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Specific stimulation of gastric acid secretion by a pentapeptide derivative of gastrin

A. M. BARRETT

The effects of gastrin, a synthetic pentapeptide (*N*-t-butyloxycarbonyl-β-Ala. Try. Met. Asp. Phe. NH_2; I.C.I. 50,123) and histamine on the secretion of gastric acid have been compared in a perfused stomach preparation using anaesthetised rats. The pentapeptide was shown to possess similar secretory activity to that of gastrin but to be 11 times less potent on a molar basis. It produced acid secretions similar to those produced by histamine when compared in several different ways. Unlike histamine, the pentapeptide was without significant effect on blood pressure or haematocrit in maximally stimulating doses.

IN a variety of clinical conditions it is necessary to assess the ability of the stomach to secrete acid. Histamine is a valuable diagnostic tool in this respect but suffers from the disadvantage that the doses required to stimulate maximal acid secretion also produce very unpleasant side-effects. In 1953, Kay proposed the concomitant use of antihistamines with histamine in his "augmented histamine test" since the action of histamine on gastric secretion is unopposed by antihistamine drugs. This technique allowed the dose of histamine to be raised to that necessary for the stimulation of maximal secretion without serious consequences for the patient.

In 1964, Gregory & Tracy announced the isolation of gastrin in pure form and described its specific effects on the gastrointestinal system. Immediate interest was aroused in the possible replacement of histamine by gastrin in the assessment of gastric secretion. A comparison of the stimulant actions of gastrin and histamine has shown potential usefulness of gastrin in this context (Mahklouf, McManus & Card, 1965). Gastrin is a polypeptide containing 17 amino-acids and although total synthesis has been achieved (Anderson, Barton, Gregory, Hardy, Kenner, MacLeod, Preston, Sheppard & Morley, 1964) it is not practicable on a commercial scale. However, in a study of the biological activity of fragments of the gastrin molecule, Tracy & Gregory (1964) found that all the physiological effects of gastrin were shared by the terminal tetrapeptide sequence Try. Met. Asp. Phe. NH_2, even though it was quantitatively less active. In a series of analogues of the active tetrapeptide, one pentapeptide (*N*-t-butyloxycarbonyl-β-Ala. Try. Met. Asp. Phe. NH_2; I.C.I. 50,123) was found to be particularly active in stimulating gastric secretion (Morley, Tracy & Gregory, 1965). This paper compares the action of this pentapeptide with that of gastrin and histamine in the stimulation of gastric acid secretion in the rat.

Experimental

METHODS

The experiments were made using male rats, 240–260 g, from the colony of specific pathogen-free albinos maintained at Alderley Park. The

From the Department of Pharmacology, Pharmaceuticals Division, I.C.I. Ltd., Alderley Park, Macclesfield.

animals were fasted for 24 hr before use but allowed free access to water. The rats were anaesthetised with urethane (8·0 ml/kg of 7·5% solution) given by the intramuscular route. They were then prepared for perfusion of the stomach by the technique of Ghosh & Schild (1958) using saline in place of buffer solution. Each rat stomach was perfused for up to 8 hr and the perfusate titrated for acidity at 10 min intervals. The rate of perfusion was 1 ml/min. Intravenous injections of stimulants were made in volumes of 0·2 ml/100 g via a cannulated jugular vein and given at intervals of not less than 90 min. Each rat received up to 5 injections and the response to each dose of stimulant was determined in 4 separate rats. Systemic arterial blood pressure was monitored in some animals by means of a Condon mercury manometer connected to a carotid artery and in others haematocrit was determined by a standard technique.

Drugs. The drugs used were gastrin II (kindly supplied by Prof. R. A. Gregory, F.R.S.), pentapeptide (prepared by Dr. J. S. Morley of the Chemistry Department at Alderley Park) and histamine acid phosphate. Doses of histamine were calculated in terms of the base.

Results and discussion

The perfused stomach preparation secreted a small but definite amount of acid throughout the whole period of perfusion. The amount secreted may vary from 2 to 6 μ-equiv. hydrochloric acid per 10 min from rat to rat but for any individual animal this background secretion is relatively constant. When gastrin, the pentapeptide or histamine is injected there is an immediate increase in acid secretion. The responses to various doses of these 3 agents are summarised in Table 1.

TABLE 1. THE EFFECT OF THE INTRAVENOUS INJECTION OF DIFFERENT DOSES OF GASTRIN, THE PENTAPEPTIDE AND HISTAMINE ON THE ACID SECRETION OF PERFUSED RAT STOMACHS. Each line shows the dose injected and the mean 10 min acid output for 10 consecutive collection periods for 4 rats

Secretory stimulant	Dose (µg/kg)	Acid secretion/10 min (µ-equiv. HCl)									
		Pre-injection			Post-injection						
		1	2	3	4	5	6	7	8	9	10
Gastrin	2	4·3	4·3	4·1	8·6	12·7	9·4	8·1	5·5	4·4	3·4
	8	5·6	4·2	4·2	8·0	14·9	13·5	10·5	8·0	5·3	4·7
	32	5·0	4·6	4·6	8·5	14·7	16·0	14·5	10·3	7·1	4·6
	128	4·5	4·4	4·4	10·2	17·2	19·1	17·3	12·5	8·1	5·8
	512	4·2	4·3	4·2	9·6	17·7	20·1	19·3	17·1	13·1	10·3
Pentapeptide	2	3·7	4·0	4·0	9·3	10·5	9·1	7·7	4·5	3·7	3·4
	8	3·7	3·4	3·3	9·0	11·7	10·4	7·3	4·5	3·4	3·2
	32	4·4	4·0	3·9	9·8	13·0	13·1	8·5	5·2	4·4	3·9
	128	4·1	4·2	4·2	11·4	17·0	14·8	8·3	5·8	5·0	4·9
	512	4·0	3·6	3·6	11·1	17·0	16·2	11·6	9·2	6·2	4·1
	2,048	5·0	4·4	4·5	10·1	16·6	17·7	16·2	13·5	10·2	7·5
Histamine	80	3·7	3·5	3·3	3·5	5·3	4·8	4·1	3·4	3·2	3·2
	320	3·2	3·1	2·5	4·0	7·7	6·4	4·9	4·2	3·4	3·1
	1,280	3·7	3·4	3·0	4·9	7·5	9·4	7·6	7·0	5·6	4·5
	5,120	3·3	3·1	2·9	4·8	11·3	14·7	14·9	14·1	12·0	10·6
	20,480	3·4	3·2	3·3	11·6	12·9	14·6	14·4	13·3	13·5	15·1

The pattern of the responses to increasing doses of gastrin and the pentapeptide is illustrated in Fig. 1. At each dose level it appears that

the increase in the first 10 min period is greater with the pentapeptide than the natural hormone although this difference is not statistically significant. However, the peak value, reached in the second 10 min period after

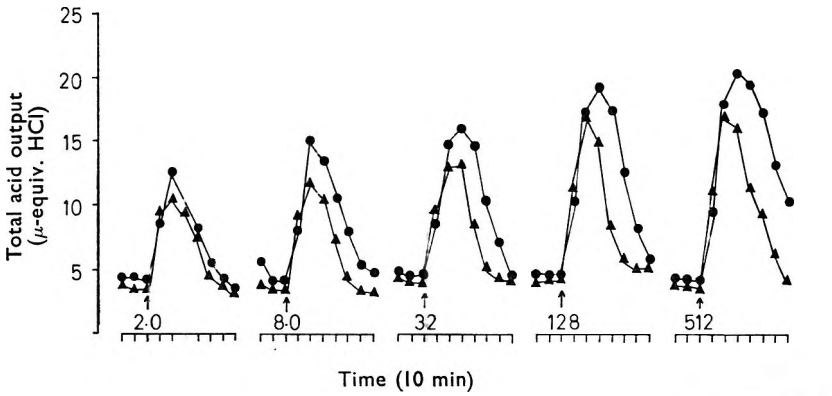

FIG. 1. The effect of gastrin and the pentapeptide on the total acid output per 10 min from perfused rat stomachs. The doses (μg/kg) were given in random order at the arrows and each point represents the mean value for 4 animals (●—● gastrin; ▲—▲ I.C.I. 50,123).

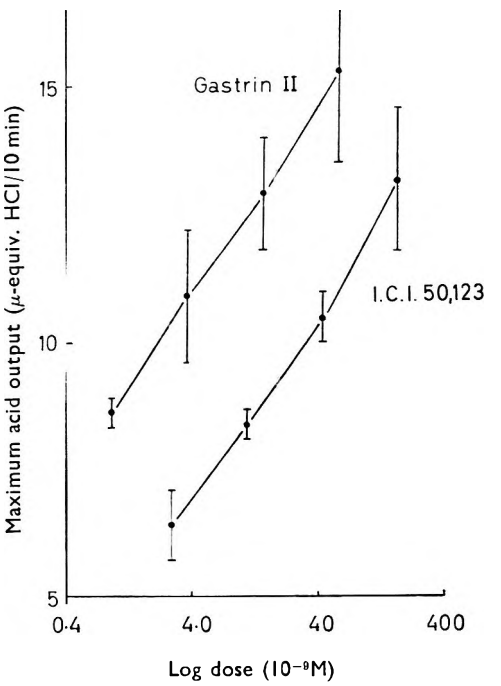

FIG. 2. Dose response curves for gastrin and the pentapeptide comparing the maximum response at different dose levels.

injection was always greater with gastrin. Further, the recovery to baseline acid secretory levels was quicker with the pentapeptide. It may be concluded therefore that gastrin has a slightly slower onset of action than equivalent doses of the pentapeptide but that it produces a larger and more sustained acid secretion. Considering that the pentapeptide represents an active fragment of gastrin, it is reasonable to conclude that the rate of access to, and removal from, the appropriate receptor sites is greater for the smaller molecule.

It was desirable to be able to compare the potency of these two agents and analysis of the results showed that the best way of doing this was to calculate the maximum response for each dose level. The mean basal level for each rat was calculated by averaging the values of 3 pre-injection and 3 post-recovery 10 min secretions and this was subtracted from the maximum acid output produced by the particular injection. When the results were summated and averaged, a plot of the maximum response against the logarithm of the dose gave two parallel straight lines over the dose range of 2 to 32 μg/kg. Because of the difference in molecular weight of gastrin (2154) and the pentapeptide (769), the acid output was plotted against the logarithm of the molar doses (Fig. 2). On a molar basis gastrin is 11 times the more active (95% confidence limits 4·8–27), but on a weight for weight basis gastrin is 4 times more potent (1·7–11·0).

Comparison of the patterns of response to the pentapeptide and histamine showed certain differences (Fig. 3). There was a marked difference in sensitivity, it being necessary to inject over 5 mg/kg of

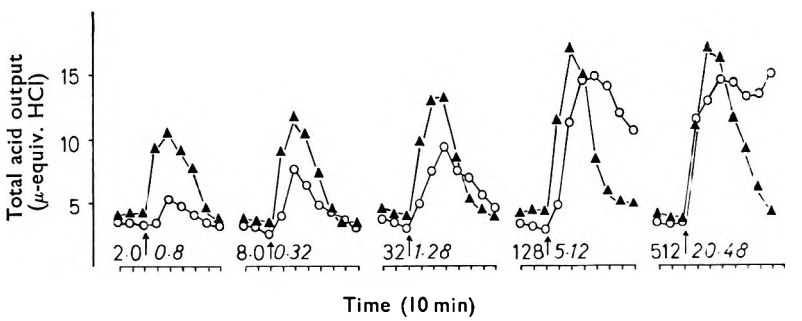

FIG. 3. The effect of histamine and the pentapeptide on the total acid output per 10 min from perfused rat stomachs. The doses, I.C.I. 50,123 in μg/kg (roman); histamine in mg/kg (italic), were given at the arrows in random order and each point represents the mean value for 4 animals (○—○histamine; ▲—▲ I.C.I. 50,123).

histamine to produce a similar acid response to that of 128 μg/kg of the pentapeptide. Further, the response to histamine was of longer duration than that to the pentapeptide. The maximum response to both substances is similar and on a weight for weight basis histamine is 80 times less active than the synthetic peptide.

The maximum acid output per 10 min is not always the most useful index of secretory activity and many workers compare stimulant activity

in terms of the peak hour output. This value is calculated by adding the two highest 10 min acid outputs, subtracting the contribution of the basal secretion and multiplying by 3. Such a comparison is presented in Fig. 4.

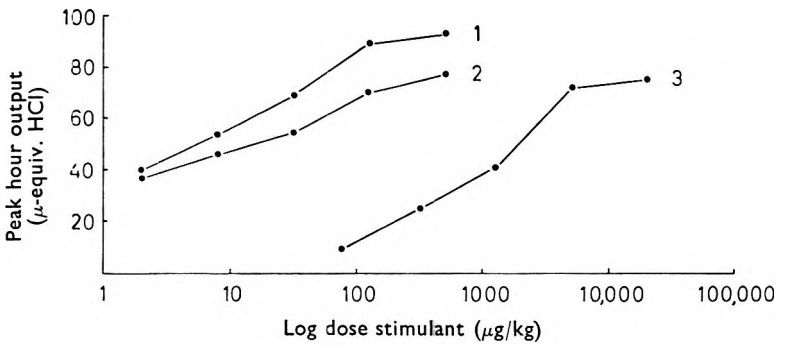

FIG. 4. Mean peak hr output of HCl (μ-equiv./hr) for gastrin (1), the pentapeptide (2) and histamine (3). (Each point represents the results from 4 rats.)

There is a clear similarity in the overall slope of the curves for the three secretagogues studied. It appears that the maximal response for histamine and the pentapeptide is only 82% of that for gastrin but these differences are not statistically significant.

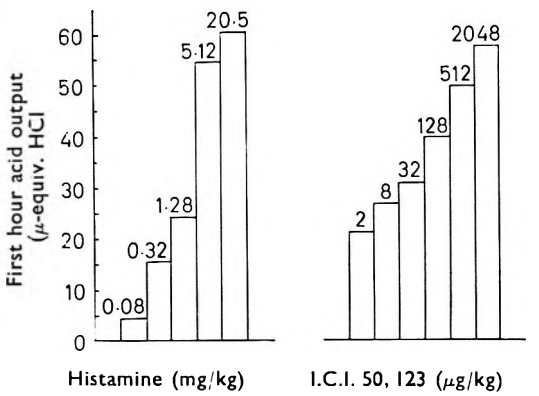

FIG. 5. Mean total acid output in the first hour after the intravenous injection of I.C.I. 50,123 and histamine (4 rats per column).

The total acid output in a period of 1 hr following injection is another widely used index of gastric secretory function. As shown in Fig. 5 the pentapeptide is capable of producing values similar to those produced by histamine.

It is well known that histamine exerts marked effects on the blood pressure. As shown in Table 2 the stimulation of high acid output by histamine was accompanied by marked falls in blood pressure. Gastrin had no effect on mean arterial pressure whereas the pentapeptide exerted slight pressor effects which appeared unrelated to dose.

Previous studies have shown that both urethane and histamine bring about marked increases in the packed cell volume. Two groups of 18 rats were sub-divided into 3 further groups of 6 rats. The main group

TABLE 2. A COMPARISON OF THE EFFECTS OF GASTRIN, THE PENTAPEPTIDE AND HISTAMINE ON GASTRIC ACID SECRETION AND BLOOD PRESSURE. The parameters were recorded in different animals, there being the mean of 4 observations ± standard error for each dose level. The mean starting blood pressure was 125 ± 8 mm Hg

Drug	Dose (μg/kg)	Peak hr output (μ-equiv. HCl)	Change in blood pressure (mm Hg)
Gastrin	2	39·9 ± 5·1	+ 2 ± 1·2
	8	54·2 ± 3·1	+ 2 ± 1·2
	32	69·0 ± 5·1	+ 3 ± 1·4
	128	89·9 ± 13·3	+ 1 ± 1·6
	512	94·1 ± 14·1	+ 4 ± 1·8
Pentapeptide ..	2	37·1 ± 3·3	+ 7 ± 2·5
	8	46·1 ± 0·9	+ 8 ± 2·1
	32	55·0 ± 1·8	+ 8 ± 2·1
	128	71·4 ± 4·2	− 7 ± 2·1
	512	78·2 ± 6·9	− 5 ± 1·0
Histamine	80	10·0 ± 2·7	− 31 ± 2·1
	320	24·8 ± 6·8	− 39 ± 3·5
	1,280	41·3 ± 10·7	− 54 ± 2·5
	5,120	73·2 ± 17·7	− 60 ± 5·1
	20,480	75·8 ± 22·4	− 65 ± 1·9

received a sham injection and the second was anaesthetised with urethane. Within the main groups, 1 hr after injection, one group received a sham injection, one histamine at 5·12 mg/kg and the third the pentapeptide at 512 μg/kg. Thirty min later the packed cell volume was determined and the results are summarised in Table 3. In conscious rats histamine

TABLE 3. THE EFFECTS OF THE PENTAPEPTIDE AND HISTAMINE ON THE PACKED CELL VOLUME (HAEMATOCRIT) OF CONSCIOUS AND ANAESTHETISED RATS. Each value denotes the mean for six rats together with the standard error of the mean

(*a*) *Conscious rats*	Treatment	Packed cell volume (%)	Increase	Change %
	Saline	43·3 ± 1·2	—	—
	I.C.I. 50,123 512 μg/kg	44·8 ± 0·5	1·5 ± 1·3	3·5 ± 3·0
	Histamine 5120 μg/kg	52·3 ± 2·3	9·0 ± 2·6	20·8 ± 6·0
(*b*) *Anaesthetised rats* (Urethane i.m.)				
	Saline	52·2 ± 1·2	8·9 ± 1·7	20·6 ± 3·9
	I.C.I. 50,123 512 μg/kg	50·2 ± 1·2	6·9 ± 1·7	16·0 ± 3·9
	Histamine 5120 μg/kg	63·5 ± 0·5	20·2 ± 1·3	46·7 ± 3·0

Estimates of significance			P value
Conscious controls	vs.	I.C.I. 50,123	N.S.
	vs.	Histamine	<0·01
	vs.	Urethane	<0·001
Anaesthetised controls	vs.	I.C.I. 50,123	N.S.
	vs.	Histamine	<0·01

increased the haematocrit by 21% whereas the pentapeptide had no effect. In rats anaesthetised with urethane there was a 21% increase in haematocrit due to the anaesthetic alone. When histamine was given to rats anaesthetised with urethane there was a further increase in packed cell volume up to 47%. The pentapeptide slightly reduced the effects of urethane on haematocrit but this effect was not significant.

From these experiments it may be concluded that I.C.I. 50,123 possesses the properties necessary to replace histamine as a diagnostic acid for the assessment of gastric secretion. The synthetic pentapeptide appears to behave similarly to the natural hormone, gastrin. It exerts a specific effect on gastric secretion and obviates the need for the concomitant administration of antihistamines which are not themselves devoid of unwanted side-effects.

Acknowledgement. The expert assistance of Robert Siddall and Maureen Wain is gratefully acknowledged.

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The effects of polyoxyethylene compounds on the hydrolysis of chlorbutol

R. A. ANDERSON AND A. H. SLADE

The rates of hydrolysis of chlorbutol are reduced by surface-active agents, but not by polyethylene glycols. The ratio of the rate-constant in simple aqueous systems to that in systems containing surface-active agents can be predicted if the distribution coefficient for chlorbutol between the micellar pseudo-phase and the aqueous phase is known and is independent of concentration.

THE hydrolysis of chlorbutol has been shown to be first-order with respect to both chlorbutol and hydroxyl ion, the overall reaction being second-order (Nair & Lach, 1959). The rate of reaction was stated to be independent of both the ionic strength and buffer species and it was concluded that the reaction was a specific hydroxyl ion catalysed reaction.

It is recognised that the presence of surface-active agents may affect the reaction rates of solutes which have been solubilised and the present experiments were made to investigate the effect of some polyoxyethylene compounds on the hydrolysis of chlorbutol.

Experimental

MATERIALS

Chlorbutol (May and Baker), polyoxyethylene lauryl ether (lauromacrogol, Brij 35 - Atlas Powder Company), polyethylene sorbitan monolaurate (polysorbate 20, Tween 20, Atlas Powder Company) and polyethylene glycol (hard macrogol, Carbowax 4000 - Union Carbide) were used as received. A single batch of each was used throughout. All other chemicals were reagent grade. Water was distilled from a Scorah all-glass still.

METHODS

Determination of chloride ion. The sample was adjusted to pH 7 by addition of acetate buffer, 1 ml 5% potassium chromate solution was added, and the solution was titrated with 0·02N silver nitrate. The end-point was taken as the first appearance of a red colour when viewed under blue light. Blank titrations were made as appropriate.

Determination of chlorbutol. The sample was allowed to stand for at least 15 min after addition of excess of a concentrated sodium hydroxide solution, and then the mixture was boiled to ensure complete hydrolysis. On cooling, the solution was neutralised to phenolphthalein with acetic acid, and the liberated chloride ion titrated with silver nitrate as described above (one molecule of chlorbutol yields three chloride ions). The surface-active agents and macrogol have no effect on the assay.

From the Pharmacy Department, University of Sydney, Australia.

Solubility of chlorbutol. The solution in which the solubility was to be determined was placed in a glass-stoppered jar and excess chlorbutol was added. The jar was maintained at 25° ± 0·05° and the contents agitated with a magnetic stirrer. Duplicate samples were withdrawn at 2 hrly intervals and assayed as described above, until no increase in concentration was found in three consecutive determinations. In buffer solutions where chlorbutol underwent hydrolysis, two pairs of samples were taken. One pair was immediately neutralised with acetic acid and assayed for free chloride ion, and the other pair assayed for chlorbutol as described above. The difference in titres was used to calculate the chlorbutol solubility.

