves. The effects of acetylcholine and methacholine on the circular muscle of the rabbit ileum must therefore be attributed to the activation of muscarinic receptors.

It remains to consider why such high concentrations of choline esters were required to activate the circular muscle of the rabbit ileum. The muscarinic receptors of the circular muscle were 10,000 times less sensitive to acetylcholine than those of the longitudinal muscle. A number of possibilities for this insensitivity must be considered. Firstly, that the trauma of preparation adversely affected the responses of the circular muscle strips. However, longitudinal muscle strips were subjected to similar procedures and yet retained a high sensitivity to acetylcholine. Secondly, a non-specific barrier to the diffusion of drugs might diminish the responses of the circular muscle to drugs. This is unlikely, since atropine and hyoscine were active in their usual low concentrations.

Finally, it is possible that the apparent insensitivity of rabbit intestinal circular muscle to acetylcholine is due to the receptors being protected by a high concentration of cholinesterase. The experiments with the anticholinesterase agents, which might have clarified this issue, did not allow assessment of the effect of acetylcholine in the presence of cholinesterase inhibition.

Considering now the effects of anticholinesterases on intestinal circular muscle, Gasser (1926) reported that eserine was not only unreliable in potentiating responses to acetylcholine, but was itself capable of stimulating circular muscle strips from the ileum of the cat which were unresponsive to acetylcholine. This latter effect of eserine was blocked by atropine. Evans & Schild (1953) also using cat intestine, but with lower concentrations of eserine than those used by Gasser (1926), found no evidence of stimulation and always observed potentiation of the responses to acetylcholine. The effect of eserine and neostigmine on circular muscle strips from the guinea-pig ileum has not been reported, but Harry (1963) observed a slowly developing irregular activity of his preparations after incubation with Mipafox. This effect was probably due to the accumulation of acetylcholine (cf. Johnson, 1963), as it was not a prominent feature of any other of his experiments. Feldberg & Lin (1949b) reported that eserine caused uncoordinated, intermittent spasms of the circular muscle of the rabbit ileum Trendelenburg preparation. These workers also reported a large output of acetylcholine from the eserinized rabbit ileum which they concluded came from non-nervous sources in the gut wall.

In the present experiments, eserine, neostigmine and even Mipafox induced vigorous activity of rabbit intestinal circular muscle strips and it was not possible to assess whether responses to acetylcholine were potentiated. The anticholinesterase-induced activity was not due to the accumulation of acetylcholine from nervous sources within the gut wall (Johnson, 1963), since it was not reduced by repeated washing, by atropine or by local anaesthetic drugs. Even if acetylcholine were arising from non-nervous sites (Feldberg & Lin, 1949b), its effects should be blocked by atropine although Cuthbert (1962) has described an atropine-resistant stimulant action of anticholinesterases upon the chick amnion. This latter effect was limited to the tertiary anticholinesterases whereas neostigmine and Mipafox were as effective as eserine in the present experiments. This is the first time that Mipafox has been reported to show significant spasmogenic activity (Harry, 1962, 1963; Cuthbert, 1962; Brownlee & Johnson, 1963; Carlyle, 1963; Johnson, 1963). It may be that the stimulant effects of the anticholinesterases in the present experiments represent a direct excitatory action independent of both cholinesterase inhibition and of the muscarinic receptor.

In the absence of satisfactory results with the anticholinesterases it is difficult to assess the failure of nicotine, DMPP, histamine and 5-HT to stimulate circular muscle strips from the rabbit ileum. Evans & Schild (1953) found that nicotine readily stimulated whole-wall circular muscle strips from the cat intestine. On the other hand, Harry (1963) and

Brownlee & Harry (1963) showed that responses of guinea-pig circular muscle strips to nicotine, 5-HT or histamine appeared only after cholinesterase inhibition by Mipafox. The failure of potassium and barium to stimulate circular muscle strips from the rabbit ileum in concentrations which were more than adequate to activate the Magnus preparation of the rabbit ileum may be explicable in terms of the membrane potential level of the two types of smooth muscle cell. The longitudinal muscle is spontaneously active and should therefore possess an unstable membrane potential (Burnstock, Holman & Prosser, 1963), which is possibly susceptible to the stimulant effect of potassium and barium. The circular muscle is quiescent and atonic and should possess a stable membrane potential which is resistant to depolarization by acetylcholine as well as by potassium or barium.

There are relatively few reports in the literature on the pharmacology of isolated intestinal circular muscle, and only one direct comparison between the longitudinal and circular muscle layers (Brownlee & Harry, 1963). However, these reports and the present work suggest that there is some basic difference between cells of the two muscle layers. Even should this prove to be untrue, it is apparent that conclusions drawn from experiments made with one of the muscle layers may not be indiscriminately applied to the other.

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Release of oxytocin contributes to the natriuretic action of aminophylline in rats

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Aminophylline, 0·25 to 2·0 mg intramuscularly, caused diuresis, natriuresis and increase in the Na/K of the urine in the 1 hr period after injection into normal hydrated or unhydrated rats. The urinary changes induced by 2 mg aminophylline and by 8 mU oxytocin equated. Salt-maintained adrenalectomized rats were fully sensitive to the diuretic, natriuretic action of aminophylline. The Na/K in the urine decreased at the 2 mg dose level. Hypophysectomy abolished and neurohypophysectomy markedly decreased the diuretic natriuretic action of aminophylline in unanaesthetized rats. Under ethanol-pentobarbitone anaesthesia the diuretic natriuretic action of 0·4 mg aminophylline intravenously lasted 30–40 min in normal rats, and for less than 10 min in neurohypophsectomized rats. The duration of the cardiovascular response to aminophylline was 7–8 min. Thioglycollate-labile oxytocic activity, not detectable in the arterial plasma of control animals, was demonstrable in the arterial plasma of normal rats 8–12 min after 0·5 mg aminophylline intravenously.

THE xanthine diuretics produce both a rise in glomerular filtration rate (GFR) and an increase in the urinary excretion of sodium in man (Howarth, McMichael & Sharpey-Schafer, 1947; Davis & Shock, 1949). Intravenous injections of theophylline-ethylenediamine cause a 35% increase in cardiac output which lasts for about 15 min. This increase in cardiac output is accompanied by a rise in GFR. The rise in GFR cannot, however, explain the total natiuresis. An increase in sodium (Na) clearance is maintained for 50–60 min and clearly outlasts the haemodynamic effects of the drug. Davis & Shock (1949) therefore suggest that the xanthine diuretics may in part produce their renal actions in man directly or indirectly through the neurohypophysis. This hypothesis has now been put to the test in rats.

Experimental

METHODS

Female Wistar rats, 160–212 g, ate a pellet diet (Lockett & Nail, 1965) and drank freely. The fluid supplied to adrenalectomized animals contained 0·7% NaCl: all other rats drank water. Preparatory operations were performed under light pentobarbitone anaesthesia. The techniques used both for adrenalectomy and for total hypophysectomy have been described (Lees, Lockett & Roberts, 1964). Neurohypophysectomy was achieved by placing an electrolytic lesion at the rostral end of the neurohypophysis using a Krieg model Stoetling stereotaxic apparatus for rats, an A.P. co-ordinate of 54·4 mm, with the electrode touching the base of the skull, in the midline. A current of 3 mA was passed for 15 sec. This lesion inflicted no instant injury to the adenohypophysis but caused damage to the vessels of the portal tract. Consequently, the size of the functional adenohypophysis became reduced

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to approximately $\frac{1}{2}$ normal and adrenal weights were found to be $\frac{1}{2}$–$\frac{2}{3}$rds normal 3 months after operation. Hence an initially dramatic state of diabetes insipidus declined: water intakes were 2–3 times normal when these rats were in use.

LONG TERM EXPERIMENTS

Rats, housed in an air-conditioned room at 24–26°, were accustomed to stomach tubes and handling before use. Each experiment was designed as a series of cross-over tests in which each animal served as its own control. Equal numbers of each treatment were allotted to each day. Tests were made every 3 or 4 days and began with a 2 hr period during which no solid food remained available. At the end of this period each rat in turn received an oral water load equivalent to 2·5% body weight and an injection and was placed in an individual metabolism cage for the collection of all urine formed in the next hour: this collection period was extended to 2 hr for adrenalectomized animals. Since, after training, almost every animal micturated spontaneously when held gently but firmly under restraint for receipt of its water load, bladders were emptied by gentle suprapubic pressure solely to terminate a collection of urine. All cross-over tests which constituted a single experiment were made at a fixed time of day since the urinary excretion of sodium by rats decreases throughout the day (Lees, Lockett & Roberts, 1954). All but two experiments were as described above, during the first hour of a water diuresis. The two remaining experiments differed as follows. Each rat received an oral dose of 3 ml 0·9% NaCl at the start of the 2 hr fast. At the end of the fast an injection was made immediately before the 1 hr period of urine collection, but no water was given.

Oxytocin (Syntocinon, Sandoz Ltd.) and aminophylline (Merck Sharp & Dohme) were dissolved in 0·1 ml 0·9% NaCl, injected subcutaneously and intramuscularly respectively, in long term experiments.

SHORT TERM EXPERIMENTS

Rats were anaesthetized by the intraperitoneal injection of 1·8 mg sodium pentobarbitone and the oral administration of 1·0 ml 12% ethanol, per 100 g. Tracheal and tail vein cannulae were inserted, and a small self-retaining cannula was stitched into the bladder. Thereafter each animal was lightly strapped on its back on a warm table tilted at 45° before an indwelling stomach tube was inserted and strapped to an upright. A steady level of anaesthesia was maintained by administration of 0·5 ml 4% ethanol per 100 g weight, every 20 min, through the stomach tube.

Diureses. When a steady low rate of urine flow had been established (usually within 80 min of induction), 4% creatinine hydrochloride (British Drug Houses Ltd) in 0·9% NaCl was injected subcutaneously, 0·6 ml/100 g wt. Serial 10 min collections of urine began 30 min later and continued for 2 hr (12 periods). One half of the animals in each group received 0·4 mg aminophylline (per rat) in 0·05 ml 0·9% NaCl washed in

with 0·05 ml 0·9% NaCl via the tail vein cannula at the end of the 3rd urine collection; 0·1 ml saline was similarly injected at the end of the 9th collection. The remaining animals received saline at the end of the 3rd and aminophylline at the end of the 6th collection of urine. Samples of venous blood, 0·25 ml were withdrawn from a femoral vein into heparinized syringes immediately before and immediately after the serial collections of urine. Since the concentrations of true creatinine in plasma and whole blood do not differ (Miller & Dubos, 1937) and the decay curve for the plasma concentration of creatinine so administered to rats is exponential from the 30th min (Lippman, 1947; 1948), log concentrations of blood creatinine were plotted against time for each animal. Mid-period plasma concentrations of creatinine were read from these individual curves.

Collection of aortic blood. Eight rats were anaesthetized and equipped with tracheal and tail vein cannulae as described above. Four received 0·1 ml heparinized 0·9% NaCl (2000 units/ml, Evans Medical Ltd) and four 0·4 mg aminophylline in 0·1 ml heparinized saline, intravenously, 30 min after induction of a steady level of anaesthesia. The abdomen was opened in the mid line to permit withdrawal of 4·5 to 5 ml arterial blood from the aorta through a sharpened polythene cannula into a cold nylon syringe during the 12th min after the injection. The plasma was immediately separated by centrifuging at 3,500 rev/min for 30 min at 5°.

EXTRACTION AND ASSAY OF OXYTOCIN FROM PLASMA

The proteins were precipitated from 2 ml samples of rat plasma without delay by addition of 10 volumes of ice cold acetone as described by Ginsburg & Smith (1959). The acetone was removed from the supernatant *in vacuo* at 40°: the resultant cloudy aqueous residue was extracted with 7 volumes of ethyl ether and was cleared of ether at 40° in a stream of air. The final clear aqueous residue was diluted 1:1 with double strength perfusion fluid before assay of the contained oxytocin on an atropinized superfused horn of the rat uterus by the method of Ginsburg & Smith (1959) as thioglycollate labile (Ames, Moore & van Dyke, 1950) uterine stimulant activity.

CHEMICAL DETERMINATIONS

The concentrations of sodium and potassium in urine were measured on an EEL flame photometer. Concentrations of creatinine in whole blood (laked by addition of 0·1 ml blood to 2 ml distilled water) and in urine were determined as described previously for plasma and urine (Davey & Lockett, 1961).

Results

THE INFLUENCE OF INTRAMUSCULAR AMINOPHYLLINE ON THE EXCRETION OF WATER, SODIUM AND POTASSIUM BY UNANAESTHETIZED RATS

Table 1 shows the results of experiments in which the effects of aminophylline on the excretion of water, sodium (Na) and potassium (K) were

TABLE 1. URINARY CHANGES INDUCED BY AMINOPHYLLINE (Am.) DURING WATER DIURESIS IN RATS

Body wt g	Injection	μequiv/100 g rat/1 hr			
		H_2O	Na	K	Na/K
Unoperated					
157 ± 3·21 (16)	Saline	2·16 ± 0·162	14·6 ± 1·77	17·6 ± 2·04	0·97 ± 0·13
	1·0 mg Am.	2·27 ± 0·157	26·0 ± 3·26**	32·6 ± 3·49**	1·01 ± 0·26
	2·0 mg Am.	2·60 ± 0·175**	38·8 ± 4·81**++	42·8 ± 8·34**+	1·15 ± 0·15
155 ± 3·93 (18)	Saline	1·95 ± 0·175	13·2 ± 1·93	17·3 ± 2·12	0·85 ± 0·13
	0·5 mg Am.	1·83 ± 0·112	25·2 ± 2·89**	21·5 ± 3·06	1·36 ± 0·17*
	1·0 mg Am.	2·14 ± 0·182	25·2 ± 4·63**	22·4 ± 3·88	1·31 ± 0·25**
	2·0 mg Am.	1·99 ± 0·179	26·1 ± 3·88**	23·8 ± 3·15*	1·14 ± 0·14*
159 ± 3·42 (18)	Saline	1·92 ± 0·141	15·9 ± 2·97	16·3 ± 2·40	0·96 ± 0·13
	8 mU oxytocin	2·34 ± 0·182*	40·7 ± 5·06**	23·0 ± 3·36*	1·70 ± 0·21**
	2·0 mg Am.	2·26 ± 0·167*	38·7 ± 6·81**	24·5 ± 3·76*	1·52 ± 0·19**
158 ± 3·62 (18)	Saline	1·76 ± 0·17	18·4 ± 1·85	14·4 ± 2·19	1·08 ± 0·18
	8 mU oxytocin	2·09 ± 0·18*	46·1 ± 4·61**	23·6 ± 3·80*	2·11 ± 0·23**
	2·0 mg Am.	2·15 ± 0·13*	35·0 ± 5·07**	16·1 ± 1·92	2·14 ± 0·26**
		mg/rat/1 hr			
		Creatinine	PAH		
159 ± 3·42 (18)	Saline	18·4 ± 1·58	4·25 ± 0·23		
	8 mU oxytocin	18·9 ± 1·49	6·07 ± 0·81*		
	2·0 mg Am.	20·8 ± 1·89	6·90 ± 0·57*		
158 ± 3·62 (18)	Saline	21·1 ± 1·34	4·67 ± 0·36		
	8 mU oxytocin	21·5 ± 1·99	7·47 ± 0·89*		
	2·0 mg Am.	21·8 ± 1·87	7·00 ± 0·77*		
Adrenalectomized salt maintained					
191 ± 5·03 (16)	Saline	1·05 ± 0·11	55·5 ± 2·41	15·4 ± 1·47	2·05 ± 0·23
	1·0 mg Am.	1·37 ± 0·12**	84·2 ± 7·60**	25·5 ± 2·73*	1·95 ± 0·31
	2·0 mg Am.	1·38 ± 0·10**	78·6 ± 4·94**	26·4 ± 1·91**	1·54 ± 0·08*
176 ± 4·74 (14)	Saline	0·98 ± 0·16	50·4 ± 5·12	16·7 ± 2·32	2·98 ± 0·17
	2·0 mg Am.	1·41 ± 0·13**	79·7 ± 6·46**	31·3 ± 4·76**	2·56 ± 0·14*
Neurohypophysectomized					
168·6 ± 3·31 (22)	Saline	2·95 ± 0·17	12·6 ± 2·11	18·8 ± 3·10	0·92 ± 0·22
	1·0 mg Am.	2·90 ± 0·17	15·3 ± 3·08	18·6 ± 2·85	0·98 ± 0·16
	2·0 mg Am.	2·76 ± 0·18	19·8 ± 3·15*	20·8 ± 3·60	1·37 ± 0·31
Hypophysectomized					
164 ± 3·9 (16)	Saline	0·89 ± 0·17	8·7 ± 0·98	6·8 ± 1·20	1·37 ± 0·32
	2·0 mg Am.	0·73 ± 0·15	9·1 ± 2·09	7·9 ± 1·47	1·62 ± 0·33
169 ± 6·0 (16)	Saline	0·83 ± 0·11	10·1 ± 2·73	13·5 ± 2·24	0·96 ± 0·37
	2·0 mg Am.	0·70 ± 0·18	8·2 ± 2·36	11·5 ± 2·26	0·99 ± 0·28
166 ± 3·8 (14)	Saline	0·97 ± 0·16	8·4 ± 0·86	7·0 ± 1·12	1·26 ± 0·29
	2·0 mg Am.	0·78 ± 0·14	9·9 ± 2·17	8·1 ± 1·36	1·59 ± 0·30
176 ± 4·8 (14)	Saline	1·03 ± 0·18	7·9 ± 1·96	11·8 ± 1·80	0·88 ± 0·26
	2·0 mg Am.	1·02 ± 0·21	11·5 ± 3·04	13·9 ± 2·59	1·04 ± 0·21

