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Some physico-chemical properties of SKF 525–A in aqueous solution

A. T. FLORENCE

*School of Pharmaceutical Sciences, University of Strathclyde, Glasgow, C.*1, *U.K.*

The properties of solutions of SKF 525–A (β-diethylaminoethyl diphenylpropylacetate hydrochloride) have been investigated using surface tension, light scattering and microelectrophoretic techniques. The interaction of the drug with cholesterol and lecithin monolayers was studied by a surface balance method. SKF 525–A is more surface-active than chlorpromazine, and forms micelles containing 22 monomers in 0·9% NaCl solution. It increases the surface pressure of L-α-lecithin monolayers spread on water and both L-α-lecithin and cholesterol monolayers spread on 0·9% NaCl solutions at 1–2 × 10^{-6}M concentration levels. Because of the surface-active properties of SKF 525–A caution is urged in the interpretation of enzyme inhibition results as surfactants are well known as a class to greatly affect enzyme systems.

SKF 525–A (β-diethylaminoethyl diphenylpropylacetate hydrochloride) is an inhibitor of many microsomal enzymatic reactions (Brodie, Gillette & La Du, 1958) and thus prolongs the action of a variety of drugs. Brodie (1956) has suggested that its action may be to decrease the permeability of the microsomal membrane and so prevent or reduce the transport of drugs to the metabolic enzymes.

It has recently been reported (Lee, Yamamura & Dixon, 1968) that SKF 525–A is surface-active and behaves in a manner similar to chlorpromazine hydrochloride as far as it affects the haemolysis and stabilization of the red cell membrane. In order to gain further insight into the properties of the compound, the present investigation has been undertaken into its surface chemistry and micellar properties. Some comparisons have been made with the behaviour in solution of chlorpromazine and proniazid.

EXPERIMENTAL

Surface tension measurements were made using a drop volume apparatus incorporating an Agla micrometer syringe thermostatted at 25° ± 0·01°. The correction factors of Harkins & Brown (1919) were used. No significant ageing of the solutions was observed. *Viscosity measurements* were made in a suspended level viscometer at 25° ± 0·01°, the relative viscosities being referred to solutions at the critical micelle concentration (CMC). *Light-scattering* measurements were made at 25° with the light-scattering photometer described by Attwood (1968), on solutions of SKF 525–A in 0·9% and 2·5% NaCl which had been filtered through 0·2 μm Millipore filters. *Electrophoretic mobilities* were determined at room temperature (22° ± 1°) using a Zeta-Meter (Zeta Meter Inc.) with glass-Teflon cell. Octadecanol was dispersed in distilled water using an MSE ultrasonic generator and the resulting suspension of spherical particles diluted into solutions of drug of varying concentration. *Surface pressure measurements* at the air–water interface were made using a

glass Langmuir trough of 2·5 litre capacity employing a conventional torsion balance. For interaction studies the cholesterol or lecithin was spread from benzene on swept water surfaces and after the surface pressure-area curve had been obtained, 1 ml of an approximately 0·2% solution of the SKF 525–A or chlorpromazine was injected beneath the surface and the resultant surface pressure-area plot obtained. *pH measurements* were made using a Pye Model 78 pH meter by titration of the drug solution into water or salt solution.

Materials

SKF 525–A was a gift from Smith Kline and French Laboratories, Welwyn Garden City and was used as supplied (Proadifen HCl purity 99·8%). Chlorpromazine HCl (Largactil, May & Baker) was a commercial sample used as received. Iproniazid (Marsilid) was a gift from Roche Laboratories and used as received. L-α-lecithin (ex egg, grade II, Koch-Light Laboratories) was a 10% solution in hexane diluted to 0·1% with benzene for spreading. Cholesterol (BDH) was a dissolved in benzene. Octadecanol (melting point 58·3°), a purified sample, was a gift of Mr J. A. Rogers. NaCl was Analar quality. Water was once distilled from glass and twice distilled from potassium permanganate for surface tension studies. Whole blood diluted in drug solution was used for determination of erythrocyte electrophoretic mobilities.

RESULTS

SKF 525–A exhibits the properties of a typical ionic surface-active agent, lowering the surface tension of water and forming micelles above a certain critical concentration, the surface activity and micelle size increasing with addition of simple electrolytes.

Surface tension results shown in Fig. 1 indicate CMC's much higher than suggested by Lee & others (1968) being closer to 10^{-2}M rather than the value of 10^{-4} quoted by

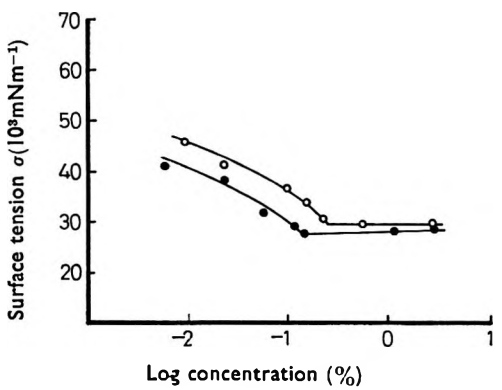

FIG. 1. Surface tension, mNm^{-1} (dynes cm^{-1}), plotted as a function of logarithm of concentration of SKF 525–A (%). ○ in 0·9% NaCl. ● 2·5% NaCl.

them, hence the onset of micellization is not connected with maximum erythrocyte membrane stabilization as they suggested, and cannot be biologically significant.

The limiting area/molecule of SKF 525–A, calculated from the surface tension values below the CMC, using the simple form of the Gibbs' equation, is 51 Å² (0·51 nm²) in 0·9% NaCl which agrees well with the value of 53 Å² (0·53 nm²) which was the area of the hydrophobic regions estimated from Catalin models. The surface tension of SKF 525–A at the CMC is lower than the corresponding values for chlorpromazine (Scholtan, 1955) indicating the greater surface activity of the former which probably results from the greater separation of the hydrophilic and hydrophobic regions in the SKF 525–A molecule.

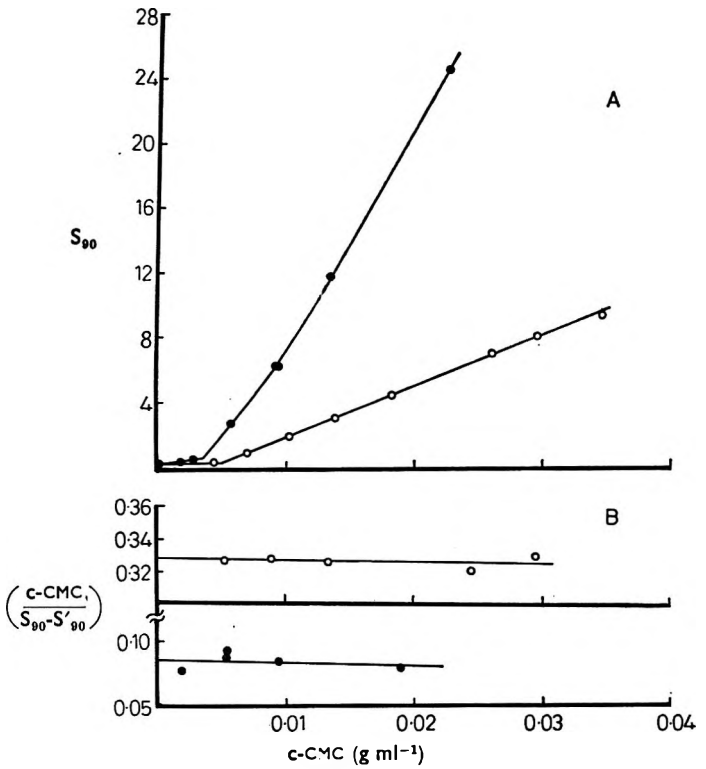

FIG. 2. A. The scattering ratio (S_{90}) as a function of concentration (g ml⁻¹) for SKF 525–A. ○ in 0·9% NaCl. ● 2·5% NaCl. B. Plots of (c–CMC)/(S_{90}—S_{90}) *vs* (c–CMC).

The high CMC's are confirmed by the light-scattering results which show distinct breaks in the scattering ratio (S_{90})–concentration (c) plots (see Fig. 2A). The light-scattering CMC's are higher than the surface tension CMC's. The latter are believed to be more accurate. Micellar weights were calculated from the reciprocal of the intercept of plots of (c-CMC)/(S_{90}–S'_{90}) *vs* (c-CMC) (Fig. 2B) which for charged surfactants in the salt solutions used is a valid procedure (Emerson & Holtzer, 1967). The micellar weights so obtained are shown in Table 1 along with CMC's determined by surface tension and light-scattering.

CMC's can also be obtained from pH *vs* log concentration plots (Lawrence & McDonald, 1957) and values from such measurements are also given in Table 1. This simple method is recommended as a rapid procedure to determine whether or

Table 1. *Limiting surface tensions, micellar weights and CMC's of SKF 525–A at 25°*

Salt concentration	Limiting σ (dynes cm^{-1}) or mNm^{-1})	Micellar weight*	Aggregation number	CMC M		
				by surface tension	by pH	by light-scattering
0	—	—	—	—	$9{\cdot}80 \times 10^{-3}$	—
0·9%	29·8	8,900	22	$5{\cdot}98 \times 10^{-3}$	$7{\cdot}88 \times 10^{-3}$	$1{\cdot}12 \times 10^{-2}$
2·5%	28·0	34,400	86	$3{\cdot}68 \times 10^{-3}$	$4{\cdot}38 \times 10^{-3}$	$8{\cdot}97 \times 10^{-3}$

* dn/dc value = 0·216, determined using a Hilger Rayleigh Interferometer at 25°.

not an ionic monobasic compound forms micelles, although the quantitative explanation of the shape of the resultant pH curves is not complete. With small micelles the CMC has to be interpolated (see Fig. 3). It is apparent from a qualitative treatment that the drug molecules are more highly dissociated in the micelle than in their free state below the CMC.

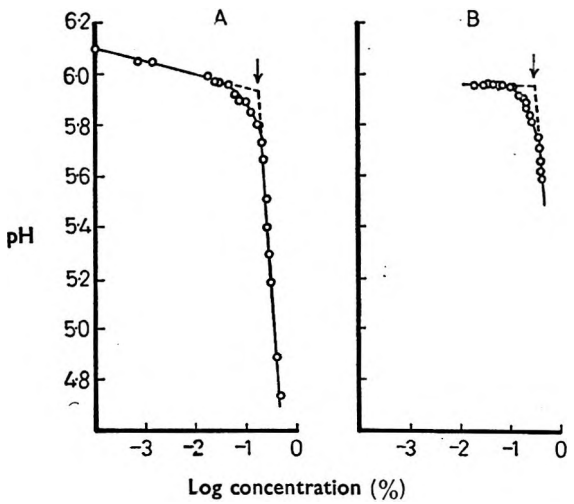

FIG. 3. Plots of pH *vs* log concentration (%) for SKF 525–A. A, in 2·5% NaCl; B, in 0·9% NaCl. The CMC is interpolated and shown by arrow. CMC values shown in Table 1 in M.

Fig. 4 shows the viscosity results plotted as reduced viscosity, $\eta_{sp}^{1}/(c\text{-CMC})$ *vs* (c-CMC). From these plots the viscosity intercept, $[\eta]$, was obtained. In both salt solutions, $[\eta]$ is 3·30 ml g^{-1} which indicates, on application of the viscosity equation, assuming spherical micelles

$$[\eta] = 2{\cdot}5\,(\bar{V}_2 + \omega V_1)$$

where $\bar{V}_2$ is the partial specific volume of the solute and V_1 of the solvent, an hydration, ω (g water/g drug) of 0·3, i.e., around 6 molecules of water per monomer which is of the same order as the hydration values of a variety of other ionic surfactants (Mukerjee, 1964).

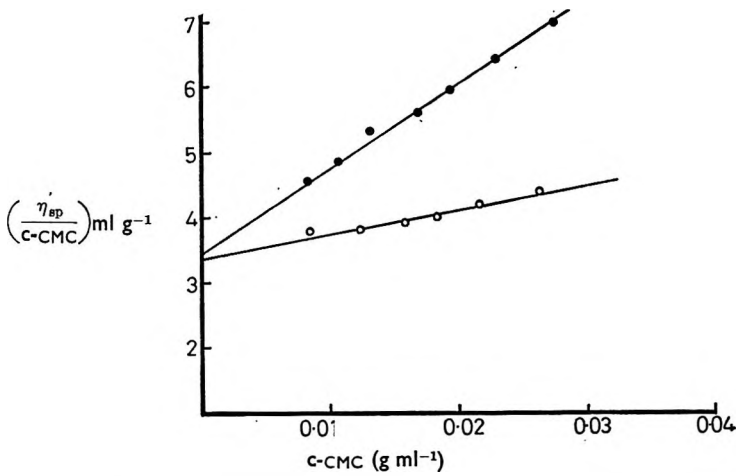

FIG. 4. Viscosity results at 25°C showing plots of $\eta^1_{sp}/(c-CMC)$ *vs* (c–CMC) in g ml^{-1}. ● SKF 525–A in 2·5% NaCl, ○ in 0·9% NaCl. Intercept, $[\eta] = 3{\cdot}3$ ml g^{-1}. η^1_{sp} = (viscosity relative to CMC – 1).

Although more surface-active than chlorpromazine at the air–0·9% NaCl interface, SKF 525–A has a higher CMC and forms smaller micelles. The micellar weight of chlorpromazine is 20,400 in 0·9% NaCl, corresponding to 64 monomers per micelle; SKF 525–A forms micelles consisting of 22 monomers in the same salt concentration. The micellar size is strongly dependent on salt concentration, increasing to four times in 2·5% NaCl. This is still lower than the micellar weight of chlorpromazine which Scholtan (1955) records as 39,800 at this salt concentration.

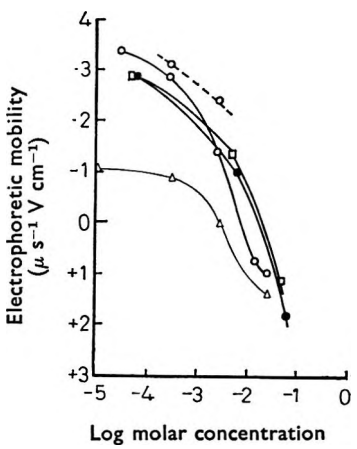

FIG. 5. Plots of electrophoretic mobilities of octadecanol in the presence of: ○ SKF 525–A, ● iproniazid, □ chlorpromazine hydrochloride. Dotted line represents results obtained by addition of HCl to the dispersion. The lowering of electrophoretic mobility caused by the drugs is more marked. △ Effect of SKF 525–A on electrophoretic mobilities of human erythrocytes.

The microelectrophoresis experiments were made to determine whether extensive adsorption of SKF 525–A at solid interfaces could be detected at very low drug

concentrations. Octadecanol was chosen as the adsorbent as it has been used as a model for a biological interface (Hollingshead, Johnson & Pethica, 1965). The sample of octadecanol dispersed in deionized water had an electrophoretic mobility of $-4{\cdot}3\ \mu s^{-1}$ $(V\ cm^{-1})^{-1}$ $(-4\,3 \times 10^{-8}\ m^2\ V^{-1}\ s^{-1})$. The adsorption of the positively charged SKF 525–A and chlorpromazine HCl can be followed in Fig. 5 by the reduction in the negative charge of the octadecanol particles. Reversal of charge occurs at $3{\cdot}96 \times 10^{-3}$M for SKF 525–A and $1{\cdot}12 \times 10^{-3}$M for chlorpromazine HCl. In both cases there is little effect on electrophoretic mobility below 10^{-5}M.

Electrophoretic mobilities of human erythrocytes in distilled water were found to be $-1{\cdot}1\ \mu s^{-1}$ $(V\ cm^{-1})^{-1}$ $(-1{\cdot}1 \times 10^{-8}\ m^2\ V^{-1}\ s^{-1})$ in good agreement with those quoted by Abramson, Moyer & Gorin (1942). SKF 525–A reduced the mobility of the erythrocytes, reversing their charge at $2{\cdot}5 \times 10^{-3}$M (see Fig. 5). These experiments were made in the absence of added salt and it is probable that in the presence of NaCl a greater lowering of electrophoretic mobility would be noted. Below 10^{-3}M SKF 525–A results in stabilization of the red cell membrane and above this concentration haemolysis ensues (Lee & others, 1968), SKF 525–A maintaining its ability to stabilize the membrane at concentrations as low as 10^{-9}M (Lee & others, 1968). This is surprising in view of the fact that the compound does not lower the surface tension noticeably until 10^{-6}M nor the electrophoretic mobility of the erythrocytes until concentrations of 10^{-4}M have been reached.

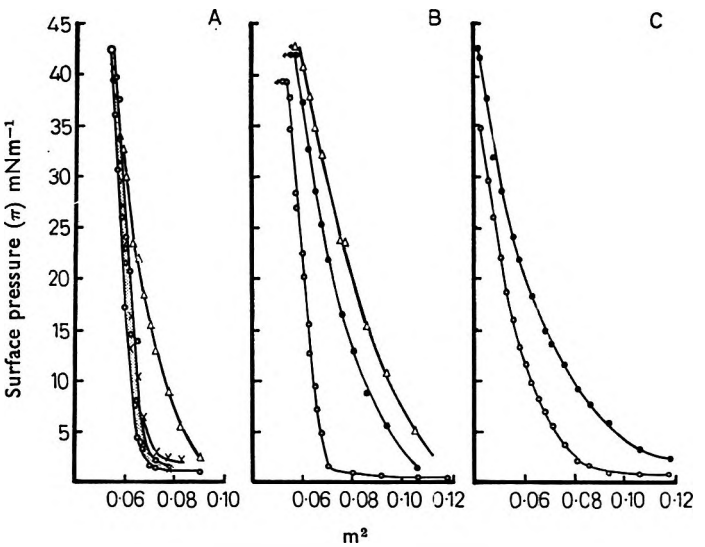

FIG. 6. Surface pressure, π (mNm^{-1}) *vs* area occupied by 10^{-3} g of film (m^2). A: ○ cholesterol film alone on water; × with $17{\cdot}6 \times 10^{-4}$ g $litre^{-1}$ SKF 525–A injected beneath the monolayer; △ with a total of $35{\cdot}5 \times 10^{-3}$ g $litre^{-1}$ SKF 525–A in substrate. B: ○ Cholesterol film alone; ● $17{\cdot}6 \times 10^{-3}$ g $litre^{-1}$ SKF 525–A after 1 h 40 min, △ total of $35{\cdot}5 \times 10^{-4}$ g $litre^{-1}$ SKF 525–A after 1 h. C: ○ Lecithin monolayer alone; ● with $17{\cdot}6 \times 10^{-4}$ g $litre^{-1}$ SKF 525–A injected beneath the monolayer. In Figs 6–7 stippled area indicates accuracy with pure monolayers.

The penetration of lipid monolayers by psycho-active drugs (Zografi & Auslander, 1965; Demel & van Deenen, 1966) and by local anaesthetics (Skou, 1961) has been studied in the belief that this should give some indication of the site of action of the drugs and an understanding of the mode of interaction of the drugs with the lipids.

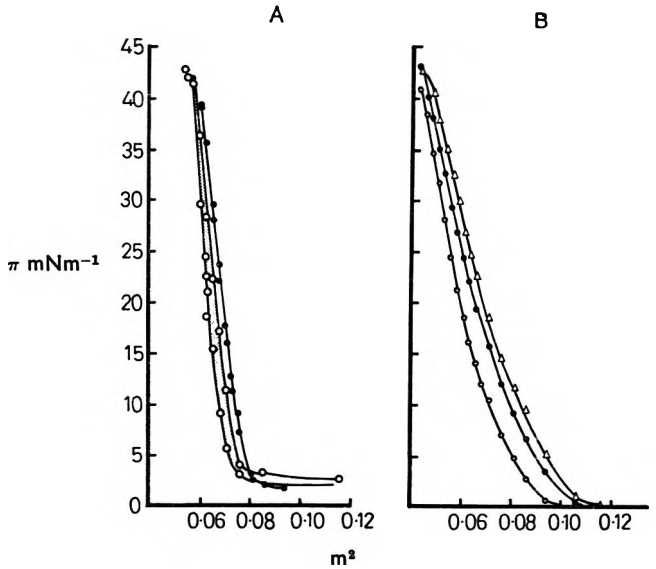

FIG. 7. A. ○ Cholesterol monolayer spread on water, ● with $17{\cdot}6 \times 10^{-4}$ g litre^{-1} chlorpromazine hydrochloride in substrate. B. ○ Lecithin monolayer spread on 0·9% NaCl; ● $17{\cdot}6 \times 10^{-4}$ g litre^{-1} chlorpromazine hydrochloride injected beneath the monolayer; △ with $35{\cdot}5 \times 10^{-4}$ g litre^{-1} chlorpromazine hydrochloride.

Because the membrane of the microsome has been implicated by Brodie (1956) as a possible site of action of SKF 525–A, studies were made with monolayers of cholesterol and lecithin spread on water and salt solutions beneath which had been injected SKF 525–A and chlorpromazine. Some results are shown in Figs 6 and 7 and Table 2. On water, SKF 525–A has no effect on cholesterol films and chlorpromazine has a limited effect (both drugs at 2×10^{-5}M). At 6×10^{-5}M SKF 525–A there was evidence of some interaction (Fig. 6). When the substrate was 0·9% NaCl much more pronounced interaction was noted between SKF 525–A and the cholesterol. On water, however, SKF 525–A interacts with the lecithin monolayer.

Table 2. *Increases in surface pressure and area caused by penetration into lecithin and cholesterol monolayers*

Compound	Monolayer	Substrate	Area at π = 30 mNm^{-1} (Å^2 = 10^{-20}m^2)*	Increase in surface pressure, at A = 0·06 m^2, dynes cm^{-1} or mNm^{-1}
SKF 525–A	Cholesterol	H_2O	37·3 Å^2 (37·9)	0
	Cholesterol	0·9% NaCl	41·2 Å^2 (37·2)	14·3
	Lecithin	H_2O	78·5 Å^2 (61·45)	12·0
	Lecithin	0·9% NaCl	65·1 Å^2 (57·3)	8·6
Chlorpromazine	Cholesterol	H_2O	41·75 Å^2 (39·8)	5
	Lecithin	0·9% NaCl	70·31 Å^2 (66·4)	5·9
Iproniazid	Cholesterol	H_2O	41·6 Å^2 (39·8)	3·0
	Lecithin	H_2O	66·7 Å^2 (61·5)	4·8

* Figures in brackets are the areas/molecule of the monolayer molecules before addition of drug.

Table 2 lists some of the results of the film penetration studies, showing the increases in surface pressure at a particular area/molecule of the original film and changes in apparent area/molecule at a surface pressure of 30 dynes cm^{-1} (mNm^{-1}). The results, in general, follow the trends observed by Zografi & Auslander (1965) in that penetration into the liquid expanded films of lecithin appears to be easier than into the condensed cholesterol films.

The penetration of both chlorpromazine and SKF 525–A into the cholesterol films changes the character of the monolayers from a condensed to a liquid expanded film (Figs 6 and 7).

Some work was done with iproniazid as it has been pointed out (Brodie, 1956) that although structurally unrelated to SKF 525–A, iproniazid inhibited the same enzyme systems as the latter. A 1% solution has a surface tension of 48 mNm^{-1} and interacts with lecithin and cholesterol monolayers to a small extent (Table 2).

In some cases (e.g., the lecithin-SKF 525–A system) the surface pressure increased over a long period (see Fig. 6). This is perhaps due to the low concentration of drug and the finite mixing time of the 1 ml of drug solution with the substrate liquid. It is not considered to be significant biologically.

DISCUSSION

The ability of SKF 525–A to lower surface tension is pronounced, the limiting surface tension being of the order of 29 mNm^{-1} which is comparable to the lowering of surface tension produced by many conventional surface-active agents. The CMC in water and saline is too high to be of biological significance, but the ability of the SKF 525–A molecules to penetrate cholesterol and lecithin monolayers at concentrations around 2×10^{-6}M is likely to be of importance in view of the proposed modes of action of the drug on the microsomal membrane. The difficulty however is to decide whether surface activity is simply incidental, i.e., the result of the amphipathic structure of the drug molecule or whether it is vital to the function of the drug (Florence, 1968). As most ionic surface-active molecules will penetrate spread insoluble monolayers it has also to be decided whether such interaction is of biological significance. As the drug affects the duration of activity of a number of drugs such as hexobarbitone it is reasonable to presume that SKF 525–A action is one on the microsomes either preventing the entry of the drug into the microsome, as Brodie suggests, or by an inhibitory effect on the particular enzyme involved. *In vitro* studies on enzyme systems are unlikely to answer this question as many ionic surfactants which are biologically inactive are known to inhibit enzyme systems, i.e., enzyme inhibition might simply be a by-product of the surface activity and is perhaps non-specific.