Dialysis. The distribution of chlorbutol between water and the micellar pseudophase was determined by the method of Patel & Kostenbauder (1958). Cells similar to those described by Patel & Foss (1964) were used with membranes of rubber latex which had been soaked in water with frequent changes until clear washings were obtained. The solution of surface-active agent was placed on one side of the membrane together with a weighed quantity of chlorbutol, and water on the other. The cells were tumbled at 25·0° for about three days when duplicate samples were taken from each side and assayed. Additional samples were taken after a further one or two days to confirm that equilibrium had been attained. (Identical results were obtained when the chlorbutol was added to the half-cell containing water.)

Determination of first-order rates. The required amount of chlorbutol was dissolved in water or aqueous surfactant solution and the solution allowed to equilibrate at 25·0°; then sufficient sodium borate and boric acid were added to give the desired buffer composition, the solution made to volume with water at 25·0°, and the zero-time sample taken.

At selected intervals, samples were withdrawn and added to sufficient acetic acid to reduce the pH to 7, and then assayed for chloride ion. Finally, duplicate samples of the solution were taken and assayed to give the original chlorbutol concentration.

Each run was duplicated and the pH was checked frequently to ensure that the pH had not fallen significantly.

Determination of second-order rates. Equal volumes of chlorbutol solution and previously standardised sodium hydroxide solution were equilibrated at 25·0° in separate wide-mouthed flasks. At zero time the two solutions were mixed by pouring the solutions quickly to one flask, then to the other and back. Samples were taken at 15 sec intervals by pouring some of the solution into previously tared beakers containing excess nitric acid. The beakers with added sample were then reweighed; the differences in weight may be used to calculate the volume of sample taken, the sample solution being found to weigh 1·00 g per ml. The contents and washings of each beaker were transferred to flasks, neutralised to pH 7, and assayed for chloride ion. Finally, duplicate samples of the solution were taken and assayed to give the original chlorbutol solution. Two experiments were made for each concentration of lauromacrogol.

Results and discussion

The solubility of chlorbutol in solutions of macrogol and lauromacrogol is shown in Figs 1 and 2. The solubility of chlorbutol in water is affected by the amount of salt present; this does not seem to affect the interaction of chlorbutol with the polyoxyethylene compounds because the solubility plots show pairs of parallel lines (changes of pH did not appear to affect solubility).

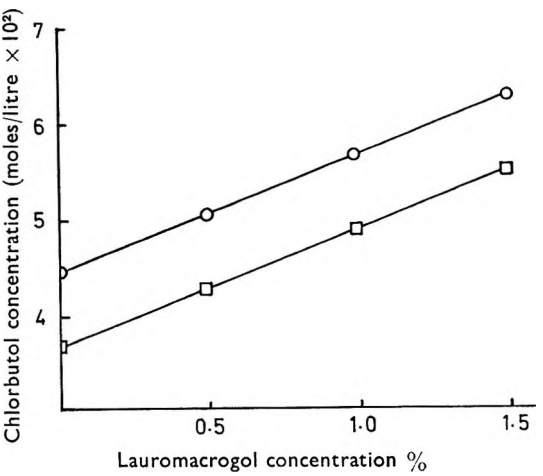

FIG. 1. Solubility of chlorbutol in lauromacrogol solutions at 25°. ○—○ Salt-free solution. □—□ 0·4 M Borate solution.

From such solubility plots, it is possible to calculate, *for saturated systems*, the ratio of free chlorbutol to that bound by the surface-active agent. The equilibrium dialysis studies showed that these ratios are independent of the degree of saturation of the system by chlorbutol and that apparent distribution coefficients may be calculated as suggested by Evans (1964).

The cloud point of polysorbate 20 is below 25·0° in systems saturated with chlorbutol and solubilities could not be determined. The dialysis data from unsaturated systems are plotted in Fig. 3 in the manner suggested by Patel & Kostenbauder (1958). The amounts of chlorbutol bound by macrogol are much less than those bound by equivalent amounts of the surface-active agents. The relatively small increases in solubility effected by polyethylene glycols have been reported for many solutes.

TABLE 1. APPARENT DISTRIBUTION COEFFICIENTS OF CHLORBUTOL IN AQUEOUS SOLUTIONS OF SURFACE-ACTIVE AGENTS

Surface-active agent	Apparent distribution coefficient	
	in absence of 0·4M borate	in presence of 0·4M borate
Lauromacrogol	27	32
Polysorbate 20	24	29

The concept of a distribution coefficient between the true aqueous phase and the micellar phase does not depend on any particular site of solubilisation, and can be applied provided the value is (essentially) independent of the concentrations of solute and surface-active agent. These conditions are met by the systems studied here, and the apparent distribution coefficients listed in Table 1 are calculated from the relationship,

$$q = \frac{(C_t - C_a)(1 - s)}{C_a \cdot s} \quad .. \quad .. \quad .. \quad (1)$$

where C_t = total chlorbutol concentration in the system expressed as moles/litre of total system; C_a = chlorbutol concentration in the true aqueous phase expressed as moles/litre of total system, and s = the weight fraction of micellar phase. The value of s has been approximated by using the w/v percentage of surface-active agent divided by 100.

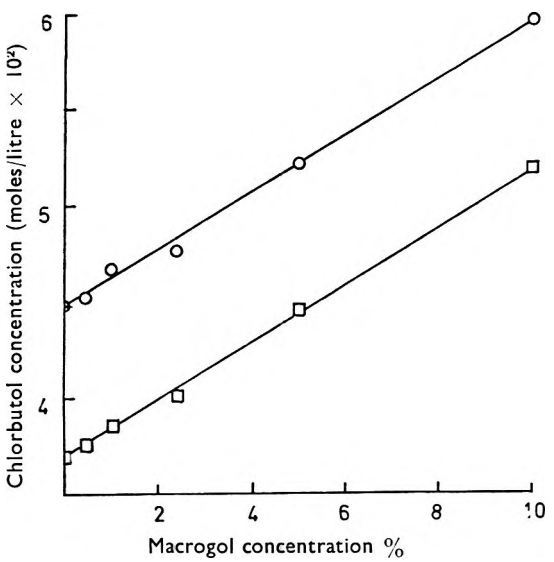

FIG. 2. Solubility of chlorbutol in macrogol solutions at 25°. ○—○ Salt-free solutions. □—□ 0·4 M Borate solution.

The hydrolysis of chlorbutol in solutions held at constant pH proceeds at first-order rates both in the presence and absence of surface-active agents. Fig. 4 shows the influence of buffer concentration on the rate of hydrolysis; the fall in rate as the buffer concentration is reduced conflicts with the conclusions of Nair & Lach (1959) who reported that buffer concentration did not affect the rate of hydrolysis. All subsequent first-order rates were determined using 0·4M buffer.

Figs 5 and 6 show plots for systems containing surface-active agents and which are initially half-saturated and one-twentieth saturated with respect to chlorbutol. The rate constants calculated from these and other

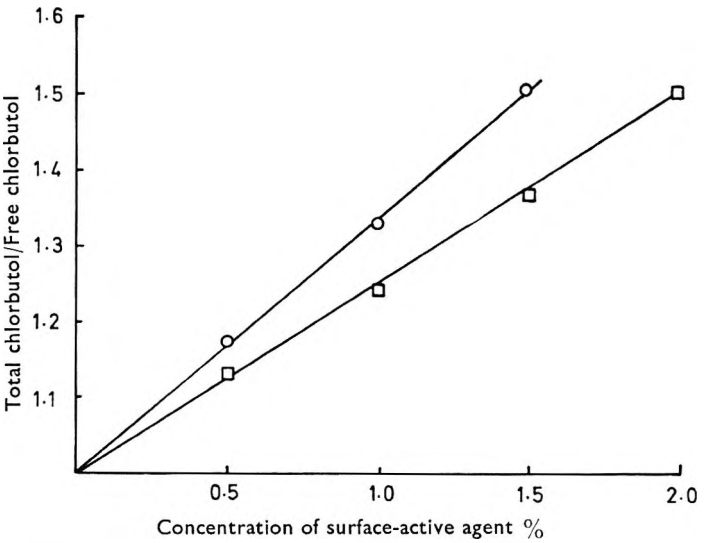

FIG. 3. Binding of chlorbutol by surface-active agents in aqueous solution at 25°. ○—○ Polysorbate 20. □—□ Lauromacrogol.

similar plots are listed in Table 2. The reaction between chlorbutol and sodium hydroxide follows second-order kinetics in the presence and absence of surface-active agents, and conventional plots (Moore, 1957) of the data are shown in Fig. 7; the second-order rate constants are listed in Table 3.

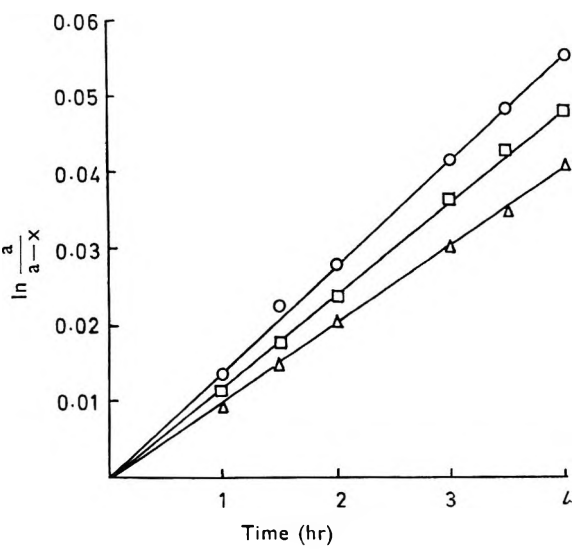

FIG. 4. Hydrolysis of chlorbutol in borate buffer systems, pH 9·2 at 25°, plotted as a first-order reaction. ○—○ 0·6 M Borate. □—□ 0·4 M Borate. △—△ 0·2 M Borate.

The rate of hydrolysis is not affected by the presence of macrogol in concentrations up to 10%. Although the solubility of chlorbutol is increased by macrogol, presumably by bonding through the hydroxyl hydrogen to an ether oxygen on the polyethylene glycol chain, the trichloromethyl groups are not shielded by the interaction and can be attacked by hydroxyl ions as readily as in simple aqueous solution.

TABLE 2. FIRST-ORDER RATE CONSTANTS FOR HYDROLYSIS OF CHLORBUTOL IN 0·4M BORATE BUFFER AT 25°

Surface-active agent and concentration	Initial concentration of chlorbutol M × 10^3	pH	Rate constant in hr^{-1}	
			Experimental	Predicted by Equation 2
Nil	3·70	8·7	0·0050	
	3·70	9·2	0·0117	
	1·85	"	0·0116	
	0·37	"	0·0117	
	0·18	"	0·0118	
			Av. 0·0117	
Lauromacrogol	4·30	8·7	0·0044	0·0043
0·5%	4·30	9·2	0·0096	
	2·15	"	0·0102	
	0·43	"	0·0092	
	0·21	"	0·0101	
			Av. 0·0098	0·0101
Lauromacrogol	4·90	8·7	0·0040	0·0038
1·0%	4·90	9·2	0·0083	
	2·45	"	0·0085	
	0·49	"	0·0081	
	0·24	"	0·0081	
			Av. 0·0082	0·0088
Lauromacrogol	5·50	8·7	0·0034	0·0034
1·5%	5·50	9·2	0·0074	
	2·75	"	0·0075	
	0·55	"	0·0065	
	0·27	"	0·0068	
			Av. 0·0070	0·0079
Polysorbate 20				
0·5%	2·31	9·2	0·0101	0·0104
2·0%	2·41	"	0·0078	0·0073

However, the presence of surface-active agents has a marked effect on the rate of hydrolysis.

The data in Tables 2 and 3 and Figs 5 and 6 show that neither the degree of saturation of the system with respect to chlorbutol nor the

TABLE 3. SECOND-ORDER RATE CONSTANTS FOR REACTION OF CHLORBUTOL WITH SODIUM HYDROXIDE AT 25·0°

Concentration of lauromacrogol	Rate constant in litre $mole^{-1}$ min^{-1}	
	Experimental	Predicted by Equation 2
Nil	8·0	
0·5%	6·8	7·1
1·0%	6·0	6·3

chlorbutol concentration affect the rate constant for the reaction. However the concentration of surface-active agent present does affect the rate constant.

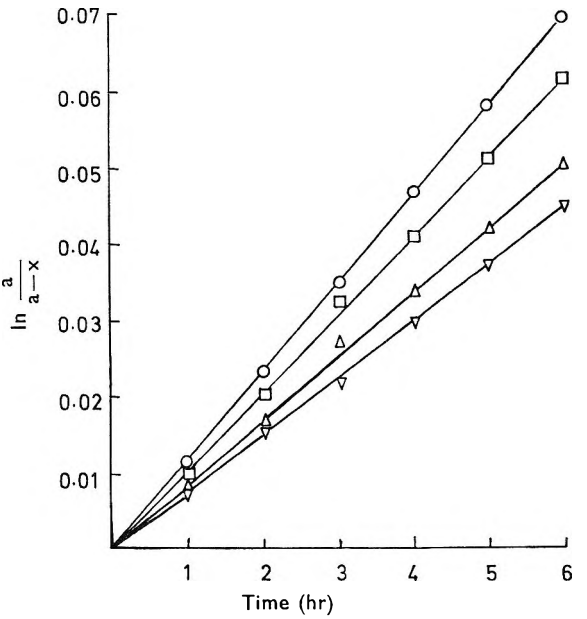

FIG. 5. Hydrolysis of chlorbutol (initially half saturated) in lauromacrogol solutions with 0·4 M borate, pH 9·2 at 25°, plotted as a first-order reaction. Initial concentrations of chlorbutol: ○—○ 1·85 × 10^{-2}M (no lauromacrogol). □—□ 2·15 × 10^{-2}M in 0·5 % lauromacrogol. △—△ 2·45 × 10^{-2}M in 1·0 % lauromacrogol. ▽—▽ 2·75 × 10^{-2}M in 1·5 % lauromacrogol.

If one makes the assumptions that: (a) only the chlorbutol free in the aqueous phase undergoes hydrolysis, and (b) the distribution ratio between the chlorbutol in the micellar and aqueous phases is instantaneously restored, it can be shown that

$$\frac{k}{k_s} = 1 + \frac{qs}{1 - s} \quad .. \quad .. \quad .. \quad (2)$$

where k = the rate constant in the absence of surface-active agent; k_s = the rate constant in the presence of surface-active agent; q = the distribution coefficient defined in equation (1); s = the weight fraction of micellar phase approximated as in equation (1).

In addition to the terms previously defined, let a = original chlorbutol concentration in the total system (moles/litre of system); x = concentration decomposed after time t (moles/litre of system); A = original chlorbutol concentration in the aqueous phase (moles/litre of water). The ratio a/A = 1 − s + qs, because a = A(1 − s) + qAs.

After time t, the concentration of chlorbutol remaining in the system is (a − x) moles/litre, and (since the distribution ratio remains constant) the concentration remaining in the aqueous phase is (a − x)A/a moles/ litre of water.

This equation has been applied to the prediction of rate constants for the chlorbutol hydrolysis in lauromacrogol and polysorbate 20 at pH 8·7 and 9·2 and the comparisons are shown in Table 2. The predicted values are within a few per cent of those found experimentally, and suggest that the assumptions and approximations made in deriving the equation are reasonable.

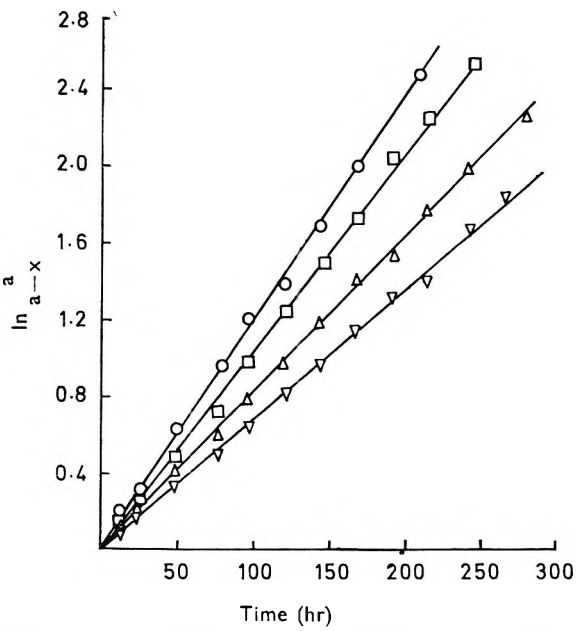

FIG. 6. Hydrolysis of chlorbutol (initially one twentieth saturated) in lauromacrogol solutions with 0·4M borate, pH 9·2 at 25°, plotted as a first-order reaction. Initial concentrations of chlorbutol: ○—○ $1{\cdot}85 \times 10^{-3}$M (no lauromacrogol). □—□ $2{\cdot}15 \times 10^{-3}$M in 0·5% lauromacrogol. △—△ $2{\cdot}45 \times 10^{-3}$M in 1·0% lauromacrogol. ▽—▽ $2{\cdot}75 \times 10^{-3}$M in 1·5% lauromacrogol.

Because the instantaneous rate of formation is first-order,

$$\frac{dx}{dt} = k \cdot \frac{(a - x)A}{a}(1 - s),$$

the factor $(1 - s)$ being introduced because dx is the concentration change with respect to total system.

Integration and rearrangement gives

$$k = \frac{a}{A(1-s)} \cdot \frac{1}{t} \ln \frac{a}{(a - x)}.$$

The experimentally determined rate constant in the presence of surface-active agent,

$$k_s = \frac{1}{t} \ln \frac{a}{(a - x)},$$

and so

$$\frac{k}{k_s} = \frac{a}{A(1 - s)} = \frac{1 - s + qs}{1 - s} = 1 + \frac{qs}{1 - s}$$

A similar procedure may be used to show the same relationship between the second-order rate constants.

The comparisons for the second-order reaction between chlorbutol and hydroxyl ions are shown in Table 3, and again the values are in reasonable agreement. These reactions are almost complete in a few minutes and might be expected to challenge the assumption that chlorbutol is released from the micelle to instantaneously restore equilibrium.

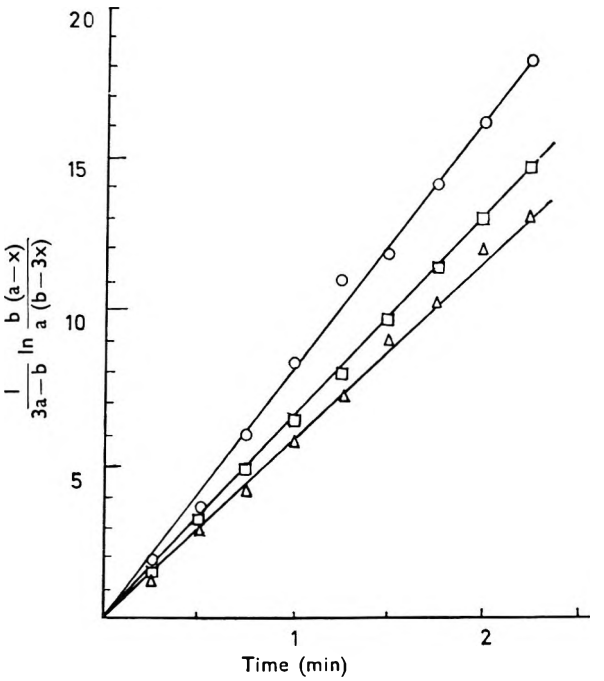

FIG. 7. Reaction between chlorbutol and hydroxyl ions in lauromacrogol solutions at 25°. ○—○ No lauromacrogol. □—□ Lauromacrogol 0·5%. △—△ Lauromacrogol 1·0%.

It seems likely that this relationship would be of general application to other solubilised systems.

Acknowledgement. We are indebted to Mr. L. R. Fisher for discussions concerning an alternative treatment of the experimental data, and to the New South Wales Pharmacy Research Trust for supporting this work.

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The effect of isoprenaline on the contraction of smooth muscle produced by histamine, acetylcholine or other agents

J. B. FARMER AND the late D. N. LEHRER

Isoprenaline given intravenously is a more potent antagonist of bronchospasm produced by histamine than of that produced by acetylcholine in the anaesthetised guinea-pig. This greater activity of isoprenaline against histamine was also observed on isolated tracheal muscle and ileum of the guinea-pig. It was also found in isolated guinea-pig ileum that stimulation of the periarterial sympathetic nerves produced a greater inhibition of contractions produced by histamine or 5-hydroxytryptamine than of those produced by acetylcholine or bradykinin. The inhibitory actions of isoprenaline were absent in tissues without β-receptors and tissues in which the β-receptors were blocked by pronethalol.

ISOPRENALINE is widely used, either alone or with other substances, as a bronchodilator. The bronchodilator action was first demonstrated in anaesthetised dogs and guinea-pigs (Konzett, 1940a, b) and has since been confirmed by many others. We have examined the inhibitory effect of isoprenaline on bronchoconstriction induced by a number of spasmogens in the anaesthetised guinea-pig and on the contractions of isolated smooth muscle preparations evoked by the same agents. Some of the results were communicated to the British Pharmacological Society in July 1965.

Experimental

In vivo EXPERIMENTS

The resistance of the guinea-pig lung to inflation was determined by the method of Konzett & Rössler (1940). Guinea-pigs, 250–350 g, were anaesthetised with urethane, 175 mg/100 g, by intraperitoneal injection. Spontaneous respiratory movements were suppressed by the additional intravenous injection of pentobarbitone. The trachea was cannulated and the lungs inflated with air by a miniature Starling pump operating on a partly closed circuit (4–6 ml stroke volume at 72 strokes/min).

Solutions of drugs in saline were injected through a cannula placed in the jugular vein. Temporary increases in bronchial resistance were produced by the intravenous injection of histamine and acetylcholine.

In vitro EXPERIMENTS

Guinea-pig ileum. A segment of ileum 2–3 cm in length was removed 20 cm from the ileocaecal junction. The segment was suspended in a 25 ml bath containing a physiological salt solution at 32° and the tissue aerated by means of a Hiflo pump. The composition of the salt solution in g/litre was $NaHCO_3$, 1·0; NaH_2PO_4, 0·32; NaCl, 8·0; glucose, 1·0; $MgCl_2$, 0·42; KCl, 0·2; $CaCl_2$, 0·4.

