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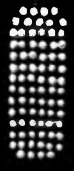

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On the mechanism of potentiation of the activity of acetylcholinesterase by some quaternary ammonium compounds

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Tetraethylammonium iodide (TEA) increases the maximum velocity of acetylcholine hydrolysis nearly 2-fold whereas it shows purely competitive kinetics when the dipropyl-analogue of acetylcholine (2-acetoxyethyldi-n-propylmethylammonium iodide) is the substrate. Unlike acetylcholine, the dipropyl-analogue does not have deacetylation as its rate determining step and this is further evidence that quaternary ammonium compounds potentiate acetylcholinesterase by accelerating the deacetylation step. The effects of 22 quaternary ammonium compounds on the rate of hydrolysis of acetylcholine, phenyl acetate and dimethylcarbamylacetylcholinesterase have been examined. The acceleration of deacylation by quaternary ammonium compounds was found to have a high degree of structural specificity. Possible mechanisms for this phenomenon are discussed in the light of the structure-action results.

Previously, Roufogalis & Thomas (1968 a, b, c) showed that some quaternary ammonium compounds and inorganic ions potentiate the hydrolytic activity of acetylcholinesterase under certain conditions. They suggested that the potentiation was by accelerating the rate of deacetylation in the hydrolytic sequence. We now provide further evidence for this mechanism and suggest a possible explanation for the acceleration of deactylation.

Belleau (1967) examined the free energy of binding of an homologous series of quaternary ammonium compounds onto acetylcholinesterase. He retained as constant the trimethylammonium head and investigated the effect of increasing chain length on the thermodynamics of binding. We have systematically altered the structure of the quaternary ammonium head, and investigated the effects of these changes on both potentiation of acetylcholinesterase and on the free energy of binding. Spiran quaternary ammonium compounds have relatively rigid structures and have proved to be useful for this purpose, since very small structural variations around the quaternary nitrogen produced more or less absolute effects on the consequences of binding of these compounds to the enzyme. The consequences of binding were determined by measuring the effect of the compounds on the deacylation step in the hydrolysis sequence.

EXPERIMENTAL

Chemistry

The synthesis of compounds I to XI (Table 2) has been previously described (Roufogalis & Thomas, 1968a). Two main methods were used for the synthesis of compounds XII to XII (Table 2).

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Method A. An α,ω-dihaloalkane (1 mol equiv) was reacted with amine (2 mo equiv) in a suitable solvent. The total concentration of reactants was kept below 5% (w/v) of the total volume of reaction mixture to promote an intramolecular cyclization, giving the required spiran quaternary ammonium halide or *NN*-dialkylcycloalkylammonium halide and amine hydrohalide. The reaction was carried out by one of two methods: (*a*) reactants were refluxed in chloroform for a suitable time (6–24 h); or (*b*) a solution of reactants in methanol was autoclaved for 50 min at 125°. The reaction solution from either *a* or *b* was distilled to dryness under reduced pressure on a water bath, the solid residue dissolved in water, sodium hydroxide (1 mol equiv) added, and the solution distilled to dryness under reduced pressure on a water bath. The resulting solid was extracted with chloroform in a Soxhlet extractor, and then purified by repeated crystallization from a suitable solvent (mixture).

Method B. The secondary amine (1 mol equiv) was refluxed with an alkyl halide (2 mol equiv) in a suitable solvent. The solution was evaporated to dryness on a water bath, the solid residue dissolved in water, sodium hydroxide (1 mol equiv) added and the resulting free amine extracted with ether, either with three separate portions or continuously. The ether was dried over anhydrous sodium sulphate and distilled on a water bath. The remaining liquid was distilled under reduced pressure in an oil bath. The resulting tertiary amine was added to a small volume of dried ethyl methyl ketone (25–100 ml) and the alkyl halide (2 mol equiv) added. The solution was either allowed to stand for 2–7 days, or refluxed for 6 h. If the solid separated out on cooling it was filtered, otherwise the ethyl methyl ketone was evaporated to dryness on a water bath. The solid was purified by repeated crystallizations.

A list of the quaternary ammonium compounds XII to XII is given in Table 1, together with analytical data, physical constants, and methods of preparation.

2-*Acetoxyethyldi-n-propylmethylammonium iodide* was prepared according to Roufogalis & Thomas (1968c).

The following compounds were used as received: tetramethylammonium iodide, tetra-n-propylammonium iodide, tetra-n-butylammonium iodide, acetylcholine perchlorate (BDH) and tetraethylammonium iodide (Hopkin and Williams).

Enzymic analyses

Acetylcholinesterase was a purified bovine erythrocyte preparation (Nutritional Biochemicals Corporation). Enzymic analyses were made by the pH stat method. A Radiometer titrator (TTTIc) equipped with recorder (SBR2c), expanded scale (pHA 630) and syringe burette (0·5 ml) with sodium hydroxide solution (0·01N, CO_2 free) as titrant, were used. The solutions were stirred mechanically throughout and dry nitrogen passed over the surface. Corrections for changes in pH caused by stirring of low ionic strength media were made (Roufogalis & Thomas, 1968b).

Procedure for determining acetylcholine hydrolysis at high substrate concentrations. The enzyme solution was made by dissolving 10 mg of acetylcholinesterase in 100 ml of glass distilled water. It was stored at 4°. Fresh solutions were made daily.

The quaternary ammonium compounds were incubated for 15 min at pH 7·4 and 37° in a medium containing glass-distilled water and 3·0 ml of the enzyme solution in a jacketed glass vessel. The volume of the incubation mixture was (20-x) ml, where x

Table 1. *Preparation of quaternary ammonium compounds XII to XXII, their physical constants and analytical data.* All compounds had C, H, N analyses within the usual limits

Compound	Amine	Alkyl halide	Method (see text)	Solvent and time of reflux	Solvent for recrystallization	m.p.[1] (°C)
XII	Piperidine	1,4-Dibromopentane[3]	Aa	Chloroform 6 h	Ethyl methyl ketone	265
XIII	2,5-Dimethylpyrrolidine[4,5]	1,5-Dibromopentane	Ab	Methanol	Chloroform	242–243
XIV	2-Methylpiperidine	1,4-Dibromobutane	Aa	Chloroform 12 h	Chloroform–ethyl methyl ketone	285–286
XV	2-Methylpiperidine	1,5-Dibromopentane	Aa	Chloroform 12 h	Chloroform	247[3]
	Piperidine	Iodoethane	B[7]	70% Ethanol 6 h	Absolute[6] ethanol	214–216[6]
XVI	*N*-Ethylpiperidine	Bromoethane	B[7]	Ethyl methyl ketone	Ethyl methyl ketone	274–275
XVII	Morpholine	Iodoethane	[8]	—	Absolute[9] ethanol–ethyl methyl ketone	246–247
XVIII	Diethylamine	1,4-Dibromobutane	Aa	Chloroform 12 h	Chloroform–ethyl methyl ketone	286
XIX	Pyrrolidine	1,4-Diiodopentane[3]	Aa	Chloroform 4 h	Ethyl methyl ketone[2]	280–281
XX	Diethylamine	1,4-Dibromopentane[3]	Aa	Chloroform 8 h	Chloroform–ethyl methyl ketone	276–277
XXI	*trans*-2,6-Dimethylpiperidine[10]	1,4-Dibromobutane	Ab	Methanol	Chloroform–ethyl methyl ketone	265–266
	Diethylamine	2-chloroethanol	B	70% Ethanol	—	b.p.[11] 160–161
XXII	2-Diethylaminoethanol	2-Iodo ethanol	B	Ethyl methyl ketone	Absolute ethanol	230–231

[1] Uncorrected m.p.
[2] Soxhlet extraction method was used for recrystallization.
[3] Prepared by J. Thomas and D. Hawley.
[4] *cis* and *trans* isomers not separated.
[5] This compound is 90–95% one isomer.
[6] Compound was isolated as *N*-ethylpiperidine and the solvent for crystallization, m.p. and analysis figures refer to the hydrobromide derivative.
[7] Repeated attempts to prepare this compound by method A (diethylamine and 1,5-dibromopentane) were unsuccessful.
[8] *NN*-Diethylmorpholinium iodide was prepared by refluxing morpholine (8·7 g) and ethyliodide (16·0 g) in methanol (100 ml) for 6 h, then sodium hydroxide (4 g) was added and stirred until dissolved. Ethyl iodide (16·0 g) was added and reaction refluxed for a further 6 h. Product worked up as in method Ba.
[9] Attempts to use absolute ethanol-chloroform were repeatedly unsuccessful.
[10] *trans* isomers separated from *cis*/*trans* mixture with a Loenco preparative GLC.
[11] Boiling point refers to 2-diethylaminoethanol, lit. b.p. 163°.

is the volume of the substrate solution. After 15 min incubation the substrate was added and the titrator started. The pH was maintained at 7·4 and the temperature at 37°. At very low substrate concentrations the reaction volume was increased to 40 ml (the amount of enzyme was also doubled to maintain a constant concentration). The resulting plots of sodium hydroxide against time were linear for at least 2·5 min and

Table 2. *The effect of quaternary ammonium compounds on the hydrolysis of acetylcholine, phenyl acetate and dimethylcarbamyl-acetylcholinesterase at low ionic strength.* "Maximum potentiation" has been defined in Methods. "V/Vo" gives the ratio of the maximum velocity in the presence of compound to that in its absence, at ionic strength less than 0·005. "*a*k3′/k3′" gives the ratio of the apparent rate of decarbamylation in the presence of compound to that in its absence, at ionic strength less than 0·005

	Compound	Maximum potentiation (Acetylcholine hydrolysis)	V/Vo (Phenyl acetate hydrolysis)	*a*k3′/k3′ (decarbamylation)
	Tetramethylammonium iodide	—	1·27 ± 0·03	1·25
	Tetraethylammonium iodide	76 ± 5%	1·94 ± 0·34	3·95
	Tetrapropylammonium iodide	—	0·62 ± 0·03[1] 0·43 ± 0·02[2]	1·3
	Tetrabutylammonium iodide	—	—	1·1
I	1,1′-Spirobipiperidinium bromide	—	—	2·7
II	*cis*-2,6-Dimethylspirobipiperidinium bromide	—	1·12 ± 0·05	1·2
III	*cis*-2,6-Dimethylspiro-(piperidine-1,1′-pyrrolidinium) bromide	52 ± 2%	2·02 ± 0·12	9·6
IV	Spiro(piperidine-1,1′-pyrrolidinium) bromide	11 ± 3%	—	0·94
V	1,1′-Spirobipyrrolidinium bromide	19 ± 4%	—	1·2
VI	Spiro(piperidine-1,4′-morpholinium) bromide	—	—	4·2
VIII	2,6-Dimethylspiro(morpholine-4,1′-piperidmium) bromide	—	1·32 ± 0·45	—
XII	2-Methylspiro(pyrrolidine-1,1′-piperidinium) bromide	0%	1·17 ± 0·28	2·55
XIII	2,5-Dimethylspiro(pyrrolidine-1,1′-piperidinium) bromide	—	1·10 ± 0·09	1·23 ± 0·27
XIV	2-Methylspiro(piperidine-1,1′-pyrrolidinium) bromide	—	—	3·5
XV	2-Methyl-1,1′-spirobipiperidinium bromide	9 ± 1%	—	2·8
XVI	1,1-Diethylpiperidinium bromide	—	—	0·93
XVII	1,1-Diethylmorpholinium iodide	24 ± 1%	—	2·1
XVIII	1,1-Diethylpyrrolidinium bromide	33 ± 2%	2·02 ± 0·38	—
XIX	2-Methylspirobipyrrolidinium iodide	11 ± 1%	—	—
XX	1,1-Diethyl-2-methylpyrrolidinium bromide	56 ± 4%	2·26 ± 0·42	—
XXI	*trans*-2,6-Dimethylspiro-(piperidine-1,1′-pyrrolidinium) bromide	50 ± 2%	—	—
XXII	Di(2-hydroxyethyl)-diethylammonium iodide	—	0·92 ± 0·06	71

[1] This result was obtained at pH 8·4. Concentration of TPA 2·21 × 10^{-6}M.
[2] This result was obtained at pH 8·4. Concentration of TPA 1·11 × 10^{-5}M.

the amount of substrate consumed during this time was less than 20% of the total available. All rates were corrected for non-enzymic hydrolysis.

Final estimates of "maximum potentiation" (Table 2) are the average of two independent determinations. The effect of quaternary ammonium compounds on acetylcholine hydrolysis at ionic strengths less than 0·005 was obtained by:

(*a*) varying the concentration of compound over a 100-fold range at an acetylcholine concentration of 2·34 × 10^{-3}M (8-fold higher than the optimum substrate concn):

(*b*) varying the concentration of acetylcholine over a 100-fold range in the presence

of a concentration of compound found to give "maximum potentiation" in (*a*) above, and again obtaining the maximum potentiation at [S] = $2{\cdot}34 \times 10^{-3}$M.

Examples of the resulting plots from (a) and (b) above may be found in Figs 2 and 7(b) in Roufogalis & Thomas (1968a).

The procedures for determining the effect of quaternary ammonium compounds on phenyl acetate hydrolysis were described by Roufogalis & Thomas (1968b), then for the kinetics of acetylcholine and 2-acetoxyethyldi-n-propylmethylammonium iodide hydrolysis at below optimum substrate concentrations by Roufogalis & Thomas (1968c) and those for the decarbamylation of dimethylcarbamylacetylcholinesterase by Roufogalis & Thomas (1969).

RESULTS AND DISCUSSION

That some quaternary ammonium compounds potentiate acetylcholinesterase by accelerating the deacetylation step in the hydrolysis sequence has been concluded from the following evidence. First, in experiments on the rate of hydrolysis *vs* substrate (acetylcholine) concentration, the presence of compounds such as tetraethylammonium iodide (TEA) causes the optimum substrate concentration to be shifted towards a higher substrate concentration, and the optimum rate of hydrolysis in the presence of these compounds is greater than the optimum rate in their absence (Roufogalis & Thomas, 1968a).

Second, the maximum velocity of phenyl acetate hydrolysis in the presence of, for example, TEA is increased (about 2-fold) over the maximum velocity obtained in the absence of such cations. Since the maximum velocity of phenyl acetate hydrolysis is directly dependent on the rate determining deacetylation step in the hydrolysis sequence, it follows that compounds such as TEA are accelerating the deacetylation step in the hydrolysis sequence (Roufogalis & Thomas, 1968b).

Third, the first-order rate of decarbamylation of dimethylcarbamyl-acetylcholinesterase is accelerated by TEA and some other quaternary ammonium compounds (Roufogalis & Thomas, 1969). Decarbamylation is considered to be analogous in many ways to deacetylation of the acetyl-enzyme and this system serves as a model for the deacetylation reaction.

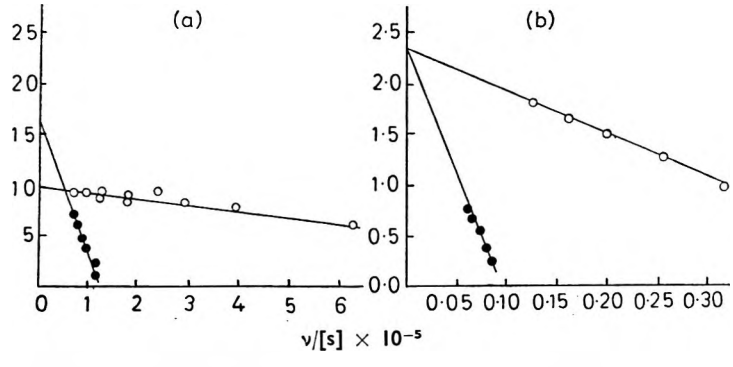


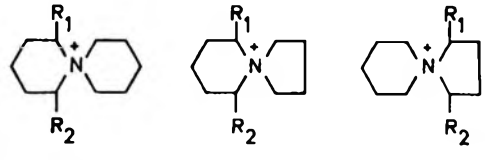
FIG. 1. Effect of TEA on the hydrolysis of (a) acetylcholine and (b) 2-acetoxyethyldi-n-propylmethylammonium iodide. The maximum velocity for the hydrolysis of acetylcholine has been arbitrarily set at 10·0, and represents a maximum velocity of approximately 20 μm/min. Substrate concentrations studied are in the range $1\text{–}15 \times 10^{-5}$M. Ionic strength in the control plots is less than 0·005. ○—○ Control. ●—● A, TEA, $1{\cdot}22 \times 10^{-3}$M. ●—● B, TEA, $4{\cdot}87 \times 10^{-4}$M.

As further evidence we have now examined the kinetics of the effect of TEA on the hydrolysis of acetylcholine, a substrate considered to have deacetylation as the rate-determining step in the enzyme-catalysed hydrolysis (Wilson & Cabib, 1956). The result is shown in Fig. 1a and it can be seen that at low substrate concentrations TEA inhibits acetylcholine hydrolysis, but the maximum velocity of hydrolysis of acetylcholine in the presence of TEA is increased 1·6-fold over that of the control. In Fig. 1b is shown the effect of TEA on the hydrolysis of 2-acetoxyethyldi-n-propylmethylammonium iodide. Comparison of the control plots of Fig. 1a and b reveals that the maximum velocity for the hydrolysis of dipropyl compound is 4·3 times slower than that for acetylcholine hydrolysis. Since both substrates are acetate esters, it follows that deacetylation, a step common to the hydrolysis of both substrates, cannot be the rate-determining step in the hydrolysis of the dipropyl compound. If quaternary ammonium compounds are acting by accelerating deacetylation, TEA would not be expected to increase the maximum velocity of the dipropyl compound, since deacetylation is not rate-determining in the hydrolysis of this substrate. Purely competitive kinetics might be expected in this case (Krupka & Laidler, 1961a). This is what is observed in Fig. 1b, which further supports the proposed mechanism.

Structure-activity relations

In an attempt to elucidate the mechanism by which some quaternary ammonium compounds accelerate deacetylation when bound, presumably to the acetylenzyme (Krupka & Laidler, 1961b; Roufogalis & Thomas 1968 a, b), the structure-activity relations among 22 quaternary ammonium compounds have been investigated. Three different systems have been used, the hydrolysis of acetylcholine at above optimum substrate concentrations, the hydrolysis of phenyl acetate, and the decarbamylation of dimethylcarbamyl-acetylcholinesterase. The results (Table 2) are discussed below.

Fig. 2 shows the effects of eight representative compounds on the rate of decarbamylation. The number after each compound is the number of times that compound



I $R_1=R_2=H$,	2·7	IV $R_1=R_2=H$,	0·94	IV $R_1=R_2=H$,	0·94
XV $R_1=H$; $R_2=Me$,	2·8	XIV $R_1=H$; $R_2=Me$,	3·5	XII $R_1=H$, $R_2=Me$,	2·6
II $R_1=R_2=Me$,	1·2	III $R_1=R_2=Me$,	9·6	XIII $R_1=R_2=Me$,	1·2

FIG. 2. Effect of some spiran quaternary ammonium compounds on the decarbamylation of dimethylcarbamyl-acetylcholinesterase. The number after each compound represents the number of times the reaction is accelerated by the compounds; a value of 1 represents no effect and values greater than 1 the amount of acceleration. The decarbamylation has been studied in the absence of added inorganic ions (see methods).

accelerates this reaction. In the middle column, as methyl groups are added to the piperidine ring of compound IV, in the position α to the nitrogen, there is a progressive increase in accelerator potency, from no effect with compound IV to 10-fold acceleration with compound III. However increase in branching does not always lead to an increase in accelerator potency, as seen in the first and third columns. In these cases progressive addition of methyl groups to the piperidine ring of compound I and the

pyrrolidine ring of compound IV does not lead to an increase in accelerator potency, since in both series the most highly branched members are inactive as accelerators of the decarbamylation reaction. Thus the structure-activity relations are subject to highly specific steric control. This is well illustrated in the third row, where it is seen that contraction of the unsubstituted piperidine ring of compound II (inactive as accelerator) by one carbon, to give compound III increases accelerator potency 10-fold. When the methyl groups of compound III are transferred to the pyrrolidine ring, as in compound XIII, the compound becomes inactive as an accelerator. The *cis*- and *trans*-isomers of 2,6-dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide have the same accelerator potency (Table 2, compounds III and XXI respectively). Tetraethylammonium is a potent accelerator of deacetylation and decarbamylation, but *NN*-diethylpiperidinium bromide (XVI) is inactive as an accelerator of decarbamylation (Table 2). On the other hand, *NN*-diethylpyrrolidinium bromide (XVIII) is a fairly potent accelerator of deacetylation and branching with one methyl group on the pyrrolidinium ring (compound XX) increases accelerator potency even further (Table 2).

It appeared possible that the compounds which do not potentiate acetylcholine hydrolysis were inhibiting deacetylation. However, the compounds which do not potentiate acetylcholine or phenyl acetate hydrolysis i.e. (TMA, II, VIII, XII, XIII, and XXII) do not block deacetylation (Roufogalis, 1968). The one exception is tetrapropylammonium iodide (TPA); this compound non-competitively decreases the maximum velocity of phenyl acetate hydrolysis, probably by blocking deacetylation (Krupka, 1965, Roufogalis & Thomas, 1968b).

The free energy of binding of quaternary ammonium compounds to acetylcholinesterase

The equilibrium constant for the dissociation of quaternary ammonium compounds from acetylcholinesterase can be characterized by K_I. This constant may be used to determine the free energy of binding of a compound to the enzyme from the relation

$$\Delta F_I = -RTL_n \frac{1}{K_I}$$

There is now a substantial body of evidence to indicate that the active site of acetylcholinesterase is non-polar in nature (see Belleau & Lacasse, 1964). The present discussion refers to the effects of the quaternary ammonium compounds on the acetyl-enzyme, and it is also considered to be non-polar.*

Belleau & Lacasse (1964) have pointed out that if an enzyme active site is non-polar, the presence of hydrophobic substituents in a substrate or inhibitor molecule will supply a significant driving force for adsorption onto the enzyme and this driving force has its origins in the entropically unfavourable hydrophobic interactions created by the non-polar substituents in water. It has been calculated from studies on the transfer of amino-acids from water to octanol (Cohn & Edsall, 1943) that the contribution of a methyl or methylene group to hydrophobic transfer is −720 cal. It is possible therefore by determining the ΔF_I of an inhibitor of acetylcholinesterase to calculate a theoretical ΔF_I for a second inhibitor which differs from the first by a

* Results obtained by Krupka (1965) support this assumption. In the series TMA, TEA, TPA, and tetra-n-butylammonium iodide (TBA), the equilibrium constant for the dissociation of these compounds with the free enzyme (K_I) and with the acetylated enzyme (K'_I) were of the same order and the variation of both K_I and K'_I with the structure of the above series of compounds followed a similar pattern.

$-CH_2-$ group. This was the approach used by Belleau & Lacasse (1964) with a series of dioxalan and tetrahydrofuran derivatives and by Belleau (1967) with the alkyltrimethylammonium series. Calculations of this type have now been made on a series of quaternary ammonium compounds and the calculated ΔF_i values compared with the experimentally determined ΔF_i values.

Table 3. *The effect of the addition of four methyl groups to* TMA *and* TEA *on the free energy of binding to free enzyme and acetyl-enzyme*

Compound	ΔFi Obsd[2]	ΔFi Calc	ΔFi Calc-Obsd (Free enzyme)	ΔFi Calc-Obsd[3] (Acetyl-enzyme)	Effect on Deacetyl-ation[1]
TMA	—4940				○
TEA	—5250	—7820	—2570	—2170	+++
TPA	—7180	—8170[4]	—990[4]	—730 [4]	−−

[1] A negative sign indicates compounds block deacetylation and the number of signs indicates the degree of blocking. ○ indicates no effect on deacetylation. + indicates acceleration of deacetylation and the number of signs indicates the degree of acceleration.
[2] Ki values calculated from Roufogalis & Thomas (1968b).
[3] Computed from the results of Krupka (1965).
[4] Calculated from the observed value of ΔFi for TEA so that ΔFi (Calc-Obsd) refers to the effect of the addition of four methyl groups to TEA.

In Table 3 this treatment has been applied to the series, TMA, TEA and TPA. The free energy of binding observed for TMA is −4940 cal. The addition of four $-CH_2-$ groups to produce TEA should increase the free energy of binding by −2880 cal (4 × 720) to a value of −7820 cal if hydrophobic transfer forces are operating optimally. In fact the ΔF_i observed for the binding of TEA to both free enzyme and the acetylated enzyme is over 2000 cal lower than the ΔF_i calculated. This suggests that the forces determining the difference in enzyme interaction in going from TMA to TEA are not simply hydrophobic and that other factors offset the hydrophobic transfer. TEA is a good potentiator. A similar analysis of the effect of the addition of four $-CH_2-$ groups to TEA to produce TPA shows that there is now a much smaller deviation between the calculated and observed values of ΔF_i for binding of TPA (−860 cal). It is also noted that TPA is not a potentiator but is an inhibitor of the deacetylation reaction. The closeness of the calculated and observed values for the binding of TPA suggests that the γ-methyl groups of TPA interact largely with a non-polar environment of the active site. Small deviations from calculated values of ΔF_i have been interpreted by Belleau & Lacasse (1964) in terms of the difficulty of getting an "ideal solution" of a non-polar group in a non-polar environment of the active site.

The treatment used above has been extended to some representative spiran quaternary ammonium compounds. The results are in Table 4 and the differences between the calculated and observed values for ΔF_i of binding for these compounds are compared with the effects of the compounds on deacetylation. The calculated values of the ΔF_i of binding for the compounds which accelerate deacetylation strongly (III, XI) are about 1500 cal different from the values observed, whereas the differences between the calculated and observed ΔF_i for the compounds which do not accelerate deacetylation (i.e. I, II, XIII, VIII) are much smaller (300 to 900 cal positive or

Table 4. *The free energy of binding of spiran quaternary ammonium compounds with acetylcholinesterase*

Compound	ΔFi Obsd[2]	ΔFi Calc[3]	ΔFi Calc-Obsd	Effect on Deacetylation[1]
IV	—2940			○ to +
I	—3420	—3660	— 240	○ to +
III	—2760	—4380	—1620	+++
II	—3920	—3480	+ 440[4]	○
		—4860	— 940[5]	
XIII	—3500[6]	—4380	— 880	○
VI	—2230			++
VIII	—4110	—3670	+ 440	○
XI[7]	—2400	—3670	—1270	++ to +++

[1] For meaning of signs see Table 3.
[2] ΔFi calculated from I50 values from Roufogalis & Thomas (1968a).
[3] ΔFi (calc) obtained by adding —720 cal per methyl or methylene group to ΔFi (Obsd) for parent compound IV or VI, unless otherwise stated.
[4] Refers to the addition of $-CH_2-$group to compound III.
[5] Refers to the addition of two methyl groups to compound I.
[6] Calculated from Ki from Roufogalis (1968).
[7] 2,6-Dimethylspiro(piperidinium-1,4′-morpholinium) iodide.

negative). In both series the compounds which are potentiators are the ones which show the greatest deviation of calculated to observed ΔF_i values. Precisely what this means in terms of a mechanism of acceleration of deacetylation is difficult to interpret. However it does seem to indicate that acceleration is related to a poor interaction between quaternary ammonium ions and the enzyme. A detailed analysis of the factors which offset the free energy change which results from hydrophobic transfer is difficult because the addition of methyl or methylene groups to the spiran quaternary ammonium compounds will alter parameters in the compounds other than their hydrophobic nature. For example, changes in the overall shape of the molecule, in steric effects due to protruding substituents or changes in the ring size would all affect the interaction with the enzyme and solvent. Coulombic interaction could also be modified as a result of changes in the radius of the cation (Robinson & Stokes, 1965) although this effect is not likely to be as important in the spirans as in the tetra-alkyl-ammonium series. Furthermore, these changes may also be sufficient to alter the site of interaction with the enzyme (O'Brien, 1969). Nevertheless despite these difficulties of interpretation, by applying this treatment to the present series, it can be seen that deviation in ΔF_i observed and calculated is different for potentiators and non-potentiators. To date this is the only property which has correlated with potentiation.

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The subcellular distribution of (±)-2,3-dehydroemetine and (−)- emetine in rat liver and changes in hepatic lipid content after treatment of rats with (±)-2,3-dehydroemetine

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Treatment of female rats with (±)-2,3-dehydroemetine leads to increases in the total lipid content of their livers, without affecting phospholipid content appreciably. The subcellular distribution of (±)-2,3-dehydroemetine or (−)-emetine in the livers of rats pretreated with these drugs, shows that each compound is associated with the mitochondria rather than the microsomal fraction or the cell sap.

