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Solubility and viscosity relations in the system ascorbic acid–water–polysorbate 80

J. R. NIXON AND B. P. S. CHAWLA*

The solubility of ascorbic acid in dispersions of polysorbate 80 has been examined. Over a large region of the solubility range liquid crystal exists, bounded by isotropic liquid phases. Excess solubilizate separates out as crystalline material. The rheological properties are complex. Within the isotropic phases the viscosity is Newtonian, but as soon as liquid crystal is produced pseudoplastic or thixotropic flow occurs. In the centre of the liquid crystal region, there were concentrations exhibiting a "yield" value followed by relaxation before the usual thixotropic loop.

THE viscosity and stability of ascorbic acid in aqueous polysorbate 20 dispersions has recently been examined by Nixon & Chawla (1965). This system, because of its hydrophilic nature, produced no liquid crystalline phases. The viscosity of the system was Newtonian throughout and rose to a maximum in an ascorbic acid-saturated dispersion containing 68% w/w polysorbate 20.

The present system exhibited far more complex relationships between solubilization and viscosity; also, liquid crystal was present. Over large regions of the "viscosity map" non-Newtonian viscosity was observed.

Experimental

Ascorbic acid. Assay (iodometric) 99%, m.p. 190–192°, $[\alpha]_D^{20}$ 2% in water +22°, pH of 2% in water 2·5.

Polysorbate 80. (Tween, Honeywill-Atlas Ltd) complied with manufacturer's specification dated October, 1956.

Determination of viscosity. The Ferranti-Shirley cone and plate viscometer was used as previously described (Nixon & Chawla, 1965). Data obtained using this viscometer allowed the preparation of stress/strain diagrams which indicated the type of flow of the system. Viscosities were calculated from these curves.

Results and discussion

The solubility of ascorbic acid decreased slowly with increases in the polysorbate 80 concentrations and it was impossible to dissolve any ascorbic acid at polysorbate 80 concentrations above 97% w/w. Liquid crystalline gel showing birefringence under polarized light occurred over a wide region. Birefringence started at a concentration of 44% w/w polysorbate 80, no ascorbic acid being present. The smallest concentration of polysorbate 80 required to produce a system containing only liquid crystal was 37·5% w/w. This system contained 9·5% w/w ascorbic acid. With concentrations of ascorbic acid increasing above this there was a single liquid crystalline phase which required increasingly larger concentrations of polysorbate 80 for its existence. In a dispersion

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saturated with ascorbic acid, the single liquid crystalline gel phase occurred at a polysorbate concentration of 52·5% w/w. Below this single gel phase a region existed in which both liquid crystal and isotropic solution occurred.

The upper limit of the liquid crystalline phase was difficult to determine because of the very high viscosity. The system could not be separated into two phases by centrifugation and appeared to pass directly from a clear isotropic solution into a liquid crystalline gel exhibiting birefringence. This, according to Winsor, is an impossible transition and a region where liquid crystal is in equilibrium with isotropic solution should always occur (Winsor, 1954). At concentrations of ascorbic acid in excess of the solubility, crystalline ascorbic acid separates out.

Dispersions of ascorbic acid–polysorbate 80–water exhibited Newtonian or non-Newtonian flow depending upon the phases present. Within the region L_1 (Fig. 1) the viscosity was Newtonian. As soon as liquid crystal

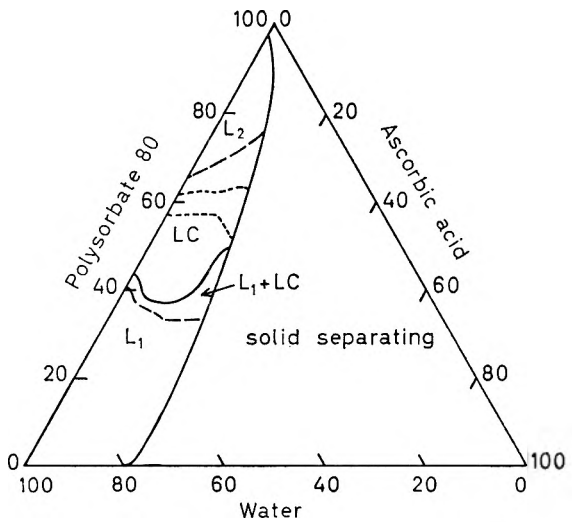

FIG. 1. The solubility of ascorbic acid in polysorbate 80 dispersions. L_1 and L_2 are isotropic liquid phases, and LC liquid crystal phases. Temperature 25° ± 0·1°; — — — upper and lower limit of existence for liquid crystal; —— lower boundary of liquid crystal gel phase; – – – boundary of liquid crystal exhibiting yield/relaxation curve before thixotropic loop. All concentrations are % w/w.

existed in contact with the L_1 phase non-Newtonian flow occurred. At the boundary of the liquid crystalline region, pseudoplastic flow was exhibited. As the proportion of liquid crystal phase increased, this pseudoplastic behaviour became more pronounced and, immediately before the liquid crystal region, the dispersions began to exhibit thixotropy. When only liquid crystal existed, thixotropy was shown by all the systems examined. Finally on passing from the liquid crystal into the L_2 phase Newtonian viscosity once more occurred.

Within the liquid crystalline phase the degree of thixotropy, as measured by the area of the thixotropic loop, increased proportionately to the

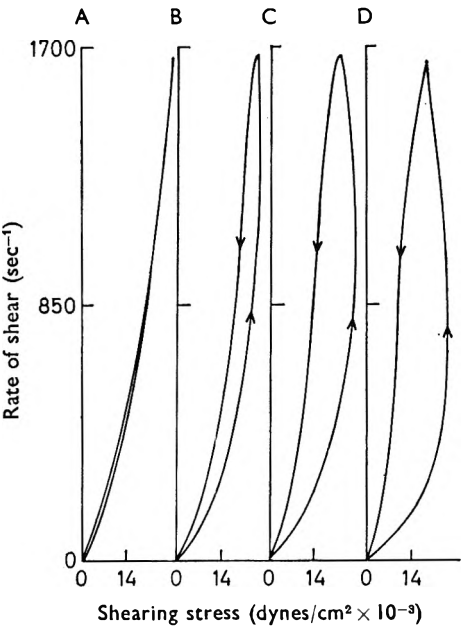

FIG. 2. The effect of ascorbic acid concentration on the degree of thixotropy. Concentration of polysorbate 80, 46% w/w; concentrations of ascorbic acid % w/w: A. 0. B. 5·2. C. 10·4. D. 14·8. Temperature 25° ± 0·1°. Cone size 2 cm. Sweep 120 sec.

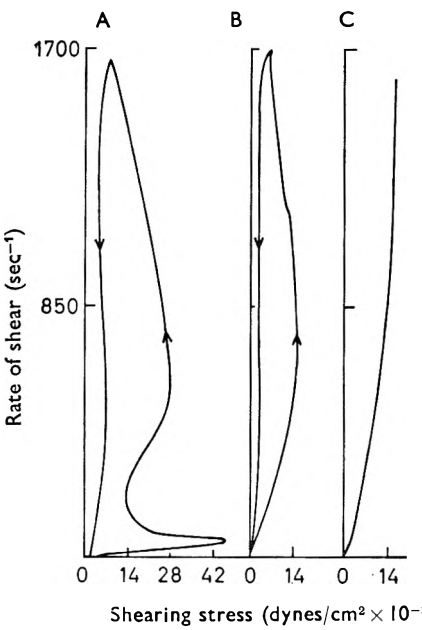

FIG. 3. Yield value before normal thixotropic loop and the effect of recycling on the liquid crystal systems. Polysorbate 80 concentration % w/w 53, ascorbic acid concentration % w/w 15. Temperature 25° ± 0·1°. A. Original system. B. Recycled once. C. Fifth recycle. Cone size 2 cm. Sweep 120 sec.

ascorbic acid present (Fig. 2). On recycling the system the thixotropic properties disappeared and pseudoplastic flow occurred. In very thixotropic dispersions, the system had to be recycled a number of times before complete pseudoplasticity was produced. This thixotropic behaviour returned on standing but the degree of thixotropy, as measured by the area of the loop, was reduced.

A centre of anomalous rheological behaviour was found within the liquid crystalline region (Fig. 1). Within this small region the thixotropic curve exhibited a preliminary "yield" value (Fig. 3). A dispersion containing 60·5% w/w polysorbate 80 and 10·8% w/w ascorbic acid showed further divergencies from the normal liquid crystal thixotropic loop. After the yield value and relaxation shown by systems within this region the hysteresis curve crossed over itself and was particularly narrow. The degree of thixotropy, as measured by the area within the thixotropic loop, decreased as the boundary between the L_2 and LC phases was approached. Once the boundary had been passed the L_2 phase exhibited Newtonian viscosity although the consistency of the dispersions was gel-like.

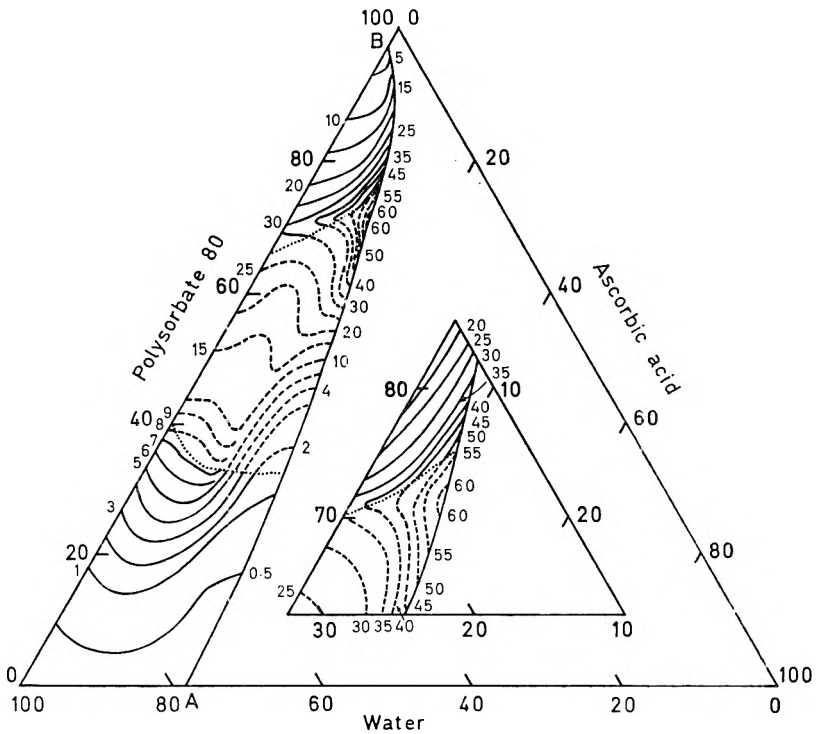

FIG. 4. Viscosity relationships in the system polysorbate 80–ascorbic acid–water. Line A–B is the solubility curve of ascorbic acid. —— Newtonian viscosity in isotropic L_1 and L_2 phases. – – – Derived apparent viscosity from recycling anisotropic L_1 + LC and LC phases. All viscosity measurements in poises. Temperature 25° ± 0·1°. Concentrations % w/w.

A viscosity co-ordinate map (Fig. 4) was prepared as previously (Nixon & Chawla, 1965). Within the L_1 and L_2 phases, where the viscosity was Newtonian, the contours represent regions of true viscosity, but within the LC and LC + L_1 phases, where non-Newtonian flow occurred, only "apparent" viscosities could be recorded. These "apparent" viscosities are calculated from the slope of the straight line portion of the pseudo-plastic curve or the linear portion obtained by recycling the thixotropic liquid crystalline gel. This viscosity map was far more complex than the one found for the system using polysorbate 20. The viscosity, particularly in the liquid crystalline and L_2 phases, was particularly high when compared with polysorbate 20 systems. Even in the isotropic L_1 phase, where in the previous system the addition of ascorbic acid resulted in an increase in viscosity, the contours did not show a simple pattern. Within this region the initial addition of the acid caused an increase in viscosity but subsequent additions, as the solubility limit was approached, resulted in a fall. This fall in viscosity was accompanied by a slight fall in pH. Angelescu & Popescu (1930), Angelescu & Ciortan (1939) and Angelescu & Manolescu (1941) have reported similar phenomena. They found that the addition of phenol or cresol to soap solutions produced an initial rise in viscosity which they attributed to an increase in the colloidal nature and solvation of the solute. The fall in viscosity on the further addition of solute was attributed to true molecular dispersion of the solute. Conductivity measurements indicated the presence of only small micelles in the less viscous systems. Bose (1952), studying the effect of added alcohol on sodium oleate solutions, also noted the existence of these viscosity maxima. In the present systems the viscosity contours in the L_1 phase appear to be amenable to the above explanation. The initial addition of ascorbic acid to an L_1 non-ionic surface-active agent solution probably results in most of the acid being taken up by the micelles. Once these are saturated, corresponding to the maximum viscosity, further additions of acid are dissolved in the water pseudo-phase.

Hyde, Langbridge & Lawrence (1954) have reported the change in viscosity on the addition of an octanoic acid to 36% Teepol solutions. At 25° the viscosity increased to a maximum in the liquid crystal region when a unimolecular ratio of additive to soap was present. This peak viscosity fell rapidly towards the end of the liquid crystal phase and the system was fluid again before all the liquid crystal had gone. These authors also found that as the chain length of the additive increased so did the size of the peak. Collison & Lawrence (1959) made a similar study of the system dodecyl sulphate-water–n-octylamine. They studied the effect of addition of the latter compound on 16·7% aqueous solutions of the soap. The viscosity maximum in the liquid crystal region at 25° was too large to measure with their viscometer. Both these studies used a rising column capillary viscometer and there is no indication whether the viscosity was Newtonian throughout or whether non-Newtonian viscosity occurred in the presence of liquid crystalline phases.

In the liquid crystal and liquid crystal + L_1 regions of the system ascorbic acid–water–polysorbate 80, non-Newtonian flow properties were

observed. Dervichian, Joly & Titchen (1954) have plotted portions of a viscosity contour diagram for the system potassium laurate–water–potassium carbonate and found the regions of viscosity and regions defined by streaming birefringence corresponded. Newtonian viscosity only occurred where birefringence was absent, as in the present system. Dervichian explained the viscosity changes within the phases of his system by suggesting they were caused by "transformations of higher order." It was suggested that these changes of state were "homogeneous" and only indicated by abrupt changes in thermodynamic properties such as entropy or compressibility. Winsor (1954) explained the phenomena in terms of the relative stability of emulsions of his S and G phases. In certain regions, the rate of shear could exert a big influence on the ratio and constitution of the phases present. This is evident from the changes in the rheological curves of the present ascorbic acid systems on recycling. Here, the thixotropic liquid crystal phase is broken down to give an emulsion of liquid crystal and isotropic solution, which at low rates of shear exhibits pseudoplastic flow properties. At high rates of shear the linearity of the stress/strain curves indicates that organized gel structure is absent.

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Molecular interactions of caffeine with *o*-, *m*- and *p*-iodobenzoic acids and *o*-, *m*- and *p*-fluorobenzoic acids

M. DONBROW AND H. BEN-SHALOM

The solubilities of *o*-, *m*- and *p*-iodo- and fluorobenzoic acids in aqueous solutions of caffeine increase linearly with caffeine concentration due to complex-formation. Insoluble complexes are formed by the *o*- and *m*-acids and soluble ones by the *p*-acids. Approximate 1 : 1 stability constants have been evaluated from the phase diagrams and their values are considered in relation to possible mechanisms of complex-formation.

IN an initial examination on the nature of the complexation reaction between aromatic substances and caffeine, Donbrow & Jan (1965) outlined some of the problems of correlating binding strength and mechanism of complexation with structural features of the aromatic compound complexed. Apart from methodological restrictions, which originated from the absence of measurable changes in the physico-chemical properties of the molecules concerned, results have been difficult to interpret because of the variety of structures studied. Hence, earlier work (Labes, 1930; Higuchi & Zuck, 1952, 1953, 1954) has not led to a clear understanding of the phenomena involved. Donbrow & Jan stressed the need to examine closely-related series of complexants selected to limit the number of structural factors which could influence the binding. Using this approach, these authors showed that there was an approximately linear relationship between log K of the caffeine complex (where K = apparent 1 : 1 stability constant) and the pK_a value of the acid for six benzoic acids; the complexes of *o*- and *p*-hydroxybenzoic acids were exceptional, probably because hydrogen-bonding was additionally involved (see Higuchi & Zuck, 1953).

We here report the extension of the studies to another six non-hydrogen-bonding monosubstituted benzoic acids.

Experimental and results

SOLUBILITY

Excess of the organic acid was shaken at 15° with quantities of caffeine in aqueous acidic solution, an aliquot portion was filtered and the organic acid content determined titrimetrically or spectrophotometrically. In the spectrophotometric determination, the caffeine was extracted by shaking with chloroform, before the determination of the acid. The general technique was the same as described previously (Donbrow & Jan, 1965).

Quantities of acid used were: o-*Iodobenzoic acid* (125 mg) in 0·002N hydrochloric acid (50 ml). Titration indicator, cresol red. Results are in Table 1.