Mechanical records were obtained by means of an isotonic lever with a frontal writing point. The lever exerted a tension of 1 g and magnified

From the Department of Pharmacology, Allen & Hanburys Ltd., Ware, Herts.

the contractions 7 times. pA_2 determinations at 2 min were made by the method of Schild (1947). In the experiments in which chlorpheniramine and atropine were used, these drugs were incorporated in the salt solution for the duration of the experiment.

The periarterial nerves were stimulated as has been previously described for the rabbit ileum (Finkelman, 1930) The preparation was stimulated at variable frequencies with rectangular pulses of 1 msec duration for periods of 10 sec before and during the administration of the spasmogen. The strength of the shocks was such that the responses for a given frequency of stimulation were maximal.

Guinea-pig tracheal chain. The tracheal chain preparation was set up as described by Castillo & de Beer (1947) except that the tracheal rings were opened by severing the cartilage (Akcasu, 1959). The tracheal chain was suspended in a salt solution (composition as above) in a manner similar to that described for the ileum.

Human myometrium. Segments of non-gravid human uteri were prepared as described by Chambers & Pickles (1958) and suspended in 50 ml of aerated salt solution at 37°. The salt solution had the following composition, g/litre: NaCl, 9·0; KCl, 0·42; $CaCl_2$, 0·24; $NaHCO_3$, 0·5; glucose, 1·0. Contractions of the muscle were recorded with an isotonic frontal writing lever which exerted a tension of approximately 1 g and magnified the contractions 10 times.

DRUGS

Histamine acid phosphate; acetylcholine chloride; pronethalol hydrochloride; papaverine hydrochloride; isoprenaline sulphate; chlorpheniramine maleate; atropine sulphate; 5-hydroxytryptamine creatinine sulphate; bradykinin (Sandoz). Doses are given in terms of these salts.

Results

EFFECT OF ISOPRENALINE ON THE BRONCHOCONSTRICTOR ACTION OF HISTAMINE AND ACETYLCHOLINE IN THE GUINEA-PIG

Fig. 1 shows the inhibitory effect of intravenously injected isoprenaline on the bronchoconstrictor action of histamine and acetylcholine in the anaesthetised guinea-pig. Responses to histamine and acetylcholine were determined before, and at 20 min intervals after injection of isoprenaline (Fig. 1). The response produced by acetylcholine returned to near normal within 200 min, whereas the response to histamine was still reduced. Much smaller doses of isoprenaline were also effective but the duration of their effects were relatively brief. The results obtained with such doses against histamine and acetylcholine are expressed graphically in Fig. 2. The degree of inhibition of the bronchoconstriction induced by histamine was 30% and 90% at doses of 0·1 and 1·0 μg/kg of isoprenaline. The doses of isoprenaline required to reduce the bronchoconstriction induced by acetylcholine to a similar degree were 1·0 and 10·0 μg/kg respectively. Thus some ten times the amount of isoprenaline is

required to antagonise bronchoconstriction induced by acetylcholine than to antagonise that produced by histamine.

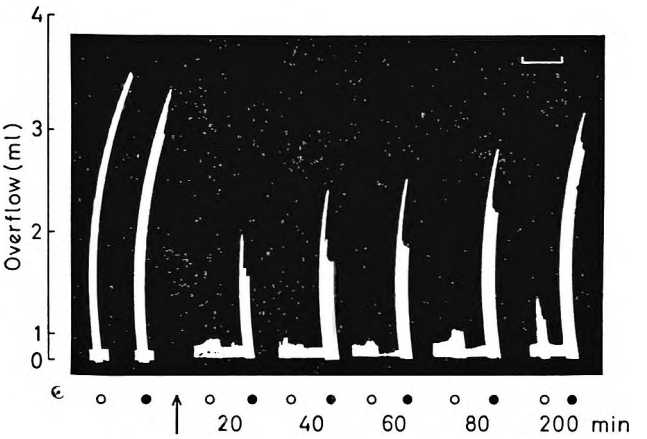

FIG. 1. Guinea-pig, 350 g. The effect of isoprenaline sulphate on the bronchoconstrictor action of histamine and acetylcholine. Histamine (○, 2·5 μg) and acetylcholine (●, 6 μg) were injected intravenously. 20 min elapsed between each injection of the same drug, acetylcholine being injected 5 min after each injection of histamine. At ↑, isoprenaline sulphate, 1 mg/kg, was injected.

MODIFICATION OF THE EFFECT OF ISOPRENALINE BY PRONETHALOL

The inhibitory action of isoprenaline against bronchospasm induced in the anaesthetised guinea-pig by histamine and acetylcholine was antagonised by the prior administration of pronethalol. The effects of isoprenaline were investigated against histamine and acetylcholine in separate experiments since much smaller doses of isoprenaline were required to antagonise the histamine induced bronchospasm. The doses of isoprenaline employed against histamine and acetylcholine were 10 and 100 μg/kg respectively, and these regularly caused complete antagonism of the histamine and acetylcholine responses. In any one experiment, constant responses to injections of histamine or acetylcholine were obtained, then an intramuscular injection of pronethalol (0·1 mg/kg) was given. Pronethalol enhanced bronchoconstrictor actions of histamine and, to a lesser extent, of acetylcholine. When the responses were constant, an intravenous injection of isoprenaline was given. After the effects of isoprenaline had subsided, a second dose of pronethalol was given (1 mg/kg) and this was followed by another dose of isoprenaline. This sequence was repeated a third time, after injection of 10 mg/kg pronethalol. The results obtained are shown in Fig. 3. The responses to histamine (open columns) and acetylcholine (closed columns) obtained 3 min after the injection of isoprenaline are expressed as a percentage of the response to histamine or acetylcholine after the injection of pronethalol but before the isoprenaline administration. In the figure, A refers to the

depressed responses produced by isoprenaline in the absence of pronethalol; B, C and D refer to the responses obtained after pronethalol (0·1, 1·0 and 10·0 mg/kg respectively). Doses of isoprenaline which were equi-effective against histamine and acetylcholine induced bronchospasm were antagonised to a similar degree by pronethalol.

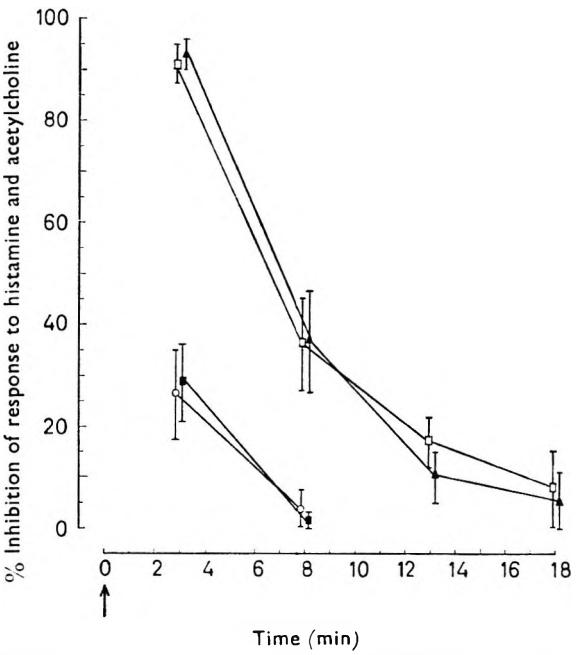

FIG. 2. The effect of graded doses of isoprenaline sulphate, 0·1 (○), 1·0 (□) and 10·0 (△) μg/kg intravenously at ↑ on the bronchoconstrictor actions of histamine (open symbols) and acetylcholine (closed symbols), in the anaesthetised guinea-pig. The points shown are mean result ± standard error for a group of 4 animals.

EFFECT OF ISOPRENALINE ON THE CONTRACTILE RESPONSES OF ISOLATED TISSUES TO HISTAMINE AND ACETYLCHOLINE

Tracheal chain preparation of the guinea-pig. The effects of isoprenaline on the contractile responses of tracheal chains to submaximal doses of histamine and acetylcholine were examined in four preparations. In all experiments, isoprenaline antagonised the response to histamine to a greater extent than it antagonised that to acetylcholine. For example it was found that concentrations of 0·5 to 1·0 ng/ml isoprenaline produced a marked reduction in the response of the tracheal chain to histamine with little or no effect on the response to acetylcholine. However 2·0 ng/ml isoprenaline was found to reduce the responses to both acetylcholine and histamine.

Guinea-pig ileum. The pA_2 value at 2 min was determined for isoprenaline against histamine and acetylcholine. These values are given as the mean value with the number of determinations in brackets. The values

found were 7·73 ± 0·11 (4) against histamine and 6·89 ± 0·05 (3) against acetylcholine. The difference in the pA_2 values was highly significant ($P < 0.001$). These pA_2 values for isoprenaline were found to be raised by the addition of atropine or chlorpheniramine to the bathing fluid.

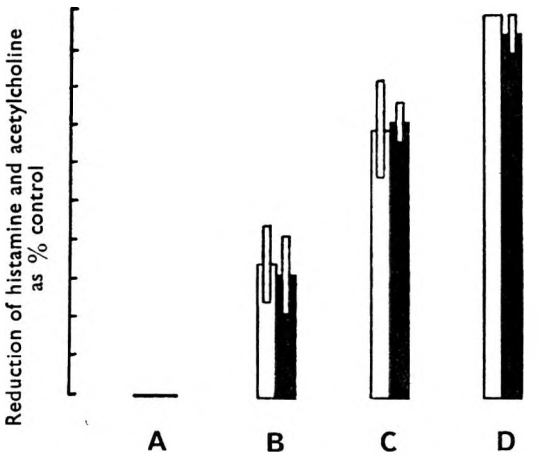

FIG. 3. The inhibitory action of isoprenaline, 10 μg/kg, on the bronchoconstrictor action of histamine (open columns) and of isoprenaline, 100 μg/kg, on the bronchoconstrictor action of acetylcholine (closed columns). Ordinate: height of response to histamine or acetylcholine 3 min after injection of isoprenaline expressed as a percentage of control (see text). A, B, C and D are mean results ± standard error for groups of 4 animals which had received prior injection of saline (A) or 0·1 (B), 1·0 (C) and 10 (D) mg/kg pronethalol intramuscularly.

Thus atropine 40 ng/ml, did not affect the response of the ileum to histamine but increased the pA_2 value for isoprenaline against histamine from 7·73 ± 0·11 (4) to 10·18 ± 0·36 (8). On the other hand, chlorpheniramine at 40 ng/ml which possessed no anti-acetylcholine action, increased the pA_2 value for isoprenaline against acetylcholine from 6·89 ± 0·05 (3) to 8·08 ± 0·19 (3). The concentrations of atropine and chlorpheniramine employed abolished the responses of the ileum to acetylcholine and histamine respectively.

The inhibitory effect of stimulation of sympathetic periarterial nerves on the response of the ileum to acetylcholine, histamine, 5-hydroxytryptamine (5-HT) and bradykinin was also examined. It did not prove practicable to examine such inhibitory effects against these four substances on any one piece of tissue, so the effect of periarterial nerve stimulation on the responses of the guinea-pig ileum to histamine, 5-HT and bradykinin was individually compared with the effect against acetylcholine in each experiment. Three experiments were made for each investigation and examples of the results are shown in Fig. 4. In Fig. 4 the effect of nerve stimulation on the responses of the ileum to 5-HT and acetylcholine were compared. The response to 5-HT was abolished, but the response to acetylcholine although markedly reduced was not abolished. Even a

four-fold increase in the concentration of 5-HT did not produce a contraction during nerve stimulation. The response to histamine was more reduced than the response to acetylcholine (Fig. 4). However the responses to acetylcholine and bradykinin were equally depressed by sympathetic nerve stimulation (Fig. 4). Thus the order of potency for the inhibitory effect of sympathetic nerve stimulation against these substances is 5-hydroxytryptamine > histamine > acetylcholine = bradykinin.

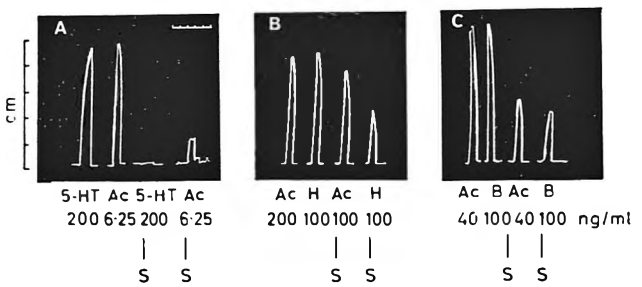

FIG. 4. The effect of periarterial sympathetic nerve stimulation (S) on the response of the guinea-pig ileum to acetylcholine (Ac), 5-hydroxytryptamine (5-HT), histamine (H) and bradykinin (B). Time scale: min.

Human myometrium. The effects of isoprenaline on spontaneous activity, and on the response of the human myometrium to histamine and acetylcholine were examined in three preparations. The results of one experiment are shown in Fig. 5. The isolated human myometrium contracted when histamine or acetylcholine was added to the bathing fluid. These responses were superimposed on the spontaneous contractions of the tissue. Concentrations of up to 40 μg/ml isoprenaline did not affect the responses to histamine or to acetylcholine, nor was there any reduction in the spontaneous motility of the tissue. Papaverine in a concentration of 10 μg/ml caused a relaxation of the tissue, decreased the spontaneous contractions and inhibited the contractions produced by histamine and acetylcholine.

Discussion

The results presented demonstrate the greater inhibitory action of isoprenaline against histamine than against acetylcholine-induced spasm of guinea-pig tissues. In unpublished experiments we have observed that this property is shared by adrenaline and noradrenaline, although on a quantitative basis these amines are less active. A similar qualitative picture was obtained for the inhibitory action of sympathetic nerve stimulation on the response of the guinea-pig ileum to histamine and acetylcholine. A related observation was made by Wilson (1964) who observed that phenylephrine, noradrenaline, adrenaline and isoprenaline were more active in antagonising the response of the guinea-pig ileum to histamine than to methacholine. Thus catecholamines show specificity in their inhibitory actions against responses to spasmogens.

Experiments were made to determine whether the inhibitory action of isoprenaline against spasmogens may be explained by its activation of β-adrenoceptive receptors. The β-blocking agent pronethalol was used to block the β-adrenoceptive receptors (Black & Stephenson, 1962).

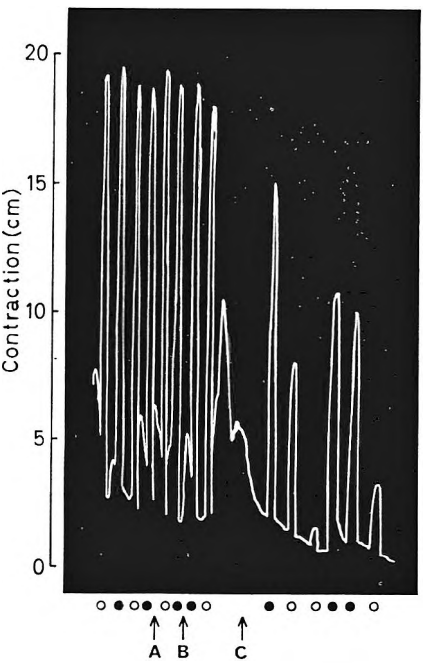

FIG. 5. Isolated human myometrium. The effect of isoprenaline 4·0 (A), 40·0 (B) μg/ml and papaverine 10 μg/ml (C) on spontaneous activity, and histamine (○) and acetylcholine (●)-induced contractions. Tissue suspended in Locke solution at 37°.

Doses of isoprenaline which produced equi-effective depressions of the bronchoconstrictor action of histamine and acetylcholine were antagonised to the same extent by equal doses of pronethalol, and it is therefore likely that only one type of β-receptor is involved. Since pronethalol completely antagonised the effect of isoprenaline on both histamine and acetylcholine, without itself suppressing the response to histamine and acetylcholine, it is unlikely that there is any direct interaction of isoprenaline with histamine and acetylcholine receptors. Secondly, evidence is available that the human myometrium contains few or no β-adrenergic receptors (Lehrer, 1965). This preparation readily contracted to histamine and acetylcholine but neither of these responses, nor the spontaneous contractions of the tissues, were reduced by isoprenaline. Papaverine (10 μg/ml) on the other hand caused relaxation of the tissue, decreased spontaneous contractions and reduced those due to histamine and acetylcholine. It was reasoned that should isoprenaline occupy histamine and to a lesser extent acetylcholine receptors, then the addition of an antihistamine might

enhance the activity of isoprenaline against acetylcholine. In fact the pA_2 value for isoprenaline versus acetylcholine was raised by the addition of chlorpheniramine to the bathing fluid, but the pA_2 value for isoprenaline versus histamine was increased to a greater extent by the presence of atropine. Clearly these results do not lend support to an action of isoprenaline on histamine or acetylcholine receptors, but they do suggest an interaction of isoprenaline with both atropine and chlorpheniramine at some other receptor sites.

Thus it would seem that the inhibitory actions of catecholamines injected, or released from sympathetic nerves show specificity in their actions against spasmogens. This is not due to any direct interaction of the catecholamines with the various receptors for these spasmogens but is a result of activation of adrenoceptic receptors.

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Effect of metal ions on the lysis of yeast cells by cationic dyes and surfactants

J. C. RIEMERSMA

Uranyl ions and thorium ions are effective in preventing cytolysis by Toluidine Blue and Azure A because they interfere with dye uptake by the cells. Neither uranyl nor thorium ions prevent the uptake of cetrimide or cetylpyridinium chloride. Cetylpyridinium chloride is capable of releasing previously bound uranyl ions from the cell surface. Anionic groups belonging to lipid constituents of the cell membrane may be involved in the uptake of uranyl and thorium ions as well as in the binding of dye and surfactants cations respectively. Possible cytolytic mechanisms involving polar interactions at the cell surface with lytic cations are discussed.

THE pronounced cytolytic effect of cationic surfactants of the quaternary alkylammonium type, as well as the effects of cationic dyes belonging to the thiazine group (Armstrong, 1957, 1958, 1963; Passow, Rothstein & Loewenstein, 1959), may be connected with the presence of anionic groups at the surface of microbial cells which are capable of binding these substances. With yeast cells, a rôle of polar interactions is suggested by the contrasting behaviour of a cationic surfactant, such as cetylpyridinium chloride, and of an anionic surfactant such as sodium dodecylsulphate (Riemersma, 1966). In yeast suspensions having a pH between 3·5 and 6·0, the cationic surfactants cetrimide and cetylpyridinium chloride were found to have strong cytolytic effects. Extensive cytolysis appeared when a critical quantity of the surfactant uptake by the cells was exceeded. An anionic surfactant, sodium dodecylsulphate, was cytolytically effective only below pH 3·2; uptake from the medium was not complete, as with cationic substances.

Metal ions capable of interaction with anionic membrane constituents might be expected to influence these cytolytic phenomena. Some observations have been reported of a protective action by uranyl salts, which appear to inhibit lysis of yeast cells by cationic dyes and surfactants (Armstrong, 1958; Passow & others, 1959). The present paper deals with the protective effects of uranyl and thorium ions.

Experimental

Fresh commercial baker's yeast (Koningsgist, Delft) was washed, aerated and freed from cell debris (Riemersma, 1966). A 35·5% stock suspension was prepared (35·5 g fresh yeast per 100 ml). Conductivity measurements were carried out with a Philips PR 9501 conductivity meter. A double-walled glass vessel was used, connected with a thermostat (21°), and the cell constant of the conductivity cell was 0·30. Cytolytic reagents were added in small quantities (usually 0·05 ml portions) from a 5 ml piston burette (Metrohm) with adequate stirring to prevent local excess of reagent. Conductivities were read after a waiting period of 2 min (in some instances after 1 min).

From the Laboratory of Medical Chemistry, University of Leiden, Holland

During conductivity experiments the concentration of the extracellular medium was analysed by withdrawing a small volume of suspension, usually 0·5 ml, by pipette at regular time intervals from the starting volume of 160 ml. This was filtered rapidly over a Millipore filter (HA, pore size 0·45 μ) to separate the medium from the cells. The filtrate was analysed colorimetrically at the absorption maximum of various lytic dyes. Surfactant analysis was made following in principle Auerbach's (1943) Bromophenol Blue method, as modified by Van Steveninck & Maas (1965). Metal ions were determined colorimetrically; uranyl ions were converted into a coloured thiocyanate complex having an absorption maximum at 365 mμ (Tucker, 1957). For thorium ions thoron was used as the reagent, the complex formed having an absorption maximum at 540 mμ (Sandell, 1959).

Results

EFFECTS OF METAL IONS IN CYTOLYSIS BY CATIONIC DYES

The conductivity changes in a yeast suspension to which a Toluidine Blue solution was added with and without uranyl ions are given in Fig. 1 (see the legend for experimental conditions). Up to a certain quantity of dye only minor conductivity changes were found. Curve A, obtained in the absence of uranyl ions, shows the characteristic conductivity changes

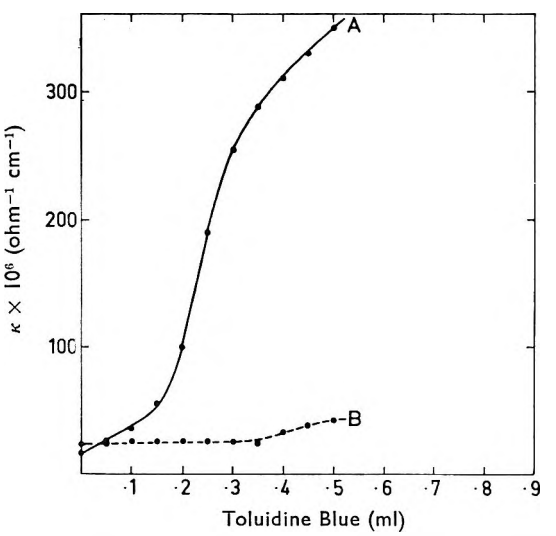

FIG. 1. Influence of uranyl ions on cytolysis caused by Toluidine Blue (21°). A. 5 ml 35·5% yeast + 27 ml water. B. 5 ml 35·5% yeast + 26 ml water + 1 ml 0·01 M uranyl nitrate.

which occurred beyond a critical dye quantity. In the presence of uranyl ions the large conductivity increase normally occurring beyond the critical dye quantity was eliminated (curve B, 3×10^{-4}M uranyl). Subsequent experiments showed that a concentration as low as 10^{-4}M uranyl

was effective in shifting the cytolytic threshold far to the right with Azure A as well as with Toluidine Blue.