We have previously examined the action of emetine (Jondorf & Szapary, 1968) and related compounds (Jondorf, Drassner & others, 1969) on protein biosynthesis in female rats of the Sprague-Dawley strain and found them to vary according to whether the experiments were conducted *in vivo* or *in vitro*. Thus, in rats killed 24 h after injection with (−)-emetine (dihydrochloride) or with (±)-2,3-dehydroemetine (dihydrochloride) at 18 μmol/kg, a non-toxic dose in this strain, there is an increase in liver size (25–30%), and increased L-amino-acid incorporation into protein *in vitro* (about 100%) by liver microsomal preparations from pretreated animals (Jondorf & others, 1969). However, short-term (2 h) pretreatment of rats with either drug inhibits the uptake of labelled amino-acid into hepatic protein *in vivo*. We have also examined the inhibition by emetine and 2,3-dehydroemetine of drug metabolism by liver microsomes in rats (Jondorf, Johnson & Donahue, 1969; Jondorf, Johnson & Drassner, 1969).

Emetine (Gimble, Davison & Smith, 1948; Davis, Dodds & Tomich, 1962) and 2,3-dehydroemetine (Schwartz & Rieder, 1961a; Schwartz & Herrero, 1965) do not appear to be metabolized and persist unchanged in various tissues such as the liver.

We have now investigated the subcellular distribution of emetine and 2,3-dehydroemetine in rat liver and have also studied changes in lipid composition of rat liver after pretreatment of 2,3-dehydroemetine.

EXPERIMENTAL

Animal treatment. Female Sprague-Dawley rats, of the same age and each weighing 160 g, had access to Purina rat chow and water in stress-free and insecticide-free standard conditions. Groups of two or more animals were pretreated with freshly made aqueous solutions of the dihydrochlorides of (±)-2,3-dehydroemetine or

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(−)-emetine at 18 μmol/kg (equivalent to 8·6 mg/kg free base), or with water, by intraperitoneal injection (injection volume 0·8 ml/160 g rat). To lower glycogen levels, food was withheld for the 24 h (9 a.m.–9 a.m.) between pretreatment and death. In some experiments the time between pre-injection and death was 1 h. Animals were killed by stunning and decapitation in the cold room (0–4°). Livers were immediately excised and treated in one of two ways after rinsing in ice-cold 0·25M sucrose and subsequent blotting.

Procedure for total lipid extraction from liver. Samples of livers were minced with scissors and were subjected to the procedure of Folch, Lees & Sloane-Stanley (1957) for the extraction of total lipids. Aliquots of the extracts (in hexane) so obtained were analysed for total lipid content by the method of Bragdon (1951) using stearic acid as the standard. Total cholesterol estimation of the total lipid extract was according to Zlatkis, Zak & Boyle (1953). Further aliquots of the total lipid extracts were analysed for phospholipid content by the method of Fiske & Subbarow (1925). Results of the phosphate analyses were compared with a disodium hydrogen phosphate standard and were then multiplied by 25, the factor for converting inorganic phosphorus to organic phosphorus (Vahouny, Moede & others, 1963). Neutral fat content could then be calculated by difference from the values obtained for total lipid and the combined values for total cholesterol and phospholipid.

Preparation of subcellular fractions from liver for spectrofluorimetric assay of emetine or 2,3-dehydroemetine. Portions (1·5 g) of freshly excised livers were set aside for the determination of alkaloid in whole liver. These portions were homogenized with five volumes of ice-cold citric acid in disodium hydrogen phosphate buffer pH 3 (Schwartz & Rieder, 1961b) in a motor-driven coaxial Teflon-glass homogenizer with a clearance of 0·84 mm, rotating at 4000 rev/min for ten complete vertical passes. The homogenates were then deep-frozen to await analysis.

The remaining portions of the liver were homogenized with five volumes of ice-cold 0·25M sucrose under the same standardized conditions. The homogenates were subjected to centrifugation at 600 *g* for 10 min to obtain the cell debris pellet. The supernatant portion from this was spun at 4°, at 15 000 *g* for 15 min to obtain the mitochondrial fraction as a pellet. The post-mitochondrial fraction after 60 min at 105 000 *g* (4°) yielded a microsomal pellet and a supernatant fraction. All the pelleted subcellular fractions were carefully surface-rinsed with standard amounts (2 ml, three times) of citric acid in disodium hydrogen phosphate buffer and were then dispersed in this buffer. All samples including the buffer rinses were then analysed for emetine or 2,3-dehydroemetine according to Schwartz & Rieder (1961b). Recoveries of known amounts of alkaloid added to samples were 91·3 (±1·8) %. All values quoted under Results are corrected for recoveries. Protein in the mitochondrial, microsomal and the 105 000 *g* supernatant fractions was measured by the method of Lowry, Rosebrough & others (1951).

RESULTS

Changes in hepatic lipid content. After injection with 2,3-dehydroemetine, there is an increase in total lipid content of livers from pretreated rats (Table 1). There is also a significant increase in total cholesterol (free and esterified) and neutral fat (triglycerides). Whilst other categories of lipids increased in quantity after pre-

treatment with 2,3-dehydroemetine, the phospholipid values remained almost at control levels.

Table 1. *Effect of pretreatment with* 2,3-*dehydroemetine* (18 μmol/kg, 24 *h*) *on the lipid composition of female rat liver*

Treatment	No. of livers	Total lipid	Cholesterol (total)[2]	Phospho-lipid	Neutral fat[3]
Control	4	45·8 ± 1·4[1]	2·1 ± 0·20	17·0 ± 3·9	26·7 ± 4·2
2,3-Dehydroemetine ..	3	71·3 ± 3·8*	5·4 ± 0·54*	19·2 ± 0·7	46·7 ± 3·9*

[1] All values are expressed as mg/g of liver (wet weight) ± standard error of the mean.
[2] Total cholesterol refers to free + esterified cholesterol.
[3] Neutral fat or triglyceride was calculated as the difference between the combined values of total cholesterol and phospholipid, and total lipid.
* Indicates a significant difference ($P < 0{\cdot}05$).

The subcellular distribution of 2,3-*dehydroemetine and emetine in the liver.* 2,3-Dehydroemetine and emetine are present in the liver at 1 h after a single injection of either drug at a dose level of 18 μmol/kg (Table 2). After 24 h, the concentration of emetine in the whole liver is more than twice that found for 2,3-dehydroemetine.

Table 2. *The concentration of either* 2,3-*dehydroemetine or emetine in whole liver and in various subcellular fractions following pretreatment of female rats with the respective compound for one or for* 24 *h*

Time of pre-treatment	Whole liver μg/g	Cell debris μg/g liver	Mitochondria μg/g liver	Mitochondria μg/mg protein	Micro-somes[1] μg/mg protein	105 000 × *g* supernatant[2] μg/mg protein
			2,3-*Dehydroemetine*			
1 h	63·2 ± 3·7[3] (5)[5]	22·0 ± 0·8[3] (3)	13·7 ± 0·4[3] (3)	1·1 ± 0·1[4] (3)	0·41 ± 0·01[4] (3)	0·27 ± 0·10[4] (6)
24 h	17·0 ± 1·8 (8)	8·0 ± 0·9 (3)	5·9 ± 0·5 (4)	0·40 ± 0·02 (4)	0·06 ± 0·01 (4)	0·11 ± 0·01 (6)
			Emetine			
1 h	79·2 ± 12·4 (3)		26·5 ± 2·6 (5)	1·7 ± 0·2 (5)	0·3 ± 0·05 (3)	0·18 ± 0·03 (3)
24 h	39·9 ± 7·1 (3)		12·6 ± 1·2 (6)	0·8 ± 0·1 (6)	0·06 ± 0·002 (3)	0·08 ± 0·01 (3)

[1] Yield, 9 mg microsomal protein per g liver. (Combined washings from surface rinsing of microsomal pellets had negligible alkaloid content.)
[2] Yield, 50 mg 105 000 × *g* supernatant protein per gram liver.
[3] All values expressed as μg free base of alkaloid ± standard error of the mean per gram of liver.
[4] All values expressed as μg free base ± standard error of the mean per mg protein.
[5] Figures in parentheses refer to number of animals.

The amount of 2,3-dehydroemetine in the cell debris (600 *g* pellet) at 1 h after pretreatment is about one third of that found in the whole liver. At 24 h after pretreatment the concentration is lower, but is half that found in the whole liver at that time. The amounts of emetine localized in the liver mitochondria at 1 h and 24 h after injecting rats (18 μmol/kg) are about double the amounts after corresponding pretreatment with 2,3-dehydroemetine.

2,3-Dehydroemetine and emetine are found only in small quantities in the 105 000 *g* supernatant fraction and in the microsomal fraction (Table 2). The differential centrifugation procedure for obtaining microsomal and 105 000 *g* supernatant fractions is essentially similar to that used for the preparation of these fractions in experiments on protein synthesis *in vitro* (Jondorf & others, 1969). The amounts of emetine or 2,3-dehydroemetine detected in the liver microsomal preparations at 24 h would be insufficient to inhibit protein synthesis *in vitro* (Jondorf & Szapary, 1968; Jondorf, Drassner & others, 1969).

We conclude, therefore, that both 2,3-dehydroemetine and emetine have a preferential affinity for liver mitochondria.

DISCUSSION

At 24 h after pretreatment of rats with 2,3-dehydroemetine, there is an increase in liver size, and an increase in liver microsomal protein synthesizing activity *in vitro* (Jondorf & others, 1969). We now find that livers obtained from 2,3-dehydroemetine-treated rats have a measurably higher total lipid content than the corresponding controls but the amount of phospholipid does not increase significantly (Table 1).

In agreement with Schwartz & Rieder (1961) the concentration of emetine found in the liver after 24 h was more than twice that found for 2,3-dehydroemetine; both are found in relatively high concentrations in the mitochondrial fraction of the liver (Table 2). This latter finding lends support to those observations (Appelt & Heim, 1964, 1965; Chang & others, 1966; Watkins & Guess, 1968) where emetine has been implicated in decreasing oxygen consumption and in inhibiting oxidative phosphorylation in a number of different tissue preparations. That pretreatment of rats with 2,3-dehydroemetine was found not to significantly affect the amount of phospholipid phosphorus in the liver, whereas other categories of lipid did increase, implies that the availability of ATP may be impaired.

It is also possible that pretreatment with 2,3-dehydroemetine (and by analogy, with emetine) might inhibit the utilization of lipid in the liver. The increased hepatic lipid content after 24 h pretreatment with 2,3-dehydroemetine may be due in part to a decrease in production of the necessary carrier proteins (Robinson & Seakins, 1962; Sabesin & Isselbacher, 1965), because of a transient inhibition of protein synthesis *in vivo* (Jondorf & Szapary, 1968; Jondorf & others, 1969), and may also be dependent on faulty utilization of the lipid material transported to the liver. If the latter is the case, the lack of availability of DPNH and TPNH generated from fatty acid oxidations would greatly impair the hepatic drug metabolism dependent on TPNH (Brodie, Maickel & Jondorf, 1958) and help to explain the observed inhibitory effects exerted by 2,3-dehydroemetine (Jondorf, Johnson & Donahue, 1969; Jondorf, Johnson & Drassner, 1969) on drug metabolizing enzyme activity in the liver.

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Inhibition by chlorpromazine of the effects of dopamine on the dog kidney

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Dopamine-induced vasodilatation in the renal artery of the dog is reversed by chlorpromazine while the vasodilatation produced by isoprenaline is not. Pronethalol has the entirely opposite actions on this preparation. The infusion of dopamine into the renal artery enhances the kidney output of water, sodium, potassium and urea. These effects are also reversed by chlorpromazine.

Chlorpromazine has been reported to block dopamine effects in the brain (Nyback, Sedvall & Kopin, 1967; Gey & Pletscher, 1968; Nyback & Sedvall, 1968). However, this action has not been unequivocally demonstrated. Dopamine causes vasodilatation when injected into the renal artery of the dog (McNay & Goldberg, 1966; Goldberg, Sonneville & McNay, 1968). Since this effect is not blocked by β-adrenergic agents or by atropine it has been suggested by these authors that dopamine acts via specific "dopaminergic" receptors in the renal vascular bed.

The present study was designed to determine whether chlorpromazine interferes with the effect of dopamine on the renal artery.

EXPERIMENTAL

Materials and methods

Mongrel dogs of either sex, 8 to 15 kg, were anaesthetized with sodium pentobarbitone (30 mg/kg, i.v.). The left renal artery was isolated by lumbar incision and retroperitoneal dissection. The left ureter was sectioned and the proximal end cannulated with a ureteral catheter, inserted up to the pelvis for collecting urine. Intra-arterial injections (1 ml) or infusions were made through a polyethylene catheter of 1·3 mm external diameter introduced through the left femoral artery up to the renal artery. Blood flow in the left renal artery was measured by means of a flow-probe connected to a Nycotron electromagnetic flow-meter. Both instantaneous and mean flows were recorded. The zero-flow base line and the electrical zero were determined immediately after application of the flow-probe and at intervals throughout each experiment by mechanical occlusion of the artery distal to the flow-probe. Sodium and potassium concentrations in the samples of urine were analysed by flame photometry; urea was measured according to Dupré. Systemic blood pressure was measured with a capacitance transducer from the femoral artery and recorded on a multichannel polygraph. The heart rate was counted from blood pressure tracings.

The following experimental design was used to examine the effect of dopamine and chlorpromazine on urine excretion. Isotonic NaCl was infused through the arterial catheter at 1 ml/min for 10 min, the urine excreted by left kidney was collected during this time. Dopamine (50 μg/ml) was then added to isotonic saline

solution which was infused for 12 min at the rate of 1 ml/min. The urine was collected starting 2 min after the beginning of the dopamine infusion.

This procedure was repeated 3 times and the values obtained were considered control values. Chlorpromazine (3 mg/kg) was then infused over 5 min. Ten min after, the chlorpromazine saline and dopamine infusions were repeated and urine was collected.

RESULTS

Inhibition by chlorpromazine of the haemodynamic effects of dopamine on the renal artery

Six dogs were used and the results are in Tables 1 and 2. The intra-arterial injections of dopamine ranging from 1 to 5 μg produced a dose-related increase in the renal blood flow; the average maximum increase was 35 $\pm$ 12%. Higher doses produced a diminished response (10 μg) or a vasoconstrictor response (20 μg).

Table 1. *Changes in the renal blood flow induced by intra-arterial dopamine, before and after chlorpromazine* (3 *mg, i.a.*)

	% Changes in blood flow produced by different doses of dopamine									
Dog No.	Before chlorpromazine					After chlorpromazine*				
	1 μg	2	5	10	20	1 μg	2	5	10	20
1	+20	+30	+38	+15	—15	0	0	0	— 5	—10
2	+12	+25	+31	+10	—22	0	0	— 5	—15	—20
3	+24	+38	+41	+21	—30	+5	0	—10	—20	—40
4	+11	+34	+26	— 5	—	0	0	— 5	—25	—
5	+13	+22	+38	+15	—10	0	0	0	—10	—
6	+20	+29	+36	+10	—15	0	0	0	—15	—
Means	+16	+29	+35	nc	nc	0	0	— 7	—15	nc
$\pm$ s.d.	8	9	12			5		3	10	

* Dopamine was injected 15 to 25 min after chlorpromazine.

Table 2. *Changes in the renal blood flow induced by intra-arterial injections of isoprenaline after chlorpromazine or pronethalol*

	% Changes in renal blood flow produced by different doses of isoprenaline								
	Isoprenaline			Isoprenaline after chlorpromazine*			Isoprenaline after pronethalol		
Dog No.	1 μg	1 μg	5 μg	1 μg	2 μg	5 μg	1 μg	2 μg	5 μg
1	+30	+50	+60	+20	+50	+60			
2	+25	+45	+55	+25	+40	+50			
3	+15	+30	+45	+15	+30	+50			
4	+20	+35	+45	+20	+30	+45			
5	+15	+25	+50	+20	+35	+45			
6	+25	+40	+55	+25	+45	+65			
Means ..	+22	+37	+51	+20	+36	+52			
s.d.	10	7	5	5	9	7			
7	+20	+35	+55				0	2	+10
8	+15	+30	+45				2	5	+10
9	+20	+40	+60				0	0	+ 5
Means ..	+18	+35	+53				0	+ 2	3·8
+ s.d. ..	3	5	7				2	2	5

* Isoprenaline was injected 15 to 20 min after chlorpromazine.

Intra-arterial injections of 5 μg of dopamine did not influence the systemic blood pressure and the heart rate. The intra-arterial injection of isoprenaline, 1 to 5 μg, produced a dose-related increase in renal blood flow (Table 2). These results are in agreement with those of McNay & Goldberg (1966).

The intra-arterial injection of chlorpromazine produced no changes in the renal blood flow, but ten to 20 min after the chlorpromazine the vasodilatating effect of dopamine was completely abolished or reversed (Table 1) while the effect of isoprenaline remained unchanged (Table 2).

On the other hand, in three experiments the vasodilating effect of isoprenaline was blocked by intra-arterial injection of pronethalol (5 mg/kg) while the vasodilatation produced by dopamine was not influenced by β-blocking agents. The vasoconstriction in the renal artery produced by high doses of dopamine was reversed by phentolamine (1 mg/kg, i.p. 5–10 min before the dopamine) but only diminished by chlorpromazine (3 mg/kg, i.a. 20 min before the dopamine) (Table 1).

Inhibition by chlorpromazine of the dopamine effect on urine excretion

These experiments were made in 6 dogs. The results are in Table 3. The infusion of dopamine (3 μg/kg min^{-1}) increased water excretion by 18% and the excretion of

Table 3. *Effect of dopamine before and after chlorpromazine on the urinary excretion of sodium, potassium and urea in dogs*

	Urine volume and composition during infusion				
Dog No.	Urine ml/10 min	Urea (g %)	Na m-equiv/litre	K	% Change in urine vol/10 min
	Saline				
1	5	10	204	12	
2	10	1·7	296	24	
3	12	5·7	255	12	
4	7	6·6	300	26	
5	7	9	168	20	
6	8	8	220	19	
means ..	8	6·8	240	19	
s.d. ..	3	5	70	7	
	Dopamine				
1	6·5	14	276	17	13
2	13	10·6	444	35	13
3	15	7·3	292	19	12·5
4	9	8·9	335	35	12
5	8	27	278	30	11·1
6	10	11	258	23	12·5
means ..	10	13	313	26	12·3
s.d. ..	4·5	4	130	9	0·8
	Dopamine after chlorpromazine				
1	4·5	10	215	12	—10
2	10	5	300	26	0
3	11	5	245	15	— 5
4	6	6	315	27	—12
5	5	7	155	18	—20
6	7	6·5	200	17	—12
means ..	7	6	221	19	— 9
s.d. ..	2	4	80	7	6

Each of the values is the average of three determinations. Chlorpromazine was given intra-arterially at the dose of 3 mg/kg 15–20 min before dopamine infusion.

urea, sodium and potassium by 100, 40 and 10% respectively. The infusion of dopamine (3 μg/kg min^{-1}) increased the renal blood flow but did not change the systemic blood pressure or the heart rate.

Chlorpromazine did not significantly modify the renal blood flow, the urinary output, and the urinary composition after saline infusion. But given before the infusion of dopamine, it decreased the blood flow by 20% while not affecting urea, sodium and potassium excretion (see Table 3).

DISCUSSION

In agreement with Goldberg & others, I found that the vasodilatation induced by dopamine in the renal artery, unlike that induced by isoprenaline, is not antagonized by β-blockade. On the other hand, the renal vasodilatation caused by dopamine is inhibited by chlorpromazine, although it does not inhibit the vasodilating effect of isoprenaline.

These results support the hypothesis that dopamine is acting upon specific receptors in the renal vascular bed and the concept that chlorpromazine inhibits dopaminergic receptors in brain (Nyback & others, 1966; Nyback & Sedvall, 1968; Gey & Pletscher, 1968). The vasoconstriction response to high doses of dopamine is probably mediated by an action upon α-adrenergic receptors. In fact, this effect is reversed by phentolamine (McNay & Goldberg, 1966). However, chlorpromazine, which is also an α-blocking agent (Courvoisier, Fournel & others, 1953; Jourdan, Duchene-Marullaz & Boisser, 1955), diminished the vasoconstriction produced by dopamine, but did not reverse it, indicating that chlorpromazine inhibits the receptors related to the vasodilatation more effectively than it does those mediating vasoconstriction. The finding that dopamine enhances water, electrolyte and urea output by its kidney deserves further investigation. That this effect is also inhibited by chlorpromazine suggest that it is mediated by specific dopaminergic receptors. Whether the dopamine effect on urine excretion is secondary to its effect on renal blood flow remains to be elucidated.

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The effect of a range of Triton non-ionic surfactants on rodent ovaries

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Cysts developing on rodent ovaries after the Triton series of non-ionic surfactants have been fed to or applied topically to the animals are subcapsular and associated with obstruction of the ostium. The ability of Triton X–100 to produce subcapsular cysts after being fed to animals has been confirmed but the mechanism by which it does so remains obscure.

We have previously reported the production of cysts in rodent ovaries after painting the non-ionic surfactant Triton X–100 on (Goldhammer & McManus, 1960) or feeding it to the animals (Goldhammer, McManus & Osborn, 1967). We have now determined the nature of the cysts and attempted to find out how they arise.

In 1960, Goldhammer & McManus reported on the toxicity of Triton X–100 when applied to various organs in the rat. We have also re-investigated these effects.

EXPERIMENTAL AND RESULTS

The dissecting microscope was used to differentiate between subcapsular and follicular cysts, to find the ostium and to assist with accurate injection of materials into the subcapsular space. Most cysts were found developing in the subcapsular space and were invariably associated with a blockage of the ostium.

Injection experiments. An Agla micrometer syringe was used to measure the volume of fluids injected. To assess the specificity of the response varying volumes of Triton X–100 undiluted and in solutions at different concentrations were injected into the subcapsular space (Table 1). The appearance of the ovary was noted after laparotomy 1 week later and the animals were killed at the times indicated in the Table. A slight accumulation of fluid was observed in some rats at the laparotomy 1 week after the subcapsular injection of undiluted Triton X–100 and subsequently there was a high incidence of subcapsular cysts in both mature and immature rats. There was a low incidence of cysts when Triton X–100 was injected in a dilute form.

The relative importance of the lipid or water solubility of the surfactants was investigated by injecting into the subcapsular space a range of Tritons, X–35, X–45, X–165 and N–100 (Table 2). All four Tritons are capable of producing cysts after subcapsular injection. The effects of xylol, liquid paraffin, distilled water and solutions of various concentrations of sodium chloride were also assessed (Table 3). Xylol obliterated the subcapsular space, liquid paraffin, water and dilute solutions of sodium chloride produced no visible changes and 3 ovaries in 14 rats showed peri-ovarian cysts after the subcapsular injection of 20% sodium chloride.

Table 1. *The effect of injection of Triton* X–100 *into the peri-ovarian space of rats.* There is a high incidence of subcapsular cysts when undiluted Triton X–100 was injected into the subcapsular spaces of mature and immature rats. There was a low incidence of cysts when Triton X–100 was injected in a dilute form.

No. of rats	Volume injected concentration (ml)	Time after injection that animal was killed	No. of subcapsular cysts found
3	0·01 undiluted	3 weeks	2
6 (1 ovary removed)	0·05 "	7 weeks	3
4	0·005 "	3 weeks	2
10 (3 weeks old)	0·005 "	3 weeks	7
3 (2 weeks old)	0·001 "	3 weeks	3
4	0·05 10%	8 weeks	1
6	0·25 1%	4 weeks	none
			18

Table 2. *The effect of the injection of various Triton surfactants into the peri-ovarian space of rats.* All of the water and lipid soluble Tritons are capable of producing subcapsular cysts after injection into the peri-ovarian space of rats.

No. of rats	Volume (ml)	Time (weeks)	No. of cysts
4	X–35 0·025	4	4
3	X–45 0·05	5	1
4	X–165 0·025	4	2
4	N–100 0·025	4	1

X–25 is water-insoluble, X–45 is water-dispersible, X–165 and N–100 are water soluble.

Table 3. *Effect of subcapsular injections of various substances on rat ovaries.* Xylol obliterated the subcapsular space, liquid paraffin, water and dilute solutions of NaCl produced no visible changes. Three ovaries in 14 rats showed subcapsular cysts after peri-ovarian injection of 20% NaCl

No. of rats	Volume (ml)	Time (weeks)	No. of cysts
7	Xylol 0·01	6	None*
6	Paraffin 0·01	6	None
6	Distilled water 0·05	5	None
14	NaCl (20%) 0·01	5	3
6	NaCl (0·9%) 0·05	5	None
3 (14 days old)	NaCl (20%) 0·001	3	None

* 3 ovaries shrunken, subcapsular space obliterated.

Two-week old animals responded to subcapsular injections of Triton X–100 and solutions of sodium chloride in the same way as mature animals.

Indian ink and carbon black experiments. A mixture of equal parts of Indian ink and either Triton X–100 0·1 ml (4 animals) or 0·9% sodium chloride (3 animals) were injected into the subcapsular space, the animals being killed after 3 (Triton)

and 4 weeks (saline). There were no cysts in the saline-treated ovaries and one on an untreated ovary of the Triton-treated animals. For the carbon black experiments the animals were given 0·02 ml of a suspension of carbon black in either Triton X–100 (5 animals) or saline (20%) (5 animals) and after killing at 6 weeks the treated animals had 3 cysts and the controls 4 cysts. These preparations leaked rapidly through the ostium and carbon particles could not subsequently be demonstrated in the subcapsular space or in cysts which had formed, though small quantities were observed in the surrounding tissues. In several experiments where no cysts were produced it was noted that the subcapsular space had been obliterated.

Feeding experiments. The experiment involving feeding of Triton X–100 to mice (Goldhammer & others, 1967) was repeated. 14 mice fed Triton X–100 at 0·3% v/w for 26 weeks had 10 cysts while 39 control mice had 1 cyst.

Goldhammer & McManus (1960) reported that the effects of surfactants varied according to the kind of tissue to which they were applied. In their experiments the amount of Triton X–100 applied was not measured, though it was applied to a known area in a known concentration. A calculation suggested that the amount applied might be in the toxic range for an intraperitoneal application and be unrelated to the site of application. The toxicity from the application of Triton X–100 to the livers of 10 rats, and kidneys of 4 rats in the dose range 0·01 to 0·08 ml was therefore compared with the toxicity of intraperitoneal injections in 28 rats. No gross difference in toxicity could be demonstrated. At autopsy, animals dying after intraperitoneal injection of Triton X–100 showed congestion of the peritoneum and ascites with lysed blood in the fluid, and there was gross haemolysis of blood taken from the heart. The dose of Triton X–100 that killed by intraperitoneal injection was much greater (50–100 fold) than that required intravenously. We concluded that much of the acute toxicity of the Triton was a consequence of the haemolysis.

Using azovan blue (2 mg/100 g) in rats and mice fed with Triton X–100 for varying periods of time, we have not observed any permeability changes which could be attributed to the Triton though permeability increases were observed in the genital tract and gonads of animals in oestrus.

DISCUSSION

We have demonstrated that the cysts are due to fluid distension of the subcapsular space and that this is invariably associated with obstruction of the ostium. These observations are in agreement with those of Alden (1942) who occluded the rat ostium with a ligature and showed that this led to the development of a peri-ovarian cyst. We surmise that the occasional cyst filled with blood stained fluid is the result of a small amount of bleeding following follicle rupture as we have only observed this in mature animals.

In the light of Alden's work, the mechanism of production of subcapsular cysts after painting or injection experiments seems fairly clear. The Triton series of surfactants are irritant when injected into tissues and will lead to damage to the peritoneal lining with increase in permeability, leakage of fibrin and some cell necrosis. Dr. R. Cummings (personal communication) has demonstrated in the guinea-pig a large increase in vascular permeability at the site of subcutaneous injections of the Triton series of surfactants even in high dilutions. Subcutaneous

injections of undiluted surfactants led to a zone of necrosis. Cummings could not show hypersensitivity to Tritons in experiments with guinea-pigs. He observed that water-soluble Tritons were more irritant that lipid-soluble Tritons. This confirms previous reports (Finnegan & Dienna, 1953).