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TABLE 1. SOLUBILITY OF *o*-IODOBENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·002N HYDROCHLORIC ACID AND *o*-FLUOROBENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·025N HYDROCHLORIC ACID AT 15°

Caffeine conc. moles × 10^2	*o*-Iodobenzoic acid conc. moles × 10^3	K $moles^{-1}$ litre	Caffeine conc. moles × 10^2	*o*-Fluorobenzoic acid conc. moles × 10^2	K $moles^{-1}$ litre
0·00	1·86	—	0·00	2·99	—
1·00	2·29	24·2	0·00	3·05	—
1·10	2·34	24·5	1·04	3·45	25·4
2·00	2·79	26·2	1·52	3·84	41·2
2·60	3·04	25·6	2·33	4·29	41·3
3·00	3·32	27·5	3·27	4·73	37·1
3·62	3·28		3·38	4·85	40·3
4·00	3·26		3·51	4·74	32·8
5·00	3·29		3·97	5·07	36·4
5·28	2·89		4·35	5·10	31·1
6·00	2·49		4·73	5·25	32·2
7·00	1·92		5·43	5·60	30·6
7·92	1·42		6·57	6·10	30·6
9·30	1·25		7·45	6·80	34·7
10·00	1·22		7·79	6·65	
11·00	1·23		8·54	6·77	
12·00	1·22		9·90	6·95	
			10·30	6·80	
			10·65	6·00	
			11·75	5·15	
			12·30	4·80	
			13·30	4·70	
K av. = 25·6 K slope = 25·7			K av. = 34·5 K slope = 31·5		

m-*Iodobenzoic acid* (125 mg) in 0·002N hydrochloric acid (50 ml). Spectrophotometric determination λ_{max} 217 mμ; ϵ_{max} 11,150. Results are in Table 2.

p-*Iodobenzoic acid* (125 mg) in 0·002N hydrochloric acid (50 ml). Spectrophotometric determination λ_{max} 257 mμ; ϵ_{max} 14,725. Results are listed in Table 3.

TABLE 2. SOLUBILITY OF *m*-IODOBENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·002N HYDROCHLORIC ACID AND OF *m*-FLUOROBENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·025N HYDROCHLORIC ACID AT 15°

Caffeine conc. moles × 10^2	*m*-Iodobenzoic acid conc. moles × 10^4	K ($moles^{-1}$ litre)	Caffeine conc. moles × 10^2	*m*-Fluorobenzoic acid conc. moles × 10^2	K ($moles^{-1}$ litre)
0·00	5·38	—	0·00	1·09	—
0·48	5·90	20·3	0·95	1·28	22·9
1·08	7·00	28·3	1·05	1·34	28·7
1·84	8·00	26·9	1·60	1·48	29·6
2·31	9·40	32·9	1·75	1·54	31·8
3·14	10·81	32·7	2·20	1·50	21·0
3·93	11·60	29·9	2·70	1·70	26·8
4·65	13·30	31·8	3·20	1·76	24·3
5·00	13·40	30·3	3·50	1·82	24·2
5·40	13·50		3·57	1·83	
6·25	13·30		4·10	1·85	
7·12	13·80		4·60	1·84	
7·41	13·50		4·73	1·87	
7·80	13·40		4·75	1·70	
8·30	12·30		5·00	1·16	
8·70	11·40		5·27	0·98	
9·20	10·40		5·50	0·98	
10·00	9·00		5·70	0·98	
			6·00	0·96	
			6·95	0·87	
			8·69	0·82	
			9·05	0·87	
			11·90	0·87	
K av. = 29·1 K slope = 30·2			K av. = 26·1 K slope = 24·5		

TABLE 3. SOLUBILITY OF *p*-IODOBENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·002N HYDROCHLORIC ACID AND OF *p*-FLUOROBENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·025N HYDROCHLORIC ACID AT 15°

Caffeine conc. moles × 10^2	*p*-Iodobenzoic acid conc. moles × 10^4	K ($moles^{-1}$ litre)	Caffeine conc. moles × 10^3	*p*-Fluorobenzoic acid conc. moles × 10^3	K ($moles^{-1}$ litre)
0·00	1·12	—	0·00	4·00	—
0·80	1·80	83·3	0·83	4·50	16·0
1·49	2·40	77·4	1·48	5·00	18·1
1·76	2·65	78·3	2·46	5·50	16·2
2·80	3·35	76·4	3·19	6·00	16·7
3·68	4·10	72·9	4·40	6·60	15·1
4·30	4·60	75·8	6·07	7·10	17·6
4·60	5·00	76·0	7·00	8·00	17·2
5·80	5·80	72·6	8·04	8·50	14·8
6·30	6·30	74·0	8·60	9·00	
6·41	6·46	75·0	8·90	8·85	
6·80	6·80	75·2	10·00	8·90	
7·20	7·00	73·5	10·50	9·00	
8·00	6·90		11·00	9·00	
9·60	6·90		11·50	9·00	
10·80	6·80				
	K av. = 75·5 K slope = 74·3			K av. = 16·5 K slope = 15·6	

o-*Fluorobenzoic acid* (250 mg) in 0·025N hydrochloric acid (25 ml). Titration indicator, phenol red. Results are listed in Table 1.

m-*Fluorobenzoic acid* (100 mg) in 0·025N hydrochloric acid (25 ml). Determination as previous acid. Results are listed in Table 2.

p-*Fluorobenzoic acid* (100 mg) in 0·025N hydrochloric acid (25 ml). Determination as previous acid. Results are listed in Table 3.

Point by point stability constants were calculated as described by Higuchi & Zuck (1952, 1953) and the mean value compared with the value obtained from the slope of the plot where the rising portion was linear (Donbrow & Jan, 1965).

Discussion

o-*Iodobenzoic acid.* Fig. 1 (●) shows the solubility curve. This has 3 stages and is typical of a system in which soluble complex formation is followed by precipitation of an insoluble complex over the plateau region, and then, when the excess solid acid is exhausted, there is a decrease in solubility due to the precipitation of complex at the expense of free acid in solution. Analysis of the solids separating in the region defined by the descending curve of Fig. 1 gave a molecular ratio of caffeine:acid of 3·4:1, whereas the calculated value from the parameters of the phase diagram was 2·5:1. However, the linearity of the initial stage indicates the formation of a mononuclear complex of caffeine (Rossotti & Rossotti, 1961); K values calculated from this stage on a 1:1 basis were: mean value 25·6, slope 25·6. The m.p. of the solid was 192–194°.

m-*Iodobenzoic acid.* The solubility curve is shown in Fig. 2 (●). It is similar to that of the *o*-iodobenzoic acid and can be interpreted in the same way. Chemical analysis of the solids isolated over the third stage gave a ratio of caffeine:acid of 3·5:1. The molecular ratio, calculated

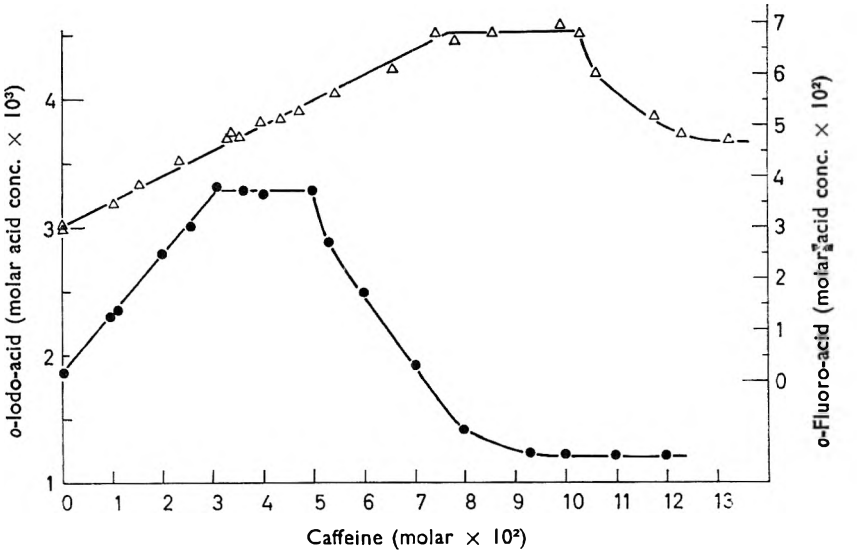

FIG. 1. Effect of caffeine on the solubility of *o*-iodo- and *o*-fluorobenzoic acid at 15° C. ●—●, Iodo-acid (solvent 0·002N HCl); △—△, fluoro-acid (solvent 0·025N HCl).

from the plateau is 2·8 : 1 and the 1 : 1 K values were 29·1 (mean) and 30·2 (slope). The m.p. of the solid was 142–144°.

p-*Iodobenzoic acid.* The solubility curve is shown in Fig. 3 (●). This acid differs from the other iodobenzoic acids in forming a complex that remains in solution. The plateau corresponds to saturation of the system with caffeine. From this point the solid phase is a mixture of excess acid with increasing amounts of solid caffeine. The initial linear

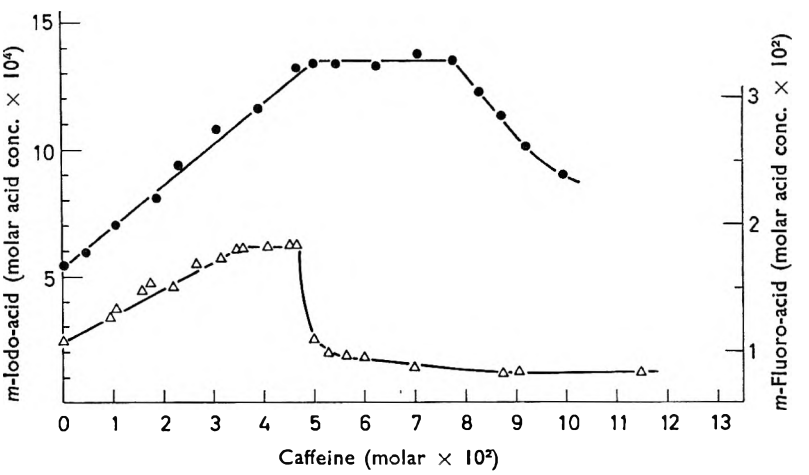

FIG. 2. Effect of caffeine on the solubility of *m*-iodo- and *m*-fluorobenzoic acids at 15° C. ●—●, Iodo-acid (solvent 0·002N HCl); △—△, fluoro-acid (solvent 0·025N HCl).

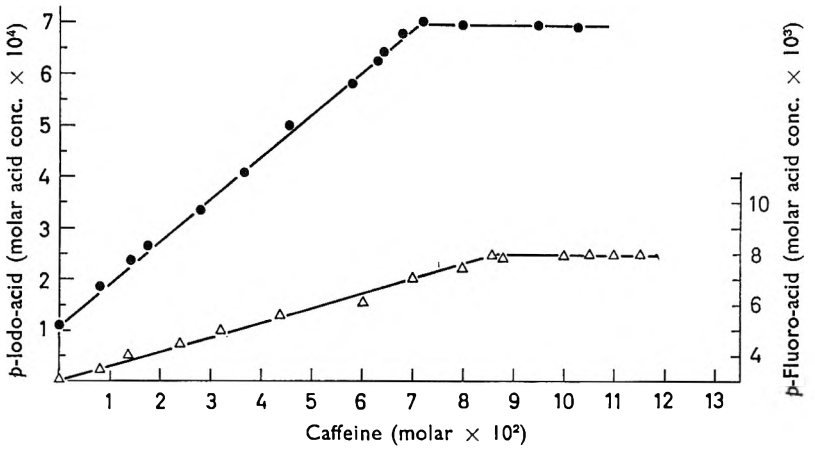

FIG. 3. Effect of caffeine on the solubility of *p*-iodo- and *p*-fluorobenzoic acids at 15° C. ●—●, Iodo-acid (solvent 0·002N HCl); △—△, fluoro-acid (solvent 0·025N HCl).

slope implies formation of a soluble complex mononuclear with respect to the caffeine. From the solubility increase of the caffeine and the acid, it was found that 6·2 moles of caffeine were solubilized for one mole of acid. Calculation of the K values on this basis did not give constant values whereas on a 1 : 1 basis values were virtually constant at 75·5 (mean) and 74·3 (slope).

o-*Fluorobenzoic acid.* The shape of the solubility curve, which is shown in Fig. 1 (△), follows the general pattern for insoluble complexes. Analysis of the solid compounds isolated in the region defined by the descending curve gave a molecular ratio of caffeine : acid of 1·2 : 1, whereas the ratio calculated from the plateau region was caffeine : acid 1·56 : 1. The m.p.'s of the solids were constant (105–108°). K values, calculated on a 1 : 1 basis, tended to fall with increase in the caffeine concentration, possibly because of the formation of higher complexes of caffeine. The mean 1 : 1 K value was 34·5 compared with 31·5 by the method of least squares.

m-*Fluorobenzoic acid.* The solubility curve is shown in Fig. 2 (△). This acid differs from the other halogen-substituted benzoic acids in showing a sharp decrease in the solubility of the acid after the plateau region. Chemical analysis of the solids precipitate gave a ratio for caffeine : acid of 1 : 1 compared with the plateau value of 1·2 : 1. Calculated on a 1 : 1 basis the K value from the slope was 24·4 and the mean value was 26·6. The m.p. of the solid was 119–121°.

p-*Fluorobenzoic acid.* The solubility surve, shown in Fig. 3 (△) is like that for the *p*-iodo acid and indicates the formation of a soluble complex. Although 2·3 moles of caffeine were solubilized per mole of acid, relatively constant K values were obtained on a 1 : 1 basis (mean 16·5, slope 15·6).

The high stoichiometric ratios observed with certain of these acids resemble those obtained in the earlier work. Were multinuclear caffeine complexes formed to any appreciable extent in solution, the solubility increase would show as a pronounced upward curvature with increasing caffeine concentration instead of the linearity actually found. Furthermore, the K values calculated on a 1 : 1 basis should be dependent on a power of the caffeine concentration because of reactions such as:

$$CA + C \leftrightharpoons C_2A$$
$$C_2A + C \rightleftharpoons C_3A, \text{ etc.}$$

(where C = caffeine; A = acid)

Since the curves and K values show no evidence of this, it must be concluded that the higher complexes make little contribution to the solubility increases.

The separation of insoluble higher complexes could be due to the formation of mixed crystals or inclusion compounds, or the very low solubility of the higher complexes, which would then precipitate in the presence of much higher concentrations of the lower complexes.* However, because of the instability of these higher complexes, their solubilities in water could not be measured.

The linearity of the curves does not preclude formation of a 1 : 2 complex, but in the iodo-acids, the low solubility, and hence low free acid concentration, is unfavourable to this. With the more soluble fluoro-acids, the validity of the 1 : 1 values is questionable but the values give a qualitative indication of the overall complexation which occurs.

For the iodo-acids, the binding strength decreases in the order *para* > *meta* > *ortho*, as in most of the benzoic acids studied hitherto, whereas the order is reversed in the fluoro-acids. Together with the previous data obtained at 15° (Donbrow & Jan, 1965), the apparent 1 : 1 constants decrease in the order: *para*, OH > I > OMe > H > NO_2 > F; *ortho*, OH > OMe ≈ F > H > I > OCOMe > NO_2. The values for the *m*-iodo- and *m*-fluoro-acids lie close to the line relating log K to pK_a of the acid (Fig. 4, Donbrow & Jan, 1965); this is not so for the other four acids. The *p*-fluoro-acid has an unexpectedly low constant and the *o*-fluoro- and *p*- and *o*-iodo-acids have unexpectedly high constants, the last being particularly surprising, since steric hindrance to the formation of a sandwich-type complex might have been anticipated; the projection of the iodine atom (van der Waals' radius 2·15 Å) outside the width of the aromatic ring (van der Waals' half-thickness 1·85 Å, see Pauling, 1960) not only fails to inhibit binding, but may possibly be associated with enhancement, which could be explained, in the three acids, by postulating either a different mechanism altogether, or a two-point attachment in which the "normal" interaction forces are supplemented by dipolar, induced dipolar or some other interaction connected with the halogen substituent. The same effect of enhancement of stability constant by *p*-iodo and weakening by *p*-fluoro has been observed by Andrews &

* "Salting-out" effects are unlikely to occur at such low concentrations of non-ionized substances.

Keefer (1949, 1950) in the complexation of substituted benzenes with aqueous silver ion, postulated as occurring by π-electron donation. The halogens do not behave regularly in a number of respects, and a plot of the ionization potentials of substituted benzenes against Taft's (1957) σ_+ constant for the *para*-substituent also shows the same kind of deviation, the iodobenzene having a much lower ionization potential and the fluorobenzene a higher one than expected (Streitweiser, 1963). This would accord with one of the theories put forward previously (Donbrow & Jan, 1965), that a donor-acceptor mechanism involving the aromatic ring electrons might be involved; the present evidence is in favour of the aromatic system as the donor. However, though there are still insufficient data to confirm the mechanism of complexation, it is apparent that complexation with caffeine is much enhanced in a number of benzoic acids substituted with mesomeric electron-donating groups, and weakened by electron attracting groups.

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Steroidal monoquaternary ammonium salts with non-depolarizing neuromuscular blocking activity

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A series of ten 2β- or 3α-steroidal monoquaternary ammonium salts, having androstane or pregnane skeletons and related in structure to acetylcholine by possession of an oxygen function on the carbon atom next but one to the quaternary nitrogen atom, were investigated for neuromuscular blocking activity *in vivo* in the cat, hen and mouse and *in vitro* on the frog rectus muscle and on the rat phrenic nerve diaphragm preparation. All compounds displayed typical non-depolarizing activity, the duration of block being significantly less than that for (+)-tubocurarine in the cat and the hen. The potency of the salts was low with the most active compound, 3α-acetoxy-2β-piperidino-5α-androstan-17-one methobromide, being 1/16th as active on a molar basis in the cat as (+)-tubocurarine.

THE ability of monoquaternary ammonium salts to act as neuromuscular blocking agents has been known since the classic experiments of Crum Brown & Fraser (1869), but in recent years, owing to the much greater potency of bis-, tris- and tetra-onium compounds (*inter alia* Bovet, 1959; Barlow, 1960; Cavallito & Gray, 1960; de Reuck, 1962; Stenlake, 1963; Edwards, Lewis & Marren, 1966 and refs cited) coupled with the fact that monoquaternary ammonium compounds may show ganglion-blocking, anti-muscarinic or acetylcholine-like (e.g. Huguenard & Martin, 1950; Hey, 1952) properties, there has been a virtual neglect of investigations of new monoquaternary ammonium salts as potential neuromuscular blocking agents. Certain observations, however, seemed to us to point to the desirability of a re-assessment of monoquaternary ammonium compounds, especially steroidal monoquaternary ammonium salts.