Adsorption of the positively charged SKF 525–A molecules at low concentrations does not reverse the charge on the surface of octadecanol or human erythrocytes. But in both cases the surface is not made positive. It is unlikely, therefore, that physical adsorption of SKF 525–A on the microsomal surface will act as a physical barrier repelling drugs of opposite charge, at low concentrations.

The penetration results show that the SKF 525–A molecule is capable of interacting with the components of cell membranes. Chlorpromazine, which has none of the enzyme inhibitory properties of SKF 525–A, also interacts with these components. Demel & van Deenan (1966) have shown that orphenadrine hydro-

chloride, which when given to rats *enhances* the inactivation of some drugs (Remmer, 1962), interacts with cholesterol monolayers but not significantly with lecithin spread on water.

Further work is necessary, perhaps with membrane diffusion cells, to determine whether the drugs alter the permeability of membranes in a specific way.

Acknowledgements

I thank Smith Kline and French Laboratories for the gift of the SKF 525–A, Roche Ltd., for the Marsilid sample and Mrs Christine Selkirk for technical assistance.

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The solubility of dodecane in water-amide mixtures

C. MCDONALD

Department of Pharmacy, The University, Manchester 13, *U.K.*

The solubility of dodecane has been determined in formamide, and in mixtures of water with formamide, *N*-methylformamide (NMF), and dimethylformamide (DMF) by a radiotracer method at 15°, 25° and 35°. The solubility of dodecane increases as the concentrations of the amides increase. The standard free energies ($\Delta G°$), partial enthalpies ($\Delta H°$), and standard entropies ($\Delta S°$), of solution of dodecane have been calculated for the various systems. Positive values of $\Delta G°$ and negative values of $\Delta H°$ and $\Delta S°$ decrease numerically as the concentrations of amides increase.

THE solubilities of hydrocarbons in water and in mixed aqueous solvents are of intrinsic interest. Solutions of hydrocarbons in such solvents also provide useful, but approximate, model systems for studying, for example, interactions between non-polar side chains of amino-acid residues in proteins, and the effects of non-polar portions of surfactants in micellization processes.

The solubility of dodecane in water-amide mixtures has been determined and the results are reported here.

EXPERIMENTAL

Materials

Dodecane (Fluka Chemicals, Purum grade) was used without further purification. Dodecane-1-^{14}C (Mallinkrodt Nuclear) was used without further purification. Formamide (B.D.H.) was stored over calcium oxide and was fractionated under reduced pressure before use and gave $n_{25}^{D} = 1{\cdot}4467$ ($n_{25}^{D} = 1{\cdot}4468$ Timmermans & Hennaut, 1935).

N-Methylformamide (NMF) (Fluka Chemicals, Purum grade) was treated as for formamide and gave $n_{25}^{D} = 1{\cdot}4315$ ($n_{19}^{D} = 1{\cdot}4313$, Davies & Thomas, 1956). Dimethylformamide (DMF) (B.D.H.) was treated as for formamide and gave $n_{25}^{D} = 1{\cdot}4271$ ($n_{25}^{D} = 1{\cdot}4269$, Ruhoff & Reid, 1937).

Solubility measurements

The mixed solvents were prepared by weight. For H_2O–formamide systems dodecane-1-^{14}C was diluted with 2 ml dodecane, and 0·5 ml aliquots of the resulting solution were transferred to separate solubility flasks where they were diluted to 2 ml with dodecane. Samples from these solutions were taken, added to 4 ml of Bray's solution (Bray, 1960) and their radioactivities, expressed as disintegrations per minute, were determined using an I.D.L. liquid scintillation counter, Type 6012.

For H_2O–NMF and H_2O–DMF systems dodecane-1-^{14}C was diluted to 5 ml with dodecane and 1 ml aliquots transferred to solubility flasks, leaving sufficient of the original solution to determine its radioactivity. Twenty ml quantities of the appropriate solvents were added to each flask. Samples were removed by syringe from

the solubility flask through an aperture closed with a vaccine bottle cap, which was previously washed in the appropriate solvent. As the aperture was below the level of the solvent–hydrocarbon interface, disturbance of this interface and possible contamination of the sample, by undissolved hydrocarbon, was avoided. The flasks were immersed in a thermostat bath at the required temperature, after allowing for vapour-saturation of the space above the liquids, and were shaken gently for 12 h. Preliminary experiments using decane and formamide had shown that saturation solubility was reached after approximately 8 h of shaking, but 12 h was thought to be a reasonable time to allow for any between solvent variations. After shaking was completed 100 μl samples were withdrawn from the flasks at regular intervals until no further change in solubility indicated that separation was complete. Solubilities were calculated from the ratios of radioactivities of hydrocarbon–saturated solvents to radioactivities of known quantities of the original dodecane-1-^{14}C-dodecane solutions. Densities were determined using a 25 ml pycnometer.

RESULTS AND DISCUSSION

The solubility of dodecane in the various solvents is shown in Table 1. It can be seen that at any one temperature, solubility increases as do the concentrations of non-aqueous solvents, and the values are much greater than the solubility of dodecane in water, $8{\cdot}9 \times 10^{-10}$ mol fraction (25°) (Franks, 1966). The solubility behaviour is obviously complex and may, to some extent, depend on interactions between water and the amides. Water is a highly structured liquid (Nemethy & Scheraga, 1962a) and irrespective of the type of structuring present in liquid water, the introduction of large quantities of non-aqueous solvents would be expected to have some effect on water and hence on its dissolving power. However, on the

Table 1. *The solubility of dodecane in water–amide mixtures* and in formamide*

Solvent	Temperature °C ± 0·1	Solubility (mol fraction)
Formamide	15°	$3{\cdot}6 \times 10^{-5} \pm 5\%$
	25°	$2{\cdot}6 \times 10^{-5} \pm 4\%$
	35°	$2{\cdot}3 \times 10^{-5} \pm 5\%$
Formamide 75% w/w ..	15°	$5{\cdot}9 \times 10^{-6} \pm 6\%$
(0·545 mol fraction)	25°	$3{\cdot}8 \times 10^{-6} \pm 7\%$
	35°	$2{\cdot}9 \times 10^{-6} \pm 8\%$
Formamide 55% w/w ..	15°	$2{\cdot}0 \times 10^{-6} \pm 6\%$
(0·328 mol fraction)	25°	$1{\cdot}1 \times 10^{-6} \pm 6\%$
	35°	$9{\cdot}5 \times 10^{-7} \pm 7\%$
NMF 50% w/w	15°	$6{\cdot}8 \times 10^{-6} \pm 3\%$
(0·234 mol fraction)	25°	$6{\cdot}0 \times 10^{-6} \pm 5\%$
	35°	$7{\cdot}3 \times 10^{-6} \pm 3\%$
NMF 25% w/w	15°	$3{\cdot}4 \times 10^{-6} \pm 5\%$
(0·092 mol fraction) ..	25°	$3{\cdot}0 \times 10^{-6} \pm 3\%$
	35°	$2{\cdot}9 \times 10^{-6} \pm 1\%$
DMF 60% w/w	15°	$1{\cdot}4 \times 10^{-5} \pm 3\%$
(0·269 mol fraction) ..	25°	$1{\cdot}2 \times 10^{-5} \pm 2\%$
	35°	$1{\cdot}6 \times 10^{-5} \pm 2\%$
DMF 30% w/w	15°	$4{\cdot}2 \times 10^{-6} \pm 3\%$
(0·095 mol fraction)	25°	$3{\cdot}6 \times 10^{-6} \pm 3\%$
	35°	$3{\cdot}4 \times 10^{-6} \pm 3\%$

* The solubilities were measured in these particular solvent mixtures to help elucidate the micellization process of some non-ionic surfactants with dodecyl hydrocarbon chains in the same solvents.

available evidence it is difficult to decide how the various amides affect, and are affected by, water. It has been suggested (Fratiello, 1963), from nuclear magnetic resonance measurements, that the breakup in the co-operative structure of water is greater in the presence of DMF than in the presence of either formamide or NMF. Further reference to Table 1 shows that, on a mol fraction basis, DMF is most effective, and formamide least effective in increasing the solubility of dodecane, which may be consistent with DMF's ability to disrupt the structure of water more than the other amides. Also, empirically, on the basis of "like dissolving like" it would be expected that DMF, with two methyl groups, would have a greater affinity for dodecane than would formamide, which has no methyl groups.

The effects of the various amides on the solubility of dodecane may be explained more rationally from a consideration of the solubility parameters of the solvents and of the solute. As solubility parameters were initially derived (Hildebrand & Scott, 1964a) to describe solubility characteristics of non-polar molecules they may not be strictly applicable to the solvents considered here. It has been shown, however (Burrell, 1955), that apparent solubility parameters of polar liquids are useful for the approximate characterizations of the solution power of such liquids. The apparent solubility parameters of H_2O, formamide, NMF and DMF are 23·4, 19·4, 16·1 and 12·2 respectively (Walker, 1952; Bauder & Gunthard, 1958; Hildebrand & Scott, 1964b) and the solubility parameter of dodecane interpolated from the data of Hildebrand & Scott (1964c) is approximately 8·0. The solubility parameter of a mixed solvent will be intermediate between those of the individual components and will depend on their concentrations, which are most conveniently expressed as either volume fractions or mol fractions (Burrell, 1955). For H_2O–amide mixtures containing the same mol fractions of formamide, NMF or DMF, the H_2O–DMF solvent would have the lowest and the H_2O-formamide solvent the highest solubility parameter, while the solubility of dodecane would be expected to be greatest in the former solvent and least in the latter, with the H_2O–NMF solvent occupying an intermediate position in terms of solubility parameter and solubility of dodecane. This is confirmed in Table 1.

It is interesting to note that for the systems studied the dielectric constants of the amides at 25° are 109·5, 182·4 and 36·7 for formamide, NMF and DMF respectively (Leader, 1951; Leader & Gormley, 1951). Thus solvents containing formamide or NMF and water would have higher dielectric constants than water (78·5, 25°), and those containing DMF would have dielectric constants less than that of water. In all cases, irrespective of the resultant dielectric constants of the solvents, addition of amides increases the solubility of dodecane.

Thermodynamics of solution

In calculating the thermodynamics of solution the standard states are taken as unit mol fractions in pure liquid hydrocarbon and in solution, at a pressure of 1 atmosphere (Nemethy & Scheraga, 1962b). By the choice of these standard states the calculated free energies and entropies can be explained on the basis of interactions between solute and solvent molecules and any changes in solvent structuring caused by these interactions (Kauzmann, 1959). The standard free energies of solution ΔG^o are calculated from the solubility values ($\Delta G^o = -RT \ln X$, X = mol fraction solubility) and partial enthalpies of solution, ΔH^o, from temperature variations of

solubility. The standard entropy of solution, ΔS^o, is calculated by combining ΔG^o and ΔH^o, (Equation 1):

$$\Delta S^o = \frac{\Delta H^o - \Delta G^o}{T} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

The thermodynamic parameters at 25° are shown in Table 2.

Table 2. *Thermodynamics of solution of dodecane in various solvents at* 25°

Solvent	ΔG^o kJ mol^{-1}	ΔH^o kJ mol^{-1}	ΔS^o J mol^{-1} deg^{-1}
Formamide	26·1	—20·1	—155
Formamide 75% w/w ..	30·9	—25·9	—193
Formamide 55% w/w ..	33·9	—33·5	—226
NMF 50% w/w	29·8	0	—100
NMF 25% w/w	31·5	—6·3	—126
DMF 60% w/w	28·0	0	— 92
DMF 30% w/w	31·0	—8·4	—134

In the formamide and H_2O–formamide systems it may be seen that favourable enthalpy values are outweighed by large negative entropies thus giving positive free energies of solution and hence low solubilities. As the concentrations of formamide increase, the values of ΔH^o and ΔS^o decrease numerically. For the solution of non-electrolytes in water, negative enthalpies and entropies are generally considered to be due to an increase in the structuring of water in the vicinity of the solute molecule (Frank & Evans, 1945). It would appear, therefore, that the addition of formamide to water results in a decrease in solvent structuring around the solute molecule. In the mixed H_2O–formamide solvents, in the vicinity of the solute, this may be brought about by replacement of water molecules by formamide molecules alone, by the formation of H_2O–formamide complexes, by a reduction in the size of the clusters of water which are thought to surround, partially, the solute molecules (Nemethy & Scheraga, 1962b), or by a combination of any or all of these factors. It is not possible to distinguish which factors are involved. In pure formamide the thermodynamic parameters still indicate the possibility of some solvent structuring around the solute molecule.

Similar thermodynamic parameters are obtained for the solution of dodecane in H_2O–NMF and H_2O–DMF solvents. For the solvents containing the lower quantities of NMF and DMF, ΔH^o and ΔS^o are negative but these values are much lower, i.e., nearer to zero, than in H_2O–formamide systems, and in the solvents which are more concentrated with respect to NMF and DMF, ΔH^o values are, within experimental error, zero. This would suggest that NMF and DMF have a greater disruptive effect on the structure of water, at least in regions close to solute molecules.

A study of the thermodynamic properties of a solution cannot give unambiguous evidence for, or against, a particular physical model for the solution, but they must be consistent, qualitatively, with the assumptions upon which the model is based. If Frank & Evans "iceberg" model for the solution of aliphatic hydrocarbons in water is taken as a starting point, the changes in the thermodynamics of solution of dodecane in the mixed solvents are consistent with a decrease in the size of the "iceberg" around the hydrocarbon. Other factors which might influence the thermodynamic parameters are the self-association of amide molecules, which will alter with

temperature (Fratiello, 1963), and possible changes in the configuration of the hydrocarbon chain in the various solvents.

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Kinetics of buccal absorption of some carboxylic acids and the correlation of the rate constants and n-heptane:aqueous phase partition coefficients

A. H. BECKETT AND A. C. MOFFAT*

Department of Pharmacy, Chelsea College (University of London), Manresa Road, London, S.W.3, U.K.

A simple model for the buccal absorption of some carboxylic acids is proposed. This model has been used, in conjunction with an analogue computer, to determine the rate constants of buccal absorption of ten acids from solutions of pH 4·0 using a single subject. These rate constants gave a positive correlation with the logarithms of previously determined n-neptane:0·1N hydrochloric acid partition coefficients (correlation coefficient 0·89).

Beckett, Boyes & Triggs (1968) studied the kinetics of buccal absorption of amphetamines using an analogue computer. Their results indicated that kinetic constants may be useful in assigning numerical values to the relative partitioning properties of drugs into the oral mucosa. Data from previous studies (Beckett & Moffat, 1968, 1969a,b), of 5 min cumulative absorptions, showed the most important criteria for the rapid absorption of drugs to be that the drugs should be in their unionized forms and that these forms should have large partition coefficients. The rate constants of absorption should therefore show a similar dependence on these two physico-chemical features. Ten acids, with previously determined pK_a values and partition coefficients (Beckett & Moffat, 1969b), were therefore used to test this hypothesis.

EXPERIMENTAL AND RESULTS

Buccal absorption

The method previously described (Beckett & Moffat, 1968) was used with the following modifications. Drug solution (25 ml McIlvaine citric acid-phosphate buffer, pH 4·00) containing 1 mg of drug was introduced into the subject's mouth for 1, 2, 3 . . . 10 min intervals. The waiting period between each test was 30 min for tests taking 1 to 5 min, increasing to 50 min for the 10 min test.

A typical result is shown in Fig. 1 where the points represent the experimentally determined absorptions of *o*-toluic acid. There was little transfer of acid from the buccal mucosa back to the oral cavity after a test, even when a buffer solution of pH 9·09 was used for 5 min as a mouthwash.

* Present address: Institute for Lipid Research, Baylor College of Medicine, Texas Medical Center, Houston, Texas, 77025, U.S.A.

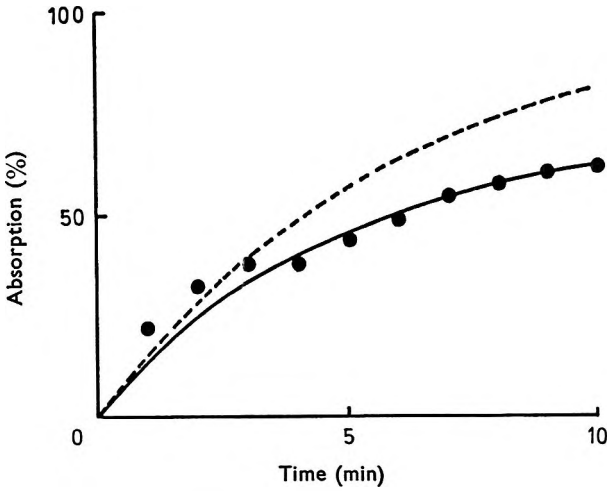

FIG. 1. Buccal absorption of *o*-toluic acid at pH 4·0. The points show the experimental results. the solid line the computer calculation and the dotted line the computer calculation ignoring the changes of pH and volume of the buffer solution.

Mathematical treatment

Apparatus. A TR–20R Analogue computer (Electronics Associates Ltd.) was used.

Method. Inspection of the above results suggested the simplest useful compartmental model for the buccal absorption of some carboxylic acids to be:

$$\underset{\text{(A)}}{\text{Drug buffer solution in mouth}} \xrightarrow{K_1} \underset{\text{(B)}}{\text{Drug in buccal mucosa}}$$

The following mathematical equations were used to describe the transfer:

$$-\frac{dA}{dt} = K_1 \frac{RA}{V}$$

$$\frac{dB}{dt} = K_1 \frac{RA}{V}$$

Where A and B = percentage of drug in the respective compartments
K_1 = rate constant governing the transfer of unionized drug molecules between compartments (ml min^{-1})
R = fraction of drug unionized at any time t
V = volume of buffer solution in the oral cavity at any time t.

To compensate for the increase in pH and volume of the buffer solution, and associated decrease in the value of R/V during the course of the experiments, a variable diode function generator was used in the computer program (Fig. 2). Although the recorded changes of R/V were for individual tests, by simulating their values with time an approximation of their values over the whole 10 min test period can be made for each drug, e.g. Fig. 3.

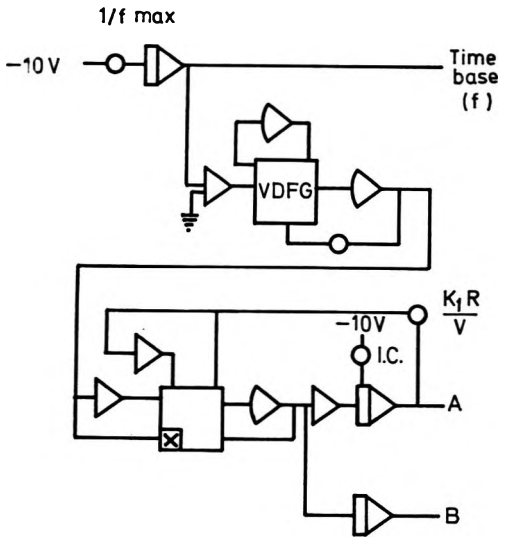

FIG. 2. Analogue computer program for the study of the kinetics of buccal absorption of some carboxylic acids.

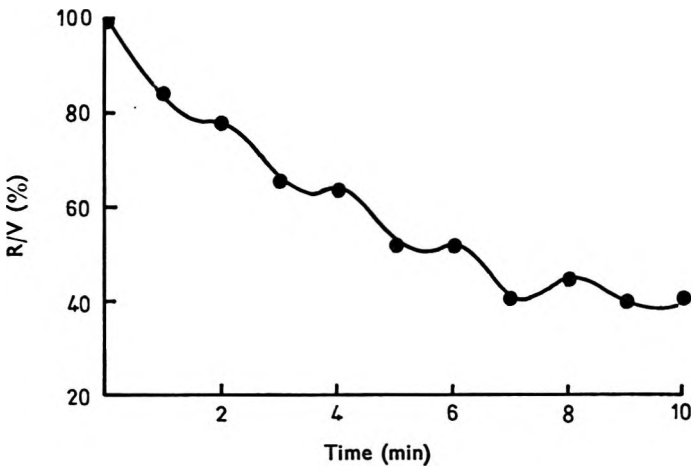

FIG. 3. Change in the fraction of unionized molecules/volume (R/V) with time during the buccal absorption test using *o*-toluic acid. The points show the experimental results, the line the variable diode function generator simulation.

The computer solutions for the absorptions of each acid were obtained by programming the variable diode function generator for each acid and by systematically altering the potentiometer representing K_1 R/V until good agreement was obtained between the computer calculations for the amount of drug absorbed and the experimental data. Since R/V values were known at the start of the test, K_1 could be calculated from the potentiometer setting.

The values of the kinetic parameters for the buccal absorption of each 'drug' are summarized in Table 1.

Table 1. *Physico-chemical constants and kinetic parameters for the buccal absorption of some carboxylic acids by one subject*

Acid	pKa at 37°[1]	At start of test: Buffer pH	At start of test: Volume (V_1) (ml)	At start of test: R_1/V_1	At start of test: K_1R/V	K_1 (ml min^{-1})	n-Heptane: 0·1N HC partition coefficient ($ml^{1/3}\mu g^{-1/3}$)[2]
Benzoic	4·21	4·00	25·0	0·0248	0·16	6·5	0·11
o-Toluic	3·92	4·00	25·0	0·0182	0·17	9·3	0·25
m-Toluic	4·24	4·00	25·0	0·0254	0·225	8·9	0·31
p-Toluic	4·33	4·00	25·0	0·0272	0·26	9·6	0·23
2,4-Dimethylbenzoic	4·28	4·00	25·0	0·0227	0·245	10·6	0·88
2,5-Dimethylbenzoic	4·05	4·00	25·0	0·0212	0·27	12·7	0·63
3,5-Dimethylbenzoic	4·31	4·00	25·0	0·0268	0·26	9·7	0·68
o-Chlorophenylacetic ..	4·07[1]	4·00	25·0	0·0216	0·11	5·1	0·03
m-Chlorophenylacetic ..	4·14[1]	4·00	25·0	0·0232	0·13	5·6	0·11
p-Chlorophenylacetic ..	4·19[1]	4·00	25·0	0·0243	0·15	6·2	0·06

[1] From Beckett & Moffat (1969b).
[2] At 25°.

DISCUSSION

A linear relation between absorption and time was not obtained after plotting the results on semi-log paper. This indicated that the absorption was apparently not a simple first order process. However, there was little return of the acid to the oral cavity after a test. Also, the increase in pH and volume of the buffer solution during a test diminished the concentration of unionized drug and its subsequent absorption. Thus, it was considered that a good approximation of the results could be obtained using a two compartmental model which incorporated volume and pH changes but a first order process.

The model is the simplest that could be devised for a system such as this. It may not be the correct one since other compartments, e.g., the blood, may need to be considered. However, good fits to the experimental points were obtained, e.g., Fig. 1. The proposed computer model is therefore a good mathematical approximation of the biological system. The necessity of including the generated function (R/V) with time is clearly seen by comparing the experimental buccal absorption data, the computer model simulation and a first order curve having the same K_1 value for *o*-toluic acid (Fig. 1). The first order curve is very much above that of the computer curve and buccal absorption data. Beckett & others (1968) used a three compartmental model for the buccal absorption of some amphetamines involving four rate constants, one of which did not exist until certain levels in other compartments had been reached. They also assumed that the volumes of the compartments did not change appreciably. Thus it is possible that by using the much simpler, more logical model now proposed, a good fit could have been obtained. It is not possible to put this to the test, since the authors had not measured volume or pH changes.

The rates of absorption of the acids studied are directly proportional to the fraction of acid in the unionized form as shown by the good fit of the experimental data to the computer simulation. This explains why buccal absorption-pH curves rise so steeply as they move from ionized to unionized molecules (see Beckett & Triggs, 1967; Beckett & Moffat, 1968, 1969a). The different rate constants for absorption of the acids are due solely to the different abilities of the unionized forms to penetrate the buccal muscosa. Plotting the rate constants for the different acids against the

logarithms of their n-heptane:0·1N hydrochloric acid partition coefficients gives a straight line (Fig. 4) (correlation coefficient 0·89), viz.

$$K_1 = 4{\cdot}42 \log K + 11{\cdot}4$$

Where K_1 = rate constant (ml min^{-1})

K = partition coefficient ($ml^{1/2}\,\mu g^{-1/2}$)

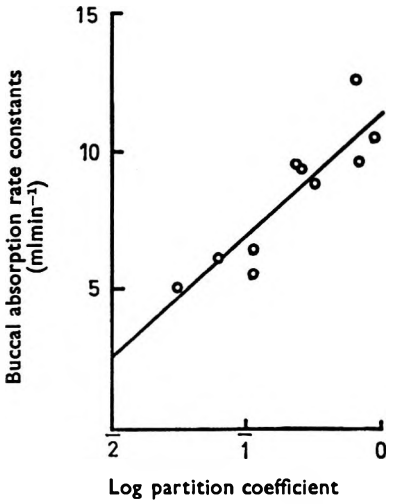

FIG. 4. Correlation of n-heptane:0·1N hydrochloric acid partition coefficients of some carboxylic acids with their rates of buccal absorption (correlation coefficient 0·89).