The mechanism of cyst formation after the feeding experiments remains obscure. Denise Madill (personal communication) using intragastric injections of tritiated Triton X–100 has shown that little labelled material was excreted in the faeces and that most of the radioactivity was recovered from the urine within 24 h. It is possible that the absorbed Triton exerts an action on serous surfaces. As the ostium is narrow and the effects of blocking it are readily observable, this may be the only site at which the effects of the Triton are grossly apparent. Tritons might exert an effect by altering the vascular permeability of the region allowing a leakage of fibrin and hence blockage of the ostium. So far we have not been able to demonstrate any alteration in permeability after azovan blue injections. The only changes observed were those associated with oestrus when there was an increase in permeability in the ovary and uterus as evidenced by a leakage of blue dye into tissues.

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The effects of some tetracyclines on synchronously growing cultures of *Escherichia coli* B/r

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Samples of synchronously growing cultures of *Escherichia coli B/r* removed at predetermined time intervals after synchronization were added to solutions containing bactericidal concentrations of tetracycline. Three active antibacterials, 7-nitro-6-demethyl-6-deoxytetracycline, 9-amino-6-demethyl-6-deoxytetracycline and 6-demethyl-6-deoxytetracycline, and one inactive compound, 7-chloroisotetracycline, were studied. Survival curves for the active agents, determined using samples of culture differing in age, were of constant slope for a given tetracycline. It is concluded that interruption of protein synthesis is the primary mode of action at bactericidal concentrations of tetracyclines, the same process as has been established for the action of the tetracyclines at the lower bacteriostatic concentrations.

Bacterial growth kinetics have been used to assess quantitatively the relative potencies of various antibacterials and also to provide insight into the possible mechanisms for action of these agents (Brown & Garrett, 1964; Jones & Morrison, 1962). Studies on tetracyclines over a wide range of concentrations suggest that at bacteriostatic concentrations these antibiotics function in one way while at bactericidal concentrations they function in another way. This suggestion is based on the observation that bacterial inhibition in the presence of bacteriostatic concentrations follows a different rate law than bacterial inactivation in the presence of bactericidal concentrations (Brown & Garrett, 1964). For low concentrations of drug there is general agreement that the primary mechanism of action is an inhibition of protein synthesis (Weisblum & Davies, 1968). With high concentrations of drug, however, evidence has been presented suggesting that inhibition of cell wall synthesis (Park, 1958), of components of the respiratory chain (Laskin, 1967), or of other biochemical systems (Laskin, 1967; Snell & Cheng, 1959), may contribute to the bactericidal effects of the tetracyclines.

Recently a number of reports dealing with the effects of various antibacterials on synchronously growing bacterial cells have appeared in the literature (Mathison, 1968; Srivastava & Thompson, 1966; Srivastava & Thompson, 1968). Cells in synchronous cultures are essentially all at the same stage in the division cycle making this technique most attractive for investigating the mode of action of the tetracyclines under bactericidal conditions. When penicillin or phenol is added as the antibacterial agent to synchronous cultures of differing age, a periodic increase and decrease in the values for the slopes of the survival curves is noted, indicating changes in the mode of action of each agent at different stages of the growth cycle (Mathison,

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1968; Srivastava & Thompson, 1966). We have looked for differences (not necessarily periodic) in the slopes of survival curves for *Escherichia coli* that could be interpreted as reflecting the inhibition of protein synthesis and one or more additional biochemical processes by tetracyclines.

EXPERIMENTAL

Materials and methods

Organism. *Escherichia coli B/r*, kindly donated by Dr. N. Yamamoto of Fels Research Institute, was used as the test organism.

Chemicals. The tetracyclines used were provided as hydrochlorides by Dr. J. H. Boothe of Lederle Laboratories. All materials used in the preparation of the media and the buffer solutions were Baker Analyzed Reagent Grade Chemicals. Agar was obtained from Bacto-Difco. The water for all solutions was distilled from an all-glass still.

Media. The liquid growth medium was K_2HPO_4 (7 g), KH_2PO_4 (3 g), $(NH_4)_2SO_4$ (3 g), $MgSO_4$ (0·08 g), Na_2SO_4 (8·5 g), Na-citrate (0·5 g), dextrose (10 g), water to 1000 ml. This was sterilized by filtration through a 0·22 μm Millipore filter under positive pressure. Solid medium was prepared by adding sterile liquid agar (sterilized by autoclaving at 15 p.s.i. for 20 min) with aseptic precautions to sterile liquid growth medium.

Diluent. A sterile phosphate buffer solution, K_2HPO_4 (7 g) and KH_2PO_4 (3 g) in 1000 ml of water (pH 7·2), was used to make dilutions for the viable cell counts.

Electrolyte solution. Suspensions of the organisms on which total counts were made using the Coulter Counter were diluted with NaCl (0·9% w/v) solution containing 0·2% (w/v) formaldehyde. This solution was filtered through two Millipore filters one of pore size 0·45 μm superimposed on one of pore size 0·22 μm for sterilization and removal of particles. The formaldehyde was included to prevent growth after sampling.

Total count method. Samples of bacterial suspension (1 ml) were diluted with electrolyte and the cells counted using a Model B Coulter Counter fitted with a 30 μm aperture tube. The operating conditions for the instrument were aperture current 0·707, amplification 1/4, lower threshold 10, upper threshold out.

Viable counting method. A spread plate technique was used. Replicate samples (0·3 ml) of suitably diluted bacterial suspensions were pipetted onto each of five overdried agar plates. The dilutions were made to obtain a maximum plate count of 500 colonies. The plates were incubated at 37° for 24 h before counting. The counts were made under direct illumination with magnification and all plates were subsequently re-examined after a further 72 h incubation for the appearance of new colonies.

Synchronization technique. Synchronized cultures were obtained using a modification of the procedure of Anderson & Pettijohn (1960). A sample of a 17 h culture was added to fresh medium at 37·5° and allowed to grow until the total count was 4×10^7 organisms/ml. This culture was then filtered under positive pressure through two 3-μm Millipore filters, one above the other, and the filtrate collected in a sterile

flask at 37·5°. The most consistent results were obtained when the filters were pre-moistened with sterile water.

Determination of bactericidal concentration. Graded concentrations (0–60 μg/ml) of each tetracycline were added to synchronous cultures and the survivors estimated by plate counts. The concentration chosen was that which reduced the number of viable organisms by a factor of 100 within 60 min (20 μg/ml).

Determination of bactericidal kinetics. Samples of a synchronized culture were removed at pre-determined times after synchronization and to these were added liquid medium containing tetracycline (at 37·5°) to give a final concentration of 20 μg/ml of drug. At timed intervals, these cultures, which were maintained at 37·5°, were sampled (1 ml) and after appropriate dilution pipetted onto agar. The number of viable organisms was estimated according to the procedure given earlier.

RESULTS

Synchronized cultures of *E. coli* at 37·5° had a doubling time of 57 min when grown in the liquid media. The synchrony could be maintained through two divisions. A representative example of the synchronization achieved is shown in Fig. 1a. Tetra-

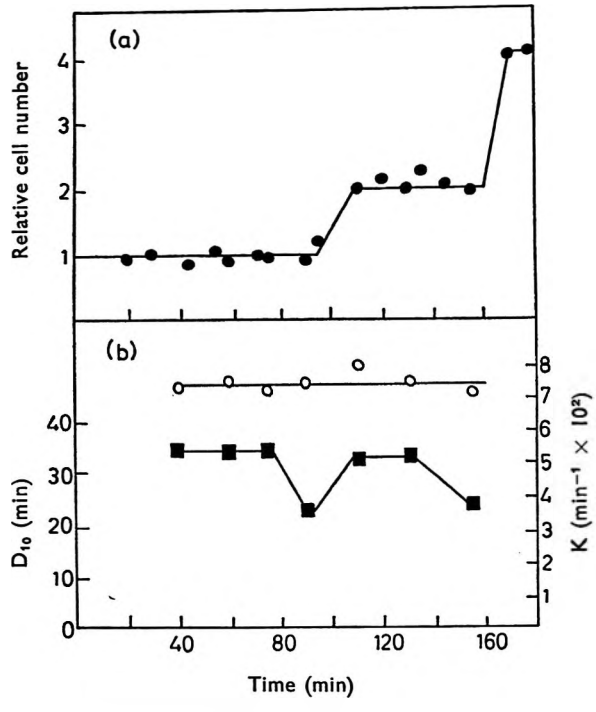

FIG. 1. (a) Cell number relative to initial innoculum at different times after synchronization; (b) Values of D_{10} (■) and apparent inactivation constants k (○) for *E. coli* B/r in the presence of 20 μg/ml of the 7-nitro-tetracycline analogue at different times after synchronization.

cyclines were not added to cultures until 30 min after synchronization since an induced lag of 30 min was consistently observed. Fig. 2 shows typical survival curves for samples of cultures exposed to the 7-nitro-substituted compound at various

times after synchronization. Survival curves for an individual tetracycline exhibited a constant lag period between the time the tetracycline was introduced into the culture sample and the time the bactericidal effects were first noted. This lag period was much shorter for those samples of culture which were taken within 5 min of division; the lag decreased by 50, 40 and 30% for the 7-nitro, 9-amino, and unsubstituted tetracyclines, respectively.

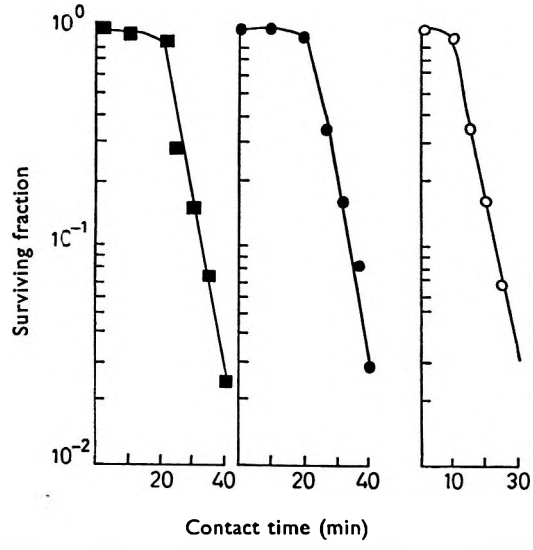

FIG. 2. Typical survival curves for synchronous *E. coli* B/r in the presence of the 7-nitro-tetracycline analogue: (■) 40 min, (●) 75 min and (○) 90 min after synchronization.

The linear portions of the survival curves were fitted by least squares regression techniques to the equation

$$\log (S/S_0) = -k\,t + \log n \qquad (1)$$

where S/S_0 is the fraction of viable cells remaining after a given contact time t, k is the apparent first-order inactivation rate constant and log n is the intercept of the extrapolated linear portion of the curve with the y-axis. A measure of the sensitivity of a given sample of culture towards the antibiotic may be expressed in terms of D_{10}, i.e. the time at which 10% of the total bacterial population still survives. Values for k, n and D_{10} appropriate to each of the tetracyclines investigated are given in Table 1.

For an individual tetracycline, the values for k did not change, showing the death rate was similar throughout the division cycle. Fig. 1b shows the relation between the age of culture after synchronization and the values for k obtained for the 7-nitro-substituted tetracycline. The values of k are in the ratio 1 : 1·57 : 2·06 for the unsubstituted, the 9-amino- and the 7-nitro-substituted tetracyclines, respectively. If the inhibition rate constants obtained by Miller, Kahlil & Martin (1970) are extrapolated to the concentration used in this study, the corresponding ratios are 1 : 1·5 : 7·9. Within the limits that the extrapolation can be considered valid, it seems that a 7-nitro-substituted tetracycline is more potent as a bacteriostatic agent than as a bactericidal agent.

Table 1. *Survival curve parameters for different tetracyclines on synchronously growing cultures of* Escherichia coli B/r *and bactericidal potencies and* π *values*

Age of culture	Tetracycline analogue[a]								
	7-Nitro-			9-Amino-			Unsubstituted		
min	D_{10}[d]	n[b]	k[c]	D_{10}[d]	n[b]	k[c]	D_{10}[d]	n[b]	k[c]
40	—	—	—	41	1·43	5·36	63	1·31	3·67
45	34	1·33	7·13	—	—	—	—	—	—
55	—	—	—	46	1·43	5·24	62	1·36	3·82
60	34	1·53	7·72	—	—	—	—	—	—
75	35	1·37	7·19	47	1·71	5·64	63	1·37	3·76
90	23	0·69	7·48	30	1·09	5·66	55	0·63	3·02
110	32	1·65	8·14	46	1·71	5·92	65	1·68	4·19
130	34	1·51	7·67	—	—	—	—	—	—
135	—	—	—	47	1·52	5·37	66	1·13	3·38
155	25	0·59	6·87	38	0·97	5·18	37	0·45	3·42
Mean value of Inactivation constant			7·45 (±0·41)			5·48 (±0·27)			3·61 (±0·37)
π			0·50			—1·10			0·00

a. 7-Chloroisotetracycline had no detectable effect on the cultures.

b. These values are to be considered as tentative since the experimental design was not ideal for the determination of extrapolation values.

c. In units of min^{-1} ($\times 10^2$).

d. In units of $min.^{-1}$

Based on D_{10} values, the sensitivity of the culture samples towards a tetracycline was unaffected by the age of the culture until 5 min before division. At this point the sensitivity increased by 23, 33 and 33% for the unsubstituted, the 9-amino- and the 7-nitro-substituted tetracyclines, respectively. The increase in sensitivity, *i.e.* the decrease in D_{10}, near division is shown in Fig. 1b for the 7-nitro-substituted tetracycline.

At times other than within 5 min of division, the mean values for n (eqn 1) are 1·37, 1·56 and 1·48 for the unsubstituted, the 9-amino- and the 7-nitro-substituted analogues, respectively. While the experimental design was not ideal for the estimation of these values (Porter, 1963) the similarity in the extrapolation values suggests that the tetracyclines tested have a common mode of action at the concentration used. Lower extrapolation values are observed for cultures that are within 5 min of division, and the similarity in the values for n at this stage is most evident for the unsubstituted and the 7-nitro-substituted compounds. The greater value for n noted at this stage for the 9-amino-derivative indicates that in this instance the culture was further from division than were the cultures in the other two instances.

DISCUSSION

From a kinetic standpoint, survival curves that differ in slope for samples of culture taken at various stages in the division cycle could be taken as evidence for at least two modes of antibacterial action. With the tetracyclines the slopes of the survival curves for a given tetracycline are essentially the same at all stages in the growth of the synchronous culture. Thus, it seems likely that the bactericidal effects of the tetracyclines are produced by the inactivation of a single biochemical process

that is continuous throughout the cell cycle. As tetracyclines in bacteriostatic concentrations are known to inhibit protein synthesis (Laskin, 1967; Snell & Cheng, 1959; Weisblum & Davies, 1968), and protein synthesis occurs continually throughout the cell cycle (Maaløe & Kjeldgaard, 1966), it appears that the bactericidal effects of the tetracyclines arise primarily because of an inactivation of protein synthesis. It is suggested that an inhibition (short of total inactivation) of protein synthesis by tetracyclines leads to bacteriostasis while the inactivation of protein synthesis by tetracyclines affords a bactericidal effect.

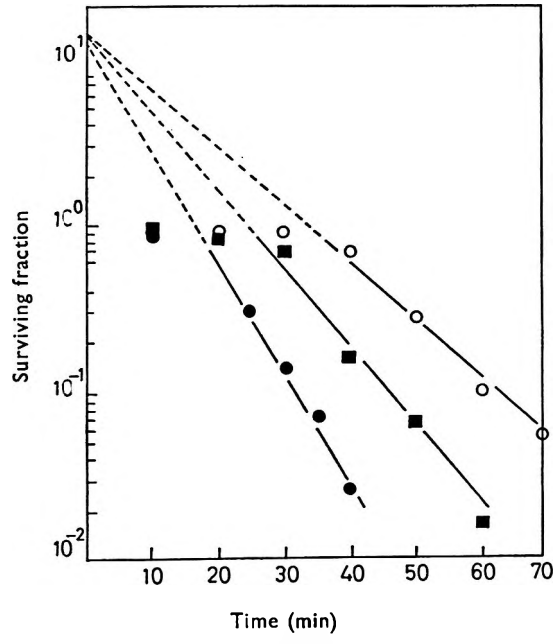

FIG. 3. Superposition of survival curves for synchronous cultures of *E. coli* B/r in the presence of 20 μg/ml, 7-nitro- (●), the 9-amino- (■) and the unsubstituted (○) tetracycline analogues, at times other than within 5 min of division.

The intercept of the linear portion of the survival curve with the line representing the initial population number (Fig. 3) may be regarded as the lag period between the time a synchronous culture is first placed in contact with a solution containing tetracycline and the ideal time when the total bacterial population first experiences the bactericidal effects of the tetracyclines. This lag time may be interpreted as the period during which a pool of available protein is utilized. It is longer for the less potent tetracyclines since these compounds are inefficient inhibitors of protein synthesis. Near division, the lag time is shortened and this reflects an increase in the rate at which protein is used. In support of this interpretation are four observations: (1) the accepted primary mode of tetracycline action is the inhibition of aminoacyl-tRNA addition to the 30s ribosomal subunit (Weisblum & Davies, 1968); (2) the bacteriostatic and bactericidal potencies of the tetracyclines are linearly related (Cammarata, Yau & others, 1969); (3) RNA synthesis continues at an unreduced rate after protein synthesis has been interrupted and diminishes in rate as the pool

of available protein becomes exhausted (Maaløe & Kjeldgaard, 1966); (4) the rate of RNA synthesis increases at division (Maaløe & Kjeldgaard, 1966).

It could be argued that the differing lag times observed for each tetracycline may be a consequence of their relative ability to penetrate the bacterial cell wall. The diminished lag time near division would then be a consequence of the increased porosity of the cell wall towards the tetracyclines at this stage. However, the slopes of the survival curves k are inversely proportional to the lag times, and there was no correlation between these inactivation constants and the π values for the substituents. [π is a measure of the lipophilicity imparted by a substituent and is defined by $\pi = \log P_X - \log P_H$ where P is the partition coefficient for a substituted member X and the parent H of a congeneric series (Hansch, 1968.)] A similar lack of correlation between the bacteriostatic potencies for 11 tetracyclines and π has been noted by Cammarata & Yau (unpublished). On this basis it appears that passive transport of the tetracyclines through the bacterial cell wall is not a primary factor determining the lag time.

Rate determining active transport through the bacterial cell wall could also account for the differing lag times, but, near division, either an increase in the rate of active transport or a change in the rate determining step must take place and the net effect would be to decrease the lag time. Then the overall mechanism of action would be changed near division and should be reflected by a change in the slope of the survival curve at this stage. Since no such change is observed, it appears likely that the inhibition of protein synthesis is the rate determining step which affects the lag time.

The lag periods reported by Mathison (1968) for chloramphenicol differ from those reported here in that his lag periods varied with stage while with the tetracyclines the lag is essentially invariant with stage up to division. It is possible that this difference may be a consequence of the different sites of action of these two antibiotics at the ribosomal level (Weisblum & Davies, 1968).

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The inhibition of the development of the spores of a spoilage mould by chemical preservatives

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The swelling characteristics of developing spores from a variety of spoilage moulds have been examined using the Coulter Counter. *P. spinulosum* spores began swelling earliest and had the most rapid swelling rate. For these reasons these spores were the most suitable for rapid electronic sizing and were used to assess preservative efficiency. The preservatives examined were propylhydroxybenzoate, Phenonip (a mixture of esters of hydroxybenzoic acid and β-phenoxyethylalcohol, Nipa Laboratories Ltd.) and benzoic acid. The first two preservatives reduce the rate of swelling of germinating spores at its onset while benzoic acid increases the time before swelling begins. Suppression of spore swelling provides early indication of preservative potential and the type of swelling curve produced indicates the mechanism of fungistasis.

The swelling of mould spores during germination and immediately preceding emergence is characteristic of most species, an exception being the *Erysiphaceae* (Brodie & Neufeld, 1942). This early development is suppressed by antimicrobial agents and measurement of changes in volume of spores has been used for rapid assessment of fungitoxicity by Mandels & Darby (1953). They determined spore volume using haematocrit tubes. Controlled conditions of deposition were essential because errors could result when the spores packed unevenly, a situation exacerbated by the production of germ tubes. Volume changes of spores during germination were determined with greater sensitivity using a microscope (Barnes & Parker, 1966).

Unlike the volume, the average diameter of developing spores increases linearly with time in agreement with the theory of mould growth kinetics of Emerson (1950), and this linear increase allows statistical and comparative estimations of fungistatic activity to be made. A refinement of the measuring technique was introduced by Barnes & Parker (1967), who used the Coulter Counter for automatic size analysis of spores.

Our previous work, confined to *Trichoderma* species, indicated that the type of swelling curve obtained in preservative systems provided insight into the mechanism of fungistasis. We now describe the swelling characteristics of spores of several species of spoilage mould and the use of one of these for fungitoxic assays. The effects of some preservatives on the chosen spore have been examined.

EXPERIMENTAL

Moulds. These were isolated from a variety of spoiled cosmetic and pharmaceutical preparations and characterized as *Penicillium spinulosum*, *Penicillium roqueforte*, *Aspergillus versicolor*, *Syncephalastrum racemosum*, *Mucor spinosus* and *Trichoderma viride*.

Spore suspensions were prepared by the method of Barnes & Parker (1968) from the moulds grown on Oxoid malt extract agar for 21 days at 25°.

Methods

Sizing of spores. Spores developing in static malt broth cultures at 25° were sized, using a Coulter Counter Model B (with a Model J plotter), as described by Parker, Barnes & Bradley (1966).

Linear growth determinations. The method of Mandels & Darby (1953) was used; the inoculum placed centrally on the surface of a wort agar (Oxoid) plate. Plates were incubated at 25° and periodic measurements of the developing colonies were made using calipers to gauge two diameters at right angles. Three replicate plates were used for each determination.

Preservatives. Solutions were prepared in sterile water of benzoic acid B.P., propylhydroxybenzoate B.P. and Phenonip (the trade name for an undefined mixture of esters of hydroxybenzoic acid and β-phenoxyethylalcohol, Nipa Laboratories Ltd.).

To measure their effects upon spore swelling, solutions of preservatives at double the overall required concentrations were mixed with aliquots of double strength malt broth as previously described (Parker & others, 1966). The pH of all reaction mixtures was measured.

To measure preservative effects upon linear growth, double strength solutions were mixed with double strength wort agar.

RESULTS

The rate of swelling of the various mould spores in static malt broth cultures is shown in Fig. 1.

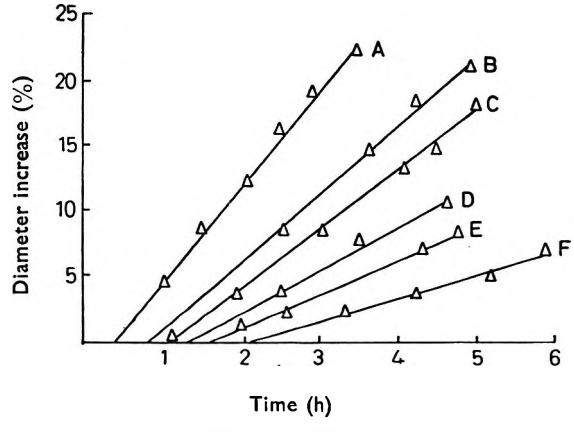

FIG. 1. Rates of spore swelling in malt broth measured with the Coulter Counter.
A, *P. spinulosum*; B, *S. racemosum*; C, *T. viride*; D, *P. roqueforte*; E, *A. versicolor*; F, *M. spinosus*.

P. spinulosum was chosen as the test organism for fungitoxic determinations because its spores showed the greatest size increase in the shortest time.

The effects of preservatives at various concentrations upon the swelling of *P. spinulosum* spores are shown in Figs 2A, B and 3. The pH values of the preservative–broth systems were all in the range 4–4·5.

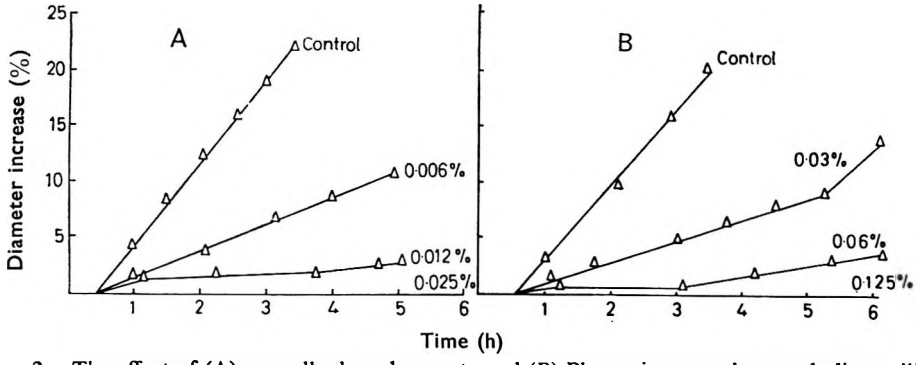

FIG. 2. The effect of (A) propylhydroxybenzoate and (B) Phenonip upon the metabolic swelling of *P. spinulosum*. Concentrations of preservative expressed as % w/v.

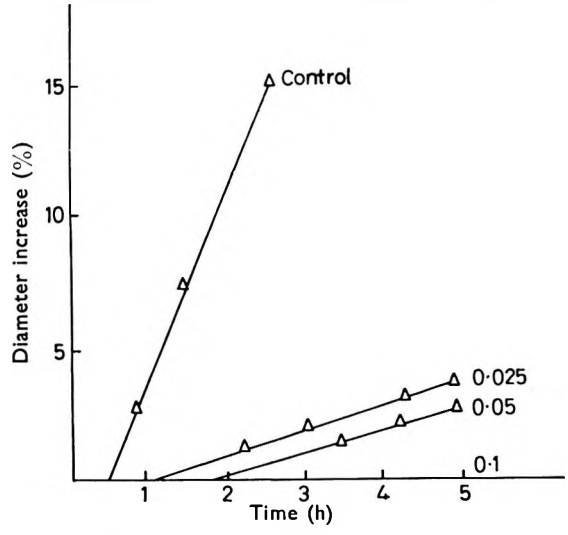

FIG. 3. The effect of benzoic acid upon metabolic swelling of *P. spinulosum*. Concentrations of preservative expressed as % w/v.

Linear growth rates of colonies from the fastest (*P. spinulosum*) and the slowest (*M. spinosus*) developing spores are shown in Fig. 4. The effect of preservatives upon linear growth rate of *P. spinulosum* is shown in Fig. 4b–d.

DISCUSSION

The spores of all the moulds examined showed a linear increase in diameter with time. Such swelling when measured optically is detected immediately (Barnes & Parker, 1966), but when using the Coulter Counter there is some delay before it is apparent. The duration of this delay ranges from 25 min for spores of *P. spinulosum* to 120 min for *M. spinosus* (Fig. 1).

It has been suggested that there is a period of spore swelling in the malt broth when the osmotic pressure of the spore contents is attaining a value isotonic with the saline electrolyte used in electronic sizing. Until this isotonicity is reached, swelling will not be detected electronically (Barnes & Parker, 1967). That this is purely an osmotic phenomenon, is indicated by the fact that the primary swelling is not suppressed by

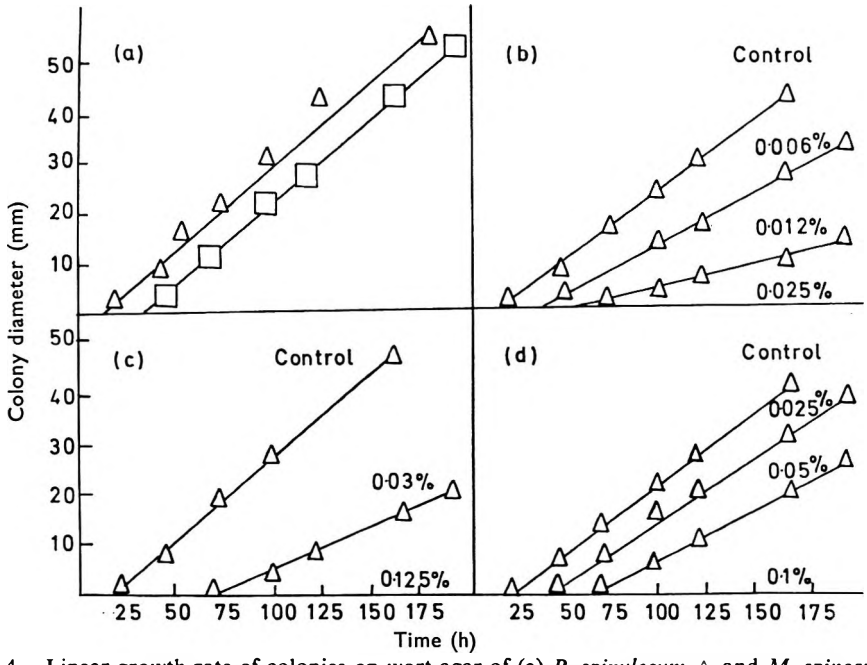

FIG. 4. Linear growth rate of colonies on wort agar of (a) *P. spinulosum* △ and *M. spinosus* □, (b) *P. spinulosum* in the presence of propylhydroxybenzoate, (c) Phenonip and (d) benzoic acid. Concentrations of preservative expressed as % w/v.

preservatives as is the subsequent swelling measured here with the Coulter Counter (Barnes & Parker, 1966). It is interesting to compare the swelling process in mould and bacterial spores, for although in the latter an osmotic phenomenon of the type described does not occur, there is an initial phase in the swelling which is insensitive to preservatives (Parker, 1969).