Thus, indications of the possible presence of desirable neuromuscular blocking properties in polycyclic monoquaternary salts are to be found in the activities of certain compounds derived from strychnidine (Karrer, Eugster & Waser, 1949) and in the possibility that the potent bisquaternary compound C-toxiferine-I could be dissociating *in vivo* into two molecules of active monoquaternary ammonium compound as suggested by its known *in vitro* hydrolysis under mild acid conditions into the metho-salt of the Wieland-Gumlich aldehyde (Battersby & Hodson, 1958, 1960). Within the steroid group, it is known that the trimethylammonium salts derived from the alkaloids funtumine (3α-amino-5α-pregnan-20-one) and funtumidine (3α-amino-5α-pregnan-20α-ol) exhibit non-depolarizing neuromuscular blocking activity (Blanpin & Bretaudeau, 1961; Blanpin & Pierre, 1961), while recent work showing the presence of potent neuromuscular blocking activity in 3α,17β- and 3β,17α-bisquaternary ammonium androstanes, in which the quaternary heads lie on opposite sides of the

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steroid nucleus (May & Baker, 1963, 1965), appears to cast doubt upon the validity of the classical two-point attachment theory of neuromuscular blockade (Barlow & Ing, 1948a,b; Paton & Zaimis, 1949) thus re-emphasizing concepts such as the adumbration theory (Loewe & Harvey, 1952) and suggesting that these compounds could be acting by way of a one-point attachment.

TABLE 1. NEUROMUSCULAR BLOCKING ACTIVITY OF STEROIDAL MONOQUATERNARY AMMONIUM SALTS IN DIFFERENT PREPARATIONS*

Compound		Molar potency ((+)-tubocurarine = 100)			
Code number	Chemical name	Cat gastro-cnemius	Hen gastro-cnemius	Frog rectus	Rat dia-phragm
B1	3α-Acetoxy-2β-piperidino-5α-androstan-17-one methobromide	6	6	37·2	0·68
B2	2β,17β-Diacetoxy-3α-piperidino-5α-androstane methobromide	2	2·5	180	1·29
B3	2β-Acetoxy-3α-piperidino-5α-androstan-17-one methobromide	1·5	2·5	40·3	0·68
B4	3α-Acetoxy-2β-dimethylamino-5α-androstan-17-one methohydroxide	1·5	7·5	7·28	0·41
B5	2β-Dimethylamino-3α-hydroxy-16α-methyl-5α-pregnan-20-one methobromide	1·5	2	70·7	
B6	2β,17β-Diacetoxy-3α-piperidino-5α-androstane methohydroxide	2	6	34·3	0·68
B7	2β-Acetoxy-3α-piperidino-5α-androstan-17-one methohydroxide	1·5	1·5	131	
B8	3α-Acetoxy-2β-pyrrolidin-1′-yl-5α-androstan-17-one methobromide	1	6·5	22·7	0·69
B9	3α-Hydroxy-2β-piperidino-5α-pregnan-20-one methobromide	2	2	50·2	2·36
B10	3α-Acetoxy-2β-pyrrolidin-1′-yl-5α-pregnan-20-one methobromide	2	4	53·9	1·43

* The molar potencies quoted, relative to (+)-tubocurarine = 100, represent the average of three determinations for each preparation.

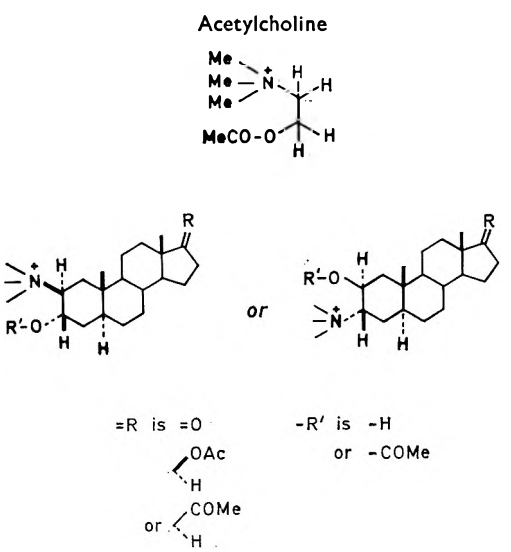

FIG. 1. General formulae of steroidal monoquaternary ammonium salts B1 to B10 showing their relationship to acetylcholine. In compounds B1, B2, B3, B6, B7, B9 the positively charged nitrogen atom is incorporated in a piperidine ring and in compounds B8 and B10 it is incorporated in a pyrrolidine ring.

In the light of these considerations and as an extension of recent interest in steroidal bisquaternary ammonium salts (May & Baker, 1963, 1965; Biggs, Davis & Wein, 1964; Khuong Huu-Lainé & Pinto-Scognamiglio, 1964; Mushin & Mapleson, 1964; Alauddin, Caddy & others, 1965) we investigated a series of ten monoquaternary ammonium salts derived from various 2β- and 3α-aminosteroids having androstane or pregnane skeletons and related in structure to acetylcholine through possession of an acetoxyl or hydroxyl substituent on the carbon atom next but one to the quaternary nitrogen atom. The general formulae of these compounds are in Fig. 1 and the individual structures are in Table 1.

Experimental

MATERIALS

The steroidal quaternary ammonium salts were kindly supplied by Organon Laboratories Ltd. Those bearing the nitrogen atom in the 2β-position were prepared as described in the patent literature (Organon Laboratories Ltd., 1966) and those bearing the nitrogen atom in the 3α-position were prepared by methods to be described by Hewett & Savage.

METHODS

Neuromuscular blocking activity

The neuromuscular blocking activity of the steroidal monoquaternary salts was evaluated using the following preparations.

Cat gastrocnemius muscle—sciatic nerve preparation. Each compound was tested in three animals. The method was a modification of that described by Bülbring & Burn (1942). Cats of either sex weighing 2–4 kg were anaesthetized by intraperitoneal injection of pentobarbitone sodium (60 mg/kg). Contractions of the gastrocnemius muscle were elicited by supramaximal stimulation of the sciatic nerve at a frequency of 6–8/min, 5–10 V, pulse width 1–2 msec. These values and the tension on the muscle (0·2–0·3 kg) were constant during any one experiment. The contractions of the muscle were recorded on smoked paper with a Brown-Schuster, spring-loaded, myograph lever. Arterial blood pressure was recorded from a common carotid artery using a mercury manometer. Drugs were dissolved in 0·9% w/v sodium chloride solution and administered intravenously into an external jugular vein.

Hen gastrocnemius muscle—sciatic nerve preparation. Each compound was tested in three animals. The method used was similar to that described above for the cat except that the hens (1–2·5 kg) were anaesthetized by injection of phenobarbitone sodium (200 mg/kg as a 10% w/v solution in saline) into a wing vein. This dose was sufficient to maintain anaesthesia throughout the experiment.

Rat phrenic nerve—diaphragm preparation. Each compound was tested on three preparations. The method was based on that of Bülbring (1946). Adult rats of either sex (150–200 g) were killed and a triangular-shaped section of the diaphragm was dissected out together with its

accompanying phrenic nerve. The preparation was then attached to a Bell's electrode and placed in an organ bath at 29° containing double glucose Tyrode solution gassed with oxygen. The contractions of the diaphragm were recorded on a moving smoked paper using a light Starling heart lever. The frequency of stimulation of the nerve was 6 square pulses/min at 5–10 V, pulse width 0·5–2 msec. The drugs were added to the bath and allowed to act for 3 min. An interval of 15 min was allowed before addition of the next dose of a drug to permit the magnitude of contraction to return to normal.

Frog rectus abdominis muscle preparation. Each compound was tested on three preparations. The method was that of Garcia de Jalon (1947). Reproducible submaximal contractions of the rectus muscle were induced by 1·0–2·0 μg/ml of acetylcholine. A suitable time interval between each dose of acetylcholine was found to be approximately 3 or 4 min. The contractions were recorded for periods of 30–90 sec. Using the same time interval between each dose of acetylcholine, each drug was added 30–60 sec before the addition of acetylcholine on the same preparation. Each drug was then quantitatively compared with (+)-tubocurarine for its antagonism to acetylcholine.

Experiments with mice. The method used was similar to that employed by Thomson (1946) for the assay of insulin in mice. Groups of ten male albino mice (18–24 g) were injected intraperitoneally at different dose levels with the drugs under test and the mice placed on a fine-mesh wire screen inclined at an angle of 60° to the horizontal. That dose of drug at which five out of the ten mice slid abruptly off the screen within 30 min of the injection was considered to be the median paralysing dose (PD 50) and was expressed as mg/kg of body weight. Similarly, the dose of drug at which five out of the ten mice died within 30 min was taken as the median lethal dose (LD 50) and expressed as mg/kg of body weight.

Anticholinesterase Activity

The acetylcholinesterase preparation was obtained from rat brain by the method of Fenwick, Barron & Watson (1957). *In vitro* anti-acetylcholinesterase activity was determined manometrically by adaptation of the method of Ammon (1933) and expressed as a pI 50 value according to the method of Blaschko, Bülbring & Chou (1949).

Ganglion Blocking Activity

Cat nictitating membrane preparation—sympathetic ganglion blockade. Cats of either sex weighing from 2·0–4·0 kg were anaesthetized by intraperitoneal pentobarbitone sodium (60 mg/kg). After the preganglionic cervical sympathetic chain was severed (Lewis & Muir, 1960), contractions of the nictitating membrane were elicited by supramaximal stimulation of the peripheral end of the chain by means of square impulses at a frequency of 800–1200/min, 8–15 V, pulse width 0·5–1·0 msec. Stimulation was for a period of 15 sec every 3 min and the drugs under test were injected 1 min before the next period of stimulation.

Peristaltic reflex of the isolated guinea-pig ileum—parasympathetic

ganglion blockade. The method employed was based on that of Trendelenburg (1917). Pieces of ileum about 7–10 cm long were removed from guinea-pigs of either sex (0·3–0·5 kg) and suspended in Tyrode solution at 30° gassed with oxygen. To prevent fatigue of the preparation, peristaltic movements were induced for 30 sec every 3 min. Drugs were added 30 sec before the initiation of peristalsis.

Results and discussion

The relative molar potencies (average of three determinations) of the compounds, as compared with (+)-tubocurarine = 100, in the cat, hen, frog and rat are shown in Table 1. Table 2 shows the PD 50, LD 50,

TABLE 2. PD 50 (± S.E.), LD 50 (±S.E.), THERAPEUTIC INDEX AND MOLAR POTENCY OF STEROIDAL MONOQUATERNARY AMMONIUM SALTS IN THE MOUSE. Observations from groups of ten animals

Code number	PD 50 mg/kg	LD 50 mg/kg	Therapeutic index LD 50/PD 50	Molar potency
B1	51 ± 3·45	64 ± 4·15	1·78	0·36
B2	46 ± 3·09	60 ± 3·30	1·30	0·47
B3	80 ± 4·49	82·3 ± 4·90	1·03	0·25
B4	136 ± 9·6	155 ± 9·00	1·14	0·11
B5	80·1 ± 4·39	102·8 ± 2·99	1·28	0·23
B6	64 ± 3·58	78·5 ± 4·09	1·23	0·31
B8	69·5 ± 2·39	71·8 ± 2·84	1·03	0·25
B9	70·2 ± 4·58	100·1 ± 4·23	1·43	0·24
B10	52 ± 2·33	59·5 ± 2·96	1·14	0·39
(+)-Tubocurarine	0·30 ± 0·03	0·54 ± 0·03	1·78	100

therapeutic index and molar potency of each compound in the mouse (with the exception of B7 of which insufficient quantities were available). Applying accepted criteria for the differentiation of depolarizing and non-depolarizing (cf. Paton & Zaimis, 1952) activities, all ten compounds were found to exhibit, on all preparations, typical non-depolarizing actions without any discernible depolarizing properties. Thus, in the cat, neuromuscular block produced by doses of 1–6 mg/kg of each steroid was intensified by (+)-tubocurarine (0·05–0·10 mg/kg) and in the hen, muscle contracture did not take place. In both the cat and the hen the block was quickly and completely reversed by neostigmine (0·02–0·10 mg/kg) and edrophonium (0·50–1·0 mg/kg). These results were supported by those obtained with the frog and rat preparations.

Although there is the possibility of an edge-on attachment of the β-acetoxy group and α-quaternary head to the receptor, the absence of depolarizing activity in all compounds incorporating a β-acetoxy-α-quaternary ammonium unit in their structure (i.e. all compounds except B5 and B9) might suggest that stereochemical factors imposed by the steroid nucleus are serving to prevent simultaneous access to both the anionic sites and the esteratic sites (e.g. Ing, 1949; Lands, 1951) of individual acetylcholine receptors, or that the quaternary ammonium groups are not permitted sufficiently close access to the anionic sites to initiate depolarization of the muscle endplate by whatever mechanism this initiation may occur (see Paton, 1961; Ariëns, 1964; Belleau, 1964, 1965).

Measurement of anticholinesterase activity revealed that the most

active of the steroids (compounds B6, B7 and B8) were more than 100 times less potent than eserine (pI50 values of 4·22 to 4·29; eserine, 6·54). It seems unlikely, however, in view of their non-depolarizing properties that they owe their neuromuscular blocking activity to an inhibition of acetylcholinesterase.

In both the cat and the hen, the time taken to reach maximal paralysis and the duration of the block were significantly less than those for (+)-tubocurarine. Moreover, as would be expected of non-depolarizing compounds (Zaimis, 1959), the potency and duration of action observed in the hen was in general comparable to that in the cat. There was, however, considerable variation in potency in other species. Thus, the sensitivity of the mouse (PD 50 values ranging from 46 $\pm$ 3·09 mg/kg to 136 $\pm$ 9·6 mg/kg) to the compounds was approximately four times (compound B8) to 17 times (compound B1) less than it was in the cat. On the other hand, the compounds were appreciably more active on the frog rectus abdominis muscle preparation in which their potency was approximately five times (compound B4) to 90 times (compound B2) as great as in the cat.

In addition to their neuromuscular blocking activity, as might have been expected (cf. Cavallito & Gray, 1960), all the monoquaternary compounds blocked both sympathetic and parasympathetic ganglia and produced a fall in the blood pressure of the pentobarbitone-anaesthetized cat. A given dose of any of the steroidal quaternary ammonium salts in each case gave greater neuromuscular blockade than sympathetic ganglion blockade where these effects were expressed as a percentage of the maximum blockade.

With the exception of compounds B2 and B7 as tested on the frog, the potency of the monoquaternary steroids as neuromuscular blocking agents was low and all were appreciably less potent than (+)-tubocurarine. The most active—compound B1—was 1/16th as active in the cat as (+)-tubocurarine on a molar basis while the least active—compound B8—was 1/100th as active. Since these two compounds are very similar in chemical constitution, differing only in the replacement of the 2β-*N*-methylpiperidinium grouping (in compound B1) by a 2β-*N*-methylpyrrolidinium grouping (in compound B8), it is probable that the variation in potency is directly ascribable to substituent effects with respect to the nitrogen atom with their attendant minor changes in the charge density, rather than to any critical change in hydrophilic to lipophilic balance (Cavallito, 1959). Further emphasis on the importance of factors other than hydrophilic to lipophilic balance in determining neuromuscular blocking activity is provided in the present work by the demonstration that compound B3 (the reversed analogue of compound B1 with respect to the acetoxyl and quaternary ammonium functions) is only 1/4th as active as compound B1.

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Some pharmacodynamic effects of the babesicidal agents quinuronium and amicarbalide

P. EYRE

The intravenous injection of a therapeutic dose of quinuronium methylsulphate (1 mg/kg) causes a fall in blood pressure in sheep, which is partly prevented by mepyramine and abolished by atropine. Larger doses of quinuronium cause more marked hypotension and inhibition of respiratory movement, which are not affected by atropine. Quinuronium strongly increases the amplitude of contraction of the isolated rabbit heart. This effect is not antagonized by atropine or mepyramine. Contractions of plain muscle in the guinea-pig and sheep, and hypersecretion of gastric acid in the rat and of saliva in the sheep were all produced by quinuronium. The responses to acetylcholine were potentiated by quinuronium, an effect which was abolished by atropine. Amicarbalide isethionate by comparison was weakly active. The drug causes no change in blood pressure, smooth muscle contraction or salivary secretion, but stimulates gastric secretion and partially inhibits the actions of acetylcholine in these preparations.

QUINURONIUM methylsulphate was first synthesized in 1933 and is still one of the principal chemotherapeutic agents for piroplasmosis. Its main disadvantage is a very low therapeutic index. Therapeutic doses (1 mg/kg) cause salivation, defaecation and urination which may be accompanied by dyspnoea. Higher doses produce cyanosis, apnoea, collapse and death (Cernaianu, Schuldner & Magureanu, 1935).

Kronfeld (1959) suggested that the toxic signs were due to central respiratory inhibition. Rümmler & Laue (1961) showed that quinuronium reduced circulating cholinesterase activity in sheep and dogs and that partial protection could be produced by atropine and by pyridine 2-aldoxime methiodide—a cholinesterase reactivator (Wilson & Ginsburg, 1955).

The anticholinesterase action of quinuronium was confirmed *in vitro* and *in vivo* in a wide variety of species (Eyre, 1966a) and the release of histamine by quinuronium demonstrated in rats, mice and sheep (Eyre, 1966b).