The values shown are means ± their standard errors. The significance of differences caused by treatments have been examined by *t*-tests in which each animal has served as its own control and is indicated by *: one, $P > 0.95$; two, $P > 0.99$. The number of animals used in each experiment is shown in brackets in the first column.

measured over the first hour of water diuresis. Aminophylline (1 to 2 mg/rat, i.m.) raised the urinary output of Na in all, and increased the excretion of water and K and the urinary Na/K ratio in three of four experiments on normal rats. The urinary changes caused by aminophylline resembled those induced by the subcutaneous injection of 8 mU oxytocin; moreover, both drugs significantly increased the excretion of *p*-aminohippuric acid but not that of creatinine. The inability of these

animals to discriminate between 1 and 2 mg aminophylline is explained by reference to Fig. 1. Maximal renal action is attained in the first hour

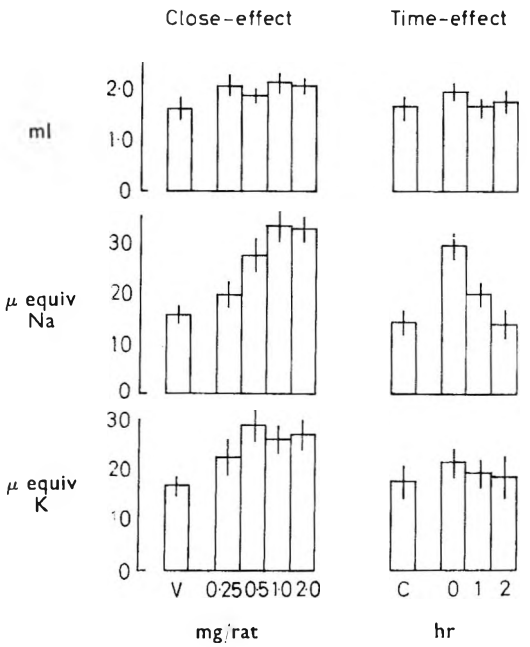

FIG. 1. Dose-effect curve (left) and time-effect curve (right) for the natriuretic action of aminophylline i.m. in water laden unanaesthetized rats. The heights of the columns depict the mean urinary outputs, per 100 g rat, measured over 1 hr period with standard errors of these means. All values were supplied by a single group of 36 rats weighing 161 ± 4·8 g. Left, dose-effect curve: aminophylline i.m. together with an oral water load equivalent to 2·5% body weight at the start of a 1 hr period of urine collection. Abscissae: V, vehicle only, dose of aminophylline i.m. per rat. Right, time-effect curve for 1 mg aminophylline per rat, i.m. Abscissae:—C, no aminophylline, then aminophylline with (0), 1 and 2 hr before water load and subsequent 1 hr period of urine collection. Both experiments were designed as 4-day cross over tests.

after intramuscular administration of this diuretic at a dose level of 1 mg/rat.

Salt-maintained adrenalectomized rats responded to aminophylline by increase in the urinary outputs of water, Na and K: in both experiments, however, the Na/K ratio of the urine was reduced by aminophylline at the 2 mg dose level. By contrast, the urinary excretion of water, Na and K was unaffected by 2 mg aminophylline in totally hypophysectomized animals. Neurohypophysectomized rats, unable to respond to 1 mg aminophylline, showed a small but just significant increase in Na excretion at the 2 mg dose level.

The urinary effects of 2 mg aminophylline in unhydrated animals closely resembled those observed in the same animals when hydrated (Fig. 2).

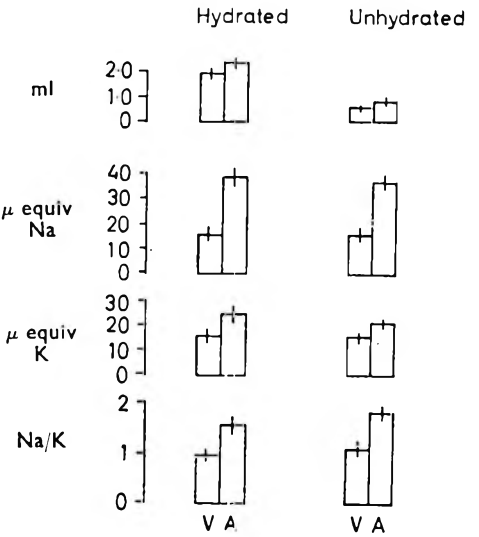

FIG. 2. Comparison of the diuretic effects of 2 mg aminophylline i.m. water laden and in unhydrated rats. The values shown were supplied by a single group of 36 rats, weighing 179 ± 2·8 g, from an experiment designed as a 4-day cross over test. Aminophylline was administered i.m. at the start of a 1 hr period of urine collection with oral water (2·5% body weight) when hydrating, and without water when unhydrated. Values shown are urinary outputs/100 g rat/hr. Ordinates: volume, ml: then μequiv Na and K respectively: Na/K in urine. Abscissae: V, vehicle only: A, aminophylline 2 mg i.m.

THE DIURETIC ACTIONS OF INTRAVENOUS AMINOPHYLLINE IN ANAESTHETIZED NORMAL AND NEUROHYPOPHYSECTOMIZED RATS

The resting rates of urine flow and of glomerular filtration found for normal and for neurohypophysectomized rats under alcohol-pentobarbitone anaesthesia did not differ (Table 2) but the rate of urinary excretion of Na and the Na/K of the urine were significantly greater for the neurohypophysectomized group. Intravenous aminophylline, 0·4 mg/rat, markedly increased ventilation and both the rate and the force of the heart beat in both groups of animals. These respiratory and cardiovascular responses developed within 30 sec of the injection, sustained maximal intensity for 2 to 3 min, then waned. Control levels of ventilation were reached in 15 min and of heart rate by the 7th to 8th min. The cardiovascular actions of the drug are therefore relevant only to the first urine collection made after aminophylline (Table 2). In the first 10 min after intravenous injection, aminophylline produced diuresis, natriuresis and raised the GFR in both normal and neurohypophysectomized animals. However, the diuresis and the increase in GFR shown by the normal animals exceeded that found for the neurohypophysectomized group ($P > 0{\cdot}99$ and $P > 0{\cdot}95$, respectively). Whereas the ratio Na/K in the urine of normal animals rose, this ratio remained unchanged in the urine of the neurohypophysectomized animals because

TABLE 2. CONTRASTS THE URINARY EFFECTS OF 0·4 MG AMINOPHYLLINE, I.V. IN NORMAL AND IN NEUROHYPOPHYSECTOMIZED RATS UNDER PENTOBARBITONE ANAESTHESIA

	Normal	Neurohypophysectomized
body weight in g (number)	163·4 ± 3·91 (6)	170·2 ± 4·90 (6)
Urine: control levels:		
ml/min	0·025 ± 0·007	0·024 ± 0·006
μequiv/min Na	1·60 ± 0·76	5·75 ± 1·35*
K	5·89 ± 1·16	9·33 ± 2·62
Na/K	0·24 ± 0·063	0·65 ± 0·128**
GFR, ml/min	1·36 ± 0·098	1·12 ± 0·059
Urine: 0–10 min after aminophylline		
ml/min	0·135 ± 0·114††	0·064 ± 0·018††
μequiv/min Na	3·83 ± 1·42†	8·22 ± 0·118†
K	7·14 ± 1·07	12·85 ± 2·16†
Na/K	0·54 ± 0·172††	0·62 ± 0·163
GFR, ml/min	1·82 ± 0·236††	1·42 ± 0·128†
Urine: 10–20 min after aminophylline		
ml/min	0·213 ± 0·090††	0·028 ± 0·006
μequiv/min Na	4·61 ± 1·95††	5·61 ± 1·26
K	7·03 ± 2·28	6·72 ± 0·97
Na/K	0·65 ± 0·153††	0·82 ± 0·150
GFR, ml/min	2·07 ± 0·282††	1·10 ± 0·038

The significance of differences between control means supplied by normal and by neurohypophysectomized animals has been examined by group *t*-tests and is indicated by *. Each animal served as its own control in *t*-tests used to assess the significance (†) of changes caused by the drug. Hence significance is indicated both by * and †: one, $P > 0.95$; two, $P > 0.99$.

the excretion of K by the latter group rose in parallel with the excretion of Na.

During the period 10 to 20 min after intraveneous aminophylline normal animals showed a further increase in diuresis, natriuresis, GFR and Na/K in the urine (Table 2). By contrast, resting levels for these parameters were found for the neurohypophysectomized group. Fig. 3

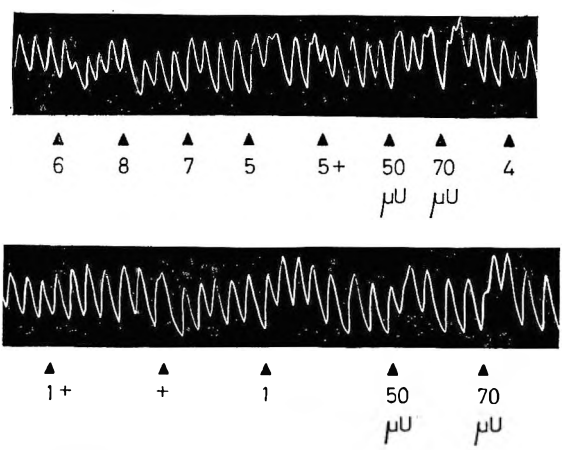

FIG. 3. Trace supplied by a superfused horn of rat uterus, responding, at signals ▲ to the following, in 0·5 ml volume:—6, 8 and 4, extracts equivalent to 2 ml arterial plasma from 3 different control rats. 1, 5 and 7, extracts equivalent to 1 ml arterial plasma withdrawn from 3 different rats 12 min after 0·5 mg aminophylline i.v. The suffix + signifies thioglycollate treatment of the extract, + alone, thioglycollate alone. μU signifies dose of oxytocin. Atropine sulphate, 1 mg/litre, in superfusion fluid.

shows that thioglycollate-labile oxytocic activity was detectable in extracts equivalent to 1 ml aortic plasma withdrawn 12 min after aminophylline (0·5 mg, i.v.) from each of 3 normal rats. By contrast, no oxytocic activity was demonstrable in any of the 3 extracts of 2 ml aortic plasma taken, in parallel, from 3 neurohypophysectomized rats 12 min after aminophylline (0·5 mg i.v.). A similar contrast was found for a fourth pair of extracts prepared from normal and neurohypophysectomized rats' plasma withdrawn 8 min after aminophylline (0·5 mg i.v.).

Discussion

Previous work, especially that of Howarth & others (1947) and Davis & Shock (1949), clearly demonstrated two component mechanisms within the diuretic action of aminophylline in man. The first component was the consequence of the cardiovascular effects which lasted for 15 min after intravenous administration of therapeutic doses. The second component was exposed as a natriuresis which outlasted the haemodynamic action by 40 to 50 min; the mechanism of this natriuresis remained obscure.

The short-lived diuresis, natriuresis and increase in GFR which resulted from intravenous administration of aminophylline to anaesthetized neurohypophysectomized rats (Table 2) synchronized with the cardiac action of the drug. Since these animals, in contrast to normal rats, showed no prolongation of natriuresis beyond the duration of the haemodynamic response, the diuretic action of aminophylline in neurohypophysectomized rats is attributed solely to the cardiovascular effects of the drug. Since the dose of aminophylline, intramuscularly, required to elicit a small increase in the 1 hr output of Na by unanaesthetized neurohypophysectomized rats was twice that maximally effective in normal animals, the cardiovascular component is the lesser contributor to the natriuresis evoked by aminophylline given by this route in this species. The failure of totally hypophysectomized rats to respond to 2 mg aminophylline intramuscularly by natriuresis suggests that the ability of the cardiovascular system to respond to aminophylline is subnormal in these animals. This defect cannot be attributed to withdrawal of corticotrophin and hence to depression of adrenal cortical function since salt-maintained adrenalectomized animals are fully sensitive to the natriuretic actions of aminophylline. The fall in the urinary Na/K which results from administration of 2 mg aminophylline intramuscularly to these adrenalectomized rats is attributed to the hyperventilation caused by the drug and the ease with which these animals develop alkalosis.

The mechanism of the long-lasting component of the natriuresis caused by aminophylline is attributed to the release of oxytocin from the neurohypophysis. The evidence in support of this conclusion is tripartite. First, this phase of the natriuresis is absent in neurohypophysectomized rats. Secondly, the diuretic effects of aminophylline (2 mg i.m.) in normal rats closely resemble those of oxytocin (8 mU s.c.).

Finally, thioglycollate-labile uterine stimulant activity was demonstrated in extracts of 1 ml arterial plasma collected from each of 4 rats 8 to 12 min after intravenous aminophylline but was not found in extracts of 2 ml arterial plasma taken from control animals.

The diuretic actions of aminophylline in water-laden and in unhydrated animals did not differ. Hence it appears that the oxytocin released by aminophylline is not accompanied by functionally significant amounts of antidiuretic hormone. A similar selective release of oxytocin has previously been encountered: the stimulus was low-pitched sound of 50–150 cycles/sec (Ogle & Lockett, 1966; Ogle, 1967).

The direct renal actions of the xanthine diuretics are well known for they have been demonstrated on the perfused dog kidney (Verney & Winton, 1930). Further work is however needed to determine the extent to which the direct effects of aminophylline on the kidney contribute to the therapeutic action of the drug in man.

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The partially inhibited growth of *Escherichia coli* in the presence of some antibacterial agents

R. M. RYE AND DAVID WISEMAN

Cultures of *Escherichia coli* were partially inhibited by treatment with tetracycline, phenol, phenylmercuric acetate or cetyltrimethylammonium bromide. The effects of these agents were investigated by measuring the cell size distributions after growth had occurred in the presence of sufficient ampicillin to suppress cellular division. Tetracycline and phenol inhibited cultures by a uniform decrease in the rate of growth of all of the cells; cetyltrimethylammonium bromide completely inhibited the growth of some of the cells whilst having no effect on the remainder; phenylmercuric acetate probably affected all the cells but inhibited each individual to a different extent. The implications of these results are discussed in terms of a general growth rate equation.

THE growth rate of bacterial cultures is reduced in the presence of sub-inhibitory concentrations of antibacterial agents. Most studies of this phenomenon have been concerned with the development of techniques for microbiological assay (Kavanagh, 1963) and few attempts to determine the mechanism of growth reduction have been made. The two mechanisms by which this overall decrease in the growth rate may be achieved are (1) a uniform inhibition of all of the cells, and (2) a non-uniform inhibition of the individual cells. The second mechanism embraces both the complete inhibition of the growth of some of the cells with the remainder growing at the normal rate (Treffers, 1956) and the situation where all the cells are growing but at widely different rates. Studies of the mechanism of inhibition have previously been based on simultaneous measurements of the total and viable cell counts (Parkinson & Pickett, 1964; Garrett & Miller, 1965) but this approach is not entirely satisfactory as organisms not multiplying under the conditions of the experiment may do so under the changed conditions used for detecting viability.

This paper describes a method for investigating the uniformity of inhibition of individual cells in partially inhibited cultures of *E. coli*. Results are given for cells treated with tetracycline, phenol, phenylmercuric acetate and cetyltrimethylammonium bromide.