Since we are concerned here with the swelling phase that is susceptible to preservative action, it will be referred to as "metabolic swelling" to distinguish it from the early non-susceptible phase.

The shorter the lag period before the onset of metabolic swelling the greater the rate of the swelling (Fig. 1). The duration of this period of osmotic adjustment may reflect some efficiency of adaptation to nutrients available since in subsequent linear growth there is a time lag of some 20 h between onset of visible growth from the fastest and slowest swelling spores (Fig. 4a).

In choosing one of the spoilage moulds as a test organism, the spores most suited to the technique will be those which show swelling detectable with the Coulter (metabolic swelling) as early as possible and then swell at a maximal rate. Apart from these criteria, based upon expediency, evidence has been cited that indicates that when moulds are developing at their optimal rate fungicides have least effect. Conversely, if any of the factors which influence growth rate are changed to lessen the rate, then the resistance of the mould is also decreased (Tomkins, 1929). *P. spinulosum* was chosen as the test organism for fungitoxic estimations since it best meets the criteria required.

In general the effect of a preservative is to depress the rate of metabolic swelling and if present in sufficient concentration to suppress it completely.

Propylhydroxybenzoate depresses the rate of swelling at 0·006% and prevents it completely at 0·025% (Fig. 2A). The intermediate concentration (0·012%) depresses the rate of swelling and imposes an apparent lag, during which no swelling is detected, before some partial recovery occurs. Any detectable swelling, however slight, will result in eventual outgrowth and colony formation on solid medium. Thus, linear growth determinations confirm that concentrations of the ester below 0·025% allow colony formation (Fig. 4b). The depression of the swelling rate of the spore is followed by the delayed appearance of mycelial development in much the same way that slow swelling spore species lag behind faster swelling forms.

Phenonip may impose an early lag in swelling (0·06%), or at a lower concentration (0·03%) it initially reduces the rate of swelling but with an indication of later recovery (Fig. 2B). Linear growth measurements again confirm that the concentration sufficient to prevent swelling (0·125%) allowed no colony formation (Fig. 4c).

Benzoic acid differed from the other preservatives in that it extended the lag period before which the onset of metabolic swelling was detected with the Coulter. Swelling then followed at a reduced rate depending upon the ambient concentration of the preservative (Fig. 3). The pH of the reaction mixture (4·2) allowed some 50% of acid to be available as the active (undissociated) molecule. Linear growth was prevented by a concentration of 0·1% and was delayed by lower levels of the preservative (Fig. 4d).

Both propylhydroxybenzoate and phenonip, which contains parabens, reduce the rate of metabolic swelling at its onset. At concentrations of these agents which almost completely suppress this swelling, the rate is so reduced that apparent lags are imposed before size increase of spores can be detected (Fig. 2A,B). This may reflect a difference in action against different phases in the swelling process (Barnes, 1968) or some degree of adaptation to the preservative.

Benzoic acid, in prolonging the period before metabolic swelling commences, delays the initiation of the animate phases of germination. After this delay, however, the subsequent vegetative growth rate is little affected by the preservative (Fig. 4d). Increased concentrations of the acid will, of course, completely suppress metabolic swelling and vegetative growth.

The method of fungitoxic assay described will rapidly provide information of effective preservative levels and can be adapted to predict effects of extraneous additives such as non-ionic (Parker & others, 1966) and anionic (Parker, McCafferty & MacBride, 1968) surfactants.

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Anti-inflammatory properties of a series of phenyl- and phenoxy-alkanoic acids

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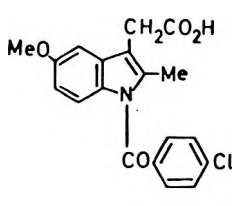
Some *o*-benzamidophenoxyacetic, phenylalkanoic and phenoxy-alkanoic acids have been synthesized. Anti-inflammatory activity was measured by the phenylbenzoquinone writhing test in mice and the rat foot oedema test. *Meta*- and *para*-substitutions in the benzamido-ring, promoting lipid solubility, enhanced the potency, whereas substitution with polar groups reduced it. Further phenyl ring substitution in the 2-(3,4-dichlorobenzamido)phenoxyacetic acids only slightly affected the potency. Side-chain modifications did not increase the activity on the three substituted phenoxyacetic acids chosen. Two phenylpropionic acids showed a good order of activity but the respective cinnamic acids were virtually inactive. From the investigations 2-(3,4-dichlorobenzamido)phenoxyacetic acid (SNR. 1804) was selected for further studies is now undergoing clinical evaluation.

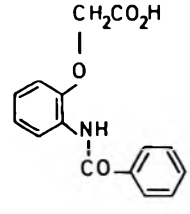
The anti-inflammatory activity of indomethacin (I) (Shen, 1963) promoted a search for this activity in related compounds in which the indole nucleus was replaced with simpler aromatic systems.

By analogy with the plant growth hormones, where activity is found both in 3-indoleacetic acids and in phenoxyacetic acids, it seemed reasonable to consider the phenoxyacetic structure as a basis for the design of potential anti-inflammatory agents, and Northover & Subramanian (1961) had already demonstrated such activity in some substituted α-phenoxypropionic acids.

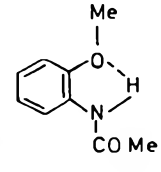
The introduction of a benzamido-group in the *ortho*-position of a phenoxyacetic acid would yield compound (II) with some formal resemblance to the structure of indomethacin (I).



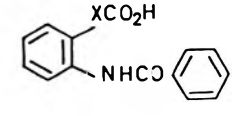
I



II



III



IV

That this structural comparison might have some validity was supported by studies by Ungnade (1954) on the ultraviolet absorption spectra of substituted acetanilides. Ungnade examined the spectra of *o*-, *m*-, and *p*-alkyl, halo- and methoxy substituted acetanilides. In these, the introduction of such groups *ortho* led to a decrease in intensity of absorption and a shift towards lower wavelength (compared with

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corresponding *m*- or *p*-compounds). This was interpreted as a steric effect in which the bulky *ortho* substituent twists the acetamido group out of the plane of the ring. Such an effect was not observed with *o*-methoxyacetanilide, and Ungnade suggested that the formation of a weak hydrogen bond stabilized the compound in the non-hindered conformation (III).

A series of *o*-benzamidophenoxyacetic acids was prepared and many of these showed anti-inflammatory activity. To investigate further the structural features necessary for activity, compounds with the general formula (IV) were prepared including phenylacetic ($X = CH_2$), β-phenylpropionic ($X = CH_2CH_2$), and cinnamic ($X = CH{=}CH$) acids, as well as extensions of the phenoxyacetic ($X = OCH_2$) series to include α- and ω-phenoxyalkanoic acids.

CHEMISTRY EXPERIMENTAL

The compounds of Tables 1 and 2 were prepared by several methods, which are illustrated in the following examples.

Method A

2-(o-*Chlorobenzamido*)*phenoxyacetic acid* (*cpd no.* 3). *o*-Nitrophenoxyacetic acid (39·2 g; 0·2 mol) was dissolved in aqueous NaOH (250 ml; 0·8N) and platinum oxide catalyst (0·5 g) was added. The solution was hydrogenated at room temperature and pressure, the solution was filtered from catalyst and aqueous NaOH (40 ml; 5N) added. To this solution was added *o*-chlorobenzoyl chloride (35·0 g; 0·2 mol), with stirring at 10° during 15 min. The solution was stirred for a further 2 h at 10–20° and extracted with $CHCl_3$ (2 × 200 ml). Evaporation gave an oil which was extracted with boiling di-n-butyl ether (300 ml). On cooling crystals, m.p. 123–5° (26·2 g), were obtained. The crude material was recrystallized from di-n-butyl ether (200 ml) and then from ethyl acetate (70 ml) to yield pure cpd no. 3 as colourless crystals, m.p. 143–4° (10·1 g; 17%). ($C_{15}H_{12}ClNO_4$) C, H, N.

Method B

2-(3,4-*Dichlorobenzamido*)-4-*methoxyphenoxyacetic acid* (*cpd no.* 34). 2-*Chloroacetamido*-4-*methoxyphenol.* To a solution of 2-amino-4-methoxyphenol (46·0 g; 0·33 mol) in dry Me_2CO (300 ml) containing Et_3N (33·5 g; 0·33 mol) was added chloracetyl chloride (37·5 g; 0·33 mol) dropwise with stirring at 5–10° during 30 min. The solution was stirred for a further 30 min at 10° and the Me_2CO removed under reduced pressure. H_2O (1 litre) was added to the solid which was filtered, washed (H_2O) and dried to give the product m.p. 173–6·5° (70·3 g; 98·5%). An analytical sample was prepared by two further recrystallizations from EtOH–H_2O (2:1 v/v) to give 2-chloroacetamido-4-methoxyphenol as pale yellow needles, m.p. 183–4°. ($C_9H_{10}ClNO_3$) C, H, N.

2,3-*Dihydro*-6-*methoxy*-1,4-*benzoxazin*-3-*one.* 2-Chloroacetamido-4-methoxyphenol (53·9 g; 0·2 mol) was dissolved in boiling EtOH (420 ml) and aqueous NaOH (560 ml; N) was added. The solution was boiled under reflux for 15 min and neutralized to pH 7 by the addition of dilute HCl (280 ml; N). After standing overnight at 5° the solid was filtered, washed with water and dried. The crude product was recrystallized from benzene (charcoal) to give 2,3-dihydro-6-methoxy-1,4-benzoxazin-3-one as colourless needles, m.p. 168–9° (31·3 g; 70%). ($C_9H_9NO_3$) C, H, N.

2-(3,4-*Dichlorobenzamido*)-4-*methoxyphenoxyacetic acid.* 2,3-Dihydro-6-methoxy-1,4-benzoxazine-3-one (8·95 g; 0·05 mol) was boiled under reflux for 2 h with aqueous NaOH (50 ml; 5N). To the solution was added H_2O (50 ml) and dilute HCl (40 ml; 5N) and the solution was cooled to 10°. A solution of 3,4-dichlorobenzoyl chloride (10·47 g; 0·05 mol) in Et_2O (50 ml) was added during 1½ h at 10° with stirring at the same time maintaining pH 7–9 by the simultaneous addition of aqueous NaOH (10 ml; 5N). During the course of the reaction the sodium salt precipitated and a further 150 ml H_2O was added to facilitate stirring. The solution was stirred at 10–20° for a further 1 h and the sodium salt was filtered, dissolved in Me_2CO–H_2O (150 ml, 1:1 v/v) and acidified to pH 3 with HCl. The solid was filtered, washed (H_2O) and dried (13·2 g), m.p. 182–7°. The crude product was recrystallized from methyl ethyl ketone to give pure cpd no. 34 as colourless needles, m.p. 195–6° (7·7 g; 42%). An analytical sample was prepared by a further recrystallization from methyl ethyl ketone to give colourless needles, m.p. 196–7°. ($C_{16}H_{13}Cl_2NO_5$) C, H, N.

Method C

γ-[2-(m-*Trifluoromethylbenzamido*)-4-*chlorophenoxy*]*butyric acid* (*cpd no.* 47). 2-(m-*Trifluoromethylbenzamido*)-4-*chlorophenol.* To a solution of 2-amino-4-chlorophenol (7·16 g; 0·05 mol) in tetrahydrofuran (THF) (100 ml), containing *NN*-dimethylaniline (5·85 g; 0·05 mol) was added a solution of *m*-trifluoromethylbenzoyl chloride (10·5 g; 0·05 mol) in THF (50 ml) at 5–10° with stirring during 45 min. The solution was stirred for a further 1 h at 10° and the THF removed under reduced pressure. The resulting oil was poured into H_2O (500 ml) containing concentrated HCl (50 ml) and the precipitate was filtered and washed (H_2O). The crude solid was dissolved in Me_2CO (200 ml) and added to aqueous NaOH (1 litre; 0·125N). To this solution was added solid CO_2 with occasional stirring to pH 6. The precipitated 2-(*m*-trifluoromethylbenzamido)-4-chlorophenol was filtered, washed with water (100 ml) and dried, m.p. 190–1° (13·9 g; 88%). An analytical sample was prepared by a further recrystallization from EtOH–H_2O (1:1 v/v) to give colourless needles, m.p. 201–2°. ($C_{14}H_9ClF_3NO_2$) C, H.

γ-(2-(m-*Trifluoromethylbenzamido*)-4-*chlorophenoxy*)*butyric acid.* Sodium (0·92 g; 0·04 mol) was dissolved in n-BuOH (200 ml), and to the warm solution was added 2-(*m*-trifluoromethylbenzamido)-4-chlorophenol (12·62 g; 0·04 mol). To this solution was added ethyl γ-chlorobutyrate (6·02 g; 0·04 mol) and sodium iodide (6·0 g; 0·04 mol) and the solution heated under reflux for 8½ h. After cooling and filtration, the solvent was removed under reduced pressure to give the crude ester. This was dissolved in EtOH (200 ml) containing aqueous NaOH (9 ml; 5N) and boiled under reflux for 1 h. The solution was cooled, poured into dilute HCl (20 ml; 5N), and the solid filtered. The crude product was dissolved in EtOAc (200 ml) and extracted three times with dilute NH_4OH (200 ml, 2 × 100 ml). The combined ammoniacal extract was acidified with concentrated HCl with cooling. The solid was filtered and washed (H_2O), dissolved in hot HOAc (100 ml) with charcoal, filtered, and H_2O (200 ml) added gradually with heating. On cooling, cpd no. 47 was obtained as colourless needles, m.p. 148–50° (4·82 g; 30%). An analytical sample was prepared by a further recrystallization from HOAc–H_2O (1:1 v/v) to give colourless needles, m.p. 150–1°. ($C_{18}H_{15}ClF_3NO_4$) C, H, N.

Method D

β-(2-(3,4-Dichlorobenzamido)-4-methylphenoxy)propionic acid (*cpd no.* 43). *β-(4-Methyl-2-nitrophenoxy)propionic acid.* To a solution of 4-methyl-2-nitrophenol (51·0 g; 0·33 mol) in aqueous NaOH (200 ml; 1·67N) at 96° was added β-propiolactone (24 g; 0·33 mol) dropwise with stirring during 30 min at 96–102°. The mixture was stirred for a further 20 min at 96°, cooled and acidified with dilute HCl. An orange oil separated, which was extracted with Et_2O (3 × 100 ml). The Et_2O solution was extracted with saturated $NaHCO_3$ solution and the aqueous layer acidified with HCl to pH 3. The yellow solid was filtered, washed and dried. The crude product was recrystallized from HOAc–H_2O (1 : 2 v/v) to give pure β-(4-methyl-2-nitrophenoxy)-propionic acid as yellow needles, m.p. 111–2° (24·2 g; 32%). ($C_{10}H_{11}NO_5$) C, H, N.

β-[2-(3,4-Dichlorobenzamido)-4-methylphenoxy]propionic acid. A solution of β-(4-methyl-2-nitrophenoxy)propionic acid (11·25 g; 0·05 mol) in aqueous NaOH (200 ml; 0·25N) was hydrogenated over 3% Pd/C (1 g), at room temperature and atmospheric pressure. The catalyst was filtered and the filtrate was treated with dilute HCl (10 ml; 5N). The H_2O was removed under reduced pressure and the resulting white solid was dried, triturated with THF (220 ml) and the NaCl filtered. To the filtrate was added *NN*-dimethylaniline (13·3 g; 0·11 mol) followed by 3,4-dichlorobenzoyl chloride (10·5 g; 0·05 mol) in THF (30 ml) dropwise over 30 min at 10–15° with stirring. The mixture was stirred for a further 2 h at 10–20°, the solvent removed under reduced pressure and the residue dissolved in EtOH (50 ml). The EtOH solution was added with stirring to dilute HCl (500 ml; 0·4N) and the solid was filtered, washed (H_2O) and dried. The crude product (14 g) was recrystallized from HOAc–H_2O (1 : 1 v/v) to give β-[2-(3,4-dichlorobenzamido)-4-methylphenoxy]-propionic acid as colourless needles, m.p. 158–9° (9·5 g; 52%). ($C_{17}H_{15}Cl_2NO_4$) C, H, N.

Method E

4-Chloro-2-(m-trifluoromethylbenzamido)phenylacetic acid (*cpd no.* 50). *Ethyl-4-chloro-2-nitrophenylacetate.* 4-Chloro-2-nitrophenylacetic acid [lit. m.p. 166–8°—Wright & Collins, 1956] (38·7 g; 0·18 mol) in EtOH (400 ml) containing conc. H_2SO_4 (1 ml) was boiled under reflux for 2½ h. The solution was poured onto crushed ice (1000 g), the suspension Et_2O extracted, the extracts combined and washed with saturated $NaHCO_3$ solution, H_2O, and then dried (Na_2SO_4). Removal of the Et_2O under reduced pressure left a solid which was crystallized from EtOH (40 ml) by cooling to 5° to give ethyl 4-chloro-2-nitrophenyl acetate (32·6 g; 74%), m.p. 41–2°. Recrystallization from EtOH gave material of m.p. 42–43°. ($C_{10}H_{10}ClNO_4$) C, H, N.

Ethyl-4-chloro-2-(m-trifluoromethylbenzamido)phenylacetate. Ethyl-4-chloro-2-nitrophenylacetate (10 g; 0·041 mol) was dissolved in EtOH (140 ml) and catalytically reduced over platinum oxide (0·1 g). The catalyst was filtered and solvent removed under reduced pressure and the residual oil was dissolved in Me_2CO (100 ml) containing Et_3N (4·15 g; 0·041 mol) and cooled to 5°. A solution of *m*-trifluoromethylbenzoyl chloride (3·4 g; 0·040 mol) in Me_2CO (50 ml) was added slowly to the amine solution over 1 h, and the mixture then boiled under reflux for 1 h, and left overnight. The resulting solution was poured onto crushed ice (1500 g), the mixture extracted with Et_2O, the extracts combined, washed with H_2O and dried (Na_2SO_4). The Et_2O was removed under reduced pressure to leave a red oil (15·5 g) which was

dissolved in light petroleum (60–80°) (50 ml) and cooled to give a buff solid. This was recrystallized from EtOAc–light petroleum (60–80°) (1:4 v/v) to give ethyl 4-chloro-2-(*m*-trifluoromethylbenzamido)phenylacetate (4·5 g; 28·5%), m.p. 83–85°. Recrystallization from EtOAc-light petroleum (1:4 v/v) gave pink needles, m.p. 86–88°. ($C_{18}H_{15}ClF_3NO_3$) C, H.

4-*Chloro*-2-(m-*trifluoromethylbenzamido*)*phenylacetic acid* (*cpd no.* 50). Ethyl-4-chloro-2-(*m*-trifluoromethylbenzamido)phenylacetate (4·18 g; 0·0108 mol) was dissolved in Me_2CO (50 ml) and aqueous KOH (7 ml; 1·18N) added with stirring, keeping the temperature below 10°. The reaction mixture was left overnight and the Me_2CO removed under reduced pressure to leave red oil. H_2O (60 ml) was added and the mixture extracted with Et_2O. The aqueous solution was acidified with HCl (5N) to give a buff coloured solid (3·61 g). Recrystallization from Me_2CO (charcoal) gave cpd no. 50 (3·08 g; 86%), m.p. 185–6°. Recrystallization from Me_2CO–light petroleum (60–80°) (30 ml; 1:1 v/v) gave material m.p. 187–188°. ($C_{16}H_{11}ClF_3NO_3$) C, H, N.

Method F

β-[4-*Chloro*-2-(m-*trifluoromethylbenzamido*)*phenyl*]*propionic acid* (*cpd no.* 53). 7-Chloro-3,4-dihydrocarbostyril (C.A. 66, 37125h, (1967) (8·35 g; 0·046 mol) was boiled under reflux for 16 h with aqueous NaOH (50 ml; 5N). The solution was diluted with H_2O (50 ml), cooled to 10°, and HCl (5N) added dropwise to bring the pH to 8–9. The mixture was treated with a solution of *m*-trifluoromethylbenzoyl chloride (9·3 g; 0·046 mol) in Me_2CO (15 ml) maintaining the temperature at 10° and the pH at 8–9 by the simultaneous addition of NaOH (N). After a further 2 h stirring the mixture was filtered, extracted with Et_2O (2 × 50 ml) and the aqueous phase acidified to pH 2 with conc. HCl. The precipitated solid was filtered, washed (H_2O) and dried to give a solid (5·65 g) which after recrystallization from Me_2CO–H_2O (2:1 v/v) gave cpd no. 53 as white plates (3·54 g; 21%), m.p. 158–60°. ($C_{17}H_{13}ClF_3NO_3$) C, H, N.

Method G

4-*Chloro*-2-(m-*trifluoromethylbenzamido*)*cinnamic acid* (*cpd no.* 56). 4-*Chloro*-2-*nitrocinnamic acid.* 4-Chloro-2-nitrobenzaldehyde (15·7 g; 0·085 mol) and malonic acid (17·6 g; 0·169 mol) were added to pyridine (30 ml) containing piperidine (2 ml). The mixture was heated for 3 h on a steam bath, cooled and poured into water (300 ml) containing conc. HCl (100 ml). The precipitated solid was filtered, washed with water and dried to give 4-chloro-2-nitrocinnamic acid (13·8 g; 72%). A portion recrystallized from benzene–light petroleum (40–60°) (1:1 v/v) gave material with m.p. 214° (Van der Lee, 1926).

2-*Amino*-4-*chlorocinnamic acid.* A solution of 4-chloro-2-nitrocinnamic acid (17·64 g; 0·078 mol) in NH_4OH (170 ml; 5N) was added dropwise with stirring to a solution of ferrous sulphate heptahydrate (174 g; 0·625 mol) in water (260 ml). NH_4OH solution (65 ml) was added and the mixture heated for 10 min on a steam bath, cooled and centrifuged. The supernatant was removed and the solid stirred with NaOH (500 ml; N) and again centrifuged. The supernatants were combined, concentrated to 400 ml under reduced pressure, the pH adjusted to 5 with HCl (5N) and the precipitate filtered, dissolved in NH_4OH (5N) and reprecipitated with HOAc to give material (11·67 g; 76% yield), m.p. 203–4°. Recrystallization from Et_2OH

gave yellow needles, m.p. 209–10°. A satisfactory analysis for 2-amino-4-chlorocinnamic acid could not be obtained.

4-*Chloro*-2-(m-*trifluoromethylbenzamido*)*cinnamic acid* (*cpd no.* 56). A solution of *m*-trifluoromethylbenzoyl chloride (5·3 g; 0·025 mol) in Me_2CO (30 ml) was added dropwise with stirring to a solution of 2-amino-4-chlorocinnamic acid (5·0 g; 0·025 mol) aqueous NaOH (40 ml; 0·625N). The pH of the reaction was maintained at 8 by the dropwise addition of an aqueous NaOH (40 ml; 0·625N). After being stirred for 30 min the reaction mixture was adjusted to pH 2 with conc. HCl. The precipitate was filtered, washed (H_2O), dried, and recrystallized from HOAc to give cpd no. 56 (0·31 g; 35% yield), m.p. 227–8°. ($C_{17}H_{11}ClF_3NO_3$) C, H, N.

PHARMACOLOGY EXPERIMENTAL

Methods

Acute toxicity. Male albino, Smith and Nephew Research (SNR) strain mice, 25–30 g were used, 4 animals per dose level. Approximate LD50 values were determined by inspection from mortalities occurring within 3 days of oral or intraperitoneal administration.

Phenylbenzoquinone (*PBQ*) *writhing test in mice.* Female albino SNR strain mice, four to six weeks old were used. A modification of the method of Siegmund, Cadmus & Lu (1957) was used. Mice were injected with PBQ 35 min after the oral administration of the anti-inflammatory compound. The mice were then observed during the 5 min period at which maximum writhing rate occurred in control animals.

(*a*) Screening test. This was a sequential test based upon the quantal responses of up to four successive groups of six mice. All compounds were screened at an oral dose of 2/5 LD50. All compounds which protected four or more mice out of twelve were examined further by a potency estimation.

(*b*) Potency. The relative potencies of the accepted compounds were determined using the method of Hendershot & Forsaith (1959) in which the dose which reduces the writhing rate by 50% is obtained from dose response curves constructed using groups of 10 mice.

Rat foot oedema (*carrageenan*) *test.* A modification of the method of Winter, Risley & Nuss (1962) was used. The initial foot volume of the rats was determined volumetrically. 0·1 ml of a suspension of carrageenan (1% in normal saline) was injected subcutaneously into the plantar region of the right hind paw 1 h after the test compounds at 50 mg/kg had been administered orally. Three h later the foot volume was again measured and the volume of oedema determined. Results were expressed as percentage inhibition related to a control oedema volume.

The oedema volume in control and treated animals was compared using students *t*-test.

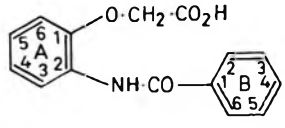
Compound no. 12 (SNR 1804; clamidoxic acid) was selected for further evaluation by the mouse tail pinch method of Bianchi & Franchescini (1954), a modified cotton wool pellet granuloma test (Winter & Porter, 1957), fever induction (Brownlee, 1939) and gastric haemorrhage in guinea-pigs (Anderson, 1963).

RESULTS AND DISCUSSION

Although the phenylbenzoquinone writhing syndrome is known to be non-specific in its response to drugs, results from this test were used to guide the synthetic

program for the following reasons. Evidence from the inflamed rat paw test, and other anti-inflammatory tests made on selected compounds suggested that in this series of compounds the activity being detected by the PBQ assay was in fact anti-inflammatory activity. Furthermore, in our hands the test has been found to yield reproducible results and good dose-response lines even with comparatively weak anti-inflammatory agents, whereas we have found it difficult to obtain reproducible quantitative data with the rat paw test.

Table 1. *2-Benzamido-phenoxyacetic acids.* Compounds were prepared by method B, except nos. 1 and 3 which were prepared by method A. All compounds had C, H, N analyses within the usual limits. All compounds except nos. 19, 21, 28 and 29 had oral LD50 doses of 1000 mg/kg or greater; those for 19, 21, 28 and 29 were 750, 750, 600 and 700 mg/kg respectively.