Amicarbalide isethionate, another babesicidal agent with a better therapeutic index than quinuronium, became available in 1960. The toxic reactions of amicarbalide so far reported include local swelling at the injection site and mild ataxia in some animals (Ashley, Berg & Lucas, 1960; Beveridge, Thwaite & Shepherd, 1960).

Amicarbalide possesses weak anticholinesterase activity in many species (Eyre, 1966a) and releases histamine in rats (Eyre, 1966b).

The purpose of these investigations was to examine more precisely the pharmacodynamic actions of quinuronium and to make comparisons with amicarbalide.

Experimental

In vivo. CAROTID BLOOD PRESSURE AND RESPIRATION IN SHEEP

Adult "south country" Cheviot sheep of mixed sexes were used.

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Anaesthesia was induced with intravenous thiopentone sodium and maintained by closed-circuit cyclopropane and oxygen. Blood pressure was recorded kymographically by a mercury manometer, simultaneously with respiratory ventilation from a tracheal cannula. Drugs were injected into the tarsal vein.

RABBIT ISOLATED HEART

The method was a modification of Langendorff's (1895) technique, and the apparatus was described by Bartlet (1963).

RABBIT PERFUSED EAR

Ears from freshly killed rabbits were perfused in air through the central artery with Krebs solution (Krebs & Henseleit, 1932) preheated to 37°, and the perfusion rate measured kymographically by a photoelectric drop recorder and a Thorpe impulse counter. Drugs were injected into the perfusion fluid.

ISOLATED SMOOTH MUSCLE

Guinea-pig ileum. Short lengths of terminal guinea-pig ileum were set up in the usual way in aerated Tyrode solution at 35°, in a 5 ml organ bath.

Guinea-pig and sheep bladder. The mucous membrane was carefully removed from longitudinal strips of bladder wall, which were set up in an isolated organ bath in oxygenated Krebs solution at 35°.

EXOCRINE SECRETIONS

Parotid salivation in sheep. Anaesthesia was induced with intravenous thiopentone sodium and maintained with small doses of pentobarbitone sodium as necessary to maintain "surgical" anaesthesia. Salivary outflow was measured from the cannulated parotid duct by means of a photoelectric drop-counter recording kymographically.

Gastric acid secretion in rats. Gastric acid was measured by a modification of the method of Ghosh & Schild (1958). Saline heated to 38° was perfused through the stomach, collected over a given time and titrated immediately against 0·01N sodium hydroxide using methyl red as indicator.

Drugs were injected intravenously.

DRUGS

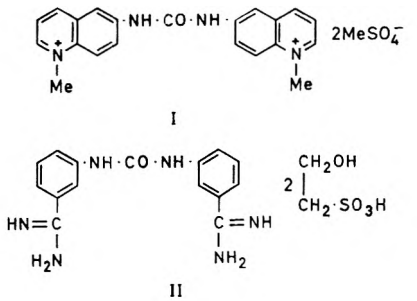
Quinuronium methylsulphate [*NN'*-diquinol-6-ylurea 1,1'-dimetho-(methylsulphate); I]; amicarbalide isethionate [*NN'*-di(3-amidinophenyl)-ureadi(2-hydroxyethanesulphonate); II]; acetylcholine chloride; histamine acid phosphate; adrenaline hydrogen tartrate; noradrenaline acid bitartrate; carbamoylcholine chloride (carbachol); atropine sulphate and mepyramine maleate were used.

Results

CAROTID BLOOD PRESSURE AND RESPIRATION IN SHEEP

Adrenaline increased, whereas acetylcholine and histamine decreased the blood pressure. Quinuronium had a variable effect in the

lower dose range (up to 1 mg/kg) whereas in therapeutic doses (1 to 2 mg/kg) there was always a fall in blood pressure, and a reduction in respiratory ventilation (Fig. 1).



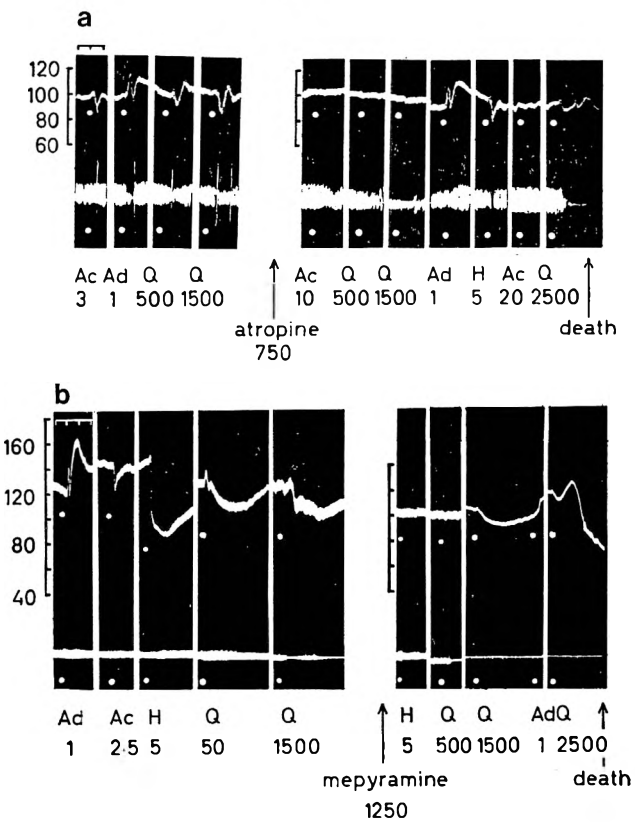


FIG. 1. Two sheep (40 kg) under cyclopropane anaesthesia. Responses of carotid blood pressure (mm Hg) (upper trace) and respiratory volume (lower trace) to intravenous injections of adrenaline (Ad), acetylcholine (Ac), histamine (H) and quinuronium (Q) before and after the administration of atropine (a) and mepyramine (b). Time scale in min. Drug doses, μg/kg i.v.

Atropine (0·75 mg/kg) antagonized the hypotensive action of quinuronium up to 1–2 mg/kg, but failed to protect above this dose.

Mepyramine (1·25 mg/kg) antagonized quinuronium up to 0·5 mg/kg. Neither atropine nor mepyramine showed any antagonism of the respiratory inhibition nor prevented death from overdosage with quinuronium.

Amicarbalide did not affect the blood pressure of sheep.

RABBIT ISOLATED HEART

Acetylcholine reduced the amplitude of contraction, whereas adrenaline, noradrenaline, histamine and quinuronium increased the contractility of the heart (Fig. 2). The doses of agonist drugs were adjusted to give

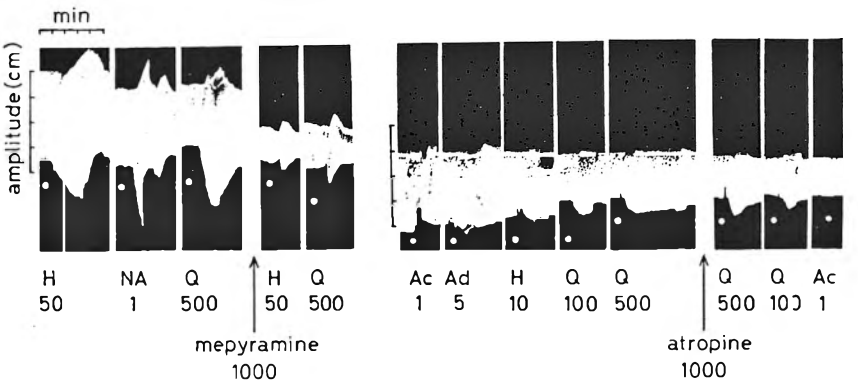


FIG. 2. The responses of isolated perfused rabbit heart to histamine (H), acetylcholine (Ac), adrenaline (Ad), noradrenaline (NA) quinuronium (Q), mepyramine and atropine, injected into the perfusion fluid. Drug doses in μg.

approximately equal responses. Atropine antagonized acetylcholine, but did not modify the action of quinuronium. Mepyramine depressed heart contractility by 60%, and did not inhibit the responses to quinuronium or histamine (Table 1). Amicarbalide had no demonstrable effect on the heart.

TABLE 1. THE RESPONSE OF ISOLATED PERFUSED RABBIT HEARTS TO ACETYLCHOLINE, ADRENALINE, NORADRENALINE, HISTAMINE, QUINURONIUM AND AMICARBALIDE AND THE INFLUENCE OF ATROPINE AND MEPYRAMINE.
The values are expressed as percentage *changes* in amplitude, and are means of four experiments. Standard errors are in parentheses.

Drug	Dose (μg)	Percentage change in amplitude of contraction		
		Drug alone	After atropine 1 mg	After mepyramine 1 mg
Acetylcholine ..	1·0	−29 (± 7)	0	
Adrenaline ..	5·0	+25 (± 6)		
Noradrenaline ..	1·0	+118 (±42)		
Histamine ..	50·0	+49 (±21)		+24 (± 8)
Quinuronium ..	100·0 500·0	+30 (±10) +78 (±28)	+55 (±16)	+76 (±23)

RABBIT PERFUSED EAR

Adrenaline, acetylcholine, histamine and quinuronium produced

vasoconstriction, but quinuronium had a much more prolonged action than the other drugs. Atropine inhibited acetylcholine and quinuronium, whereas mepyramine inhibited histamine but not quinuronium. Amicarbalide did not produce any effect on vascular resistance, but had some antagonism against acetylcholine and quinuronium (Fig. 3).

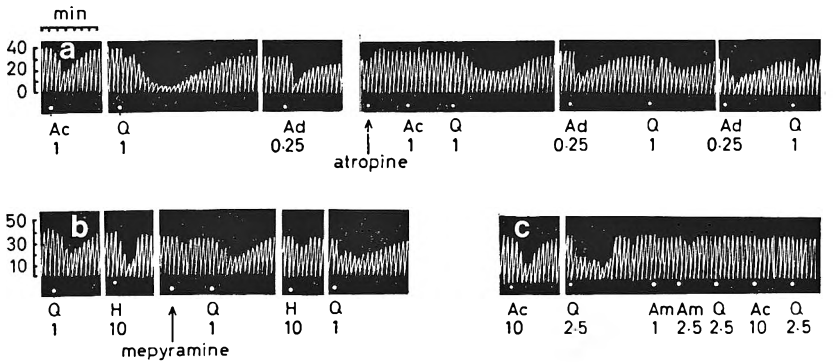


FIG. 3. Records of venous outflow from three perfused rabbit ears in response to intra-arterial injections of acetylcholine (Ac), adrenaline (Ad), histamine (H), quinuronium (Q), before and after atropine 1 mg (a) and mepyramine 2·0 mg (b); and of amicarbalide (Am) (c). Doses in μg for acetylcholine, adrenaline and histamine; in mg for quinuronium. Vertical scale: No. of drops.

ISOLATED SMOOTH MUSCLE

Guinea-pig ileum. A shortening of the ileum occurred in response to concentrations of quinuronium, $3{\cdot}5 \times 10^{-9}$ to $3{\cdot}5 \times 10^{-6}$ M. This increased tone was accompanied by increased spontaneous movement, illustrated in Fig. 4. The ileum is here contracting to alternate doses of 0·30 μg and 0·15 μg acetylcholine. Quinuronium, $3{\cdot}5 \times 10^{-9}$ to $3{\cdot}5 \times 10^{-7}$ M, produced in addition to increased tonus, a potentiation of the responses to acetylcholine, with a maximum in both phenomena at quinuronium, $3{\cdot}5 \times 10^{-8}$ M.

After a period of 5 to 10 min exposure to quinuronium, the muscle tone decreased to a level only slightly above the control. The spontaneous movement persisted however, but the responses to acetylcholine were inhibited. On washing out the quinuronium, the tone returned to normal and spontaneous movement disappeared almost immediately. The acetylcholine responses were restored to normal within 5 to 10 min with repeated washings.

In the presence of quinuronium in concentrations greater than 10^{-6} M there was little or no increase in muscle tone and the responses to acetylcholine and histamine (Fig. 4d, f) were depressed and finally abolished at a quinuronium concentration of $3{\cdot}5 \times 10^{-4}$ M. It became more difficult with increasing concentrations to "wash out" the quinuronium. After concentrations 10^{-5} and 10^{-4} the acetylcholine responses did not return to normal within an hour of repeated washing. Amicarbalide isethionate did not contract the ileum or potentiate acetylcholine at any concentration.

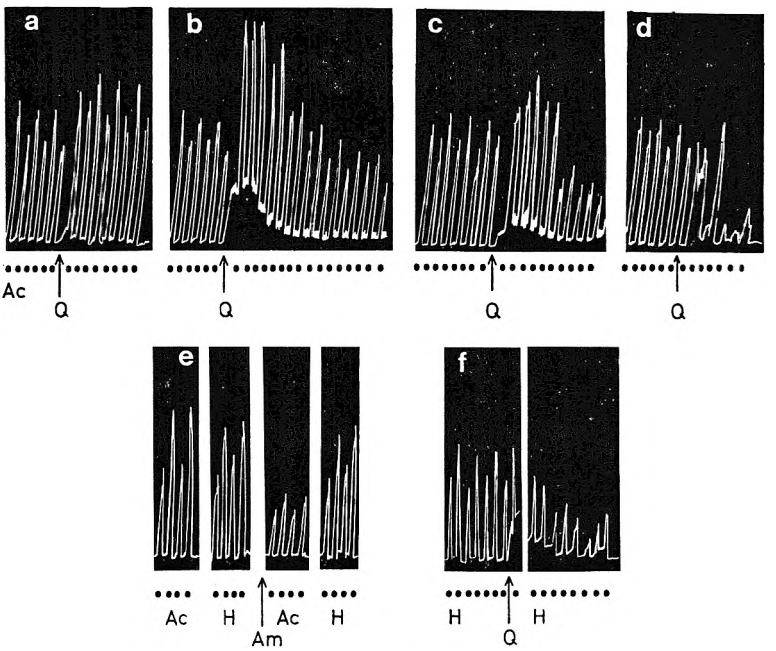


FIG. 4. (a), (b), (c) and (d). Isolated guinea-pig ileum in aerated Tyrode at 35°, contracting to alternate doses of 0·30 and 0·15 μg acetylcholine (Ac) added to the bath for 30 sec at 2 min intervals, showing the effects of increasing molar concentrations of quinuronium (Q) on muscle tone and the responses to acetylcholine. (a) Q = $3{\cdot}5 \times 10^{-9}$M, (b) Q = $3{\cdot}5 \times 10^{-8}$M, (c) Q = $3{\cdot}5 \times 10^{-7}$M, (d) Q = $3{\cdot}5 \times 10^{-6}$M. (e) Guinea-pig ileum contracting to alternate doses of 0·10 and 0·20 μg acetylcholine (Ac) and to alternate doses of 0·05 and 0·10 μg histamine (H), showing the effect of adding amicarbalide $2{\cdot}1 \times 10^{-4}$M (Am). (f) Guinea-pig ileum contracting to alternate doses of 0·04 and 0·08 μg histamine (H), showing the inhibitory action of $1{\cdot}0 \times 10^{-6}$M quinuronium (Q).

Large concentrations (above 10^{-4} M) inhibited responses to acetylcholine (Fig. 4e).

Guinea-pig and sheep bladder. Bladder strips from both species contracted strongly to histamine and acetylcholine. Quinuronium had a

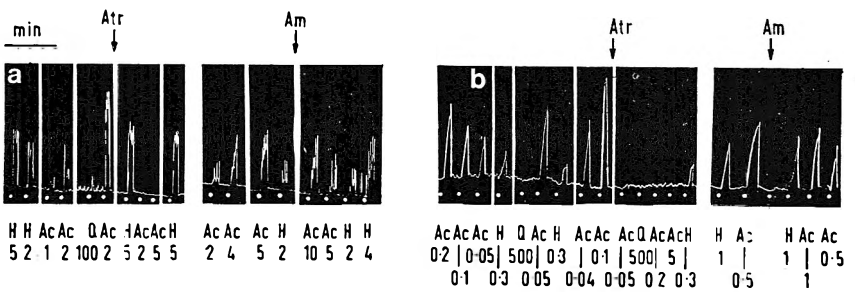


FIG. 5. Isolated longitudinal strips of (a) guinea-pig bladder and (b) sheep bladder in oxygenated Krebs, contracting to acetylcholine (Ac), histamine (H), quinuronium (Q), each added to the bath for 60 sec at 5 min intervals. Atropine (Atr) and amicarbalide (Am) added to the bath at the points indicated. Drug concentrations μg/ml.

variable and inconsistent effect. In all but one of six preparations from the guinea-pig there was muscle contraction and increased spontaneous movement, whereas in only one of five preparations from sheep was there a slight increase in tone. Repeated washings over a period of 5 to 15 min were required before the effects of quinuronium passed off, during which time the responses to acetylcholine were potentiated (Fig. 5). Atropine abolished the actions of acetylcholine and quinuronium.

Amicarbalide produced no contraction of the bladder muscle, but at high concentrations the compound partially inhibited the acetylcholine responses.

EXOCRINE SECRETIONS

Parotid salivation in sheep. A hypotensive dose of acetylcholine produced a brief increase in salivation, usually persisting for less than 1 min. A dose of histamine which was equally hypotensive produced little or no change in salivary flow. Doses of quinuronium less than 10 μg/kg produced no change, but above 100 μg/kg there was prolonged salivation which persisted for 10 to 40 min. Immediately after the effect of quinuronium had passed off, the responses to acetylcholine (both blood pressure and salivation) were potentiated. Atropine antagonized this effect (Table 2).

TABLE 2. THE ACTION OF ADRENALINE, HISTAMINE, QUINURONIUM AND ATROPINE ON PAROTID SALIVATION IN SHEEP.
Sheep, 30–40 kg, anaesthetized with thiopentone and pentobarbitone. Parotid salivary outflow in response to i.v. drug injections measured kymographically and expressed as mean *increases* (ml/min).