Thorium had similar effects. Calcium ions had a pronounced effect in shifting the break of the conductivity plot to the right, but only in a concentration of 10^{-3}M. Sodium and potassium had to be present in a 10^{-1}M concentration to give a corresponding effect.

Analysis of the extracellular medium at several points of the conductivity curve, following Millipore filtration of 0·5 ml samples taken from the suspension, showed that uranyl and thorium ions (3×10^{-4}M) prevented the uptake of Azure A by the cells. Normally the whole quantity of added dye is bound by the cells. With uranyl or thorium ions present the dye was completely recovered from the medium.

It proved possible to remove previously bound Azure A by adding a uranyl solution, in the following way. A small volume of yeast suspension to which a sub-critical quantity of dye had been added was filtered through a Millipore filter. Subsequently a few ml of 0·001M uranyl nitrate solution was added to the packed cells on the filter. Filtration of this liquid gave a dark blue filtrate containing the total dye quantity originally bound by the cells.

EFFECTS OF METAL IONS IN CYTOLYSIS BY SURFACTANT

Uranyl ions inhibited yeast cytolysis by cetrimide (see Fig. 2). The critical quantity of cetrimide at which extensive cytolysis began was shifted to the right. Thorium ions had a similar effect, postponing cytolysis to even higher cetrimide quantities. Uranyl and thorium ions exerted a similar protective action with cetylpyridinium chloride.

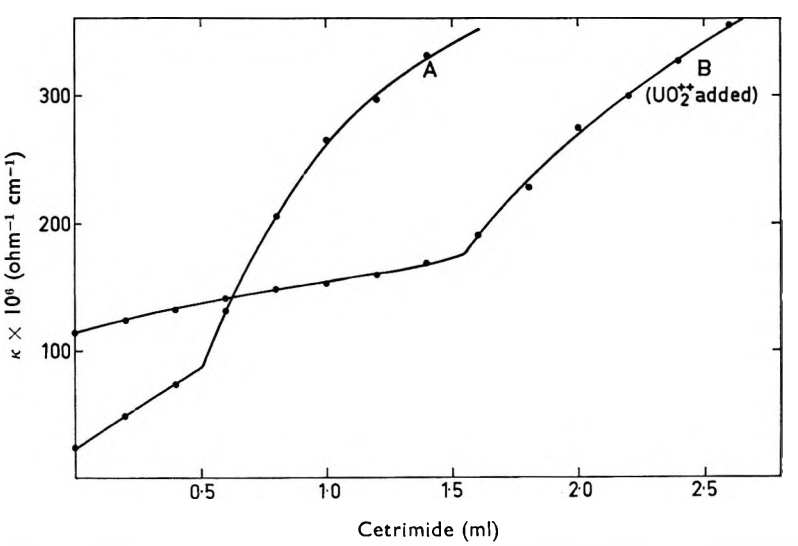

FIG. 2. Influence of uranyl ions on cytolysis by cetrimide (21°). A. 7 ml 35·5% yeast + 25 ml water. B. 7 ml yeast + 24 ml water + 1 ml 0·01 M uranyl nitrate.

Other divalent ions were less effective in shifting the cytolytic surfactant quantity to higher values. Calcium ions had little protective effect, in contrast to what was observed with the two dyes. Sodium and potassium ions also had a negligible influence on the conductivity curves, even in high concentrations.

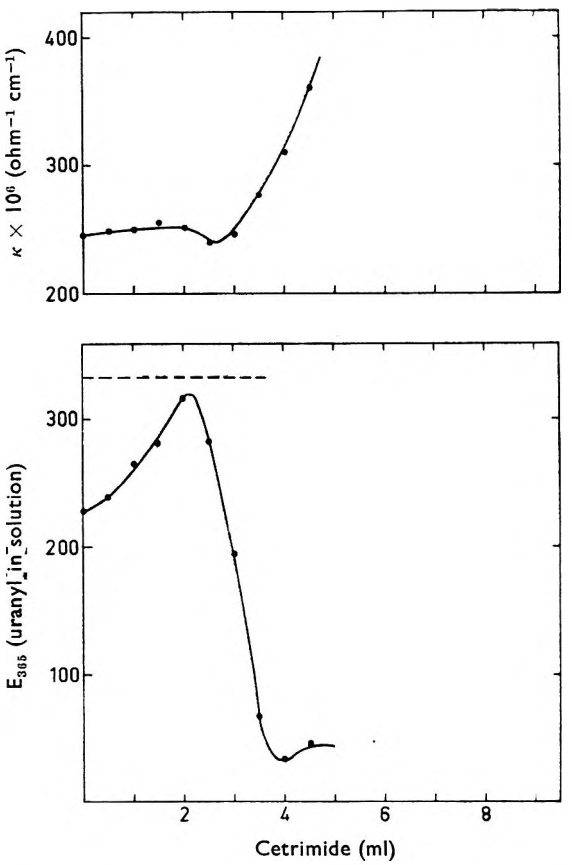

FIG. 3. Relation between uranyl concentration in the extra-cellular medium (measured as the absorbance of a thiocyanate complex E_{365}) and the specific conductivity changes of a yeast suspension during stepwise addition of 0·02M cetrimide (21°). Conditions: 28 ml 35·5% yeast + 94 ml water + 6 ml 0·01M uranyl nitrate. Uranyl determined as described in the text. Dotted line indicates the value of E_{365} if all the uranyl ions present are free in solution; removal of uranyl from the cells is incomplete.

Surfactant determinations in the extracellular medium, obtained after Millipore filtration of cell suspension samples, showed that virtually all the added surfactant cations are bound by the cells, whether or not uranyl or thorium ions are present. Apparently these metal ions do not prevent uptake of *surfactant* cations, as they do *dye* cations.

In fact rather than preventing surfactant ion uptake, uranyl was itself removed from the cell surface by successive surfactant additions. In the

experiment in Fig. 3, for instance, first a small uranyl nitrate quantity was added to a yeast cell suspension and subsequently the effect of a series of cetrimide-additions on conductivity, and on the extracellular uranyl ion concentration, was determined. Under the conditions specified, the uranyl concentration in the medium gradually rose to a value corresponding to an almost complete removal of uranyl ions from the cells, at which point lysis occurred. Lysis was followed by uranyl ion binding by released cytoplasmic material, and a corresponding drop in the uranyl concentration. Similar experiments with cell suspensions to which thorium nitrate was added showed that no comparable removal of thorium ions is effected. This ion evidently was bound more strongly at the cell surface than uranyl.

METAL ION BINDING BY YEAST CELLS

By adding increasing quantities of uranyl salt to a constant quantity of yeast, and by determining unbound uranyl after centrifugation, the existence of a definite plateau for uranyl uptake was confirmed (see also Rothstein, Frenkel & Larrabee, 1948b; Van Steveninck & Booij, 1964). Thereby an approximate value for the uptake capacity of the cell surface was obtained. To obtain a more precise figure, a constant uranyl quantity above the saturation level was added to a series of increasing yeast quantities and the quantity of bound uranyl was determined as a function of the yeast quantity. In 8 volumetric flasks (50 ml) were placed respectively, in duplicate, 1, 2, 5, and 10 ml 14·2% yeast suspension, 0·15 ml 0·15M hydrochloric acid, and 1 ml 0·01M uranyl nitrate; the volume was made up to 50 ml with water. After mixing and standing 10 min this suspension was centrifuged for 10 min at 300 rpm, 25 ml supernatant was transferred to a series of Erlenmeyer flasks, to which subsequently 10 ml ascorbic acid (2%) and 15 ml ammonium thiocyanate (50%) were added. After standing for 1 hr the absorbance was measured in 10 mm cuvettes at 365 mμ. A straight-line dependence was found between the quantity of yeast and the quantity of uranyl bound. The slope of this line gave a binding capacity of 3·5, 3·3 and 2·8 μ-equiv. UO_2/gram yeast, in three separate series.

A similar procedure was adopted to determine the binding capacity for thorium ions. In a series of 16 volumetric flasks (50 ml) respectively, and in duplicate, were placed 1, 3, 5, 10, 15, 20, 25, 30 ml 7·1% yeast suspension, 1·0 ml hydrochloric acid 0·15M and 2 ml thorium nitrate (1000 μg thorium/ml). After adding water to 50 ml, mixing and standing for 5–10 min the suspensions were centrifuged. 5 ml amounts of supernatant were transferred to a series of volumetric flasks (50 ml), to which 2·5 ml 6M hydrochloric acid and 5 ml 0·1% thorin [2-(2-hydroxy-3,6-disulpho-1-naphthylazo)benzene arsonic acid] were added. The volume was brought to 50 ml with water and the absorbance was measured at 545 mμ. The slope of the line obtained for bound thorium as a function of the yeast quantity gave respectively 4·7, 5·4, and 5·9 μ-equiv. Th/g yeast in three separate series. The differences reflect variations among batches of yeast cells rather than errors inherent in the analytical procedure, since the difference between duplicate determinations was generally not

more than 1–2%; the same holds for the figures regarding the binding capacity for uranyl.

Rothstein & others (1948) gave for uranyl ions a binding capacity of 2·2 μ-equiv. uranyl ion/g yeast, while Van Steveninck & Booij (1964) found 4·5 to 6·2 μ-equiv./g. For thorium ions Rothstein & others gave no data, while Van Steveninck & others found an uptake of 8·5 to 10·4 μ-equiv./g yeast. Variations in the culture conditions and in pre-treatment may in part account for the divergence of these figures.

Discussion

The critical quantity of cationic dye beyond which extensive cytolysis occurs in a yeast suspension is greatly increased in suspensions containing uranyl or thorium ions (Fig. 1). Experiments with cationic surfactants as lytic substances also demonstrated a pronounced effect of uranyl or thorium ions (Fig. 2). Uranyl and other metal ions in fact interfered with the uptake of basic dyes by the yeast cells.

The uptake of cationic surfactants, on the other hand, occurred whether or not metal ions were present. Here the protective action of metal ions is less pronounced; it is restricted to only a few metal ions such as uranyl and thorium. Postponement of cytolysis to higher surfactant quantities may in these instances be the result of an increased stability of the cell membrane due to bound metal ions. The membrane can apparently take up relatively larger quantities of the cationic surfactant in the presence of uranyl or thorium than in their absence, before becoming unstable. In the case where uranyl ions are present, surfactant uptake is accompanied by a removal of uranyl from the cell surface. Cetyltrimethylammonium ions have a large enough affinity to certain anionic sites to displace uranyl ions. This affinity cannot be explained solely on the basis of Coulomb interactions between these quaternary ammonium ions and negative groups. The strong and apparently irreversible binding of surfactant cations by the cell surface suggests, apart from Coulomb interactions between ionic groups, an important contribution of van der Waals' interactions between alkyl chains. One way in which such interactions could occur is by the penetration of surfactant alkyl chains into the membrane lipid bilayer. Another, although less likely way, is an accumulation of associated surfactant cations against the cell membrane. With dye cations, given their removability from the cell surface by heavy metal ions, a less intimate combination with membrane constituents is suggested.

Studies by earlier authors have led to the hypothesis that phosphate groups at the yeast cell surface are responsible for binding thorium and uranyl ions (Booij, 1940; Rothstein & Larrabee, 1948; Rothstein & others 1948; Rothstein & Hayes, 1956; Van Steveninck & Booij, 1964). The competitive character of cationic surfactant binding and uranyl binding, as discussed in this paper, suggests that the same phosphate groups might also be involved in surfactant binding. Given the analytical data about the lipid composition of yeast cells, it is tempting to regard membrane phospholipids as directly involved in surfactant binding (Riemersma, 1966).

Phosphate groups belonging to phosphoinositides, phosphatidic acid, and other anionic lipids could combine with the "head" of the surfactant cation, while the surfactant "tail" could enter into the hydrophobic layer of the membrane. In this way the membrane bilayer would form a mixed micelle with surfactant cations, which could be transformed into globular micelles once a certain critical uptake quantity was passed. This type of mechanism is in agreement with an all-or-none type of cytolysis, and with the relatively large surfactant uptake required to reach the cytolytic threshold (of the same order as the quantity of membrane lipids). In the case of the cationic dyes, a dye accumulation in the form of a counter-micelle against the membrane must be assumed which leads to membrane breakdown once a certain critical quantity is passed.

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Decarboxylation and demethylation of some phenolic benzoic acid derivatives by rat caecal contents

RONALD R. SCHELINE

Rat caecal contents decarboxylate phenolic benzoic acid derivatives when a free hydroxyl group is in the *para* position but the presence of substituents adjacent to this group or the carboxyl group reduce or abolish the reaction. Compounds containing a hydroxyl group in the *ortho* or *meta* position but lacking one in the *para* position are not decarboxylated. Some methoxy-derivatives are demethylated. The possible relationship between these findings and urinary phenols is discussed.

URINARY pyrogallol (1,2,3-trihydroxybenzene) is probably derived from gallic acid (3,4,5-trihydroxybenzoic acid) by decarboxylation in the alimentary tract (Tompsett, 1958). Booth, Masri, Robbins, Emerson, Jones & DeEds (1959) reported that rabbits fed a diet containing gallic acid excreted pyrogallol, which was isolated from the acid-hydrolysed urine (see also Watanabe & Oshima, 1965). That the decarboxylation may occur in the alimentary tract is supported by the finding of Booth & Williams (1963) that protocatechuic acid (3,4-dihydroxybenzoic acid) is decarboxylated to catechol (1,2-dihydroxybenzene) by rat faecal and caecal extracts. Gallic acid and protocatechuic acid are partially decarboxylated in the rat when given orally but not when given intraperitoneally (Scheline, 1966). Decarboxylation also occurs when these acids are incubated with extracts of rat intestinal contents or faeces. Since treatment of the rats or the incubation mixtures with oxytetracycline or neomycin greatly reduced or abolished the reaction it was concluded that the decarboxylation of gallic acid and protocatechuic acid was effected by the intestinal microflora.

Other phenolic benzoic acid derivatives might also be decarboxylated. The present report describes a study of the decarboxylation and also the demethylation of some benzoic acid derivatives by the rat caecal microflora.

Experimental

COMPOUNDS

1,3-Dihydroxy-2-methoxybenzene (Schöpf & Winterhalder, 1940), 3-hydroxy-4-methoxybenzoic acid (Perkin & Stoyle, 1923), 3,4-dihydroxy-5-methoxybenzoic acid (Jurd, 1959) and 3,5-dihydroxy-4-methoxybenzoic acid (Geissman & Mojé, 1951) were prepared. Other compounds were obtained commercially. The compounds were checked for purity chromatographically and recrystallised if required.

METHODS

The incubation medium used consisted of 0·5% yeast extract (Difco) and 0·5% peptone (Difco) in 0·1M phosphate buffer (pH 7·4). The

From the Department of Pharmacology, University of Bergen, Haukelandsveien 10, Bergen, Norway.

medium (10 ml) in a 15 × 150 mm test tube was placed in a boiling water-bath for 15 min. The test substances were dissolved in water with the aid of a minimum amount of solid sodium bicarbonate to give a concentration of 10 mg/ml. This solution (1 ml) was added to the cooled medium. Caeca were obtained from adult male albino rats fed a diet obtained from Felleskjöpet, Oslo. The entire caecal contents were well mixed with five volumes of medium and the resulting suspension centrifuged at low speed. The supernatant liquid (1 ml) was added to the sample tube, which was then flushed with nitrogen, stoppered, mixed by inverting a few times and incubated at 37° for 22 hr. The incubation mixture was then acidified with concentrated hydrochloric acid (1 ml) and extracted twice with 25-ml portions of ether. The ether extract was dried over anhydrous sodium sulphate, evaporated to dryness and the residue dissolved in acetone (1 ml). Controls were prepared similarly except that the caecal extract was omitted.

TABLE 1. THIN-LAYER CHROMATOGRAPHY AND COLOUR REACTIONS OF SOME PHENOLIC COMPOUNDS

Compound		Rf		Colour with:	
Chemical name	Trivial name(s)	Solvent 1	Solvent 2	Fast blue B salt	Diazotised sulphanilamide
2-Hydroxybenzoic acid	Salicylic acid	0·94	0·49	Pale yellow-brown	Pale yellow-brown
3-Hydroxybenzoic acid	—	0·35	0·45	Orange	Yellow
4-Hydroxybenzoic acid	—	0·35	0·46	Orange-brown	Yellow-orange
2,3-Dihydroxybenzoic acid	α-Pyrocatechuic acid	0·35	0·42	White-brown	Pink-tan
2,4-Dihydroxybenzoic acid	β-Resorcylic acid	0·24	0·31	Violet	Yellow
2,5-Dihydroxybenzoic acid	Gentisic acid	0·16	0·43	White-grey	Pale-tan
2,6-Dihydroxybenzoic acid	γ-Resorcylic acid	0·18	0·38	Red-brown	Yellow
3,4-Dihydroxybenzoic acid	Protocatechuic acid	0·06	0·34	White-tan	Pink-tan
3,5-Dihydroxybenzoic acid	α-Resorcylic acid	0·02	0·33	Red-purple	Grey-yellow
2,3,4-Trihydroxybenzoic acid	—	0·05	0·28	Pale-brown	Pale-brown
3,4,5-Trihydroxybenzoic acid	Gallic acid	0·00	0·28	Pink-brown	Green-brown
2,4,6-Trihydroxybenzoic acid	—	0·02	0·21	Purple	Yellow-brown
3-Hydroxy-4-methoxybenzoic acid	Isovanillic acid	0·56	0·33	Pink-brown	Orange
4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	0·79	0·37	Pink-brown	Orange
3,4-Dihydroxy-5-methoxybenzoic acid	3-*O*-Methylgallic acid	0·10	0·29	White-grey	Pink-grey
3,5-Dihydroxy-4-methoxybenzoic acid	4-*O*-Methylgallic acid	0·07	0·40	Red-purple	Brown-yellow
3,5-Dimethoxy-4-hydroxybenzoic acid	Syringic acid	0·75	0·31	Pink-brown	Red
Phenol	—	0·88	0·69	Red-orange	Yellow-orange
1,2-Dihydroxybenzene	Catechol, pyrocatechol	0·30	0·62	Pink-grey	Pink-grey
1,3-Dihydroxybenzene	Resorcinol	0·11	0·56	Red-purple	Grey-yellow
1,4-Dihydroxybenzene	Hydroquinone, quinol	0·10	0·63	Grey-brown	Brown
1,2,3-Trihydroxybenzene	Pyrogallol	0·04	0·55	Pink-brown	Brown
1,3,5-Trihydroxybenzene	Phloroglucinol	0·00	0·42	Purple	Yellow-brown
2-Methoxyphenol	Guaiacol	0·98	—	Violet	Orange
1,2-Dihydroxy-3-methoxybenzene	1-*O*-Methylpyrogallol	0·54	0·53	White-grey	Pink-grey
1,3-Dihydroxy-2-methoxybenzene	2-*O*-Methylpyrogallol	0·43	0·63	Violet	Grey-yellow
2,6-Dimethoxyphenol	—	0·96	0·54	Purple	Red

Solvent 1: benzene - glacial acetic acid - H_2O (6:7:3, upper layer); Whatman CC.41 cellulose.
Solvent 2: 20% aqueous potassium chloride - glacial acetic acid (100:1); Macherey, Nagel & Co. MN 300 cellulose.

CHROMATOGRAPHY

The above acetone solutions, together with appropriate standards, were examined by thin-layer chromatography on 0·5-mm thick layers of cellulose [CC.41 (Whatman) or MN 300 (Macherey, Nagel and Co.)]. Rf values and colour reactions of the phenolic compounds are shown in Table 1. They were detected by spraying with fast blue B salt and diazotised sulphanilic acid solutions. Whatman CC.41 cellulose was used with solvent 1, as it was found to give sharper separations. Salicylic acid gave a very pale colour with the two spray reagents but was readily detected with ultraviolet light (254 mμ).

Results and discussion

The main findings are summarised in Table 2. Decarboxylation occurred only when a free hydroxyl group was present in the *para* position. This is best shown by 4-hydroxybenzoic acid, which was decarboxylated to phenol under these conditions. Initial experiments using 0·1M

TABLE 2. METABOLISM OF PHENOLIC BENZOIC ACID DERIVATIVES BY RAT CAECAL EXTRACTS

Compound	No. of experiments	Decarboxylation	Demethylation	Observations
2-Hydroxybenzoic acid	5*	—**	...	—
3-Hydroxybenzoic acid	4	—	...	—
4-Hydroxybenzoic acid	6	+++	...	Trace of unchanged compound in 2/6, none in 4/6. Large amounts of phenol in 6/6
2,3-Dihydroxybenzoic acid	4	—	...	—
2,4-Dihydroxybenzoic acid	5	—†	...	—
2,5-Dihydroxybenzoic acid	4	—	...	—
2,6-Dihydroxybenzoic acid	4	—	...	—
3,4-Dihydroxybenzoic acid	5	+++	...	Unchanged compound in 4/5. Large amounts of catechol in 5/5.
3,5-Dihydroxybenzoic acid	4	—	...	—
2,3,4-Trihydroxybenzoic acid	4	—	...	—
3,4,5-Trihydroxybenzoic acid	8	+++	...	Unchanged compound in 4/8. Pyrogallol in 5/8. Resorcinol in 7/8.
2,4,6-Trihydroxybenzoic acid	2	—†	...	—
3-Hydroxy-4-methoxybenzoic acid	6	—†	++	Unchanged compound in 6/6. 3,4-Dihydroxybenzoic acid in 2/6. Catechol in 6/6.
4-Hydroxy-3-methoxybenzoic acid	6	++	++	Unchanged compound in 6/6. Guaiacol in 5/6. 3,4-Dihydroxybenzoic acid in 3/6. Catechol in 6/6.
3,4-Dihydroxy-5-methoxybenzoic acid	5	+	+	Unchanged compound in 5/5. 1-*O*-Methylpyrogallol in 4/5. Resorcinol in 4/5.
3,5-Dihydroxy-4-methoxybenzoic acid	5	—†	+	Unchanged compound in 5/5. Pyrogallol in 1/5. Resorcinol in 4/5.
3,5-Dimethoxy-4-hydroxybenzoic acid	5	—†	+	Unchanged compound in 5/5. 3-*O*-Methylgallic acid in 5/5. Resorcinol in 1/5.