Comp. No.	Subt. Ring A	Subt. Ring B	m.p. °C	Formula	PBQ test (oral) Mice protected at 2/5 LD50	PBQ test (oral) 50% reduction in writhing dose (mg/kg)	Rat foot test*
1	—	—	164	$C_{15}H_{13}NO_4$	4/12	140	19—
2	—	4-Me	198	$C_{16}H_{15}NO_4$	19/24	40	22—
3	—	2-Cl	143	$C_{15}H_{12}ClNO_4$	0/6	NT	0
4	—	3-Cl	161	$C_{15}H_{12}ClNO_4$	15/18	40	0
5	—	4-Cl	215	$C_{15}H_{12}ClNO_4$	9/18	100	0
6	—	3-Br	158	$C_{15}H_{12}BrNO_4$	10/12	40	24+
7	—	4-Br	210	$C_{15}H_{12}BrNO_4$	11/12	75	11—
8	—	4-F	183	$C_{15}H_{12}FNO_4$	11/12	40	8—
9	—	4-I	192	$C_{15}H_{12}INO_4$	5/6	35	0
10	—	2,4-Cl_2	196	$C_{15}H_{11}Cl_2NO_4$	0/6	NT	0
11	—	2,5-Cl_2	188	$C_{15}H_{11}Cl_2NO_4$	0/6	NT	0
12	—	3,4-Cl_2	218	$C_{15}H_{11}Cl_2NO_4$	12/12	31	39+
13	—	3,5-Cl_2	237	$C_{15}H_{11}Cl_2NO_4$	10/12	15	2—
14	—	3-CF_3	160	$C_{16}H_{12}F_3NO_4$	14/18	29	0
15	—	2-OH	162	$C_{15}H_{13}NO_5$	8/12	49	0
16	—	4-OH	162	$C_{15}H_{13}NO_5$	3/12	NT	0
17	—	4-$MeSO_2$	177	$C_{16}H_{15}NO_6S$	2/12	NT	0
18	4-Me	—	147	$C_{16}H_{15}NO_4$	8/12	50	8—
19	4-Me	4-Cl	174	$C_{16}H_{14}ClNO_4$	11/12	11	5—
20	4-Me	3,4-Cl_2	218	$C_{16}H_{13}Cl_2NO_4$	12/12	40	37+
21	4-Me	3-CF_3	161	$C_{17}H_{14}F_3NO_4$	12/12	27	0
22	5-Me	3,4-Cl_2	233	$C_{16}H_{13}Cl_2NO_4$	4/12	49	0
23	4-Et	3,4-Cl_2	209	$C_{17}H_{15}Cl_2NO_4$	12/12	19	29+
24	4-CH<Me, Et	3,4-Cl_2	138	$C_{19}H_{19}Cl_2NO_4$	12/12	38	11—
25	4-CMe_3	3,4-Cl_2	182	$C_{19}H_{19}Cl_2NO_4$	19/24	50	0
26	3,5-Me_2	3,4-Cl_2	229	$C_{17}H_{15}Cl_2NO_4$	6/18	62	0
27	4,5-Me_2	3,4-Cl_2	235	$C_{17}H_{15}Cl_2NO_4$	7/12	95	0
28	4-Cl	—	166	$C_{15}H_{12}ClNO_4$	15/18	85	46+
29	4-Cl	4-Cl	164	$C_{15}H_{11}Cl_2NO_4$	16/24	150	35±
30	4-Cl	3,4-Cl_2	208	$C_{15}H_{10}Cl_3NO_4$	12/12	29	56+
31	4-Cl	3-CF_3	159	$C_{16}H_{11}ClF_3NO_4$	11/12	32	49+
32	4-Cl	4-MeO	195	$C_{16}H_{14}ClNO_5$	15/24	100	24+
33	4-Br	3,4-Cl_2	200	$C_{15}H_{10}BrCl_2NO_4$	7/12	32	24±
34	4-MeO	3,4-Cl_2	196	$C_{16}H_{13}Cl_2NO_5$	7/12	37	14±
35	5-MeO	4-Cl	207	$C_{16}H_{14}ClNO_5$	11/12	67	0
36	5-MeO	3,4-Cl_2	222	$C_{16}H_{13}Cl_2NO_5$	8/12	77	0
37	4-OH	3,4-Cl_2	254	$C_{15}H_{11}Cl_2NO_5$	6/12	130	0
38	5-OH	3,4-Cl_2	221	$C_{15}H_{11}Cl_2NO_5$	0/12	NT	0

* Oral, % reduction in oedema at 50 mg/kg.
NT = not tested.

Anti-inflammatory activity was found in most of the basic structural types examined. The unsubstituted *o*-benzamidophenoxyacetic acid (cpd 1, Table 1) showed activity of about the same order as aspirin or phenylbutazone, and the most active of the substituted derivatives (cpd 13, 19, 50, 52, 53) were approximately ten times as potent.

Substitution in Ring B

The introduction of a single halogen atom in Ring B in the *m*- and *p*-positions generally increased potency up to three fold (Table 1, cpd 4, 5, 6, 7, 8, 9) whereas similar substitution in the *o*-position destroyed activity (cpd 3). In a series of dichloro-derivatives the presence of a Cl atom in the *o*-position again yielded inactive compounds (cpd 3, 10, 11) whereas the 3,4- and 3,5-compounds (12, 13) had 10 and 4 times the potency of the unsubstituted parent. Of other substituents examined, those promoting lipid solubility yielded compounds with increased activity, whereas polar substituents gave inactive compounds (16, 17). A simple regression analysis was made on the first 17 compounds of Table 1 excluding those with an *o*-substituent (cpd 3, 10, 11, 15), using log 1/ID50 as an expression of biological activity, against the Hansch substituent constant, π (Hansch & Fujita, 1964). The equation obtained was $\log 1/ID50 = 0{\cdot}55\pi - 2{\cdot}14$ with correlation coefficient = 0·87.

Substitution in Ring A

Only two compounds (18, 28) were prepared with substitution (Me, Cl) in Ring A alone. Both of these were more potent than the unsubstituted parent, suggesting that here also groups promoting lipid solubility yielded compounds with increased potency. Ring A variations were studied more extensively in a group of compounds with 3,4-Cl_2 substitution in Ring B. The introduction of further substituents (alkyl, halogen, methoxy) affected activity only slightly and generally adversely, but again the introduction of a hydroxyl group (cpd 37, 38) reduced activity markedly.

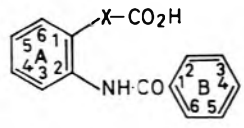
Modification of side-chain

To examine the effect of variations in side-chain linking Ring A to the carboxyl group, three representative substituted phenoxyacetic acids were chosen (cpd 12, 20, 31), as bases for modification. α-Alkyl substitution had little effect on activity (cpd 39–41) (Table 2) whereas lengthening of the side-chain, as in the β-phenoxypropionic acids (cpd 42–44) and γ-phenoxybutyric acids (cpd 45–47) caused a significant reduction in activity, the magnitude of the effect depending on the individual ring substituents.

Removal of the ether oxygen atom yielded phenylacetic (cpd 48–50) and phenylpropionic (cpd 51–53) acids. Two of the latter compounds, in which the "chain length" was similar to that of the phenoxyacetic acids, were among the most potent of the series. In contrast, the cinnamic acid analogues (cpd 54–56) were virtually inactive. These latter compounds would be expected to have a relatively rigid *trans*-configuration thus fixing the position of the carboxyl group relative to the planar ring skeleton, and lack of activity suggests that the position and overall separation of the carboxyl group relative to the ring system has a considerable bearing on the level of potency within this class of compound.

Clamidoxic acid was shown, using the mouse tail pinch method of Bianchi & Franchescini (1954) not to possess central anti-nociceptive activity and it seems reasonable

Table 2. *2-Benzamidophenylalkanoic and -phenoxyalkanoic acids.* All compounds had C, H, N analyses within the usual limits. All compounds except nos. 41, 50 and 56 had LD50 doses of 1000 mg/kg or greater; those for 41, 50 and 56 were 700, 750 and 250 mg/kg respectively.



Compound No.† method	Subt. Ring A	Subt. Ring B	X	m.p.	Formula	PBQ test (oral) Mice protected at 2/5 LD50	PBQ test (oral) 50% reduction writhing dose (mg/kg)	Rat foot test‡
39 B*	—	3,4-Cl_2	–O–CH(Me)	171°	$C_{16}H_{13}Cl_2NO_4$	8/12	47	0
40 B*	4-Me	3,4-Cl_2	–O–CH(Me)	175°	$C_{17}H_{15}Cl_2NO_4$	12/12	39	0
41 C	4-Cl	3-CF_3	–O–CH(Et)	132°	$C_{18}H_{15}ClF_3NO_4$	11/12	40	19–
42 D	—	3,4-Cl_2	–O–$[CH_2]_3$–	160°	$C_{16}H_{13}Cl_2NO_4$	11/12	55	0
43 D	4-Me	3,4-Cl_2	–O–$[CH_2]_3$–	158°	$C_{17}H_{15}Cl_2NO_4$	8/12	120	10–
44 D†	4-Cl	3-CF_3	–O–$[CH_2]_3$–	197°	$C_{17}H_{12}ClF_3NO_4$	2/12	NT	9–
45 C	—	3,4-Cl_2	–O–$[CH_2]_2$–	126°	$C_{17}H_{15}Cl_2NO_4$	16/18	80	0
46 C	4-Me	3,4-Cl_2	–O–$[CH_2]_2$–	150°	$C_{18}H_{17}Cl_2NO_4$	8/12	40	17–
47 C	4-Cl	3-CF_3	–O–$[CH_2]_2$–	150°	$C_{18}H_{14}ClF_3NO_4$	16/18	70	0
48 E	—	3,4-Cl_2	$-CH_2-$	189°	$C_{16}H_{11}Cl_2NO_3$	13/18	80	0
49 E	4-Me	3,4-Cl_2	$-CH_2-$	178°	$C_{16}H_{13}Cl_2NO_3$	11/12	40	19–
50 E	4-Cl	3-CF_3	$-CH_2-$	188°	$C_{16}H_{11}ClF_3NO_3$	11/12	14	7–
51 F	—	3,4-Cl_2	$-CH_2-CH_2-$	185°	$C_{16}H_{13}Cl_2NO_3$	2/12	NT	12–
52 F	4-Me	3,4-Cl_2	$-CH_2-CH_2-$	183°	$C_{17}H_{15}Cl_2NO_3$	12/12	6	37+
53 F	4-Cl	3-CF_3	$-CH_2-CH_2-$	159°	$C_{17}H_{12}ClF_3NO_3$	11/12	15	68+
54 G	—	3,4-Cl_2	–CH=CH–	275°	$C_{16}H_{11}Cl_2NO_3$	4/12	160	0
55 G	4-Me	3,4-Cl_2	–CH=CH–	263°	$C_{17}H_{13}Cl_2NO_3$	2/12	NT	0
56 G	4-Cl	3-CF_3	–CH=CH–	227°	$C_{17}H_{11}ClF_3NO_3$	3/12	NT	0
	Aspirin					—	110	27+
	Phenylbutazone					—	100	26+
	Indomethacin					—	1·3	27+§

*α-Chloropropionylchloride was used for these two compounds.
† The intermediate 3-(4-chloro-2-nitrophenoxy)-propionic acid was reduced with sodium dithionite instead of catalytically.
‡ Oral, % reduction in oedema at 50 mg/kg. *P* values for *t*-tests on rat foot oedema data are as follows: + = $P<0·05$ ± = $P<0·1$ to $>0·05$ – = $P>0·1$.
§ Indomethacin dose = 1·0 mg/kg orally.
NT = not tested.

to suggest that its activity in the PBQ writhing test is due to its anti-inflammatory activity. In this test clamidoxic acid was found to be more active than phenylbutazone, acetylsalicylic acid and flufenamic acid and less active than indomethacin.

The order of activity of clamidoxic acid was also established in the carrageenan rat foot oedema test where it was shown to be more active than acetylsalicylic acid, equivalent to phenylbutazone and much less active than indomethacin. Clamidoxic acid maintained its activity in the carrageenan foot test in adrenalectomized rats, indicating that its anti-inflammatory activity is not mediated via the adrenal cortex.

The acute response to clamidoxic acid in the carrageenan rat foot test was not modified by pretreatment of the animals with clamidoxic acid.

A modification of the cotton wool pellet granuloma test (Winter & Porter, 1957) was used to grade the activity of clamidoxic acid which was approximately equivalent to phenylbutazone and less active than indomethacin.

Clamidoxic acid was half as effective as aspirin and one quarter as active as phenylbutazone as an antipyretic agent in the fever test in rats (Brownlee, 1939).

Clamidoxic acid does not produce gastric haemorrhages in guinea-pigs to the same extent as acetylsalicylic acid and indomethacin (Anderson, 1963) and was significantly less toxic than acetylsalicylic acid in subacute toxicity tests in rats.

This compound was therefore selected to undergo intensive pharmacological and toxicological testing and is now undergoing clinical evaluation in rheumatic and allied inflammatory conditions.

Table 3. *Intermediates not listed in the literature*

Intermediate for compound	Compound	m.p.	Molecular formula	Analyses
23	2,3-Dihydro-6-ethyl-1,4-benzoxazin-3-one	160–1°	$C_{10}H_{11}NO_2$	C, H, N
	2-Chloracetamido-4-ethylphenol	153–4°	$C_{10}H_{12}ClNO_2$	C, H, N
24	2,3-Dihydro-6-s-butyl-1,4-benzoxazin-3-one	85–6°	$C_{12}H_{15}NO_2$	C, H, N
	2-Chloracetamido-4-s-butylphenol	193–4°	$C_{12}H_{16}ClNO_2$	C, H, N
25	2,3-Dihydro-6-t-butyl-1,4-benzoxazin-3-one	151–2°	$C_{12}H_{15}NO_2$	C, H, N
	2-Chloracetamido-4-t-butylphenol	225–6°	$C_{12}H_{16}ClNO_2$	C, H, N
26	2,3-Dihydro-5,7-dimethyl-1,4-benzoxazin-3-one	205–9°	$C_{10}H_{11}NO_2$	C, H
27	2,3-Dihydro-6,7-dimethyl-1,4-benzoxazin-3-one	250–1°	$C_{10}H_{11}NO_2$	C, H, N
40	α-Chloro-2-hydroxy-5-methyl-propionanilide	121–2°	$C_{10}H_{12}ClNO_2$	C, H, N
41	2-(*m*-Trifluoromethylbenzamido)-4-chlorophenol	201–2°	$C_{14}H_9ClF_3NO_2$	C, H, N
43	β-(4-Methyl-2-nitrophenoxy)propionic acid	111–2°	$C_{10}H_{11}NO_5$	C, H, N
44	β-(4-Chloro-2-nitrophenoxy)propionic acid	127–8°	$C_9H_8ClNO_5$	C, H, N
45	2-(3,4-Dichlorobenzamido)phenol	193–5°	$C_{13}H_9Cl_2NO_2$	C, H, N
46	2-(3,4-Dichlorobenzamido)-4-methyl-phenol	230–1°	$C_{14}H_{11}Cl_2NO_2$	C, H, N
48	Ethyl 2-(3,4-dichlorobenzamido)-phenylacetate	124–5°	$C_{17}H_{15}Cl_2NO_3$	C, H, N
49	Ethyl 2-(3,4-dichlorobenzamido)-4-methylphenylacetate	134–5°	$C_{18}H_{17}Cl_2NO_3$	C, H, N
50	Ethyl-4-chloro-2-(*m*-trifluoromethyl-benzamido)phenylacetate	86–8°	$C_{18}H_{15}ClF_3NO_3$	C, H, N
56	2-Amino-4-chlorocinnamic acid	209–10°	$C_9H_8ClNO_2$	C, H

The respective phenols required for the synthesis of compounds 26, 27 and 33 were not isolated. The phenol required for compound 47 was the same as used for compound 41.

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The preparation and intravenous anaesthetic activity of tetrahydrofuran-3-ols

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A number of tetrahydrofuran-3-ols have been prepared and examined for intravenous anaesthetic activity. The compounds studied had low intrinsic anaesthetic activity and there was an inverse relation between anaesthetic activity or toxicity and solubility in water. It was considered unlikely that compounds comparable in activity to thiopentone or methohexitone would be found in this series.

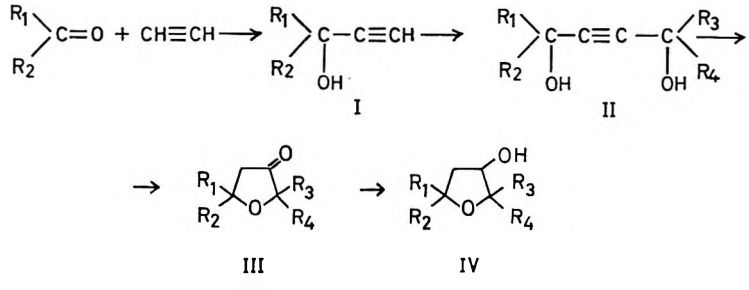
Many unrelated chemical compounds cause general anaesthesia in animals, and attempts have been made to relate their physical properties to their anaesthetic activity (Butler, 1950; Miller, Paton & Smith, 1965). From these studies it may be concluded that any anaesthetic compound must be both soluble in lipid materials and sufficiently soluble in water to enable it to reach its site of action. Thus, any anaesthetic compound could be given intravenously provided it was sufficiently soluble in water.

The general anaesthetic, diethyl ether, which is usually inhaled, can cause anaesthesia when injected intravenously in aqueous solution (Adams, 1944; Butt, Ochs & others, 1965). But diethyl ether is not very potent and only poorly soluble in water. Tetrahydrofuran (THF), the cyclic analogue of diethyl ether, also causes anaesthesia when inhaled (Stoughton & Robbins, 1936; Henderson & Smith, 1936) or injected intravenously in aqueous solution (see this paper); unlike diethyl ether, THF is very soluble in water. Accordingly, we decided to prepare a number of novel substituted derivatives of THF with the object of increasing anaesthetic activity and examining the relation between solubility in water and activity. Simple alkyl substituted THF's were insoluble in water, but the addition of a hydroxyl group to the ring overcame this problem, and resulted in the series of tetrahydrofuran-3-ols now examined.

CHEMISTRY EXPERIMENTAL

Synthetic methods

Preparation of the tetrahydrofuranols.



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The foregoing general method which was used for the synthesis of the furanols can lead to a number of isomeric forms. At the ring closure stage hydration of the triple bond takes place and this can lead, when at least one of R_1, R_2, R_3 and R_4 are different, to two position isomers where the ketone group is either in the 3 or 4 position. Reduction of the ketones to alcohols can lead to a further series of geometrical isomers.

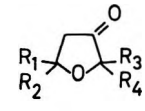
General preparative methods

Ethynyl alcohols (I). These were prepared by the literature methods.

Acetylenic diols (II). These were prepared by the general method of Dupont (1913). Most of these intermediates have been described in the literature; those that were new were generally cyclized to the furanone without purification.

Tetrahydrofuranones (III). The acetylenic diols were ring closed to the furanones (Table 1) by the general method of Dupont (1913).

Table 1. *The chemical characteristics of some tetrahydrofuran-3-ones.* Analyses for C and H are within the usual limits



R_1	R_2 (R_4 = H)	R_3	b.p. °C/mm Hg	Formula
Me	H	Pent	104–110/10	$C_{10}H_{18}O_2$
Et	Et	Et	120–130/80	$C_8H_{14}O_2$
Me	isoBu	H	85–89/10	$C_9H_{16}O_2$
Me	Pr	H	70–73/10	$C_8H_{14}O_2$
Me	H	Bu	98–102/16	*
Pr	Pr	H	95–98/10	$C_{10}H_{18}O_2$
Pr^i	Pr^i	H	85–90/10	$C_{10}H_{18}O_2$
Me	Nonyl	H	150–154/8	$C_{14}H_{16}O_2$
Me	Me	Et	99–103/90	*
Et	Et	Me	75–80/21	$C_9H_{16}O_2$
Me	Et	Me	105–110/50	$C_9H_{16}O_2$
Me	Me	Pr	67–71/10	$C_9H_{16}O_2$
Me	Me	Pr^i	64/10	$C_9H_{16}O_2$
Et	Et	Et	96–101/18	$C_{10}H_{18}O_2$
Me	Me	Bu	84/9	$C_{10}H_{18}O_2$
Me	Et	Pr	214–218/760	$C_{10}H_{18}O_2$
Et	Pr	Me	98–111/20	$C_{10}H_{18}O_2$
Me	Pr	Et	95–98/17	$C_{10}H_{18}O_2$
Spirocyclopentyl		Me		*
Et	Et	Me (R_4 = Me)	100–110/20	*

* Used without purification.

Tetrahydrofuranols (IV). Most of the tetrahydrofuranols were prepared by hydrogenation of the corresponding tetrahydrofuranone in ethanol in the presence of a suitable catalyst.

3-*Ethyl*-2,2,5-*trimethyl-tetrahydrofuran*-3-*ol* (19) was prepared by the action of ethyl magnesium bromide on 2,2,5-trimethyl-tetrahydrofuran-3-one.

2,2-*Diethyl*-5-*methyl-tetrahydrofuran*-3,4-*diol* (27). 2,2-Diethyl-5-methyl-tetrahydrofuran-3-one was brominated at steam bath temperature with 4 mol of bromine. The reaction mixture was steam distilled to give a heavy oil. The crude distillate, 4,4-dibromo-2,2-diethyl-5-methyl-tetrahydrofuran-3-one, was refluxed until complete solution was obtained. This solution was extracted with ether to give 3-ethyl-2,2,5-trimethyl-tetrahydrofuran-3,4-dione, b.p. 140–144°/15 mm. Found: C, 62·8; H, 8·3. $C_9H_{14}O_3$ requires C, 63·5; H, 8·2. The tetrahydrofuran-3,4-dione was catalytically reduced to give the diol (27).

3-Ethynyl-2,2,5,5-tetramethyl-tetrahydrofuran-3-ol (42). 2,2,5,5-Tetramethyl tetrahydrofuran-3-one was reacted with ethynyl magnesium bromide to give the corresponding furan-3-ol (42).

2,2,5,5-Tetramethyl-tetrahydro-3-hydroxy-3-furanyl methyl ketone (43). 3-Ethynyl-2,2,5,5-tetramethyl-tetrahydrofuran-3-ol was hydrated by the method of Dupont (1913) to give the corresponding methyl ketone (43).

Solubility in water. Solubility in water was determined at room temperature (20–24°).

Partition coefficients. These were determined in carbon tetrachloride-water at room temperature (20–24°).

PHARMACOLOGY EXPERIMENTAL

Intravenous anaesthetic activity in mice

The technique adopted was similar to that used by Wirth & Hoffmeister (1965) in the rabbit.

Anaesthesia was assessed in mice by observing the loss of the righting reflex, the fore and hind limb toe pinch reflexes and the corneal reflex. Anaesthesia was defined as the loss of all these reflexes. The time taken for each reflex to return was noted during the experiment. Side-effects were also noted and scored.

All compounds were dissolved in distilled water and used immediately. Injections were given into a lateral tail vein in a volume of 0·2 ml/20 g of mouse. Using 10 mice per dose level, the dose which caused anaesthesia in 50% of mice injected (AD50), and the dose which killed 50% of mice injected (LD50) within 2 h was determined. The figures were calculated using the method of Litchfield & Wilcoxon (1949). The therapeutic index was defined as the ratio of the LD50 to the AD50.

The results were analysed using a PDP.8 computer. Regression lines were calculated using a least squares fit assuming the relation between the data would be expressed by a polynomial equation of the form

$$Y = X(0) + X(2)x + X(2)x^2 \ldots + X(m)x^m$$

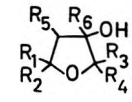
for powers 0 to 7. Using the coefficients determined for each equation, the variance of the points about each line was calculated for each power. The variances were then compared for significant diminution in size.

RESULTS

The anaesthetic activity (AD50), acute intravenous toxicity (LD50) and therapeutic index of each compound were determined in mice. The results have been summarized in Table 2. Data on diethyl ether, THF and two barbiturate intravenous anaesthetics, methohexitone and thiopentone, have been included for comparison purposes.

Most of the compounds prepared possessed anaesthetic activity and some, 5, 9 and 18, were found to have particularly high therapeutic indexes in comparison with thiopentone and methohexitone. However, the tetrahydrofuran-3-ols were much less active than the barbiturate anaesthetics. All the compounds examined induced anaesthesia very rapidly. Often the animal lost its righting reflex before the injection was completed. Induction was usually accompanied by convulsive side-effects which varied in severity from compound to compound (Table 2). Recovery from anaesthesia was also rapid; most animals recovered their righting reflex within 15

Table 2. *The chemical characteristics and intravenous anaesthetic activity in the mouse of some tetrahydrofuran-3-ols.* Data on diethyl ether, tetrahydrofuran, methohexitone and thiopentone are included for comparison purposes. T.I. = therapeutic index, S.E. = side-effects, Sol. = solubility in water (%). Analyses for C and H are within the usual limits



No.	R_1	R_2	R_3 ($R_4=R_5=R_6=H$)	b.p. °C/mm Hg	Formula	AD50	LD50	T.I.	S.E.	Sol.
	Diethyl ether					202	440	2·2	+	7
	Tetrahydrofuran					607	759	1·2	++	100
	Thiopentone sodium					54·5	68·0	1·2	+	>10
	Methohexitone					17·0	36·5	2·1	+	>10
1	H	H	H	Wynberg, 1958		3850	3850	1·0	+++	100
2[1]	H	H	H	Hanschke, 1955		1340	1760	1·3	+++	20
3	Me	H	H	Curtis & others, 1962		2400	4150	1·7	+++	20
4	H	H	Me	96–97/25	$C_5H_{10}O_2$	4000	4360	1·1	+++	>10
5	Me	H	Me	185/760	$C_6H_{12}O_2$	550	1750	3·2	++	20
6	Me	H	Pent	74–75/0·06	$C_{10}H_{20}O_2$	54	64	1·2	++++	0·2
7	Pr	H	Pr	118–121/15	$C_{10}H_{20}O_2$	69	75	1·1	+++	0·2
8	Me	Me	H	Colonge & others, 1958		900	1460	1·6	+++	10
9	Me	Et	H	Colonge & others, 1958		305	890	2·9	+++	10
10	Et	Et	H	95–98/11	$C_8H_{16}O_2$	185	310	1·7	+++	2
11	Me	Bu[i]	H	109/9	$C_9H_{18}O_2$	175	175	1·0	+	1
12	Me	Pr	H	103–104/10	$C_8H_{16}O_2$	224	318	1·4	++	2
13	Me	Bu	H	117–119/20	$C_9H_{18}O_2$	77	138	1·8	++	0·4
14	Spirocyclohexyl		H	Colonge & others, 1958		225	360	1·6	++	2
15	Pr	Pr	H	121/10	$C_{10}H_{20}O_2$	84	124	1·5	++	1
16	Pr[i]	Pr[i]	H	118/10	$C_{10}H_{20}O_2$	138	138	1·0	+	1
17	Me	Nonyl	H	110/0·1	$C_{14}H_{28}O_2$	188	188	1·0	+	•
18	Me	Me	Me	82–83/30	$C_7H_{14}O_2$	650	1900	2·9	+	>25
19[14]	Me	H	Me	55–62/0·15	$C_6H_{12}O_2$	387	620	1·6	++	7
20	Me	Me	Et	80–88/11	$C_8H_{16}O_2$	625	1530	2·4	++	5
21	Me	Et	Me	117–119/38	$C_8H_{16}O_2$	340	920	2·7	++	100
22	Et	Et	Me	103/5	$C_9H_{18}O_2$	177	220	1·2	+	0·5
23[3]	Et	H	Et	120/35	$C_6H_{12}O_2$	204	385	1·9	+	2
24	Me	Me	Pr	92–95/6	$C_9H_{18}O_2$	130	300	2·3	+	2
25	Me	Me	Pr[i]	90/12	$C_9H_{18}O_2$	190	292	1·5	++	5
26	Me	Pr	Me	102/10	$C_9H_{18}O_2$	180	225	1·4	+−	3
27[2]	Et	Et	Me	86–90/0·2	$C_9H_{18}O_2$	470	530	1·4	+	20
28	Et	Et	Et	99–103/9	$C_{10}H_{20}O_2$	116	186	1·6	+	1
29	Me	Me	Bu	101/10	$C_{10}H_{20}O_2$	99	137	1·4	+++	0·5
30	Me	Et	Pr	108/8	$C_{10}H_{20}O_2$	130	175	1·3	++++	1
31	Et	Pr	Me	115–119/22	$C_{10}H_{20}O_2$	195	235	1·2	++	•
32	Me	Pr	Et	114/19	$C_{10}H_{20}O_2$	130	240	1·8	+	1
33	Spirocyclopentyl		Me	117–120/8	$C_9H_{16}O_2$	330	490	1·5	++	20
34[4]	Me	Me	Me	Korbitsyna & others, 1960		720	1250	1·7	+	10
35[5]	Me	Me	Me	Heuberger & Owen, 1952		5000	5000	1·0	+	10
36[6]	Me	Me	Me	m.p. 207–209°	$C_9H_{17}O_3$	4000	4000	1·0	++	10
37[7]	Me	Me	Me	Sulzbacher & Bergmann, 1953		746	1030	1·4	+++	10
38[8]	Me	Me	Me	m.p. 131–135°	$C_7H_{14}O_2$	3230	3230	1·0	+	2
39[9]	Me	Me	Me	m.p. 80–84°	$C_9H_{16}O_2$	480	480	1·0	+	1
40[10]	Et	Et	Me	110–115/10	$C_{10}H_{20}O_2$	125	145	1·2	+++	0·5
41[11]	Me	Et	Me	Dupont, 1913		133	250	1·9	+	2
42[12]	Me	Me	Me	m.p. 86–88°	$C_{10}H_{16}O_2$	220	410	1·8	+	2
43[13]	Me	Me	Me	m.p. 46–52°	$C_{10}H_{18}O_3$	390	715	1·8	+	5
44[14]	Me	Me	Me	Sulzbacher & Bergmann, 1953		300	300	1·0	+−+	7
45[15]	Et	Et	Et	128-129/16	$C_{13}H_{24}O_2$	58	82	1·4	++	0·3
46[16]	Spirocyclohexyl			Korbitsyna & others, 1960		2·5	2·5	1·0	+++	0·7

* Dissolved in 10% Cremophor E.L.
† = No side-effects. + = ataxia, slight tremors. ++ = slight convulsions, marked ataxia on recovery. +++ = convulsions, marked ataxia on recovery. ++++ = severe convulsions, rigidity and gross ataxia on recovery.
[1] R_5 = Me. [2] R_4 = Me. [3] R_5 = OH. [4] R_4 = Me. [5] R_4 = Me, R_5 = OH. [6] R_4 = Me, R_5 = $CONH_2$, R_6 = OH. [7] R_4 = R_6 = Me. [8] R_4 = Me, R_5 = CH_2OH. [9] R_4 = R_5 = Me. [10] R_4 = Me. [11] R_4 = Et. [12] R_4 = Me, R_6 = −C≡CH. [13] R_4 = Me, R_6 = COMe. [14] R_4 = Me, R_6 = Et. [15] R_4 = Et. [16] R_4 = spirohexyl.

min and appeared normal 30–45 min later. Animals which had received lethal doses of these compounds died of respiratory failure. However, there was no indication that anaesthesia was due to anoxia when sub-lethal doses were administered.