Experimental

Escherichia coli (NCTC 1013) was used. The conditions of culture, media and methods used to measure absorbance and to prepare cell suspensions have been described previously (Rye & Wiseman, 1966). Freshly prepared solutions of the following antibacterial agents in glucose-free medium were used; phenol B.P., cetyltrimethylammonium bromide (CTAB, cetrimide), phenylmercuric acetate, and tetracycline B.P.

PREPARATION AND TREATMENT OF CELL SUSPENSIONS

Exponentially growing cells were harvested by membrane filtration and suspended in glucose-free medium at 37° to give an absorbance of 0·200.

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Fifteen min after harvesting, equal volumes of the suspensions and of solutions of the antibacterial agents were mixed and maintained at 37°. After a further 15 min, glucose (1 mg/ml) together with sufficient ampicillin B.P. to produce a final concentration of 2 μg/ml were added and the growth of the cells followed by absorbance measurements.

TOTAL CELL COUNTS AND SIZE (VOLUME) DISTRIBUTIONS

These were obtained using a model B Coulter electronic particle counter at various times after the addition of glucose and ampicillin to the treated suspensions. Details of the methods used have been described previously (Rye & Wiseman, 1967a). Size distributions are represented graphically or characterized by the parameters mode, median and mean cell volumes and coefficient of variation. The coefficient of variation is the ratio of the calculated standard deviation of the size distribution to the mean cell volume and is a measure of the spread of cell sizes in a suspension (Koch, 1966).

Results

Preliminary experiments were performed to determine the concentrations of tetracycline, phenol, phenylmercuric acetate and CTAB required to partially inhibit the growth of cultures of *E. coli*. The addition of 2 μg/ml of ampicillin to such treated cultures and to untreated controls was found to prevent cellular division but to have no significant effect upon the rates of increase in absorbance. No evidence of cell lysis was observed in any of the experiments reported.

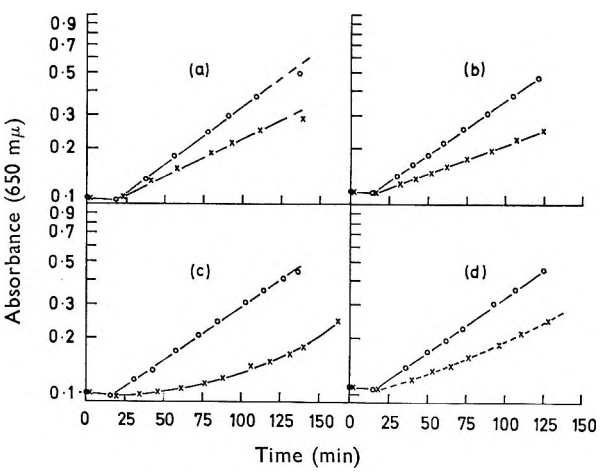

FIG. 1. Changes in the absorbance of suspensions of *E. coli* in glucose-free medium at 37° after the addition of glucose (1 mg/ml) and ampicillin (2 μg/ml) in the absence ○——○ and the presence ×——× of (a) 0·25 μg/ml tetracycline, (b) 1·0 mg/ml phenol, (c) 0·03, μg/ml phenylmercuric acetate and (d) 1 μg/ml CTAB. These antibacterial agents were added at time zero and the glucose and ampicillin after 15 min. The broken line in (d) is the theoretical change in absorbance calculated using equation (1) with $\alpha = 0{\cdot}4$ and $k^1 = k$.

Fig. 1 shows the changes in absorbance which occurred after the addition of glucose (1 mg/ml) and ampicillin (2 μg/ml) to resting suspensions of *E. coli* containing (*a*) 0·25 μg/ml tetracycline, (*b*) 1·0 mg/ml phenol, (*c*) 0·03 μg/ml phenylmercuric acetate and (*d*) 1 μg/ml CTAB, and to untreated control suspensions. Growth in the presence of phenol or tetracycline was exponential but occurred at a slower rate than in the control suspensions. With phenylmercuric acetate or CTAB-treated cells, the graph of log absorbance with time was convex to the time axis.

At various times, measurements were made of the distributions of cell sizes in the suspensions described in Fig. 1 and the results are given in Figs 2–4 and in Table 1.

TABLE 1. THE PARAMETERS OF THE SIZE DISTRIBUTIONS OF THE CONTROL AND PHENOL-TREATED CELLS OF FIG. 1(b), BEFORE AND 80 MIN AFTER THE ADDITION OF GLUCOSE AND AMPICILLIN

	Time (min) after the addition of glucose and ampicillin	Cell volume (μ^3)			Coefficient of variation
		Mean	Median	Mode	
Control cells ..	0	0·93	0·85	0·74	0·351
Control cells ..	80	2·31	2·10	1·80	0·391
Phenol-treated cells	80	1·41	1·32	1·15	0·358

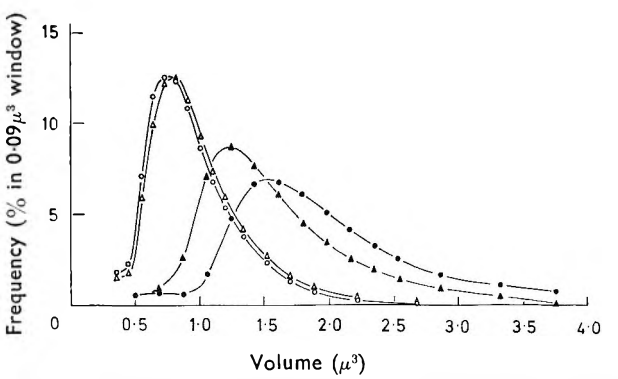

FIG. 2. The size distributions of the control ○——○ and tetracycline-treated cells △——△ of Fig. 1(a) before (open symbols) and 60 min after (full symbols) the addition of glucose and ampicillin.

Fig. 2 compares the cell size distributions of tetracycline-treated cells with those of the control suspension before and 60 min after the addition of glucose and ampicillin to the cells. During this period the mean cell size of the tetracycline-treated cells increased from 1·00 to 1·62 μ^3 and that of the control from 0·97 to 1·99 μ^3. No significant change in the coefficients of variation of the size distributions occurred, the values changing from 0·387 to 0·374 and 0·385 to 0·375 for the tetracycline-treated cells and controls respectively. In both cultures virtually no cells were left in the smallest size ranges after 60 min.

Similar results were obtained with phenol-treated cells and Table 1 compares the parameters of the size distributions obtained after 80 min

growth in the presence of 1·0 mg/ml phenol with those of the control suspension.

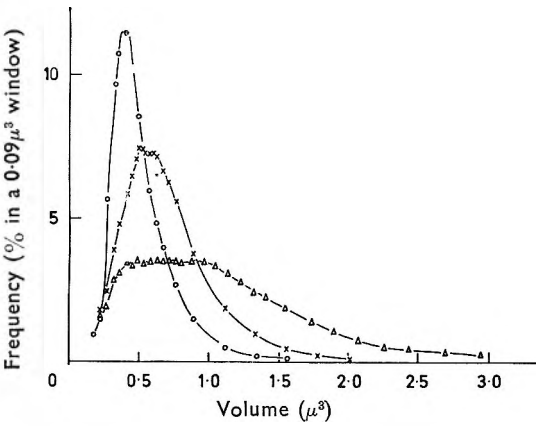

FIG. 3. The size distributions of the phenylmercuric acetate-treated cells of Fig. 1(c), before ○——○, 100 min ×——×, 220 min △——△ after the addition of glucose and ampicillin.

Fig. 3 shows the size distributions of phenylmercuric acetate-treated cells before and 100 and 220 min after the addition of glucose and ampicillin. Both the mean cell volume and the coefficients of variation of the distributions increased during growth, and even after 220 min some cells still remained in the smallest size ranges.

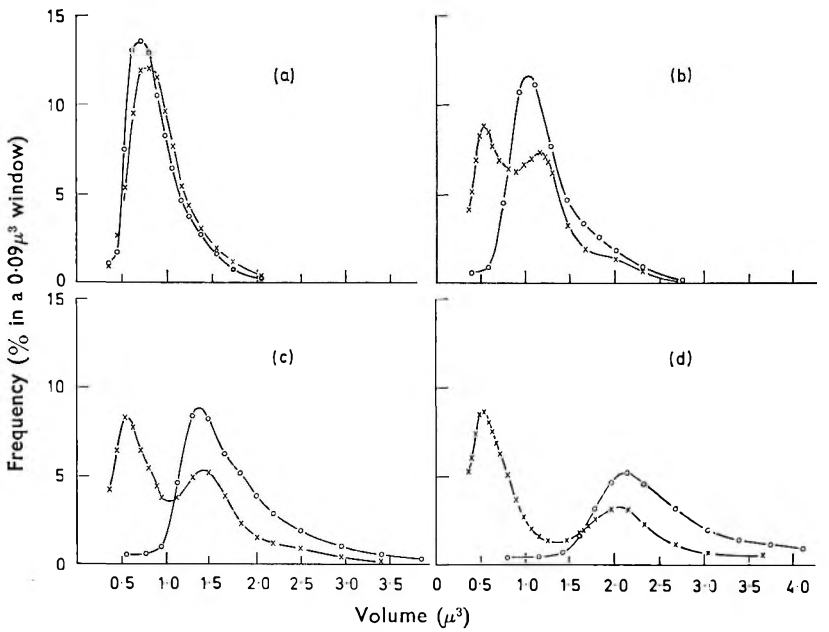

FIG. 4. The size distributions of the control ○——○ and CTAB-treated cells ×——× of Fig. 1(d), (a) before, (b) 34, (c) 60 and (d) 95 min after the addition of glucose and ampicillin.

Fig. 4 shows the size distributions of control and CTAB-treated suspensions (*a*) before, (*b*) 34, (*c*) 60 and (*d*) 95 min after the addition of glucose and ampicillin. The mean cell size of the control suspension increased throughout this period with little increase in the coefficient of variation. In the CTAB-treated suspension, the size distribution widened during growth and resolved itself into a bimodal distribution. From these distributions it was calculated that approximately 60% of the cells had remained at their original size whilst the remainder had increased in size at about the same rate as those in the control suspension.

Discussion

Kinetic studies of the effects of antibacterial agents on micro-organisms at concentrations below those required for the total inhibition of cellular growth can yield information on their mechanisms of action (Garrett, Miller & Brown, 1966). Studies using chloramphenicol and tetracycline indicated that the overall growth rate constant of cultures is reduced by low concentrations of these antibiotics (Ciak & Hahn, 1958). Garrett & Miller (1965) found that the total and viable cell counts coincided during the partially inhibited growth of *E. coli* in the presence of chloramphenicol or tetracycline and they suggested that this resulted from a general inhibition of growth of all of the cells rather than to some of the cells not growing. But with other antibacterial agents, the reduced growth rate of cultures may result from a non-uniform inhibition of the individual cells. This possibility was discussed by Treffers (1956), and Parkinson & Pickett (1964) showed that although the main effect of sub-bacteriostatic concentrations of phenol was to increase the generation time of *E. coli*, some loss in viability occurred in the growing cultures at the higher concentrations above 30°. Studies in this field have previously been complicated by the absence of a method for determining whether or not the individual cells in such partially inhibited cultures are inhibited to the same extent and we believe that this information is essential in order to understand fully the reasons for the overall decrease in growth rate.

The presence of low concentrations of ampicillin prevents the increase in numbers which normally follows the addition of glucose to suspensions of *E. coli* in glucose-free medium but has no effect upon the rate of increase in cell mass (Rye & Wiseman, 1967b). Similar results were obtained during this investigation when ampicillin was added to cultures partially inhibited by tetracycline, phenol, phenylmercuric acetate or CTAB. In these cultures where cellular division is prevented by ampicillin, those cells that are able to grow simply increase in size at rates dependent on their individual growth rates. After some growth has occurred, a comparison of the size distribution of these partially inhibited cells with that of a control suspension containing ampicillin alone, gives information both on the overall extent of growth and on the distribution of the individual cell growth rates in the presence of these antibacterial agents.

The coefficients of variation of the size distributions obtained when using phenol- or tetracycline-treated cells are no greater than in the control

suspensions. This indicates that the decreased overall growth of these cultures results from an extremely uniform inhibition of growth of all the individual cells. In the culture treated with phenylmercuric acetate however, the gradual widening of the size distribution and the continual but slow decrease in the number of cells in the lower size ranges suggests that all the cells are growing but at widely different rates. Phenylmercuric acetate thus appears to inhibit the individual cells in a culture to different extents either by causing a non-uniform decrease in their rates of growth or by imposing a lag period on the cells the duration of which varies from cell to cell. The bimodal distribution of cell sizes developing in the presence of CTAB shows the presence of two kinds of cell. The smaller cells which retain the size characteristic of glucose-starved cells are clearly not growing, whereas the larger cells appear to be growing at the same rate as the untreated control cells. The relative number of the two types of cell remained constant and it was estimated from the distributions that 60% of the cells were not growing. The partially inhibited growth of CTAB-treated cultures unlike that of tetracycline- or phenol-treated cultures thus results from a reduction in the actual number of cells growing and not from a general decrease in the growth rate of the cells.

The results which we have described in this paper suggest that the growth of bacterial cultures may be represented by the equation:

$$D = \alpha D_0 e^{k^1 t} + (1 - \alpha) D_0 \quad .. \quad .. \quad (1)$$

where D_0 represents the total bacterial population in terms of either mass or numbers and D the total population after time t, α is the fraction of the initial population that is actually growing, and k^1 is a growth rate constant for mass or numbers as appropriate. In an uninhibited, exponentially growing culture $\alpha = 1$ and k^1 becomes the first order growth rate constant k. The equation then simplifies to the generally accepted growth equation:

$$D = D_0 e^{kt} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

When growth is partially inhibited by the addition of an antibacterial agent, the overall decrease in growth rate may be due to one or both of two effects: (1) the complete inhibition of growth of some of the population i.e., a decrease in α, and (2) a decrease in the value of the growth rate constant k.

If the values of α or k^1 alter as the bacterial population increases, equation (1) will become inapplicable.

The chief factor governing the type of growth inhibition which occurs may be the extent of uptake of the antibacterial agent being used. Thus if only a small proportion of the antibacterial agent is adsorbed out of solution by the cells then the increase in cell population during growth will cause little or no change in its effective concentration and the inhibiting effects will remain constant with time. It is probable that in these circumstances the agent will be uniformly distributed amongst the cells and each cell will be inhibited to about the same extent. Where however most of the agent is taken out of solution by the cells it may be unevenly distributed amongst the cell population and the individual cells

inhibited to different extents. In addition, any growth which occurs will reduce the effective concentration of agent per unit of cell population and if the agent is not firmly bound then a decrease in the number of cells inhibited or an increase in their growth rate constants may occur.

Under the conditions of our experiments tetracycline and phenol act solely by decreasing the value of the growth rate constant, their effects are relatively uniform on all the cells and are in no way reduced by the increase in cell population during the first 2 hr of growth. These observations suggest that the proportion of these agents taken up is small at the concentrations used. The data presented by Judis (1964) and Bean & Das (1966) for the uptake of phenol confirm this conclusion. CTAB however decreases the proportion of the cell population able to grow and, as the number of inhibited cells did not decrease during the time span of our experiments, its uptake and inhibitory effects appear to be irreversible. The results obtained by McQuillen (1950) and Salton (1951) for the uptake of CTAB by bacteria indicate that at the low concentrations used in our experiments most of the agent would be taken up by the cells. McQuillen (1950) also showed that in dilute solutions of this agent the electrophoretic mobilities of the individual cells were affected to different extents. Phenylmercuric acetate affects all the cells in a culture but to different extents and as α and k^1 both appear to alter during growth its effects must be reversible.

Acknowledgement. We are grateful to Mrs. Sheena Kaye for technical assistance.

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The determination of bupivacaine, lignocaine and mepivacaine in human blood

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A specific method for the quantitative analysis of bupivacaine, lignocaine and mepivacaine in blood, in clinically occurring concentrations, using gas-liquid chromatography, is described. The extraction procedure yields a recovery of 97·5 (±5)%, and the chromatographic conditions allow concentrations as low as 0·04 μg/ml of local anaesthetic in 2 ml of whole blood to be measured. The method has a standard deviation of 5·4%. No interference is encountered from commonly used premedicant or general anaesthetic drugs.