Drug	Dose (μg/kg)	Increase in mean salivary flow (ml/min)		
		Drug alone	After quinuronium	After atropine
Adrenaline	2	0·35	—	—
Histamine	4	0	—	0
	10	0	—	0
Acetylcholine	1	0	0·51	—
	2	0·20	0·60	0
	4	0·50	1·04	0
	15	—	—	0
Quinuronium	10	0	—	—
	100	0·32	—	—
	500	1·66	—	0
	1,000	2·10	—	0

Amicarbalide was without effect on this preparation.

Gastric acid secretion in rats. Histamine and acetylcholine stimulated the secretion of gastric acid for periods varying between 10 and 40 min. Doses of quinuronium less than 50 μg/kg induced no increase in secretion but potentiated the action of acetylcholine. Quinuronium at a rate greater than 100 μg/kg induced marked and prolonged acid secretion, which persisted for 1–2 hr. The response to acetylcholine was potentiated when the drug was given immediately after the effects of quinuronium had passed off (Table 3).

TABLE 3. THE ACTION ON GASTRIC SECRETION IN RATS OF ACETYLCHOLINE, HISTAMINE, QUINURONIUM, AMICARBALIDE, ATROPINE AND MEPYRAMINE
Rats, 150–250 g, anaesthetized with urethane. Gastric acid secretion in response to i.v. injection of drugs, expressed as percentage of resting secretion. Values are means with standard errors and numbers of experiments are in parentheses.

Drug	Dose µg/min for 10 min	Percentage increase in the mean gastric acid output per min				
		Drug alone	After atropine (1 mg/kg)	After mepyramine (2 mg/kg)	After quinuronium	After amicarbalide
Acetylcholine	10	485 ± 39 (20)	0 (4)	561 ± 65 (4)	826 ± 24 (4)	273 ± 33 (4)
Histamine ..	25	563 ± 37 (18)	553 ± 20 (4)	580 ± 28 (6)	550 ± 19 (4)	545 ± 41 (4)
Quinuronium	30	3128 ± 201 (8)	320 ± 50 (4)	3606 ± 350 (4)	—	—
Amicarbalide	200	433 ± 86 (8)	517 ± 103 (4)	540 ± 123 (4)	—	—
Mepyramine..	200	349 ± 110 (18)	—	—	—	—

Atropine abolished the activity of acetylcholine and partially antagonized quinuronium. Mepyramine showed no antagonism against any of the drugs used.

Amicarbalide stimulated gastric secretion and was not affected by the presence of atropine. After amicarbalide, the response to acetylcholine was diminished.

Discussion

Therapeutic doses (1 mg/kg) of quinuronium caused hypotension in anaesthetized sheep, which was almost completely prevented by atropine and partially prevented by mepyramine. However, neither atropine nor mepyramine prevented or alleviated the respiratory inhibition, or the hypotensive action of larger doses of quinuronium (>1 mg/kg).

Quinuronium produced vasoconstriction of the rabbit ear which was completely inhibited by atropine and not at all by mepyramine, suggesting that the activity was "muscarinic" and that histamine effects were not involved in the action of quinuronium in this preparation. While amicarbalide itself showed no vascular action, the compound appeared to possess atropine-like activity, shown by its antagonism against acetylcholine and quinuronium.

In the isolated heart, quinuronium always produced an *increase* in amplitude of contraction which was unaffected by atropine. Both observations indicate that this action on the heart is not cholinergic, and is probably due to an action of quinuronium distinct from anticholinesterase activity. Mepyramine did not inhibit the stimulant effects of either quinuronium or histamine.

In 1910, Dale & Laidlaw showed that histamine stimulated the heart of cats and rabbits, and Went & Lissack (1935) found that histamine also stimulated the hearts of guinea-pigs.

Recently several authors have reported that the common antihistamine agents do not antagonize the effect of histamine on the heart of guinea-pigs (Lockett & Bartlet, 1956; Trendelenburg, 1960), and although Mannaioni (1960) reported that diphenhydramine antagonized the cardiac action of

histamine, this has not been confirmed (Bartlet, 1963). It is possible, therefore, that the cardiac action of quinuronium might involve some histamine-like activity, but in the absence of an antagonist this could neither be established nor denied.

The peripheral vasoconstriction and myocardial stimulation will not contribute to the production of hypotension by quinuronium. Other experiments designed to show the action of the drug on vascular resistance in the intestine, limb, and pulmonary bed of sheep have been negative (unpublished observations). Thus the results of experiments on the cardiovascular system have not as yet explained the fall in blood pressure which is such a prominent feature of quinuronium intoxication. A possibility is that cardiovascular changes occur as a consequence of the action of quinuronium on the central nervous system (Kronfeld, 1959) mediated for example through the vagus nerve. This possibly has not been investigated.

The actions of quinuronium on the ileum and urinary bladder were varied. In the ileum, small concentrations produced increased tonus and spontaneous movements invariably; whereas the effects on bladder muscle were less consistent.

In both muscles the responses to acetylcholine were potentiated by small concentrations of quinuronium (10^{-9} to 10^{-7} M). Larger concentrations ($>10^{-6}$ M) produced smaller increases in tone and the responses of the ileum to acetylcholine and histamine were inhibited.

Similar observations have been made by Heathcote (1932) and Shelley (1955) using eserine. It seems likely that the inhibitory action of quinuronium on the intestine, like that of eserine, is due to a factor not concerned with cholinesterase inhibition.

Amicarbalide is, comparatively, a very weak anticholinesterase (Eyre, 1966a). Shelley (1955) showed that increase in tonus of the intestine and acetylcholine potentiation occurred when less than 20% of the true cholinesterase activity of the tissue was inhibited. High concentrations of amicarbalide may inhibit cholinesterase to this extent (Eyre, 1966a), but in a series of experiments on the guinea-pig ileum, using a range of amicarbalide concentrations between 10^{-9} and 10^{-3} M, no increase in tone or acetylcholine potentiation has been observed before the onset of inhibition of the ileum. Amicarbalide thus inhibits the ileum in a manner which is almost certainly not related to anticholinesterase activity and which is different from the inhibition of the ileum by quinuronium, in that it is more specific for acetylcholine and is not preceded by contraction or potentiation of the responses to acetylcholine.

Comparable results were obtained when recording parotid salivation in sheep. Quinuronium itself induced prolonged salivation and potentiated the responses to acetylcholine.

Atropine effectively antagonized acetylcholine and quinuronium on plain muscle and on salivation.

Quinuronium increased gastric acid secretion in rats, an effect which could not be completely antagonized even by very large doses of atropine (approximately 5 mg/kg). Amicarbalide caused a mild transient hyper-

acidity which was atropine-resistant. It is known that atropine does not abolish gastric acid secretion (Gray & Ivy, 1937; Code, 1951); but in these experiments, since atropine completely antagonized the effect of acetylcholine, it may be that the atropine-resistant action of quinuronium on gastric secretion was non-cholinergic, although more evidence would be needed to establish the point. Both babesicides have been shown to release histamine in rats (Eyre, 1966b), but in the absence of an antagonist for the gastric secretory action of histamine it was not possible to show whether either compound stimulated gastric secretion by releasing histamine. [Many authors have reported that antihistamine compounds do not antagonize the gastric effects of histamine (Ashford, Heller & Smart, 1949; Loew, 1950; Paton & Schachter, 1951) and Mota & Da Silva (1960) showed that mepyramine released histamine *in vitro*.]

It was interesting that amicarbalide, although itself stimulating gastric acid secretion, appeared partly to antagonize the actions of acetylcholine but not of histamine. This observation is consistent with the activity of amicarbalide on smooth muscle.

The data presented confirm the many reports of the toxicity of therapeutic doses of quinuronium (Cernaianu & others, 1935; Egerov, 1951; Kronfeld, 1959; Rümmler & Laue, 1961; Eyre, 1966c), namely the signs of hypotension, dyspnoea, defaecation, micturition and salivation. Moreover they explain the rationale of using atropine during therapy with quinuronium sulphate.

The failure of atropine to alleviate the apnoea, collapse and death which characterizes more severe intoxication with quinuronium is not readily explained and will require further investigation. Kronfeld (1959) attributed the "respiratory-type death" to a failure of cellular respiration in the central nervous system (CNS) and (presumably) elsewhere. The observations may also be explained in part by the anticholinesterase activity of quinuronium (Rümmler & Laue, 1961; Eyre, 1966a) possibly causing bronchoconstriction initially, followed by CNS depression not antagonized by atropine (Modell & Krop, 1946). Atropine would prevent bronchoconstriction but at high doses of quinuronium the central effects would supervene.

In view of the quaternary nature of quinuronium sulphate it is unlikely to diffuse readily into the CNS and it may be a metabolite which causes the effects. This question has not been investigated, but if the respiratory effect of the drug were due principally to a metabolite there would probably be a time lag in the onset of respiratory inhibition. This is not so. In fact apnoea may be observed before the full hypotensive effect of the drug and the action is more likely due to quinuronium as such.

It has been observed through a large number of acute experiments in several species that after repeated dosage with quinuronium there is always a progressive deterioration in blood pressure and respiration which cannot be prevented or alleviated (Fig. 1). Although the metabolism of quinuronium has still to be described, the *in vivo* study of cholinesterase inhibition by quinuronium (Eyre, 1966a) showed that the enzyme activity remained depressed for up to 24 hr after a single injection of the drug,

which suggests either that the metabolism of quinuronium is comparatively slow or that a metabolite persists which also inhibits cholinesterase. The ultimate cardiovascular and respiratory failure is thus probably the manifestation of the persistence and/or accumulation of quinuronium and/or its metabolite(s) in the CNS and elsewhere, which would be consistent both with Kronfeld's (1959) explanation of cytotoxicity and with cholinesterase inhibition (Rümmler & Laue, 1961; Eyre, 1966a).

Quinuronium sulphate has thus been shown to possess a number of pharmacodynamic actions. The results support the conclusion that most of the toxic effects caused by therapeutic doses of quinuronium may be attributed to cholinesterase inhibition, the symptoms of which are largely prevented by atropine. Severe intoxication with quinuronium produces irreversible cardiovascular and respiratory depression, the precise mechanisms of which are not known.

Amicarbalide isethionate is, comparatively, much less active and produces no marked reactions even in high doses, the inhibition of cholinergic (muscarinic) responses being the only consistent finding.

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Degraded and undegraded carrageenans and experimental gastric and duodenal ulceration

W. ANDERSON AND P. D. SOMAN

The prevention of histamine-induced gastric and duodenal ulceration in the guinea-pig has been examined using a series of undegraded and degraded carrageenans. Undegraded carrageenans were active at lower doses than degraded carrageenans. The high viscosity of the undegraded carrageenans in solution prevented their use in larger doses. Degradation of carrageenan without serious loss of sulphate, gives a product which allows the dose to be increased to an extent that its effect more than offsets the slight loss in activity caused by the degradation. No single feature of carrageenan structure can be related to anti-ulcer activity although degradation, and hence reduction of molecular size, generally reduces activity. Sulphate contents over 30% have little apparent effect on activity; κ-carrageenans were not consistently different in anti-ulcer activity from λ-carrageenans. This contrasts with the antipeptic activity of carrageenans where κ-carrageenans are less active than their λ-counterparts. As with antipeptic activity, the degree of anti-ulcer activity is probably determined by a combination of structural features which includes molecular size and polyanionic properties.

PREVENTION of experimental histamine gastroduodenal ulceration in guinea-pigs by carrageenan (Anderson & Watt, 1959) has been confirmed using several animal species (Houck, Bhayana & Lee, 1960; Misaki, Okita, & others, 1965; Lambelin, 1966). It is also known that carrageenan has antipeptic activity (Anderson & Watt, 1959; Bonfils, Dubrasquet & Lambling, 1959, 1960; Anderson, 1961; Anderson & Harthill, 1967), but neither the role of the proteolytic component of gastric secretion in peptic ulceration, nor the relation between antipeptic and anti-ulcer effects of sulphated polysaccharides is understood. It is likely, however, that the elucidation of one of these actions could help our understanding of the other.

The examination of a series of different carrageenans (Anderson & Harthill, 1967) revealed differences in antipeptic activity. The present report deals with differences between the anti-ulcer activities of selected carrageenans of the series.

Experimental

MATERIALS AND METHODS

Carrageenans. Selection was made on the basis of antipeptic activity and availability. The code names correspond to those used by Anderson (1967). A degraded κ-carrageenan from *C. crispus* was also included, CRF-κ-D2 (SO_3Na, 28·6%; η_{inh}, 0·90; antipeptic activity 30, 11 by methods A and B, respectively). Antipeptic activities, determined at the lower carrageenan concentrations by methods A and B (Anderson, 1967) showed that, of the undegraded carrageenans, CNS-κ was less active than GP-λ and CY-λ ($P < 0·01$). Differences in antipeptic activity did not exist ($P > 0·1$) between the degraded carrageenans.

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Gastric ulceration. Gastric ulceration was produced in the pylorus-ligated guinea-pig of suitable strain (Anderson & Soman, 1963). Guinea-pigs, fasted 24 hr, with water *ad lib.*, were kept individually in cages with raised grids of suitable mesh to minimize coprophagy. They were anaesthetized with pentobarbitone (30 mg/kg) intraperitoneally and the gastroduodenal junction ligated. Saline (1 ml) alone (control) or containing half the dose of carrageenan in solution was injected intraduodenally 0·5 hr before, and repeated immediately after, the injection of histamine acid phosphate (5 mg/kg, subcutaneously in saline, 1 ml/kg). The stomach was not emptied since the resting volumes were small and reasonably uniform, and complete empyting without disturbance of, and the risk of mucosal damage to, the stomach is very difficult; the procedure may also stimulate secretion. The animals were killed 1 hr after administration of histamine; ulceration was scored on a 4+ scale (Anderson & Soman, 1965a) and averaged for each group. Reduction in average ulceration in the test groups was expressed as a percentage of the average ulceration for the control group. Secretion volumes were measured and converted to volume per kg body weight; free and total acidities were titrated using Topfer's reagent and phenolphthalein respectively.

Duodenal ulceration. Histamine acid phosphate (10 mg/kg), suspended in a beeswax–arachis oil (10:90) base (1 mg/ml), was administered intramuscularly to intact fasted guinea-pigs. This consistently produced duodenal ulceration (incidence = 100%) at the end of 24 hr. Ulceration was assessed on a 4+ scale and averaged for each group (Anderson & Soman, 1965b). In this experiment, the carrageenan was administered in the drinking water. In the test groups, carrageenan solution was offered in place of the usual drinking water during the 24 hr preceding the histamine and again during the 24 hr following the histamine. The volumes consumed were recorded. Degraded carrageenans were used at 1% or 5% concentration in the drinking water; undegraded carrageenans had to be used at 0·5% because of their viscosity in solution.

Reduction in average duodenal ulceration in test groups was expressed as a percentage of the ulceration occurring in the control groups, which were offered normal drinking water.

Results and discussion

The results are in the Tables. For both gastric and duodenal ulceration a 30% reduction in ulcer score is considered to be the smallest acceptable for anti-ulcer activity.

Concerning the aetiology of the two types of experimental ulceration used in this work, we have contended (Anderson & Soman, 1966a) that the lesion appearing in the duodenum is caused by simple exposure to the passing of the copious, acid gastric secretion resulting from histamine stimulation, whereas the ulceration appearing in the stomach is caused by a lesser secretion acting on an exhausted, devitalized mucosa. Evidence of devitalization is that gastric secretion increases on increasing the dosage of histamine without the appearance of ulcers until a stage is reached when

further increase in histamine dosage results in lowered volumes of secretion. It is at this stage that ulceration appears. The lowered volumes of gastric secretion are taken as evidence of a devitalized mucosa. In this condition, together with the angiotoxic effects of histamine, the mucosa is susceptible to the erosive gastric secretion.

We have used both types of ulceration to demonstrate ulcer prevention. For duodenal ulcers we have made use of the antisecretory effects of carrageenan (Anderson, Marcus & Watt, 1962; Anderson & Soman, 1965c). But for gastric ulcers, the protection is of a different (and unknown) nature from the simple protection by surface contact postulated earlier (Anderson & Watt, 1959) although the mucosa is obviously protected when ulceration is prevented. In the present antigastric ulcer experiment carrageenan does not appear on the mucosal surface.

Comparison of antiduodenal ulcer activities in the present experiments involves the assumption that the animals follow the same drinking pattern throughout the 48 hr. Drinking carrageenan solutions before histamine stimulation confers greater protection than drinking after (Anderson & Soman, 1965b); drinking before and after is better still. In general, there was a tendency to drink more before the histamine than after it. Carrageenan solution was offered only before histamine in the case of CY-λ and CNS-κ and this probably accounts for the slightly lower protection compared with GP-λ where carrageenan solutions were offered both before and after the histamine injections.

The undegraded carrageenans showed anti-ulcer activity by both methods even at the low doses used. These doses were the highest practicable. In the duodenal ulceration experiments the guinea-pigs were reluctant to drink solutions more viscous than those used although they appeared to drink non-viscous carrageenan solutions at least as avidly as they drink water; in the gastric ulceration experiments highly viscous solutions could not easily be injected into the duodenum.

UNDEGRADED CARRAGEENANS

No evidence of marked difference between the activities of undegraded κ- and λ-carrageenans in the anti-ulcer experiments was found. This contrasts with the anticoagulant and antipeptic activities of undegraded κ- and λ-carrageenans where λ-carrageenans are generally more active than the corresponding κ-carrageenans (Hawkins & Leonard, 1962; Anderson & Duncan, 1965; Anderson & Harthill, 1967).