Although the relation is empirical it shows that n-heptane has similar properties to the lipid membrane of the buccal mucosa with respect to absorptions of the acids used in these experiments, which is in agreement with our previous findings concerning the good positive correlations of n-heptane:aqueous phase partition coefficients with buccal absorption data using acids and amines at 1 and 10% levels of unionization.

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Further structure-activity relations of heterocyclic analogues of hemicholinium-3

F. W. BENZ AND J. P. LONG

Department of Pharmacology, University of Iowa, Iowa City, Iowa, U.S.A.

The importance of the 3-methyl group on the pyridinium ring of bis-quaternary nitrogen salts for hemicholinium-3-like activity having been conditionally established (Benz & Long, 1969a,b), derivatives containing classical isosteres of the 3-methyl group and the three oxidation states of the 3-methyl group were examined. Oxidation of the 3-methyl group to $-CH_2OH$ decreased activity tenfold. Subsequent oxidation to –CHO and –COOH further decreased activity. When the 3-methyl group was replaced by a halogen, activity was maintained by the iodo-derivative but decreased as the size of the halogen decreased and as the electronegativity increased. Substitution of an ethyl group for the 3-methyl decreased activity twofold, whereas replacement with –OH eliminated activity.

It has previously been shown (Benz & Long, 1969a) that the hemiketal moiety of hemicholinium-3 (HC-3) can be replaced by the 3-methylpyridinium cationic substituent without loss of activity. That the *m*-methyl group enhanced activity was demonstrated by the fact that both the *ortho* and *para* isomers were inactive as was the unsubstituted pyridinium ring. It was hoped that the same structural specificity would extend to the isomeric piperidinium series. However, this was not found to be so. The 4-methylpiperidinium derivative was equipotent to HC-3, whereas the 3-methylpiperidinium analogue was much less active. The low activity of the 3-methylpiperidinium analogue was tentatively explained on the basis of enantiomeric dilution coupled with a possible low yield of the active conformer on quaternization (Benz & Long, 1969a). This hypothesis is presently being investigated using nmr spectroscopy.

To further investigate the structural requirements for activity, a series of dimethylalkylammonium and dimethylpyridinium analogues was synthesized (Benz & Long, 1969b). It was thought that perhaps the carbon unit linking the quaternary nitrogen to the *m*-methyl group on the 3-methylpyridinium system could be mimicked by a simple alkyl chain. Thus, a three carbon chain with one double bond should be optimal for activity. This expectation was realized since highest activity was observed with the allyl derivative. Activity decreased as the carbon chain was lengthened, shortened or if the double bond was deleted. In the dimethylpyridinium series it was shown that addition of a second methyl group to the ring, in addition to the 3-methyl group, decreased activity in all cases. This was especially surprising for the 3,5-dimethylpyridinium derivative. This compound had a methyl group in each of its *meta*-positions, thereby increasing the statistical likelihood of the *m*-methyl group coming in contact with its receptor. However, this compound was only 1/20 as active as its monomethyl isomer.

The purpose of this communication is to report the results of our experiments with another series of heterocyclic HC-3 analogues, and to discuss their structure-activity relation.

EXPERIMENTAL

Chemistry

The compounds were synthesized according to the procedure described for hemicholinium (Long & Schueler, 1954). Carbon, hydrogen and nitrogen analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y., or by Galbraith Laboratories, Inc., Knoxville, Tenn. All combustion analyses fell within $\pm 0{\cdot}3\%$ of the calculated percentages. Infrared spectra were obtained with a Beckman model IR-10 recording infrared spectrometer as 1% mixtures of the quaternary salt in potassium bromide. Nuclear magnetic resonance spectra were obtained on a Varian A-60 spectrometer. Compounds were run as 1 to 10% solutions in d_6-dimethyl sulphoxide with tetramethylsilane as an internal standard.

Pharmacology

The 24-h LD50 for each compound was determined using three groups of 10 mice, each weighing 20–25 g. The animals received the compound intraperitoneally and were observed for the type and time for onset of symptoms. The LD50 and its 95% fiducial limits were estimated by the method of Litchfield & Wilcoxon (1948).

Two frequencies of nerve stimulation were used in the sciatic nerve-gastrocnemius muscle preparation of Duch rabbits. The rabbits, weighing 1 to 2 kg, were anaesthetized with phenobarbitone sodium, 200 mg/kg, administered intravenously. Interrupted tetanic stimulation (high frequency) of 250 Hz, with a pulse duration of 1 ms and maximal voltage (10–15 V), was applied to the sciatic nerve for 0·2 s every 10 s. A Grass stimulator (model S-4C) with appropriate circuit interrupter was used in these studies. The other experiments (low frequency) utilized similar preparations, except single shock stimulations every 10 s with a pulse duration of 5 ms and a voltage of 10–15 V were used. Application of a stimulus with these parameters elicited maximal muscle contraction. Only the most active compounds at the higher frequency of stimulation were tested with single shock. The relative potencies and 95% fiducial limits for the higher frequency stimulations were calculated by the method of Finney (1952) using a 2×2 parallel line assay. In these assays five animals were used for each dose and the doses were varied by a 0·48 log interval. The ED50 was estimated by using a log dose-probability plot. The ED50 was not determined at the lower frequency of stimulation since our only purpose for testing the most active agents was to qualitatively demonstrate their relative inactivity at single shock with respect to their activity at interrupted tetanic stimulation. All drugs were administered via the marginal ear vein.

The ability of the compounds to inhibit human red blood cell acetylcholinesterase was measured with a thermostatic recording pH stat (S-30240, E. H. Sargent). The specific methods have been published previously (Benz & Long, 1969a,b).

RESULTS

Neuromuscular inhibition in rabbit

In this preparation HC-3 causes a neuromuscular blockade characterized by its slow onset (approximately 5 min after i.v. administration), long duration (approximately 2–3 h), dependence on the frequency of nerve stimulation (the higher the frequency, the greater the blockade) and specific antagonism by choline (1–5 mg/kg).

Any compound causing a neuromuscular blockade with these characteristics will be termed HC-3-like.

Compound 1 showed only weak HC-3-like activity. Maximum blockade occurred 15–30 min after intravenous administration with a duration of action of about 1h. Its effects were only partially antagonized by choline, 10 mg/kg. An initial drop in twitch tension occurred immediately, followed by the slow developing HC-3 effect. Compounds 2 and 3 demonstrated qualitatively the same effects.

In the halogen series, HC-3-like properties became more pronounced as the atomic size of the halogen was increased to iodine. The time for maximum blockade was 10–20 min for the chloro-(compound 4), 30–40 min for the bromo-(compound 5) and 35–45 min for the iodo-derivative (compound 6). The effect of the iodo-derivative was readily reversed by choline, 1–2 mg/kg, whereas the effect of the chloro-derivative was less so. The slowly developing blockade caused by compounds 4 and 5 was always preceded by a small initial drop in twitch tension. This was not true with compound 6. However, compound 6 did elicit the initial drop in twitch tension at higher doses. This transient initial phase of action has been observed previously (Benz & Long, 1969a,b) and is thought to be due to the compounds' ability to inhibit acetylcholinesterase. Compounds 7 and 10 exhibited HC-3-like properties, but compounds 8, 9 and 11 did not.

At high frequency nerve stimulation, compounds 6 and 7 were the most active agents studied. Their activity was evaluated in seven preparations at low frequency nerve stimulation and was found to be significantly less, as would be expected for an HC-3-like agent. Doses of 1 mg/kg were necessary to produce a blockade which was rapid in onset and was accompanied by salivation, lacrimation and defaecation. At lower doses these compounds enhanced neuromuscular transmission. The latter two effects are explainable on the basis of the compounds' ability to inhibit acetylcholinesterase (Table 1).

Mouse toxicity (Table 1)

Toxicity in mice paralleled the various compounds' ability to block neuromuscular transmission in the rabbit. The most active HC-3-like agents in the rabbit elicited symptoms of HC-3 poisoning upon intraperitoneal administration to mice as outlined by Long & Schueler in 1954. The most active acetylcholinesterase inhibitors caused marked salivation, urination, defaecation and fasciculations before death occurred. Fasciculations continued even after respiratory movements ceased.

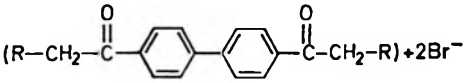
Acetylcholinesterase inhibition (Table 1)

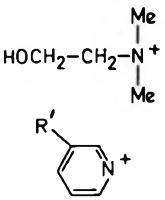
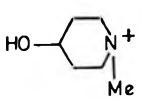
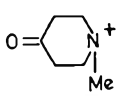
All compounds demonstrated the ability to inhibit human red blood cell acetylcholinesterase. A qualititative difference in the manner in which these compounds inhibited the enzyme, as compared to neostigmine, was observed. Neostigmine required an induction period of a few minutes before maximum inhibition occurred, whereas the present compounds inhibit maximally, immediately upon being added to the incubation medium.

DISCUSSION

It has previously been shown that placement of a methyl group specifically in the 3-position on the pyridinium ring or in the 4-position on the piperidinium ring greatly

Table 1. *Biological data for substituted nitrogen heterocyclic derivatives of* HC-3



Compound	Structure R =	Neuromuscular inhibition in rabbit Interrupted tetanic R.P.[a]	Neuromuscular inhibition in rabbit Interrupted tetanic ED50 (mg/kg)	Toxicity in mice LD50 (mg/kg)	Acetylcholinesterase inhibition ID50 (M)
		1·0	0·013	0·12 (0·09–0·17)	
1	R′ = CH_2OH	0·10 (0·09–0·13)	0·142	1·3 (1·1–1·5)	$3·8 \times 10^{-8}$
2	R′ = CHO	0·05 (0·03–0·07)	0·290	2·3 (1·4–3·7)	$1·3 \times 10^{-7}$
3	R′ = COOH	<0·03	>0·5	>20·0	
4	R′ = Cl	0·19 (0·06–0·32)	0·072	0·35 (0·31–0·41)	$1·2 \times 10^{-8}$
5	R′ = Br	0·24 (0·13–0·82)	0·053	0·27 (0·20–0·35)	$2·5 \times 10^{-8}$
6	R′ = I	0·98 (0·56–1·68)	0·013	0·13 (0·11–0·14)	$1·5 \times 10^{-8}$
7	R′ = Et	0·49 (0·22–1·43)	0·027	0·21 (0·15–0·28)	$2·2 \times 10^{-8}$
8	R′ = OH	<0·03	>0·5	>20·0	$5·6 \times 10^{-6}$
9		<0·03	>0·5	6·3 (5·6–7·1)	$3·8 \times 10^{-7}$
10		0·09 (0·04–0·25)	0·150	1·10 (0·77–1·57)	$6·9 \times 10^{-7}$
11		<0·03	>0·5	3·00 (2·5–3·5)	$2·1 \times 10^{-8}$
	Neostigmine	—	—	—	$5·2 \times 10^{-7}$

[a] R.P. = relative potency with respect to HC-3 = 1·00 and 95% fiducial limits.

enhanced the HC-3-like neuromuscular blocking activity of these cationic substituents (Benz & Long, 1969a). To rule out the possibility that one of the oxidation products of the methyl group was responsible for the activity, compounds 1–3 were evaluated. The most active agent was compound 1. Its activity was approximately 1/10 that of the 3-methylpyridinium analogue (compound 2, Benz & Long, 1969a), which is equipotent to HC-3. None of these products can account for the high activity of the 3-methylpyridinium system. Another series of this type has been studied previously (Marshall & Long, 1959).

Since the methyl group may be regarded as an inert space filler (Schatz, 1960), it was of interest to apply the principles of bioisosterism to this series. Four classical isosteres of the methyl group, three halogens and the hydroxyl group, were evaluated. Activity increased from the chloro-derivative (compound 4) which was 1/5 as active, through the bromo-derivative (compound 5) which was 1/4 as active, to the iodo derivative (compound 6) which was equipotent to HC-3. In the literature, it has been estimated that the methyl and chloro-groups occupy similar volumes (Burger & Foggio, 1956). However, the van der Waals radii for the various halogens and the methyl group should give a better indication of the sphere of influence of these groups, since this is the optimum distance from the nucleus beyond which the electron cloud of a non-bonded atom cannot easily advance. The van der Waals radius of a methyl group has been found by a number of investigations to approach 0·2 nm (Cromer, Ihde & Ritter, 1951; Steinfink, Post & Fankuchen, 1955). The van der Waals radii of chlorine, bromine and iodine are 0·180, 0·195 and 0·215 nm respectively.* Thus, bromine or perhaps iodine would best represent the volume of a methyl group. However, since bromine is more electronegative than iodine, it is not unreasonable to expect highest activity with 3-iodo-derivative (compound 6), since it would be most capable of mimicking a methyl group as an inert space filler.

The inactivity of the *m*-hydroxy isomer (compound 8) can be similarly explained. It is well known that phenols are stronger acids than alcohols, due to the fact that the oxygen anion can delocalize into the ring system. This is even more true for compound 8 since the hydroxyl group is *meta* to a quaternary nitrogen atom which will withdraw electrons from the oxygen atom by induction, rendering the hydroxyl group more acidic. A model compound for comparison would be 3-hydroxy-*N*-methylpyridinium which has a $pK_a = 4{\cdot}99$ (Bridges, Davies & Williams, 1966). Since this molecule is ionized at pH 7·4, compound 8 may be also. The oxygen anion thus formed may be too polar and thus, does not mimic the methyl group.

From the above discussion, and as one would expect, the receptor site for the methyl group seems to be hydrophobic in nature, as compounds with non-polar substituents on the ring have higher activity. This theory was further confirmed by the relatively high activity of the 3-ethylpyridinium analogue (compound 7). This compound was approximately 1/2 as active as the 3-methyl derivative, which is equipotent to HC-3. It is apparent that the receptor can accept the longer 3-ethyl group, but its activity is less than that of the 3-methyl derivative. Compound 7 presented the necessary hydrophobic group to the methyl receptor, but this group was too large for ideal binding and therefore its activity was reduced.

From the low activity exhibited by the piperidinium analogues (compounds 9–10), it appears that the binding site for the 4-methyl group on the piperidinium system

* From Framework Molecular Models, Prentice Hall, Inc., Englewood Cliffs, N.J.

(compound 6, Benz & Long, 1969a) is also specific, lending further evidence that its binding site and that of the 3-methyl group on the pyridinium system may be the same or of the same type.

It has previously been suggested (Benz & Long, 1969a) that another factor may be considered in relating structure with biological activity in compounds of this type. This hypothesis focuses on the acidity of the protons on the methylene group joining the carbonyl group to the quaternary nitrogen. According to this hypothesis one might conjecture that activity may be associated with the acidity of such protons or with an interaction of the anion thus formed.

The importance of increased acidity of the methylene protons, which join the carbonyl and quaternary nitrogen head, in relation to the biological activity of the molecule is minimized when one reflects on the relative activity of the various analogues in this and the previous series. In the monomethyl pyridinium and piperidinium series (Benz & Long, 1969a) this theory would predict highest activity for the unsubstituted heterocycles, when in fact the 3-methylpyridinium and 4-methylpiperidinium analogues were found to be the most active. Likewise, in the present series, this theory would predict high activity for the 3-hydroxy (compound 8) and the 3-chloro (compound 4) derivatives since they would decrease the electron density about the quaternary nitrogen, thereby increasing the acidity of the methylene protons the most. Yet, the 3-hydroxy derivative was inactive and the 3-chloro- was much less active than the 3-iodi-derivative. The 3-iodo- and 3-methyl analogues were equipotent biologically, yet the former exerted a −Inductive effect on the quaternary nitrogen, whereas the 3-methyl exerted a +Inductive effect.

These inductive effects of the halogens and the methyl group on the pyridinium ring system have been adequately demonstrated by Brown and co-workers (Brown & McDaniel, 1955). Therefore, it seems unlikely that the heterocyclic ring substituents exert their effects via increasing or decreasing electron density on the quaternary head which in turn would influence acidity of the methylene group. Stereochemical and volume factors of the ring substituents appear to be more important.

Acknowledgements

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Some pharmacodynamic effects of the nematocides: methyridine, tetramisole and pyrantel

P. EYRE*

Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh 9, U.K.

Methyridine, tetramisole and pyrantel affect several cholinergic mechanisms. In the rabbit, methyridine and tetramisole cause hypotension which is partially antagonized by atropine. In the cat, tetramisole causes an increase in blood pressure and causes prolonged contraction of the nictitating membrane. The evidence indicates that tetramisole may release catecholamines from the adrenal medulla. Pyrantel contracts the nictitating membrane; an effect which is abolished completely by hexamethonium. The three anthelmintics block neuromuscular transmission in a way which is characteristic of non-competitive depolarizing agents. They also inhibit cholinesterases. These pharmacological data correlate closely with the reported clinical signs of the toxicity of these agents.

The compounds methyridrine, tetramisole and pyrantel are of relatively recent introduction to antinematodal chemotherapy. Methyridine [2-(2 methoxyethyl) pyridine], a colourless water-miscible liquid, was introduced in 1961. It was the first anthelmintic to possess the then novel characteristic of being more efficient when given subcutaneously than when given by mouth (Broome & Greenhalgh, 1961). The drug is moderately well-tolerated at approximately 200 mg/kg (Walley, 1961) Dullness and anorexia have been reported but the most marked effect is local oedema at the site of injection, followed by necrosis (Thorpe, 1962). Broome (1961) described the distribution of the drug in the body and also presented evidence of depolarizing neuromuscular block in the parasite. Catarsini & Gagliano (1963) observed that methyridine inhibited blood cholinesterases in sheep. In view of these reports it was of interest to study further the actions of methyridine on cholinergic mechanisms.

Tetramisole {(±)-1,2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole HCl}, a more recently introduced nematocide (Thienpont, Vanparijs & others, 1966), is a white water-soluble powder, most frequently given orally but may be injected subcutaneously. Its efficacy was established by Walley (1966). The therapeutic dosage (15 mg/kg orally or 12·5 mg/kg subcutaneously) produces depression of the central nervous system and occasional "muscular twitching" (Forsyth, 1966; Walley, 1966). Doses greater than this produced salivation and more marked and frequent muscular tremors. Kaemmerer & Budden (1966) demonstrated that small doses of tetramisole produced a pressor effect, while larger doses were depressor in the cat. In cattle and sheep there was lachrymation, salivation, increased gut activity and clonic muscular spasms at doses from 15–80 mg/kg subcutaneously. A feature in cattle

* Present address: Section of Pharmacology, Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada.

was spastic tail erection similar to the morphine "Straub" phenomenon in mice. In view of these side-effects it seemed important to investigate the action of tetramisole on autonomic mechanisms.

Another nematocide, pyrantel tartrate {*trans*-1,4,5,6-tetrahydro-1-methyl-2-[2-(thienyl)vinyl]pyrimidine}, has been used in gastrointestinal parasitism in sheep (Austin, Courtney & others, 1966; Cornwell, 1966; Cornwell, Berry & others, 1966a, b). It is a water-soluble white powder and is administered orally at a dose of 25 mg/kg. Cornwell (1966) did not report any untoward side-effects at the dose used.

EXPERIMENTAL

Guinea-pig isolated ileum

Short lengths of guinea-pig terminal ileum were set up in the usual way in aerated Tyrode solution at 35° in a 15 ml organ bath. Isotonic contractions were recorded with a linear motion transducer and a "Grass" polygraph. Acetylcholine, histamine and the anthelmintics were added to the bath for 30 s every 2 min.

Cat nictitating membrane preparation

Experiments were made on 6 cats anaesthetized with sodium pentobarbitone (30 mg/kg, i.v. or i.p). The cats were prepared as described by Bowman, Callingham & Cuthbert (1964). Semi-isometric contractions of the nictitating membrane were elicited by stimulating supramaximally the preganglionic sympathetic trunk with 10 rectangular shocks/s (0·5 ms duration). Contraction of the membrane, femoral arterial blood pressure and respiratory volume were recorded with appropriate electronic transducers and a Grass polygraph. Drugs, dissolved in isotonic saline were injected via a cannula in the femoral vein.

Two cats were pre-treated with reserpine (CIBA) intramuscularly. Cat No. 1 received 0·5, 1·0, 2·0 and 3·0 mg/kg, respectively, 6, 5, 4 and 3 days before the day of experiment. Cat No. 2 received the same doses but, in addition, 3 mg/kg of reserpine was injected intramuscularly 12h before the experiment.

Rat isolated phrenic nerve-diaphragm

Hemidiaphragms, prepared as described by Bülbring (1946), were suspended in a 50 ml bath containing Krebs (1932) solution at 35°, gassed with 5% carbon dioxide in oxygen. The muscle was supramaximally stimulated, using the modified electrode of Cooper & Marshall (1962), with 8 rectangular impulses/min; alternately 4 shocks through the nerve and 4 directly. Isometric contractions were recorded kymographically.

Quantitative comparisons between the anthelmintics and tubocurarine were made. Log molar concentration-response curves were constructed and the molar concentration of each compound producing 50% neuromuscular block (EC50) was estimated graphically (Blackman & Ray, 1964).

Chick isolated biventer nerve-muscle preparation

The chick biventer was prepared according to Ginsborg & Warriner (1960). The nerve-tendon was stimulated in similar conditions to those described for the rat diaphragm, and semi-isometric contractions were recorded.

Chick isolated semispinalis muscle

The muscles, obtained from chickens less than 7 days old, were set up according to Child & Zaimis (1960) in a 5 ml organ bath containing Krebs solution at 35°, gassed with 5% carbon dioxide in oxygen, and isotonic contractions recorded.

In vivo: *peroneal nerve-digital extensor muscles, respiration and carotid blood pressure of the rabbit*

Experiments were made on rabbits of mixed breed and sex, weighing 1 to 2·5 kg, anaesthetized with urethane described by Eyre & Goff (1968). The centrally ligated peroneal nerve was supramaximally stimulated with 8 rectangular impulses/min at 5 to 10 V. Isometric muscle contractions were recorded kymographically, simultaneously with carotid blood pressure and respiratory movement (Eyre, 1967). Drugs were administered intravenously.

Cholinesterase activity

Cholinesterase activity was measured colorimetrically as described by Katsch (1955) and Eyre (1966). Blood was collected with heparin, by jugular venepuncture of sheep and horses. Horse plasma was used as the source of butyrylcholinesterase and sheep erythrocytes (washed three times with equal volumes of isotonic sodium chloride solution) were the source of acetylcholinesterase.

Log molar concentration-response (% inhibition) curves were constructed for each anthelmintic agent and eserine. The relative potency of the compounds was determined by estimating pI50 values graphically. The pI50 value is the logarithm of the reciprocal molar concentration of a drug which reduces enzyme activity by 50% (Blaschko, Bülbring & Chou, 1949).

Drugs

The drugs used were acetylcholine chloride, histamine acid phosphate; atropine sulphate; hexamethonium bromide; eserine (physostigmine) salicylate; tubocurarine chloride; decamethonium iodide; dibenamine hydrochloride, propranolol hydrochloride; tetramisole hydrochloride; pyrantel tartrate. Drug-solutions for injection were made in isotonic sodium chloride solution; other solutions were in distilled water.

RESULTS

Guinea-pig isolated ileum

Methyridine (10 to 20 μg/ml) caused slight spontaneous movement of the ileum. Tetramisole (5 to 10 μg/ml) produced more pronounced irregular spontaneous activity (Fig. 1a). Pyrantel (5 μg/ml) elicited a marked sustained contraction of the ileum which was followed by a number of spontaneous "spikes" for some 5 to 10 min after washing the preparation with fresh Tyrode (Fig. 1b).

Contractions produced by acetylcholine (0·02 to 0·20 μg/ml) were potentiated following treatment with pyrantel (Fig. 1b) whereas pretreatment with methyridine or tetramisole did not cause cholinergic potentiation. The responses due to histamine (0·03 to 0·20 μg/ml) were unaffected by the anthelmintics at the concentrations employed.