THE local anaesthetics bupivacaine, lignocaine or mepivacaine may be administered in single or repeated doses to achieve local anaesthesia for surgery, or for pain relief in labour. Lignocaine is also used intravenously, in intermittent doses or by infusion, in the treatment of cardiac arrythmias. The present method was devised to allow blood concentrations of these anaesthetics to be measured after separate or simultaneous administration. The procedures developed are based in part on those described by Boyes (1967) for lignocaine and on those of Pratt, Warrington & Grego (1967) for mepivacaine.

Experimental

MATERIALS AND APPARATUS

5N sodium hydroxide. 0·1N hydrochloric acid. Analar diethyl ether, freshly distilled. Internal marker: methadone hydrochloride, 0·5 μg/ml in distilled water. Centrifuge tubes with well-fitting stoppers. 15 ml stoppered evaporating tubes with finely tapered bases (Beckett, 1966) Mechanical shaker: see-saw type mixer, 30–40 rocks/min. 10 μl Hamilton syringe.

CHROMATOGRAPHY

A Perkin Elmer F 11 gas chromatograph with a flame-ionization detector. Column: 2 metre $\frac{1}{4}$ inch o.d. glass. Solid support: Chromasorb G. 80–100 mesh. Liquid phase: $2\frac{1}{2}$% silicone gum rubber, S.E. 30 (or E 301). Oven temperature: 210°. Nitrogen (carrier gas) flow rate: 70 ml/min (15 lb/inch^2). Hydrogen pressure: 20 lb/inch^2. Air pressure: 25 lb/inch^2. Packed column held under operating conditions for 24 hr, and silanized *in situ* with 2 × 5 μl hexamethyl disilazane, before use.

PROCEDURE

To 1 ml of internal marker solution in a centrifuge tube, add 2 ml of blood, 2 ml of water, 0·5 ml of 5N NaOH and 2·5 ml of ether. Stopper

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the tube, invert briskly twice, relieve the pressure, stopper and shake mechanically for 5 min. Transfer the supernatant ether layer only to a second stoppered centrifuge tube, and make three further ether extractions. Combine the four ether extracts, shake for 5 min with 5 ml 0·1N hydrochloric acid and discard the ether phase. Add 0·5 ml 5N NaOH and extract with 3 × 2·5 ml ether. Combine the ether extracts in an evaporating tube, add a glass bead and concentrate the extract in a water bath at 42°. Remove the tube from the bath and insert the stopper just as the ether vapour ceases to moisten the ground glass neck. Place the tube in crushed ice, thereby washing the inner walls with condensed ether; a volume of about 50 μl results. Evaporate further to about 20 μl by placing the tube with its base in the bath, then remove, stopper, and replace it in crushed ice (sensitivity may be increased by repeating the evaporation/condensation cycle). Mix the liquid by syringe, finally inject about 2 μl into the column of the chromatograph.

CALCULATION

Construct a calibration curve from the results obtained by adding local anaesthetics to blank anticoagulated blood to achieve concentrations found clinically (0·0625–1·0 μg/ml of each for mixtures of the anaesthetics or bupivacaine alone; 0·1–5·0 μg/ml for mepivacaine or lignocaine alone); add internal marker and proceed as described. Calculate the ratio of peak heights (PHR) of local anaesthetic to internal marker and construct a calibration curve of PHR against concentration of local anaesthetic.

The concentrations of local anaesthetics in an unknown sample are obtained by calculation of PHR and relating it to the calibration curve.

DETERMINATION OF THE PERCENTAGE RECOVERY OF THE LOCAL ANAESTHETICS AND METHADONE

The following solutions were used: (*a*) 1 μg/ml of each of the local anaesthetic bases in ether; (*b*) 2 μg/ml of methadone base in ether; (*c*) local anaesthetics added to blood equivalent to 1 μg/ml free base of each; (*d*) methadone in water equivalent to 2 μg/ml free base.

I. 2 ml of (*a*) were mixed with 1 ml of (*b*) in each of four tubes, and the solutions concentrated and subjected to gas-liquid chromatography as above.

II. 4 tubes, each containing 2 ml of (*c*) were treated as described in the general extraction procedure, but with the addition of 1 ml of (*b*) to the final ether extract before concentration.

III. 4 tubes, each containing 2 ml of (*c*) and 1 ml of (*d*) were treated as in the general extraction procedure.

Percentage recoveries of local anaesthetics were calculated by comparison of PHRs obtained in I with those in II, and that of methadone by comparison of PHRs in II and III.

SAMPLING PROCEDURE

Arterial blood samples were obtained, using a Bradley arterial catheter (Portland Plastics) and a three-way tap, flushed intermittently with heparinized saline. Venous blood samples were obtained via a Bardic angiocath, size 16, and a three-way tap, kept open with a slow infusion of physiological saline containing heparin 0·01 mg/ml. Blood samples were heparinized (about 0·2 mg/ml blood) and refrigerated pending sampling.

Results and discussion

Of the liquid phases investigated, 2½% S.E. 30 (or E 301) gave the sharpest and most readily reproducible peaks. The retention times (min) under these conditions were: lignocaine, 2·2; mepivacaine, 4·0; methadone, 5·0; bupivacaine 7·6.

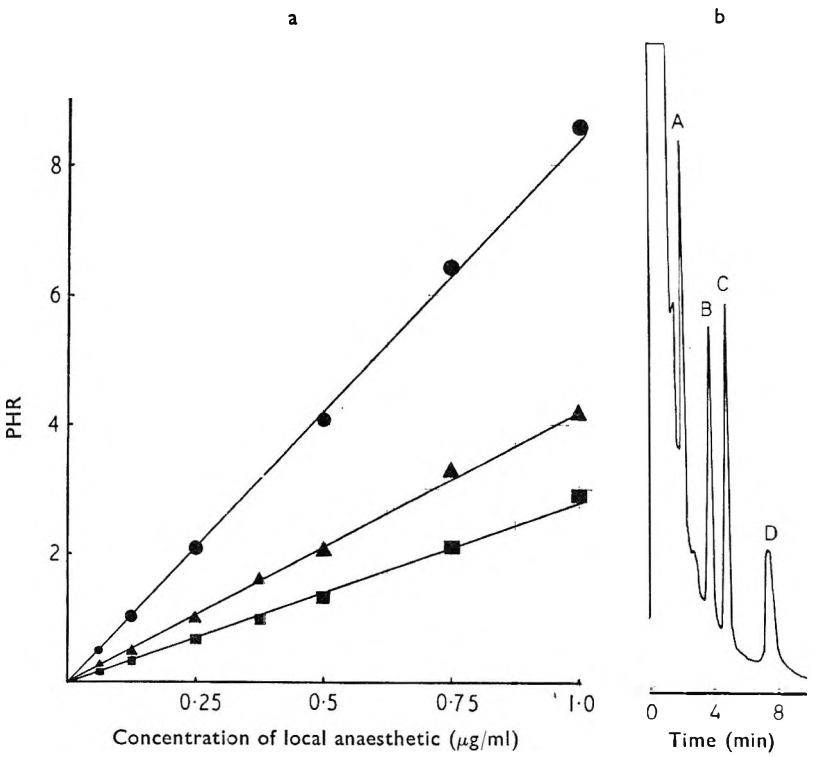

FIG. 1a. Calibration curves of bupivacaine (■), lignocaine (●) and mepivacaine (▲). PHR = Ratio, of peak height of local anaesthetic: peak height of internal marker.

b. Gas-liquid chromatogram from blood, found to contain lignocaine 0·10 μg/ml (A), mepivacaine 0·21 μg/ml (B), and bupivacaine 0·12 μg/ml (D). Methadone marker 0·5 μg (C). This figure shows lignocaine at the same sensitivity as the other drugs. The lignocaine peak height (wt for wt the greatest) is more accurately measured if the recording is made at a lower sensitivity, when the base line is flatter.

The temperature of 210° was necessary to produce an adequately sharp peak of bupivacaine at low concentrations. With a lower temperature, the peak became too blunt and wide for accurate measurement.

A linear calibration curve was obtained for each drug in whole blood (Fig. 1a). The standard deviation of eight samples containing 0·1 μg/ml of bupivacaine base (for which the analysis is least sensitive) was found to be $\pm 5{\cdot}4\%$. As little as 0·04 μg/ml of any of the drugs could be measured with reasonable accuracy, provided duplicate analyses were made. Consistent results were obtained with many different blood samples. Fig. 1b is a chromatogram obtained from the blood of a subject who had had 0·56 mg/kg of each local anaesthetic, intravenously, $1\frac{3}{4}$ hr before the blood sample was taken.

TABLE 1. PERCENTAGE RECOVERIES OF MIXED LOCAL ANAESTHETICS AND OF METHADONE, FROM ONE SAMPLE OF WHOLE BLOOD

	Bupivacaine		Lignocaine		Mepivacaine		Methadone
	PHR	Recovery %	PHR	Recovery %	PHR	Recovery %	Recovery %
I. Ethereal standard (mean of 4)	0·543	Represents (100%)	1·582	Represents (100%)	0·722	Represents (100%)	
II. Whole blood	0·55	101·3	1·70	107·5	0·76	105	97
	0·51	94	1·57	99	0·70	97	95
	0·483	89	1·48	93·5	0·71	98	101
	0·483	89	1·485	94	0·70	97	95
Average recovery % ± s.d.	93·26 ± 5·4		98·5 ± 6·5		99·25 ± 3·8		97 ± 2·8

The recoveries for the extraction procedure are in Table 1. The mean percentage recovery (97·5%) is much greater than that reported by Pratt & others (1967) for mepivacaine in blood (56%).

The drugs were stable in acid-citrate dextrose blood at 4° for up to one month. But, blood samples placed in heparin tubes sometimes coagulated within one week. More rapid coagulation took place if the sampling cannula was kept open by 5% dextrose instead of by physiological saline.

POSSIBLE SOURCES OF ERROR

(i) *Incomplete recovery.* Four ether extractions were found necessary. Errors were minimized by the addition of internal marker before the extraction was made.

(ii) *Contamination from the evaporating tubes.* Errors due to contamination were overcome by scrupulous washing with detergent, rinsing, chromic acid 20% and re-rinsing.

(iii) *Chromatographic interference.*

(*a*) If, during the pipetting off of the supernatant ether in the initial extractions, any of the blood phase was included, an irregular baseline was obtained.

(*b*) After injecting an extract from blood a very wide peak emerged, with a retention time of 80 min. It was therefore necessary to limit the number of samples injected in sequence to eight, with 9–10 min/sample, and then to wait for at least 80 min after the last injection to allow for eight wide peaks to emerge.

(*c*) When constructing a calibration curve from data obtained using stored acid-citrate-dextrose blood, a constant interference peak emerged, with a retention time of $14\frac{1}{2}$ min. Serial injections could be timed so that this peak emerged between that of methadone and that of bupivacaine in the subsequent sample. Fresh blood did not produce this peak.

(iv) *Interference from drugs.* When stored blood is used in the preparation of a calibration curve, the local anaesthetic being measured must not have been given to the donor at the time of taking the blood. (Procaine may be used for local infiltration where necessary without interference.) No commonly encountered premedicant or other drug was found to interfere with the assay. The only other drug that emerged on the chromatogram was pethidine, but this has a retention time of 1·6 min, and therefore did not interfere.

Acknowledgements. We thank Dr. R. N. Boyes for his advice in the development of the method, Professor R. S. Stacey for his helpful criticism and support, and Messrs. Duncan, Flockhart & Evans for supplies of bupivacaine.

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The solubilities of the lower testosterone esters

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The solubilities of the formate to valerate esters of testosterone have been determined in water and several organic solvents. The aqueous solubilities decrease logarithmically as the homologous series is ascended, but the acetate is less soluble than anticipated in the organic solvents. The variation in solubility from ester to ester can be predicted in the organic solvents from thermodynamic data, and is a reflection of the differences in melting point. The melting point differences are explained from the space group dimensions and the area of α to α face contact in the crystals.

IT is well established that the intensity and duration of biological action of testosterone are enhanced by esterification, and vary from ester to ester (Miescher, Wettstein & Tschopp, 1936; Parkes, 1936). The postulate that this is due to slow release of testosterone by hydrolysis has been tested by Pesez & Bartos (1962) and Schenck & Junkmann (1955) with limited success, but no attempt has been made to relate the changes in biological activity to solubility. The solubilities of the lower testosterone esters from formate to valerate have therefore been determined as a preliminary to such an investigation.

Experimental and results

Materials. The testosterone esters were from British Drug Houses Ltd. Organic solvents were fractionally distilled and their purity checked by refractive index.

Determination of solubility in water. An excess of ester was stirred with water at 25° until a saturated solution was obtained. About 500 ml was filtered off, weighed and continuously extracted with a small volume of hexane, which was subsequently adjusted to 50 or 100 ml. The concentration of the saturated aqueous solution was then calculated from the extinction of the hexane extract measured at 229·5 mμ. This procedure was necessary because the aqueous solutions were too dilute to yield reliable spectrophotometer readings. Results are given in Table 1. The presence of colloidal dispersed material was eliminated by subjecting saturated solutions to high speed centrifugation. The extinctions, measured at 244 mμ in a 40 mm cell, did not change after 4 hr at 100,000 *g*.

Determination of solubilities in organic solvents. Saturated solutions were prepared at 25° by percolating about 0·25 ml of solvent through a column of about 500 mg of solute, supported on cotton wool. The eluate was returned to the top of the column until a saturated solution was obtained. An aliquot of the solution was assayed by weighing the residue after evaporation at 80°; vacuum was necessary to remove nitrobenzene. Results are given in Table 1.

Calorimetric determinations. Heat capacities and heats of fusion were measured on a Perkin Elmer D.S.C.1 differential scanning calorimeter.

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TABLE 1. SOLUBILITIES OF THE TESTOSTERONE ESTERS IN VARIOUS SOLVENTS (25°)

Ester	Mole fraction solubility						
	Water	Ethanol	Toluene	Benzene	Chloro-form	1,2-Di-chloro-ethane	Nitro-benzene
Formate	$2{\cdot}50 \times 10^{-7}$	$2{\cdot}31 \times 10^{-2}$	0·211	0·283	0·381	0·315	0·245
Acetate	$1{\cdot}28 \times 10^{-7}$	$1{\cdot}70 \times 10^{-2}$	0·133	0·165	0·290	0·227	0·147
Propionate ..	$7{\cdot}74 \times 10^{-8}$	$2{\cdot}79 \times 10^{-2}$	0·199	0·260	0·354	0·294	0·220
Butyrate	$2{\cdot}53 \times 10^{-8}$	$1{\cdot}60 \times 10^{-2}$	0·197	0·237	0·339	0·250	0·153
Valerate	$1{\cdot}40 \times 10^{-8}$	$6{\cdot}85 \times 10^{-3}$	0·124	0·169	0·278	0·188	0·124

TABLE 2. THERMAL DATA FOR TESTOSTERONE ESTERS

Ester	m.p. °C	ΔH_M^F kcal mole^{-1}	$\Delta Cp(T_M - T)$ kcal mole^{-1}	$\Delta H_{25°}^F$ kcal mole^{-1}	Ideal mole fraction solubility (25°)	
					Integrated method	By mean heat of fusion
Formate ..	125	4·33	3·31	1·02	0·182	0·322
Acetate ..	140	5·38	4·06	1·32	0·087	0·204
Propionate ..	120	5·29	3·85	1·44	0·149	0·254
Butyrate ..	109	6·05	3·03	3·02	0·172	0·192
Valerate ..	107	7·40	2·25	5·15	0·127	0·102

The technique has been described elsewhere (Watson, O'Neill & others, 1964). Calibration curves were constructed for heat of fusion with metallic tin and for heat capacity with sapphire. The instrument is claimed by its manufacturer to yield heat capacity results within $\pm 2\%$ of those obtained by conventional means. Heat capacities were measured to about 50° above the melting point. Results are shown in Table 2.

Determination of densities of saturated solutions. A drop of saturated solution in chloroform was added to a concentrated solution of cadmium chloride in water, saturated with chloroform and the relevant testosterone ester. The concentration of the aqueous solution was adjusted until the drop neither rose nor fell, and the density of the aqueous phase then determined by hydrometer and density bottle.