Because the use of higher doses of undegraded carrageenans was prevented by the high viscosity of their solutions, there remains the possibility that greater activity might be shown if more substance could be administered. Even oral administration in solid dosage form is unsatisfactory because of the low solubility, poor dispersibility and the slow rate of dissolution of undegraded carrageenans after administration.

DEGRADED CARRAGEENANS

Degradation of the carrageenans allows higher and more frequent dosage to be administered conveniently, and the results show that higher

TABLE 1. ANTI-GASTRIC ULCER ACTIVITY AND THE EFFECTS ON GASTRIC SECRETION OF UNDEGRADED AND DEGRADED CARRAGEENANS

Carrageenan*	Number of animals	Reduction in gastric ulceration % score	Total dose of carrageenan, intraduodenally mg	Gastric secretion		
				Average volume mg/kg	Average acidity m-equiv./litre	
					Free	Total
Undegraded						
CY-λ	16	46	20	31	96	104
CNS-κ	11	57	20	32	99	107
GP-λ	8	38	20	32	103	111
Degraded						
C16	8	12	200	29	97	105
"	14	60	400	21	83	101
GP-λ-D2	4	36	50	31	105	113
"	4	47	150	29	96	104
CRF-κ-D2	4	20	50	35	101	110
"	6	60	150	30	116	124
CY-λ-D5	4	36	150	33	91	100
CY-λ-D6	4	89	"	40	115	124
CY-λ-D7	5	55	"	35	101	110
CY-λ-D8	5	22	"	27	89	97
CY-λ-D9	4	4	"	27	72	82
CY-λ-D10	6	73	"	42	109	116
CY-λ-D11	4	57	"	29	98	106
GP-λ-D3	4	47	150	32	100	108
GP-λ-D4	4	0	"	21	80	90
GP-λ-D5	4	49	"	36	99	109
GP-λ-D6	4	39	"	45	103	111
GP-λ-D7	4	49	"	44	101	109
ED3	6	44	150	37	83	93
ED4	5	6	200	40	89	98
ED5	6	0	200	33	92	100
ED6	6	4	200	36	89	98
Controls	27	Average ulceration = 3·74 ± (±0·10)	1 ml saline intraduodenally, twice	33 (±2)	99 (±3·6)	107 (±3·4)

* See Anderson & Harthill (1967), Table 1, for the code.

TABLE 2. ANTI-DUODENAL ULCER ACTIVITY OF UNDEGRADED AND DEGRADED CARRAGEENANS

Carrageenan*	Number of animals	Reduction in duodenal ulceration % score	Dose of carrageenan (from drinking water) mg		
			Pre-histamine	Post-histamine	Total
Undegraded					
CY-λ	12	27	115	—	115
CNS-κ	12	36	100	—	100
GP-λ	12	44	110	85	195
Degraded					
C16	11	39	380	280	660
"	6	52	1150	900	2050
"	21	44	—	2150	2150
"	21	73	2400	—	2400
GP-λ-D2	10	73	330	260	590
CRF-κ-D2	5	0	300	180	480
CY-κ-D10	4	44	150	150	300
GP-κ-D4	4	47	220	230	450
GP-λ-D6	4	47	220	270	490
GP-λ-D7	4	39	250	310	560
Controls	60	Average ulceration = 3·76 ± (±0·07)			

For undegraded carrageenans, 0·5% solutions were used; for degraded carrageenans, 1% solutions were used, with the exception of the three highest doses of C16, where a 5% solution was used.
CY-λ-D7, CY-λ-D8, CY-λ-D9, CY-λ-D11, GP-λ-D3 and GP-λ-D5, all gave less than 10% protection.
* See Anderson & Harthill (1967), Table 1, for the code.

activity can thereby be obtained. Thus C16, CRF-κ-D2, and probably also GP-λ-D2, have increasing anti-gastric ulcer activity with increasing dosage (Table 1); a similar response is seen for the anti-duodenal ulcer activity of C16 (Table 2). Although the increased dosage made possible by degradation is valuable in therapeutic use, degradation, even without sulphate hydrolysis, appears to result in substances with lower activity than the parent substance. In some instances degradation causes a loss of all anti-ulcer activity.

SULPHATE CONTENT

It is noteworthy that sulphate content remained fairly constant through the degraded series (Anderson & Harthill, 1967: Table 1). This suggests that even a content of 30–40% sulphate does not assure anti-ulcer activity, although we have never encountered (in an unreported screening programme) anti-ulcer, or indeed marked antipeptic, activity, either in non-sulphated polysaccharides from a variety of vegetable and animal sources, or in carrageenans with sulphate of less than 25%. It would appear that sulphate content is not a lone determinant of the quantity of biological activity although the possession of adequate sulphate appears necessary for some activity. A resolution of the relation between sulphate content and biological activity is therefore not even possible amongst carrageenans, although the polysaccharide contains only one principal sugar—galactose, either in the undegraded state [compare CY-λ, CNS-κ, GP-λ, Tables 1 and 2; sulphate contents are in Table 1 (Anderson & Harthill, 1967)] or in the degraded state.

ANTI-ULCER ACTIVITY

Anti-ulcer activity was sufficiently indiscriminate to preclude the use of one or other ulcerous condition to differentiate between undegraded κ- and λ-carrageenan, the only differentiation being between a degraded κ- and a degraded λ-carrageenan for antiduodenal ulcer activity (GF-λ-D2 and CRF-κ-D2, Table 2).

Eucheuma carrageenan, a κ-type carrageenan, yielded a degraded series of which only ED3 has activity (Table 1); ED4-6 although having similar percentages of sulphate, have lower viscosities (Table 1, Anderson, 1967). Degradation in this series was essentially by mild acid treatment which Black, Blakemore & others (1965) found sufficient to split the acid labile 3,6-anhydrogalactosidic link. However, the anti-ulcer activity of λ-carrageenans, with their naturally low content of 3,6-anhydrogalactose links, discounts dependence on this link for activity, a conclusion also reached by Anderson & Harthill (1967) for antipeptic activity.

It is not clear at present why only certain of the degraded CY-λ and GP-λ series should possess antiduodenal ulcer activity. The results suggest that sulphate content and molecular characteristics (and antipeptic activity) cannot be taken as unfailing indication of anti-ulcer activity of both types.

EVALUATION OF THE RESPONSE

If percentage reductions in gastric ulceration scores (Table 1) are grouped into those below 30% (8) and those above 50% (7) and the corresponding means for volume and free acidity of secretion compared, no significant difference emerges between the mean volumes for the two groups, but the free acid is higher for the group experiencing greater protection (103 m-equiv./litre) than for the group where protection was less than 30% (89 m-equiv./litre) ($P < 0{\cdot}01$). Thus the protection afforded by carrageenan against ulceration appears to be accompanied by a fully functional secretory mechanism which fails to some extent if protection is less than 30%. A similar effect is also seen when degraded carrageenans are administered intravenously (Anderson & Soman, 1967).

THE RELATION OF ANTIPEPTIC AND ANTI-ULCER ACTIVITY

We conclude that for the antipeptic and antiulcer activities of carrageenan, whether in the undegraded or degraded state, there is a combination of as yet undefined molecular features, including sulphate, which determines the amount of activity. On the other hand, activity appears to accompany not only the high molecular weight carrageenan but (in smaller degree) even relatively small molecules, such as C16 and GP-λ-D2 which have weight average molecular weights of around 25,000 and possess 30–40% sulphate.

While there is, in the undegraded series, a systematic difference between the antipeptic activities of the κ- and λ-components, there is no real differentiation between the anti-ulcer activity of κ- and λ-carrageenans. However, it is not clear whether such a differentiation is to be expected; nor is it clear that refinement in method (if that proves to be possible) will reveal differences.

In the case of degraded carrageenan, where absorption from the gastrointestinal tract appears to occur, only a small fraction of the administered dose appears to be absorbed (Anderson & Soman, 1966b). Intravenous administration of an amount equivalent to the fraction absorbed gives anti-ulcer activity (Anderson & Soman, 1967) but, again, κ- and λ-carrageenans are not differentiated. It may well be that anti-ulcer activity can be indirectly mediated, by some humoral mechanism, which can be triggered by κ- or λ-carrageenan (degraded or undegraded) in the duodenum. This would also explain the anti-ulcer activity of undegraded carrageenan (at even smaller dosage than certain degraded carrageenans) which is apparently not absorbed from the intestine of the guinea-pig. If this hypothesis is correct there is, as yet, no apparent reason why any differences between the antipeptic activity of κ- and λ-fractions, as demonstrated *in vitro*, should correlate with corresponding observed differences in anti-ulcer activity.

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Viscosity of phospholipid sols

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The viscosities of lysolecithin sols in water were found to be Newtonian at the concentrations investigated. A Couette viscometer was used. In the mixed sols containing both lysolecithin and lecithin, thixotropy was absent at all ratios and only the sol with a high lecithin fraction showed some non-Newtonian behaviour. The axial ratio of the mixed micelles calculated by the Simha method varies with the lecithin/lysolecithin ratio, rising to a maximum when the phospholipid mixture is of one mole lecithin to two moles lysolecithin. This phenomenon is probably due to the packing of the various hydrophobic groups in the micelle.

IN the work reported here the apparatus, techniques, and interpretation of results and preparation of lecithin were those described previously (Perrin & Saunders, 1966).

PREPARATION OF LYSOLECITHIN

Lysolecithin was prepared by a modification (Saunders, 1957) of Hanahan's method (Hanahan, Rodbell & Turner, 1954). The procedure described for lecithin (Perrin & Saunders, 1966) was followed as far as the ion-exchange treatment. The solution from the Dowex 1 × 4 ion exchange resin was evaporated to dryness and the residue taken up in 1 litre of ether. Water (10 ml) containing 5 mg of Russell viper venom was added and the flask shaken gently for 1 min. The flask was then allowed to stand for 2–3 hr until precipitation of the lysolecithin was complete. The supernatant ethereal solution was decanted and the lysolecithin was washed with three portions of 500 ml ether and finally with 500 ml acetone. The lysolecithin was dissolved in a small quantity of chloroform and precipitated with 6 volumes of ether. This procedure was repeated four times. The lysolecithin was dissolved in 200 ml ethanol and centrifuged. The solution was evaporated to dryness, the residue being washed with acetone. This purified lysolecithin was again taken up in 200 ml absolute ethanol and the solution centrifuged. The lysolecithin was stored at 0° in this clear alcoholic solution, when crystals of lysolecithin appeared on standing. The yield was 2 g from 12 eggs.

Typical analysis. Total phosphorus, 6·02; total nitrogen, 2·75; sugar, 0·0; choline, 22·8; ester, 52·5; iodine number, 0. Ratio $\frac{\text{nitrogen}}{\text{phosphorus}} = 1{\cdot}01$ (theoretical = 1·00).

Preparation of sol. Lysolecithin was dried at the vacuum pump and dissolved in ion-free water by shaking. The optically clear sol was then freed of dust by spinning at 6000 rev/min in the laboratory centrifuge.

Viscosity measurements. All sols examined behaved in a Newtonian manner and data derived from the measurements are shown in Table 1. The density of lysolecithin was taken as the 1·095 g/ml found by Thomas (1958).

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DISCUSSION

Lysolecithin sols have very low relative viscosities in water, and for the application of Simha's equations (1940) for ellipsoidal particles, measurements must be made on very dilute solutions. However, the

TABLE 1. VISCOSITIES OF LYSOLECITHIN SOLS

conc.	$\phi \times 10^2$	η_{rel}	η_{sp}	η_{sp}/ϕ
7·88	7·71	1·38	0·383	5·33
7·27	7·12	1·32	0·321	4·84
6·72	6·58	1·26	0·258	4·20
6·21	6·08	1·24	0·244	4·31
5·52	5·41	1·19	0·190	3·75
4·99	4·88	1·15	0·154	3·38
4·41	4·32	1·15	0·150	3·73
3·92	3·86	1·13	0·128	3·57
3·39	3·32	1·12	0·124	4·00
2·98	2·92	1·09	0·086	3·16
2·65	2·59	1·07	0·075	3·10

Couette viscometer is not suitable for the measurement of relative viscosities less than 1·10, and so in the present work measurements were made on sols more concentrated than is desirable for Simha's interpretation. The results indicate that the value of $\eta_{sp/\phi}$ is close to the 2·5 (Einstein, 1906) value required for spherical particles. Such particles are stable to shearing and orientation effects do not occur, so accounting for the lack of thixotropy and variation of viscosity with low shear rates. Robinson (1961) and Thomas (1958) found $\eta_{sp/\phi}$ to be slightly greater than 2·5 in capillary viscometers, the discrepancy being due to hydration of the particles. Correlation of viscosity data with data from diffusion, light scattering and ultracentrifugation have been reported elsewhere by these authors (Perrin & Saunders, 1964) and give an anhydrous micellar weight of 95,000 for lysolecithin in aqueous solution.

MIXED SOLS CONTAINING LECITHIN AND LYSOLECITHIN

Preparation of mixed sols

The two phospholipids were prepared as described above. A volume of alcoholic lecithin was evaporated almost to dryness in a weighed flask warmed to 35° on a bath using a vacuum pump. The lecithin was precipitated from the syrupy liquid with acetone and then evaporated to complete dryness overnight. After weighing, the procedure was repeated after the addition of alcoholic lysolecithin solution, but in this instance the drying was completed in under 2 hr. The preparation of the sol from the dried mixed phospholipid depended on the percentage of lysolecithin in the mixed phospholipid. All concentrations are expressed as weight in weight.

(*a*) *Sol containing less than* 40% *lysolecithin in the mixed phospholipid.* The mixed phospholipid was dispersed in a small quantity of ether (lecithin is soluble in ether, lysolecithin is ether insoluble). Ion-free water was then added and the ether gently removed by warming, bubbling with nitrogen, and shaking. The nitrogen was only used when small quantities of lysolecithin were present, in order to avoid the formation of a stable foam. After about an hour the flask was reweighed and the

sol aged for 24 hr in accordance with the findings of Thomas & Saunders (1958).

(*b*) *Sol containing more than* 40% *lysolecithin of mixed phopholipid.* The phospholipids were dried as above and were dispersed in the ion-free water by shaking and warming for an hour. After reweighing the sol was aged for 24 hr.

Viscosity measurements

All measurements were made in the Couette viscometer as previously described (Perrin & Saunders, 1966). Ten major sols were prepared as above, and their viscosities measured after testing for thixotropy. All these sols were sheared at about 9 rev/min (shear rate of approximately 9/sec) and no fall in deflection was noted after shearing for 1 hr, so demonstrating a lack of thixotropy at these slow shear rates. This absence of thixotropy enabled all revolutions to be timed against a stop watch. The viscosities of dilutions of the major sols were measured to enable the viscosity factor γ ($\eta_{sp/\phi}$ at zero concentration) to be extrapolated.

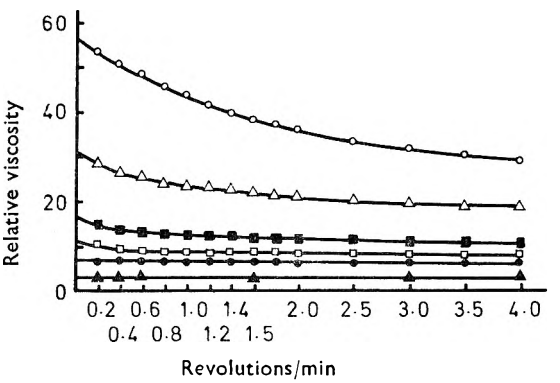

FIG. 1. Change of relative viscosity with shear rate for a sol containing phospholipid mixed in the ratio 87·4% lecithin and 12·6% lysolecithin. Concentrations are total phospholipid weight in weight in the sol. ○ = 4·925, △ = 4·471, ■ = 3·953, □ = 3·607, ● = 3·304, ▲ = 2·3022.

Non-Newtonian behaviour

In some instances the major sols showed very slight non-Newtonian behaviour (especially the more viscous ones), but only with the sol containing 87·4% lecithin of the mixed phospholipid was the phenomenon very marked. Fig. 1 shows the plot of relative viscosity against shear rate for this sol containing mainly lecithin in the phospholipid fraction, and the values of η_{rel} at zero shear obtained from the graph are:

Sol conc.	Relative viscosity at zero shear
4·925	56·7
4·471	31·5
3·953	16·7
3·607	11·5
3·304	6·9

On further dilution the viscous behaviour became Newtonian.

Newtonian behaviour

Most major sols exhibited Newtonian or near Newtonian behaviour as shown in Fig. 2. The more viscous sols had to be much diluted so that

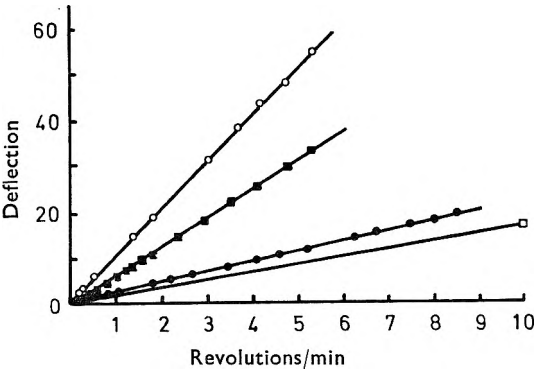

FIG. 2. Flow curves of a sol containing phospholipid in the ratio 77·6% lecithin and 22·4% lysolecithin. ○ = 5·96% phosphatide, ■ = 4·99, ● = 1·94; □ is pure water.

measurements could be taken with a capillary viscometer. Sols were diluted until measurable relative viscosities (less than 15) were obtained. Values of η_{sp}/ϕ were obtained and plotted against ϕ so that a viscosity factor γ was found by extrapolation to zero concentration as shown in Fig. 3.