Hexamethonium (1·0 μg/ml) blocked the activity of pyrantel on the ileum (Fig. 1c) but did not antagonize the action of tetramisole. Atropine (0·1 to 0·2 μg/ml)

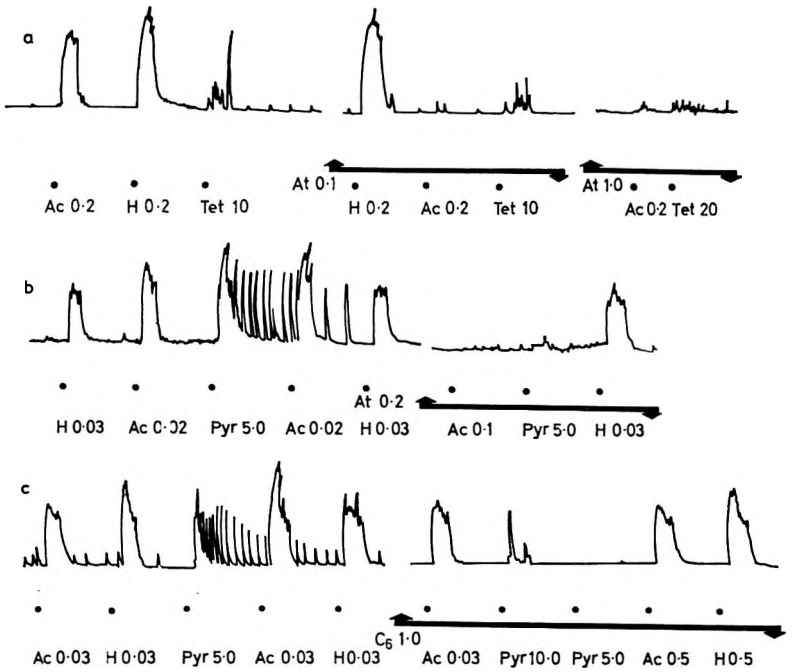

FIG. 1. Contractions of guinea-pig isolated ileum in 15 ml aerated Tyrode at 35°. H = histamine. Ac = acetylcholine. Tet = tetramisole. Pyr = pyrantel. At = atropine and C_6 = hexamethonium present between the arrows. Doses in μg/ml.

antagonized pyrantel completely and tetramisole partially. Atropine (1·0 μg/ml) abolished the response due to tetramisole (Fig. 1a).

Cat nictitating membrane preparation

Pyrantel (2 mg/kg, i.v.) contracted the nictitating membrane with a delay less than 10 s. At the same time there was a pronounced rise in blood pressure and transient dyspnoea. Hexamethonium (5 mg/kg) abolished both the contraction of the membrane due to preganglionic electrical stimulation and also the membrane contraction and the rise in blood pressure due to pyrantel (Fig. 2). Tetramisole (2 mg/kg, i.v.), after a delay of 20 s, produced a strong contraction of the nictitating membrane which was accompanied by a small brief fall in blood pressure followed by a sustained rise that persisted for up to 20 min. Respiration was affected only minimally, usually brief hyperventilation (Fig. 3). The actions of tetramisole were similar to, but more sustained than those of adrenaline (2 to 5 μg/kg, i.v.). Hexamethonium (5 to 10 mg/kg), while abolishing the effect of electrical stimulation of the membrane, did not inhibit the actions of adrenaline or tetramisole. Dibenamine (5 mg/kg), in contrast, reversed the pressor responses of adrenaline and tetramisole and partly inhibited their action on the nictitating membrane (Fig. 3). Bretylium (1 mg/kg) or propranolol (1 mg/kg) did not antagonize tetramisole.

In two reserpinized cats, tetramisole (2 mg/kg) and adrenaline (2 to 5 μg/kg) elicited a greater contractile response in the membrane than in non-reserpinized animals.

Methyridine was not examined.

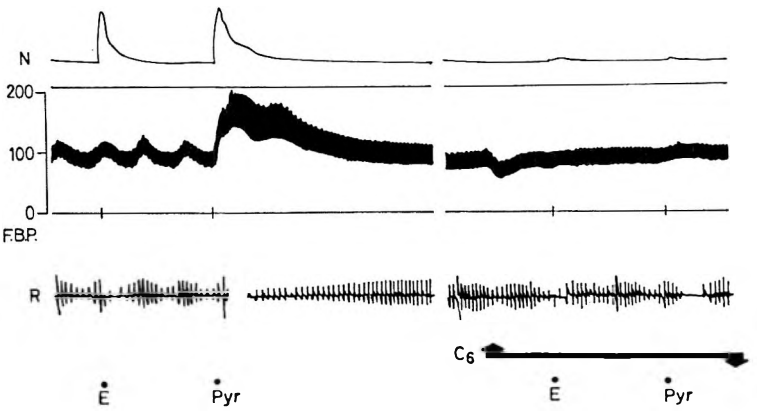

FIG. 2. Cat anaesthetized with sodium pentobarbitone. Top tracing = contraction of the nicitating membrane (N). Middle tracing = femoral arterial blood pressure (F.B.P.) mm Hg. Bottom tracing = respiratory volume (R) taken from a tracheal cannula. E = preganglionic stimulation, Pyr = pyrantel 2 mg/kg i.v. Hexamethonium (C_6, 5 mg/kg i.v. present between the arrows) abolishes the effects of electrical stimulation and those of injected pyrantel.

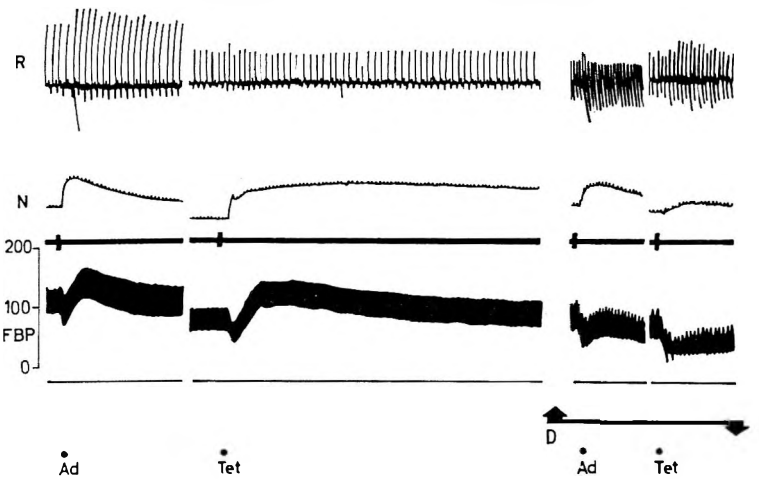

FIG. 3. Cat anaesthetized with sodium pentobarbitone. Top tracing = respiratory volume (R) recorded from a tracheal tube. Middle tracing = contraction of the nictitating membrane (N) Bottom tracing = femoral arterial blood pressure (F.B.P.) mmHg. Ad = adrenaline 3 μg/kg, i.v. Tet = tetramisole 2 mg/kg, i.v. Dibenamine (D, 5 mg/kg, i.v. present between the arrows) partially inhibits the actions of adrenaline and almost abolishes those of injected tetramisole.

Rat isolated phrenic nerve-diaphragm

Tetramisole (10^{-5} to 10^{-4}M) and pyrantel (10^{-6} to 10^{-5}M) augmented the twitch responses of both the directly and indirectly stimulated muscle, whereas methyridine did not (Fig. 4a).

Methyridine (10^{-8}M), tetramisole (10^{-4}M) and pyrantel (10^{-5}M) produced partial neuromuscular block which was not affected by a tetanus or by potassium or eserine. The anthelmintics reduced slightly the responses to direct stimulation (Fig.4b.) Tetramisole (10^{-5} to 10^{-4}M) or pyrantel (10^{-6} to 10^{-5}M) added during a partial neuromuscular block by tubocurarine (3×10^{-6}M) partly relieved the block (Fig. 4c).

Quantitative comparison showed that the molar EC/50 values were: decamethonium

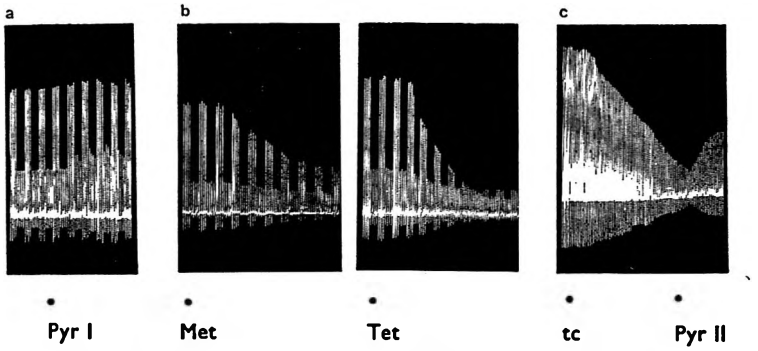

FIG. 4: a, b. Contractions of the isolated rat hemidiaphragm in 50 ml O_2 + CO_2—aerated Krebs at 35°. Muscle stimulated supramaximally with 8 rectangular shocks/min—alternately 4 shocks through the nerve (larger twitches) and 4 directly into the muscle (smaller twitches). c. Muscle stimulated 8 shocks/min. indirectly through the nerve.
Pyr = pyrantel I 9×10^{-6}, II 1×10^{-5}M. Met = methyridine 7×10^{-3}M. Tet = tetramisole 3×10^{-4}M tc = (+)-tubocurarine 3×10^{-6}M.

8×10^{-5}, tubocurarine $1{\cdot}6 \times 10^{-6}$, tetramisole $1{\cdot}4 \times 10^{-4}$, pyrantel $1{\cdot}2 \times 10^{-4}$ methyridine $5{\cdot}7 \times 10^{-3}$.

Chick isolated biventer nerve-muscle

Tetramisole (10^{-5} to 10^{-4}M) and pyrantel (10^{-6} to 10^{-5}M) increased the twitch response of the preparation and reversed the blocking action of tubocurarine. Methyridine did not show these effects.

Tetramisole (10^{-4}M), pyrantel (2×10^{-5}M) and methyridine (1×10^{-3}M) produced reversible neuromuscular block and contracture of the muscle, indicative of depolarization (Fig. 5).

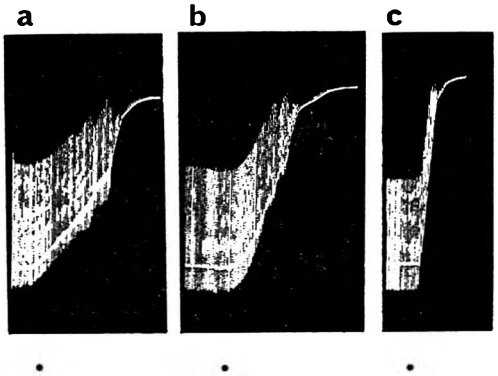

FIG. 5. Contractions of the chick isolated biventer nerve-muscle preparations in 20 ml O_2 + CO_2 —aerated Krebs at 35°. Muscle stimulated supramaximally with 8 rectangular shocks/min through the nerve. Effects of: a. ● Methyridine, $2{\cdot}4 \times 10^{-3}$M. b. ● Tetramisole. 1×10^{-4}M c. ● Pyrantel 1×10^{-5}M.

Chick isolated semispinalis muscle

Tetramisole (6×10^{-5}M), pyrantel ($1{\cdot}5 \times 10^{-5}$M) and methyridine ($2{\cdot}8 \times 10^{-3}$M) produced contractions of the semispinalis which were inhibited by tubocurarine ($1{\cdot}5 \times 10^{-7}$M) (Fig. 6). Pyrantel showed marked tachyphylaxis and inhibited slightly the responses to decamethonium and nicotine.

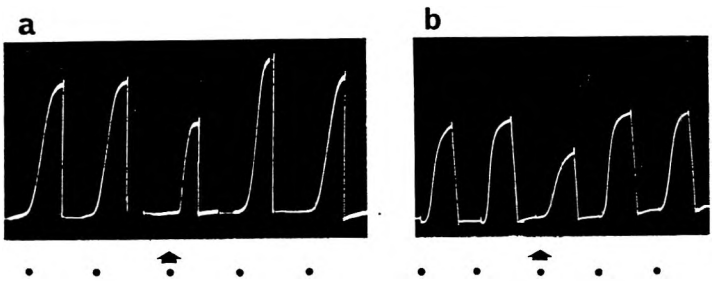

FIG. 6. Contractions of the semispinalis muscle of the chick in 10 ml O_2 + CO_2—aerated Krebs at 35°. a. ● Contractions caused by methyridine $2{\cdot}8 \times 10^{-3}$M. (+)-Tubocurarine ($1{\cdot}5 \times 10^{-7}$M) added at the arrow. b. ● Contractions due to tetramisole 6×10^{-6}M. Tubocurarine (5×10^{-7}M) added at the arrow.

In vivo *peroneal nerve-digital extensor muscles, respiration and carotid blood pressure of rabbit*

A small intravenous dose of methyridine (10–50 mg/kg) increased slightly the twitch-height of the extensor muscles and caused a precipitous fall in carotid blood pressure which was partially prevented by atropine 1 mg/kg (Fig. 7). Respiratory

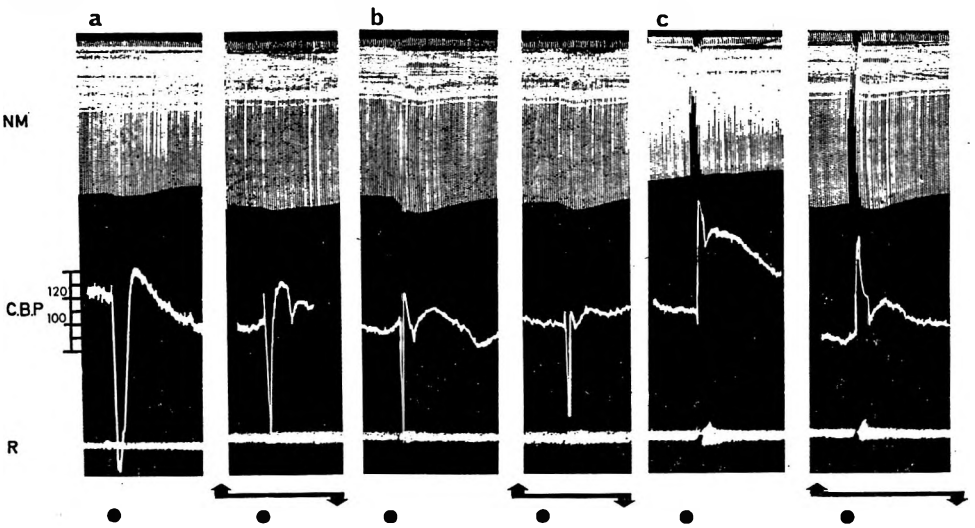

FIG. 7. Rabbit anaesthetized with urethane. Top tracing: (NM) isometric contractions of the digital extensor muscles due to peroneal nerve stimulation at 8 impulses/min. Second tracing: carotid blood pressure (C.B.P.) in mmHg. Bottom tracing: respiratory volume (R) from tracheal cannula. a. ● Methyridine 20 mg/kg, i.v.; between arrows—atropine 1 mg/kg, i.v. b. ● tetramisole 10 mg/kg, i.v.; between arrows—atropine 1 mg/kg, i.v. c. ● Pyrantel 2 mg/kg, i.v.; between arrows—hexamethonium 1 mg/kg, i.v.

volume and rate were not altered. A therapeutic dose of methyridine (200 mg/kg, s.c.) produced a sustained hypotension and partial neuromuscular block.

Tetramisole (5–10 mg/kg, i.v.) caused a sharp transient fall in blood pressure which was partially antagonized by atropine. Respiratory volume was either reduced briefly or unaffected. Neuromuscular transmission was briefly enhanced; an effect which diminished after repeated dosing in the same animal (Fig. 7) A therapeutic dose of tetramisole (10–25 mg/kg, s.c.) caused a prolonged fall in blood pressure.

Neuromuscular transmission was enhanced. Doses greater than 30 mg/kg caused profound, sustained hypotension and apnoea together with neuromuscular block. The animal could be kept alive only by means of a respiration pump.

Pyrantel (2–10 mg/kg, i.v.) caused an immediate brief increase in carotid blood pressure followed by a more sustained pressor response. The secondary hypertension was almost abolished by hexamethonium (1 mg/kg) (Fig. 7). Transient apnoea and neuromuscular block coincided with the first phase of hypertension.

A therapeutic dose of pyrantel (25 mg/kg, s.c.) produced a transient small hypotension followed by a sustained rise in blood pressure. Neuromuscular transmission was slightly enhanced but respiration was unaffected. Pyrantel (50–100 mg/kg) given subcutaneously caused total neuromuscular block and apnoea together with oscillating rises and falls of blood pressure. Animals died of cardiac arrest despite artificial respiration.

Cholinesterase activity

Acetylcholinesterase of sheep erythrocytes. The pI50 of eserine was 6·4. Tetramisole was the most active anthelmintic compound (5·0) and pyrantel was next (4·5). The activity of methyridine was very small (1·9).

Butyrylcholinesterase of horse plasma. The pI50 of eserine was 7·0. Tetramisole and pyrantel were weaker inhibitors of the plasma enzyme than of erythrocytes—having values respectively of 4·4 and 4·0. Methyridine had a greater inhibitory action on the plasma (2·3) enzyme than on erythrocytes (1·9).

DISCUSSION

The results show that the three nematocides evoke several pharmacological responses.

Tetramisole and pyrantel produced reproducible contractions of the ileum which were abolished by atropine; showing that muscarinic receptors were stimulated either directly or indirectly to induce contraction. Pyrantel caused more marked, more regular and more prolonged spontaneous contractions and also potentiated acetylcholine. Hexamethonium abolished the responses due to pyrantel but not those of tetramisole; which suggested that tetramisole may have a direct muscarinic action whereas pyrantel was acting by stimulating nicotinic receptors of the ganglionic synapses of the intestine.

This possibility was studied further using the cat superior cervical ganglion-nictitating membrane preparation, in which the membrane contracted strongly following intravenous injection of pyrantel. Pyrantel produced a pronounced pressor response which was confirmed in another group of experiments in the rabbit. Premedication with hexamethonium abolished the contractile effect of pyrantel and of preganglionic electrical stimulation on the nictitating membrane, whereas the action of adrenaline on the membrane was unchanged. Similarly, hexamethonium antagonized the hypertensive action of pyrantel in the cat and rabbit.

The effect of tetramisole on the nictitating membrane was different. Tetramisole caused a contraction of the organ which was slower in onset and more prolonged in duration. The arterial pressure responded with a small sharp fall followed by a prolonged pressor phase. These responses of tetramisole resembled those of adrenaline. Hexamethonium, at the dosage used, did not inhibit the action of tetramisole

on the nictitating membrane—an effect which was partly inhibited by dibenamine but not by propranolol. It seemed, therefore, that tetramisole may not have been acting on nicotinic receptors in the ganglion, but in some way the anthelmintic caused stimulation of adrenoceptive mechanisms in the nictitating membrane. Bretylium failed to reduce the action of tetramisole on the membrane and in two cats which had been chronically treated with reserpine, there was no diminution of the action of tetramisole.

It is possible that tetramisole stimulates the cat nictitating membrane (a) by direct stimulation of adrenoceptive receptors or (b) by releasing adrenaline from the adrenal medulla; an effect which would be unaffected by bretylium and incompletely inhibited by reserpinization. The fact that the pressor effect of tetramisole is biphasic, and there is a delay in onset of responses followed by prolonged hypertension and membrane contraction, tends to support the probability of adrenaline release from the adrenal gland. This is further supported by the fact that dibenamine reverses the pressor responses of both adrenaline and tetramisole (since the adrenal gland contains principally adrenaline).

The failure of hexamethonium to block the effect of tetramisole in contrast to pyrantel, may be due to tetramisole being a more potent nicotinic stimulant of the adrenal medulla or to it having some direct action on adrenoceptive receptors. The present evidence does not allow the latter possibility to be ruled out.

Tetramisole and pyrantel both consistently augmented skeletal neuromuscular transmission and antagonized the myoneural blocking action of (+)-tubocurarine. These effects may be ascribed both to the depolarizing action and to cholinesterase inhibition by the anthelmintics. Methyridine, which is a weaker depolarizer and has a much smaller anticholinesterase action, had little effect on the ileum and showed little or no tendency to augment neuromuscular transmission or to antagonize curare. Increasing concentrations of the three drugs caused neuromuscular block *in vitro* and *in vivo*. In the rat diaphragm *in vitro*, this blocking action was not overcome by the addition of potassium or eserine, or by tetanic stimulation. Furthermore, when the anthelmintics were tested on the semispinalis and biventer muscles of the chicken, contractions were produced which were antagonized by tubocurarine. These results suggest strongly that the three nematocidal compounds cause neuromuscular paralysis which is of the non-competitive type and is accompanied by depolarization in a manner characteristic of nicotine, succinylcholine or decamethonium.

The myoneural actions of the three nematocides *in vitro* were confirmed *in vivo* in the rabbit. Methyridine caused a fall in blood pressure in rabbits. Tetramisole had a depressor action in the rabbit whereas in the cat the response was always pressor. Atropine partly inhibited the hypotension caused by methyridine and by tetramisole in the rabbit, and it would appear that methyridine and tetramisole act by exerting both muscarinic and nicotinic effects. Pyrantel appears to excite mainly nicotinic receptors.

The results of these experiments are consistent with the main features of the toxicity of the drugs which have been reported from the field. Broome (1961) showed that methyridine paralysed the parasitic helminth and suggested that depolarization may be a principal feature of the drug. This was confirmed. Catarsini & Gagliano (1963) showed some depression of circulating cholinesterase activity in

sheep. It seems likely that owing to the relatively small action of methyridine on cholinesterases, that direct depolarization may be more important.

The pharmacodynamics of tetramisole agree with the symptoms of salivation, defaecation and muscular contraction which have been described (Forsyth, 1966; Walley, 1966; Kaemmerer & Budden, 1966). Forsyth was the first to suggest a similarity between tetramisole intoxication and organophosphorus poisoning, but he did not investigate the implications of his observation. Some of the toxicity of this drug may be concerned with cholinesterase inhibition leading to manifestations of the muscarinic and nicotinic actions of acetylcholine; in addition to which there is depolarizing neuromuscular block which might explain Kaemmerer's observations of spastic tail erection and muscle tremors.

However, the actions of tetramisole on sympathetic elements have not been described hitherto and these effects do not "fit" the picture of toxicity of tetramisole which has become recognized. It must be assumed that in outward symptoms the cholinergic manifestations predominate.

On first consideration it is curious that no particular "clinical" syndrome of toxicity has been reported for pyrantel. This may be due to the fact that pyrantel is always given orally and is poorly absorbed from the intestine. Hence general systemic pharmacological effects will not occur in the normal conditions of usage of the drug Tetramisole on the other hand may be injected or, when given by mouth, is well-absorbed into the circulation, thus giving rise to the pharmacodynamic actions reported in the literature.

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Potentiation of the effects of noradrenaline and of sympathetic stimulation of the perfused rat caudal artery by angiotensin

T. E. NICHOLAS

The Department of Pharmacology, The University of Western Australia, Nedlands, Western Australia

The isolated perfused caudal artery of the rat develops tachyphyllaxis rapidly to the direct constrictor action of angiotensin but not towards the potentiation of responses to submaximally effective periarterial stimulation or to noradrenaline. These potentiations persist in the presence of maximally effective concentrations of cocaine, are unaffected by increased sodium concentration and are enhanced by raised concentrations of calcium. Thus, angiotensin potentiates the effects of sympathetic nervous activity by an influence on the role of calcium in the contractile process. Hydrochlorothiazide did not affect arterial tone, the responses of the artery, or the potentiation to noradrenaline caused by angiotensin.

The reduction in the pressor effect of injected noradrenaline which is caused in rats by the oral administration of hydrochlorothiazide precedes both the antihypertensive and the diuretic effects of this drug by 1 h, and the cardiovascular actions of hydrochlorothiazide are abolished by nephrectomy (Lockett & Nicholas, 1968). It is possible that these cardiovascular actions of hydrochlorothiazide are mediated by the renin-angiotensin system since the interaction of angiotensin with contractile responses of smooth muscle after sympathetic stimulation are well known. Angiotensin greatly increases the contraction of the vas deferens in response to stimulation of the hypogastric nerves (Benelli, Della Bella & Gandini, 1964) and this effect was attributed to increase by angiotensin of the quantity of noradrenaline released from the terminals of the postganglionic sympathetic fibres per nerve impulse. Support for this interpretation was provided by Zimmerman & Gomez, 1965; and Zimmerman & Gisslen, 1968) who worked on the responses of the cutaneous and renal vascular beds to sympathetic stimulation. Moreover, angiotensin has been shown to release catecholamines from the adrenal medulla (Renson, Barac & Bacq, 1959; Feldberg & Lewis, 1963) and to block the uptake of noradrenaline by blood vessels (Palaič & Khairallah, 1967). The present purpose has been to examine the potentiation by angiotensin of the response of the isolated caudal artery of the rat (Nicholas, 1969) to sympathetic stimulation and to exclude the possibility that hydrochlorothiazide influences this potentiation by a direct effect on the artery itself.