Discussion

The solubilities of members of an homologous series normally decrease logarithmically with the addition of each successive methylene group (Butler & Ranchandani, 1935). The testosterone esters examined here behaved in this way in water, but in all the other solvents, minimal solubility was found with the acetate. The validity of this observation was first tested by calculating ideal solubilities from thermodynamic data. Ideal solubility assumes uniform intermolecular attraction involving no energy change when the components are brought together, except that necessary to liquify the solute, and can be calculated from,

$$\frac{d}{dT} \ln a_2 = \frac{\Delta H^F}{RT^2} \qquad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where a is the ideal solubility, and the subscript 2 represents solute. If ΔH^F, the molar heat of fusion, is considered constant, integration gives,

$$-\ln.a_2 = \frac{\Delta H^F}{R}\left[\frac{T_M - T}{T_M T}\right] \quad .. \quad .. \quad .. \quad (2)$$

where T_M is melting point. ΔH^F, the mean molar heat of fusion over the range T to T_M, is given by the equations,

$$\Delta H^F = \tfrac{1}{2}(\Delta H^F_T + \Delta H^F_M) \quad .. \quad .. \quad .. \quad (3)$$

where ΔH^F_T and ΔH^F_M are molar heats of fusion at the temperature of interest and the melting point respectively, and,

$$\Delta H^F_T = \Delta H^F_M - \Delta C_p\,(T_M - T) \quad .. \quad .. \quad (4)$$

ΔC_p is the difference between the heat capacities of the solid and supercooled liquid. ΔC_p is not necessarily independent of temperature, and the calorimeter results indicated that ΔC_p varied with temperature. The correction equivalent to $\Delta C_p\,(T_M - T)$ was therefore obtained by extrapolating the liquid enthalpy line from above the melting point at 25° and measuring the area between this line and that for the solid. This is illustrated in Fig. 1.

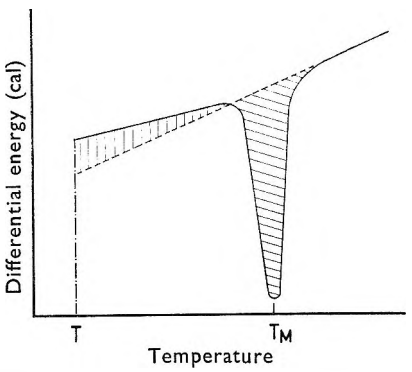

FIG. 1. Determination of heat capacity and heat of fusion. Vertical hatching, $\Delta Cp\Delta T$. Diagonal shading, ΔH^F_M.

Solubilities were also calculated using the integrated value of ΔH^F between T_M and T. Heat capacity can be expressed as,

$$\Delta C_p = a + b\,(T_M - T) \quad .. \quad .. \quad .. \quad (5)$$

where a and b are constants, evaluated by plotting ΔC_p against $(T_M - T)$.

Since,

$$\frac{d}{dT}\Delta H^F = \Delta C_p \quad .. \quad .. \quad .. \quad .. \quad (6)$$

substitution for ΔC_p from equation (5) and integration between T_M and T gives,

$$\Delta H^F = \Delta H^F_M - a\,(T_M - T) - b/2\,(T_M - T)^2 \quad .. \quad (7)$$

which, when substituted in equation (1), yields, on integration between the same limits,

$$\ln.a_2 = \frac{1}{R}\left[\frac{(-\Delta H^F_M + aT_M + b/2\,T_M^2)\,(T_M - T)}{T_M T} - (a + bT_M)\ln.\frac{T_M}{T} + b/2\,(T_M - T)\right] \quad .. \quad .. \quad .. \quad (8)$$

Heats of fusion and calculated solubilities are shown in Table 2. Both sets of results confirm that the solubility should pass through a minimum at the acetate, and indicate that it is a reflection of the high melting point of this ester.

At the melting point vibrational energy exceeds the intermolecular attraction, which in testosterone esters results from London forces. These are significant only with those groups which are close to neighbouring molecules, since the forces decrease in proportion to the sixth power of distance. Intermolecular forces can be estimated from the space group dimensions, reproduced in Table 3 from Griffiths, James &

TABLE 3. CRYSTALOGRAPHIC DATA FOR TESTOSTERONE ESTERS

Ester	Angstrom units			Area of α face adjacent to those of neighbouring molecules (Å²)	Area / m.p.
	a	b	c		
Acetate	12·6	18·1	7·8	77	0·55
Propionate ..	12·6	20·3	7·6	63	0·53
Butyrate	12·3	16·3	10·3	88	0·81
Valerate	12·3	16·7	10·3	89	0·83

Rees (1965). Courtauld models were fitted into scaled up space groups of these dimensions, and indicated that the alkyl chains of the acetate and propionate continue along the b axis, but those of the butyrate and valerate are folded back over the molecule. This produced a shortening of the b axis and an increase in the c axis due to the alkyl chain acting as a wedge. The inference from this is that the acetate should have a similar melting point to the propionate, and the butyrate a similar melting point to the valerate, those of the former pair being higher than those of the latter pair.

The difference between the melting points of acetate and propionate is probably due to α to α face attraction. The testosterone molecule is essentially planar. One side of the plane, the β face, has two angular methyl groups projecting from it, while the other side, the α face, is substituted entirely with hydrogen atoms. The α face therefore presents a large comparatively flat surface, and the area of α to α face contact is probably the largest contributing factor towards the melting points. The β face would be of lesser importance because the projecting methyl groups allow fewer points of contact. This theory is supported by the fact that the ratio of the area per molecule of α face adjacent to the α face of a neighbouring molecule, measured from the models, to the melting point, is the same for acetate and propionate. A similar relationship obtains with butyrate and valerate. These are shown in Table 3. The formate could not be considered in this way because it belongs to a different space group.

Restaino & Martin (1964) have shown that benzoate esters form regular solutions (Hildebrand & Scott, 1962) in low polarity solvents, and therefore testosterone esters were expected to behave in the same

way. Ideal solubility is the product of the regular solubility and the activity coefficient (γ), which can be calculated from the equation,

$$\ln\gamma_2 = \frac{\phi_1^2 V_2 (\delta_1 - \delta_2)^2}{RT} \quad .. \quad .. \quad .. \quad (9)$$

where ϕ is the volume fraction, and V the molar volume. The subscript 1 denotes solvent and 2 solute. δ is the solubility parameter and is a measure of escaping tendency. When $\delta_1 = \delta_2$ solute and solvent have the highest affinity for each other, the activity coefficient becomes unity, and the solution is said to be ideal.

The solubility parameter of a solute can be determined by plotting solubilities in a series of solvents against solvent solubility parameter (Chertkoff & Martin, 1960). Equation (9) predicts that solubility is maximal when $\delta_1 = \delta_2$ so that the peak of the graph coincides with the solubility parameter of the solute. The method has the disadvantages that the maximum is not sharp and the solute solubility parameter can not be fixed accurately. More precise results were obtained here, by plotting the logarithms of the solubilities against the solubility parameters of the solvents. Two intersecting straight lines were obtained near the solubility maximum, so that the solubility parameter could be determined to within 0·1 $cal^{\frac{1}{2}}$ $cm^{-3/2}$. A typical plot is shown in Fig. 2.

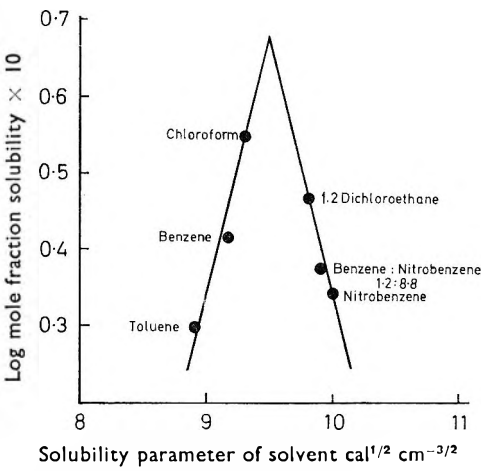

FIG. 2. Determination of solubility parameters.

All 5 esters, and testosterone, gave a solubility parameter of 9·5 $cal^{\frac{1}{2}}$ $cm^{-3/2}$. A difference between the solubility parameters of testosterone and its esters would be anticipated if the polarities of ester and hydroxy groups had a significant effect on intermolecular attraction. Hildebrand & Scott (1962) consider that regular solutions are formed by esters, only "when the dipole is reasonably well buried within the molecule". It appears that the bulky steroid nucleus in the testosterone esters has caused such an effect.

Solutions in chloroform, because its solubility parameter is close to 9·5 $cal^{\frac{1}{2}}\ cm^{-3/2}$ can, as an approximation, be assumed to be ideal. Evaluation of the activity coefficient gave a value close to unity, confirming that this was a reasonable simplification. V_2 was calculated from,

$$\frac{x_1M_1 + x_2M_2}{\text{Density of saturated solution}} = x_1V_1 + x_2V_2 \quad .. \quad (10)$$

where M is molecular weight, and ϕ_1 from,

$$\phi_1 = \frac{x_1V_1}{x_1V_1 + x_2V_2} \quad .. \quad .. \quad .. \quad .. \quad (11)$$

Thus for testosterone acetate, which was found to give a saturated solution of density 1·28, calculation yielded a molar volume of 286 and an activity coefficient of 1·003. The other esters gave similar results. The observed solubilities in chloroform, shown in Table 1, should therefore agree with the ideal solubilities, calculated from thermodynamic data, and shown in Table 2. The poor correlation could be due to the long extrapolation in obtaining $\Delta C_p\ \Delta T$, or the large difference in molar volumes of solute and solvent which does not permit the random distribution assumed in regular solution theory. These possibilities are being investigated.

The fact that the aqueous solubilities did not follow the same pattern as in the organic solvents can be attributed to deviation from regular solution behaviour.

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Peptic inhibition by macroanions

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Heparin, dextran sulphate, dextran phosphate, chondroitin sulphate and degraded λ-carrageenan inhibit peptic activity by substrate occlusion or depletion. This holds for various substrates and enzyme preparations, but the amount of inhibition observed varies with method and inhibitor used. The most active inhibitors of the series are disulphated on at least alternate sugar residues and in addition to disulphation high molecular weight confers, in certain conditions, greatest activity. High and low molecular weight macroanions have different inhibition characteristics and it is concluded that activity may depend upon the structure of the substrate-inhibitor complexes formed. Inhibition is not observed when substrate-inhibitor interaction does not occur, as when pepsin or *N*-acetyl-L-phenylalanine-L-diiodotyrosine (APDT) are used as substrate.

INHIBITION of the proteolytic activity of acid gastric secretion by high and low molecular weight carrageenans is caused by occlusion or depletion of available substrate from the digestion system as a result of substrate-inhibitor interaction (Anderson, 1961; Anderson & Baillie, 1967). The effect of pH, order of addition of reagents, ratios and nature of interacting species, on the inhibition suggested that, to elucidate conditions existing *in vivo* with therapeutic or physiological sulphated polysaccharides, systems containing different substrates and macroanions should be investigated.

We used alternative substrates, various macroanions (heparin, dextran sulphate, dextran phosphate, chondroitin sulphate, degraded λ-carrageenan), and purified separated human gastric enzymes.

Experimental

MATERIALS AND METHODS

Heparin was highly purified sodium heparin (150·1 units/mg, batch H35460). *Chondroitin sulphate* was prepared from bovine trachea and contained S, 5·26; N, 3·6; sulphated ash, 24·0; and Na, 7·4% (batch 9). *Dextran sulphate* was PDS 242 11/256 (Glaxo). It contained S, 19·5%; specific rotation +90·7; average molecular weight was given as 6,000–8,000. *Dextran phosphate* was H 15 F1 (Glaxo). It contained P, 9·8; Na, 11%, 85% occurred as the monoester, average molecular weight was given as 9,000. *Carrageenans.* Undegraded λ-carrageenan (CY-λ) contained SO_3Na (37·3%), and was prepared from *Chondrus crispus*. Degraded carrageenans were: (a) degraded λ-carrageenan (GP–λ–D2), containing SO_3Na, 37·7% and prepared from the λ-carrageenan of *Gigartina pistillata* by hypochlorite degradation (Black, Blakemore & others, 1965); and (b) degraded *Eucheuma* carrageenan containing SO_3Na, 36·1%, and prepared from the κ-like carrageenan of *E. spinosum* by mild mineral acid degradation. Degraded λ-carrageenan and degraded *Eucheuma* carrageenan both have similar molecular weights (about 25,000, determined by light scattering). Undegraded and degraded λ-carrageenans and degraded *Eucheuma* carrageenans have been described

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by Black & others, 1965; Anderson & Harthill, 1967; Anderson & Baillie, 1967). *Pepsin.* Twice crystallized pig pepsin (Light-Koch) was used in the autodigestion experiments. *Haemoglobin* was haemoglobin substrate powder for proteolytic enzyme assay (Armour Laboratories Ltd.). *Synthetic pepsin substrate. N*-Acetyl-L-phenylalanine-L-diiodotyrosine (APDT) was obtained from Yeda Research and Development Co. Ltd., Rehovoth, Israel. *Plasma protein substrate* was dried human plasma protein. *Human gastric proteolytic enzymes.* The proteolytic activities of human gastric secretion having pH optima at pH 2·2 and 3·2 and referred to as human pepsin and gastricsin respectively, were separated from bulked gastric secretions obtained during augmented histamine tests according to Tang, Wolf & others (1959).

PROETOLYTIC ACTIVITY AND INHIBITION

The inhibition of peptic activity by heparin, chondroitin sulphate, dextran phosphate and sulphate and degraded λ-carrageenan was determined at pH 2·2 as described by Anderson & Baillie (1967) in which the macroanion is added tc the enzyme before digestion (method *a*), or to the substrate before addition of the enzyme (method *b*).

Concentrations of plasma protein substrate (S) and inhibitor (I) in the 6 ml digest were varied as follows: with (I) constant at 10 mg, (S) ranged from 50–250 mg; with (S) constant at 250 mg, (I) ranged from 5–20 mg for all inhibitors except chondroitin sulphate, for which the range was 10–40 mg.

The effects of undegraded λ-carrageenan and degraded *Eucheuma* carrageenan (examples of high and low molecular weight macroanions respectively) on human pepsin and gastricsin were studied as follows. Proteolytic activity was determined in 0·1M citrate–HCl buffers at pH 2·2 and 3·2 at 37°. To 0·8 ml enzyme solution (1·5 mg pepsin/100 ml; 1 mg gastricsin/100 ml), 2·2 ml of the appropriate carrageenan solution was added. The reaction was started by the addition of 1 ml haemoglobin solution in buffer at appropriate pH and the mixture was incubated 50 min. Trichloroacetic acid (2 ml; 10% w/v) was then added, the extinction of the filtrate read at 280 mμ and converted to equivalent mg/ml tyrosine from a standard reference curve. Appropriate blanks were included.

Variations of (S) and (I) in the 4 ml digest were as follows: (a) with (I) constant at 4 mg, (S) ranged from 10–35 mg; (b) with (S) constant at 25 mg (I) ranged from 0–6 mg (undegraded carrageenan) and 0–10 mg (degraded carrageenan).

There was no substrate inhibition at the substrate concentrations used and the uninhibited reaction progressed linearly with time during the digestion period.

Inhibition, $i = 1 - V_I/V$, where V_I and V represent digestion with and without inhibitor respectively.

The effect of undegraded λ- and degraded *Eucheuma* carrageenans on the peptic hydrolysis of APDT was studied using the methods of Chiang,

Sanchez-Chiang & others (1966) except that 30% isopropanol was substituted for 50% isopropanol. The carrageenan was dissolved in the diluted gastric juice before admixture with substrate.

Carrageenan and autodigestion of pepsin. 100 mg pig pepsin was dissolved in 6 ml of HCl (pH 2·2) with or without carrageenan (up to 100 mg degraded *Eucheuma*; up to 24 mg undegraded λ-carrageenan). Aliquots of 0·5 ml were removed at intervals for 24 hr and mixed with 5 ml trichloroacetic acid (10% w/v) the mixture being left in the water bath for 15 min. Filtration (Whatman No. 1) was followed by measurement of the extinction of the filtrate at 280 mμ. Appropriate blanks were included and the adjusted increase in extinction was used as a measure of autodigestion.

Results

The shapes of the double reciprocal plots (Figs 1A, 1B), plots (Figs 2A, 2B) of $1/V_1$ against I (Dixon 1953) and plots (Figs 3A, 3B) of I against $i/(1 - i)$ (Reiner, 1959) indicate a substrate-depletion mechanism of inhibition in all cases. In the absence of inhibitor, double reciprocal plots (compare Figs 1A, 1B) were linear over the substrate range studied. Figs 4A, 4B reveal the quantitative differences in activity between the macroanions under the conditions of study.

Effect on APDT hydrolysis. Undegraded λ-carrageenan, at up to 3 mg/ml and degraded *Eucheuma* carrageenan at up to 20 mg/ml failed to inhibit the peptic digestion of APDT.