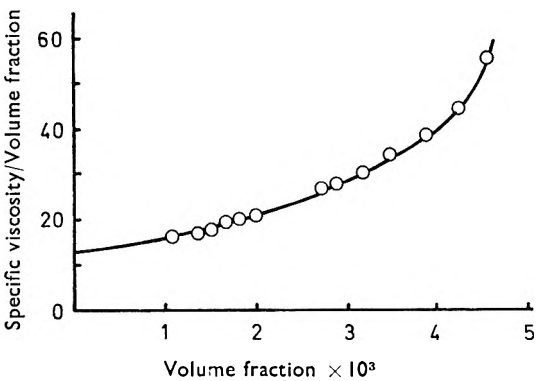

FIG. 3. Graph to obtain the value of specific viscosity/volume fraction at zero volume fraction for a sol containing phospholipid in the ratio 77·6% lecithin and 22·4% lysolecithin.

Table 2 shows the data obtained from the 10 major sols and gives the viscosity factors obtained and the axial ratios computed using Simha's equations, derived assuming ellipsoidal particles.

Fig. 4 shows a plot of viscosity factor against percentage weight in weight lecithin in the mixed phosphatide.

TABLE 2. VISCOSITIES OF THE MIXED SOLS

Lecithin in mixed phospholipid	ε_{sp}/ϕ ($\phi \to 0$)	Axial ratios	
		Prolate	Oblate
87·4	36·0	19·0	52
77·6	11·7	9·0	15·0
65·0	4·0	3·5	4·0
55·4	26·0	15·5	37
50·3	89·5	33	120
44·5	280	66	412
36·7	870	125	1280
27·7	475	87	700
19·4	72	29	104
10·0	3·0	2·25	2·3

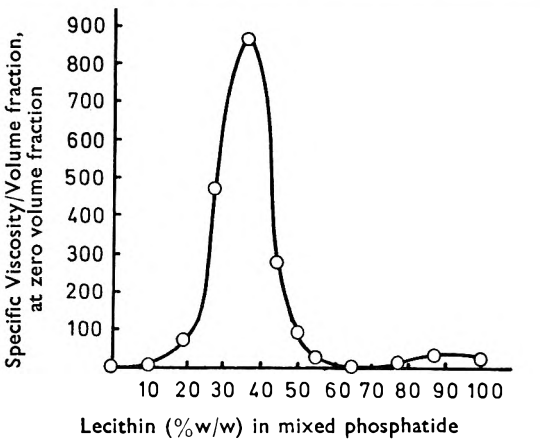

FIG. 4. Effect of phosphatide mixture on specific viscosity/volume fraction at zero volume fraction.

DISCUSSION

Thixotropy is completely absent from the mixed sols at these very low shear rates (less than 10/sec), whereas Thomas & Saunders (1958) found considerable thixotropy in mixed sols sheared at between 20 and 20,000/sec in a Ferranti-Shirley cone and plate viscometer. The addition of 12% lysolecithin solubilizes lecithin sufficiently to prevent breakdown of the micelles on shearing. The non-Newtonian characteristics are also diminished or lost on addition of lysolecithin, the only mixed sol showing change of η_{rel} with shear rate contained a high proportion of lecithin (87·4%) in the mixed phospholipid. The Table of axial ratios calculated from the viscosity factor by the Simha method for ellipsoids does not take into account hydration, and particularly in the region 50–20% lecithin of mixed phospholipid the sols are gels and probably much solvent binding has occurred. The change of viscosity factor with phosphatide composition in Fig. 4 suggests variation from almost spherical particles to asymmetric discs or rods. The curve shows a minimum in the region of 65% lecithin of total phospholipid and this minimum corresponds to axial ratios of 3·5 for a rod or 4·0 for a disc. This can be compared to an equimolar mixture of lecithin and lysolecithin which has a lecithin fraction of 60–62%. After this minimum the viscosity factor increases

with decreasing lecithin percentage until a maximum is reached in the region of 40% lecithin, giving axial ratios of 125 for a rod and over 1200 for a disc. A phospholipid mixture of 1 mole lecithin and 2 moles of lysolecithin would have a lecithin percentage of between 40 and 44. After this maximum the viscosity factor falls sharply until at 10% lecithin content axial ratios of approximately 2·3 are obtained assuming either rods or discs.

Thomas (1958) studied the diffusion of mixed phospholipids containing up to 30% lecithin and found that sols containing up to 15% lecithin had diffusion coefficients only slightly lower than that of lysolecithin. These lower diffusion coefficients suggest asymmetry or hydration causing an increased micellar weight and are in agreement with the viscosity findings. Robinson (1961) examined a mixed micelle containing approximately 14% lecithin by the light-scattering technique and he quotes a dissymmetry of 2·27 for the mixed phosphatide compared with values of 1·44 for lecithin and 1·08 for lysolecithin. The expected value of viscosity factor for a sol of this composition would be nearly 30, a value only slightly greater than that of lecithin. With sols containing a bigger percentage of lecithin in the mixed phosphatide, Thomas was unable to obtain diffusion coefficients because of difficulty in forming boundaries and anomalous flow properties. Robinson did not investigate sols containing a bigger fraction of lecithin than 14·3% because the sols were no longer optically clear. Thomas & Saunders (1958) studied the complete range of mixed micelles in Cannon-Fenske viscometers and obtained a similar plot of viscosity against lecithin content of mixed phosphatide. However, they obtained no minimum at 65% lecithin and their maximum occurred at approximately 45% lecithin instead of at 40% as reported here. The values of $\eta_{sp/\phi}$ given by Thomas are different from those quoted here, probably because of (*a*) variation in the preparation of the sols, (*b*) higher and varying shear rates are used in viscosity measurements in the capillary viscometers. Thomas found his sols to be thixotropic and so the viscosity would depend upon their previous history.

The addition of lecithin (two fatty acid chains) to lysolecithin (one fatty acid chain) would alter the packing of the molecules in the micelle because the bulkier hydrophobic groups of the lecithin molecule would interfere with the arrangement of the wedge-shaped lysolecithin molecules so causing the production of asymmetric micelles, the degree of asymmetry varying with the molar ratio of lecithin to lysolecithin. This effect is fully discussed by Saunders (1966).

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Uncoupling of oxidative phosphorylation by glycyrrhetic acid, fusidic acid and some related triterpenoid acids

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Some new derivatives of 18α- and 18β-glycyrrhetic acid and oleanolic acid were tested for their ability to inhibit phosphorylation coupled to succinate oxidation in rat liver mitochondria. Glycyrrhetic acid is a potent uncoupler of oxidative phosphorylation (approaching 2,4-dinitrophenol in potency); uncoupling activity is dependent upon each of the following functional groups: 3-hydroxy, 11-oxo and 30-carboxyl groups. Inversion of the configuration at C-18 (D/E ring junction) or replacement of the 11-oxo-12-ene system in ring C by the 9(11),12-diene system in glycyrrhetic acid abolished uncoupling activity. By contrast, the hemisuccinates (3-*O*-carboxypropionyl derivatives) of 18α-glycyrrhetic acid and of the 18β-9,12-diene acid were moderately potent uncoupling agents but less active than 18β-glycyrrhetic acid. The uncoupling activity of these hemisuccinates could not be attributed to the carboxypropionyl group alone. Structural analogues of various glycyrrhetic acid derivatives prepared from oleanolic acid (with the carboxyl group at C-28) were less active in uncoupling oxidative phosphorylation than the corresponding compounds in the glycyrrhetic acid series (with the carboxyl group at C-30). The uncoupling activity of some derivatives of two naturally occurring tetracyclic triterpenoid acids, polyporenic acid A and fusidic acid, was also investigated and found to largely depend upon their chemical structure. The possible application of these compounds as drugs in man is discussed.

GLYCYRRHETIC acid (glycyrrhetinic acid, Fig. 1A), the aglycone of glycyrrhizin (from liquorice) uncouples oxidative phosphorylation, that is, it inhibits the mitochondrial biosynthesis of adenosine 5′-triphosphate without inhibiting mitochondrial respiration.

Polyporenic acid A (ungulinic acid) is a tetracyclic triterpenoid (trimethyl sterol), one of several polyisoprenoids produced by the birch fungus *Polyporus betulinus* (Jones & Halsall, 1954; Fieser & Fieser, 1959; Ourisson, Crabbe & Rodig, 1964). It is 3α,12α-dihydroxy-24-methylene-5α,25ξ-lanost-8-en-26-oic acid (Fig. 1B) (Halsall, Hodges & Jones, 1953; Halsall & Hodges, 1954). Polyporenic acid A uncouples oxidative phosphorylation in liver mitochondria (Whitehouse, 1963) but does not exhibit anti-inflammatory activity in rats when assayed by the carrageenan granuloma test (private communication from Dr. R. M. A. Atkinson, Glaxo Laboratories).

Fusidic acid (Fusidin) is an antibiotic originally obtained from *Fusidium coccineum* (Godtfredsen, Roholt & Tybring, 1962; Newman, Bhat & others, 1962) and subsequently shown to be identical with ramycin, isolated from several cephalosporia and a phycomycete (Vanderhaeghe, Van Dijck & De Somer, 1965). Most features of its structure have now been elucidated (Fig. 1C) (Godtfredsen & Vangedal, 1962; Arigoni,

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Daehne & others, 1963; Bucourt & Legrand, 1964; Godtfredsen, Daehne & others, 1965). It has recently been found to alleviate arthritis in man (Dr. W. O. Godtfredsen, private communication).

We have now examined the relationship between chemical structure and uncoupling activity of several derivatives and analogues of glycyrrhetic acid, polyporenic acid A and fusidic acid.

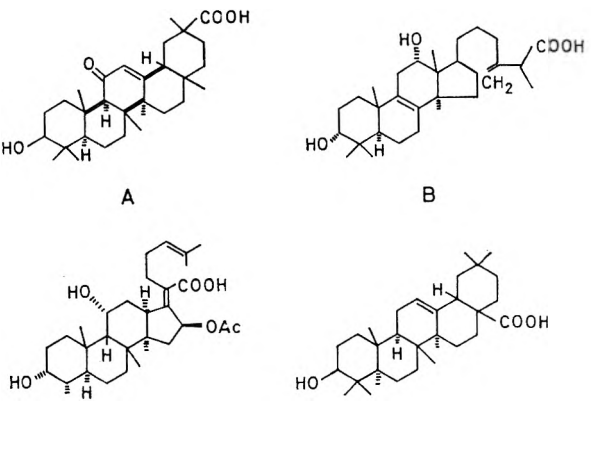

FIG. 1. Structure of some triterpenoid acids. A. 18β-Glycyrrhetic acid. B. Polyporenic acid A. C. Fusidic acid. D. Oleanolic acid.

Experimental

Materials. The following compounds were kindly made available by Dr. S. Gottfried & the late Prof. E. E. Turner, F.R.S. (Biorex Laboratories, London, E.C.1): oleanolic acid, purified 18α- and 18β-glycyrrhetic acid, the corresponding 3-*O*-hemisuccinate (β-carboxypropionyl) esters, the 3-*O*-sulphate ester (as the Na salt) of the 18β-acid, 3-dehydro- and 11-deoxo-18β-glycyrrhetic acid, 3-*O*-acetyl- and 3-*O*-propionyl-18β-glycyrrhetic acid, certain conjugates of oleanoic acid and glycyrrhetic acid with anthranilic acid and *p*-aminobenzoic acid, and a highly purified preparation of glycyrrhizin (originally obtained from Ihei Matsumoto & Co., Ltd., Tokyo). A commercial sample of ammonium glycyrrhizate was obtained from L. Light & Co., Colnbrook, Bucks.

Other derivatives of the glycyrrhetic acids and derivatives of oleanolic acid were synthesized as described (Dean, Halsall & Whitehouse, 1967).

Polyporenic acids and their derivatives were prepared according to Curtis, Heilbron & others (1953); Jones & Woods (1953) and Halsall & Hodges (1954). Eburicoic acid was isolated from *Polyporus eucalyptorura*. 3β-Hydroxycholest-5-en-26-oic acid was synthesized from 25-oxonorcholesterol (Dean & Whitehouse, 1966). Fusidic acid and some of its derivatives (Godtfredsen & Vangedal, 1962) were supplied by Dr. W. O. Godtfredsen (Leo Pharmaceutical Products, Copenhagen). Tumulosic acid was donated by Dr. J. E. E. Holker, University of Liverpool and cholesterol 3-*O*-(hydrogen sulphate) by Mr. P. R. H. Raggatt.

METHODS

Rat liver mitochondria were isolated and incubated at 30° with various oxidizable substrates, glucose and yeast hexokinase at pH 6·8 as detailed by Skidmore & Whitehouse (1965a). Oxygen uptake was measured manometrically; phosphate uptake was measured colorimetrically (Fiske & Subbarow, 1925). Compounds to be tested for their effect upon oxidative phosphorylation were added to the main compartment of Warburg vessels either in 100 μl of neutralized aqueous solution or in 25 μl of dimethylformamide together with the theoretical equivalent of aqueous sodium bicarbonate.

The activity of yeast hexokinase as a glucose phosphorylase in the presence of these triterpenoids was determined by the method of Darrow & Colowick (1955).

Drug binding to ϵ-amino-groups of bovine plasma albumen was determined colorimetrically using 2,4,6-trinitrobenzaldehyde (Skidmore & Whitehouse, 1965b; 1966); *N*-benzoyl-L-arginine ethyl ester was substrate.

Drug action on a thiol enzyme (papain) was studied by Whitehouse & Leader (1967).

Results

Glycyrrhetic acid (100 μM) and polyporenic acid A (200 μM) were fairly potent uncouplers of oxidative phosphorylation. A higher concentration of fusidic acid (600 μM) was needed for the same effect *in vitro*. At these concentrations these triterpenoid acids abolished the phosphorylation of ADP (i.e. uptake of inorganic phosphate) accompanying the mitochondrial oxidation of succinate, pyruvate, glutamate and citrate. Also, they had no effect on mitochondrial respiration or on either the glucose-phosphorylating or the ATP-ase activity (de la Fuente & Sols, 1963) of the yeast hexokinase which was added, with glucose, to the phosphorylating mitochondrial preparations to trap the newly incorporated labile γ-phosphate group of ATP (as the stable ester-phosphate group in glucose 6-phosphate). The uncoupling action of these acids was not reversed by co-incubation with 5 mM cysteamine or mercaptoethanol but was reversed on diluting the drug after preincubation with mitrochondria.

Further evidence that these acids were uncouplers of oxidative phosphorylation was found as follows. Slices of cattle tracheal cartilage were incubated with sodium [^{32}P]phosphate and with sodium [^{35}S]sulphate and the incorporation of ^{32}P into organic phosphates and the ATP-dependent incorporation of ^{35}S into the polysaccharide sulphates of the cartilage tissue were each determined in the presence and in the absence of these triterpenoids (Whitehouse & Haslam, 1962; Whitehouse, 1964a). Glycyrrhetic acid (100 μM) and polyporenic acid A (200 μM) each inhibited these biosynthetic processes by more than 50% without impairing the oxidation of [$^{14}C_u$]glucose and [$^{14}C_1$]octanoate to [^{14}C]carbon dioxide by these cartilage slices. Oleanolic acid (300 μM) and 11-deoxo-18β-glycyrrhetic acid (comp. 12; Table 1) (150 μM), which do not uncouple oxidative phosphorylation in liver mitochondria (see below), had no effect on either ^{32}P or ^{35}S incorporation by cartilage slices.

The relationship of chemical structure to uncoupling activity was explored with selected derivatives of glycyrrhetic, oleanolic, polyporenic and fusidic acids, using rat liver mitochondria incubated with succinate as the test system. A compound which lowered the P/O ratio to $\leqslant 85\%$ that of the P/O ratio obtained in parallel incubations without added triterpenoids, was considered to be capable of uncoupling oxidative phosphorylation. The P/O ratio in these drug-free controls (assigned a value of 100%) actually varied with each mitochondrial preparation but fell within the range 1·2–1·7 (see Skidmore & Whitehouse, 1965a).

With this biological test system, a fairly reproducible response was obtained to a given concentration of a triterpenoid. Thus in duplicate assays for 75 μM glycyrrhetic acid with 20 successive mitochondrial preparations, succinate oxidation was inhibited by no more than 20% and the P/O ratio was 32 $\pm$ 7 (s.e.m.)% of the controls; the range of P/O values being actually 10–55% of the controls.