EXPERIMENTAL

Methods

The method used for the isolation and perfusion of the caudal arteries of the rat has been described (Nicholas, 1969). Periarterial stimulation, previously shown to activate solely postganglionic sympathetic neurons, was by platinum electrodes

placed closely adjacent to and on either side of the proximal 1 cm length of the preparation. Rectangular pulses, 1 ms in duration were delivered 2 to 10/s for 3 s, each min at 15 V from a Grass stimulator (S4K). Krebs bicarbonate solution (Umbreit, Burris & Stauffer, 1964), saturated with 5% carbon dioxide in oxygen, was used both as bath medium and perfusate. Depolarization was effected by doubling the concentration of KCl in this fluid. All drugs except hydrochlorothiazide were dissolved in Krebs bicarbonate solution. Hydrochlorothiazide was dissolved in 0·5 M NaOH and was then adjusted to pH 9·0 by addition of N HCl. Administration was either by close arterial injection into the perfusion fluid immediately before its entry into the preparation, or by solution in the perfusion fluid or by addition to the bath fluid (15 ml) surrounding the artery.

Hydrochlorothiazide was received as a gift from Merck, Sharp & Dohme. (—)-noradrenaline (Winthrop Laboratories), angiotensin II val^5 asp.-β-amide (Ciba Laboratories), Vasopressin (Parke Davis & Co. Ltd.) and cocaine hydrochloride (Macfarlane Smith Ltd.) were obtained commercially.

RESULTS

Arteries initially responded to single injections of 1 to 2 ng angiotensin II by constriction and the effects of submaximal periarterial stimulation were potentiated by the drug, Fig. 1 (upper). Whereas tachyphyllaxis developed rapidly to the

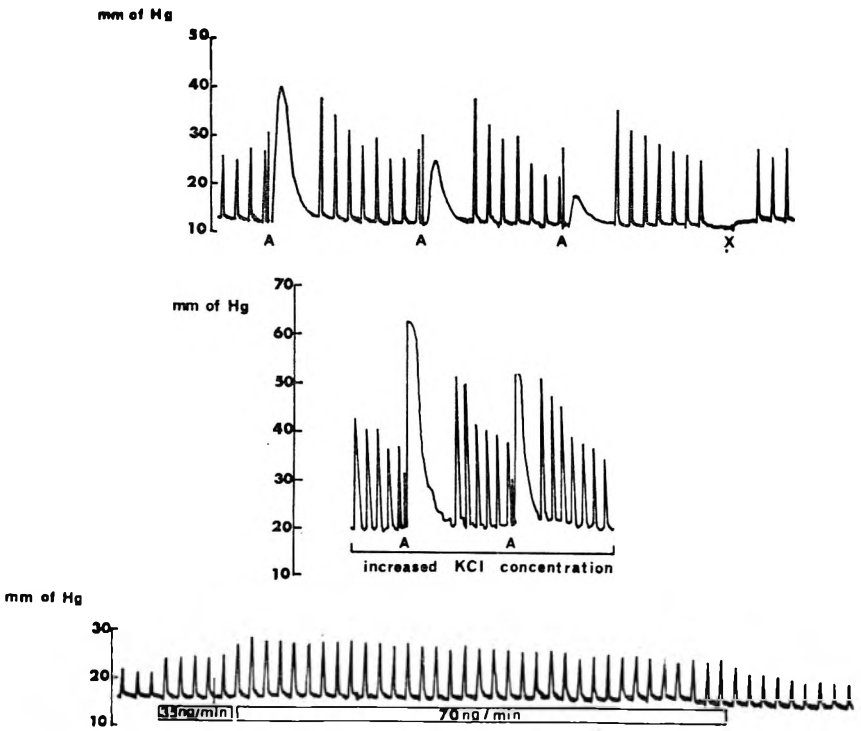

FIG. 1. Responses of perfused rat caudal artery. *Upper:* Tachyphyllaxis develops to 25 ng angiotensin II (A) but not to the potentiation of the response to periarterial stimulation which A causes. X signifies 3 min without stimulation. *Middle:* Doubling the concentration of KCl does not affect the potentiation of the response to stimulation caused by A. *Lower:* Infusions of angiotensin 32 and 70 ng/min cause sustained potentiation of the response to stimulation.

constrictor action of angiotensin, the potentiation persisted unmodified for many hours. Depolarization of the artery did not alter the effects of angiotensin on the preparations, Fig. 1 (middle). Potentiation of the response of the artery to submaxial stimulation could also be produced by infusions of angiotensin in preparations completely tachyphyllactic to the constrictor actions of the drug. Under these conditions the potentiation produced by angiotensin slowly waned; invariably, however, the stimulus response decreased abruptly when the infusion ended. Sometimes the response to periarterial stimulation appeared depressed after a long infusion, Fig. 1 (lower).

The extent of the potentiation of the arterial response to stimulation was directly related to the dose of angiotensin when stimulus rate was kept constant. The degree of the potentiation was also directly related to the frequency of stimulation when the dose of angiotensin was fixed (Fig. 2). Angiotensin still potentiated the responses of the artery to noradrenaline and to stimulation during the maximum action of cocaine (Fig. 3).

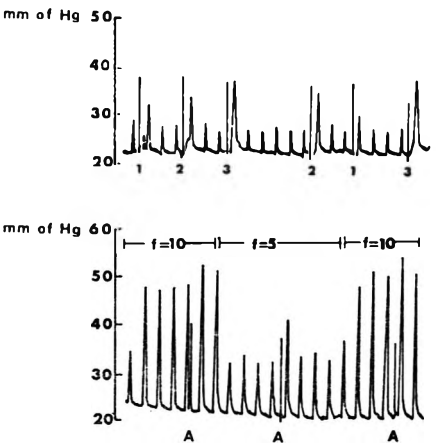

FIG. 2. Responses of a perfused rat caudal artery. *Above:* Potentiation of the response to periarterial stimulation (2/s) caused by (1) 6·25, (2) 12·5 and (3) 25·0 ng angiotensin II, respectively. *Below:* Effect of change in frequency of stimulation on the potentiation caused by 25 ng angiotensin II (A).

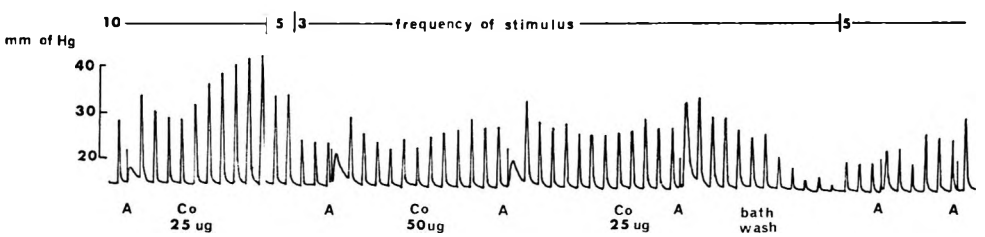

FIG. 3. Responses of a perfused rat caudal artery, showing the effect of 5 ng angiotensin II (A) on the responses to periarterial stimulation during the effect of cocaine (Co) in the bath fluid.

Infusion of isotonic calcium chloride at 1 ml/min into perfusate entering the artery at 3 ml/min increased the response of the artery to stimulation approximately threefold. When reduction in stimulus frequency during the infusion of calcium chloride

had reduced the response of the artery to match the initial pre-calcium effect, the potentiation caused by angiotensin was much greater than that initially observed. Single injections of 0·1 ml isotonic $CaCl_2$ caused transient and reproducible increases in perfusion pressure which were unaffected by injections of angiotensin. Fig. 4

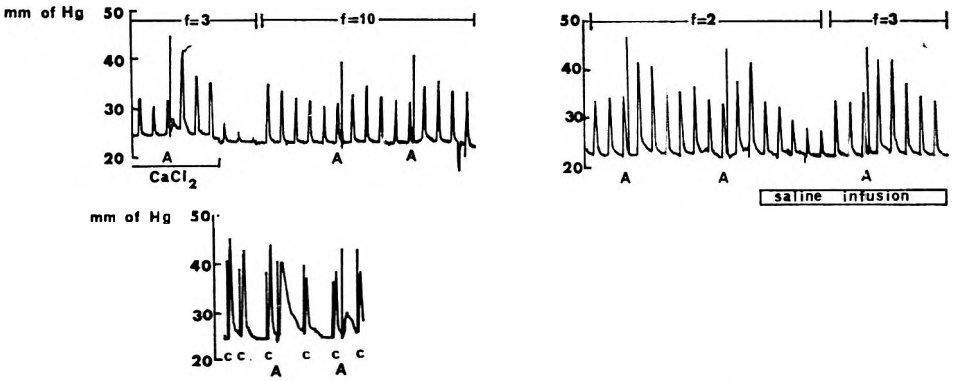

FIG. 4. Responses of a perfused rat caudal artery. *Above left:* The effect of infused isotonic $CaCl_2$, 0·2 ml/min, on the constriction caused by periarterial stimulation and its potentiation by 2·5 ng angiotensin II (A). *Below left:* The effect of injections of 5 ng angiotensin II (A) on the constrictor action of 0·1 ml injected isotonic CaCl(c.) *Above right:* the effect of doubling the NaCl concentration in the perfusate on the response to periarterial stimulation and its potentiation by 5 ng angiotensin II (A).

shows that on cessation of the infusion of calcium chloride, the augmented response to periarterial stimulation rapidly declined, as does the potentiation of the stimulus effect by angiotensin.

The maximum potentiation of the effects of periarterial stimulation attainable by single injections of angiotensin II were approximately 200 to 250% of the original responses. Corresponding figures for minor potentiations caused by noradrenaline and by vasopressin were 27 and 36% respectively.

Doubling the concentration of NaCl in the perfusion and the bath fluid depressed the response of the artery to electrical stimulation, increased the constrictor effect of angiotensin and did not affect the potentiation of the response to stimulation caused by angiotensin (Fig. 5).

Exposure of the artery to hydrochlorothiazide in the bath fluid (35 μg/ml) or in normal or high sodium perfusate (20 μg/ml) for 30 min, or both, did not alter the perfusion pressure. The responses of the artery to electrical stimulation, potentiation of these responses by angiotensin and the constrictor effects of angiotensin remained unchanged.

DISCUSSION

Interaction between angiotensin and sympathetic nervous activity is well established. McCubbin & Page (1962) demonstrated potentiation of pressor responses to tyramine, ephedrine and dimethylphenylpiperazinium by infusions of angiotensin in dogs. Although these substances act indirectly by releasing noradrenaline, the angiotensin did not potentiate the pressor effects of injected noradrenaline. Angiotensin does, however, potentiate the responses of both the vas deferens (Bennelli & others, 1964) and the renal vascular bed (Zimmerman & Gisslen, 1968) to sympathetic

stimulation. By contrast Day & Owen (1968) found that angiotensin inhibited the constrictor response of the perfused central artery of the rabbit ear to sympathetic stimulation. On the other hand, the present work demonstrates that angiotensin invariably potentiates the responses of the perfused caudal artery of the rat both to noradrenaline and to sympathetic stimulation.

Potentiation by angiotensin of the effects of sympathetic stimulation on the perfused caudal artery of the rat cannot be due to an increase in the amount of noradrenaline liberated per nerve impulse since the effects of exogenous noradrenaline are similarly increased. If potentiation by angiotensin of constrictions induced both by endogenous and by exogenous noradrenaline is to be attributed to reduction in uptake into the terminal fibrils, then the uptake component blocked has been shown to be insensitive to inhibition by cocaine. It is unlikely the potentiations are a consequence of any membrane action of angiotensin since these potentiations are demonstrable after depolarization of the vascular smooth muscle. There can be no close linkage between the mechanisms of the vasoconstrictor action of angiotensin and of the potentiation of noradrenaline vasoconstriction by this polypeptide. Whereas a rise in the sodium concentration of the bath fluid increases the effect of angiotensin on smooth muscle (Blair-West, Harding & McKenzie, 1967), potentiation of the action of noradrenaline by angiotensin is independent of sodium concentration. It it possible that angiotensin potentiates the constrictor effects of noradrenaline on vascular smooth muscle by an influence at the site of action of calcium in the contractile mechanism, since infusions of calcium chloride increase the effects of exogenous and endogenous noradrenaline.

It is of interest that hydrochlorothiazide had no effect on the tone of the caudal arterial smooth muscle, on the responses of the artery to sympathetic stimulation and to exogenous noradrenaline or on the potentiation of responses to noradrenaline caused by angiotensin. Hence the antihypertensive action of this diuretic is not attributable to an effect on any interaction between angiotensin and endogenous noradrenaline in vascular smooth muscle.

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The effects of P-2-AM on the release of acetylcholine from the isolated diaphragm of the rat

ROBERT G. GOYER

School of Pharmacy, University of Montreal, Montreal, Canada

Release of acetylcholine from rat isolated diaphragm preparations stimulated through the phrenic nerve was investigated in the presence of P–2–AM 0·01–100 mM. At 1 mM, the release of acetylcholine was increased by 157%, while higher concentrations resulted in a decrease. The findings indicate that the biphasic action of P–2–AM can be explained, at least in part, by variations in the quantal release of acetylcholine—an effect involving a presynaptic action.

Wilson & Ginsburg (1955) and Child, Davies & others (1955) reported that pyridine-2 aldoxime methochloride or methiodide (P–2–AM or PAM) can reactivate phosphorylated cholinesterases. Fleisher, Corrigan & Howard (1958), Holmes & Robins (1955) and Koelle (1957) demonstrated that such reactivation is observed at the motor end plates of skeletal muscles. Many authors have shown that, in addition to reactivation, PAM has a facilitating, and also a depressing or blocking action at the neuromuscular junction. The facilitation has been reported to result from a depolarizing effect (Wills, O'Leary & Oikemus, 1959), from an anticholinesterase activity (Holmes & Robins, 1955; Wagley, 1957; Fleisher & others, 1958; Fleisher, Moen & Ellingson, 1965; Goyer, 1968; Fleisher & Ellingson, unpublished results) or from an increase in the quantal release of acetylcholine (Edwards & Ikeda, 1962). The depressing effect on muscular contraction has been assumed to result from a direct toxic action on the muscle fibres (Holmes & Robins, 1955) or from a (+)-tubocurarine-like activity (Grob & Johns, 1958; Fleisher & others, 1958, 1965; Goyer, 1968; Fleisher & Ellingson, unpublished results). Recently I have found (unpublished results) that the antagonism of PAM to acetylcholine is non-competitive.

I have now examined whether the two effects of PAM at the neuromuscular junction could also be related to a presynaptic site of action, if so, the release of the chemical transmitter (acetylcholine) would then be modified. During these experiments, the rat phrenic nerve-diaphragm muscle preparation was used, as many workers (Burgen, Dickens & Zatman, 1949; Brooks, 1954; Straughan, 1960; Krnjević & Mitchell, 1961; Mitchell & Silver, 1963) have demonstrated that acetylcholine is released by this preparation when the motor nerve is stimulated.

EXPERIMENTAL

Innervated diaphragm and collection of acetylcholine

Phrenic nerve-diaphragm muscle preparations, from male Wistar rats, 200–250 g, were set up as described by Bulbring (1946), in a 7 ml bath containing Krebs solution of the following composition (g/litre): NaCl 6·92, KCl 0·35, $CaCl_2$ 0·28, $NaHCO_3$ 2·1, KH_2PO_4 0·16, $MgSO_4.7H_2O$ 0·30, glucose 2·0. The preparation was gassed

with 5% carbon dioxide in oxygen and the temperature was kept constant at 37 ± 0·1°. The phrenic nerve was stimulated by means of a Grass stimulator (S-8) delivering supramaximal rectangular pulses of 0·2 ms duration at a frequency of 0·1 or 25 Hz; the isotonic contractions were recorded by means of an E & M Physiograph. To prevent hydrolysis of acetylcholine released during nerve stimulation, the cholinesterases were inhibited by adding 5 μM neostigmine methylsulphate to the Krebs solution. Under the experimental conditions, it has been verified that such a concentration of neostigmine totally inhibits cholinesterases. Before each experiment, the preparation was allowed to rest for 30 min. The procedure was that of Straughan (1960), as modified by Cheymol, Bourillet & Ogura (1962).

(1) *Adaptation period*: the phrenic nerve was stimulated at a low frequency (0·1 Hz) for 10 min; the preparation was then allowed to rest for 20 min, after which time it was washed twice.

(2) *Stimulation period I*: the phrenic nerve was stimulated at 0·1 Hz for 3 min and at 25 Hz for an additional 20 min. The bath solution was then collected for the biological assay of acetylcholine.

(3) *Rest period*: the preparation was washed twice and allowed to rest for 10 min, after which the bath solution was discarded and the preparation was washed again.

(4) *Stimulation period II*: same as (2). (5) *Rest period*: same as (3).

(6) *Action of PAM*: without prior stimulation of the phrenic nerve, PAM was added to the bath in an amount to yield the required final concentration, and left in contact with the preparation for 20 min. The bath solution was then discarded and the preparation was washed once.

(7) *Stimulation period III*: same as (2), immediately after (6). (8) *Rest period*: same as (3). (9) *Stimulation period IV (recovery period)*: same as (2).

Estimation of acetylcholine

After each sample collection, the bath solution was refrigerated at 0°. Acetylcholine was estimated by the rat blood pressure preparation (male Wistar rats, 200–250 g), as described by Straughan (1958, 1960). The activity of 0·1 ml of the bath solution was evaluated against 0·1 ml of known concentrations of acetylcholine in Krebs solution containing neostigmine. The biological assay for acetylcholine was made on the same day as, and immediately after, collection of the test substance. The values for acetylcholine were expressed as ng of ions.

PAM was used at 0·01, 0·1, 1·0, 10 and 100 mM. A total of 31 experiments were made, with a minimum of three assays for each concentration of PAM.

RESULTS

Under my experimental conditions, the amount of acetylcholine (mean ± s.e.) released during stimulation period II and expressed as ng of ions is 52·5 ± 2·7. This compares with those of Straughan (1960) and Cheymol & others (1962). At concentrations of 0·01 and 0·1 mM (Fig. 1 and Table 1), PAM decreases the release of acetylcholine by 10·0 and 13·9% respectively; but these figures are not significant (P, 0·05). At 1·0 mM, the amount of acetylcholine released during nerve stimulation is greatly enhanced, the mean values for stimulation periods II and III being 48·0 and 123·2 ng, respectively, an increase of 157%. With higher concentrations

of PAM, the amount of acetylcholine released during nerve stimulation is decreased. At 10 mM, a 54·5% decrease was observed; with 100 mM a complete block occurred

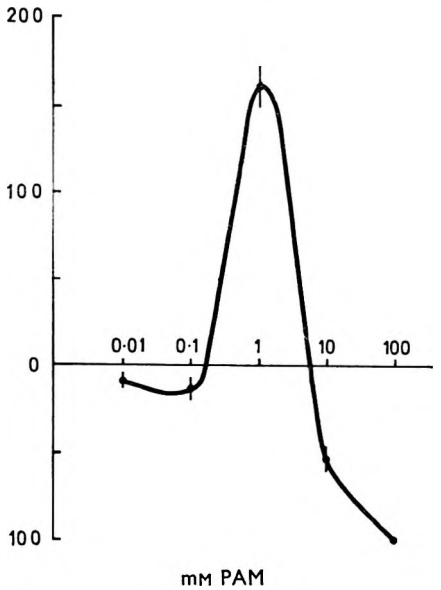

FIG. 1. Influence of various concentrations of PAM on the release of acetylcholine from rat isolated diaphragm preparations stimulated through the phrenic nerve. The amount of acetylcholine released is expressed as percentage of decrease or increase with respect to control experiments.

Table 1. *Release of acetylcholine (ng of ions) from rat phrenic nerve-diaphragm muscle preparations in presence of various concentrations of PAM*

Concentration of PAM (mM)	Stimulation period II $\bar{X}$	s.e.	Stimulation period III $\bar{X}$	s.e.	Stimulation period IV $\bar{X}$	s.e.	Value of *P* for II and III
0·01	50·400	3·960	45·920	5·429	45·280	4·613	>0·05
0·10	53·500	4·504	46·600	5·744	50·532	5·520	>0·05
1·00	48·000	8·092	123·200	21·200	50·000	7·776	<0·01
10·00	56·532	5·748	27·235	4·884	53·760	2·240	<0·01
100·00	48·532	3·732	0	0	50·400	5·600	<0·01

If the results obtained during stimulation period II and IV are compared (Table 1), there is no significant difference in the release of acetylcholine, regardless of the concentration of PAM used and the nature of the effect observed (increase or decrease in acetylcholine release).

DISCUSSION

As mentioned previously, many authors have demonstrated that PAM exerts a biphasic action at the neuromuscular junction. Most of these authors have explained the facilitating effect by an anticholinesterase activity, while they have interpreted the depressing effect as being caused by a (+)-tubocurarine-like activity. It appears that the anticholinesterase and tubocurarine-like activities do not serve as an adequate or the sole explanation for the facilitating or the depressing effect. Indeed, as shown by Goyer (1968), PAM (6 mM) did not influence muscular contractions, while it considerably inhibited the cholinesterases (60%). In the same series of experiments, PAM was at least 16 times less active as a cholinesterase inhibitor

in vitro than tubocurarine. Moreover, the tubocurarine-like activity appears when the cholinesterases are almost completely inhibited. It is probable that an additional mechanism or site of action is responsible for this biphasic activity.

According to Edwards & Ikeda (1962), PAM has a presynaptic site of action. In fact, these authors demonstrated on the frog nerve-muscle preparation that PAM (0·2–4·0 mM) increases the quantal release of acetylcholine. No decrease in its release was observed, as no concentration higher than 4 mM was used. At similar concentrations, our results confirm those of Edwards & Ikeda (1962), in that PAM (1·0 mM) increased the release of acetylcholine by 157% on the rat phrenic nerve-diaphragm muscle preparation and by 180% on the frog nerve-muscle preparation. These effects are only transient, however, as recovery is rapid and complete.

The concentrations of PAM that produce a neuromuscular block on the phrenic nerve-diaphragm muscle preparation of the rat (Goyer, 1968) and those that decrease the release of acetylcholine on the same preparation, are similar: with 10 mM, the amplitude of contraction is reduced by 20%, while the release of acetylcholine is decreased by 54·5%. At 25 mM, the amplitude of contraction is reduced by 100%, if the preparation is stimulated through its phrenic nerve, whereas the amplitude of contraction of the directly stimulated preparation is not modified.

The biphasic action of PAM can thus be explained, at least in part, by a presynaptic effect. The postsynaptic effects, namely, an anticholinesterase activity and a tubocurarine-like effect, are not the only nor perhaps even the major factors involved in the neuromuscular action of PAM. The prejunctional effects appear to play an important role.

Acknowledgements

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A new preparation of the isolated intact trachea of the guinea-pig

J. B. FARMER AND R. A. COLEMAN

Department of Pharmacology, Allen and Hanburys Ltd., Ware, Herts., U.K.

A preparation is described in which the guinea-pig isolated intact trachea is subjected to repeated transmural electrical stimulation with alternating square wave pulses. The intraluminal pressure is continuously sensed by a pressure transducer. An increase in the intraluminal pressure is the predominant response to electrical stimulation. This response is prevented by low concentrations of atropine. After the initial rise a small fall in intraluminal pressure occurs; this is prevented by low concentrations of propranolol. The sensitivity of the preparation to various drugs is described.

Various preparations of guinea-pig isolated tracheal smooth muscle are used for the assessment of bronchodilator activity of drugs. The trachea may be cut into rings which are sewn together (Castillo & de Beer, 1947), or the rings severed through the cartilage and subsequently joined (Akcasu, 1959). Alternatively the trachea may be cut spirally to form a continuous preparation. Contractions or relaxations of these tissues may be measured isometrically or isotonically. Intact isolated tracheas have been used (Jamieson, 1962) and volume changes in response to transmural stimulation were studied by Foster (1964) and Carlyle (1964). Wellens (1966) also described an intact trachea preparation in which the effects of drugs on resting intraluminal pressure were measured by a pressure transducer.