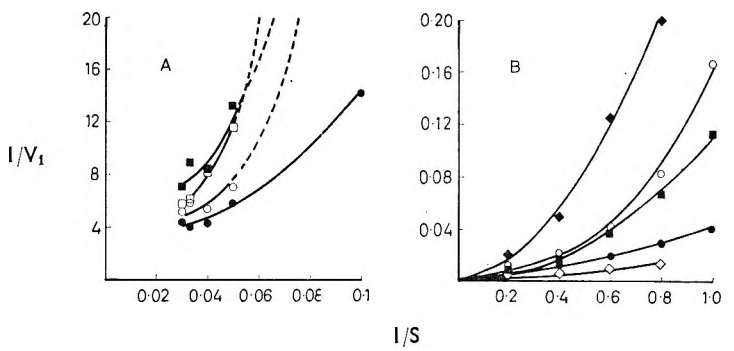

FIG. 1A. Plot of $1/V_1$ against 1/S. (I) constant at 4 mg/4 ml. Broken lines extend to $1/V_1$ values for 1/S = 0·1 (undegraded carrageenan at pH 2·2, $1/V_1 = 100$; degraded carrageenan at pH 2·2, $1/V_1 = 125$; degraded carrageenan at pH 3·2, $1/V_1 = 40$).

Key to FIGS 1A–4A (purified enzyme/haemoglobin system), ○ = undegraded carrageenan at pH 2·2 (pepsin); ● = undegraded carrageenan at pH 3·2 (gastricsin); □ = degraded carrageenan at pH 2·2 (pepsin); ■ = degraded carrageenan at pH 3·2 (gastricsin).

FIG. 1B. Plot of $1/V_1$ against 1/S. (I) constant at 10 mg/6 ml.

Key to FIGS 1B–4B (gastric juice/plasma protein system), ◆ = dextran sulphate; ○ = heparin; ■ = degraded λ-carrageenan; ◇ = dextran phosphate; ● = chondroitin sulphate.

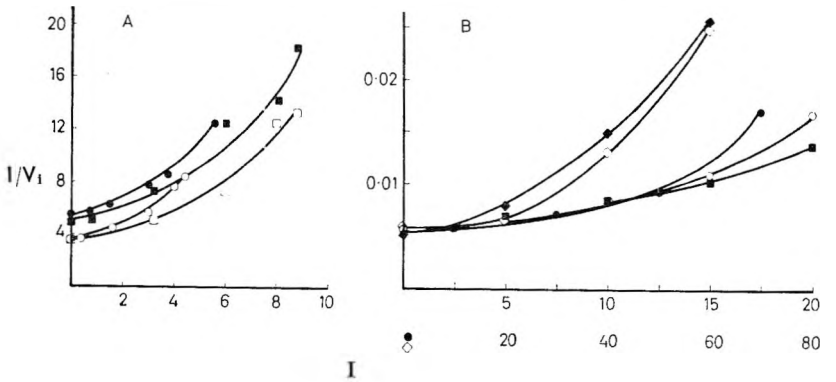

FIG. 2A. Plot of $1/V_1$ against I (mg/4 ml digest). (S) constant at 25 mg/4 ml.
FIG. 2B. Plot of $1/V_1$ against I (mg/6 ml digest). (S) constant at 250 mg/6 ml.

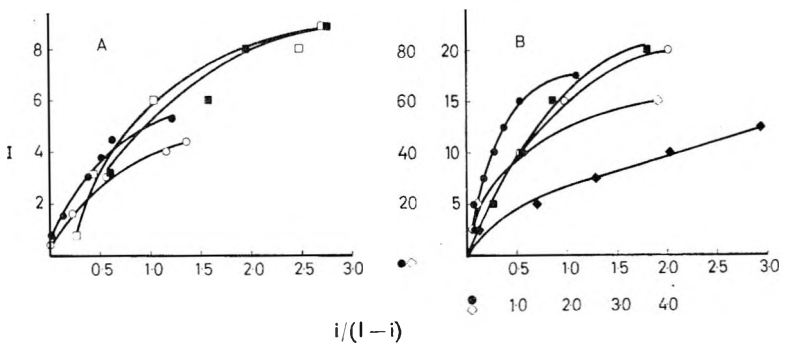

FIG. 3A. Plot of I (mg/4 ml digest) against $i/(1-i)$.
FIG. 3B. Plot of I (mg/6 ml digest) against $i/(1-i)$.

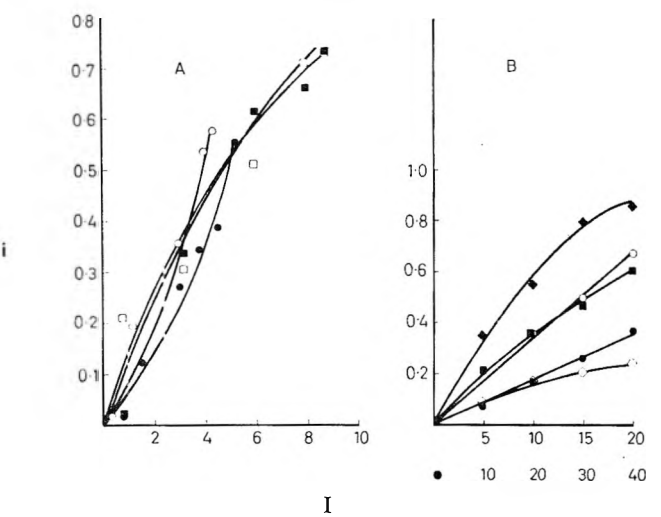

FIG. 4A. Plot of i against I (mg/4 ml digest).
FIG. 4B. Plot of i against I (mg/6 ml digest).

Autodigestion. Failure to inhibit peptic autodigestion was observed with both undegraded λ-carrageenan and degraded *Eucheuma* carrageenan.

Discussion

Anderson & Baillie (1967) showed the mechanism of inhibition of peptic activity by high and low molecular weight carrageenans to be depletion or protection of substrate, following interaction with inhibitor, when a plasma protein substrate is used. The present results (Figs 1–3 show that inhibition of haemoglobin proteolysis is also of this type and that other macroanions with different anionic groups and polysaccharide structure are similar in antipeptic action to carrageenan, indicating the general nature of this type of activity.

The results with purified pepsin and gastricsin were similar to those where whole gastric juice was used, showing that the non-enzymic components of gastric juice have no marked effect. The results in Figs 1–4 and the absence of evidence for direct anti-gastriscin activity indicate an inhibition based on a substrate-inhibitor interaction at pH 3·2 for this enzyme. Also, protein interaction with carrageenan at this pH is the same as that at pH 2, suggesting that differences in antipeptic activity of carrageenan with changing pH reflect differences in the structure of the substrate-inhibitor product. The inhibition of the proteolytic activity of human acid gastric juice, pepsin and gastricsin by sulphated polysaccharides is accomplished by substrate depletion and may be a common mechanism for the gastric protease spectrum.

Differences between methods. The high and low molecular weight carrageenans have similar antipeptic activity when observed in the pepsin-haemoglobin or gastricsin-haemoglobin systems (Fig. 4) whereas in the gastric juice-plasma protein system the undegraded carrageenan (high molecular weight) was previously observed to be more active. However at low substrate concentrations (Fig. 1) in the present haemoglobin system, undegraded carrageenan can appear less active than degraded carrageenan, especially at the higher pH. Decrease in the activity of undegraded carrageenan with increase in pH also occurred in a plasma protein system (Anderson & Baillie, 1967). The activity of undegraded carrageenan is therefore more readily affected by type of substrate protein and pH than degraded carrageenan. This influence of method on activity has been observed for a series including both types of carrageenans (Anderson & Harthill, 1967). In the plasma protein system the difference between the undegraded and degraded carrageenans was attributed to the structure of the substrate protein-polysaccharide complex being different; the present observations on the two carrageenans can be adequately explained similarly.

The mechanism of peptic inhibition by macroanions is common to all systems so far studied and the practical implications of the observed effects are twofold: first, the protective activity of low molecular weight macroanions *in vivo* would be expected to be affected less than that of

the higher molecular weight group by variation of conditions of free acidity and protein present; second, care is required in comparison of quantitative results of antipeptic activity when different methods of examination are used.

Inhibition by different macroanions. Dextran sulphate and phosphate, heparin, degraded λ-carrageenan and chondroitin sulphate gave increasing inhibition of peptic activity with increase in concentration (Fig. 4b). Each substance effects similar inhibition whether added to the enzyme or to the substrate first. In both these respects the activity of this group therefore conforms to low molecular weight macroanion inhibition typified (Anderson & Baillie, 1967) by degraded carrageenan. Although differences in activity emerge between members of the low molecular weight group a pattern is not readily discernible; dextran sulphate might be expected to be more active than dextran phosphate of similar molecular weight, on the basis that the sulphate ester groups are more electronegative than the phosphate ester group, resulting in a substrate complex which is less accessible to proteolysis. Chondroitin sulphate, even though it has the highest molecular weight of all members of the group, is the least active. Matthews (1964) has found that chondroitin sulphate B has a much greater binding affinity than chondroitin sulphate A or C, except when carboxyl group ionization is strongly suppressed, when binding affinities become similar. Hence, it is doubtful if the isomeric chondroitin sulphates would exhibit enhanced antipeptic activity or substrate interaction in experiments of the present type. The low activity of chondroitin sulphate reflecting low substrate binding, is adequately explained on the basis of its low sulphate ester content, which provides only one sulphate ester group per repeating disaccharide unit, less than the other macroanions in the group. Degraded λ-carrageenan and heparin, of similar activity, have one 2,6-disulphated sugar per disaccharide unit with occasional sulphation in the other sugar, whereas degraded *Eucheuma* carrageenan, being a κ-type with high 3,6-anhydro-D-galactose content, features 2,6-disulphated galactose in place of the 3,6-anhydride only occasionally, the more usual repeating unit being 3-linked β-D-galactose 4-sulphate with 4-linked 3,6-anhydro-α-galactose 2-sulphate. The influence of 2,6-disulphation is seen in the requirement of 15 mg of degraded λ-carrageenan and heparin for $i = 0{\cdot}5$ whereas with degraded *Eucheuma* carrageenan 20 mg was required (Anderson & Baillie, 1967). More active (9 mg for $i = 0{\cdot}5$) than these is dextran sulphate, completely sulphated (S, 19·5%) and of molecular weight about 8,000, but, on the other hand undegraded λ-carrageenan (S, 11·5%), which is less highly sulphated than dextran sulphate, requires just under 2 mg (Anderson & Baillie, 1967) thus indicating the profound effect of high molecular weight (800,000) in this particular method, in the presence of 2,6-disulphate esterification.

Interaction with protein. Differences in the extent of interaction of the macroanions with protein can be measured by determining the ratio mg protein : mg macroanion at which free macroanion appears in solution

(Anderson & Baillie, 1967). The ratios observed, heparin 5·5, dextran sulphate 5, dextran phosphate 4·5, chondroitin sulphate 4·5, degraded λ-carrageenan 3, give no indication of the order of antipeptic activity, suggesting that the structure of the inhibitor-substrate complex is important in determining the antipeptic activity, rather than the reacting quantities.

Activity in systems where substrate interaction is low or absent. Absence of antipeptic activity is observed in systems where either pepsin or APDT is used as substrate. Interaction between degraded or undegraded carrageenan and pepsin or APDT could not be demonstrated. This is to be expected in the case of pepsin with an isoelectric point at or below pH 1 precluding the assumption of active cationic character for the enzyme in the digestion system and therefore eliminating the possibility of interaction with the anionic groups of the sulphated polysaccharides. Massive concentrations of degraded carrageenan can be shown to cause slight inhibition in these systems but this is interpreted as a simple ionic effect causing incomplete association between enzyme and substrate and can be demonstrated even for microions. The absence of inhibition in these experiments is further evidence that interaction with the enzyme does not occur and supports the interpretation of other data that inhibition of peptic activity by sulphated polysaccharides is brought about solely by protection of the substrate from digestion.

The study of macroanionic inhibition in the strongly acid conditions of the peptic digest, where the enzyme itself behaves as an anion, provides a special case of the type of inhibition described by Spensley & Rogers (1954) for enzyme inhibition by macroanions in systems where the enzyme behaved as a macroion and is consequently engaged in direct interaction with the inhibitor. Also, the reduced (or even absence of) inhibition observed when undegraded carrageenan is added to substrate first constitutes an exception to the principle (Levey & Sheinfeld, 1954; Dellert & Stahmann, 1955; Kornguth & Stahmann, 1960), that this suggests interaction with the enzyme; the reduction in this instance can be explained by the substrate - inhibitor interaction.

This mechanism of inhibition for sulphated polysaccharides probably reflects a physiological function, for example in conditions of excessive pepsin secretion or mucosal susceptibility, and it is of interest to consider the recent demonstration of chondroitin sulphate A- or C-like substance in the chief cells of the gastric mucosa and the fundic gastric juice of the dog (Gerald, DeGraeff & others, 1967). The present work shows that degree of antipeptic activity of sulphated polysaccharides can vary according to conditions in the digest, and the order of activity reported for *in vitro* systems may not necessarily hold for *in vivo* or intracellular systems, for example, those involving chondroitin sulphate. Nevertheless, the mechanism of activity, namely protection of protein from peptic hydrolysis without destroying or inactivating a physiological enzyme has been shown to hold in all the systems studied. This type of inhibition of peptic activity differs fundamentally from direct pepsin inhibition

observed *in vitro* for the natural pepsin inhibitor (Herriott, 1941), poly-amino-acids (Katchalski, Berger & Neumann, 1954; Dellert & Stahmann, 1955), α-keto analogues of amino-acids (Geratz, 1965) and substrate-like irreversible inhibitors (Ong & Perlmann, 1967).

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A note on insulin tolerance during mebanazine treatment

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In contrast to previous findings, mebanazine, a hydrazine monoamine oxidase inhibitor, administered for 21 days had no effect on insulin sensitivity in the rabbit. Insulin sensitivity was increased in mebanazine pretreated 24 hr starved rats but unchanged in mebanazine pretreated fed rats. Muscle and liver glycogen in the rat was unaffected by mebanazine treatment.

INCREASE in the hypoglycaemic action of insulin has been reported in rats (Barrett, 1965) and rabbits (Cooper & Ashcroft, 1966) during treatment with mebanazine (α-methylbenzylhydrazine), a hydrazine monoamine oxidase inhibitor. This phenomenon has been further investigated.

Experimental

Mebanazine oxalate 10 mg/ml and soluble insulin (Burroughs Wellcome & Co.) were made up in 0·9 g/100 ml saline immediately before use. Animals were 2·5–3·0 kg male rabbits and 200–300 g male Wistar rats. Blood glucose was estimated by glucose oxidase (Marks, 1959) and glycogen by the method of Krisman (1962).

Insulin tolerance in rabbits. Each experiment was on a different group of four animals. Blood was taken from an ear vein resting and at intervals after intravenous insulin. An initial insulin tolerance was undertaken (control 1), a second after treatment for 21 days with mebanazine (treated) and a third (control 2) 21 days after mebanazine withdrawal.

Insulin tolerance in rats. Rats treated with mebanazine 10 mg/kg subcutaneously for 21 days were housed individually and the weight of food consumed by each animal was given daily to a weight-matched individually housed control. The insulin tolerance of control and treated groups was determined on day 22. The animals were stunned, the abdomen opened rapidly and blood taken from the abdominal aorta. An equal number of treated and control rats were killed at each time interval to determine mean blood glucose.

Tissue glycogen. Glycogen was measured in liver and thigh muscle of mebanazine-treated and pair-fed control rats and in liver from normal rabbits.

Results

Unless otherwise stated, statistical comparison was by the Mann-Whitney U-test (Siegel, 1956).

Insulin tolerance in rabbits. Control and treated insulin tolerance curves for mebanazine (12 mg/kg s.c.), using insulin (0·24 unit/kg) after 2 hr food deprivation showed the insulin sensitivity to be unchanged

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during mebanazine treatment. Similar results were found with mebanazine, 3 mg/kg. In other experiments, hypoglycaemic stress was increased by doubling the dose of insulin and insulin tolerance was assessed in rabbits pretreated with mebanazine (12 mg/kg, i.m.) and then starved for 24 hr. In no experiment was insulin sensitivity increased during mebanazine treatment.