A number of these compounds were examined in the rabbit, cat or monkey but in all species side-effects, similar to those seen in the mouse, were observed.

The partition coefficients (carbon tetrachloride–water) of ether and tetrahydrofuran were 11·0 and 3·0 respectively. Tetrahydrofuran, despite its high solubility in water, had a partition coefficient approximately one third that of ether.

DISCUSSION

It is generally accepted that a suitable balance of lipophilic and hydrophilic properties is essential for any compound to cause anaesthesia. THF appeared to fulfil this requirement as it was both soluble in carbon tetrachloride and water and had a reasonably high partition coefficient in the system carbon tetrachloride–water. We therefore hoped that alkyl substituted derivatives of tetrahydrofuran would have more anaesthetic activity than the parent compound whilst retaining high solubility in water.

In the series of tetrahydrofuran-3-ols examined, there was an inverse relation between solubility in water and anaesthetic activity (AD50) (Fig. 1A) or toxicity

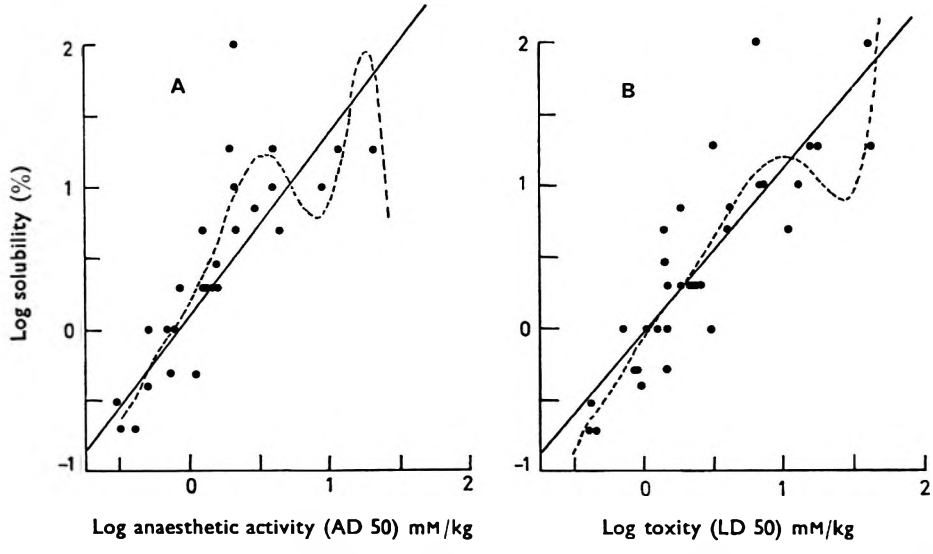


FIG. 1. A. Log anaesthetic activity (AD50) versus log per cent solubility in water. The solid line represents the normal regression line and is of unit slope. The broken line represents the regression line calculated for a 7th power polynomial equation. The variance about the normal regression line was not significantly reduced by including higher powers.

B. As above except that log toxicity (LD50) is plotted against log solubility in water. The solid line is of unit slope.

(LD50) (Fig. 1B). Similar findings have been reported for paraffin hydrocarbons (Fuhner, 1921) and a series of ethers (Cone, Forman & Krantz, 1941) though Miller & others (1965) found no such relation among the compounds they investigated. A regression line of the form

$$Y = X(0) + X(1)x$$

gave a line with a variance which was not significantly reduced by including higher powers of x. Thus if it is assumed that the thermodynamic activity of each compound is proportional to its solubility in water, then the tetrahydrofuran-3-ols adhere quite closely to Ferguson's principle (Ferguson, 1939). But this in no way precludes any

relations between activity and lipid solubility (Meyer, 1937), or solubility in organic solvents (Miller & others, 1965), or surface activity (Butler, 1950) or some other physical property. However, it does suggest that the tetrahydrofuran-3-ols cause anaesthesia in a non-specific manner. An investigation of the anaesthetic activity of the configurational isomers of some of the 2,5-disubstituted derivatives would confirm this conclusion.

Further analysis of the data obtained from this series showed that there was no relation between therapeutic index and solubility in water or anaesthetic activity.

In view of the low intrinsic anaesthetic activity of the tetrahydrofuran-3-ols and the inverse relation between activity and solubility in water, we concluded that it was unlikely that compounds comparable in activity to thiopentone or methohexitone would be found in this series.

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The distribution of chloroquine in man after fatal poisoning

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Detailed analysis of autopsy specimens from two fatal cases of chloroquine poisoning is reported. Post-mortem blood levels of 1·60 and 1·24 mg chloroquine/100 ml blood were found; results for liver blood, urine, stomach contents, liver, lungs and kidneys are given. The highest chloroquine concentration was in liver and the presence of a metabolite was demonstrated in the tissues.

Published information on the distribution of chloroquine [7-chloro-4-(4′-diethylamino-1′-methylbutylamino)quinoline] in the tissues and body fluids of man is incomplete, as it relates only to plasma concentrations of the drug after oral dosage, or to occasional reports of post-mortem concentrations in some tissues. We have examined the distribution of the drug in blood, urine, stomach contents, lung, liver, kidneys, heart and brain in two acute fatal overdose cases.*

Chloroquine is not a common suicidal agent (in the U.K.) but its use as an alleged abortifacient has resulted in fatalities (Kiel, 1964).

EXPERIMENTAL

Material

Post-mortem blood, liver blood, urine, stomach contents, lung, liver, kidneys, heart and brain was from a 33 year old female who had swallowed an estimated 12 tablets containing chloroquine at 5 am. She was taken unconscious to hospital at 6 am where she died 24 h later without regaining consciousness and despite treatment by dialysis. (Case No. 1).*

Ante-mortem blood, post-mortem blood, liver blood, urine, stomach contents, lung, liver and kidneys was from a 24 year old female who had taken an estimated 40 tablets containing chloroquine phosphate at about 4 am and later a few tablets of paracetamol and of aspirin. She collapsed while walking into hospital and died at 8.30 am after a cardiac arrest. (Case No. 2).

Direct extraction of body fluid specimens. The specimen (5 ml) was made alkaline with strong ammonia solution and extracted with ether (75 ml). Basic substances were recovered by extraction of the ether with dilute hydrochloric acid (5 ml, 0·1N).

Extraction of tissue. Minced tissue (10 g) was suspended in sodium hydroxide solution (5 ml, 20% w/v) and heated on a boiling water-bath for 90 min. The cooled hydrolysates were extracted with 100 ml of ether. The ether extract was washed free from alkali and the bases were extracted into 0·1N hydrochloric acid. A portion of blood (5 ml) was similarly treated for comparison.

Extraction of body fluids containing paracetamol and salicylates (*Case No.* 2). An aliquot (5 ml) of body fluid was extracted with 50 ml of chloroform. Salicylate was

* Details of case histories will appear in Medicine, Science and the Law, 1970.

recovered from the chloroform by extraction into 1% w/v sodium bicarbonate solution and assayed using Trinder reagent (Trinder, 1954). The chloroform extract was again extracted with sodium hydroxide solution (0·45N) to recover the paracetamol for spectrophotometric assay at 265 nm before and after the addition of acid. The chloroform was then washed free from alkali and the chloroquine recovered by extraction with 0·1N hydrochloric acid.

Determination of chloroquine. The absorption spectrum of each acid extract was recorded using a Unicam SP800 spectrophotometer over the range 220–360 nm and the chloroquine content was determined on the basis of the absorption at 343 nm.

Infrared absorption spectrum. The basic substances in the total stomach content specimen from Case No. 1 were extracted into ether and the solvent evaporated. The infrared absorption spectrum of the oily residue was examined as a liquid film in a Grubb Parsons spectrophotometer.

Thin-layer chromatography (TLC). Basic substances from the acid solutions used for assay were recovered into chloroform and the solvent evaporated. The residue was chromatographed on 250 μm silica gel plates with chloroform–ethanol–ammonia (80:20:1 by volume) as the solvent. Potassium iodoplatinate solution was used to detect basic substances on the chromatograms [chloroquine (Rf 0·75–0·80) and the metabolite (Rf 0·38) gave brown colour reaction].

Gas liquid chromatography (Case No. 1). The residues of the chloroquine extracts used for thin-layer chromatography were dissolved in about 100 μl volume of chloroform and aliquots were injected into an F & M model 400 gas chromatograph equipped with a 4 ft glass column packed with 3·8% SE 30 on 80–100 mesh Diatoport S. A flame ionization detector was used. The instrument was programmed to operate the oven isothermally at 195° and, after 10 min to increase the temperature by 5°/min up to 245°. Nitrogen was used as the carrier gas.

RESULTS

Chloroquine. The infrared spectrum from an extract of the stomach contents of the first case was comparable with that of authentic chloroquine similarly treated, and resembled the spectrum published by Kuroda (1962).

The quantitative results from the ultraviolet absorption analysis are in Table 1. There was no significant difference between results obtained by direct extraction of

Table 1. *Tissue levels of chloroquine*

Specimen	Chloroquine base mg/100 ml or /100 g	
	Case 1	Case 2
Ante-mortem blood	—	0·86
Post-mortem blood	1·60	1·24
Liver blood	9·00	4·40
Urine	2·00	6·84
Stomach contents*	1·80	55·20
Lung	3·80	9·80
Liver	17·50	34·40
Kidneys	7·00	30·00
Heart	5·70	—
Brain	1·60	—

* Volume of stomach contents of Case No. 2—125 ml.

the body fluids or by extraction after alkaline hydrolysis. Recovery experiments in which a known amount of chloroquine was added to tissue and taken through the entire procedure showed that the recovery of the chloroquine was better than 95%.

Results were calculated as total chloroquine base; this probably included a metabolite of chloroquine corresponding with that identified as a mono-des-ethylated compound (McChesney, McAuliff & others, 1954; Kuroda, 1962).

There was less metabolite than unchanged drug present in specimens of post-mortem blood and urine. The stomach contents from the second case contained mostly unchanged drug and a much smaller quantity of the metabolite the unexpected presence of which might be the result of gastric secretion. The absence of the metabolite from the ante-mortem blood sample only of all the specimens analysed, may reflect the time interval before the formation of detectable amounts of metabolite.

In the first case the metabolite was present only in the liver, kidney and lung. A qualitative examination of the TLC residues from this case by gas-liquid chromatography gave a major peak for each extract which had retention times in the range of 19–23 min and corresponded with chloroquine. In addition to the major peak, the tissue extracts produced between 4–7 minor peaks, while extracts from the body fluids produced four minor peaks. Most of these peaks may be attributed to tissue constituents.

DISCUSSION

Absorption of chloroquine in therapeutic dosage is almost complete from the gastrointestinal tract (Goodman & Gilman, 1965) but our findings for stomach contents and liver blood, compared with peripheral blood, indicated that absorption of the drug was incomplete at death in both cases. The post-mortem blood concentrations of paracetamol and salicylates did not suggest that excessive amounts of these drugs had been taken.

A chloroquine metabolite, the properties of which corresponded with 7-chloro-4-(4′-ethylamino-1′-methylbutylamino)quinoline, was present in most extracts, indicating that the detoxifying enzymes were still functional in both cases. Several chromatographic systems failed to demonstrate more than the one metabolite.

The post-mortem blood concentrations in both cases and the ante-mortem concentration in the second case greatly exceeded the peak concentrations reported after therapeutic dosage with the drug. Thus Alving, Eichelberger & others (1948) reported that peak plasma concentrations 6 h after one 500 mg dose never exceed 3·5 μg of chloroquine/100 ml, while Berliner, Earle & others (1948) found peak plasma concentrations ranging from 2·2 μg/100 ml after 50 mg to 21·7 μg/100 ml after 400 mg dose of the drug. Hoole (1966) however, reported the chloroquine blood concentration of a 45 year old male found dead as 9·9 mg/100 ml.

The tissue concentrations in the second case were also higher than the ranges reported by Prouty & Kuroda (1958) for eight non-suicide cases. Present data are compared with those published by others in Table 2. Apart from the post-mortem blood chloroquine concentration reported by Hoole (1966), there are no reported data for body fluids and tissues other than liver, kidney, heart and brain.

During therapeutic dosage, chloroquine is excreted partly unchanged in the urine (50–70% of the dose) and partly as the mono-des-ethylated metabolite (25–50% of the dose) according to McChesney, Conway & others (1966). An earlier study

Table 2. *Published reported tissue levels of chloroquine in man compared with levels found in the same tissues of the two cases reported in the present text*

	Prouty & Kuroda (1958)			Kiel (1964)	Hoole (1966)	This paper†	
	8 Non Suicides	2 Suicides		13 Suicides*	1 Overdose	Case 1	Case 2
	mg/100 g	mg/100 g		mg/100 g	mg/100 g	mg/100 g	
Liver	0·43–4·8	—	90·0	0·23–75·0	88·3	17·5	34·4
Kidney	0·06–0·58	—	47·0	11·0–64·0	18·8	7·00	30·0
Heart	0·41–2·0	8·4	—	4·0	—	5·70	—
Brain	0·07–0·73	1·0	1·10	0·04–5·0	—	1·60	—

* In only one case are all of the tissue levels reported.
† Data for other specimens are given in Table 1.

(McChesney & others, 1954) based on solvent soluble dye complex assays showed that 8% of the daily oral dose was excreted in the faeces and 14% in the urine (range 10–25%): the urinary excretion would be expected to be increased or decreased by administration of acid cr alkali. Chloroquine was present in the urine of both cases at post-mortem and significant concentrations of the drug, accompanied by the metabolite, were found in the kidneys (Table 2).

The ratio of kidney : liver levels probably reflects the combination of the time interval and treatment between ingestion of the drug and death: for the first case the kidney/liver ratio is 0·4 and the survival time was 24 h; for the second case the ratio is 0·87 and the time interval was 6 h but renal damage might have prevented excretion of the drug. The highest concentration of the drug was in the liver, which is consistent with previous reports, including those where overdosage was not the immediate cause of death.

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LETTERS TO THE EDITOR

Flow decrease through rat hind limb vasculature by (±)-carnitine, (±)-acetylcarnitine and (±)-chloroacetylcarnitine chlorides

The occurrence of carnitine and various acyl carnitine derivatives in mammalian tissues is well documented (Pearson & Tubbs, 1964); however, the pharmacological effects of these biosubstrates have not yet been clearly elucidated. (±)-Acetylcarnitine (Dallemagne, Philippot & others, 1955; Fritz, 1963) and carnitine (Charlier, 1954; Yoshimi, Takaori & Shimamoto, 1965) produce cholinergic effects. We synthesized (±)- acetylcarnitine chloride (II) and (±)-chloroacetylcarnitine chloride (III) [$Me_3N^+{\cdot}CH_2{\cdot}CH(CH_2{\cdot}COOH)O\text{-}XCl^-$: I, X-H; II, X = COMe; III, X = $COCH_2Cl$] for comparison of the effect of these derivatives with (±)-carnitine chloride (I) on rat hind limb vasculature preparations *in situ*. Experiments now reported suggest that (±)-carnitine, (±)-acetylcarnitine and (±)-chloroacetylcarnitine possess potent vasoconstrictive properties.

Female albino Sprague Dawley rats, 250–300 g, were used for *in situ* hind limb vasculature preparations. (±)-Carnitine, (±)-acetylcarnitine and (±)-chloroacetylcarnitine chlorides significantly reduced flow rate through hind limb vasculature (Table 1). Reductions in flow rates were related to amounts of test compounds infused. At concentrations producing no discernible effects, test compounds potentiated the inhibitory effect of adrenaline on the isolated duodenum of the rabbit. To see if these compounds could potentiate the effect of noradrenaline on arterial smooth musculature, we examined the effect of (±)-acetylcarnitine on perfusate flow through rat caudal artery preparations (Kosegarten, De Feo & De Fanti, 1969, 1970). The flow rate response to noradrenaline was not altered by (±)-acetylcarnitine (5×10^{-8} g/ml). (±)-Carnitine alone elicited no demonstrable effect on arterial smooth musculature preparations at 1×10^{-8} and 5×10^{-9} g/ml.

Our evidence constrasts with published reports (Dallemagne & others, 1955; Fritz, 1963; Charlier, 1954; Yoshimi & others, 1965) of *in vivo* and *in vitro* acetylcholine-

Table 1. *Inhibition of flow through rat hind limb vasculature by carnitine and carnitine derivatives.* Each point represents the mean of at least two determinations. Animals were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.). Perfusate, collected from a polyethylene (PE 90) cannula in the inferior vena cava, was recorded using an automatic drop counter and E & M Physiograph. Test compounds were added directly to the perfusion fluid following control flow rate determination (29·3 ± 2·5, mean drops ± s.e.). Responses of all preparations were standardized with methacholine, acetylcholine and adrenaline.

Amount infused (mg)	Flow decrease (%)		
	(±)-Carnitine	(±)-Acetylcarnitine	(±)-Chloroacetyl-carnitine
2·0	78	50	76
4·0	63	48	—
6·0	—	—	86
8·0	63	88	—
10·0	—	—	—
12·0	—	100	—
20·0	—	—	100

like activities of carnitine and acyl carnitine derivatives. Since these compounds have been administered to man (Gravina & Gravina-Sanvitale, 1969) further experiments are being made on the physiological significance of these findings.

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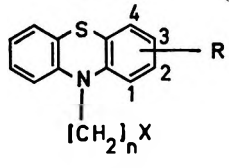
On the local anaesthetic action of chlorpromazine and some non-tranquillizing analogues

Chlorpromazine has been found in several tests to be a more potent local anaesthetic than procaine (Courvoisier, Fournel & others, 1953; Kopera & Armitage, 1954; Rosenberg & Ehrenpreis, 1961). In man, chlorpromazine has been successfully used to produce long-lasting local anaesthesia for the relief of post-surgical pain (Terrier, 1953), its subsequent abandonment for this purpose being possibly due to side-effects such as orthostatic hypotension. Other phenothiazines also cause local anaesthesia. In man promethazine is slightly less active than procaine, but it has been recommended for use in patients hypersensitive to local anaesthetics of the procaine type (Kalz & Fekete, 1960; Meltzer, 1960). The tranquillizer prochlorperazine has been reported to be ten times as potent as xylocaine in blocking conduction along single myelinated nerve fibres from the frog (Hille, 1966). It is not known whether the central depressant and local anaesthetic properties of these drugs have a common underlying mechanism.

The structural requirements for potent tranquillizing activity in the aminoalkylphenothiazine series (formula below) are well defined (Gordon, 1967). The ring



	R	n	X
I (chlorpromazine)	2-Cl	3	NMe_2
II (prochlorperazine)	2-Cl	3	N NMe
III	4-Cl	3	NMe_2
IV	2-Cl	4	NMe_2

substituent (R) must be in the 2-position, and the tertiary amino-group (X) must be separated from the ring nitrogen by a trimethylene chain (n = 3). To test whether the local anaesthetic and tranquillizing properties in this series are related, the local anaesthetic activities of two tranquillizers, chlorpromazine (I) and prochlorperazine (II), have been compared in mice, using the tail clip method of Bianchi (1956), with those of two closely-related chlorpromazine analogues (III and IV), whose structures do not conform to the above requirements for tranquillizing activity and which cause only slight central depression in animals (Green, 1967).

The drugs, together with adrenaline tartrate (15 μg/ml), were dissolved in 0·9% NaCl. Four groups of ten adult mice were used for each compound, and the potency was assessed from the approximate concentration (EC50) required to cause a loss of withdrawal reflex to an artery clip on the tail in 50% of the mice 15 min after subcutaneous injection of 0·1 ml of the drug solution near the root of the tail. All four phenothiazines, irrespective of whether or not they were tranquillizers, had about the same EC50 (0·03 to 0·05%). All were more active than procaine (EC50, 0·1%) and all had a more prolonged action than procaine.

Higher concentrations (0·1 and 0·2%, total dose 3–6 mg/kg) of chlorpromazine and prochlorperazine ultimately caused marked sedation. However, whereas this sedation took 2–4 h to reach its maximum extent, the local anaesthesia had declined by this time (EC50 about 0·1%), hence the failure of the mice to respond to the artery clip on the tail after treatment with these drugs is unlikely to be attributable solely to a depressant action of central origin. The phenothiazines (III) and (IV) caused no sedation at these concentrations.

These results suggest that aminoalkylphenothiazines which do not have the requisite structural features to be potent tranquillizers may be a fruitful source of new long-lasting local anaesthetics. They also indicate that the action of chlorpromazine-like compounds on nerve cell membranes, which results in a depression of nerve conduction, is not the primary mechanism behind their tranquillizing effect.

The phenothiazine derivatives were kindly supplied by Smith Kline and French Laboratories, Philadelphia.

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N-(Perhydroazepinoalkynyl)- and *N*-(perhydroazocinoalkynyl) succinimides as oxotremorine antagonists

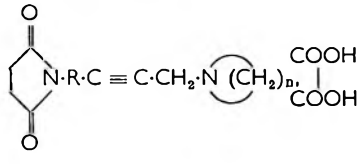
We have reported that some *N*-(4-t-amino-2-butynyl)-substituted succinimides had a blocking action on the motor effects of oxotremorine, but were less active on the peripheral cholinergic effects (Dahlbom, Karlén & others, 1966 a,b). We also found that if the 2-butyne chain was branched with one or two methyl groups or lengthened with a methylene group between the acetylenic bond and the imide nitrogen, the tremorolytic activity was enhanced, some compounds exceeding atropine in this respect (Karlén, Lindeke & others, 1970).

Lévy & Michel-Ber (1967) reported that *N*-(perhydroazepino-2-butynyl)succinimide was an oxotremorine antagonist, and we have investigated analogous modifications of the intermediate 2-butynyl chain of this compound and also some eight-membered cyclic amines. This report deals with the synthesis and pharmacological properties of a series of *N*-(perhydroazepinoalkynyl)- and *N*-(perhydroazocinoalkynyl)succinimides, some of which are much more active than atropine as oxotremorine antagonists.

The compounds were prepared via the Mannich reaction by refluxing a mixture of the appropriate *N*-alkynylsuccinimide (Karlén, Lindeke & others, 1970), formaldehyde, and the cyclic amine in dioxane in the presence of catalytic amounts of cuprous chloride, and they were isolated and purified as the oxalate salt. The compounds prepared and the results of the pharmacological tests for central and peripheral anticholinergic activity are presented in Table 1.

Antagonism of tremor induced by oxotremorine was estimated by determining the median effective dose of oxotremorine necessary to produce an intermittent spontaneous tremor (grade 2 tremor). The intensity of the tremor was graded visually

Table 1. *Physical and pharmacological data for* N-(*perhydroazepinoalkynyl*) *and* N-(*perhydroazocinoalkynyl*)*succinimides*



Compound	R	n	M.p. °C	Formula	*In vivo* dose (μmol/kg) in mice required to produce Oxotremorine blockade†	Mydriasis§
1*	CH_2	6	143–145	$C_{16}H_{22}N_2O_6$	3·9	
2	CHMe	6	113–115	$C_{17}H_{24}N_2O_6$	0·45	5·6
3	CHMe	7	141–143	$C_{18}H_{26}N_2O_6$	4·6	23
4	CMe_2	6	144–146	$C_{18}H_{26}N_2O_6$	1·2	7·7
5	CMe_2	7	122–124	$C_{19}H_{28}N_2O_6$	1·5	9
6	$(CH_2)_2$	6	84–86	$C_{17}H_{24}N_2O_6 \cdot H_2O$	15	>150
7	$(CH_2)_2$	7	140–142	$C_{18}H_{26}N_2O_6$	27	‡
8	$(CH_2)_3$	6	118–119	$C_{18}H_{26}N_2O_6$	22	>75
9	$(CH_2)_3$	7	113–115	$C_{19}H_{28}N_2O_6$	26	180
Atropine					2·8	0·29

* Reported by Levy & Michel-Ber (1967) and included for comparison.

† Dose of test compound required to double the dose of oxotremorine inducing a grade 2 tremor in 50% of the mice.

‡ This compound produced miosis over the entire dosage range tested.

§ Dose of test compound required to double the pupil size relative to the control.

according to a three point system earlier described (Cho & Jenden, 1964). The "up and down" method for small samples described by Dixon (1965) was used to estimate the median effective dose of oxotremorine. Each compound was screened to determine its effective dose range and then four linearly spaced doses including zero were chosen. Female mice, 22 to 26 g in groups of six were given oxotremorine intravenously with or without the test compound (given i.p. 10 min previously)and the median effective dose of oxotremorine determined using a logarithmic series of doses with a spacing of 0·1 units in the $\log_{10}$ dose scale. Tremors were graded 3 min after the oxotremorine injection. Animals with a grade 2 tremor or more were designated positive; others were negative. The median effective dose of oxotremorine was then plotted against the dose of the test compound, and the dose of antagonist which doubled the median effective dose of oxotremorine was estimated graphically.

Mydriatic activity was estimated on mice (groups of 6) by measuring the pupillary diameter before, and 10 min after, the intraperitoneal injection of the test compound. The measurements were made under constant light source using a binocular dissecting microscope with a calibrated eyepiece. The mydriatic dose was estimated graphically as that required to double the pupil size relative to the control.

All the compounds were active in blocking the motor effects of oxotremorine (Table 1), Nos 2, 4 and 5 were more active than atropine, the most active (No. 2) being about six times more potent. The dose producing oxotremorine blockade was always less than that producing mydriasis. This is in marked contrast to atropine, which is less effective in blocking oxotremorine than in producing mydriasis. Consequently the compounds reported here can be regarded as anti-acetylcholine agents with a greater selectivity for the central nervous system than atropine. This property has been shown to be partly due to the low base strength of these amines which favours their distribution to the brain (Karlén & Jenden, 1970).

Activity is enhanced if the parent 2-butyne chain is branched with one or two methyl groups between the acetylenic bond and the imide nitrogen, whereas lengthening of the chain diminishes the activity. The perhydroazepino compounds seem to be more active than the corresponding perhydroazocino compounds.

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Effect of SKF 525A on the fate of thiopentone

The initial rapid decline in thiopentone blood concentrations in all species after intravenous injection is attributed mainly to redistribution either into lean tissue (Price, Kevnat & others, 1959), or lean tissue and fat (Mark & Brand, 1963). The further slow decline of thiopentone blood levels may be due to further redistribution or to a combination of the two.

Mark, Brand & others (1965) in man, and Saidman & Eger (1966) in the dog, have demonstrated an arterial hepatic venous difference in thiopentone concentrations and have claimed that metabolism by the liver is of importance in lowering the blood concentrations of thiopentone.

Many of the estimations of thiopentone metabolism by the liver both *in vivo* and *in vitro* (Winters, Spector & others, 1955; Spector & Shideman, 1959) are of doubtful validity because they use methods which result in the degradation of thiopentone to pentobarbitone (Bush, Mazel & others 1961); thus, the rate of thiopentone metabolism has been variously put at 2, to 40%/h.

A method of demonstrating the contribution of metabolism in the termination of the anaesthetic action of thiopentone would be by measuring the effect of a liver enzyme inhibitor.