* Equal to number of rats used.
** Symbols: —none, +minor, ++moderate, +++ extensive, ... not applicable.
† See text.

phosphate buffer showed that the reaction was quantitative over the pH range of 6·6–8·0 but fell to 54% at pH 6·2 and 9% at pH 5·8. Other examples of this decarboxylation to the corresponding phenol are seen

with protocatechuic acid, vanillic acid (4-hydroxy-3-methoxybenzoic acid) and 3-*O*-methylgallic acid (3,4-dihydroxy-5-methoxybenzoic acid), which were largely or partly metabolised to catechol, guaiacol and 1-*O*-methylpyrogallol, respectively.

With gallic acid, decarboxylation gave rise to pyrogallol, which was found in most of the samples. However it was sometimes absent and in these instances large amounts of resorcinol were observed on the chromatograms. Dehydroxylation to resorcinol was also seen when pyrogallol itself was incubated with the caecal extract. Pyrogallol was not dehydroxylated to catechol in these experiments. The other trihydric phenols were similarly studied and it was found that hydroxyquinol (1,2,4-trihydroxybenzene) but not phloroglucinol (1.3,5-trihydroxybenzene) was partly dehydroxylated to resorcinol. None of the dihydric phenols (catechol, resorcinol and quinol) were dehydroxylated by the caecal extracts.

The extent of resorcinol formation in the gallic acid experiments was variable and this may reflect differences in the intestinal microflora of the rats used. This variation has also been seen in *in vivo* experiments where a conjugate of resorcinol was found in the urine of some but not all rats given gallic acid orally (Scheline, 1966). A similar finding in man has been reported by Curzon (1957) and Curzon & Pratt (1964) who found that resorcinol sulphate was excreted by some subjects. They suggested that it originated from the action of particular intestinal bacteria on dietary tea polyphenols.

Several of the acids containing a free hydroxyl group in the *para* position underwent little or no decarboxylation and it appears that certain substituents adjacent to either this group or the carboxyl group greatly reduce this reaction. Although decarboxylation was not appreciably affected by adjacent hydroxyl groups as in protocatechuic acid and gallic acid, methoxyl groups adjacent to the hydroxyl group in the *para* position as in vanillic acid, 3-*O*-methylgallic acid and syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), reduced or abolished decarboxylation. Thus moderate amounts of guaiacol were formed from vanillic acid and small amounts of 1-*O*-methylpyrogallol were formed from 3-*O*-methylgallic acid, but decarboxylation did not occur with syringic acid in which two methoxyl groups are adjacent to the hydroxyl group in the *para* position.

The inhibitory effect of substituents adjacent to the carboxyl group was even more pronounced and only the unchanged compound was found in the incubation mixtures containing 2,3,4-trihydroxybenzoic acid. Small amounts of resorcinol were formed from 2,4-dihydroxybenzoic acid but this was not due to the caecal extract, as decarboxylation occurred to the same extent in control samples without caecal extract. This spontaneous decarboxylation was also seen with 2,4,6-trihydroxybenzoic acid, as phloroglucinol was seen in equal amounts in the incubation mixtures with and without caecal extract. Instability of the other compounds was not encountered.

All five of the methylated derivatives in Table 2 underwent demethylation to some extent when incubated with the caecal extracts. Vanillic acid was partly decarboxylated to guaiacol and partly demethylated to

protocatechuic acid, which was then decarboxylated to catechol. Isovanillic acid (3-hydroxy-4-methoxybenzoic acid) was not decarboxylated, as no guaiacol was detected. However, it was partly demethylated but less so than vanillic acid. The presence of a carboxyl group in the molecule was not essential for demethylation as guaiacol was metabolised to catechol to a small extent.

The resorcinol in the incubation mixtures containing 3-*O*-methyl- or 4-*O*-methyl-gallic acid can be accounted for by their demethylation to gallic acid, which would be decarboxylated to pyrogallol and finally dehydroxylated to resorcinol. As demethylation is a minor reaction with these compounds, it is not surprising that the intermediates were usually not detected. Resorcinol could arise similarly from syringic acid, via 3-*O*-methylgallic acid.

The demethylation of several non-phenolic methoxy-acids was examined using *o*-anisic acid (2-methoxybenzoic acid), *m*-anisic acid (3-methoxybenzoic acid), anisic acid (4-methoxybenzoic acid), veratric acid (3,4-dimethoxybenzoic acid) and 3,4,5-trimethoxybenzoic acid. Except when veratric acid was used, no phenolic metabolites were detected when any of these components were incubated with the caecal extracts. Veratric acid was demethylated to a small extent at both the *meta*- and *para*-positions, as traces of vanillic acid were found in three, and traces of isovanillic acid in two of six samples.

The decarboxylation of 4-hydroxybenzoic acid, protocatechuic acid and gallic acid was prevented when oxytetracycline was added to the incubation mixtures at a level of 4 μg/ml.

The present results demonstrate the ability of rat intestinal microflora to decarboxylate and demethylate benzoic acid derivatives. The significance of this effect on the metabolism of such compounds when ingested by man or animals remains to be seen, although two of the compounds, protocatechuic acid and gallic acid, have already been found to be partially decarboxylated when administered orally to rats (Scheline, 1966). The finding by Booth & others (1959) and Watanabe & Oshima (1965) that pyrogallol is excreted in the urine by rabbits given gallic acid orally, indicates that decarboxylation also occurs in this species, possibly by the intestinal flora. Harborne & Simmonds (1964) have stated that many of the simple phenolic acids, including 4-hydroxybenzoic acid, protocatechuic acid and vanillic acid, are widely distributed in plants. These compounds are therefore normal dietary components for man and many animals. From the evidence now available it seems reasonable to assume that decarboxylation and demethylation of phenolic compounds by the intestinal microflora can explain the presence of some of the phenols normally excreted in urine (see Williams, 1959). Scheline (1966) has suggested that decarboxylation of protocatechuic acid contained in plant material is responsible for some of the catechol found in the urine of animals. The present results indicate that vanillic acid may also be a precursor of urinary catechol and suggest that guaiacol may be expected in urine. Urinary phenol is thought to arise from tyrosine in the gastrointestinal tract by the action of bacteria (Bray & Thorpe, 1954; Rogers, Burdick

& Burnett, 1955). The finding that 4-hydroxybenzoic acid is readily decarboxylated to phenol by the caecal microflora indicates that another pathway for phenol formation is available, although the relative importance of these two routes remains to be determined.

Diet can play an important role in influencing the nature of the intestinal flora, for rats fed on different diets showed considerable differences in the types and numbers of micro-organisms found in the alimentary tract (Smith, 1965). Diet could, therefore, influence the pattern of urinary metabolites of those compounds which are susceptible to metabolism by intestinal microflora.

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Neuromuscular blocking agents: branched-chain tetra-onium compounds

D. EDWARDS,* the late J. J. LEWIS† AND GLADYS MARREN†

The compounds tri(6-dimethylaminohexyl)amine, tri(6-diethylaminohexyl)amine and tri(6-dipropylaminohexyl)amine have been synthesised and eight tetra-onium derivatives prepared from these bases have been tested for neuromuscular blocking activity on the cat, hen, frog, rat and mouse. Two of the compounds possessed ganglion blocking activity and this was much weaker than in hexamethonium. All the compounds had tubocurarine-like properties, the most active being 7-ethyl-7-(5-triethylhexylammonium)-7-azoniatridecamethylenebis(triethylammonium) tetra-iodide The results of the pharmacological tests give little support for a one-point theory of receptor attachment.

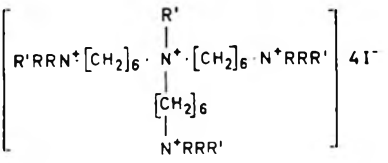
RECENT discoveries of potent neuromuscular blocking activity in certain mono- and bis(quaternary ammonium) steroids has served to cast some doubt upon the validity of the classical concept of a two-point theory of receptor attachment (Barlow & Ing, 1948; Paton & Zaimis, 1949) and to re-emphasise the possibility of one-point drug-receptor interaction, together with the importance of other physico-chemical factors (e.g. adumbration and water/fat solubility ratios) known to influence this type of pharmacological activity (Cavallito & Gray, 1960). Thus certain 3,17-bis(quaternary ammonium) androstanes (May & Baker, 1963; Martin-Smith & Sugrue, 1964) in which the quaternary heads lie on opposite sides of the steroid nucleus, possess potent neuromuscular blocking properties while qualitatively similar, weaker, non-depolarising effects are observed among several monoquaternary steroids (Martin-Smith & Sugrue, 1964; Blanpin & Bretaudeau, 1961; Ross & Lewis, 1965, personal communication). It is interesting to note that to explain the activity of a series of tetra(dimethylaminomethyl)methane tetramethobromide derivatives possessing neuromuscular blocking properties, a one-point receptor-site attachment has also been suggested (Kensler, Langemann & Zirkle, 1954). Additionally, using a series of bis-choline ether salts, evidence has been obtained to support a theory of a one-point receptor attachment for ganglion blocking activity (Fakstorp, Pedersen, Poulsen & Schilling, 1957; Fakstorp & Pedersen, 1957).

The present investigation, having a bearing on the problem of a one-point attachment, concerned a series of branched-chain tetra-onium compounds where one of the four quaternary nitrogen atoms was separated from each of the other three by a chain of six methylene groups (I). The structure of these compounds would permit drug-receptor interaction to be maintained either by a single (one-point) onium head or alternatively by a two-point attachment involving the central onium head and one of the terminal ones, or by two of the terminal onium heads.

In addition to their possible neuromuscular blocking potential, suggested by their structural similarity to several poly-onium derivatives of established activity, the present series of compounds was also tested for ganglion

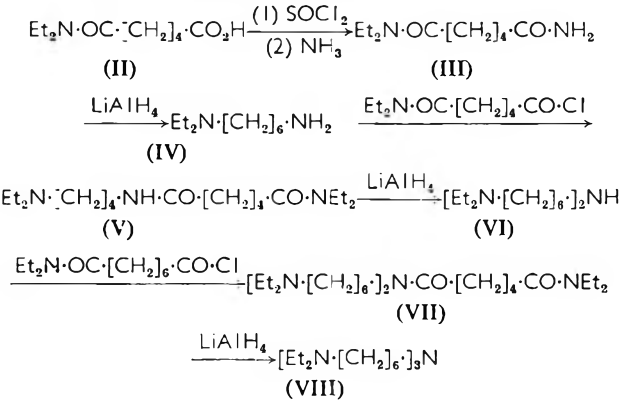
*From the School of Pharmacy, Robert Gordon's Institute of Technology, Aberdeen.

† From the Experimental Pharmacology Division, Institute of Physiology, University of Glasgow.



(I)

blocking properties, since they resembled hexamethonium in that a hexamethylene chain separated the central nitrogen atom from each of the terminal ones. In the course of both investigations the well-known influence on biological activity brought about by altering alkyl groups attached to the onium centres was also examined.



CHEMICAL

The compounds described in the experimental section were synthesised as shown in the flow sheet and the final bases (VIII) quaternised with the appropriate alkyl halides to yield the tetra-onium compounds (I)—see also Table 1.

EXPERIMENTAL

Methyl NN-*dimethyladipamate* was prepared from methyl hydrogen adipate (241 g) by the method described for the preparation of ethyl *NN*-diethyladipamate (Edwards & Stenlake, 1955), except that the solution of the acid chloride was added to the solution of dimethylamine. The *product* was obtained as a straw-coloured oil, b.p. 119–121°/0·3 mm, $n_D^{22.5}$ 1·4588 (275 g, 97·6%). Found C, 57·9; H, 8·85; N, 7·7; $C_9H_{17}NO_3$ requires C, 57·75; H, 9·2; N, 7·5%.

Methyl NN-*dipropyladipamate*, prepared from methyl hydrogen adipate (124·5 g) by the above method, was obtained as a yellow oil, b.p. 137°/0·5 mm, n_D^{11} 1·4605 (177·5 g, 93·9%). Found: C, 63·5; H, 10·2; N, 5·9. $C_{13}H_{25}NO_3$ requires C, 64·2; H, 10·4; N, 5·8%.

NN-*Dimethyladipamic acid*, prepared from methyl *NN*-dimethyladipamate (187 g) by the method described for the preparation of *NN*-diethyladipamic acid (Carey, Edwards, Lewis & Stenlake, 1959), was

obtained as a colourless oil, b.p. 164°/0·25 mm, which solidified to a greyish-white hygroscopic solid, m.p. 45° (138 g, 79·7%). Carey, Furst, Lewis and Stenlake (1964) report b.p. 178°/0·05 mm. Found: C, 54·8; H, 8·9; equiv. 172·7. Calc. for $C_8H_{15}NO_3$: C, 55·5; H, 8·7%; equiv. 173·2.

NN-*Dipropyladipamic acid*, prepared from methyl *NN*-dipropyladipamate (177·5 g) by the above method, was obtained as a yellow viscous oil, b.p. 186°/0·2 mm, $n_D^{13·5}$ 1·4763 (158·2 g, 94·6%). Carey, Edwards, Lewis & Stenlake, 1959, found b.p. 198°/0·5 mm, n_D^{25} 1·4723).

NN-*Diethylhexamethylenediamine*. *NN*-Diethyladipamic acid (41·8 g) (Carey & others, 1959), in benzene (30 ml), was refluxed with excess of thionyl chloride (20 ml) for 15 min. After removal of the solvent and excess of reagent, the acid chloride in benzene (20 ml), was added slowly (45 min) to a stirred excess of strong ammonia solution (80 ml, sp.gr. 0·880), cooled to 0°. After standing for 18 hr, the mixture was evaporated to dryness under reduced pressure, the residue extracted with ethanol, and the extract filtered and evaporated. Extraction of this residue with hot benzene gave crude *NN*-diethyladipamide as a brown solid (38 g). The amide, in hot benzene (40 ml) was added slowly (25 min) to a stirred refluxing suspension of lithium aluminium hydride (16 g) in ether (800 ml) and the refluxing continued for 22 hr. The mixture was worked up and extracted in the usual manner to yield *NN*-diethylhexamethylenediamine as a colourless oil, b.p. 155°/15 mm, n_D^{19} 1·4545 (16·6 g, 46·4%). Breslow & Houser (1945) report b.p. 103–105°/10 mm. Found: C, 69·5; H, 13·8. Calc. for $C_{10}H_{24}N_2$: C, 69·8; H, 13·95%.

NN-*Dimethylhexamethylenediamine*, prepared from *NN*-dimethyladipamic acid (88·5 g) by the above method, was obtained as a colourless oil, b.p. 89–91°/13 mm, n_D^{16} 1·4440 (30·4 g, 42%). Short, Biermacher, Dunnigan & Leth (1963) report b.p. 108-109°/32 mm, n_D^{25} 1·4423. Found: C, 66·6; H, 14·0. Calc. for $C_8H_{20}N_2$: C, 66·5; H, 13·9%.

NN-*Dipropylhexamethylenediamine*, prepared from *NN*-dipropyladipamic acid (70 g) by the above method, was obtained as a colourless oil, b.p. 140–143°/15 mm, n_D^{20} 1·4545 (28·2 g, 46·1%). French, Ugnade, Poe & Eilers (1945) report b.p. 97–99°/1 mm. Found: C, 72·3; H, 14·1; N, 13·7. Calc. for $C_{12}H_{28}N_2$: C, 72·1; H, 14·0; N, 14·0%.

Di(6-diethylaminohexyl)amine. *NN*-Diethyladipamic acid (28·7 g), in benzene (21 ml), was refluxed with excess of thionyl chloride (14 ml) for 20 min, and the solvent and excess of reagent evaporated off under reduced pressure. An excess of *NN*-diethylhexamethylenediamine (24·5 g), in benzene (50 ml), was added slowly (30 min) to a stirred solution of the acid chloride in benzene (50 ml) and the mixture refluxed for 30 min. After cooling, water (50 ml) was added, the mixture basified by the addition of an excess of sodium hydroxide solution and then extracted with benzene. The benzene extracts, after drying (Na_2SO_4) and removal of solvent and most of the unchanged *NN*-diethylhexamethylenediamine, yielded crude *N*-diethylaminohexyl-*N'N'*-diethyladipamide (22 g), which was reduced in the normal manner with lithium aluminium hydride in ether. Fractional distillation of the product gave di(6-*diethylaminohexyl*) *amine* as a colourless oil, b.p. 182–184°/1 mm, $n_D^{21·5}$ 1·4620 (14·4 g,

30·8%). Found: C, 73·4; H, 13·7; N, 12·8. $C_{20}H_{45}N_3$ requires C, 73·3; H, 13·7; N, 12·8%.

Di(6-dimethylaminohexyl)amine, prepared from *NN*-dimethyladipamic acid (17·7 g) and excess of *NN*-dimethylhexamethylenediamine (29 g) by the above method, was obtained as a colourless oil, b.p. 141–145°/0·3 mm, n_D^{20} 1·4595 (11 6 g, 41·8%). Found: C. 70·5 H, 13·7. $C_{16}H_{33}N_3$ requires C, 70·8; H, 13·6%.

Di(6-dipropylaminohexyl)amine, prepared from *NN*-dipropyladipamic acid (22·3 g) and excess of *NN*-dipropylhexamethylenediamine (39 g) by the above method, was obtained as a colourless oil, b.p. 190–195°/0·3 mm, n_D^{17} 1·4628 (22·7 g, 60·8%). Found: C, 74·5; H,13·7; N, 11·1. $C_{24}H_{53}N_3$ requires C, 75·0; H, 13·8; N, 10·9%.

Tri(6-diethylaminohexyl)amine was prepared from *NN*-diethyladipamic acid (8·6 g) and di (6-diethylaminohexyl)amine (14 g) by a similar method to that described above for the preparation of the latter compound. Fractional distillation of the reduction product yielded *tri(6-diethylaminohexyl)amine* as a colourless oil, b.p. 215–220°/0·7 mm, $n_D^{12·5}$ 1·4678 (7·7 g, 37·3%). Found: C, 74·95; H, 13·65; N, 11·5; equiv. 120·3. $C_{30}H_{66}N_4$ requires C, 74·6; H, 13·7; N, 11·6%; equiv. 120·7.

Tri(6-dimethylaminohexyl)amine, prepared from *NN*-dimethyladipamic acid (7·5 g) and di(6-dimethylaminohexyl)amine (11·5 g) by the above method, was obtained as a colourless oil, b.p. 173–176°/0·2 mm, n_D^{18} 1·4656 (3.5 g 20·3%). Found: C, 71·95; H, 13·4, $C_{24}H_{54}N_4$ requires C, 72·4; H, 13·6%.

Tri(6-dipropylaminohexyl)amine, prepared from *NN*-dipropyladipamic acid (13·2 g) and di(6-dipropylaminohexyl)amine (22·2 g) by the above method, was obtained as a colourless oil, b.p. 250–260° (bath)/0·1 mm, $n_D^{15·5}$ 1·4668 (14·8 g, 45·3%). Found: C, 76·0; H,14·1; N, 9·8. $C_{36}H_{78}N_4$ requires C, 76·3; H, 13·8; N, 9·9%.

Tetra-onium compounds were prepared from either tri(6-diethylaminohexyl)amine, tri(6-dimethylaminohexyl)amine or tri(6-dipropylaminohexyl)amine by refluxing with the appropriate alkyl halide in ethanol, evaporating off the solvent and crystallising the product. Reflux time, crystallisation solvent and yields are indicated for each compound in that order, in parenthesis.

7-(6-*Diethylnexylmethylammonium*)-7-*methyl*-7-*azoniatridecamethylene-bis(diethylmethylammonium) tetraiodide*. (25 min; ethanol; 97%), m.p. 262°. Found: I, 48·5; N, 5·5; $C_{34}H_{78}I_4N_4$ requires I, 48·3; N, 5·3%.

7-*Ethyl*-7-(6-*triethylhexylammonium*)-7-*azoniatridecamethylenebis(triethylammonium) tetraiodide*. (50 min; ethanol-ether; 81%), m.p. 266°. Found: I, 45·7; N, 5·1. $C_{38}H_{86}I_4N_4$ requires I, 45·85; N, 5·1%.

7-(6-*Diethylnexylpropylammonium*)-7-*propyl*-7-*azoniatridecamethylene-bis(diethylpropylammonium) tetraiodide*. (1 hr; acetone-ethanol-ether; 60%), m.p. 167°. Found: C, 43·2; H, 7·9; I, 43·25; N, 4·8. $C_{42}H_{94}I_4N_4$ requires C, 43·4; H, 8·1; I, 43·6; N, 4·8%.

7-(6-*Hexyltrimethylammonium*)-7-*methyl*-7-*azoniatridecamethylenebis(trimethylammonium) tetraiodide*. (5 min, ethanol; 70%), m.p. 227° (hygro-

scopic). Found: C, 34·1; H, 7·1; I, 51·9, N, 5·25. $C_{28}H_{66}I_4N_4$ requires C, 34·8; H, 6·9; I, 52·5; N, 5·2%.