β-Adrenoreceptor agonists cause relaxation of tracheal smooth muscle but quantitative determination of this activity is not easy because tracheal muscle preparations possess little intrinsic tone. This paper describes a preparation in which the isolated intact trachea is subjected to repeated transmural electrical stimulation with alternating square wave pulses to induce regular contraction of the tissue. The intraluminal pressure is continuously sensed by a pressure transducer.

EXPERIMENTAL

Methods and materials

Guinea-pigs were killed by a blow on the head and the trachea excised. By dividing the trachea halfway along its length, two preparations could be made from one animal. Each portion was mounted on the apparatus illustrated in Fig. 1 by tying the ends over blocks D and E. The physiological salt solution was in contact with the inner and outer surface of the trachea, although there was no direct contact between these fluids. It had the following composition in g/litre: NaCl, 8·0; $NaHCO_3$, 1·0; NaH_2PO_4, 0·32; glucose, 1·0; $MgCl_2$, 0·42; KCl, 0·2; $CaCl_2$, 0·4. The bath temperature was maintained at 37° and the solution gassed with air.

The apparatus consisted of a Perspex rod (A) 10 cm long, 6 mm diameter, on which was attached a foot (B) 1·5 cm long by 3 mm depth. Mounted on rod A was an adjustable collar (C) with clamping screw. Each end of the trachea was

tied to mounting blocks (D and E). The mounting block (D) was drilled to give two outlets, one for measurement of intraluminal pressure, the other for removal of air from the system. Platinum wire electrodes were mounted as shown and connected to an electronic stimulator by shielded copper wire let into the sides of rod A. Square wave alternating pulses of 1 ms duration and supramaximal voltage were applied to the tissue for varying durations (usually 7 s) at any given frequency.

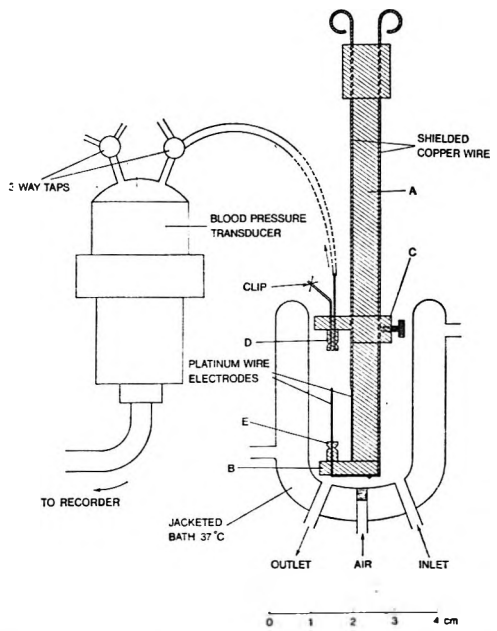

FIG. 1. Diagram of apparatus used to record pressure changes in the guinea-pig trachea induced by transmural electrical stimulation. A: Perspex rod. B: foot. C: adjustable collar. D and E: mounting blocks for trachea.

The following drugs were used: atropine methonitrate (BDH), isoprenaline sulphate (Burroughs Wellcome), adrenaline bitartrate (BDH), noradrenaline bitartrate (Bayer), papaverine hydrochloride (BDH), oxymetazoline hydrochloride (A & H), choline theophyllinate (A & H), phenylephrine hydrochloride (A & H), hemicholinium-3 (Aldrich), hexamethonium iodide (Koch-Light).

RESULTS

Nature of response. The application of the electrical stimulus to the tissue resulted in a rapid increase in intraluminal pressure followed by a decrease and a slow return to normal (Fig. 2a). The rise in pressure could be antagonized by atropine 10 ng/ml and the decrease in pressure by propranolol 50 ng/ml (Fig. 2a, b). A complete block in the rise but not the fall in intraluminal pressure was obtained with 10 μg/ml hemicholinium-3 which was not reversed by choline 400 μg/ml but was reversed by repeated washing of the preparation. Neither the rise nor the fall in intraluminal pressure was reduced by hexamethonium 10 μg/ml. With regular stimulation (20 Hz every 1 min) the resting intraluminal pressure became negative with respect to atmospheric pressure. If lower stimulation rates were applied in

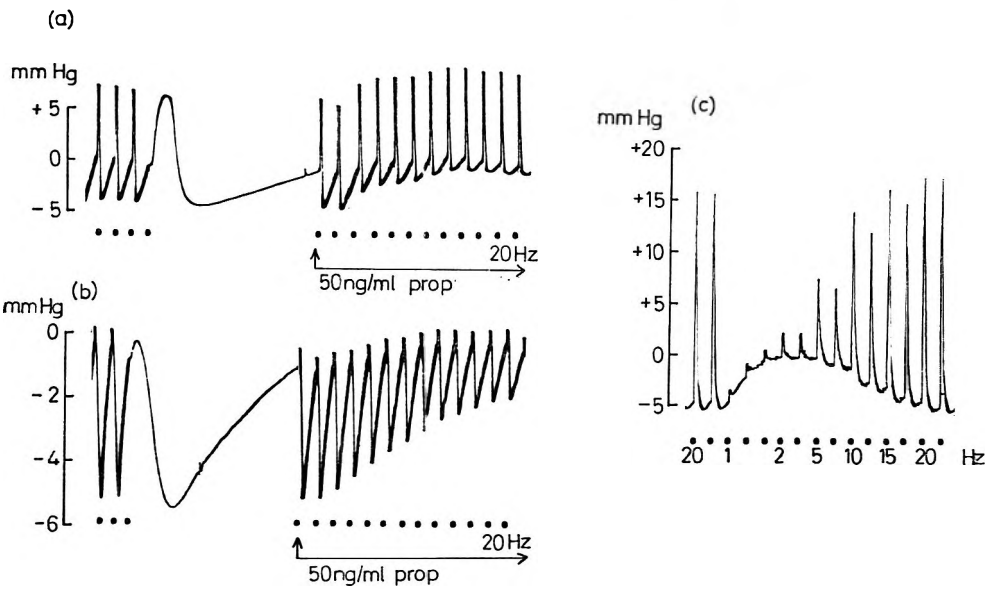

FIG. 2. Typical responses of the isolated intact trachea to transmural electrical stimulation. (a) Repeated stimulations at 20 Hz every 1 min, expanded part of record shows clearly the two components of the response. The decrease in intraluminal pressure is blocked by propranolol 50 ng/ml (prop). (b) Responses to stimulation at 20 Hz after the addition of 10 ng/ml atropine to the bath. Only a relaxation of the tissue is observed. This could be antagonized by propranolol 50 ng/ml. The sensitivity of the recording system was increased to demonstrate clearly the relaxation of the tissue. (c) Responses to stimulation at frequencies of 1, 2, 5, 10 and 20 Hz. Note the tendency of the resting intraluminal pressure to return to zero when lower rates of stimulation are employed. All drugs applied to outer surface.

order to show the relations between frequency and response (Fig. 2c), or stimulation was withheld, the resting intraluminal pressure returned to atmospheric pressure.

Effects of drugs on the rise in intraluminal pressure. The rise in intraluminal pressure could be prevented by drugs possessing anticholinergic, sympathomimetic or spasmolytic activity. Cumulative dose-effect curves for the inhibitory action of atropine, isoprenaline, adrenaline, noradrenaline, papaverine, oxymetazoline, choline theophyllinate and phenylephrine on the rise in intraluminal pressure are shown in Fig. 3. The inhibitory effect of isoprenaline was prevented by propranolol. Cumulative dose-effect curves were obtained for isoprenaline before and after the addition of 1, 5 and 25 ng/ml of propranolol. The dose-effect curve for isoprenaline took 50–60 min to prepare and was started 15 min after the addition of propranolol. The pA_2 value (contact time 45 min) was determined by the method of Arunlakshana & Schild (1959). The mean value $\pm$s.e. for three determinations was $8{\cdot}59 \pm 0{\cdot}78$.

DISCUSSION

A preparation of airway smooth muscle was required for the rapid evaluation of β-adrenoreceptor agonists. Most preparations to date have disadvantages due to the method of measurement or lack of intrinsic tone, or both. The whole trachea preparation described can be subjected to repeated periods of stimulation at selected frequencies by use of an automated gating device on the stimulator, and the intraluminal pressure continuously sensed and recorded. It was found necessary to use

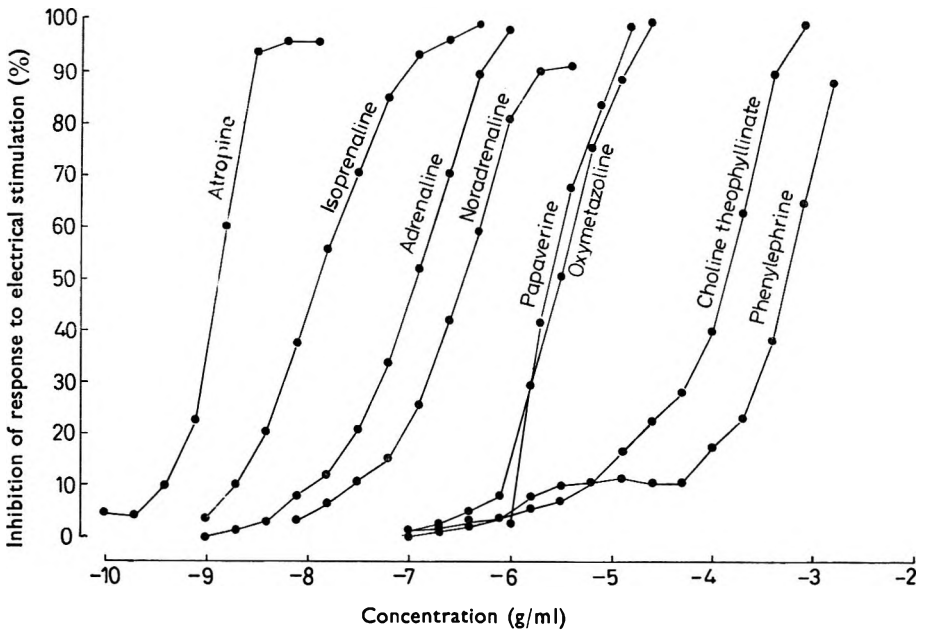

FIG. 3. Dose-effect curves for the inhibitory actions of drugs on the rise in intraluminal pressure induced by transmural stimulation of the isolated trachea. Frequency of stimulation 20 Hz. Drugs applied to the outer surface.

alternating square wave pulses since monophasic pulses resulted in gas production at the electrode surfaces. The gas production on the intraluminal electrode caused an increase in intraluminal pressure and deterioration of the preparation. The preparation gave a rise and then a fall in intraluminal pressure in response to stimulation. The rise in intraluminal pressure was usually very much greater than the subsequent fall. In some preparations no fall in intraluminal pressure was observed. The preparation of whole trachea of the guinea-pig as described by Foster (1964) and Carlyle (1964) gave a small contraction superceded by a rapid relaxation in response to electrical stimulation. These workers used a narrower pulse width for stimulation than was used here, which may have been a contributing factor. The pulse width of 1 ms used in the present preparation was found to be optimum for a contractile response. Lower pulse width resulted in a smaller contraction whilst greater pulse width did not increase the size of the contraction.

The contraction was due to excitation of postganglionic parasympathetic nerves since the response was blocked by atropine and hemicholinium-3 but not by hexamethonium. Similarly, the relaxation was due to stimulation of postganglionic sympathetic nerves since the response was reduced by propranolol and not by hexamethonium or hemicholinium-3. The preparation showed the expected sensitivity to a variety of drugs known to inhibit contractile responses of tracheobronchial smooth muscle. Of particular interest was the quantitative aspects of the interaction of propranolol with isoprenaline on the β-adrenoreceptors in this tissue. The trachea contains β-2 type receptors (Lands, Arnold & others, 1967) and propranolol gave a pA_2 value of 8·59 against isoprenaline on these receptors. Blinks (1967) gave a pA_2 value of 8·8 for propranolol against isoprenaline on the

β-1 type receptor the isolated driven left atria of the guinea-pig. Thus propranolol can be used to demonstrate the presence of β-adrenoreceptors in a tissue but it does not differentiate between β-1 type and β-2 type receptors.

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We are grateful to Mr. S. W. Smith for constructing the apparatus.

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Effects of salicylate on the incorporation of orotic acid into nucleic acids of mouse tissues *in vivo*

K. JANAKIDEVI AND M. J. H. SMITH

Department of Biochemical Pharmacology, King's College Hospital Medical School, Denmark Hill, London, S.E.5, U.K.

In mice, given intraperitoneal injections of orotic acid-5-^{3}H, two peaks of incorporation of radioactivity at 30 min and 6 h occurred in the RNA of kidney and of liver and its subcellular components. The concurrent administration of salicylate, in doses of 200 mg/kg body weight and above, significantly inhibited the incorporation of the labelled orotic acid at 30 min but not at 6 h.

Salicylate, in concentrations of 3 mM and above, inhibits the activities of RNA and DNA polymerases prepared from rat liver (Janakidevi & Smith, 1969). These observations suggested that the drug may interfere with the biosynthesis of nucleic acids *in vivo*. The present paper describes the effects of the injection of salicylate on the incorporation of radioactivity from tritiurated orotic acid into nucleic acid fractions of mouse liver and kidney.

EXPERIMENTAL

Materials and methods

Male albino mice, 25–30 g, maintained on MRC modified cube diet no. 41B were used. Orotic acid-5-^{3}H (specific activity 1000 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks., RNA from Boehringer Corporation (London) Ltd. and orcinol from the Sigma Chemical Co., St. Louis. The sodium salicylate was of British Pharmacopoeial grade, all other chemicals were of analytical grade and glass distilled water was used throughout. Each mouse received an intraperitoneal injection (0·5 ml) containing 25 μCi of orotic acid-5-^{3}H plus either sodium salicylate at dose levels of either 50, 100, 200 or 400 mg/kg body weight or sufficient sodium chloride to contain the same final concentration of sodium. Groups, each of three mice, were killed by cervical fracture at appropriate time intervals (see Results section) and duplicate samples (approximately 0·25 to 0·5 g) of the excised liver and kidney were immediately homogenized, using an all glass pestle and mortar, at 0° with 10 volumes of 3% (v/v) $HClO_4$. The homogenates were centrifuged at 3000 *g* for 15 min at 0° and the sediment resuspended and centrifuged with two further quantities (5 vol) of perchloric acid and one of distilled water (5 vol). The original supernatant together with the supernatants from the subsequent acid and water extractions of the sediment were combined as the acid-soluble fraction. The final sediment was washed with two quantities of ice cold ethanol–ether mixture (3:1) to remove lipids, and the RNA was then extracted according to the directions of Tata & Widnell (1966). The specific activity of the extracted RNA was measured by estimating the RNA content by the orcinol method

(Hurlbert, Schmitz & others, 1954) and the radioactivity as described below. Sufficient M KOH was added to the acid-soluble fraction to adjust the pH to 8·0, the mixture was allowed to stand for 2 h at 0° and then centrifuged at 3000 *g* to remove the deposited $KClO_4$. Aliquots (0·1 ml) of the supernatant were removed for measurement of radioactivity and the pH of the remaining supernatant was adjusted to pH 1 with 6M HCl (Munro, Jackson & Korner, 1964). The extinction at 260 nm was measured in this mixture using a Unicam SP 800 spectrophotometer and taken to represent the acid-soluble nucleotide fraction. All radioactive counting was performed on samples dried on GF/A (2·1 cm) glass fibre discs in a Beckman LS 200B liquid scintillation system

In some experiments the whole liver was homogenized in 10 vol of 0·32 M sucrose containing 3 mM $MgCl_2$ and fractionated by differential centrifugation. The crude nuclear pellet, obtained at 700 *g*, was further purified (Janakidevi & Smith, 1969), the mitochondrial fraction being separated at 15000 *g* for 20 min, the microsome fraction at 105 000 *g* for 1 h and the residual supernatant taken as the cell sap. The acid-soluble components and the RNA were extracted from each subcellular fraction as described above.

RESULTS

The incorporation of radioactivity into the acid-soluble fraction and into the RNA of mouse liver at 5, 10, 15, 30 min, 1, 3, 6, 12 and 24 h after the intraperitoneal injection of orotic acid-5-^{3}H are given in Fig. 1. The results show that the incorporation of radioactivity into both fractions reached a peak at 30 min, and

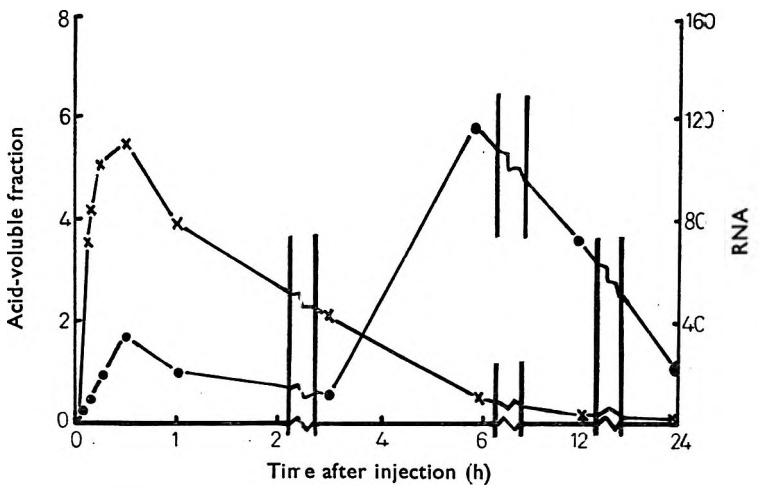

FIG. 1. Incorporation of orotic acid-5-^{3}H into the acid soluble fraction and RNA of mouse liver. The labelled orotic acid (25 μCi) given by intraperitoneal injection at 0 min. Individual results represent the mean values from three mice. ×, incorporation of ^{3}H into acid soluble fraction, expressed as counts/min × 10^{-2} per μg equivalent of RNA. ●, incorporation of ^{3}H into RNA, expressed as counts/min × 10^{-2} per mg of RNA isolated.

subsequently declined for the acid-soluble fraction but that the initial peak at 30 min for the RNA was succeeded by a second peak at 6 h. Similar results were also observed with the mouse kidney.

The effects of an intraperitoneal injection of 400 mg/kg body weight of salicylate, given at the same time as the labelled orotic acid, on the incorporation of radioactivity into the acid-soluble fraction and into the RNA of mouse liver and kidney at 30 min and at 6 h are given in Table 1. The results show that the salicylate

Table 1. *Effect of the injection of salicylate on the incorporation of orotic acid-5-^3H into the acid-soluble fraction and RNA of mouse liver and kidney.* The labelled orotic acid and the salicylate (400 mg/kg body weight), when present, were given by intraperitoneal injection at 0 min. The results are expressed as counts/min per μg RNA equivalent for the acid-soluble fraction and counts/min per mg RNA isolated for the RNA. Each value is given as the mean ± standard deviation for each group of three animals.

Organ	Treatment	Radioactivity incorporated 30 min after injection		6 h after injection	
		Acid-soluble fraction	RNA	Acid-soluble fraction	RNA
Liver	Control	518 ± 103	2268 ± 377	715 ± 116	13528 ± 4744
	Salicylate	267 ± 96*	732 ± 118*	546 ± 124	14816 ± 5716
Kidney	Control	4336 ± 754	72290 ± 5193	1190 ± 278	194237 ± 22661
	Salicylate	2459 ± 1000*	46018 ± 5772*	1493 ± 301	236255 ± 54738

* Statistically significant difference between control and salicylate results. $P < 0{\cdot}05$.

injection caused significant inhibition of the incorporation of radioactivity into the acid soluble fraction and into the RNA of both the liver and kidney at 30 min but not at 6 h. The results of further experiments, in which the labelled orotic acid was administered at zero time and the salicylate injected after 5½ h, showed that the drug did not affect the incorporation of tritium into either the acid-soluble fraction or into the RNA in the liver and kidney of animals killed at 6 h.

The results in Table 2 show the effects of the injection of 400 mg/kg body weight of salicylate on the incorporation of isotope into the acid-soluble fraction and into

Table 2. *Effect of the injection of salicylate on the incorporation of orotic acid-5-^3H into the acid-soluble fraction and RNA of subcellular fractions of mouse liver.* Experimental details as for Table 1

Subcellular fraction	Treatment	Radioactivity incorporated 30 min after injection		6 h after injection	
		Acid-soluble fraction	RNA	Acid-soluble fraction	RNA
Nuclei	Control	99 ± 23	36600 ± 3961	116 ± 25	50175 ± 5392
	Salicylate	31 ± 14*	14216 ± 9599*	87 ± 16	74066 ± 6890
Mitochondria	Control	193 ± 23	259 ± 22	82 ± 22	12421 ± 1690
	Salicylate	98 ± 21*	138 ± 40*	64 ± 12	9400 ± 1596
Microsomes	Control	154 ± 36	670 ± 128	21 ± 10	16415 ± 1560
	Salicylate	103 ± 15	194 ± 64*	42 ± 15	15240 ± 1468
Cell sap	Control	193 ± 70	767 ± 155	60 ± 21	3306 ± 456
	Salicylate	146 ± 46	266 ± 67*	54 ± 14	3589 ± 520

* Incorporation of ^3H significantly decreased in salicylate-treated animals. $P < 0{\cdot}05$.

the RNA of the subcellular components of liver from mice killed after 30 min and 6 h. In general, the drug caused significant inhibition of incorporation of radioactivity at 30 min in all the fractions except the acid-soluble fraction of the microsomes and cell sap, but did not inhibit the corresponding incorporations at 6 h. In Table 3, the effects of varying the size of the injected dose of the salicylate on the

Table 3. *Effect of varying the size of the injected dose of salicylate on the incorporation of orotic acid-5-^3H into the acid-soluble fraction and RNA of mouse liver.* Experimental details as for Table 1

Organ	Salicylate mg/kg body wt	Radioactivity incorporated 30 min after injection Acid-soluble fraction	RNA
	0	641 ± 85	2203 ± 345
	50	583 ± 146	1870 ± 264
	0	723 ± 48	2709 ± 440
	100	654 ± 106	2642 ± 502
Liver	0	742 ± 69	2085 ± 355
	200	248 ± 38*	812 ± 115*
	0	518 ± 103	2268 ± 377
	400	267 ± 96*	732 ± 118*
	0	7547 ± 1399	68027 ± 6059
	50	5732 ± 1899	68891 ± 10470
	0	6956 ± 403	59211 ± 8622
	100	6223 ± 1142	51337 ± 8045
Kidney	0	7909 ± 1736	90265 ± 3423
	200	3765 ± 708*	68646 ± 1422*
	0	4336 ± 754	72290 ± 5193
	400	2459 ± 1000*	46018 ± 5772*

* Statistically significant difference between control and salicylate results. $P < 0.05$.

30 min incorporation into the acid-soluble fraction and the RNA of whole liver and kidney are given. Significant inhibition was only observed with salicylate doses of 200 mg/kg body weight and above.

DISCUSSION

Orotic acid-5-^3H is incorporated into the acid-soluble fraction and into the RNA of mouse liver and kidney *in vivo*, the initial peak of incorporation occurring 30 min after administration of the labelled precursor. The concurrent administration of salicylate, in doses of 200 mg/kg body weight and above, significantly inhibits the 30 min incorporation into the two organs and into the main subcellular fractions of the liver. Packman, Esterly & Peterson (1969) have recently reported that salicylate inhibits the incorporation of tritiurated uridine into the nucleic acids of human peripheral lymphocytes.

Salicylate could produce this effect by one or more of several mechanisms. It has been shown (Janakidevi & Smith, 1969) that the drug, in concentrations of 3 mM and above, inhibits the activities *in vitro* of nucleic acid polymerases prepared from rat liver. In the initial experiments reported in the present paper a dose of 400 mg/kg body weight was chosen because the salicylate concentration in the liver and kidneys of mice 30 min after intraperitoneal injection of this dose was approximately 2 mM (Sturman, Dawkins & others, 1968). It is therefore possible that

salicylate inhibits these enzyme activities *in vivo* and therefore prevents the incorporation of the orotic acid. The failure of the salicylate injection to effect the 6 h incorporation of the orotic acid could have been due to the lower salicylate concentrations (0·5 mM) present in the organs at the longer time interval. However, the 6 h incorporation was not affected by a dose of 400 mg/kg salicylate being given 5½ h after the orotate injection when salicylate concentration of about 2 mM would have been present in the liver and kidney. It is possible that salicylate only inhibits the biosynthesis of certain types of nucleic acids, e.g., messenger RNA and not the formation of other RNA species, e.g., ribosomal RNA. A second mechanism is that salicylate could interfere with the series of phosphorylation reactions concerned in the conversion of orotic acid to UTP and related nucleotide triphosphates. The drug is known to uncouple oxidative phosphorylation reactions in respiring mitochondrial preparations in concentrations above 0·5 mM (Brody, 1956) and salicylate concentrations as low as 0·1 mM decrease the formation of ATP in the isolated rat diaphragm (Smith & Jeffrey, 1956). The present results do not exclude the possibility that the salicylate may have interfered with the transport of the labelled orotic acid from the injection site into the circulation and hence to the liver and kidney.