TABLE 1. MEAN BLOOD GLUCOSE, RESTING AND AT INTERVALS AFTER INSULIN IN RATS TREATED WITH MEBANAZINE (10 MG/KG S.C.) FOR 21 DAYS AND IN PAIR-FED CONTROLS

Experiment	Treatment	Food deprivation	Insulin (unit/kg, s.c.)	Mean blood glucose (mg/100 ml)				Number of deaths
				Resting	90 min	150 min	300 min	
1	Mebanazine	none	0·075	82·2 (5)	—	29·2 (5)	60·0 (10)	none (10)
	Pair-fed	none	0·075	75·9 (5)	—	24·9 (5)	51·7 (10)	none (10)
2	Mebanazine	24 hr	0·05	87·0 (8)	15·5 (4)	9·3 (4)	—	9 (9)
	Pair-fed	24 hr	0·05	62·8 (8)	21·8 (4)	19·5 (4)	27·6 (9)	none (9)
3	Mebanazine	24 hr	0·1	87·0 (8)	14·0 (4)	8·0 (4)	—	6 (6)
	Pair-fed	24 hr	0·1	62·8 (8)	19·0 (4)	15·5 (4)	15·0 (5)	1 (6)

Figures in parentheses are the number of rats from which the values are derived.

Insulin tolerance in rats (Table 1). In experiment 1 rats were allowed access to food until the insulin tolerance test. There was no significant difference between mebanazine-treated and pair-fed groups.

In rats starved for 24 hr (expt 2 and 3) fasting blood glucose was higher after mebanazine pretreatment ($P = 0{\cdot}003$); but after insulin 0·05 units/kg (expt 2), blood glucose was lower at 90 min ($P = 0{\cdot}029$) and 150 min ($P = 0{\cdot}029$) in the mebanazine-treated group. Of nine control and nine pretreated animals to be killed at 300 min, all pretreated rats died between 150 and 300 min after convulsions, and with a blood glucose in each animal of less than 9 mg/100 ml. No controls died and this difference in mortality is significant ($P < 0{\cdot}005$, Fisher exact probability test).

In starved rats given insulin 0·1 unit/kg (expt 3), blood glucose was again lower in the mebanazine-treated group at 90 min ($P = 0{\cdot}057$) and

TABLE 2. MUSCLE AND LIVER GLYCOGEN FOR MEBANAZINE-TREATED AND PAIR-FED RATS UNDER FED AND 24 HR STARVED CONDITIONS AND LIVER GLYCOGEN FOR FED AND 24 HR STARVED NORMAL RABBITS

Animals	Treatment	Food deprivation	Glycogen (mean)	
			muscle (mg/g)	Liver (mg/100 mg)
Rats (5)	Mebanazine	none	0·84	1·06
Rats (5)	Pair-fed	none	0·84	1·01
Rats (5)	Mebanazine	24 hr	0·62	0·055
Rats (5)	Pair-fed	24 hr	0·62	0·053
Rabbits (4) ..	Normal	none	—	9·0
Rabbits (4) ..	Normal	24 hr	—	0·040

150 min ($P = 0{\cdot}014$). Of six rats in each group to be killed at 300 min, all pretreated animals died between 150 and 300 min after convulsions, and with a blood glucose in each animal of less than 7 mg/100 ml. One control died and the difference in mortality is significant ($P < 0{\cdot}05$, Fisher exact probability test).

Tissue glycogen. Neither starvation nor mebanazine treatment caused a significant reduction in the muscle glycogen of rats ($P > 0{\cdot}05$). In mebanazine-treated and pair-fed rats as well as in normal rabbits, there was subtotal hepatic glycogen depletion after starvation for 24 hr (Table 2).

Discussion

Impaired glucose tolerance (Goldblatt & Ellis, 1932), insulin resistance (Tiitso, 1925) and hepatic glycogen depletion (Lawrence & McCance, 1931) have been described in animals deprived of food. For this reason control rats were pair-fed and the increased insulin sensitivity in mebanazine-treated starved rats cannot be due to changes in food intake brought about by treatment.

Barrett (1965) found increased tolbutamide hypoglycaemia in starved rats and increased insulin hypoglycaemia in fed rats after mebanazine treatment. I have found increased insulin hypoglycaemia only in starved animals. These animals have over 95% depletion of hepatic glycogen with sufficient remaining to produce less than 10 mg of glucose. The animals depend upon glucose production from other sources to restore blood glucose from hypoglycaemic levels.

Since mebanazine increased insulin hypoglycaemia under conditions where compensatory hepatic glycogenolysis can play little part, mebanazine cannot act by impairment of hepatic glycogenolysis. In the absence of liver glycogen, glucose production must be by muscle glycogenolysis or gluconeogenesis and mebanazine probably interferes with glucose production from these sources. In fed rats, hepatic glycogenolysis can predominate in restoration of blood glucose, impairment of muscle glycogenolysis or gluconeogenesis would be masked, and this could explain why mebanazine caused no detectable increase in insulin sensitivity in the fed animal.

Muscle glycogen was normal during mebanazine treatment but this may or may not be readily available. Conversion to glucose is at least in part dependent upon lactic acid dehydrogenase and *in vitro* inhibition of dehydrogenases by hydrazine monoamine oxidase inhibitors has been described (Redetzki & O'Bourke, 1961). These inhibitors also inhibit some pyridoxal requiring enzymes (Robinson, 1966) and it may be significant that transaminases, concerned in gluconeogenesis, require pyridoxal (Weber, Singal & others, 1964).

A possible explanation for the finding that mebanazine had no effect upon insulin hypoglycaemia in the rabbit would be that the larger animal always maintained its liver glycogen and so behaved in the same way as the fed rat, even after starvation. It can be seen from Table 2 that this is not so.

The present results in rabbits differ from the other report about this species (Cooper & Ashcroft, 1966). The discrepancy is difficult to explain. I used male rabbits and a sex difference in monoamine oxidase activity has been described (Zeller, 1966). This may result in sex differences in response to monoamine oxidase inhibitors. A strain difference in behaviour cannot be excluded and in man there is a genetically controlled variation in the rate of acetylation of the closely related drug isonicotinic acid hydrazide (Brodie, 1964). In the report of Cooper & Ashcroft (1966), mebanazine was administered to 3 rabbits. Twenty animals were used in the present investigation which should yield a more representative result.

The manner in which the insulin tolerance tests were made was entirely different in the two species but the results do raise the possibility of a species difference in response to mebanazine.

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The penetration of catechol and pyrogallol into mouse brain and the effect on cerebral monoamine levels

SIR,—It has previously been reported (Angel & Rogers, 1968) that catechol (1,2-dihydroxybenzene), when administered parenterally to mice readily evokes convulsions, whereas pyrogallol (1,2,3-trihydroxybenzene) does so only in near lethal doses. Since Matsuoka, Yoshida & Imaizumi (1962) have suggested that pyrogallol does not pass the blood-brain barrier, we decided to measure the penetration of both pyrogallol and catechol into the brain and also to investigate the effect of these compounds on the levels of cerebral monoamines, as both have been shown to inhibit cerebral catechol-*O*-methyl transferase *in vivo* (Crout, Crevelin & Udenfriend, 1961; Ross & Haljasmaa, 1964).

Male mice (18–22 g) of an inbred strain were decapitated at suitable times after the intraperitoneal injection of catechol (60 mg/kg) or pyrogallol (120 mg/kg). The phenols in trichloroacetic acid brain extracts were estimated by a modification of the method of Swain & Hillis (1959). The solvent system described by Shore & Olin (1958) was used in the determination of cerebral noradrenaline (Shore & Olin, 1958), dopamine (Carlsson & Waldeck, 1958) and 5-hydroxytryptamine (Weigand & Perry, 1961). Two mouse brains were pooled for each determination. Groups of 10 mice (5 pairs) were used at each dose level.

Spontaneous motor activity of the mice in a container was measured by suspending it from a strain gauge, the output of which was rectified and integrated over time intervals of 2 sec. The integrated activity at the end of each 2 sec period was sampled, stored in a digital computer (Biomac Data Laboratories) and transferred to paper tape for subsequent analysis.

The time course of changes in motor activity and in the cerebral concentration of the phenolic compounds are represented in Figs 1 and 2. Catechol (Fig. 1) elicits convulsions consisting of violent jerks and tremors, which commence within 15–20 sec of injection. The duration of this convulsive activity is transient, lasting approximately 8 min and the peak convulsive activity occurs at 2–3 min. It can be seen that the time course of catechol penetrations into the brain follows closely the time course of the convulsive activity. Mice receiving 120 mg/kg of pyrogallol show no change in motor activity (Fig. 2) even though this dose of pyrogallol is sufficient to produce cerebral concentrations which are about the same as those produced by the convulsive dose of catechol.

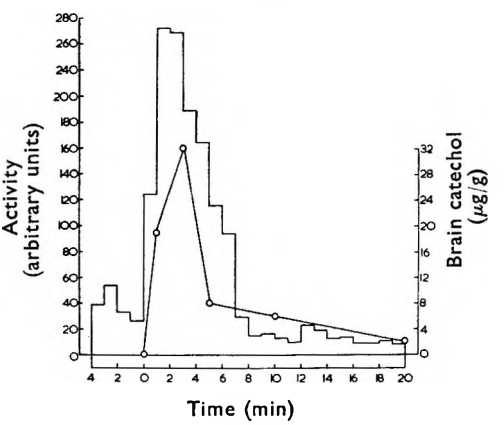

FIG. 1. Penetration of catechol into mouse brain (○) and the effect on motor activity (histogram). Catechol (60 mg/kg) injected intraperitoneally at time zero.

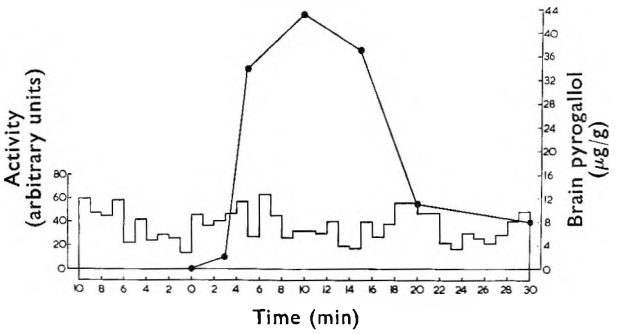

FIG. 2. Penetration of pyrogallol into mouse brain (●) and the effect on motor activity (histogram). Pyrogallol (120 mg/kg) injected at zero time.

TABLE 1. EFFECTS OF CATECHOL (60 MG/KG) AND PYROGALLOL (120 MG/KG) ON THE CONCENTRATION OF MONOAMINES IN MOUSE BRAIN

Time after injection (min)	5-HT μg/g ± s.e.	Noradrenaline μg/g ± s.e.	Dopamine μg/g ± s.e.
Catechol—			
0	0·53 ± 0·02	0·41 ± 0·02	0·58 ± 0·02
5	0·53 ± 0·03	0·40 ± 0·04	0·56 ± 0·02
10	0·51 ± 0·04	0·41 ± 0·02	0·56 ± 0·03
30	0·56 ± 0·02	0·45 ± 0·02	0·61 ± 0·03
Pyrogallol—			
0	0·53 ± 0·02	0·41 ± 0·02	0·58 ± 0·02
10	0·56 ± 0·06	0·36 ± 0·03	0·57 ± 0·03
30	0·60 ± 0·02	0·42 ± 0·02	0·58 ± 0·03

The concentrations of noradrenaline, dopamine and 5-hydroxytryptamine in the brains of mice treated with either catechol or pyrogallol (Table 1) were found to be not significantly different from the levels in control animals. Pyrogallol, administered parenterally to rats has previously been shown to produce no change in the cerebral concentration of catecholamines (Crout & others, 1961; Maitre, 1966) although intra-cisternal injection of this compound has been reported to increase the levels of catecholamines in rabbit brain (Matsuoka & others, 1962). Despite the fact that both catechol and pyrogallol are potent inhibitors of catechol-*O*-methyltransferase it is reasonable to assume that the levels of cerebral catecholamines may be unaltered by these compounds since re-uptake mechanisms are largely responsible for the "removal" of noradrenaline liberated at nerve terminals (see Iversen, 1967).

In conclusion therefore, the results indicate that both catechol and pyrogallol enter the brains of mice after intraperitoneal injection, catechol alone evoking convulsions. Neither of these hydroxyphenolic catechol-*O*-methyltransferase inhibitors produce changes in the gross levels of noradrenaline, dopamine or 5-hydroxytryptamine in mouse brain.

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The effect of electroconvulsive shock on the cerebral metabolism of dopamine and 5-hydroxytryptamine

SIR,—Previous reports concerning the effects of convulsive treatment on the levels of 5-HT in brain have been at variance. While Garattini & Valzelli (1956, 1957), Jori, Valsecchi & Valzelli (1957), Fresia, Valsecchi & Valzelli (1957), Garratini, Kato & others (1960) and Breitner, Picchioni & Chin (1964) found significant increases in brain 5-HT concentration after a single electroconvulsive stimulation (ECS) in the rat and other species, Bonnycastle, Giarman & Paasonen (1957) and Bertaccini (1959) could detect no significant increase in 5-HT in rat brain after similar experiments.

Using dogs we have studied the effect of a course of electroconvulsive shock on the cerebral metabolism of 5-HT and dopamine by estimating the concentrations of their amino-acid precursors, tryptophan and tyrosine, and acid metabolites, 5-hydroxyindol-3-ylacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylacetic acid (HVA), in samples of cerebrospinal fluid (CSF) drawn serially from the lateral ventricle. On each occasion that electroconvulsive shock was given, the dog was lightly anaesthetized with intravenous sodium thiopentone and 0·7 ml samples of CSF were withdrawn through a needle introduced percutaneously into a cannula previously implanted in the skull (Ashcroft, Crawford & others, 1968). The samples of CSF were taken at 0, 60, 120 and 150 min and an electroconvulsive shock of 150 V for 1 sec, was administered through bitemporal leads at 90 min. 5-HIAA and HVA were estimated by a modification (Ashcroft, Crawford & others, 1968) of the methods of Ashcroft & Sharman (1962) and Andén, Roos & Werdinius (1963); tryptophan and tyrosine were

TABLE 1. CONCENTRATIONS (μG/ML) OF 5-HYDROXYINDOL-3-YLACETIC ACID (5-HIAA) AND 3-METHOXY-4-HYDROXYPHENYLACETIC ACID (HVA) IN DOG LATERAL VENTRICULAR CSF DURING A SERIES OF ELECTROCONVULSIVE SHOCKS (ECS)

Day	Treatment	Dog 1		Dog 2	
		5-HIAA	HVA	5-HIAA	HVA
	Pre-ECS. Mean of two estimates at 0 and 60 min	0·18	1·21	0·17	1·28
1	Post-ECS. Mean of two estimates at 120 and 150 min	0·15	1·02	0·17	1·19
3	Pre-ECS	—	1·06	0·27	1·41
	Post-ECS	—	1·03	0·27	1·82
8	Pre-ECS	0·31	1·44	0·29	1·43
	Post-ECS	0·31	1·51	0·24	1·43
10	Pre-ECS	0·31	1·35	Methodological difficulties	
	Post-ECS	0·32	1·33		
15	Pre-ECS	0·30	1·28		
	Post-ECS	0·30	1·15		
17	Pre-ECS	0·30	1·19		
	Post-ECS	0·32	1·24		

estimated by a modification (Moir, 1967) of the method of Hess & Udenfriend (1959) as applied by Guroff & Udenfriend (1962).

Throughout the experiments no significant changes were detected in the concentrations of tryptophan and tyrosine in CSF. There were no significant differences between the acid metabolite concentrations at 0 and 60 min, or between those at 120 and 150 min. Table 1 shows the mean concentrations of 5-HIAA and HVA in CSF before and after each shock treatment. While there was no alteration in the concentrations of 5-HIAA or HVA during a single shock experiment, the concentrations of both acids rose throughout the course of treatment (a comparison of the metabolite concentrations obtained on day 8 with those of day 1 showed significant alterations, 5-HIAA, $P < 0{\cdot}001$, HVA $P < 0{\cdot}02$), the increase in 5-HIAA concentration being particularly marked and well maintained.

These findings may indicate an increase in the permeability of the brain–CSF barrier to these acids following electroconvulsive shock treatment (Aird, 1958; Rosenblatt, Chanley & others, 1960) although no corresponding changes were observed in the concentrations of either tryptophan or tyrosine. An alternative explanation of our results may be that the shock facilitates the intracerebral hydroxylation of tryptophan, the normal rate determining step in the cerebral metabolism of 5-HT (Moir & Eccleston, 1968), thus inducing an increase in the turnover rate of 5-HT. This hypothesis fits well with the clinical data of Ashcroft, Crawford & others (1966), who found that the abnormally low concentrations of 5-HIAA in the lumbar CSF from patients with endogenous depression rose to levels in the normal range when clinical remission was induced by electroconvulsive shock or other means.