7-*Ethyl*-7-(6-*ethylhexyldimethylammonium*)-7-*azoniatridecamethylenebis*-(*ethyldimethylammonium*) *tetraiodide*. (35 min, ethanol; 74%), m.p. 228°. Found: C, 36·7; H, 7·5; I, 49·6; N, 5·4. $C_{32}H_{74}I_4N_4$ requires C, 37·6; H, 7·3; I, 49·6; N, 5·5%.

7-(6-*Hexyldimethylpropylammonium*)-7-*propyl*-7-*azoniatridecamethylenebis*(*dimethylpropylammonium*) *tetraidodide*. (1 hr; acetone-ethanol-ether; 84%), m.p. 184°. Found: C, 39·7; H, 7·7; I, 46·8. $C_{36}H_{82}I_4N_4$ requires C, 40·1; H, 7·7; I, 47·0%.

7-(6-*Hexylmethyldipropylammonium*)-7-*methyl*-7-*azoniatridecamethylenebis*(*methyldipropylammonium*) *tetraiodide*. (18 hr without refluxing; ethanol; 95%), m.p. 220°. Found: C, 42·6; H, 8·5; I, 44·4; N, 4·8. $C_{30}H_{90}I_4N_4$ requires C, 42·3; H, 8·0; I, 44·7; N, 4·9%.

7-*Ethyl*-7-(6-*ethylhexyldipropylammonium*)-7-*azoniatridecamethylenebis*-(*ethyldipropylammonium*) *tetraiodide*. (40 min; acetone-ethanol-ether; 24%), m.p. 197°. Found: C, 43·9; H, 8·1; I, 42·1; N, 4·8. $C_{43}H_{98}I_4N_4$ requires C, 44·4; H, 8·3; I, 42·6; N, 4·7%.

PHARMACOLOGICAL

Methods. The evaluation of neuromuscular blocking activity was made using conventional techniques previously described, including (Edwards, Lewis, Stenlake & Zoha, 1957, 1958; Edwards, Lewis, Stenlake & Stothers, 1961) the cat and hen gastrocnemius muscle-sciatic-nerve, the rat phrenic-nerve diaphragm, the frog rectus abdominis muscle and the mouse inclined-screen method (Thomson, 1946). Sympathetic ganglion-blocking activity was estimated using the nictitating membrane preparation of the cat and parasympathetic ganglion blockade by the guinea-pig ileum peristaltic reflex experiment (Trendelenburg) (Feldberg & Lin, 1949). Toxicity measurements on mice employed the inclined-screen method.

Results. Table 1 shows the comparative molar potencies of the compounds compared with tubocurarine (TC) on the frog, the rat, the mouse, the cat and the hen.

All eight compounds possessed varying degrees of muscle relaxant activity of the non-depolarising variety, there being no evidence of any depolarising properties. Even the methyl analogue (compound IA) caused no contracture of the hen gastrocnemius muscle when used in doses producing approximately 85% inhibition of twitch height. None of the compounds showed significant effects on the blood pressure of the urethane/pentobarbitone anaesthetised rat. Weak ganglion blocking activity existed in only two of the compounds investigated (compounds IB and IC). The all-methyl derivative (compound IA) was not active in this respect, and the active compounds were approximately 2·5–13% as active as hexamethonium on a molar basis.

The therapeutic ratio (LD50/PD50) for all the compounds tested was higher than that for tubocurarine and variation in the response of different species to any one compound was marked. The general order of increasing sensitivity to the compounds was rat, mouse, frog, cat, hen.

Discussion

The compounds investigated bore some structural and pharmacological resemblance to those of Kensler, Zirkle, Matallana & Condouris (1954) (IX, n = 2,3 or 4 and R = Me or Et). In keeping with these and numerous other observations conducted on linear tri- and tetra-onium compounds (Carey & others, 1959; Edwards, Lewis, McPhail, Muir & Stenlake, 1960), the all-ethyl compound (IE) (Table 1) was found to be the most active in the majority of species examined. Its high potency in the hen again emphasises the marked variation between mammalian and avian species (Blaber & Bowan, 1962).

$$[(RRRN^{+}\cdot[CH_2]_{n}\cdot)_3CH]3Br^{-} \qquad \text{(IX)}$$

Replacement of ethyl groups by methyl or propyl groups led to a reduction in neuromuscular activity in all species except rodents (Table 1),

TABLE 1. PHARMACOLOGICAL ACTIVITY

$$[(RRR'N^{+}\cdot[CH_{2_{6}}]\cdot)_3N^{+}R']4I^{-}$$

Compound	Substituents		Relative molar potencies (TC = 100)					Type of activity
	R	R'	Cat	Hen	Frog	Rat	Mouse	
IA	Me	Me	14	63	18	0·42	9·1	TC-like
IB	Me	Et	30	190	34	0·45	17	TC-like
IC	Et	Me	13	45	31	1·0	5·9	TC-like
ID	Me	Pr	26	78	15	0·71	14	TC-like
IE	Et	Et	79	815	35	0·67	12	TC-like
IF	Pr	Me	27	98	12	0·46	14	TC-like
IG	Et	Pr	66	310	22	0·87	49	TC-like
IH	Pr	Et	51	272	11	0·19	16	TC-like

but none of the compounds, not even the trimethyl compound (IA), showed decamethonium-like properties. This lack of depolarising activity in related compounds, where ethyl groups have replaced methyl groups, has been attributed (Carey & others, 1959) to the considerable shielding of the charge on the nitrogen atom which would prevent the close approach to the receptor required for this type of activity (Paton, 1961).

Using Courtauld atomic models the maximum distance measured between the central nitrogen atom and any other nitrogen atom is about 9Å and between terminal nitrogen atoms, with a tetrahedral arrangement of groups attached to the central nitrogen atom, about 14·7 Å. Since it seems unlikely that the effect on internitrogen distance of alkyl substituents in the onium heads in the present series will be as pronounced as that in some bis-quaternary compounds (Elworthy, 1963, 1964), and since the molecules are not rigid structures, two-point attachment could be made on anionic receptors at any distance up to 9 Å by means of a central nitrogen atom and one of the terminal ones, or up to 14·7 Å by any two of the terminal ones. Although the distance of 9 Å approaches the mean distance of 9·5 Å calculated by Elworthy (1963) for decamethonium, the structure of these molecules being as they are, it cannot be assumed that drug-receptor interaction is occurring at a central nitrogen atom and one of the terminal ones.

The virtual absence of ganglion blocking activity in these compounds is

in agreement with a similar lack of this activity in related compounds (Edwards & others 1958; Carey & others, 1959).

It is interesting to note that the most potent member was the diethylmethyl derivative (IC); this is unlike hexamethonium analogues where the ethyldimethyl derivative was found to be the most potent (Wien, Mason, Edge & Langston, 1952).

The present results give little supporting evidence for a one-point theory of receptor attachment. Neither the all-methyl (compound IA) nor the all-ethyl (compound IE) derivative shows properties resembling respectively those of the classic mono-quaternary compounds tetramethylammonium or tetraethylammonium. Although the adumbrating effect of the remainder of the molecule must be taken into account, it is difficult with a one-point theory of receptor attachment to explain the absence of ganglion blockage in compound IE and of ganglion stimulating properties in compound IA.

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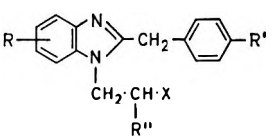
Ionisation constants and partition coefficients of some analgesically active 2-benzylbenzimidazole derivatives and related compounds

A. F. CASY* AND J. WRIGHT

The ionisation constants (in aqueous ethanol) and the partition coefficients (in a water-cyclohexane system) of some analgesically active 2-benzylbenzimidazole derivatives and related compounds are reported. The results are discussed in terms of structure-activity relationships.

IN 1957 a novel class of analgesics (I), related to 2-benzylbenzimidazole, was reported (Hunger, Kebrle, Rossi & Hoffman, 1957, 1960). Certain of these compounds were highly active, their potencies in animal tests far exceeding that of morphine and of any synthetic analgesic known at that time** (e.g. Ia-d). The action of these derivatives appears to be typically morphine-like. Thus, their side-effects in man include respiratory depression and constipation (Bromig, 1958), while, in addicts, the two derivatives (Ia and f) have addiction potentials comparable with that of morphine (Fraser, Isbell & Wolbach, 1960).



	R	R'	Activity† (morphine = 1)
a	5-NO_2	OEt	1000
b	5-NO_2	OisoPr	500
c	5-NO_2	OMe	100
d	H	OEt	70
e	H	OMe	1
f	5-NO_2	Cl	3
g	6-NO_2	Cl	0·1

† in mice, tail pressure method (Hunger & others, 1960).

In common with other classes, analgesics based upon 2-benzylbenzimidazole possess a flat aromatic system linked to a 2-aminoethyl side-chain via a non-hydrogen bearing atom [features considered essential for fit at the proposed analgesic receptor site (Beckett & Casy, 1954, 1965)], their distinctive structural features being as follows: (1) two basic centres (most analgesics are monobasic), (2) a bicyclic aromatic nucleus (most analgesic molecules contain a single benzene ring or two isolated 5-or 6-membered

From the School of Pharmacy, Chelsea College of Science and Technology, London, S.W.3.

* Present address: Faculty of Pharmacy, University of Alberta, Edmonton, Alberta.

** Since 1957 synthetic analgesics of similarly high potencies have been reported, e.g. t-alcohols derived from the thebaine-vinyl methyl ketone adduct (Bentley & Hardy, 1963).

aromatic rings), (3) a 4- alkoxy substituent in the benzyl and a 5-nitro-group in the benzimidazole fragments of the more active derivatives. One or more of these features may be partly responsible for the high potencies observed in these derivatives, either by a direct influence upon the intrinsic activity of the molecule, or by one upon factors involved in the transport of the drug molecule to its locus of action [such transport is considered to be a simple diffusion process which is governed principally by the dissociation constant of the drug and by the lipid solubility of the unionised form of the drug (Brodie & Hogben, 1957; Brodie, Kurz & Shanker 1960)]. The present physico-chemical study was undertaken with a view to making a preliminary assessment of certain structure-activity relationships in 2-benzylbenzimidazole analgesics, with particular reference to the influence of structure upon drug transport.

Experimental

All benzimidazole derivatives used were prepared by reported methods (Hunger & others, 1960; Casy & Wright, 1966). 2-Benzyl-5-nitro-(2-diethylaminoethyl) benzimidazole formed a monohydrochloride monohydrate, m.p. 199–200° (found: C, 58·6; H, 6·75; Cl, 9·05. $C_{20}H_{25}ClN_4O_2 \cdot H_2O$ requires: C, 59·05; H, 6·6; Cl, 8·7%), ν_{max} 3500 cm^{-1} (H_2O) and 1-(2-diethylaminoethyl)-2-(4-ethoxybenzyl)-5-nitrobenzimidazole, a monohydrochloride monohydrate, m.p. 160° (found: C, 57·9; H, 6·8. $C_{22}H_{29}ClN_4O_3 \cdot H_2O$ requires: C, 58·6; H, 6·9%), ν_{max} 3400 cm^{-1} (H_2O), when treated with excess of ethanolic hydrogen chloride.

Ionisation constants. The pK'_a values of acids conjugate to the bases* (the superscript indicates that values are uncorrected for ionic strength) were determined in 50% water (ion-free)-ethanol by the method of Albert & Serjeant (1962) (water could not be used because the bases were sparingly soluble in this solvent). The base hydrochloride (0·25 mmole) was dissolved in the solvent mixture (47·5 ml) and the stirred solution titrated under nitrogen with 0·05N sodium hydroxide (carbonate free) at 25°. The titrant was added in ten equal portions of 0·5 ml and the pH of the mixture recorded after each addition as soon as equilibrium had been reached. A Pye Dynacap pH meter (with a calomel and a screened glass electrode) was used to make these measurements; the glass electrode was standardised by 0·05 M potassium hydrogen phthalate (pH 4·00) and 0·05 M borax (pH 9·15) buffers, and the accuracy of the apparatus and technique were checked by measuring the pK_a of benzoic acid [pK'_a 4·09 in water, Albert & Serjeant (1962) give 4·12]. pK_a values were calculated by applying Henderson's equation to each of the nine pH values obtained during the potentiometric titrations, and averaging the results. Average values were only acceptable if the scatter did not exceed $\pm$ 0·08 pK'_a unit.

Partition coefficients. The partition coefficients of the benzimidazole derivatives were determined between cyclohexane (spectroscopic grade) and an aqueous buffer of pH 7·4, prepared by mixing 0·1 N sodium hydroxide (39·5 ml) with 0·1 M potassium dihydrogen phosphate (50 ml)

* Henceforth for brevity referred to as the pK'_a values of the bases.

and adjusting the volume to 100 ml (Lange, 1952). For the partitioning, cyclohexane saturated with buffer solution and buffer solution saturated with cyclohexane were used. Analysis of the concentrations of partitioned substances were made using a Uvispek H. 700/304 spectrophotometer. The absorption peaks near 248 mμ (for non-nitrobenzimidazoles) and 240 mμ (for nitrobenzimidazoles) were used in these analyses, absorption determinations being made of a suitable dilution of the aqueous layer after partitioning. In the case of morphine, the catechol chromophore (λ_{max} 287 mμ, ϵ 2700) was employed for spectroscopic measurements. The small amount of cyclohexane dissolved in the water had no effect on the absorption curves at wavelengths higher than 220 mμ (in contrast, ethylene dichloride had a marked effect and could not be used as the organic phase on this account). The amount of sample and the volume of the two phases chosen was such that a onefold dilution of the aqueous phase gave an absorbance of between 0·2 and 0·8 using a 1 cm cell. Calibration curves were drawn for each compound studied (Beer's Law was obeyed over this absorbance range in all cases).

Procedure. Ten ml of each liquid phase was pipetted into a Quickfit tube, and after the addition of about 5 mg of the sample (accurately weighed), the tube was sealed and shaken horizontally for 24 hr at 37°. After the two layers had separated, the aqueous phase was run off and a 5 ml pipetted sample diluted to 10 ml with 0·002 N hydrochloric acid before measuring its absorbance. Each determination was made in duplicate.

The ratio C_1/C_2, where C_1 is the solute concentration in cyclohexane and C_2, that in the aqueous buffer, gives the apparent partition coefficient (P′) (Reese, Irwin, Dittert, Chong & Swintosky, 1964), since C_2 includes both ionised and unionised forms of the benzimidazole derivatives. The true partition coefficient (P) involves only molecular species common to both phases and is obtained from the expression

$$P = \frac{C_1}{C_2\,(1-\alpha)}$$

where α is the degree of ionisation of the bases at pH 7·4 [α is calculated from the equation

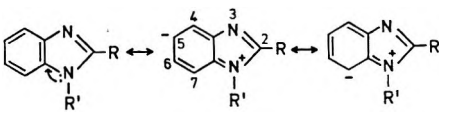
$$\text{Per cent ionised} = \frac{100}{1 + \text{antilog}\,(7{\cdot}4 - pK_a)}$$ (Albert, 1960)].

P values (for unionised base) have been calculated on the assumption that the pK_a values of the bases in water are 0·5 pH units higher than those in 50% ethanol-water*, and that the bases are completely unionised in cyclohexane.

* Although anomalies may arise from the use of mixed solvents in some cases (Albert & Serjeant, 1962), a number of workers (Mizutani, 1925; Hall & Sprinkle, 1932) have found that, in a series of related bases, the use of 50% aqueous ethanol consistently caused a depression of approximately 0·5 units in the pK_a compared to the value obtained in water. This was also found to be so with a series of benzimidazole derivatives studied by Davies, Mamalis, Petrow & Sturgeon (1951).

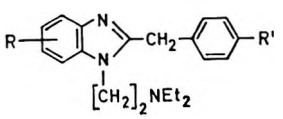
Results

2-Benzylbenzimidazole, the parent molecule of the derivatives studied here, is a weak base, its pK'_a value (5·01) being similar to that of benzimidazole itself [4·98 in 50% aqueous ethanol (Rabiger & Joullié, 1964); 5·53 in water (Schwarzenbach & Lutz, 1940)]. Corresponding 5(6)- and 4(7)-nitro-derivatives have pK'_a values approximately two units lower than that of the unsubstituted molecule (e.g. Table 1, Nos 2–5), the base-weakening influence of the nitro-group probably being a result of its stabilising resonance forms such as (II and III) with a consequent drift



II III

TABLE 1. IONISATION CONSTANTS[1] AND PARTITION COEFFICIENTS[2] OF SOME 2-BENZYLBENZIMIDAZOLE DERIVATIVES



I

No	R	R′	R″	X	pK′a		% ionised at pH 7·4[7]	Partition coefficient	
					Side-chain nitrogen	Heterocyclic nitrogen		Apparent (P′)	Corrected (P)
1[3]	H	H	—	—	—	5·01[4]	—	—	—
2[3]	5(6)-NO_2	H	—	—	—	2·94	—	—	—
3[3]	4(7)-NO_2	H	—	—	—	2·84	—	—	—
4	5-NO_2	H	H	OH	—	2·84	—	—	—
5	5-NO_2	H	Me	OH	—	3·10	—	—	—
6	H	H	H	NMe_2	6·49	4·12	29	4·3	6
7	H	H	Me	NMe_2	6·51	[5]	—	—	—
8	H	OEt	Me	NMe_2	6·80	4·05	44	11	20
9	H	OEt	Me	NEt_2	6·90	4·10	50	15	30
10	5-NO_2	H	H	NEt_2	6·34	2·83[4]	22	57	73
11	6-NO_2	H	H	NEt_2	6·35	3·71	22	89	114
12	5-NO_2	H	Me	NEt_2	6·70	3·07	—	—	—
13	5-NO_2	OEt	H	NEt_2	6·36	2·86[6]	22	76	97
14	5-NO_2	OEt	Me	NEt_2	6·67	3·03	40	78	130

[1] in 50% aqueous ethanol at 25°
[2] in an aqueous buffer (pH 7·4)—cyclohexane system
[3] N(1) side-chain absent
[4] Rabiger & Joullié (1964) give pKa 5·7 in 95% aqueous ethanol
[5] not measured
[6] pKa measured by adding excess of acid and back titrating (dihydrochloride could not be isolated)
[7] calculated from the expression

$$\% \text{ ionised} = \frac{100}{1 + \text{antilog}\,[7{\cdot}4 - \text{pKa}\,(H_2O)]}$$ (Albert, 1960);

it is assumed that pKa (H_2O) = pKa (50% aqueous-ethanol) + 0·5.

of electrons away from the basic centre. 1-Dialkylaminoethyl-2-benzylbenzimidazoles have two basic centres, that of the side-chain nitrogen being the stronger by a factor of almost 10^3 (e.g. Table 1, Nos 8 and 9). The heterocyclic basic centre (pK'_a near 4) in such derivatives is approximately ten times weaker than that of 2-benzylbenzimidazole, proton uptake at this centre being opposed by a positive charge on the side-chain nitrogen. [Nuclear magnetic resonance and ultraviolet spectral evidence for N(1) rather than N(3), being the site of protonation of the 1-substituted benzimidazole derivatives will be presented elsewhere.] In 1-(2-dialklyaminoethyl)-2-benzylnitrobenzimidazoles, the nitro-substituent lowers the pK'_a of the heterocyclic centre to values similar to those of the nitro-derivatives discussed previously, but has little influence upon the pK'_a of the side-chain nitrogen atom (Table 1, Nos 10, 12–14). When the nitro-group is in the 6-position (Table 1, No. 11) the pK'_a of the heterocyclic nitrogen atom is 0·7–0·9 units higher than that of corresponding 5-nitro-derivatives (Table 1, No 10, 12–14). This result is interpreted in terms of the 6-nitro-group being less effective than a 5-nitro-group in stabilising resonance contributors such as (II and III) because resonance interactions between a 6-nitro-group and a negative charge at C(5) or (7) are not possible; hence the electron withdrawing influence of the nitro-group may only operate by the less effective inductive mechanism. As a result, its base-weakening influence is less than that of a 5-nitro-group, the pK'_a of the heterocyclic nitrogen in the derivative (Table 1, No 11) being close to those of analogues lacking a nitro-group (Table 1, Nos 6, 8 and 9). A 2-methyl substituent in the aminoethyl side-chain appears to have a small base-strengthening effect upon both centres (cf. Table 1, Nos 10 and 12; 13 and 14), and it may be of significance, in this respect, that while the 2-methylethyl derivatives (Table 1, Nos 12 and 14) formed dihydrochlorides when treated with excess of ethanolic hydrogen chloride, the unbranched analogues (Table 1, Nos 10 and 13) formed monohydrochlorides under the same conditions.

In the partition work, the distributions of benzimidazole derivatives between an aqueous buffer at pH 7·4 (close to physiological pH) and cyclohexane (an organic solvent intended to simulate a lipid membrane) were determined. The apparent partition coefficients (P′) and those corrected for ionisation (P) are given in Table 1; the latter values are larger because all the derivatives are partially ionised at pH 7·4. The derivative (Table 1, No. 6), which lacks both nitro- and ethoxy groups, has the lowest partition coefficient of the series. An ethoxy group increases the affinity of the molecule for the organic phase (cf. Table 1, Nos 6 and 8), as does also an increase in size of the side-chain basic group (cf. Table 1, Nos 8 and 9). A 5-nitro-group enhances lipid solubility to a pronounced degree while the effects of nitro- and ethoxy groups appear to be additive in this respect (Table 1, Nos 13 and 14). The influence of a 6-nitro-group outweighs the combined effects of 5-nitro- and ethoxy, the derivative (Table 1, No. 11) having the highest partition coefficient of all the compounds examined. Morphine, in the same solvent system, had an apparent partition coefficient of 0·15 and a corrected one of 0·8 [assuming a pK'_a of 8·05 (Farmilo, Oestreicher & Levi, 1954) in water].