Shideman, Kelly & Adams (1947) have shown that carbon tetrachloride markedly prolongs thiopentone sleeping times in rats and this was assumed to arise from the hepatotoxicity of carbon tetrachloride. Megirian (1964) however, has since shown that carbon tetrachloride alters the distribution of thiopentone and that its action in prolonging sleeping times may be due to this effect.

SKF 525A (β-diethylaminodiphenylpropyl acetate), an inhibitor of liver microsomal enzymes, has been shown to prolong the sleeping times in rats produced by hexobarbitone and other barbiturates (Fouts & Brodie, 1956). It was of interest, therefore, to measure the effect of SKF 525A on thiopentone blood concentrations and on thiopentone sleeping times.

Five dogs (3 greyhounds, 2 terriers) were given SKF 525A (10 mg/μg) intravenously and after 30 min thiopentone (30 mg/kg) intravenously. Blood samples were taken at intervals.

Thiopentone blood concentrations were measured by the method of Brodie & others (1950) except that ethylene dichloride was used as the extraction solvent, whereby less than 1% degradation of thiopentone occurs in the extraction procedure.

It should be noted that the greyhounds maintained higher blood concentrations of thiopentone, presumably through lack of body fat, and were correspondingly anaesthetized and ataxic for longer than the other two dogs.

SKF 525A did not produce any alteration in the rate of decline of thiopentone blood levels in the five dogs.

Hooded inbred rats were injected intraperitoneally with SKF 525A (10 mg/kg) 30 min before thiopentone. A control group of rats were given saline. The rats were then injected with thiopentone (25 mg/kg) in a tail vein and the sleeping times measured.

SKF 525A did not produce any significant difference in thiopentone sleeping times in rats ($52{\cdot}2 \pm 10{\cdot}8$, $n = 13$) compared with animals given thiopentone alone ($47{\cdot}8 \pm 5{\cdot}5$, $n = 12$) ($P > 0{\cdot}4$).

These results would indicate that hepatic metabolism is not important in the termination of the anaesthetic action of thiopentone.

SKF 525A was kindly provided by Smith, Kline and French Laboratories, Kimpton, Herts.

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2-Mercaptobenzothiazole, an inhibitor of dopamine β-hydroxylase

The presence of copper in purified preparations of dopamine β-hydroxylase and the functional role of cupric ions in the oxidative conversion of dopamine to noradrenaline have been reported (Friedman & Kaufman, 1965). The critical role of cupric ions in the activity of dopamine β-hydroxylase renders this enzyme vulnerable to inhibition by copper chelating agents. Chelation of the cupric ion is the probable mechanism for the inhibition of this enzyme by inhibitors which include disulfiram (Goldstein, Anagnoste & others, 1964), phenylethyldithiocarbamate (Jonsson, Grobecker & Gunne, 1967), tropolone (Goldstein, Lauber & McKereghan, 1964) and various aromatic and alkyl thioureas, including U-14,624 [1-phenyl-3-(2-thiazolyl)-2-thiourea] (Johnson, Boukma & Kim, 1969, 1970). The irreversible inhibition of a banana polyphenoloxidase, also a copper enzyme, by 2-mercaptobenzothiazole (MBT) (Palmer & Roberts, 1967) prompted our investigation of this drug as a potential inhibitor of dopamine β-hydroxylase.

In vitro inhibition of dopamine β-hydroxylase isolated from bovine adrenal medulla (Friedman & Kaufman, 1965) was measured (Goldstein, Prochoroff & Sirlin, 1965). The animals were CF-1 male mice, 18–22 g, and Upjohn Sprague-Dawley male rats, 180–190 g. The drugs were dissolved or suspended in 0·25% aqueous methylcellulose before intraperitoneal administration. Noradrenaline and dopamine in paired mouse brains were measured (Veldkamp, Johnson & Keasling, 1968). The repletion of rat myocardial noradrenaline from exogenous dopamine after the depletion of noradrenaline with metaraminol was examined as described by Nikodijevic, Creveling, & Udenfriend (1963). Myocardial noradrenaline was adsorbed onto alumina (Anton & Sayre, 1962), eluted with 0·5M acetic acid and assayed (von Euler & Floding, 1958).

Spontaneous motor activity was recorded in actophotometer cages (Woodward Research Corp.). Mice received MBT (300 mg/kg, i.p.) or vehicle and two mice from the same treatment group were placed in each cage. After an initial 10 min acclimation period, activity was recorded in each 30 min interval for 4 h.

MBT inhibited non-competitively dopamine β-hydroxylase *in vitro* 72% at 10^{-5}M and 47% at 5×10^{-6}M. In the same assay disulfiram produced 45% inhibition at 2×10^{-7} M and benzyloxyamine 30% at 5×10^{-4} M.

MBT (300 mg/kg, i.p.), lowered noradrenaline to approximately 60% of control after 1 and 2 h (Table 1). Dopamine levels were raised 24% above control concentrations at 2 h. Both noradrenaline and dopamine returned to control values at

Table 1. *Mouse brain catecholamine levels* 1, 2, *and* 4 *h after* 2-*mercaptobenzothiazole* (MBT), 300 mg/kg, i.p. All values are expressed as μg/g wet weight whole brain tissue and are the average of at least three determinations ±s.e.

		Noradrenaline	Dopamine
Diluent treated controls		0·43 ± 0·03	0·78 ± 0·06
MBT	1 h	0·25 ± 0·01*	0·86 ± 0·02
	2 h	0·27 ± 0·02*	0·96 ± 0·03†
	4 h	0·42 ± 0·00	0·81 ± 0·02

* = $P < 0.01$ † = $P < 0.05$

Table 2. *Effect of* 2-*mercaptobenzothiazole* (MBT) *on the repletion of rat myocardial noradrenaline from exogenous dopamine after its depletion with metaraminol.* Rats were pretreated with metaraminol bitartrate, 5 mg/kg, 18 h before each received MBT, 300 mg/kg, or diluent. Dopamine hydrochloride, 35 mg/kg, or diluent was administered 30 min later and all rats were killed 3 h later. All values are expressed as μg/g and are the average of at least three determinations ±s.e.

	Myocardial noradrenaline
Diluent treated controls	0·88 ± 0·05
Metaraminol	0·15 ± 0·01
Metaraminol + dopamine	0·52 ± 0·09*
Metaraminol + MBT	0·18 ± 0·03
Metaraminol + MBT + dopamine	0·17 ± 0·02

* Significantly different from each of the other values, $P < 0.05$.

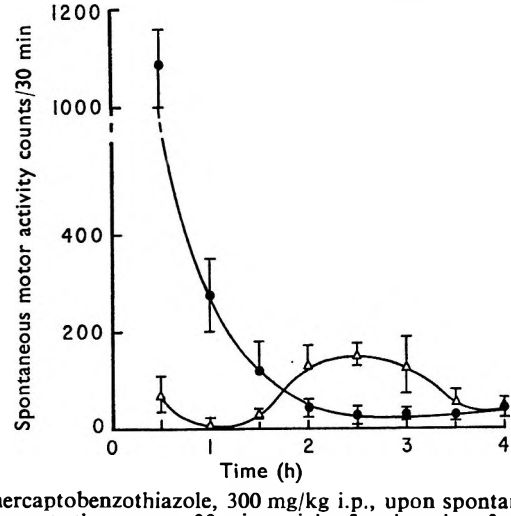

FIG. 1. Effect of 2-mercaptobenzothiazole, 300 mg/kg i.p., upon spontaneous motor activity in mice. Each point represents the average 30 min activity for six pairs of mice ± s.e. ——●——, control; ——△——, MBT.

4 h. Overtly, the treated mice were extremely depressed shortly after the administration of the drug and the depression accompanied by marked ptosis extended after 2 h. The return of amine levels to control concentrations paralleled a loss of overt depression. After 4 h the mice appeared normal.

We also treated rats with metaraminol bitartrate (5 mg/kg, i.p.) to deplete heart noradrenaline stores. After 18 h, rats were dosed with MBT (300 mg/kg) or diluent and 30 min later with dopamine (35 mg/kg). All rats were killed 3 h after the dopamine. The effect of MBT upon the repletion of rat myocardial noradrenaline from exogenous dopamine is summarized in Table 2. Exogenous dopamine restored the myocardial noradrenaline concentratations to 60% of the pre-drug level. At 300 mg/kg, MBT totally blocked the conversion of dopamine to newly synthesized noradrenaline. The effect of MBT upon spontaneous motor activity is shown in Fig. 1. Motor activity in control mice fell rapidly in the first hour and the mice remained relatively inactive for the remainder of the experiment. Initial exploratory activity was absent in MBT-treated mice. The increase in the spontaneous activity of treated mice at 2 h coincided with the termination of dopamine β-hydroxylase inhibitory activity in the brain as indicated by the recovery of brain noradrenaline stores between 2 and 4 h.

Thus, MBT effectively inhibits dopamine β-hydroxylase *in vitro* and *in vivo*. *In vitro*, MBT had 1/25th the inhibitory activity of disulfiram. *In vivo*, the compound altered mouse brain catecholamines in a manner consistent with that expected for inhibition of the enzyme in brain. In addition, the initial rate of decline of mouse brain noradrenaline ($0{\cdot}18\ \mu g/g/h^{-1}$) after MBT compares favourably with similar results we have obtained in mice after treatment with disulfiram and U-14,624 (Johnson & others, 1970). The effectiveness of the *in vivo* inhibition of noradrenaline synthesis by MBT was also clearly demonstrated by the complete block of the conversion of exogenous dopamine to noradrenaline in the rat myocardium. The recovery of both brain noradrenaline and dopamine to control levels by 4 h reflects a short term inhibition by MBT of mouse brain dopamine β-hydroxylase.

This compound should serve as an additional pharmacologic tool to study the effects of depleted noradrenaline levels concurrent with the short term *in vivo* inhibition of dopamine β-hydroxylase upon animal behaviour.

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Inhibition of hepatic ribonuclease activity by chronic administration of phenobarbitone

Acute or chronic administration of phenobarbitone enhances the biotransformation of other drug molecules (Conney, Davison & others, 1960) and increases the incorporation of radiolabelled amino-acids into microsomal protein in *in vitro* and *in vivo* systems (Kato, Jondorf & others, 1966; Kuriyama, Omura & others, 1969; Shuster & Jick, 1966). Although the molecular basis for this phenomenon has not been established it may be due to an enhanced DNA-RNA polymerase activity, since it has been shown that actinomycin D inhibits phenobarbitone-induced enzyme activity (Orrenius, Ericsson & Ernster, 1965). This increase in rate of transcription may be attributed to an inhibition of repressor molecules synthesized by regulator genes (Jacob & Monod, 1961a, b). Alternatively, phenobarbitone may cause enzyme activation by interacting with the endoplasmic reticulum in such a way as to enhance the translation process (Conney, 1967). In addition, a decrease of hepatic ribonuclease (RNase) activity by phenobarbitone may be related in part to enzyme induction, since an inhibition of this enzyme by triamcinolone was associated with an increase in amino-transferase activity (Sarkar, 1969). Therefore, the purpose of this communication is to describe the effect of phenobarbitone on RNase activity.

Male Sprague-Dawley rats, 160 to 200 g, were pretreated with either 100 mg/kg phenobarbitone intraperitoneally, or equivalent volumes of physiologic saline on a daily basis for 2, 6 and 8 injections. Liver, excised from these animals fasted for 24 h after the last injection, was homogenized in 9 volumes of cold 0·24 M sucrose; 50mM tris-HCl, pH 7·6; 12mM $MgCl_2$; and 100mM KCl solution (medium I). Liver microsome and postmicrosomal supernatant fractions were prepared by the method of Zomzely, Roberts & Rapaport (1964). Ribonuclease activity of these fractions was measured (Barondes & Nirenberg, 1962, as modified by Zomzely, Roberts & others, 1968). Approximately 1 mg of microsomal or 0·7 mg of postmicrosomal supernatant protein was used in the incubation system. The protein concentration was measured by the method of Lowry, Rosenbrough & others (1951). Undergraded [^{14}C] polyuridylic acid (Poly U) was collected on Millipore filters (A-AWR-025-00, AAO-8μ), air dried, placed in scintillation vials with 5 ml of a toluene scintillation system composed of 0·5% PBD (phenylbiphenylyloxadiazole, Packard) and 0·01% dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazoyl) benzene, Packard), and counted in a Packard Tri-Carb scintillation counter.

Six or 8 injections of a 100 mg/kg does of phenobarbitone causes an 80 and 60% reduction of RNase activity in hepatic microsomal and postmicrosomal supernatant fractions respectively, at the 30 min incubation interval (Table 1). There was also no difference in RNase activity of the latter fraction between control animals and rats

Table 1. *The effect of phenobarbitone on hepatic ribonuclease activity*

Number of phenobarbitone injections	Postmicrosomal supernatant			Microsomes		
	5 min	15 min	30 min	5 min	15 min	30 min
0	1250*	970	550	350	280	180
2	1450	1020	550	880	380	230
6	1590	1470	1120	1400	1200	900
8	1590	1470	1200	1400	1200	900

* The recovery of [^{14}C]Poly U in counts/min mg^{-1} protein. Each mean value is based on three independent incubations in duplicate.

receiving two injections of the barbiturate at all designated time intervals. However, at the 5 and 15 min interval, the recovery of [^{14}C]Poly U by microsomes from the latter group was greater than the controls. Therefore it appears that the degree of RNase inhibition is dependent on the number of injections of drug. Additional experiments in our laboratory on *N*-demethylation of aminopyrine (McMahon & Easton, 1962) indicates a positive correlation of enzyme activity and the number of phenobarbitone injections, which confirms Orrenius & Ernster's (1964) findings on *N*-demethylase and NADPH-cytochrome C reductase activity.

Sarkar (1969) has also shown a positive correlation of an increase in aspartate and alanine aminotransferase activity, and a decrease in RNase activity in rats pretreated with triamcinolone. These observations indicate that RNase inhibition may be associated with hepatic enzyme induction.

In vitro addition of phenobarbitone in concentrations up to 1×10^{-3} M to the RNase incubation system of control fractions did not alter the rate of [^{14}C]Poly U degradation. These results indicate that phenobarbitone did not inhibit RNase activity by direct action. It may be possible this inhibition is caused by induced inhibitor formation.

Our experiments suggest that ribonuclease activity inhibited in hepatic microsomal and postmicrosomal supernatant fractions upon chronic phenobarbitone pretreatment was not caused by direct action of the barbiturate and may in part play an essential role in enzyme induction.

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The inhibition of catecholamine biosynthesis by apomorphine

Apomorphine stimulates the dopamine receptors in the striatum (Ernst, 1967; Ernst & Smelik, 1966; Andén, Rubenson & others, 1967). More recently, apomorphine was also used in the treatment of parkinsonism (Cotzias, Papavasiliou & others, 1970). Biochemical studies have shown that apomorphine retards the depletion of the central dopamine stores, but not noradrenaline stores, in animals pretreated with tyrosine hydroxylase inhibitors (Andén & others, 1967). Apomorphine reduces the impulse flow of the dopamine neurons, probably by a negative feedback mechanism arising from dopamine receptor stimulation. But no evidence exists on whether apomorphine has a direct effect on tyrosine hydroxylase activity. Apomorphine contains a catechol group and catechols are known to be inhibitors of tyrosine hydroxylase (Nagatsu, Levitt & Udenfriend 1964; Goldstein, Gang & Anagoste, 1967). We have now investigated the effects of apomorphine on tyrosine hydroxylase activity and on dopamine biosynthesis *in vitro* and *in vivo*.

Male Sprague-Dawley rats, 250–300 g, were decapitated and the striata immediately dissected, sliced and incubated at 37° in Krebs-Henseleit medium. The incubation procedure and the determination of [^{14}C]catecholamines was done as previously described (Goldstein, Ohi & Backstrom, 1970). In some experiments the animals were treated with apomorphine 25 mg/kg subcutaneously and 30 min later [^{14}C]L-tyrosine (U) (6·25 μCi/rat; specific activity 450 mCi/mol) was administered intracisternally. Haloperidol (2 mg/kg, i.p.) was given 30 min before the apomorphine. Tyrosine hydroxylase activity was measured according to Nagatsu & others (1964).

The effects of apomorphine on tyrosine hydroxylase activity at different concentrations of the substrate 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) are in Table 1. Apomorphine 10^{-4}M significantly inhibits tyrosine hydroxylase activity. At lower concentrations of the pteridine the inhibition by apomorphine is more effective. However, at 10^{-6}M, apomorphine does not inhibit significantly tyrosine hydrolyase activity even at low $DMPH_4$ concentrations.

Apomorphine inhibits effectively the biosynthesis of [^{14}C]dopamine from [^{14}C] tyrosine in slices of rat striatum (Table 2) even at 10^{-6} and 10^{-7}M. Thus, striatal slices are more sensitive to the inhibition of [^{14}C]dopamine biosynthesis by apomorphine than tyrosine hydroxylase preparations obtained either from bovine adrenal glands or from the striatum of rats. The addition of haloperidol to the media in which the striatal slices were incubated did not affect the inhibitory activity of apomorphine.

In separate experiments the effects of apomorphine on [^{14}C]catecholamines were investigated after intraventricular injection of [^{14}C]tyrosine. Treatment with apomor-

Table 1. *The effect of apomorphine on tyrosine hydroxylase activity at different concentrations of* $DMPH_4$. The results represent averages from 3 experiments with a standard deviation of $\pm 5\%$. Enzyme preparations at the $(NH_4)_2SO_4$ purification step obtained from bovine adrenal glands and from the striatum of rats were used. The enzyme was preincubated at room temperature (20°) for 5 min with apomorphine and the incubations were according to Nagatsu & others, (1964).

Concentration of apomorphine (M)	Activity % of control Concentration of $DMPH_4$ (μmol) 0·5	0·25	0·1
Control	100·0	100·0	100·0
10^{-6}	100·0	95·5	90·0
10^{-5}	90·0	76·0	70·5
10^{-4}	59·0	51·5	42·5

Table 2. *The effect of apomorphine on* [^{14}C]*dopamine biosynthesis from* [^{14}C]*tyrosine in slices obtained from the striatum of rats.* In all experiments the slices were incubated for 20 min. Results are the mean ± s.e. from 5 experiments and are expressed as counts/min × 10^{-3} per incubation. Each incubation contained 70 mg of slices.

Concentration of apomorphine (M) in the incubation medium	[^{14}C]Dopamine formed slices	medium	% Inhibition
Control	55·0 ± 1·00	6·8 ± 0·30	——
10^{-7}	44·5 ± 0·70	5·1 ± 0·25	19·8
10^{-6}	22·0 ± 0·50	3·1 ± 0·20	59·4
10^{-5}*	8·5 ± 0·35	2·0 ± 0·20	83·0

* Haloperidol 5 × 10^{-6} M had no effect on the inhibition of [^{14}C]dopamine synthesis by apomorphine.

phine results in approximately 50% decrease of [^{14}C]catecholamine biosynthesis from [^{14}C]tyrosine in the telencephalon and in the brain stem of rats. The *in vivo* inhibitory activity of apomorphine was not affected by pretreatment of the rats with haloperidol.

Apomorphine, like other catechols, inhibits tyrosine hydroxylase activity *in vitro* and *in vivo*. The findings that striatal slices are more sensitive to the inhibition of dopamine biosynthesis by apomorphine than tyrosine hydroxylase preparations *in vitro* suggest that apomorphine accumulates in the striatal dopaminergic neurons or that some other mechanisms are responsible for the effective inhibition in the striatal slices. The experiments with haloperidol, a drug known to block the dopamine receptors, indicate that the inhibitory effectiveness of apomorphine is not due to its stimulation of dopamine receptors. Thus, the inhibitory effectiveness of apomorphine in the striatal slices is most likely due to the accumulation of the drug in the dopaminergic neurons.

Although apomorphine inhibits dopamine formation *in vivo*, it is unlikely that the functional changes produced by the drug are associated with its inhibitory properties. The functional changes are observed after the administration of low doses of apomorphine (Butcher & Andén, 1969), while the inhibition of dopamine biosynthesis *in vivo* requires a higher dose.

The inhibition of catecholamine biosynthesis by apomorphine may limit the usefulness of this drug or of some apomorphine type derivatives in treatment of parkinsonism. The stimulation of the dopamine receptors with a concomitant decrease in catecholamine levels might produce some undesirable effects.

Finally, the present findings suggest an interpretation other than that previously presented for the acceleration of noradrenaline disappearance by apomorphine after tyrosine hydroxylase inhibition (Persson & Waldeck, 1970). The accelerated noradrenaline disappearance produced by apomorphine after tyrosine hydroxylase inhibition is most likely due to the potentiation of tyrosine hydroxylase inhibition and not as previously postulated, to the stimulation of dopamine receptors.

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Sensitivity changes to noradrenaline in the guinea-pig vas deferens induced by amphetamine, cocaine and denervation

Recently de Moraes & Carvalho (1968) and Carvalho, Martins & de Moraes (1970) provided strong evidence that amphetamine is an indirectly-acting sympathomimetic amine that induces presynaptic supersensitivity to noradrenaline. Amphetamine is known to inhibit noradrenaline uptake (Axelrod, Hertting & Potter, 1962; Burgen & Iversen, 1965; Häggendal & Hamberger, 1967). The current theory of the action of cocaine is that the drug produces competitive saturation of the noradrenaline uptake into adrenergic nerves (Furchgott, Kirpekar & others 1963; Draskóczy & Trendelenburg, 1968) by impairing amine uptake by the adrenergic nerves (Langer & Trendelenburg, 1969). On the other hand, the sensitizing action of cocaine cannot be attributed solely to this action

Guinea-pigs 450–600 g were killed by a blow on the back of the neck and decapitated. The vas deferens was suspended in a water-jacketed bath containing 18 ml of modified Krebs-bicarbonate solution (Huković, 1961), maintained at 31° and bubbled with 5% carbon dioxide in oxygen. Dose-response curves for noradrenaline were obtained by the single dose method and constructed from recording of isotonic contractions obtained by means of a frontal writing level on a kymograph. Two control dose-response curves were always determined on each vas deferens before the treatment of the tissue with the sensitizing agent. Tissues were sensitized to noradrenaline with amphetamine or cocaine during 20 min. Repetition of dose-response curves at intervals less than 20 min occasionally resulted in erratic responses. (−)-Noradrenaline bitartrate (+)-amphetamine sulphate and cocaine hydrochloride were dissolved in distilled demineralized water which contains 0·02 mM of ascorbic acid. Noradrenaline, cocaine and amphetamine were expressed as molar concentrations of the bases. In some of the animals the vas deferens was denervated according to Birmingham (1967). Fourteen days after surgical sympathectomy the animals were killed and the vas deferens prepared as described.

The dose-response curves to noradrenaline determined on the preparation before and after the exposure to amphetamine 10^{-4} M, and to cocaine (10^{-5} M) and after surgical denervation and amphetamine (10^{-4} M) are shown in Fig. 1. It is apparent that after the treatment with amphetamine the dose-response curve of the preparation to noradrenaline is shifted to the left by more than 2 log units without increase in the maximum control response; this we have found before (Carvalho, Martins & de Moraes, 1970). After treatment with cocaine or surgical denervation, amphetamine shifts the dose-response curve to noradrenaline to the left only by factors of 20 and 18 respectively, although cocaine and surgical sympathectomy increased the maximum control response (Table 1).

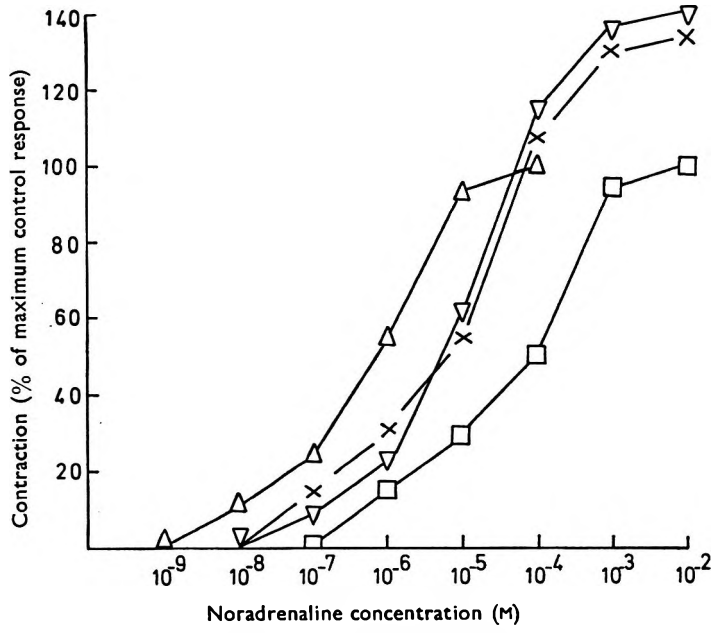

FIG. 1. Dose-response curves of noradrenaline determined in the isolated guinea-pig vas deferens. (□) control; (△) after exposure to 10^{-4} M amphetamine; (▽)after 10^{-5} M cocaine followed by 10^{-4} M amphetamine (×) 2 weeks after surgical denervation followed by 10^{-4} M amphetamine.

TABLE 1. *The effect of various procedures on the response of the guinea-pig isolated vas deferens to noradrenaline*

Agent and procedure	n	EC50 (Mean ± s.e.)	Relative sensitivity to nor adrenaline	Maximum response (mean ± s.e.) mm
Control	10	3·986 ± 0·028	1	48·6 ± 0·6
10^{-4} M Amphetamine	5	6·150 ± 0·035	146	47·4 ± 1·9[a]
10^{-5} M Cocaine	5	4·950 ± 0·080	9·5	123·3 ± 1·1[b]
2 weeks after surgical denervation	8	4·700 ± 0·018	5	120·5 ± 2·6[b]
10^{-5} M Cocaine and 10^{-5} M amphetamine	5	5·300 ± 0·086	20	124·2 ± 1·8[b]
2 weeks after surgical denervation and 10^{-4} M amphetamine	8	5·250 ± 0·015	18	119·4 ± 2:1[b]

n Number of experiments.
[a] Value not significantly different from control ($P > 0.05$).
[b] Values significantly different from control values ($P < 0.05$) EC50 molar concentration of noradrenaline producing 50% of the maximum effect.

The sum of evidence presented strongly favours the conclusion that amphetamine induces presynaptic supersensitivity to noradrenaline in the guinea-pig isolated vas deferens. The sensitizing action of the drug was reduced by cocaine and is dependent on the functional integrity of the adrenergic nerves. It has been suggested that cocaine produces supersensitivity to noradrenaline not only by inhibiting the noradrenaline uptake but also by changing the conformation of the receptor area and thus, probably increasing the efficiency of the drug-receptor complex (Barnett, Greenhouse & Taber, 1968; Reiffenstein, 1968; Varma & McCullough, 1969). In view of these facts it is suggested that the site of the sensitizing action of amphetamine

is presynaptic, there is probably an impairment of the noradrenaline uptake into the adrenergic nerves. Cocaine and surgical denervation inhibit the uptake of noradrenaline but they also seem to sensitize the isolated vas deferens by a "deformation" of the receptor area, thus altering receptor kinetics to allow increased receptor utilization.

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Pentazocine and nikethamide antagonism

Pentazocine, a benzomorphan derivative, is in increasing use for the relief of pain. Like all analgesics it is capable of producing respiratory depression in man and possibly in some other species also. Because pentazocine is itself an opiate antagonist, respiratory depression produced by it cannot be reversed by nalorphine. It therefore seemed worth while to examine the possibility of using nikethamide to reverse the respiratory depression produced by pentazocine.

The respiratory minute volume of rabbits was measured with the aid of the Gaddum Respiration Recorder by methods described previously (Hunter, Pleuvry & Rees, 1968).

In a preliminary trial, pentazocine, 4 mg/kg, produced in rabbits significant respiratory depression in most animals. The administration of nikethamide, 25 mg/kg, produced a sharp increase in respiratory minute volume and a less distinct increase in respiratory rate in animals given 4 mg/kg of pentazocine 7 min previously (Table 1). The simultaneous administration of nikethamide and pentazocine produced significantly less depression of respiratory minute volume, but not of rate, than pentazocine alone. A dose of nikethamide of 25 mg/kg was also capable of producing an increase in respiratory minute volume and rate of respiration in an animal depressed by morphine (Table 1).

These findings point the way to the treatment of an emergency in which the administration of pentazocine produces an unexpectedly severe depression of respiration in the human subject.