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The influence of cardiac glycosides on membrane permeability in guinea-pig atrial tissue, determined by means of ^{86}Rb

SIR,—It seems that *toxic* doses of cardiac glycosides provoke a reduction of intracellular potassium, accompanied by an increase of the sodium concentration in cardiac tissue, thus explaining cardiac arrhythmia during digitalis intoxication (Holland, 1964; Klaus, 1964). But, with lower non-toxic doses, there is disagreement about the effects on membrane permeability. Tracer experiments by Klaus, Kuschinsky & Lüllman (1962) have shown that therapeutic concentrations of digitoxigenin neither affect the intracellular K-concentration nor the ^{42}K-efflux in guinea-pig isolated atria. However, Greeff, Meng & Moog (1962) observed a small increase in the K-efflux from guinea-pig isolated perfused hearts upon treatment with non-toxic ouabain doses. According to Repke (1963) and to Piechowski, Grobecker & Greeff (1963) ouabain in low concentrations may influence the K-Na sensitive membrane ATPase in myocardial tissue, although it should be emphasized that experimental evidence for this assumption is mainly based on investigations with isolated red cells.

We have found that ^{86}Rb may be used conveniently to measure changes in permeability towards ions, ^{86}Rb being suitable as a tracer for ion movements in atrial tissue (Zwieten, 1968) and easier to handle than ^{42}K.

Isolated atria were loaded with ^{86}Rb upon incubation in a K-free Tyrode solution that contained an equivalent amount of RbCl (2·7 mmole/litre), part of the Rb^+ ions being radioactive (^{86}Rb). The atria thus loaded (2 hr) were attached in a small organ bath (volume 1·8 ml) that was perfused continuously with K-free, Rb-Tyrode solution (see above). By means of an infusion pump the perfusate was drawn with a constant rate of 1·5 ml/min through a plastic tube, wound within the counting vial of an ECKO N664C liquid scintillation spectrometer. The β-radiation of the ^{86}Rb in the perfusate penetrated the wall of the plastic tube with a loss of 25%, and provoked light impulses in the counting vial that was filled with a liquid scintillation phosphor. The impulses were counted, integrated and recorded continuously by means of a Rikadenki device. The curve thus obtained reflected the ^{86}Rb content of the perfusate, i.e., the amount of ^{86}Rb released by the isolated atrium. The bath fluid was gassed with 5% carbon dioxide in oxygen and kept at 30°. The atria were stimulated electrically with a frequency of 180/min (Grass S4H). Mechanical activity was recorded continuously by a transducer and Helco-scriptor recording device (Zwieten, 1968). Isolated atria incubated in K-free, Rb-containing Tyrode solution show unimpaired mechanical activity, whereas the response to electrical stimulation and to cardioactive drugs of various categories is the same as that found during incubation of the organs in normal, K-containing Muralt-Tyrode-solution (Zwieten, 1968).

The loss of ^{86}Rb from atria, beating with a frequency of 180/min occurs by means of two different rate constants, i.e., $22{\cdot}2 \times 10^{-4}$ and $2{\cdot}0 \times 10^{-4}$ sec^{-1}, the slower process becoming predominant after a perfusion of about 20 min. The straight line obtained upon plotting the ^{86}Rb level of the perfusate towards the perfusion time is a direct measure for the rate of ^{86}Rb release by the atrial tissue. The straight line, fitted by the method of the least squares had the general equation $\log Y = bX + a$, where $Y = {}^{86}Rb$-content of the perfusate, expressed in % of initial, maximal value and X the perfusion time in min (see Table 1). To determine the effect of cardiac glycosides in therapeutic dosage, the drugs were added to the perfusion Tyrode solution.

The following cardiac glycosides were studied: ouabain, digoxin, peruvoside (the α-thevetoside of cannogenin) and digitoxin. In the dosage used the

drugs caused a pronounced therapeutic effect without provoking any cardiac arrhythmia or contracture. Again, the rate of ^{86}Rb release was characterized by means of the regression line, representing the slow process of wash-out. The results are summarized in Table 1. The influence of each drug has been studied on 5–10 different atria.

TABLE 1. INFLUENCE OF CARDIAC GLYCOSIDES IN THERAPEUTIC CONCENTRATIONS ON THE RATE OF ^{86}Rb-RELEASE. General equation of the regression lines, representing the rate of ^{86}Rb-release: $\log Y = bX + a$. (a and b are given)

Cardiac glycoside	Concentration in perfusion Tyrode solution (M)	Increase in contractile force in % (mean ± s.e.)	b	a (± S log Y)
Controls	—	—	$5{\cdot}35 \times 10^{-2}$	1·6787 ± 0·1802
Ouabain	$3{\cdot}4 \times 10^{-7}$	56 ± 8·3	$6{\cdot}33 \times 10^{-2}$	1·8120 ± 0·2054
Digoxin	$3{\cdot}2 \times 10^{-7}$	50 ± 6·9	$5{\cdot}69 \times 10^{-2}$	1·7136 ± 0·1765
Peruvoside	$3{\cdot}6 \times 10^{-7}$	60 ± 6·9	$7{\cdot}01 \times 10^{-2}$	1·7795 ± 0·2632
Digitoxin	$3{\cdot}3 \times 10^{-7}$	46 ± 2·2	$5{\cdot}34 \times 10^{-2}$	1·6715 ± 0·2123

None of the equations shown in Table 1 are significantly different from that representing the ^{86}Rb-release under control circumstances. Accordingly, the rate of ^{86}Rb release is not changed by cardiac glycosides in therapeutic concentrations. Therefore, the Rb-efflux also remains unchanged, since this parameter is directly proportional to the rate constant of the release process.

Toxic concentrations of the cardiac glycosides caused arrhythmia and finally contracture after an initial positive inotropic action. The following doses were used: ouabain $1{\cdot}7 \times 10^{-6}$M; digoxin $6{\cdot}4 \times 10^{-6}$M; peruvoside $4{\cdot}3 \times 10^{-6}$M; digitoxin $6{\cdot}5 \times 10^{-6}$M. During the initial positive inotropic effect no increase in ^{86}Rb release could be observed. However, an irregular though large acceleration of the release process accompanied the toxic symptoms of the glycoside effect. A quantitative evaluation of the accelerated ^{86}Rb-release could not be given since the irregular contractile activity of the atria in this stage also enhanced the release of $^{86}Rb^{+}$-ions, the arrhythmia being accompanied by an increased frequency of beating. Evidence has been given that the release of ^{86}Rb is directly related to the number of contractions (Zwieten, 1968). Consequently, the membrane permeability in atrial tissue is increased by toxic concentrations of cardiac glycosides, whereas therapeutic concentrations of the same drugs do not affect this parameter. This finding is in satisfactory agreement with the studies using ^{42}K as a tracer (Klaus, Kuschinsky & Lüllman, 1962).

The ouabain-induced increase in ^{86}Rb-release, and also the mechanical arrhythmia, could be antagonized by the simultaneous infusion of diphenylhydantoin-sodium into the organ bath. This drug has proved of some use in the therapy of digitalis-induced arrhythmia in patients (see, e.g., Delius, 1966). Electrophysiological studies by Lüllman & Weber (1968) suggest that a reduction of passive ion fluxes on the membrane may explain the therapeutic effect of diphenylhydantoin-sodium. The investigations with ^{86}Rb also demonstrate that the ouabain-induced increase in membrane permeability may be antagonized by the hydantoin.

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Effects of some spasmolytic agents on the lipid-facilitated transport of calcium ions

SIR,—Depolarization of smooth muscle cells is accompanied by an influx of calcium ions. Woolley (1963) proposed that lipids were involved in the transport of calcium ions and phospholipids extracted from skeletal and cardiac muscle and nervous tissue were found to facilitate the transport of calcium ions from an aqueous to the chloroform phase (Feinstein, 1964; Nayler, 1966a). Several substances were found to elicit effects on this simple system consistent with their pharmacological properties (Feinstein, 1964; Blaustein & Goldman, 1966; Nayler, 1966a,b; Sandow & Isaacson, 1966; Blaustein, 1967; Piccinini & Pomarelli, 1967). We (Santi, Ferrari & Contessa, 1964; Tóth, Ferrari & others, 1966) implicated two effects in the mechanism of action of spasmolytics: (i) the inhibition of oxidative phosphorylation shared by papaverine and its main oxy-alkyl-benzylisoquinoline derivatives, and (ii) interference with calcium ions presumably assuming a prominent importance in the myolytic activity of other compounds devoid of inhibitory effects on energy production (Ferrari & Gaspa, 1965; Tóth & others, 1966). We now describe the effects of some myolytic agents on lipid-facilitated calcium transport.

Phospholipids were extracted (1 hr) from a homogenate of calf stomach muscle with chloroform–methanol (2:1) solution (1·5 ml)/g wet weight (Feinstein, 1964; Blaustein, 1967). The extract was washed and diluted with chloroform–methanol (2:1) to obtain a phospholipid concentration of 1·5 mg/ml (Folch, Lees & Stanley, 1957). The drugs (papaverine hydrochloride, eupaverin hydrochloride, isoxsuprine hydrochloride and aminopromazine hydrochloride) were dissolved at concentrations ranging from 0·1 to 2 mM in a medium containing 116 mM NaCl, 2·5 mM KCl, 0·5 mM $CaCl_2$, 0·2 μc/ml $^{45}CaCl_2$. Samples (0·5 ml) of this solution were added to 1 ml of chloroform–methanol phospholipid extract; the mixture was shaken for 1 min in a cyclomixer and then centrifuged for 10 min at 2500 *g*. Aliquots of 0·2 ml of the chloroform phase were tested for radioactivity in an end window Geiger counter.

Under these experimental conditions it was observed that aminopromazine, papaverine, eupaverin and isoxsuprine inhibit the lipid-facilitated calcium transport from the aqueous to the chloroform phase. Aminopromazine is the

TABLE 1. EFFECTS OF VARIOUS CONCENTRATIONS OF SPASMOLYTIC DRUGS ON THE LIPID-FACILITATED TRANSPORT OF CALCIUM IONS FROM AN AQUEOUS TO CHLOROFORM PHASE

Drugs	Concentration (mM)	% inhibition (± s.e.) of the lipid facilitated calcium transport
Papaverine hydrochloride	0·5	27·00 ± 2·06
	1·0	57·20 ± 1·28
	1·5	85·00 ± 3·07
	2·0	92·40 ± 0·60
Eupaverin hydrochloride	0·5	26·60 ± 0·73
	1·0	64·00 ± 5·90
	1·5	89·30 ± 4·10
	2·0	96·60 ± 3·30
Isoxsuprine hydrochloride	0·5	19·90 ± 2·90
	1·0	39·50 ± 5·00
	1·5	57·50 ± 0·03
	2·0	70·40 ± 2·32
Aminopromazine hydrochloride	0·1	8·00 ± 4·37
	0·2	32·50 ± 2·50
	0·5	91·50 ± 3·50
	1·0	100·00 ± 0·00

most effective compound: its activity is about four times greater than that of papaverine and eupaverin, which are more active than isoxsuprine (Table 1).

There seems to be a similarity of effects between the drugs tested and some local anaesthetics (Feinstein, 1964), and we suggest that like these anaesthetics, certain spasmolytic agents may interact with phospholipids, thus impairing their calcium binding ability. If phospholipids are effectively involved in calcium transport at membrane level, it seems of interest to mention the results of our previous investigations indicating that some myolytic agents prevent calcium uptake by red cell membranes (Carpenedo, 1966) and that a clear antagonism occurs between spasmolytic drugs and calcium ions both in polarized (Ferrari, 1964; Ferrari & Gaspa, 1965) and K-depolarized intestinal smooth muscle (Ferrari & Carpenedo, 1968).

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A new peripheral and coronary vasodilator substance of the β-aminoketone group

SIR,—The pharmacological effects of β-aminoketones are many. Thus, their action on the central nervous system (Brit. Pat. 1961), their analgesic (Ruschig, Schmitt & Meixner, 1962), adrenergic neuron blocking (de Stevens, 1961), antinicotinic (Nádor & Porszász, 1958), antibacterial (Magarian & Nobles, 1967) properties have been investigated. In the course of clinical trials of an antinicotinic action of 2-methyl-1-piperidino-3-(*p*-tolyl)-propan-3-one hydrochloride (Mydocalm), a vasodilator effect was observed (Molnár, 1961). This was substantiated by animal experiments (Solti, Iskun & others, 1964). This effect of the aminoketone has been investigated in a series of new compounds and 3-(*p*-octylphenyl)-1-piperidinopropan-3-one hydrochloride (N-1113) proved to be a significant peripheral and coronary vasodilator.

Table 1 illustrates the increase of blood flow following different doses of papaverine and N-1113 in the femoral artery of cats weighing 3 to 3·5 kg anaesthetized with pentobarbitone (40 mg/kg i.p.). Measurements were made with a Nycotron electromagnetic blood flow meter. The substances were administered in a 0·2 ml volume intra-arterially distally from the flow probe

TABLE 1. VASODILATOR EFFECT ON THE FEMORAL ARTERY OF THE ANAESTHETIZED CAT

Drug	Dose mg i.a.	n	Flow increase Mean ± s.e. (ml)	Flow increase %*
Papaverine	0·09	4	2·9 ± 0·3	40
	0·27	9	4·8 ± 1·4	44
	0·37	3	7·0 ± 2·2	96
	0·50	3	8·2 ± 2·6	112
	0·81	12	9·0 ± 1·5	124
	1·12	4	12·5 ± 2·9	170
	2·43	6	12·6 ± 1·6	173
N-1113	0·03	3	3·5 ± 0·3	48
	0·06	4	4·1 ± 0·3	56
	0·09	7	5·5 ± 1·3	75
	0·12	6	7·9 ± 1·1	95
	0·27	14	10·4 ± 1·8	142
	0·81	8	14·5 ± 3·0	198

* Value related to the basic flow during the period of the effect.

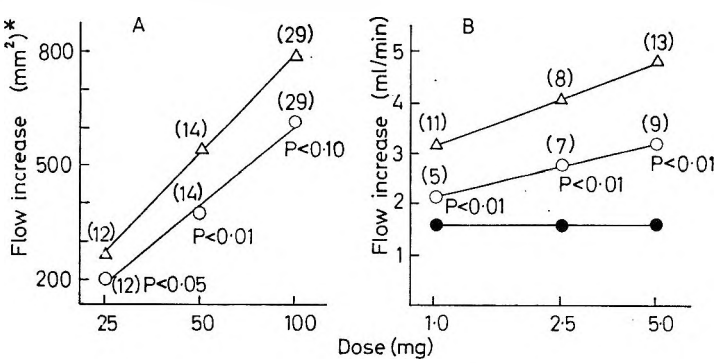

FIG. 1. Coronary flow increase on isolated heart (A) and anaesthetized animal (B) on the effect of Prenylamine (○—○) and N-1113 (△—△). ●—●, Basic flow. Figures in parenthesis indicate the number of experiments on which the mean was based. P = statistical significance of identical doses.

* Increase of perfusion was evaluated planimetrically.

in a fine polyethylene cannula inserted into a side branch of the artery. In this manner femoral arterial flow was not interrupted. The increased flow following the effect of the drug is obtained by subtracting from the total flow from the beginning of the effect, the basic flow for the same period.

The dose response curves derived from the results in Table 1 are parallel, thus a similar mode of action may be assumed and in a weight basis N-1113 has some three times the potency of papaverine. Increase of flow by both substances at these doses subsides within 1 to 3 min.

Coronary flow was measured on the cat isolated heart (Langendorff) and also by measuring with a drop counter the quantity of blood flowing from the cannula inserted in the coronary sinus of an anaesthetized cat with open thorax. In the heart preparation the drugs were administered in the perfusion fluid in a volume of 1 ml, in the anaesthetized animal, into the femoral vein.

Fig. 1 shows that the effect of N-1113 exerted on the amount of blood flowing through the coronary artery in *in vivo* and *in vitro* experiments is significantly greater than that caused by prenylamine in the same dose. The duration of action of N-1113 is 6 to 7 min, which is about 2·5 to 3 times that of prenylamine.

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Letters to the Editor

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