Discussion

The pK'_a values of the 2-benzylbenzimidazole derivatives of Table 1 (some of which are potent analgesics) in 50% aqueous ethanol, lie in the ranges 6·3–6·9 (side chain nitrogen) and 2·9–4·1 (heterocyclic nitrogen); corresponding values in water are assumed to be approximately 0·5 pK_a units higher (see footnote, p. 679). Farmilo & others (1954) have reported the pK'_a values of several different types of analgesics, most of which had values within the range 7·8–8·9 in water or aqueous ethanol (this range corresponding to the bases being approximately 90% ionised as cations at physiological pH). Hence, although exact comparisons cannot be made, the analgesically active derivatives of Table 1, and probably related active derivatives also, are somewhat less ionised than many other classes of analgesic. This is probably due to the fact that the side-chain nitrogen atom in benzimidazole derivatives is linked to the aromatic nucleus by a second nitrogen atom (of high electronegativity), whereas, in most other classes of analgesic the link is a quaternary carbon atom which has a smaller base-weakening influence. Nevertheless, in view of the high activities observed, the extent to which they are ionised at physiological pH (20–50% as monocations–ionisation as dications is negligible at pH 7·4) appears to be high enough to provide sufficient protonated molecules [considered the active species (Beckett & Casy, 1965)] in the vicinity of the receptor site. Hence the dibasic character and the lower (monocation) pK'_a values of benzimidazole analgesics, compared with those of other classes, is probably not significant in regard to the intrinsic activities of these compounds. However, since the penetration of lipid barriers involves non-ionised, rather than ionised, molecules (Brodie & others 1957, 1960), the reduced extents to which benzimidazole analgesics are ionised at body pH, may have an important influence upon the transport of such molecules to their site of action.

All the benzimidazole derivatives (Table 1) have apparent partition coefficients* in the aqueous buffer (pH 7·4)—cyclohexane system, greater than that of morphine (taken as a typical analgesic molecule since it is a monobase and contains one aromatic ring). Differences are particularly extreme between morphine and 2-benzylbenzimidazole derivatives containing nitro- and nitro-ethoxy substituents. The highly potent derivative (No 13, Table 1) for example, is more than 500 times more soluble than morphine in the organic phase.

Assuming the distribution of a compound in cyclohexane-water (pH 7·4) to reflect its partition between physiological lipid and non-lipid phases, these results suggest that the benzimidazole derivatives (I), especially those containing ethoxy and nitro-substituents, will be rapidly transported across lipid barriers to their site of action, once they have been administered. A drug which penetrates the central nervous system rapidly has a greater

* Apparent, rather than corrected, partition coefficients are considered of more value in assessing the influence of the water-lipid distribution properties of drug molecules upon activity because the former constant takes into account ionisation as well as partition factors.

chance of reaching its site of action than one of similar pharmacology whose rate of penetration is slow, because the former drug is less likely to be metabolised or excreted during the time interval elapsing between drug administration and response. Hence, *other factors being equal*, the more potent of two drugs will be the one which more readily penetrates lipid barriers. For these reasons it may be inferred that the extremely high potencies of 1-(2-diethylaminoethyl)-2-(*p*-ethoxybenzyl)-5-nitro benzimidazole and similar derivatives are related to their probable ease of penetration of the central nervous system.

However, transport considerations alone are inadequate in accounting for the influence of ethoxy and nitro-substituents upon activity in this series. The ethoxy derivative (Id), for example, is 70 times more active in mice than the methoxy analogue (Ie) although the two derivatives probably have similar lipid solubilities, this fact reflecting the well-known structural specificity of the analgesic receptor site. The receptor is also sensitive towards the position of substitution of the lipophilic nitro-group. While a 5-nitro-group allows (and may enhance) drug-receptor interaction, a 6-nitro-group (more effective than 5-nitro in increasing lipid solubilities—cf. Table 1, Nos 10 and 11) impedes the same association as is evident from the relative activities of the derivatives (If and g).

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A note on the use of the isolated stomach preparation of the Syrian golden hamster

E. MIKOŚ

The sensitivity of the isolated stomach strip of the Syrian golden hamster to some neurohormones has been assessed. Acetylcholine (5×10^{-9} g/ml), histamine (10^{-8} g/ml), bradykinin (10^{-9} g/ml) and angiotensin (10^{-9} g/ml) contracted the preparation. The action of bradykinin and angiotensin was probably direct although some results for angiotensin suggested a neurogenic component. The sensitivity of this preparation to angiotensin or bradykinin is only a little less than that of the ascending or descending colon of the rat.

THE Syrian golden hamster (*Cricetus, Mesocricetus auratus*) is widely used in bacteriological and virological work. I have explored the possibility of its use for pharmacodynamic purposes.

A preliminary investigation of the isolated stomach of the Syrian golden hamster showed it to be sensitive to some pharmacologically active drugs (Mikoś, 1966). This paper reports the sensitivity of the dose response curve of this preparation to some neurohormones.

Methods

Syrian golden hamsters of either sex were killed by a blow on the head. The stomach was dissected from the abdomen and the fundus was prepared (Vane, 1957). The fundus strip was suspended in a bath of 10 ml capacity filled with Tyrode or Krebs solution at a temperature of 35–36° and gassed with oxygen 95% and carbon dioxide 5%. The movements of the stomach strip were recorded on smoked paper using a frontal writing auxotonic lever (Paton, 1957) with 16:1 magnification and a load on the tissue of 1·5–2 g. The drugs were dissolved in nutrient fluid and added to the bath in volumes of 0·1–0·2 ml, for 60–90 sec; the bath was then washed out three times. A cycle of 3–5 min was used.

The stomach strips usually showed small spontaneous movements in Tyrode or Krebs solution. Constant responses to drugs were obtained about 1 hr after setting up the preparation.

Drugs

Drugs used were: acetylcholine bromide, adrenaline hydrochloride, hyoscine bromide, histamine acid phosphate, nicotine tartrate, morphine sulphate (all BDH), bradykinin, synthetic vasopressin (Sandoz), hexamethonium bromide, 5-hydroxytryptamine creatinine sulphate, noradrenaline tartrate (all M & B), angiotensin (Ciba), oxytocin (Richter; Parke Davis), phenasoline (Polfa).

Results

Acetylcholine, histamine, bradykinin or angiotensin contracted the stomach strips in the concentrations shown in Table 1. 5-Hydroxytryptamine (5-HT), 10^{-9}–10^{-4} g/ml, sometimes contracted the stomach strips, but the contraction was small and did not increase with the increase in concentration of drug. Sometimes the stomach was completely insensitive to 5-HT. Oxytocin sometimes caused contraction in a concentration

From the Department of Pharmacology, Medical Academy of Kraków, ul. Grzegórzecka, 16, Kraków, Poland.

TABLE 1. THE INFLUENCE OF DRUGS ON THE ISOLATED STOMACH STRIP OF THE SYRIAN GOLDEN HAMSTER

Drug	Conc. g/ml	Conc. causing contraction (c) or relaxation (r)	Antagonistic action to:
Acetylcholine bromide	10^{-9}–10^{-4}	5×10^{-9} (c)	—
Adrenaline hydrochloride	10^{-10}–10^{-4}	10^{-8} (r)	10^{-9}–10^{-8} to all contracting drugs
Hyoscine bromide	10^{-7}	—	10^{-7} to acetylcholine
Histamine acid phosphate	10^{-9}–10^{-4}	10^{-8} (c)	—
Bradykinin	10^{-9}–10^{-6}	10^{-9} (c)	—
Hexamethonium bromide	10^{-5}	—	—
5-Hydroxytryptamine creatinine sulphate	10^{-9}–10^{-4}	10^{-8}–10^{-4} (c) not always	—
Angiotensin-hypertensin	10^{-10}–10^{-7}	10^{-9} (c)	—
Noradrenaline bitartrate	10^{-10}–10^{-4}	10^{-8} (r)	10^{-7}–10^{-6} to all contracting drugs
Nicotine bitartrate	10^{-5}	—	—
Morphine sulphate	10^{-6}	—	—
Oxytocin	1 mU–1 U	0·1 U–1 U (c or r)	—
Phenasoline	10^{-7}–10^{-5}	—	2×10^{-5} –$5 \times 10^{-}$ to histamine
Vasopressin (synthetic)	1 mU–1 U/ml	0·02 U (c)	

of 0·1–1 units/ml, synthetic oxytocin (Parke Davis) never caused a contraction, though in a concentration of 1 unit/ml a small relaxation occurred probably due to chlorbutol in the injection. Vasopressin in a concentration of 0·02 unit/ml to 1 unit/ml did not always cause a contraction.

The contractions caused by acetylcholine or histamine were quick and monophasic, taking up to 1 min to reach a maximum and returning rapidly to the baseline. The contractions caused by bradykinin or angiotensin were slower, gradually increasing and taking up to 1 min to reach a maximum (Fig. 1*A*).

Dose response curves for acetylcholine, histamine, bradykinin, or angiotensin, were constructed (Fig. 1*B*).

Histamine and acetylcholine contractions were antagonised by atropine, hyoscine or phenasoline. Adrenaline and noradrenaline antagonised contractions to all the drugs used (Table 1).

The mode of action of angiotensin and bradykinin was investigated in the presence of some blocking agents.

Hyoscine in concentrations of 10^{-7} g/ml and morphine, 10^{-6} g/ml, did not block or diminish the response to bradykinin or angiotensin. Hexamethonium or nicotine, 10^{-5} g/ml, did not reduce the effect of bradykinin, but in 50% of the experiments diminished the response of angiotensin.

Discussion

The site of action of bradykinin and angiotensin in different preparations is not clear. Wiegershaussen, Stopp & Eichstädt (1964) working on the

guinea-pig isolated ileum found that the contraction of bradykinin could be diminished by atropine and morphine and increased by eserine. They suggested that bradykinin, besides having a direct action on muscle, also had an indirect action involving release of acetylcholine.

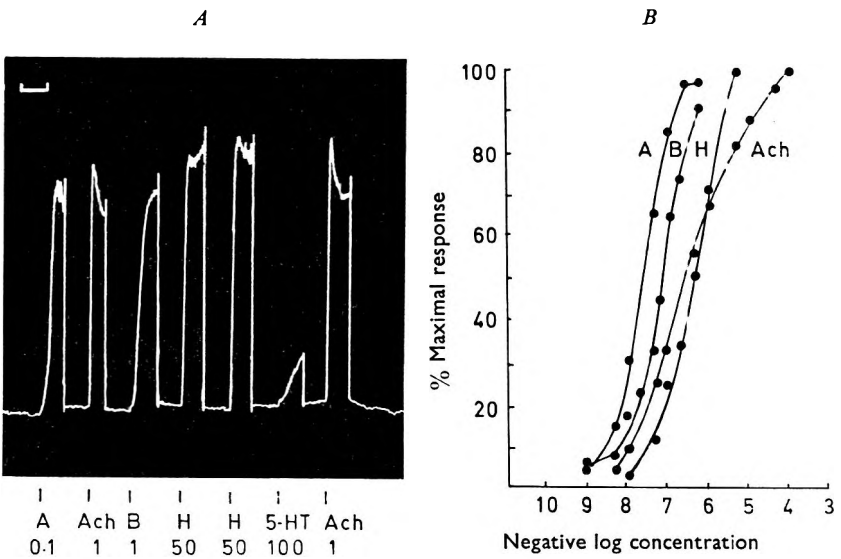

FIG. 1. Isolated stomach strip of Syrian golden hamster, *A* showing contractions and *B* dose response curves caused by angiotensin (A), acetylcholine (Ach), bradykinin (B), histamine (H) and 5-hydroxytryptamine (5-HT). Drug concentrations in μg to 10 ml bath. Drug contact time 1 min. The drugs were injected every 3 min. Time scale = 5 min.

On the hamster stomach the effect of bradykinin was not changed by hyoscine, morphine, hexamethonium or nicotine. Thus bradykinin probably acts directly, as observed by Khairallach & Page (1951) on the guinea-pig ileum. In the present investigation the response to angiotensin was slightly diminished by hexamethonium and nicotine in half of the experiments, but hyoscine or morphine were without effect. Thus angiotensin probably has a direct action on this tissue. The diminution of the response by hexamethonium or nicotine in some experiments suggests a small neurogenic component.

The sensitivity of this preparation to angiotensin or bradykinin is only a little less than that of the ascending or descending colon of the rat.

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Lack of effect of yeast RNA upon two types of conditioning

SIR,—We have already shown (Boissier, Simon, Tillement & Privat de Garilhe, 1965) that a ribonucleic acid (RNA) with well known physical and chemical properties* did not increase the acquisition or retention of a Mowrer-Miller type of conditioned reflex in rats. Cook, Davidson, Davis, Green & Fellows (1963) were able to show an increase in the rate of acquisition and a decrease in the rate of extinction of a pole climbing test by rats after chronic administration of RNA.

We have repeated our first experiment with a RNA of a different origin†. Three groups of 12 male rats weighing 110–130 g were used. The first group received 160 mg/kg (10 ml/kg) i.p. RNA 15 min before each of the daily trials. The second group were given the same treatment for 53 days before the experiment, and before each trial. The third, a control group, received saline in a similar manner. There were no significant differences between the performance of treated and control rats either after acute or chronic pretreatment.

The effect of this RNA was also studied on the rate of learning a maze which had red painted blind alleys and a correct path coloured green. This procedure enabled us to omit pretraining. The rats were deprived of water but not food, and drank only once each day in the goal box of the maze.

A group of 15 rats was given 50 mg/kg i.p. (10 ml/kg) RNA, 15 min before each trial but showed no increased rate of learning or running speed when compared to 15 controls injected with saline.

These results confirm our earlier observations and the more recent findings of Cohen & Barondes (1966).

Department of Neuropsychopharmacology,
Institute of Pharmacology,
Faculty of Medicine,
75-Paris 6°, France
June 15, 1966

J. R. BOISSIER
P. SIMON
J. M. LWOFF

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* Yeast RNA No. 6015-045 provided by "Industrie Biologique Française", Genne villiers, France.

† Yeast RNA No. 2217 provided by Schwartz Fould Springer, Maisons-Alfort, France.

Methixene: a non-competitive antagonist of bradykinin

SIR,—Garcia Leme & Rocha e Silva (1965) recently reported methixene [9-(1-methylpiperid-3-ylmethyl)thiaxanthen] to be a competitive antagonist of bradykinin in contrast to most of the similar tricyclic compounds they tested.

We have also studied the antagonistic effect of methixene, using the cumulative dose-response procedure according to Rossum (1963), Rossum & Brink (1963) and Ariëns Simonis (1964). Guinea-pig ileum was bathed in a 10 ml bath with a Tyrode solution containing 10^{-7} g/ml atropine sulphate and saturated with a mixture of oxygen 95% and carbon dioxide 5% at 37°.

Two cumulative dose response curves with bradykinin were made and if these curves differed less than 10% the actual experiment was begun. The guinea-pig ileum was incubated with methixene added to the medium to a final concentration of 5×10^{-8}, 10^{-7}, 5×10^{-7}, 10^{-6} g/ml for 30 min, after which period cumulative doses response curves with bradykinin in the presence of the inhibitor were made. The guinea-pig ileum was then washed several times with a fresh Tyrode solution during 30 min and a cumulative dose response curve with bradykinin was then made again. The same preparation was always used for three successive experiments with methixene, the sequence of methixene doses was chosen at random. Only when the last cumulative dose response curve with bradykinin without methixene was within 15% of the first two, was the experiment accepted.

The bradykinin curves were readily reproducible. The lowest dose of bradykinin which induced a concentraction of the guinea-pig ileum in these conditions had a final concentration in the bathing medium of 0·05 μg/ml. The maximum

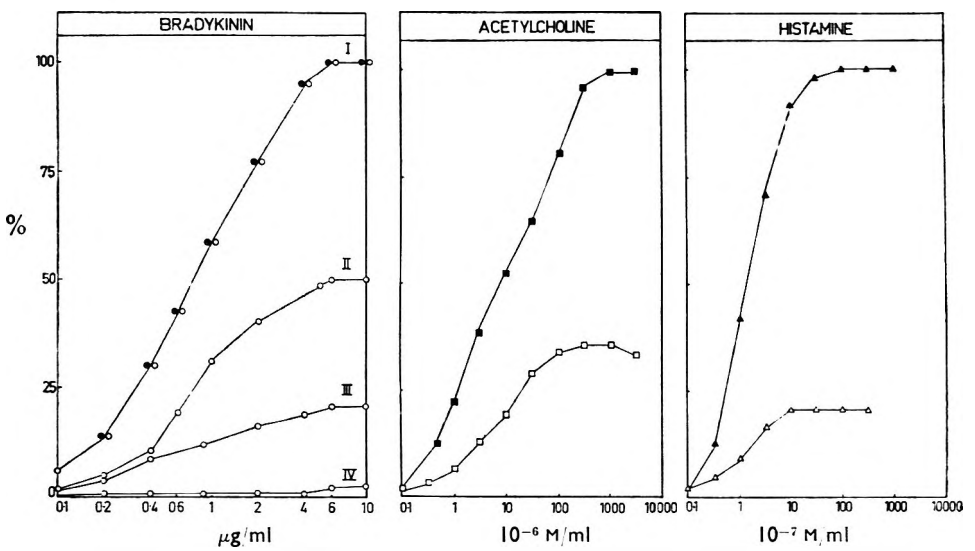

FIG. 1. Cumulative dose response curves of contractions of guinea-pig ileum in Tyrode solution saturated with oxygen 95% and carbon dioxide 5% at 37° (plus 10^{-7} g/ml atropine sulphate in the bradykinin experiments). All values means of 4 curves except where stated. ● Bradykinin controls (mean of 29 curves). ○ Bradykinin in the presence of I, 5×10^{-8}; II, 10^{-7}; III, 5×10^{-7}; IV, 10^{-6} (mean of 2 curves) g/ml of methixene. ■ Acetylcholine controls. □ Acetylcholine and 5×10^{-7} g/ml methixene. ▲ Histamine controls. △ Histamine and 5×10^{-7} g/ml methixene.

contraction, after which a tenfold increase of concentration did not induce a further increase in the contraction of the ileum, was found regularly at 10 μg/ml.

In the presence of 5×10^{-8} g/ml methixene no antagonistic effect was seen (Fig. 1). However, 10^{-7} g/ml methixene decreased the effect of bradykinin, but not the affinity. This was more pronounced with the 5×10^{-7} g/ml concentration of methixene. In the final concentration of 10^{-6} g/ml methixene the inhibition was almost completed (Fig. 1). These results suggest that methixene is a noncompetitive antagonist of bradykinin. This is not in agreement with the findings of Garcia Leme & Rocha e Silva who used a three dose technique on the guinea-pig ileum.

Additional experiments supported our findings of the non-specificity of the methixene blocking effect of bradykinin. In the urethane anaesthetised guinea-pig the effect of bradykinin on the bronchioles was examined with a modification of the method of Konzett & Rössler (1940). Methixene did not antagonise the bronchoconstrictor effect elicited by bradykinin in any of the doses given (0·01–10 mg/100 g i.v.).

TABLE 1. EFFECTS OF METHIXENE ON THE VASODILATOR RESPONSE OF RATS IN URETHANE ANAESTHESIA (1·1 ml 12·5% i.p. 100 g) TO BRADYKININ INJECTED INTO THE TAIL VEIN. Pressure recorded from the carotid artery. Measurements to the nearest 5 mm Hg accurate with Statham P 23 transducers and Grass polygraph inkwriting recorder. Number of observations in brackets. Blood pressure given in mm Hg.

		Methixene (μg)			
Bradykinin μg	control	45	150	450	1500
0·25	5 (8)	10 (9)	15 (5)	25 (10)	40 (4)
0·5	10 (3)	—	—	25 (3)	40 (2)

The blood pressure decrease caused by the vasodilator effect of bradykinin recorded in the urethane anaesthetised rat was even potentiated by methixene in a dose dependent manner (Table 1). In addition, methixene was equally active as a non-competitive antagonist of the contractions of the guinea-pig ileum caused by cumulative doses of histamine and acetylcholine (Fig. 1) and also of the contractions caused by 10^{-5} M/ml 5-hydroxytryptamine and 1 U/ml substance P, which were 80% inhibited by 5×10^{-7} g/ml methixene. The contractions caused by 10^{-3} M/ml barium chloride on the guinea-pig ileum were also inhibited by the same concentration of methixene.

In conclusion methixene appears to be a non-competitive inhibitor of bradykinin and of several other agonists on the guinea-pig ileum.

Acknowledgements. The bradykinin was kindly supplied by Sandoz A. G. Bazel, the methixene by Wander A.G. Bern. For technical assistance we thank Miss E. Bettink.

H. VAN RIEZEN

University of Utrecht,
Department of Pharmacology,
Vondellaan 6, Utrecht,
The Netherlands

July 12, 1966

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Effect of solanaceous alkaloids on the 5-hydroxytryptamine content of rat brain

SIR,—The central stimulatory actions of atropine in high doses causing excitement, tremors and hallucinations are well known. These actions in relation to brain acetylcholine (Giarman & Pepeu, 1964) and cholinesterase (Lullmann, Forster & Westerman, 1952) have been investigated. Some similarities in the actions of atropine and lysergic acid diethylamide (LSD) like facial flush, hyperthermia, rise in blood pressure and mydriasis are known (Pfeiffer, 1959). Further, LSD has been shown to cause an increase in the 5-HT content of rat brain (Freedman, 1961; Freedman & Giarman, 1962). We now report the effects of atropine, hyoscine and the total alkaloids of *Datura alba* on the 5-HT content of rat brain.

Adult albino rats, 100–150 g, were injected intraperitoneally with the drugs (25 mg/kg) in 0·5 ml of 0·9% saline. Control animals had normal saline only. The rats were killed ½ hr after the administration of drugs and the brain (excluding the olfactory lobe, cerebellum and pituitary glands) removed quickly. The 5-HT was extracted by the method of Amin, Crawford & Gaddum (1954), and assayed on the oestrous uterus of rat (Parratt & West, 1957). The alkaloids of *Datura alba* were extracted by the B.P. (1963) method.