Since this work was begun Kallos & Smith (1968) showed that naloxone can reverse the respiratory depression produced by pentazocine in human volunteers, and this finding is amply backed by experimental evidence in animals. Until naloxone

Table 1. *The effect of nikethamide* (25 mg/kg) *on the respiratory depression produced by pentazocine* (4 mg/kg) *and morphine* (8 mg/kg). Figures are means and standard errors of means, recorded immediately before and after the injection of nikethamide.

Drug	No. animals	Respiratory minute volume (% control) Before nikethamide	After nikethamide	Significance of difference *P*	Respiratory rate (% control) Before nikethamide	After nikethamide	Significance of difference *P*
Pentazocine	5	86 ± 3·2	110 ± 4·9	<0·01	79 ± 5·1	104 ± 4·8	<0·01
Morphine	5	16 ± 1·8	69 ± 10	<0·01	22 ± 1·6	35 ± 4·6	<0·05

Table 2. *The effect of giving nikethamide simultaneously with pentazocine.* Figures are means and standard errors of means.

	No. animals	Respiratory minute volume (% control) With nikethamide	Without nikethamide	Significance of difference *P*	Respiratory rate (% control) With nikethamide	Without nikethamide	Significance of difference *P*
7 min after injection	5	105 ± 2·4	92 ± 4·0	0·05	89 ± 3·0	91 ± 3·2	N.S. >0·05
15 min after injection	5	105 ± 2·9	93 ± 3·9	0·05	98 ± 2·3	96 ± 6·5	N.S. >0·05

becomes available for use in the United Kingdom, nikethamide remains a convenien antagonist should respiratory depression arise after the administration of pentazocine.

We are indebted to Dr. R. A. P. Burt for reading this text, to Mrs. H. R. Lane for help with the statistical analysis, and to the Sterling-Winthrop Research Division for generous supplies of pentazocine.

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The time course of the carrageenan-induced oedema of the paw of the rat

The oedema of the rat paw induced by carrageenan injection develops slowly and reaches its peak in 4 h (Winter, Risley & Nuss, 1962). It is uninfluenced by pretreatment with antihistamine agents but is inhibited by salicylate congeners (Bonta, 1965). On the other hand, the paw oedema induced by dextran, yeast or egg albumin develops rapidly, reaching its maximum in ½ to 1 h and then slowly subsides (Winter & others, 1964). It is blocked by pretreatment with antihistamine and anti-5-hydroxytryptamine agents (Bonta, 1965). Thus oedema following carrageenan is attributable to capillary permeability from the release of kinins, while oedema from dextran, yeast or egg albumin mainly follows release of histamine and 5-hydroxytryptamine. We have now examined the time course of the oedema induced by carrageenan.

Adult albino rats, 80–100 g, were divided into groups of six. Carrageenan (0·05 ml of 1% suspension in normal saline) was injected subcutaneously into the hind paw. The volume of the paw was measured before and every ½ h after injection for 3 h by the micropipette method of Buttle, D'Arcy & others (1957). One group of rats served as control and one group each was treated with intraperitoneal injections of test drugs. Mepyramine (5 mg/kg, i.p.), 2-bromolysergic acid diethylamide (3·2 mg/kg, i.p.), acetylsalicylic acid (50 mg/kg, i.p.) and trasylol (20,000 units/kg, i.p.) were injected 1 h before the carrageenan injection, while compound 48/80 (1 mg/kg, i.p.) was injected for 3 days before the experiment to deplete histamine (Parratt & West, 1957).

In control animals the oedema developed slowly, although an increase in volume was measured after ½ h, and reached its maximum in 3 h.

Both mepyramine (5 mg/kg, i.p.) and compound 48/80 (1 mg/kg, i.p. × 3 days) pretreatment reduced the early (P 0·001 and 0·01 respectively) and the delayed phase of swelling at 3 h ($P < 0·01$ and 0·001 respectively). Bromolysergic acid (3·2 mg/kg, i.p.) pretreatment failed to inhibit the early phase of rat paw oedema at the end of ½ h (P 0·1) but it significantly reduced delayed phase of the oedema at the end of 3 h (P 0·01).

Acetylsalicylic acid (50 mg/kg) or trasylol (20,000 units/kg) failed to inhibit the oedema after ½ h (P 0·2 and 0·9 respectively), but did so at 3 h ($P < 0·01$).

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Absorption of (−)-nicotine-1′-*N*-oxide in man and its reduction in the gastrointestinal tract

(−)-Nicotine is metabolized to (−)-nicotine-1′-*N*-oxide and (−)-cotinine in man (Bowman, Turnbull & McKennis, 1959; Booth & Boyland, 1970). Nicotine, cotinine and the highly water soluble, but virtually lipid insoluble, nicotine-1′-*N*-oxide* are excreted in urine. Nicotine-1′-*N*-oxide has negligible lipid solubility over the pH range from 2 to 9 (Badgett, Eisner & Walens, 1952) and little absorption from an oral dose would therefore be predicted.

However, oral administration of nicotine-1′-*N*-oxide* results in peak urinary excretion of this *N*-oxide within 2 h of administration (Fig. 1); neither of the two metabolites, nicotine nor cotinine, could be detected until 4·5 h. The amounts recovered as (−)-nicotine-1′-*N*-oxide, (−)-nicotine and (−)-cotinine are shown in Table 1.

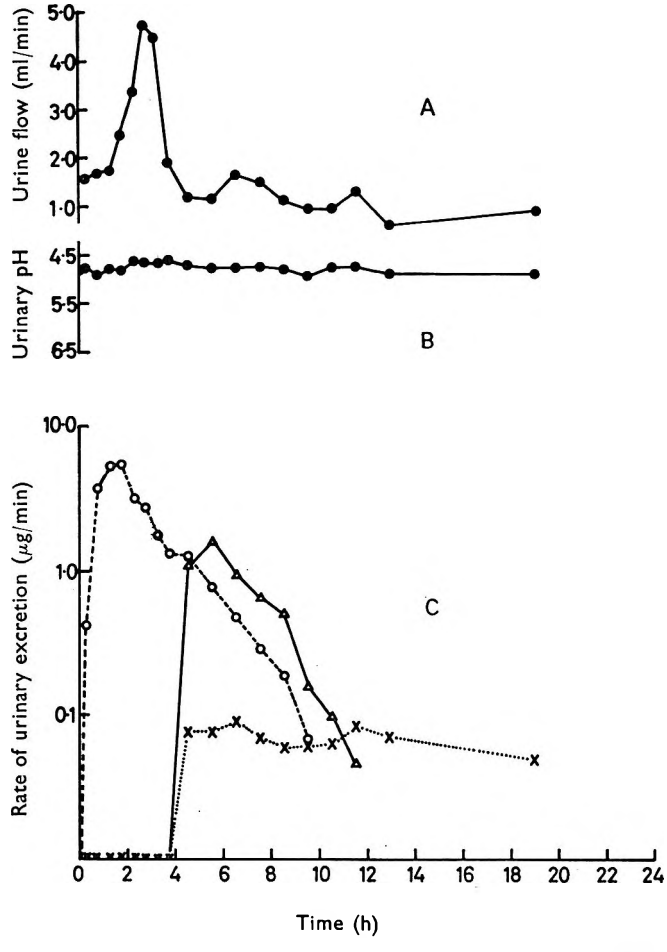

FIG. 1. The urinary excretion of nicotine, cotinine and nicotine-1′-*N*-oxide after oral administration of 2·6 mg nicotine-1′-*N*-oxide under conditions of acidic urinary pH. – – ○ – – Nicotine-1′-*N*-oxide. ——△—— Nicotine. . . X . . Cotinine.

* Mixture of two diastereoisomers.

Table 1. *The urinary recoveries of nicotine-1′-N-oxide, nicotine and cotinine after oral, intravenous and rectal administration of nicotine-1′-N-oxide to subjects with acidic urinary pH (pH 4·8 ± 0·2)*

	% urinary recovery after								
	Oral administration 2·6 mg			Intravenous administration 1 mg			Rectal administration* 2·6 mg		
Subject	*N*-Oxide	Nicotine	Cotinine	*N*-Oxide	Nicotine	Cotinine	*N*-Oxide	Nicotine	Cotinine
1	44·6	15·3	3·4	103	0	0	0·7	8·3	6·3
2	37·7	17·0	3·9	104	0	0	7·0	15·0	7·6
3	29·5	10·5	11·5	—	—	—	—	—	—
4	22·4	15·0	15·0	—	—	—	—	—	—

* Subject 2 was able to retain the solution in the rectum for 5 h but subject 1 for only 1 h.

Approximately twice as much nicotine was recovered in the urine, after oral administration of the *N*-oxide, than when the corresponding dose of nicotine was administered orally. On the other hand, intravenous administration of the *N*-oxide lead to quantitative recovery of this compound in the urine; nicotine and cotinine could not be detected (Table 1).

The results show that circulating levels of nicotine-1′-oxide in the blood are not reduced to nicotine metabolically. The results from oral administration are interpreted by assuming that the highly water soluble *N*-oxide is rendered lipid soluble by ion pair formation under acidic conditions with chloride ions in the stomach. This would account for the rapid peak levels observed in the urine. Some of the material escapes into the duodenum where it is not absorbed due to changes in pH. As the material proceeds down the intestinal tract, reductases in the gut contents or flora, reduce the *N*-oxide to nicotine which is absorbed as such and part of which is then metabolized to cotinine in the liver. Because the nicotine becomes available for absorption further along the gastrointestinal tract than when an oral dose of nicotine itself is given, higher recoveries of nicotine are obtained in the former case since a partial first bypass of the hepatic drug-metabolizing enzymes occurs.

This explanation is supported by the fact that rectal administration of an aqueous solution of nicotine-1′-*N*-oxide results in negligible absorption of the *N*-oxide itself, but the appearance of substantial amounts of nicotine and cotinine in the urine within 0·5 h of administration of the *N*-oxide (Table 1).

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A neutron diffraction study of an acetylcholine analogue-*erythro*(±)-α,β-dimethylacetylcholine iodide

Nuclear magnetic resonance (Culvenor & Ham, 1966) and x-ray diffraction studies (Canepa, Pauling & Sörum, 1966) of acetylcholine have established that the conformation of the choline residue (i.e., N^+-C-C-O atoms) is synclinal (*gauche*) both in deuterium oxide and in the solid state. It has been suggested (Canepa & others, 1966; Martin-Smith, Smail & Stenlake, 1967a) that this spatial arrangement owes at least part of its stability to N^+-C-H . . . O hydrogen bonding of the type proposed by Sutor (1963). Infrared data (Martin-Smith & others, 1967b) on this molecule have been interpreted to be consistent with the presence of C-H . . . O hydrogen bonds. The nmr spectra does not, however, substantiate the existence of such an interaction. X-ray studies of acetylcholine and many of its analogues have never been of sufficient accuracy to locate the protons and thus clarify this question. A neutron diffraction study of an acetylcholine analogue (*erythro*-(±) α,β-dimethylacetylcholine iodide) was undertaken to establish whether the synclinal conformation of the N^+-C-C-O system is in fact stabilized by C-H . . . O hydrogen bonding. The non-hydrogen atom structure of this molecule has been studied by x-ray methods (Shefter, Sackman & others, 1970) and shown to have a similar N^+-C-C-O conformation to that of acetylcholine.

Crystals of sufficient size for a neutron diffraction study (approximately 4 × 2 × 2 mm) were grown from an ethanol–ether solution. The crystallographic parameters obtained for these monoclinic crystals by x-ray diffraction (Shefter & others, 1970) were used in the neutron study. Intensity data were collected on an automated four-circle diffractometer with a neutron wavelength of 1·038 Å and to a maximum *two theta* value of 85°. These data were processed in the usual manner correcting for the Lorentz factor and absorption to obtain structure amplitudes. An extinction correction was applied during the structure refinement. All of the hydrogen positions were located from a difference Fourier synthesis using the non-hydrogen positions determined by x-ray diffraction as the phasing model. Positional and thermal parameters have been refined by a full matrix least squares method to a current R factor (usual reliability index) of 0·080 for 2084 observed reflections. The standard errors obtained for the bond lengths are on the average 0·008 Å for those involving non-hydrogen atoms and about 0·02 Å for the non-hydrogen to hydrogen distances. A detailed structural report will be published.

The upper limit for the distance between a hydrogen and an oxygen atom at which the two atomic species might be considered as forming a hydrogen bond is 2·4 Å (Hamilton, 1968). This value is 0·2 Å less than the sum of the van der Waals radii of

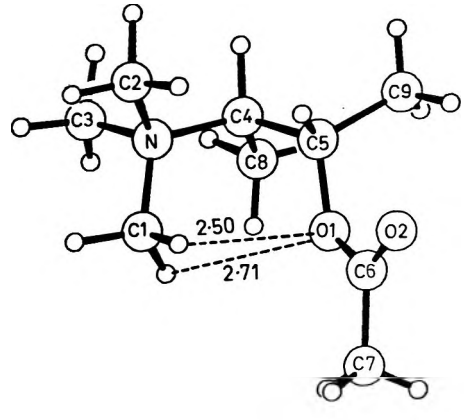

FIG. 1

the respective atoms. The neutron diffraction study (Coppens, 1964) of *o*-nitrobenzaldehyde provides experimental justification for the use of this limiting distance.

The hydrogen atoms which are closest to the acetoxy oxygen [O(1)] are clearly denoted in Fig. 1. The H . . . O distances indicate that hydrogen bonding between the cationic portion of the molecule and the ester linkage is not involved in the stabilization of the conformation of the choline moiety. This is further supported by the C(1)-H . . . O(1) angles which deviate substantially from linearity (100° and 88°). The stabilization of the *synclinal* arrangement of the N^+-C-C-O grouping is in all probability greatly influenced by the electrostatic interaction between the basic portion of the molecule (ester linkage) and the acidic quarternary nitrogen (Shefter & Mautner, 1969).

Mathieson (1965) has shown that the preferred conformation for secondary esters has a synperiplanat (0° ± 30°) distribution of H-C-O-C angles [H-C (5)-O(1)-C(6) in this structure]. The possibility has been raised that this conformation might be stabilized by a C(5)-H . . .O(2) hydrogen bond. This is based on the fact that a torsion angle of 0° would make the H to O(2) distance approximately 2·2 Å (well within the correct range for such an interaction). In the *erythro*-acetylcholine analogue the O(2) . . . H distance is 2·46 Å and the torsion angle [H-C (5)-O(1)-C(6)] is −35° for the $\alpha(R)$ $\beta(S)$ isomer. Again the possibility of C-H . . . O hydrogen bonding as a mode of conformational stabilization has been eliminated. In fact, the hydrogen has a repulsive effect on the conformation of the C(5)-O(2) ester bond.

The relevance of the conformational parameters to cholinergic activity of this molecule has been discussed at some length (Shefter & others, 1970; Shefter, 1970) and requires no further comment here.

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Accumulation of 5-hydroxytryptophan in mouse brain after decarboxylase inhibition

The amount of 5-hydroxytryptophan (5-HTP) normally present in the brain is too small to be detected by any of the methods so far available. Wiegand & Scherfling (1962) have reported a content of 5-HTP in mouse brain of less than 0·1 μg/g and Lindqvist (unpublished) has found a value of less than 0·07 μg/g rat brain and less than 0·03 μg/g mouse brain. We have now investigated the accumulation of 5-HTP in mouse brain after decarboxylase inhibition.

Groups of 13–14 white female mice (NMRI), 18–24 g, were injected with a single dose of the aromatic amino-acid decarboxylase inhibitor Ro 4-4602[N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)hydrazine], 800 mg/kg intraperitoneally. The animals were decapitated at time intervals after the injection, the brains quickly dissected and each brain immediately homogenized with ice-cold perchloric acid containing ascorbic acid and EDTA (disodium ethylenediamine tetra-acetate). The interval between killing an animal and homogenization of the brain was less than 15 s. 5-HTP was isolated on a Dowex 50, X-4 column according to an unpublished method of Lindqvist.

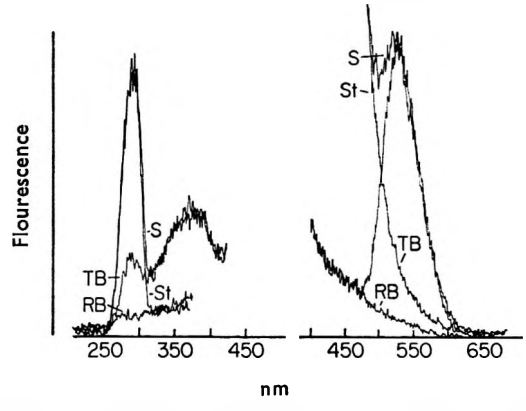

FIG. 1. Activation (left) and fluorescence (right) spectra of mouse brain samples. Ro 4-4602, 800 mg/kg i.p., was given 2 h before killing the animals. The activation spectrum was recorded at a fluorescence wavelength of 545 nm. The fluorescence wavelength was recorded at an activating wavelength of 285 nm. An ultraviolet filter was placed in front of the photocell for elimination of the second order light scatter peak at 570 nm. St = 0·1 μg authentic 5-HTP per 1·6 ml. S = sample. TB = tissue blank. RB = reagent blank.

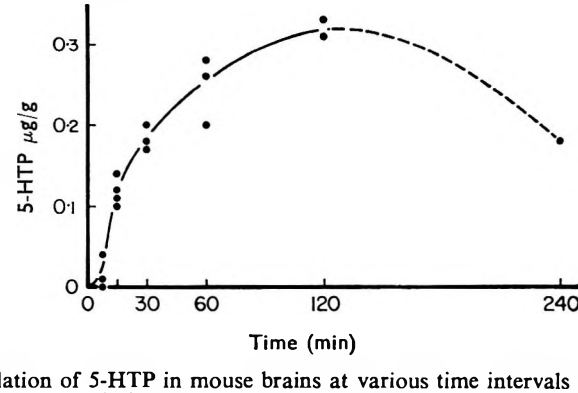

FIG. 2. Accumulation of 5-HTP in mouse brains at various time intervals after injection of Ro 4-4602, 800 mg/kg i.p. Each dot represents one determination on 13–14 pooled brains.

The fluorimetric assay was as for 5-hydroxytryptamine (5-HT) (Andén & Magnusson, 1967). Fig. 1 shows typical spectra of 5-HTP in brain samples of mice treated with Ro 4-4602, 4 h before death.

The concentrations of 5-HTP in mouse brains at various times after Ro 4-4602, 800 mg/kg, are shown in Fig. 2. A rapid accumulation of 5-HTP was seen between 7·5 and 15 min after the injection of the decarboxylase inhibitor. The highest amounts were found after 2 h, about 0·3 μg/g brain. After 4 h the values had declined, probably because of the short duration of action of the inhibitor. The accumulation of 5-HTP between 7·5 and 15 min (about 0·1 μg/g) corresponds to a synthesis rate of 5-HT of 0·8 μg/g h^{-1}. Other authors have reported a turnover rate of 5-HT in rat brain of 0·3 μg/g h^{-1} (Diaz, Ngai & Costa, 1968). Whether this difference represents a real discrepancy, remains to be elucidated.

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The quantitative analysis of alkyl polyoxyethylene glycol monoethers with mass spectrometry and proton magnetic resonance spectroscopy in combination

The most recent spectroscopic method to be applied to the analysis of non-ionic surfactants is proton magnetic resonance (pmr) spectroscopy which, apart from qualitative data, has the added advantage of providing quantitative data without the use of standard compounds, provided a suitable internal standard, like the aromatic protons of a polyoxyethylene alkylphenol, is present in the molecule.

We now report the use of a combination of pmr spectroscopy and mass spectrometry in the examination of the purity of samples of dodecyl tetra-, hexa- and octa- oxyethylene glycol monoethers ($C_{12}E_4$, $C_{12}E_6$ and $C_{12}E_8$, respectively) which were prepared (Corkill, Goodman & Ottewill, 1961) for experiments reported elsewhere (McDonald, 1969). The mass spectra of these samples showed, as expected, molecular ions at m/e = 362, 450 and 538, respectively, and the expected fragmentations by stepwise loss of CH_2 and ($O{\cdot}CH_2{\cdot}CH_2$) units, indicated by a series of peaks at $M^+ - 14$, $M^+ - 28$, $M^+ - 42$, etc. and $M^+ - 44$, $M^+ - 88$, etc., respectively, in each spectrum. Small peaks at m/e = 376 ($M^+ + 14$) (13% of the molecular ion peak) and at m/e = 390 ($M^+ + 28$) (5% of the molecular ion peak) were present in the spectrum of $C_{12}E_4$, indicating the presence of homologous impurities ($C_{12}E_4 + CH_2$) and ($C_{12}E_4 + 2CH_2$), respectively, in the sample. Similarly, small peaks at m/e = 464 ($M^+ + 14$) (1% of the molecular ion peak) and at m/e = 478 ($M^+ + 28$) (5% of the molecular peak) were present in the spectrum of $C_{12}E_6$, indicating the presence of impurities of molecular formula ($C_{12}E_6 + CH_2$) and ($C_{12}E_6 + 2CH_2$), respectively, whereas the spectrum of $C_{12}E_8$ had no peaks of

greater m/e than its molecular ion. Since there were no peaks other than those mentioned above in these mass spectra at higher m/e than the molecular ion peaks, there are no impurities in the samples containing additional ethylene oxide moieties. The possibility that the sample contained impurities with a lower number of ethylene oxide moieties cannot, of course, be ruled out from the mass spectral evidence alone. However, this spectral evidence coupled with the results of the classical method of analysis for ethylene oxide moieties (Siggia, Starke & others, 1958), which agreed with the nominal values for pure samples, make this unlikely. Consequently, we can now use the signal caused by the protons of the CH_2–O group in the pmr spectra of these samples as an internal molecular standard in a quantitative analysis of these compounds: this was not possible in earlier pmr measurements (Flanagan, Greff & Smith, 1963) where mass spectral data was not available.

The pmr spectra were recorded in deuterochloroform solution on a Varian A 60 spectrophotometer with tetramethylsilane as internal standard and checking each integral over three runs. From a table of spectra (Table 1) the combined integrals of

Table 1. *The proton magnetic resonance spectral data of* $C_{12}E_4$, $C_{12}E_6$ and $C_{12}E_8$.

Protons giving rise to signal	Sample $C_{12}E_4$ Signal (τ)	Sample $C_{12}E_6$ Signal (τ)	Sample $C_{12}E_8$ Signal (τ)
$-O-(CH_2)_2-O$	6·34 (s)	6·33 (s)	6·37 (s)
Dodecyl CH_2–O	6·57 (t) (J = 7Hz) } 26[a]	6·53 (t) (J = 7Hz) } 27[a]	6·57 (t) (J = 7Hz) } 26[a]
Dodecyl C–CH_2–C	8·74 (s)[c] 30[b]	8·72 (s)[c] 21[b]	8·75 (s)[c] 15[b]
Dodecyl CH_3–C	9·13 (t) (J = 6Hz)	9·12 (t) (J = 6Hz)	9·12 (t) (J = 6Hz)
–OH	6·89 (s, broad)	6·98 (s, broad)	Not observable

[a] Combined integral. [b] Integral. [c] Strong singlet masks, but incorporates the quartet caused by the methylene protons of the ethyl group of the dodecyl moiety.

the low-field singlet and low-field triplet of $C_{12}E_4$, $C_{12}E_6$ and $C_{12}E_8$ (i.e. 26, 27 and 26, respectively) must correspond to 18, 26 and 34 protons, respectively (i.e. 2m + 2 where m is the number of methylene groups in the polyoxyethylene chain). Thus it can be seen readily that the corresponding integrals of the high-field singlet (i.e. 30, 21 and 15, respectively) are equivalent to 20·8, 20·2 and 19·6 protons ($\pm 2\%$ owing to errors in determining integral values), compared with 20 protons (i.e. 2n-2 where n is the number of methylene groups in the dodecyl moiety) which would be expected for pure compounds.

If in the above mass spectra $M^+ + 14$, $M^+ + 28$, etc. peaks were absent but $M^+ + 44$, $M^+ + 88$, etc. peaks were present, the high-field singlet in the pmr spectrum would then have acted as internal molecular standard, assuming lower homologues than dodecyl ethers were absent. If, however, the mass spectra indicated the presence of both series of peaks above M^+ then no internal molecular standard would be available (the OH and CH_3 signals are relatively too weak and ill-defined) and the above approach would not be valid.

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Journal of Pharmacy and Pharmacology

Volume 22 Number 9 September 1970

Original Papers

649–658 B. D. ROUFOGALIS, J. THOMAS
On the mechanism of potentiation of the activity of acetylcholinesterase by some quaternary ammonium compounds

659–663 H. H. MILLER, W. R. JONDORF
The subcellular distribution of (±)-2,3-dehydroemetine and (−)-emetine in rat liver and changes in hepatic lipid content after treatment of rats with (±)-2,3,dehydroemetine

664–667 GIOVANNI BROTZU
Inhibition by chlorpromazine of the effects of dopamine on the dog kidney

668–671 HELEN GOLDHAMMER, W. R. MCMANUS, R. A. OSBORN
The effect of a range of Triton non-ionic surfactants on rodent ovaries

672–678 JOHN H. COLLETT, CATHERINE COLLETT, ALFRED N. MARTIN, ARTHUR CAMMARATA
The effects of some tetracyclines on synchronously growing cultures of *Escherichia coli* B/r

679–683 MARGARET MCCAFFERTY, MALCOLM S. PARKER
The inhibition of the development of the spores of a spoilage mould by chemical preservatives

684–693 D. J. DRAIN, M. J. DALY, B. DAVY, M. HORLINGTON, J. G. B. HOWES, J. M. SCRUTION, R. A. SELWAY
Anti-inflammatory properties of a series of phenyl- and phenoxy-alkanoic acids

694–699 D. G. BAMFORD, D. F. BIGGS, M. F. CUTHBERT, H. N. GRANT, G. E. LEE, D. W. PULSFORD, W. R. WRAGG
The preparation and intravenous anaesthetic activity of tetrahydrofuran-3-ols

700–703 ANN E. ROBINSON, ARNOLD I. COFFER, FRANCIS E. CAMPS
The distribution of chloroquine in man after fatal poisoning

Letters to the Editor

704–705 R. T. LOUIS-FERDINAND, K. R. CUTRONEO, D. C. KOSEGARTEN, R. C. VASAVADA, J. G. TURCOTTE, D. R. DEFANTI
Flow decrease through rat hind limb vasculature by (±)-carnitine, (±)-acetylcarnitine and (±)-chloroacetylcarnitine

705–706 A. L. GREEN
On the local anaesthetic action of chlorpromazine and some non-tranquillizing analogues

707–708 A. LINDQVIST, S. LINDGREN, B. LINDEKE, B. KARLÉN, R. DAHLBOM, M. R. BLAIR, JR.
N-(Perhydroazepinoalkynyl)- and *N*-(perhydroazocinoalkynyl) succinimides as oxotremorine antagonists

709–710 JAMES BOGAN
Effect of SKF-525A on the fate of thiopentone

710–712 G. A. JOHNSON, S. J. BOUKMA, P. A. PLATZ
2-Mercaptobenzothiazole, an inhibitor of dopamine β-hydroxylase

713–714 SHELDON STOLMAN, HORACE H. LOH
Inhibition of hepatic ribonuclease activity by chronic administration of phenobarbitone

715–717 MENEK GOLDSTEIN, LEWIS S. FREEDMAN, TONY BACKSTROM
The inhibition of catecholamine biosynthesis by apomorphine

717–719 S. DE MORAES, F. V. CARVALHO, R. D. WEHRLE
Sensitivity changes to noradrenaline in the guinea-pig vas deferens induced by amphetamine, cocaine and denervation

719–720 A. R. HUNTER, C. T. MAJOR
Pentazocine and nikethamide antagonism

721 T. N. BHALLA, K. K. TANGRI
The time course of the carrageenan-induced oedema of the paw of the rat

722–723 A. H. BECKETT, J. W. GORROD, P. JENNER
Absorption of (−)-nicotine-1′-*N*-oxide in man and its reduction in the gastrointestinal tract

724–725 THOMAS F. BRENNAN, FRED K. ROSS, WALTER C. HAMILTON, ELI SHEFTER
A neutron diffraction study of an acetylcholine analogue-*erythro* (±)-α,β-dimethylacetylcholine iodide

726–727 ARVID CARLSSON, MARGIT LINDQVIST
Accumulation of 5-hydroxytryptophan in mouse brain after decarboxylase inhibition

727–728 C. MCDONALD, B. ROBINSON
The quantitative analysis of alkyl polyoxyethylene glycol monoethers with mass spectrometry and proton magnetic resonance spectroscopy in combination