If salicylate inhibits the biosynthesis of nucleic acids *in vivo* this may explain, at least in part, the effects of the drug in retarding growth in young animals (see Limbeck, Conger & others, 1966) and in wheat coleoptiles (Reid, 1957) and its teratogenic effects in rodents (see Larsson, Bostrom & Ericson, 1963).

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LETTERS TO THE EDITOR

The effect of pH on the bactericidal activity of pentafluorophenol

It is generally accepted that the undissociated molecule is the predominantly toxic form of antimicrobially active weak acids (Ordal, 1941; Winsley & Walters, 1965; Albert, 1968). However, pentachlorophenate ions have been shown to have high bactericidal activity (Allawala & Riegelman, 1954; Hueck, Adema & Wiegmann, 1966). During a study of the activities of ring-substituted fluorine derivatives of phenol (Pinney & Walters, 1967, 1969), we have investigated pentafluorophenol solutions of varying pH to determine whether ionic activity also occurred in this pentahalogenated phenol.

Solutions of 0·15% pentafluorophenol were prepared in 0·04M McIlvaine citric acid-phosphate buffer (McIlvaine, 1921), and 0·1% survivor times of *Escherichia coli* NCTC 5933 in these solutions were determined as before (Pinney & Walters, 1967, 1969). Buffer alone at pH 3·60 produced a 70% kill of the organisms after 5 h, but at pH 5·46, the loss of viability was only 20% after the same period (Fig. 1a). The percentages of ionized molecules at each pH tested were calculated from the Henderson-Hasselbalch equation, the pK_a of pentafluorophenol being 5·33 (Birchall & Haszeldine, 1959). Results are presented in Fig 1b and c.

It is apparent from Fig. 1b, that the intrinsic bactericidal activity of pentafluorophenol in solution decreases markedly with increasing ionization. If the bactericidal

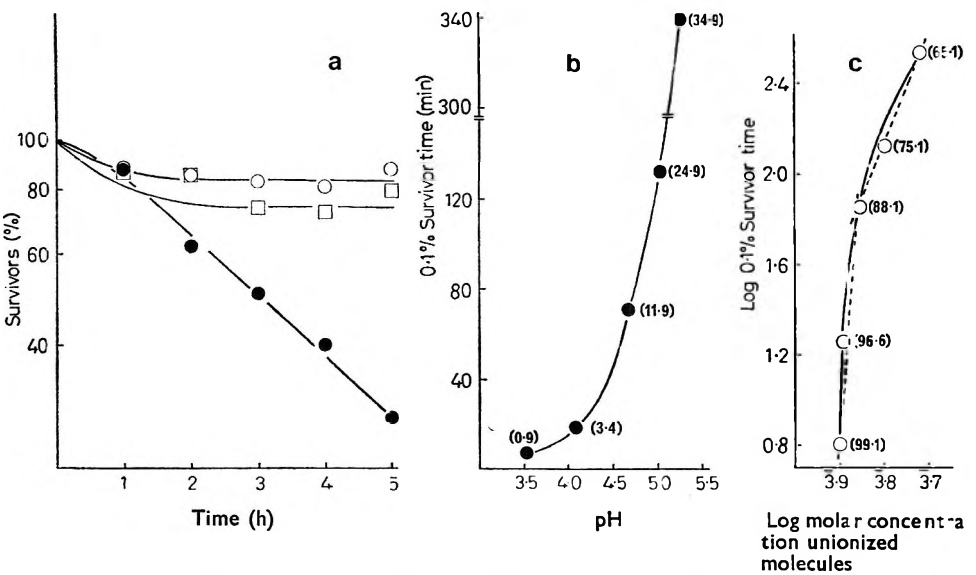

FIG. 1. a. Log survivor-time curves for *E. coli* in water and in citric acid-phosphate buffer. ○ = Water. □ = Buffer, pH 5·46. ● = Buffer, pH 3·60.
b. Relation between pH and 0·1% survivor time for *E. coli* in solutions of 0·15% pentafluorophenol. Figures in brackets are the percentages of ionized molecules.
c. Concentration exponent plot of unionized molecules of 0·15% pentafluorophenol at various pH values against *E. coli*. Figures in brackets are the percentages of unionized molecules. Dashed lines correspond to calculated regressions (slope = n). For 99·1% to 88·1% unionized molecules n = 19·2; for 88·1% to 65·1% unionized molecules n = 5·7.

activity is due only to the undissociated molecules, the concentration exponent plot for unionized molecules (Fig. 1c) should be linear with a slope corresponding to the dilution coefficient (n) of the compound in water. The calculated slope of the regression of log 0·1% survivor time on log molar concentration of unionized molecules (Fig. 1c) for pH 3·54–4·66 (99·1%–88·1% unionized molecules) is 19·2; for pH 4·66–5·22 (88·1%–65·1% unionized molecules) it is 5·7. This latter figure corresponds well with the n value of 5·1 for pentafluorophenol in water (Pinney & Walters, 1969), from which it may be concluded that over this pH range, the antibacterial activity is due to the unionized molecules. Over the lower pH range, the bactericidal effect is greater than would be expected if the unionized molecules alone were responsible for toxicity. The 0·1% survivor time for 0·15% pentafluorophenol in buffer at pH 3·54 (99·1% unionized molecules) is 6·4 min, and for the same concentration in water (pH 3·80 = 98·2% unionized molecules) it is 24·5 min. It is therefore evident that the activity is increased by the presence of buffer as was found with the monofluorophenols (Pinney & Walters, 1967). However, for the buffer to be completely responsible for the enhanced activity at the lower pH, it would need to exert an unequal synergistic toxic effect over the pH range 3·5 to 5·3. Such a mechanism is less likely to be the case than that the increased activity is due to a combination of both the buffer effect and the greater concentration of hydrogen ions at the lower pH values; thus Fig. 1a shows that the toxicity of buffer alone is greater at pH 3·60 than at pH 5·46.

Therefore whilst the precise cause of the biphasic nature of Fig. 1c is not known, it is clear from the results that unlike pentachlorophenol, the toxic form of pentafluorophenol is the unionized molecule; pentafluorophenate ions have little activity.

Department of Pharmaceutics,
The School of Pharmacy,
Brunswick Square,
*London, W.C.*1, *England.*

R. J. PINNEY
V. WALTERS

October 10, 1969

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Differential inhibition of RNA polymerase activities by salicylate *in vitro*

The final stage in the biosynthesis of RNA is the polymerization of nucleoside triphosphates by a polymerase enzyme (nucleoside triphosphate: RNA nucleotidyltransferase EC 2.7.7.6.). It was reported by Widnell & Tata (1966) that the activity of the mammalian nuclear polymerase, with respect to the types of RNA synthesized, depended on whether the assay system contained either magnesium ions or ammonium sulphate plus manganese ions. Several hypotheses have been formulated to explain this observation (see Stirpe & Fiume, 1967) but recent work strongly suggests that there are two forms of RNA polymerase with different specificities towards the DNA templates and different requirements for divalent cations (Liao, Sagher, & others, 1969). Base composition analysis of the reaction products showed that the Mn^{2+}: ammonium sulphate-activated enzyme synthesized DNA-like RNA whereas the Mg^{2+}-activated enzyme caused the formation of ribosomal RNA (Widnell & Tata, 1966).

Salicylate, in concentrations of 3mM and above, significantly inhibits the activity of rat liver nuclear RNA polymerase in the presence of Mn^{2+} and ammonium sulphate (Janakidevi & Smith, 1969). The present results (Table 1) confirm this finding but also show that inhibition does not occur with the Mg^{2+}-activated enzyme in rat liver nuclei either incubated with 3 mM salicylate *in vitro* or obtained from rats killed 30 min after injection with 400 mg/kg body weight of sodium salicylate. Similar results were obtained in the mouse and with a nuclear fraction obtained from whole 13 day and 16 day rat foetuses.

Table 1. *Effects of salicylate on the nuclear RNA polymerases*

Treatment	p mol nucleotide incorporated per mg DNA	
	Mn^{2+}: $(NH_4)_2SO_4$ activated enzyme	Mg^{2+}-activated enzyme
Incubated with 3 mM salicylate	890 ± 38 (5)	166 ± 30 (5)
Control	* 991 ± 42 (4)	161 ± 18 (5)
Obtained from animals injected with 400 mg/kg body weight salicylate	505 ± 62 (3)	177 ± 22 (3)
Control from animals injected with saline	* 1052 ± 152 (3)	174 ± 20 (3)

Incubation mixtures according to the directions of Widnell & Tata (1966) except that the nucleoside triphosphate concentrations were 0·4 mM, ATP-8-^{14}C being used as the labelled precursor, the Mn^{2+}: $(NH_4)_2SO_4$ activated enzyme being incubated for 1 h at 17° and the Mg^{2+}-activated enzyme for 15 min at 37°. The polymerase activities were assayed as described previously (Janakidevi & Smith, 1969). Each value is given as the mean ± standard deviation, the number of experiments being given in parentheses. The results have been analysed by the *t*-test and * indicates a statistically significant difference ($P < 0.05$) between the control and salicylate values.

These observations support the view that there are two forms of RNA polymerase in rodent tissues. Salicylate resembles α-amanitin in only inhibiting the Mn^{2+}: ammonium sulphate enzyme and differs from other inhibitors of RNA polymerase, such as actinomycin D and aflatoxin B_1, which are more inhibitory for the Mg^{2+}-activated enzyme (Stirpe & Fiume, 1967). They also suggest that salicylate preferentially interferes with the biosynthesis of some species of RNA. The formation of DNA-like RNA, including messenger RNA, but not of ribosomal RNA would be expected to be inhibited by the drug both *in vitro* and *in vivo*. This could explain the finding that the intraperitoneal injection of salicylate into adult mice decreases the incorporation of radioactive orotic acid in the RNA of liver and kidney at 30 min but not at 6 h (Janakidevi & Smith, 1970).

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Department of Biochemical Pharmacology,
King's College Hospital Medical School,
Denmark Hill,
London, S.E.5, England.

K. JANAKIDEVI
M. J. H. SMITH

July 31, 1969

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The effect of oxotremorine on the acetylcholine content of different parts of cat brain

Tremorine (Pepeu, 1953; Holmstedt, Lundgren & Sundwall, 1963) and also its active metabolite oxotremorine (Holmstedt & Lundgren, 1966) cause an increase of brain acetylcholine and tremor in rats. A causal relation between the two effects was proposed (Holmstedt & Lundgren, 1966) but a number of substantial objections have been recently presented (Cox & Potkonjak, 1969a).

We have now investigated whether oxotremorine also increases brain acetylcholine in rats and whether the increase occurs uniformly in all brain regions.

Of eight young cats, each weighing about 1 kg, four received an intraperitoneal injection of saline and four 1·0 mg/kg of oxotremorine. Within a few minutes of giving the drug the cats showed intense tremor, salivation, miosis and behaviour similar to false rage. Fifteen min after the injection the cats were killed under light halothane anaesthesia, the skull opened, the brain removed and placed on ice. From each brain 3 samples were prepared: (I) about 500 mg of cortex were excised from the frontal lobes; (II) the diencephalon and the upper part of the midbrain were dissected following the lateral ventricles, the head of the caudate nucleus and a plane from the posterior colliculi to the rostral border of the pons. The weight of this sample was about 2·0 g; (III) the caudal part of the brain stem including the pons and the medulla oblongata; its weight was about 1·5 g.

Acetylcholine was extracted by the method of Smallman & Fisher (1958) modified by Bartolini & Bedarida-Jarach (1965), and assayed on the dorsal muscle of the leech. Recovery of added acetylcholine was 90%.

The results are reported in Table 1. The values of acetylcholine content found in the control cats are in good agreement with previous observations (Macintosh, 1941; Pepeu, 1966).

Table 1. *The influence of oxotremorine* (1 *mg/kg, i.p.*) *on the acetylcholine content of the cat cortex* (*I*), *diencephalon and upper part of midbrain* (*II*) *and the caudal part of the brain stem* (*III*). (Means ± s.e. of four experiments)

	Acetylcholine (μg/g)			
	Controls	After oxotremorine	% Increase	*P*
I	1·12 ± 0·26	1·43 ± 0·33	27	N.S.
II	2·53 ± 0·28	4·79 ± 0·49	89	<0·01
III ..	3·32 ± 0·61	3·45 ± 0·58	4	N.S.

It appears that oxotremorine increases the content of brain acetylcholine in the cat as it did in rats, but the increase is limited to some parts of the brain. There is an almost two fold rise of concentration in sample II which includes the diencephalon and part of the midbrain, a small but not significant rise in the cortex and no change in the lower brain stem.

The mechanism of action is not yet known. An effect on cholinesterases and on cholinoacetylase seems to be excluded (Holmstedt, Lundgren & others, 1965) and the hypothesis that the drug might act by mobilizing acetylcholine from an otherwise undetected store has been proposed (Holmstedt, 1967). Oxotremorine is known to cause a rise in total acetylcholine also when added to a brain homogenate (Lundgren & Malberg, 1968).

The largest increase in acetylcholine content occurs in the region which includes the caudate nucleus, the substantia nigra and the globus pallidus. Direct injections of tremorine in these nuclei have been shown to produce tremor in the rat (Cox & Potkonjak, 1969b). Before ruling out a causal relation between the effect of oxotremorine on acetylcholine content and the onset of tremor, the possibility of a rapid and strictly localized rise of acetylcholine level in the areas of the extrapyramidal system involved in the control of movement and posture should be excluded.

Institute of Pharmacology & Pharmacognosy,
Faculty of Pharmacy,
University of Cagliari, Italy.

A. BARTOLINI
R. BARTOLINI
G. C. PEPEU

October 24, 1969

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A comparison of the β-adrenoreceptor stimulant properties of isoprenaline, with those of orciprenaline, salbutamol, soterenol and trimetoquinol on isolated atria and trachea of the guinea-pig

In a previous publication (Cullum, Farmer & others, 1969) the β-adrenoreceptor agonist activities of isoprenaline, orciprenaline and salbutamol on the force of contraction of isolated whole atria and on the tone of tracheal chain preparations of the guinea-pig were reported. We have now examined the activities of these drugs and those of soterenol and trimetoquinol on isolated right and left atria and on isolated whole trachea of the guinea-pig.

Guinea-pigs were killed by a blow on the head and the trachea and heart removed. The trachea was mounted on an apparatus which allowed the measurement of the changes in intraluminal pressure of the trachea in response to transmural electrical stimulation (Farmer & Coleman, 1969). Right atrial strips were prepared for the measurement of the rate of contraction according to the method of Black, Duncan & Shanks (1965). The strips were attached to an isometric strain gauge, the output of which was used to drive an instantaneous rate meter. The left atrium was prepared for measurement of isometric tension and was driven electrically by supramaxial square wave pulses of 0·5 ms duration, every second (Blinks, 1967). Cumulative dose response curves for the above drugs were determined on these three preparations and the results are illustrated in Fig. 1A–C. Activities shown are relative to isoprenaline. The maximum effect obtained with isoprenaline was taken as 100%.

Isoprenaline, salbutamol, soterenol and orciprenaline produced dose-dependent decreases in the response of the isolated trachea to electrical stimulation. The slope of the dose effect curve and maximum response achieved were similar for each drug. Trimetoquinol however produced a plateau effect at the 75% level of inhibition which is probably attributable to its β-adrenergic activity. With further and much larger increases in concentration, 100% inhibition was obtained. This latter effect may be due to a non-adrenergic spasmolytic action which would not be surprising in a drug which is chemically related to papaverine. The dose ratios for 50% inhibition were: isoprenaline 1, trimetoquinol 2, soterenol 5, salbutamol 6 and orciprenaline 144.

On the isolated atria preparations, only isoprenaline and orciprenaline had dose effect curves of similar slope and magnitude on both rate and force of contractions. Soterenol was a full agonist with respect to rate but a partial agonist with respect to force. Salbutamol and trimetoquinol were partial agonists with respect to both force and rate although salbutamol had fuller agonist activity on rate than trimetoquinol. The dose ratios with respect to rate (50% maximum) for these drugs were: isoprenaline 1, soterenol 3·3, orciprenaline 125, salbutamol 500. A dose ratio for trimetoquinol could not be calculated. The dose ratios with respect to force were: isoprenaline 1, orciprenaline 63, salbutamol 2500 and for soterenol and trimetoquinol $<10{,}000$.

Lands, Arnold & others (1967) showed that small modification of the chemical structure of isoprenaline at or near the nitrogen atom could produce compounds with high selectivity for cardiac or bronchial β-receptors. They postulated that the β-receptors in these tissues were different and classified those in heart muscle as β-1 type and those in the bronchial muscle as β-2 type. The activities of new β-receptor agonists which do not have catechol functions, e.g. salbutamol and soterenol, or which are not phenylethanolamines e.g. trimetoquinol show very clearly that these receptors are not the same. Salbutamol and trimetoquinol show selectivity for the β-2 type receptors in the trachea. Soterenol has high activity on β-2 type but also on β-1 type

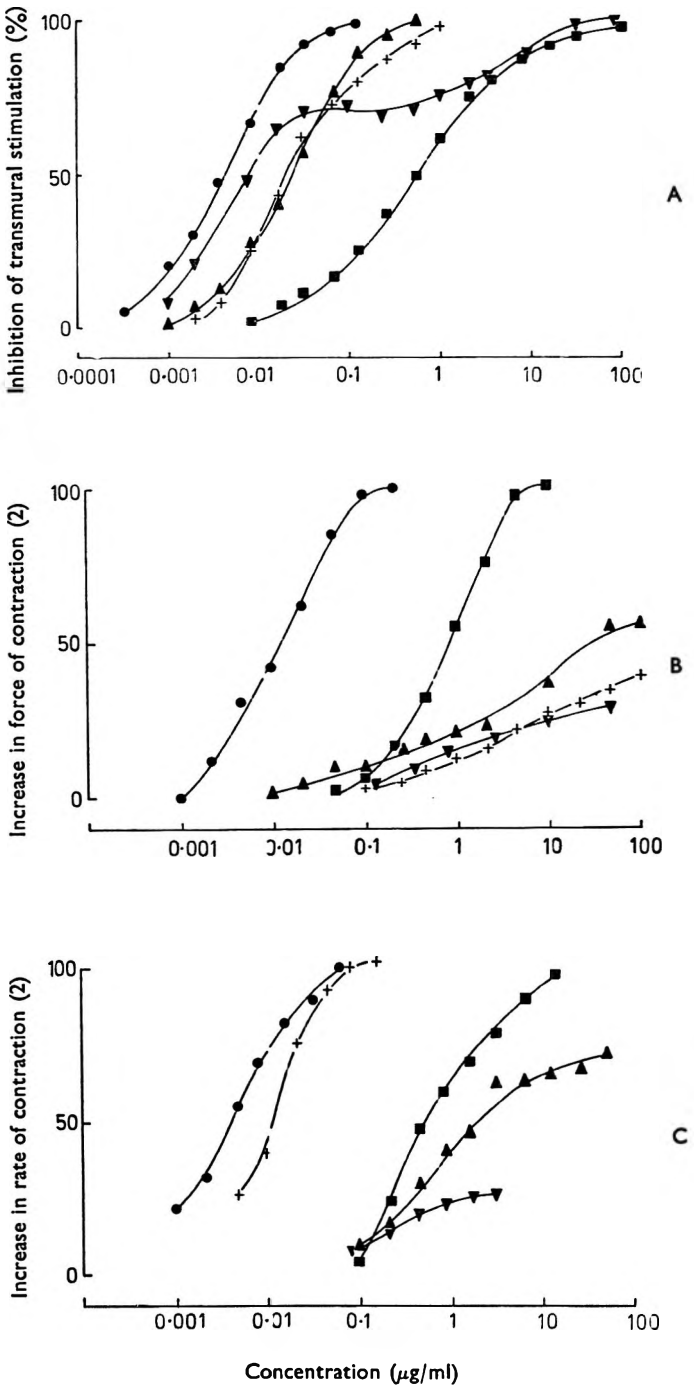

FIG. 1. Dose-response curves for isoprenaline (●), salbutamol (▲), soterenol (+), orciprenaline (■) and trimetoquinol (▼) A, in inhibiting increases in intraluminal pressure induced by transmural electrical stimulation of isolated guinea-pig trachea; B, on the force of contraction of the electrically driven isolated left atrium of the guinea-pig; C, on the rate of contraction of the spontaneously beating right atrial strip of the guinea-pig.

(rate) but not β-1 type (force) of the atria. This result suggests that β-1 type receptors are not homogeneous. Orciprenaline is not selective and has equal but low activity on β-1 and β-2 type receptors. The activities described for these new compounds further substantiate the classification proposed by Lands and co-workers (1967) for β-receptors in cardiac and bronchial muscle.

Department of Pharmacology,
Allen and Hanburys Ltd.,
Ware, Herts.
September 25, 1969

J. B. FARMER
I. KENNEDY
G. P. LEVY
R. J. MARSHALL

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The absence of a significant histamine receptor reserve in vascular smooth muscle

The existence of a receptor reserve was first postulated by Nickerson (1956) on the basis of the parallel shift in the dose response curve for histamine, produced by partial blockade with GD-131, an irreversible antagonist of the β-haloalkylamine class. This phenomenon, which was observed in the guinea-pig ileum preparation, can readily be explained by supposing that only a small fraction of the total number of available receptors need to interact with the agonist in order to elicit the maximum response. Such a receptor reserve has been observed in cholinergic systems (Ariens, van Rossum & Koopman, 1960) but appears to be absent or insignificant in adrenergic systems (Moran, May & others, 1967a, 1967b; Moran, Triggle & Triggle, 1969).

To determine whether this parallel shift of the dose-response curve after partial blockade with a β-haloalkylamine is a characteristic of other histamine receptor systems, the nature of the progressive blockade of the histamine-induced contractions of rabbit aortic strips has been observed, using phenoxybenzamine as the irreversible antagonist.

Rabbit aortic strips prepared as described by Furchgott & Bhadrakom (1953) were suspended in organ baths of 15 ml working volume and allowed to equilibrate at 37° for 3 h in Krebs bicarbonate solution containing 0·05 M glucose. The resting tension was maintained at 1 g. A cumulative dose response curve to histamine was then obtained; contractions were recorded by means of a force-displacement transducer (Grass FT03) connected to a Grass model 5P1 polygraph. The preparations were then washed with Krebs bicarbonate solution at frequent intervals until they had returned to the resting tension. Phenoxybenzamine hydrochloride was dissolved in normal saline containing 0·01 M hydrochloric acid and, after this solution had been kept at room temperature for 10 min, aliquots were added to the organ baths to achieve the required concentration. After 5 min exposure to phenoxybenzamine the tissues were washed twice and washed thereafter at 15 min intervals for 2½ h. A further cumulative dose-response curve to histamine was then obtained. Since this preparation is known to increase in sensitivity with time, the experiment was repeated

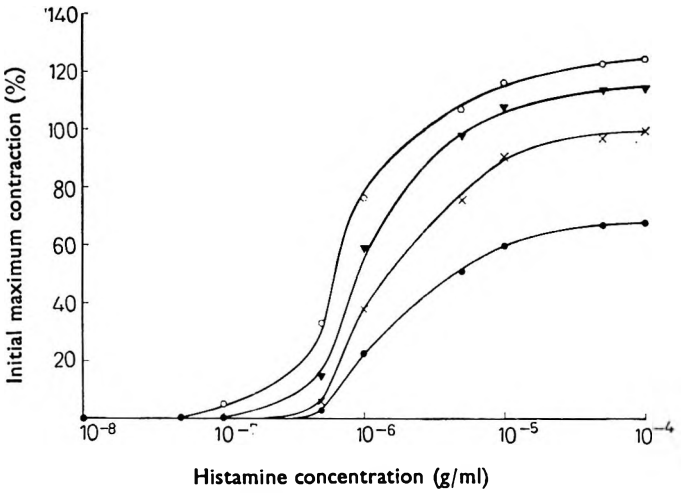

FIG. 1. Dose-response curves for histamine. ○——○ control, ▼——▼ after phenoxybenzamine (10^{-8} g/ml), ×——× after phenoxybenzamine ($2{\cdot}5 \times 10^{-8}$ g/ml), ●——● after phenoxybenzamine (5×10^{-8} g/ml).

a number of times with the omission of the phenoxybenzamine blockade, such preparations being regarded as controls. The histamine phosphate used in these studies was obtained from Fisher Scientific and the phenoxybenzamine hydrochloride was generously donated by Smith Kline and French; drug concentrations are expressed as grams of the salt/ml of bathing medium.