TABLE 1. EFFECT OF ATROPINE, HYOSCINE AND TOTAL ALKALOIDS OF *Datura alba* ON THE 5-HT CONTENT OF RAT BRAIN

Drugs 25 mg/kg	Time interval in hr	No. of rats	Brain 5-HT content in µg/g ± s.e.
Control	½	10	0·40 ± 0·04
Atropine	½	5	0·59 ± 0·03
Hyoscine	½	5	0·71 ± 0·05
Total alkaloid of *Datura alba* ..	½	6	0·76 ± 0·007

Atropine, hyoscine and the total alkaloid of *Datura alba* cause an increase in the 5-HT content of rat brain (Table 1). This rise in the 5-HT content also resembles the action of LSD referred earlier (Freedman, 1961) and may be related to the central effects like tremor, excitement and ataxia (Udenfriend, Weissbach & Bogdanski, 1957), commonly seen in atropine and datura poisoning.

Department of Pharmacology,
Mahatma Gandhi Memorial Medical College,
Indore, M.P.
India.

B. C. BOSE
M. A. MATIN
R. VIJAYVARGIYA
M. LAHIRY

June 22, 1966

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The stimulation of β-adrenergic receptors by macusine B

SIR,—Macusine B, an alkaloid isolated by Battersby, Binks, Hodson & Yeowell (1960) from *Strychnos toxifera*, inhibits both α-adrenergic and tryptamine receptors in a number of isolated tissues (Leonard, 1965a). There is also evidence from its action both *in vivo* and *in vitro* that it stimulates β-adrenergic receptors (Leonard, 1965b). An assessment has now been made of its stimulant action on β-adrenergic receptors.

Isolated rabbit auricles were suspended in a 50 ml bath of Ringer Locke solution, at 30° (Burn, 1952). Macusine B increased both the heart rate and the amplitude of contraction of the auricles (Fig. 1), but only in high doses (200 μg/ml bath fluid) compared with an approximately equipotent dose of isoprenaline (0·02 μg/ml). The effects of both macusine B and of isoprenaline were completely blocked by pronethalol. The weak β-stimulating effect of macusine B is further suggested by its effect on the heart rate of mice.

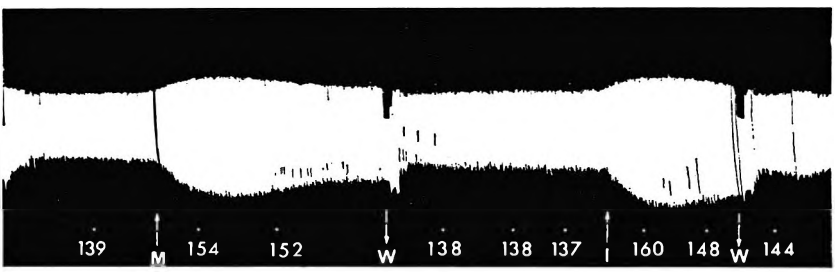

FIG. 1. Effect of macusine B on the rate and amplitude of contraction of the rabbit isolated auricles. Auricles suspended in oxygenated Ringer Locke solution at 30°. At ↑ M, 200 μg/ml macusine B and at ↑ I, 0·02 μg/ml isoprenaline sulphate were added to the bath. The bath was washed out at ↓ W. The figures indicate the rate/min.

Ten albino mice (20–30 g) were lightly anaesthetised with anaesthetic ether and the resting heart rate recorded with an electrocardiogram. The animals were then allowed to recover, and 1 hr later were injected with macusine B (10 mg/kg i.p.). Thirty min after injection the mice were again lightly anaesthetised and the heart rate counted. Macusine B, in a dose that is approximately 0·25 LD50 dose caused a 14% increase in the heart rate but this was not significantly different from the control value ($P > 0·1$).

It is evident from these results that macusine B has only weak β-adrenergic stimulant activity; its main action appears to arise from its α-adrenergic and tryptamine blocking activity (Leonard, 1965a).

Acknowledgement. I wish to thank Professor A. R. Battersby for the sample of macusine B.

Pharmacy Department,
The University,
Nottingham.

B. E. LEONARD

July 14, 1966

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Effects of hexobarbitone, ether, morphine, and urethane upon the acute toxicity of propranolol and D-(−)-INPEA

SIR,—Recently, we reported that propranolol increased the acute toxicity of hexobarbitone (Murmann, Almirante & Saccani-Guelfi, 1966). The interaction of these drugs was attributed to the central nervous system depressant properties of propranolol since D(−)-2-isopropylamino-1-(*p*-nitrophenyl)ethanol(INPEA) another adrenergic β-receptor antagonist (Almirante & Murmann, 1966; Somani, 1966), did not interact with hexobarbitone, and D(−)-INPEA is known to be free from CNS depressant actions (Murmann, Almirante & Saccani-Guelfi, 1956). We have now extended these observations to include the interactions of propranolol and D-(−)-INPEA with ether, morphine and urethane.

To measure the median lethal doses of the various drugs, groups of twenty adult, male mice, NMRI strain, in a quiet room at 25 ± 0·2°, were given propranolol and D-(−)-INPEA subcutaneously and hexobarbitone (0·4% v/w) in 0·1N NaOH, ether (4% v/v), morphine (1·15% w/v) or urethane (7% w/v) in saline.

TABLE 1. EFFECT OF HEXOBARBITONE, ETHER, MORPHINE AND URETHANE ON THE TOXICITY OF TWO DIFFERENT β-ADRENERGIC BLOCKING AGENTS

Interval between β-blocker and test compound (min)	LD 50* mg/kg s.c.	Slope function	Time of death (range)	LD 50* mg/kg s.c.	Slope function	Time of death (range)
	Hexobarbitone: 40 mg/kg 0·6 LD 50 (intravenously) LD 50 = 65·6 (53·6–80·4) S = 1·39 (1·12–1·72)			Ether: 0·4 ml/kg 0·6 LD 50 (intravenously) LD 50 = 0·70 (0·63–0·78) S = 1·13 (0·87–1·46)		
Propranolol (control)	187·0 (164·5–212·6)	1·16 (1·03–1·31)	>1 <72 hr	240·0 (192·8–298·8)	1·42 (1·17–1·73)	>0·5 <2 hr
Simultan.	216·0 (186·1–250·8)	1·41 (1·06–1·89)	>1 <48 hr	279·0 (202·9–383·6)	1·86 (1·23–2·83)	>3 min <48 hr
15	35·2 (24·4–50·9)	3·33 (1·44–7·64)	>0·5 <7 min	11·4 (5·5–23·6)	5·20 (2·17–12·48)	>3 <7 min
30	41·8 (29·9–58·5)	1·92 (1·28–2·87)	>0·5 <6 min	25·2 (14·4–44·1)	2·97 (1·36–6·48)	>2 <7 min
60	38·5 (27·4–54·1)	1·75 (1·21–2·54)	>0·5 <5 min	47·0 (28·0–90·0)	2·28 (1·13–4·60)	>2 <12 min
120	36·0 (26·6–48·8)	1·64 (1·23–2·20)	>1 <6 min	52·0 (33·6–80·6)	1·68 (1·02–2·77)	>3 <6 min
180	50·8 (38·3–67·3)	1·38 (1·16–1·64)	>3 <11 min	78·0 (50·7–120·1)	2·37 (0·97–5·78)	>3 <7 min
300	73·5 (58·8–91·9)	1·29 (1·10–1·52)	>2 <11 min	>150		
480	122·5 (106·5–140·9)	1·18 (1·03–1·35)	>2 <16 min			
D(−)-INPEA (control)	322·0 (272·9–378·0)	1·39 (1·25–1·54)	>5 min <3 hr	290·0 (267·3–314·7)	1·24 (1·16–1·32)	>20 <60 min
Simultaneously	359·0 (320·5–402·1)	1·20 (1·10–1·31)	>5 min <16 hr	268·0 (245·9–292·1)	1·15 (1·08–1·23)	>2 <47 min
15	240·0 (210·7–273·4)	1·29 (1·06–1·58)	>3 <27 min	>200		
30	209·0 (166·5–262·3)	1·31 (1·05–1·63)	>1 <8 min	>200		
60	>300			>150		
120	>300					
	Morphine: 115 mg/kg 0·6 LD 50 (intravenously) LD 50 = 184·0 (160·7–210·7) S = 1·16 (1·02–1·33)			Urethane: 700 mg/kg 0·6 LD 50 (intravenously) LD 50 = 1130·0 (918·7–1389·9) S = 1·25 (1·03–1·52)		
Propranolol (control)	217·0 (192·9–244·1)	1·32 (1·21–1·43)	>0·5 <24 hr	217·0 (192·9–244·1)	1·32 (1·21–1·43)	>0·5 <24 hr
Simultaneously	107·0 (66·9–171·2)	2·85 (1·30–6·27)	>2 min <18 hr	116·0 (121·2–227·4)	1·44 (1·09–1·90)	>2 <80 min
15	31·5 (17·4–57·0)	3·83 (1·42–10·33)	>2 <8 min	0·228 (0·125–0·417)	3·27 (2·02–5·30)	>1 <6 min
30	41·0 (21·6–77·9)	3·66 (1·38–9·70)	>3 <10 min	1·000 (0·658–1·520)	2·28 (1·19–4·38)	>2 <7 min
60	55·8 (39·6–78·7)	1·98 (1·04–3·79)	>2 <8 min	0·740 (0·339–1·613)	6·64 (1·71–26·63	>1 <5 min
120	63·0 (38·4–103·3)	3·02 (1·26–7·24)	>2 <11 min	10·00 (6·64–15·05)	2·26 (1·51–3·39)	>1 <5 min
180	85·0 (49·4–168·3)	2·95 (1·33–6·54)	>1 <9 min	11·70 (5·04–27·14)	3·93 (1·40–11·00)	>2 <7 min
300	114·0 (81·4–159·6)	1·73 (1·19–2·53)	>2 <99 min	39·8 (26·53–59·7)	1·97 (1·47–2·65)	>1 <6 min
480				68·5 (51·7–90·8)	1·38 (1·11–1·62)	>3 <6 min
D(−)-INPEA (control)	322·0 (272·9–378·0)	1·39 (1·25–1·54)	>5 min <3 hr	322·0 (272·9–378·0)	1·39 (1·25–1·54)	>5 min <3 hr
Simultaneously	76·5 (51·0–114·8)	2·22 (1·11–4·44)	>2 min <18 hr	413·0 (384·2–444·0)	1·12 (1·02–1·24)	>25 <70 min
15	65·8 (47·2–91·8)	1·46 (1·15–1·86)	>1 min <24 hr	>300		
30	73·5 (51·7–104·7)	1·78 (1·11–2·85)	>2 <7 min	>300		
60	>150			>300		
120	>300					
180	>300					

* Results calculated from experimental data by the method of Litchfield & Wilcoxon (1949).

were given intravenously at the rate of 0·01 ml/sec. These agents were given in equivalent sub-lethal doses (0·6 LD50) either simultaneously or 15, 30, 60, 120, 180, 300, or 480 min after graded doses of propranolol or D(−)-INPEA; the LD50 estimates were calculated (Litchfield & Wilcoxon, 1949).

The three anaesthetics and morphine increased the toxicity of propranolol (Table 1). The enhanced toxicity of these drug combinations appeared to be associated with the interaction of CNS depressant actions. There was a significant increase in the sleeping times of the surviving animals when the anaesthetics and morphine were given after propranolol. By contrast, D(−)-INPEA is a CNS stimulant and greatly reduced the sleeping times they produced. While the anaesthetics did not enhance the toxicity of D-(−)-INPEA there was a clear interaction with morphine. This appeared to be due to the summation of their central excitatory actions. The central excitatory action of D-(−)-INPEA alone often led to convulsions and death when the higher dosed animals were handled for the administration of the test substances but when it was combined with the central excitatory action of morphine death by convulsions occurred at much lower doses of D(−)-INPEA. The interaction with morphine was thus quite different from the interactions of propranolol and the various test substances.

The electrocardiograms of mice were recorded to investigate the sudden deaths which occurred when the test substances were given after propranolol. The average heart rate of 25 normal, untreated, mice was 741 ± 12 beats per min. Doses of propranolol between 3–100 mg/kg subcutaneously reduced the heart rate of mice to between 350 and 450 beats per min. However, when the test substances were given 15 min after propranolol, an extreme bradycardia, ending in cardiac arrest, developed within a few minutes in each case.

Atropine was used to try to find the extent of vagal involvement in this bradycardia. Groups of 50 mice were treated with propranolol 2 or 3 mg/kg s.c. + urethane 700 mg/kg i.v. given 15 min after propranolol with or without atropine 2 mg/kg i.v. given at the same time as the propranolol. The atropine reduced but did not completely prevent the acute mortality produced by the urethane-propranolol combination (from 50 to 20% or from 86 to 40%). Since this dose of atropine would be expected to produce complete vagal blockade in the mouse, it would appear that either there is a substantial non-vagal component in the bradycardia or alternatively primary cardiac arrest is not the only cause of death. Propranolol is known to have both local anaesthetic actions and quinidine-like actions on the heart and so the effects of urethane on quinidine and xylocaine toxicity were investigated. As shown in Table 2, urethane

TABLE 2. EFFECT OF URETHANE ON THE TOXICITY OF QUINIDINE AND XYLOCAINE

	Urethane: 700 mg/kg 0.6 LD50 (intravenously) LD50 = 1130.0 (918.7–1389.9) S = 1.25 (1.03–1.52)			
Interval between administration of drugs (min)	LD 50* mg/kg s.c.	Slope function	Time of death (range)	
Quinidine (control) ..	465·0 (414·8–521·3)	1·20 (1·09–1·33)	>1	<16 hr
Simultaneously	389·0 (329·1–459·8)	1·21 (1·03–1·43)	>2	<24 hr
15	260·0 (208·0–325·0)	1·66 (1·33–2·07)	>2 min	<15 hr
30	235·0 (171·5–322·0)	1·58 (1·29–1·93)	>2 min	<9 min
Xylocaine (control) ..	264·0 (216·4–322·1)	1·25 (1·08–1·44)	>10	<90 min
Simultaneously	279·0 (216·3–359·9)	1·34 (1·02–1·76)	>5	<97 min
15	209·0 (185·8–235·1)	1·17 (1·04–1·32)	>2	<9 min
30	227·0 (174·6–295·1)	1·38 (1·02–1·86)	>2	<4 min

* Results calculated from experimental data by the method of Litchfield & Wilcoxon (1949).

produced a significant increase in quinidine toxicity but there was a barely significant interaction with xylocaine.

In conclusion, a marked synergism has been shown to occur between propranolol and various anaesthetics and morphine. This synergism was not seen with D(−)-INPEA. Unlike propranolol, D(−)-INPEA does not have depressant actions on the central nervous system nor quinidine-like actions on the heart—effects of propranolol which may be involved in its synergism with the anaesthetics and morphine.

Research Department,
Selvi e C.,
Laboratorio Bioterapico Milanese,
Via Gallarate, 184,
Milan, Italy.
July 29, 1966

W. MURMANN
L. ALMIRANTE
M. SACCANI-GUELFI

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The effects of morphine, pethidine and nalorphine on the isolated frog skin preparation

SIR,—Although morphine has a long history of clinical use, the mechanism by which it exerts its important and complex effects upon the central nervous system is still obscure. As an alternative to direct studies on the neuraxis, experimentally simpler systems shown to be affected by morphine have been examined, e.g. guinea-pig ileum (Paton, 1957; Cox & Weinstock, 1966) and superior cervical ganglion (Kosterlitz & Wallis, 1966). The present work arose from the chance observation that morphine produced effects upon the frog skin preparation. The actions of some nitrogenous bases on the transport of sodium ions across this membrane have been studied by Kirschner (1953) and Skou (1961). In the following experiments, morphine, pethidine and nalorphine were applied to the isolated skin.

A circle of washed abdominal skin of *Rana temporaria* separated frog Ringer solution (pH 7·65) contained in two adjacent 15 ml cells at room temperature. The preparation was left for 2 hr to equilibrate and then the short-circuit current (SCC) which had to be applied to reduce the skin potential to zero was measured. The current was maintained continuously for the rest of the experimental period, adjustments and readings being made at 5 min intervals. Drugs were applied to either surface of the membrane and any changes in the SCC noted.

Fig. 1 shows typical results following application to the inside of the skin. The three drugs produced significant falls in SCC, approximately equipotent doses being morphine sulphate 10 mg, pethidine hydrochloride 1 mg and nalorphine hydrobromide 5 mg (corresponding to the following final concentrations in the bathing solution in terms of the free bases: morphine 1·75 mM, pethidine 0·23 mM and nalorphine 0·85 mM). Although different preparations varied in sensitivity, the initial value of the SCC did not appear to be critical provided that it was greater than 80 μA/4 cm^2. When the same drugs were applied to the outer surface of the frog skin, larger doses (4–10 times) were required to produce significant falls in the SCC. It will be seen from Fig. 1

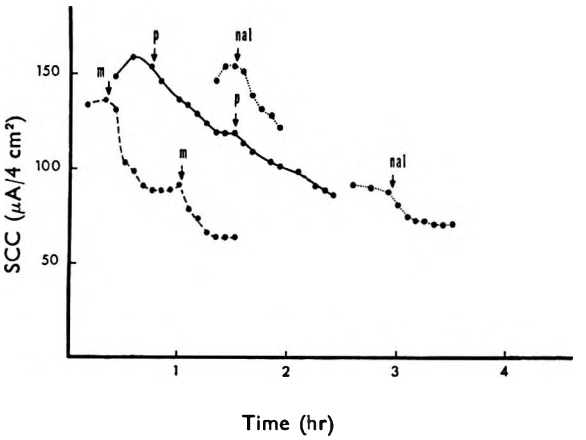

FIG. 1. The effects on the short-circuit current (SCC) across the isolated frog skin of two separate doses of morphine (m, 10 mg, dashed line), pethidine (p, 1 mg, unbroken line) or nalorphine (nal, 5 mg, dotted lines) applied to the inner surface.

that a second dose of morphine or pethidine given 40–45 min after the original dose of the same drug produced a similar quantitative effect. The responses to 10 mg morphine or 1 mg pethidine were not markedly modified by a single dose of 5 mg nalorphine given either simultaneously or in the preceding or the following 30 min. The concentrations of morphine necessary to produce the above effects are much larger than those required to depress transmission at post-ganglionic neuro-effector junctions (Cox & Weinstock, 1966) or at autonomic ganglia (Kosterlitz & Wallis, 1966) but of a similar order to those found to be effective for the actions of amines on the isolated frog skin preparation (Skou, 1961).

Skou (1961) found that the action of amines applied to the outside of the frog skin was pH dependent, i.e. it varied with the degree of ionic dissociation of the drug. As morphine and pethidine are weak bases with pK_a values above 7·65 (Beckett, 1956), in frog Ringer solution they are mainly present in the ionised form. The pK_a value and the % ionisation at pH 7·65 and 9·05 are respectively for pethidine, 8·72, 92, 32, and for morphine, 8·05, 72, 9. Thus to observe what happened when excess of the unionised base was present at the inner surface of the membrane, the experiments were repeated in frog Ringer solution buffered to pH 9·05 by the addition of 50 mM Tris[2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride] and 5 mM HCl. To avoid calcium precipitation at such an alkaline pH, the concentration of this ion was reduced to one-tenth of the usual: a control experiment showed that this alone did not affect the drug responses. At pH 9·05 the responses to 10 mg morphine and to 1 mg pethidine were almost identical with those obtained at pH 7·65. There is no evidence, therefore, that the ionised and unionised forms of morphine or pethidine have significantly different effects when applied to the inside of the frog skin preparation.

On the accepted model of the frog skin (Koefoed-Johnsen & Ussing, 1958), vasopressin is considered specifically to increase the permeability of the membrane selectively permeable to sodium at the outer surface of the basal epithelial cell ("sodium permselective membrane") and ouabain specifically to depress the

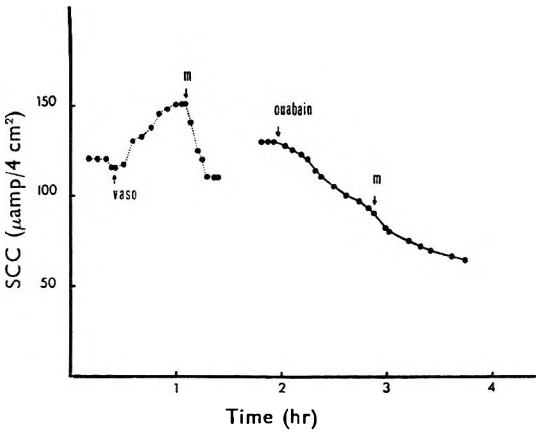

FIG. 2. The effects of morphine (m, 10 mg) on the short-circuit current (scc across the isolated frog skin following either vasopressin (vaso, 10 units, dotted line or ouabain (1 μg, unbroken line). All drugs applied to the inner surface of the membrane.

Na^+ K^+ Mg^{++}-dependent ATPase ("sodium pump") located in the inward-facing surface of the same cell. Therefore, in an attempt to define more precisely the action of morphine on this preparation, the drug was applied after either vasopressin (10 units) or ouabain (1 μg). Typical results are shown in Fig. 2, from which it will be seen that whereas after vasopressin the morphine effect was *practically unchanged*, after ouabain the response was insignificant. These findings suggest that morphine may be acting primarily on the sodium pump mechanism. However, the effect of morphine on the frog skin scc may well be a non-specific one in view of the large concentrations of drug necessary to produce it in comparison with the minimal effective doses at postganglionic neuro-effector junctions (Cox & Weinstock, 1966; Kosterlitz & Wallis, 1966).

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Department of Pharmacology,
Downing Street,
Cambridge.
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H. F. GRUNDY

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Contents

Letters to the Editor

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