The dose-response curves obtained from these experiments are shown in Fig. 1. The contractions are expressed as the percentage of the maximum response obtained initially, and each curve represents the mean of at least seven experiments. It can be seen that progressive blockade is not accompanied by a shift in the dose-response curve and both the maximum contraction and the slopes of the dose-response curves are depressed after blockade with phenoxybenzamine. This situation would be anticipated in the absence of a significant fraction of spare receptors and it is, therefore, concluded that there is no significant receptor reserve for histamine in rabbit aortic strip.

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Department of Pharmacology,
University of Alberta,
Edmonton 7, Alberta, Canada.

DAVID A. COOK

October 3, 1969

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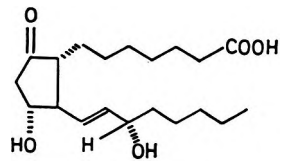
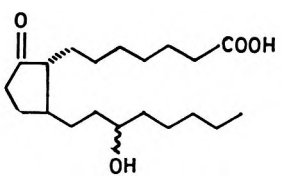
Inhibition of gastric acid secretion by a potent synthetic prostaglandin

The synthetic prostaglandin, 9-oxo-15ξ-hydroxyprostanoic acid (11-deoxy-13,14-dihydroprostaglandin) (AY-22,093), has been examined for its effect on gastric acid secretion in rats using the Shay procedure (Shay, Sun & Gruenstein, 1954) as described by Lippmann (1969) and in rats given repeated small doses of pentagastrin.



PGE_1



AY-22,093

AY-22,093 inhibited basal gastric acid secretion and the activity was dose-dependent (Table 1). At 0·8 mg/kg the inhibition was 52%. Similar activity was observed with PGE_1 at 0·4 mg/kg. Thus, AY-22,093 was about one-half as active as PGE_1.

Pentagastrin, a gastrin-like agent, causes an increase in gastric acid secretion (Barrett, 1966). In a continuous stomach perfusion in anaesthetized rats, subcutaneous injections of pentagastrin made every 20 min gave a steady plateau of gastric acid secretion (Barrett, Raventos & Siddall, 1966). I have examined the effects of repeated injections of a small dose of pentagastrin on gastric acid secretion in rats which were ligated under ether anaesthesia at the pyloric end of the stomach and also at the oesophagus (Levine, 1965). After pyloric ligation the stomachs were washed with 0·9% NaCl until clear. An intraperitoneal injection of 0·5 ml water was given immediately after the oesophageal ligation. Pentagastrin (or vehicle) treatment (1 μg/kg, s.c.) was begun 20 min after the oesophageal ligation and continued at 20 min intervals; the animals were killed by a blow on the head 20 min after the last injection, i.e. 2 h after ligation. The stomachs were removed and the gastric contents emptied into centrifuge tubes. The stomachs were rinsed with twice distilled water to yield a final volume of 5 ml. The samples were centrifuged and titrated (14–17 per group) against 0·01N sodium hydroxide in a direct reading pH meter to pH 7·0 to obtain the total acid. The increase in gastric acid secretion reached a plateau after 4 injections; less variation among the values was observed after 5 injections. The maximum stimulation of gastric acid secretion was about 4 times that of the controls.

The above procedure, employing 5 injections of pentagastrin, was used to determine the effect of AY-22,093 on the induced increase in gastric acid secretion. All animals

Table 1. *Inhibition of basal gastric acid secretion in the rat by AY-22,093*

Compound	Dose mg/kg, s.c.	Gastric acid secretion: m mol acid 4 h ± s.e.	*P*	Gastric acid secretion: % of control
None		0·49 ± 0·04		
AY-22,093	1·6	0·11 ± 0·03	<0·001	23
	0·8	0·24 ± 0·03	<0·001	48
	0·4	0·43 ± 0·06	>0·30	88
PGE_1..	0·4	0·22 ± 0·04	<0·001	44
	0·2	0·31 ± 0·04	<0·01	63

Table 2. *Inhibition of pentagastrin-induced gastric acid secretion in the rat by AY-22,093*

Compound	Dose mg/kg	Gastric acid secretion: μ mol acid/2 h $\pm$ s.e.	*P*	% of control
None		16 $\pm$ 4		
Pentagastrin	0·001 (5x), s.c.	70 $\pm$ 16	<0·02	438
AY-22,093	6·4, s.c.	14 $\pm$ 1	>0·50	88
+ pentagastrin	0·001 (5x), s.c.		<0·01*	
AY-22,093	3·2, s.c.	38 $\pm$ 4	<0·01	238
+ pentagastrin	0·001 (5x), s.c.		>0·10*	
Imipramine	10·0, i.p.	17 $\pm$ 3	>0·90	106
+ pentagastrin	0·001 (5x), s.c.		<0·02*	

* Versus pentagastrin.

received an injection of the test compound, or the corresponding vehicle, and the appropriate injection of the other vehicle immediately after oesophageal ligation; there were 7–9 animals in each group. AY-22,093 prevented the increase in gastric acid secretion at 6·4 mg/kg, s.c., but not at 3·2 mg/kg, s.c. (Table 2). Imipramine, in aqueous solution, (10 mg/kg, i.p.) also inhibited the pentagastrin-induced increase.

The synthetic prostaglandin (PGE), AY-22,093, is a potent inhibitor of basal gastric acid secretion. The presence of a saturated bond at C-13,14 in this type of compound is of importance for the high activity since AY-22,093 is 5 times more active than the previously-reported unsaturated derivative (Lippmann, 1969). In contrast to AY-22,093 the naturally-occurring PGE_1 is unsaturated at C-13,14; PGE_1 also contains a hydroxyl group on C-11. The basal gastric acid secretion-inhibitory activity of PGE_1 reported here is similar to that reported previously (Robert, Nezamis & Phillips, 1968; Lippmann, 1969).

The synthetic PGE also inhibits the increase in gastric acid secretion caused by pentagastrin. This type of activity is also exhibited by PGE_1 (Shaw & Ramwell, 1968a,b). Imipramine prevents the pentagastrin-induced increase; imipramine also has been shown to prevent the induced increase caused by reserpine (Lippmann, 1968). I have observed also that imipramine in half the dose used in the present experiments does not prevent the pentagastrin-induced gastric acid secretion. The total dose (5 μg/kg) of pentagastrin used in the present experiments to induce gastric acid secretion is similar to that used (6 μg/kg) in the experiments made in man (Abernethy, Gillespie & others, 1967).

PGE_1 has recently been reported not to inhibit the pentagastrin-induced gastric acid secretion in man (Horton, Main & others, 1968). However, in this respect, human gastric mucosa contains another prostaglandin, PGE_2, (Bennett, Murray & Wylie, 1968). Thus, it is possible that an appropriate synthetic PGE, in contrast to PGE_1, might exhibit an inhibitory activity in man.

Unlike PGE_1, which is optically pure, the synthetic PGE is a racemate with 4 possible isomers and one of these might prove even more active.

AY-22,093 was synthesized by Dr J. F. Bagli of Ayerst Laboratories.

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Biogenic Amines Laboratory,
Ayerst Laboratories,
Montreal, Quebec,
Canada.

W. LIPPMANN

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The formation of 5-hydroxytryptophol from exogenous 5-hydroxytryptamine in cat spinal cord *in vivo*

5-Hydroxytryptophol was identified in 1962 as one of the main metabolites of exogenous 5-hydroxytryptamine (5-HT) in rats (Kveder, Iskrić & Keglević, 1962). Its occurrence in human urine (Davis, Cashaw & others, 1966) shows that it is also a metabolite of endogenous 5-HT in man. However, in the central nervous system—where the metabolism of 5-HT is quite vigorous (cf. Bulat & Supek, 1968)—5-hydroxytryptophol has been found, so far, only in the pineal body (McIsaac, Farrell & others, 1965). Several authors (Feldstein & Wong, 1965; Eccleston, Moir & others, 1966) have shown that the rat brain tissue is able to metabolize 5-HT into 5-hydroxytryptophol *in vitro*. The present communication reports the formation of 5-hydroxytryptophol from 5-HT in the spinal cord *in vivo*.

We have chosen the spinal cord for studying the metabolism of 5-HT because lumbar and sacral cord show the highest density of 5-HT nerve terminals in mammalian central nervous system (Fuxe, Hökfelt & Ungerstedt, 1969). The experiments were made with adult cats lightly anaesthetized with thiopentone sodium. The lumbosacral cord was exposed and two fine polyethylene tubes were inserted subarachnoidally, one at L_1 segment (inflow) and the other at S_4 segment (outflow). A closed subarachnoid space was formed by tying the thread around dura at L_1 and S_4 segment. To remove cerebrospinal fluid the subarachnoid space was first washed with 5-HT creatinine sulphate in Krebs-Ringer buffer (1 mg of free base/ml), and then it was filled with the same solution (*ca* 0·5 ml) which was left in contact with the spinal tissue for 90 min. After, the solution was collected and the subarachnoid space washed with 2 ml of Krebs-Ringer buffer. Then, both solutions ("superfusate") were pooled and deproteinized with perchloric acid. The portion of the spinal cord from L_1 to S_4 segment (about 2·27 g) was dissected, dura and arachnoid stripped off, spinal tissue washed with Krebs-Ringer to remove the adsorbed 5-HT and homogenized with 3 volumes of 0·5M perchloric acid.

Table 1. *5-Hydroxyindoles found in tissue and superfusate after 90 min exposure of the spinal cord segment (approximately 2·27 g) to 5-HT solution (0·5 ml; 1 mg free base/ml Krebs-Ringer buffer) applied subarachnoidally. The amounts are expressed as μg/g of wet tissue*

	Spinal tissue		Superfusate	
5-Hydroxyindoles	Cat I	Cat II	Cat I	Cat II
5-Hydroxytryptamine	5·13	12·92	40·52	53·09
5-Hydroxyindoleacetic acid ..	3·64	4·03	2·53	4·20
5-Hydroxytryptophol	0·48	0·64	0·69	0·84

The excess of perchloric acid from superfusate and homogenate was removed with KOH, both fractions evaporated to a small volume and subjected for qualitative analysis to thin-layer chromatography on silica gel G. The chromatograms were sprayed with Ehrlich reagent for the detection of indolic compounds. Besides unmetabolized 5-HT and 5-hydroxyindoleacetic acid, 5-hydroxytryptophol was detected and its identity confirmed by chromatography with an authentic sample [Rf —0·66; 0·81; 0·46 in solvents: chloroform : acetic acid : methanol (75 : 5 : 20); isopropanol : ammonia : ethyl acetate (35 : 20 : 45); ethyl acetate : chloroform (75 : 25)].

Deproteinized superfusate and homogenate were also subjected to successive column chromatography for the separation of 5-HT and its metabolites as described previously (Iskrić, Stančić & Kveder, 1969) and the indolic compounds were quantitatively determined by spectrofluorometry. Besides 5-HT, its two metabolites—5-hydroxyindoleacetic acid and 5-hydroxytryptophol—were found in measurable quantities. The results of two typical experiments are given in Table 1. To assure that the fluorescence measured is really caused by 5-hydroxytryptophol, the appropriate fractions were pooled and subjected to thin-layer chromatography. The spot assigned to 5-hydroxytryptophol was chromatographically identical to that of the authentic sample.

These preliminary experiments show that the described preparation of the cat spinal cord is a practical and simple experimental model for studying 5-HT metabolism in the central nervous system *in vivo*, since fairly large amounts of the substrate, needed for the detection of some metabolites, can be applied. Such large amounts applied in the brain would cause toxic or lethal effects.

Institute "Ruder Bošković",
Zagreb, Yugoslavia.

October 20, 1969

M. BULAT
S. ISKRIĆ
L. STANČIĆ
S. KVEDER
B. ŽIVKOVIĆ

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Inhibition of neuromuscular transmission in the intact rat by emetine

Whether the skeletal muscular weakness which often complicates treatment with emetine hydrochloride (Goodman & Gillman, 1965) arises from a direct action on the muscle (Klatskin & Friedman, 1948) or is secondary to an action on the nerve (Brown, 1935) is still uncertain. Young & Tudhope (1926), in experiments on the rat, found no evidence of inflammation in the nerve but observed degenerative changes in muscle; and recently Ng (1966) showed that the drug blocked the indirectly elicited contraction of the rat isolated diaphragm without significantly affecting the directly elicited contraction. This problem has been further investigated by studying the effect of emetine on neuromuscular transmission in the intact rat using the sciatic nerve-gastrocnemius muscle preparation. Emetine is already known to produce morphological as well as *in vitro* functional changes in the neuromuscular system of the rat.

Twelve rats were given subcutaneous injections of emetine, 1 mg/kg (comparable to the human dose), daily for ten days. Twenty-four h after the last injection, a sciatic nerve-gastrocnemius muscle preparation was made. Similar nerve-muscle preparations were also made in 12 untreated rats. The nerve was stimulated supramaximally with square pulses of 100 to 1000 μs duration. Single twitches of the muscles were elicited at a frequency of 8/min, and, at intervals, tetani were elicited by increasing the stimulation rate to 64/s for 15 s.

Nerve stimulation in treated rats produced twitches of the muscle in the usual manner. However, unlike the normal rat preparation, the twitches were often of irregular height, and after 2–5 min there was a progressive fall in twitch height with

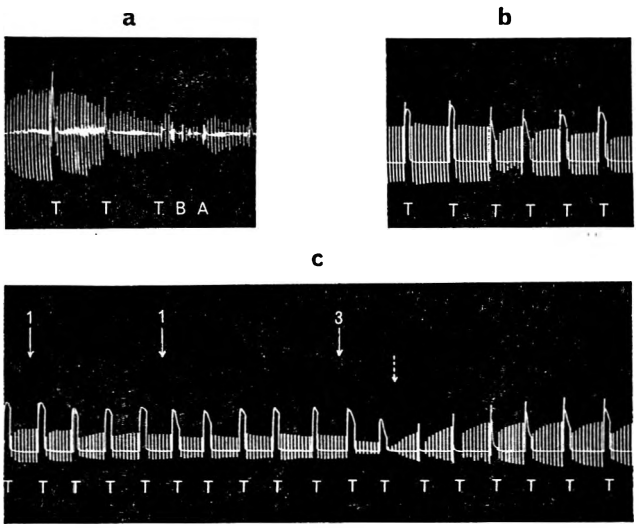

FIG. 1a. Rat 225 g. Treated with emetine hydrochloride 1 mg/kg subcutaneously daily for 10 days. Indirect maximal twitches and tetani (T) of the left gastrocnemius muscle. At B pulse strength was doubled with no effect on twitch. At A, transient recovery of twitch occurred after stopping stimulation for 5 min.

b. Control rat 225 g. Sciatic nerve-gastrocnemius muscle preparation. Indirect maximal single twitches and tetani (T) of the left gastrocnemius muscle. Emetine hydrochloride (1 mg/kg) was given intravenously between the second and third tetani.

c. Control rat 250 g. Indirect maximal twitches and tetani (T) of the left gastrocnemius. At unbroken arrows, emetine hydrochloride was given intravenously. The dose given (in mg/kg) is shown on top of each arrow. At the broken arrow, prostigmine (5 mg) was given intravenously.

complete absence of response after 10–20 min (Fig. 1). Increasing the strength of the impulse did not prevent this abolition of the muscle response, but if nerve stimulation was at this juncture stopped for 5 min or longer, a transient recovery of twitch occurred. Also, in contrast to normal rats, the treated animals failed to maintain muscle tetanus (Fig. 1a).

Intravenous injection of emetine (1 mg/kg) in preparations from untreated rats produced little effect on single twitches, but caused a definite fall in the height of the tetanus with inability to maintain it at a steady height for the duration of the stimuli (Fig. 1b). Onset of the effect as well as recovery from it was rapid, and the effect could be reproduced by repeating the dose of emetine (Fig. 1c). With increasing doses of the drug, single twitches were also inhibited but the effect on tetanus was always greater than that on single twitches. A dose of 3 mg/kg produced complete inhibition of both single twitches and tetanus. The inhibitory effect of the drug was antagonized by prostigmine, the effect on single twitches being more readily antagonized than that on tetanus (Fig. 1c)

The results of these *in situ* studies thus support the *in vitro* findings of Ng (1966) and suggest that emetine has a tubocurarine-like effect on neuromuscular transmission in the rat.

Department of Pharmacology,
University of Ibadan,
Ibadan, Nigeria.

L. A. SALAKO

September 9, 1969

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Treatment of experimental lymphoedema with coumarin

Experimental lymphoedema can be treated with coumarin. Investigations were made in groups of 30 rats, 200 ± 30 g. Lymphoedema was induced by extensive ligation of cervical lymph nodes, with careful sparing of blood vessels and nerves; the extent of the lymphoedema of the head and of the neck was measured plethysmographically on the 4th postoperative day.

Control animals were treated with 0·9% NaCl solution and the experimental group was injected with 5 mg coumarin per kg daily. The controls showed an increase in the volume of the head and the neck of 21%, the coumarin-treated animals an increase of 10%. The difference between the treated and the untreated group is significant $P < 0{\cdot}02$. The explanation of the therapeutic effect of coumarin remains to be elucidated.

Second Department of Medicine,
University Medical School,
Szeged, Hungary.

E. BÖRCSÖK
K. FÖLDI
I. GYÖRI
Ö. T. ZOLTÁN
M. FÖLDI

October 1, 1969

Interactions between calcium and some mylotic agents on depolarized vascular smooth muscle

A mutual antagonism occurs between calcium ions and papaverine-like drugs, both on polarized and KCl-depolarized smooth muscle preparations (Ferrari, 1964; Ferrari & Gaspa, 1965; Toth, Ferrari & others, 1966; Ferrari & Carpenedo, 1968). In view of the essential role played by calcium in muscular contraction, such antagonism was considered of great importance in the mechanism of action of spasmolytics. However, besides the antagonism with calcium ions, papaverine and some derivatives exert other effects related to the mechanism of their spasmolytic action: in fact, these drugs strongly inhibit oxidative phosphorylation and elicit on isolated smooth muscle preparations similar effects to those induced by anoxia or by metabolic inhibitors (Santi, Contessa & Ferrari, 1963; Santi, Ferrari & Contessa, 1964). Also, these metabolic inhibitors are spasmolytic, the most closely related to papaverine being the fish poison rotenone: it has the same site of action in the respiratory chain as papaverine, it shares several pharmacological properties with papaverine but is more active both pharmacologically and biochemically (Santi, Ferrari & Toth, 1963; 1966). We have now compared on depolarized vascular smooth muscle the antagonism of rotenone, papaverine and some congeners by calcium ions.

The investigations were made by testing the effects of rotenone (0·33, 1·65 $\times$ 10^{-7}M), papaverine (1·17, 2·34, 4·68 $\times$ 10^{-5}M), eupaverin (1·17, 2·34, 4·68 $\times$ 10^{-5}M), aminopromazine (0·76, 1·52 $\times$ 10^{-6}M) and iproveratril (0·85, 1·70 $\times$ 10^{-7}M) on the vasoconstriction induced by $CaCl_2$ on the rabbit isolated ear preparation perfused with a depolarizing solution (Ringer KCl). After an initial perfusion (lasting about 20 min) with calcium-free Tyrode medium, the preparation was perfused with calcium-free KCl-Ringer. $CaCl_2$ was added subsequently and its concentration was gradually

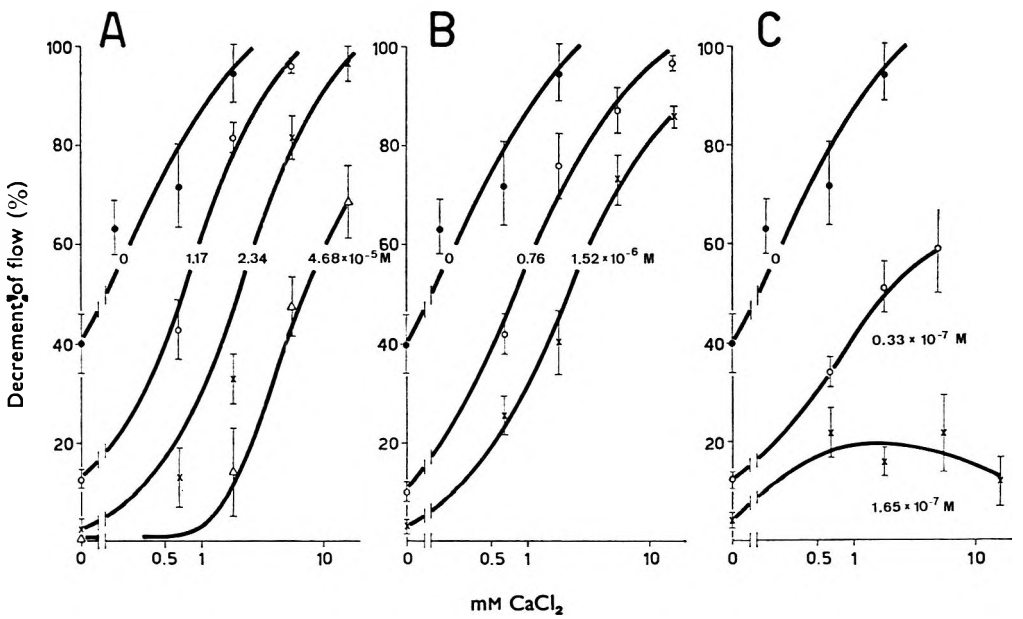

FIG. 1. Rabbit isolated ear perfused with KCl-Ringer at room temperature: cumulative log concentration-response curve for calcium ions in the presence of various concentrations of papaverine (in A), aminopromazine (in B) and rotenone (in C).

increased, every 3 min, following a cumulative sequence. In control experiments $CaCl_2$ concentrations ranged between 0 and 2mM; 2mM $CaCl_2$ constantly induced a complete vasoconstriction. In the presence of myolytic agents, higher $CaCl_2$ concentrations were employed and ranged from 0 to 16 mM. Flow values were recorded by means of a Jacquet drop-counter. The drugs were employed under continuous perfusion, in the presence of different calcium concentrations. Their effects were recorded after a 3 min perfusion and evaluated by van Rossum's method (1963).

Under these experimental conditions the drugs tested retain a clear vasodilating activity. The increase of calcium concentration poorly affects the myolytic activity of rotenone but fully counteracts the vasodilation induced by papaverine, eupaverin, aminopromazine and iproveratril: when plotted as % decrement of flow versus log $CaCl_2$ concentration, the results show (Fig. 1) that in the presence of papaverine or aminopromazine the dose-response curves assume a parallel displacement compared with the controls. The pattern of antagonism of eupaverin and iproveratril by calcium closely parallels that observed with papaverine and aminopromazine.

These findings suggest that, at least within a limited range of concentrations, the effects of papaverine-like drugs may be mainly ascribed to an impairment of calcium availability to the contractile system. Rotenone instead is not counteracted by calcium; this is consistent with the previous hypothesis that its myolytic activity should be entirely ascribed to metabolic inhibitory effects (Santi, Ferrari & Toth, 1966).

Institute of Pharmacology,
University of Padua, Italy.

M. FERRARI

September 23, 1969

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Amphotericin
Azathioprine
Beclomethasone Dipropionate
Bethanidine Sulphate
Bupivacaine Hydrochloride
Carbenicillin Sodium
Chlormadinone Acetate
Clofibrate
Diazepam
Dihydrocodeine Tartrate
Ecothiopate Iodide
Ethacrynic Acid
Ethynodiol Diacetate
Fluocortolone Hexanoate
Fluocortolone Pivalate
Fluphenazine Hydrochloride
Haloperidol
Idoxuridine
Inulin
Lithium Carbonate
Mefenamic Acid
Megestrol Acetate
Metformin Hydrochloride
Methaqualone
Methotrexate
Pnenazocine Hydrobromide
Thiotepa
Tolnaftate

Other new monographs include Eltor Vaccine, Mixed Cholera Vaccine, Human Albumin, and Sodium Iodide (^{125}I) Solution.

The Addendum 1969 is official from June 1, 1970, except for amendments to the monograph on Sterilised Surgical Catgut and the Appendix on Uniformity of Diameter of Tablets. On January 1, 1971, a new decimal system of gauging is introduced for catgut and on June 1, 1971, new metric requirements for the diameters of tablets will come into force. Full details of the changes are provided.

ADDENDUM 1969 to B.P. 1968
120 pages. £3 5s. post free.

BRITISH PHARMACOPOEIA 1968
1454 pages. £8 post free

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