UNCOUPLING ACTIVITY OF GLYCYRRHETIC ACID DERIVATIVES

Table 1 indicates the relative uncoupling potencies of 18β-glycyrrhetic acid and 17 of its derivatives. Simple derivatives lacking the ionized carboxyl group at C-30 were too water-insoluble for testing. Several

TABLE 1. UNCOUPLING ACTIVITY OF SOME DERIVATIVES OF 18α- AND 18β-GLYCYRRHETIC ACID AND RELATED COMPOUNDS

No.	Compound	Conc. $\times 10^{-4}$M	% Control P/O ratio
—	None	—	100
1	18α-Glycyrrhetic acid(isoglycyrrhetic acid)	1·5	92
2	18β-Glycyrrhetic acid	0·75	35
		1·5	5
3	3-Dehydro-18β-glycyrrhetic acid	0·75	83
4	Methyl 18β-glycyrrhetate	1·0*	86
5	3-*O*-Acetyl-18β-glycyrrhetic acid	0·75	73
		1·5	38
6	3-*O*-Propionyl-18β-glycyrrhetic acid	0·75	85
7	3-*O*-(β-Carboxypropionyl)-18α-glycyrrhetic acid	2·2	0
		1·2	43
8	3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid	2·2	34
		1·2	70
9	Methyl 3-*O*-(β-carboxypropionyl)-18α-glycyrrhetate	1·2	84
10	Methyl 3-*O*-(β-carboxypropionyl)-18β-glycyrrhetate	1·5	100
11	3-*O*-Acetyl-18β-glycyrrhetamide	0·75	100
12	11-Deoxo-18β-glycyrrhetic acid	1·5	96
13	3-*O*-(β-Carboxypropionyl)-11-deoxo-18β-glycyrrhetic acid	2·0	80
14	9,11-Dehydro-11-deoxo-18β-glycyrrhetic acid	0·75	100
15	3-*O*-(β-Carboxypropionyl)-9,11-dehydro-11-deoxy-18β-glycyrrhetic acid	2·0	5
		1·0	25
16	Glycyrrhizin (purified Na salt)	7·5	92
17	Glycyrrhizin (commercial NH_4^+ salt)	5·0	74
18	18β-Glycyrrhetic acid 3-*O*-(hydrogen sulphate)	2·5	0–70†
19	*N*-(18β-Glycyrrhetyl)glycine	1·5	65
20	*o*-(18β-Glycyrrhetamido)benzoic acid	1·5	53
21	*o*-[3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetamido]benzoic acid	0·75	55
22	*o*-(3-*O*-Acetyl-18β-glycyrrhetamido)benzoic acid	0·75	30
		0·4	55
23	*p*-(3-*O*-Acetyl-18β-glycyrrhetamido)benzoic acid	0·75	95
24	*o*-Cholamidobenzoic acid	4·0	45
25	Cortisone 21-sulphate (Na salt)	2·5	98
26	Cholesterol 3-*O*-(hydrogen sulphate) (K salt)	2·5	100

* Conc. uncertain due to limited solubility.
† Variable activity, see text.

other derivatives, which retained this carboxyl group, were too insoluble in the presence of magnesium ions (4·5 mM) at pH 6·8 to be tested adequately. In either instance, when this happened with compounds containing a 3β-hydroxyl group, it was usually possible to test their 3-*O*-(β-carboxypropionyl derivatives ("hemisuccinate esters") for uncoupling activity instead, these particular derivatives being much more soluble than their parent alcohols in aqueous media containing magnesium ions.

3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid (comp. 8) was approximately one-third as potent as uncoupling agent as the parent alcohol, i.e. 18β-glycyrrhetic acid (comp. 2). The corresponding hemisuccinate of 18α-glycyrrhetic acid (comp. 7) was more active in uncoupling oxidative phosphorylation than the hemisuccinate of the acid (comp. 8). By contrast, the parent alcohol (comp. 1) was inactive; it was certainly sufficiently water-soluble in the presence of magnesium ions at pH 6·8 to be compared with the unsubstituted 18β-acid (comp. 2). In two other instances it was likewise found that hemisuccinates uncoupled oxidative phosphorylation (comp. 13 and 15), but the corresponding 3-hydroxy compounds did not (comp. 12 and 14).

Some "quasi-physiological" partition experiments were made in attempts to find an explanation for these findings. The removal of ultraviolet absorbing 3-hydroxy compounds and hemisuccinates from the aqueous salt solution pH 6·8 by liver mitochondria during a 30 min incubation at 30° was measured as follows. The residual ultraviolet absorption at 250 mμ [due to the 11-oxo-12-ene and the 9(11),12-diene chromophores respectively] in the aqueous phase was determined after removal of the mitochondrial lipid phase (at 8,500 *g*, 15 min) and subsequent partition (concentration) of the ultraviolet absorbing triterpenoids into methylene dichloride at pH 6·8; parallel assays were made on solutions of the triterpenoids similarly incubated without mitochondria.

These experiments showed that the parent 18α- and 18β-acids were both removed from the aqueous phase to approximately the same extent (ca. 80%) by thick mitochondrial suspensions (ca. 20% by volume); only 20% of the hemisuccinate of the 18α-acid was removed under the same conditions. Whilst these results indicate why the uncoupling activity of the hemisuccinate of the 18β- acid was only 1/4 to 1/3 that of the parent 18β-acid, they give no indication why the parent 18α-acid should be inactive while its hemisuccinate is a potent uncoupling agent. The hemisuccinate group itself apparently had no uncoupling activity; at least 5 of the other hemisuccinates of triterpenes tested had no effect upon oxidative phosphorylation (see below).

Replacement of the ionized carboxyl group at C-30 effectively abolished uncoupling activity; the methyl esters of 18β-glycyrrhetic acid (comp. 4) and of the hemisuccinates of 18α- and 18β-glycyrrhetic acids (comp. 9 and 10) and the amides of both the 18β-glycyrrhetic acid and the 3-*O*-acetyl-18β-acid (comp. 11), were each without any effect on oxidative phosphorylation [18β-glycyrrhetamide was not pure: it was tested in admixture with methyl glycyrrhetate (which is inactive) from which it was prepared by ammonolysis in liquid ammonia; the amide has a higher

Rf value than the ester on thin-layer plates developed with ethyl acetate].

Other more soluble derivatives of glycyrrhetinamide were also examined for uncoupling activity. Only the *N*-anthranilic acid (comp. 20) and *N*-glycine conjugates (comp. 19) had any significant activity. Esterification of the 3-hydroxyl group of the anthranilic acid conjugate (to give comp. 21 and 22) actually increased the uncoupling activity, perhaps by increasing the solubility.

Modification of the 3β-hydroxyl group of 18β-glycyrrhetic acid, either by oxidation to the ketone (comp. 3) or by esterification (comp. 5,6,8 and 18) diminished but did not abolish the uncoupling activity. The 3-*O*-acetyl, 3-*O*-(hydrogen sulphate) and 3-*O*-(β-carboxypropionyl) derivatives (comp. 5, 18, 8), though differing greatly in the ease with which they partition between liver mitochondria and aqueous solutions of pH 6·8 (the acetyl ester resembling the unesterified acid, see above) were all less active than the parent 3β-hydroxyl compound (comp. 2). The hydrogen sulphate showed variable uncoupling activity (relative to the parent acid; comp. 2) in different experiments, which may reflect some hydrolysis to the parent acid. This ester is labile, particularly in acidic media (Dr. J. C. Turner, private communication). The very water-soluble 3-glycoside, glycyrrhizin (comp. 16 and 17) was inactive. These observations indicate that the presence of an unsubstituted hydroxyl function at C-3, though not essential for uncoupling activity, nevertheless determines the absolute activity of this type of compound in uncoupling oxidative phosphorylation.

Removal of the 11-keto-group of 18β-glycyrrhetic acid, with concomitant reduction of C-11 to a methylene group, virtually abolished the uncoupling activity (cf. comp. 12 and 13 with comp. 2 and 8). However, when the 11-oxo-12-ene grouping in 18β-glycyrrhetic acid was replaced by the 9(11),12-diene grouping the uncoupling activity was apparently retained in one instance (cf. comp. 15 with comp. 8) but not in another (cf. comp. 14 and 2). This situation corresponded to that found with the hemisuccinate of glycyrrhetic acid (active) and the unsubstituted 18α-acid (inactive); in each instance the parent 3β-hydroxy compounds were inactive whilst the 3-carboxypropionyl esters were active.

UNCOUPLING ACTIVITY OF OLEANOLIC ACID DERIVATIVES AND OTHER PENTACYCLIC ACIDS

Oleanolic acid (comp. 27, Table 2) is an isomer of 11-deoxo-18β-glycyrrhetic acid in which the carboxyl group is at C-28 (in the junction of the D and E rings) instead of at C-30 as in the glycyrrhetic acid series.

Neither 18β-oleanolic acid nor its hemisuccinate (comp. 28) uncoupled oxidative phosphorylation (Table 2). The hemisuccinate of 11-deoxo-glycyrrhetic acid (comp. 13), though non-absorbing at 250 mμ (i.e. 11-oxo-12-ene grouping was absent) always showed some slight but significant uncoupling activity. Other analogues of active compounds in the glycyrrhetic acid series were prepared from oleanolic acid, e.g. 11-oxo-18β-oleanolic acid, for comparison with 18β-glycyrrhetic acid (Fig. 1A). These oleanolic acids (comp. 29, 30, 31, Table 2) were less potent uncoupling agents than the corresponding glycyrrhetic acids

TABLE 2. UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME OLEANOLIC ACID DERIVATIVES

No.	Compound	Conc. $\times 10^{-4}$M	P/O ratio (% controls)
—	None	—	100
27	18β-Oleanolic acid	1·5	100
28	3-*O*-(β-Carboxypropionyl)-18β-oleanolic acid	3	92
29	11-Oxo-18β-oleanolic acid	1·5	83
30	3-*O*-Acetyl-11-oxo-18β-oleanolic acid	1·5	83
31	3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid	2·2	72
32	3-*O*-(β-Carboxypropionyl)-9,11-dehydro-18β-oleanolic acid	2	15
33	3-*O*-(β-Carboxyproprionyl)11-oxo-oleanolyl (28 → 13)lactone	2	100
34	*o*-(Oleanolamido)benzoic acid	1·5	45

(comp. 1, 5, 8, 15, Table 1). These findings show that the position of the carboxyl group in this series of compounds (4 oleanolic acids and 4 glycyrrhetic acids) largely governs the uncoupling activity. The most active oleanolic acid analogue encountered in this study was the hemi-succinate of 9,11-dehydro-18β-oleanolic acid (comp. 32) which contains the 9(11),12-diene system.

The inactivity of the water-soluble lactone (comp. 33) again shows the requirement for an unsubstituted carboxyl group for uncoupling activity.

Other pentacyclic triterpenoid acids tested (ursolic, boswellic, morolic and 11-oxoboswellic acids) did not uncouple oxidative phosphorylation when tested at 150 μM.

UNCOUPLING ACTIVITY OF UNSATURATED STEROIDS AND TRITERPENOIDS

Some conjugated unsaturated ketones and water-soluble conjugated dienes were tested but found not to uncouple phosphorylation linked to succinate oxidation. Compounds tested included cholest-4-en-3-one (70 μM), 11-deoxycorticosterone (deoxycortone) (100 μM), cortisol-21-aldehyde (hydrocortisone 21-aldehyde) (200 μM), 3-β-carboxypropionyl)-ergosterol (100 μM). Uncoupling activity is evidently not associated with these unsaturated groups *per se*, in the absence of a nuclear carboxyl group. Other steroid sulphates (comp. 25, 26) did not mimic glycyrrhetic acid 3-*O*-sulphate (comp. 18) in uncoupling phosphorylation (Table 1).

UNCOUPLING ACTIVITY OF SOME TETRACYCLIC TRITERPENOID ACIDS

A few derivatives of polyporenic acid A, a βγ-unsaturated acid, were examined for uncoupling activity (Table 3). One of the αβ-unsaturated

TABLE 3. UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME TETRACYCLIC TERPENOID (TRIMETHYL STEROID) ACIDS

No.	Compound	Conc. $\times 10^{-4}$M	P/O ratio (% control)
35	Polyporenic acid A	1·5	30
36	Polyporenic acid C	3	100
37	Eburicoic acid	3	100
38	Fusidic acid	3	75
39	ψ-Polyporenic acid (25-isopolyporenic acid)	5	45
40	3,12-Dehydropolyporenic acid	1·5	40
41	24,28-Dihydropolyporenic acid A	1·5	50
42	3β-Hydroxycholest-5-en-26-oic acid	1·5	55
43	Tumulosic acid	3	92

isomers ψ-polyporenic acid (comp. 39) and 24,28-dihydropolyporenic acid (comp. 41) were both less active than polyporenic acid A in uncoupling oxidative phosphorylation, indicating that the double bond between C-24 and C-28 may possibly contribute to the uncoupling activity of polyporenic acid A. The diketo-derivative (comp. 40) was less active than polyporenic acid A (cf. 3-dehydro-18β-glycyrrhetic acid and 18β-glycyrrhetic acid itself (comp. 3, 2)]. A crude preparation of the naturally occurring conjugate (glycoside?) of polyporenic acid A (containing a small quantity of the free acid) was much less potent than polyporenic acid A itself on a weight for weight basis. Unless this unknown conjugate has a molecular weight at least 5 times that of the free acid, this observation suggests that the naturally occurring form(s) of polyporenic acid A is less active in uncoupling phosphorylation than the free acid (cf. glycyrrhizin and glycyrrhetic acid).

Polyporenic acid C (16α-hydroxy-24-methylene-3-oxo-5α-lanosta-7,9(11)-dien-20-oic acid) and tumulosic acid (3β,16α-dihydroxy-24-methylene-5α-lanost-8-en-20-oic acid), which are both also obtained from *Polyporus betulinus*, and eburicoic acid (3β-hydroxy-24-methylene-5α-lanost-8-en-20-oic acid) did not uncouple oxidative phosphorylation at concentrations at which they were soluble in the buffered salt medium.

Little variation in uncoupling activity was found when the fusidic acid derivatives described by Godtfredsen & Vangedal, (1962) were tested.

RELATIVE BINDING TO ALBUMEN

There was some correlation between the solubility of a terpenoid acid in the presence of 0·1 mM bovine plasma albumen and its uncoupling activity, for example eburicoic acid, oleanolic acid, 18α-glycyrrhetic acid and 11-deoxo-18β-glycyrrhetic acid did not bind sufficiently strongly to albumen to give 1 mM solutions but polyporenic acid A, 18β-glycyrrhetic acid and fusidic acid readily gave mM solutions in the presence of 0·1mM

TABLE 4. RELATIVE BINDING OF SOME ACIDIC POLYISOPRENOIDS TO ALBUMEN (LYSYL) AMINO-GROUPS DETERMINED COLORIMETRICALLY WITH 2,4,6-TRINITROBENZALDEHYDE. Inhibitors by 1 mM drug of colour developed when reagent (0·1 mM) reacts with bovine plasma albumen (0·1 m at pH 7·4 (0·1M phosphate), determined at 425 mμ.

No.	Compound	Inhibition %
—	None	—
2	18β-Glycyrrhetic acid	35
3	3-Dehydro-18β-glycyrrhetic acid	40
5	3-*O*-Acetyl-18β-glycyrrhetic acid	38
19	*N*-(18β-Glycyrrhetyl)glycine	23
18	18β-Glycyrrhetic acid 3-*O*-(hydrogen sulphate)	48
16	Glycyrrhizin (purified Na salt)	0
17	Glycyrrhizin (commercial NH_4^+ salt)	0
8	3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid	60
21	*o*-[3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetamido]benzoic acid	74
7	3-*O*-(β-Carboxypropionyl)-18α-glycyrrhetic acid	70
31	3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid	75
35	Polyporenic acid A	65
41	24,28-Dihydropolyporenic acid A	44
38	Fusidic acid	20
	3,7,12-Trioxocholanic acid	0
	Deoxycholic acid	38
	3,12-Dioxocholanic acid	32

albumen. Within this latter class of albumen-soluble acids, uncoupling activity approximately paralleled the affinity of these compounds for the albumen (lysyl) ϵ-amino-groups as indicated by their ability to prevent 2,4,6-trinitrobenzaldehyde from reacting with these amino-groups and so quenching the red colouration formed by the albumen-trinitrobenzaldehyde interaction. [A similar correlation has been found in studies of aromatic anti-inflammatory acidic drugs (Skidmore & Whitehouse, 1965b).] Table 4 shows that this albumen-binding is primarily a property of the 30-carboxyl group in the glycyrrhetic acid series but is enhanced by the 3-(β-carboxypropionyl) (hemisuccinate) and 3-*O*-sulphate groups. The 3-*O*-(β-carboxypropionyl)-18α-glycyrrhetic acid (comp. 7) which is more potent than the hemisuccinate of the 18β-acid (comp. 8) in uncoupling phosphorylation, also binds more strongly to these albumen amino-groups. 3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid (comp. 31) binds more strongly to albumen amino-groups than 3-*O*-(β-carboxypropionyl)-18β-oleanolic acid (comp. 28) but neither of these compounds significantly uncouples phosphorylation, showing that albumen-binding is of itself no absolute guide to potential uncoupling activity, though it may provide an indication of the relative potency within a given series of compounds with intrinsic uncoupling activity.

When these uncoupling acids were co-incubated with bovine plasma albumen and phosphorylating mitochondria, it was found that 75 μM 18β-glycyrrhetic acid no longer uncoupled phosphorylation in the presence of 1 mg/ml albumen. The hemisuccinates of 18α- and 18β-glycyrrhetic acids (comp. 7, 8) and glycyrrhetic acid 3-*O*-sulphate (comp. 18) (all at 25 μM) and sodium fusidate (comp. 38) (500 μM) still retained some uncoupling activity in the presence of 5 mg/ml albumen and polyporenic acid A (120 μM) retained some activity in the presence of 2·5 mg/ml albumen, but the potency of these acids was reduced (approx. 50–70%).

Discussion

These studies have been limited by the poor solubility of many of the compounds, but it is possible to draw the following conclusions.

(1) The ability of certain triterpenoid acids to uncouple oxidative phosphorylation must now be added to the list of known biological properties of terpenes (reviewed by Martin-Smith & Khatoon, 1963). The anti-inflammatory activity of glycyrrhetic acid in small animals and the inhibition of neoplastic cell growth by the triterpenoid fractions from *Polyporus betulinus* (Utzig & Samborski, 1957; Wandokanty & Utzig, 1958) may depend upon the uncoupling of oxidative phosphorylation in the subcutaneous or neoplastic tissue by glycyrrhetic and polyporenic acid A respectively. The antibacterial activity of fusidic acid (Godtfredsen & others 1962), like that of halophenols, salicyclic acid and many natural products (antibiotics) such as gramicidin and usnic acid, may also be a consequence of its uncoupling activity. The uncoupling activity of fusidic acid exceeds that of salicylic acid (Whitehouse, 1964a) whilst that of glycyrrhetic acid actually approaches the activity of 2,4-dinitrophenol

(almost complete uncoupling at 50 μM) and certainly exceeds the uncoupling activity of the most potent steroidal (cholanic) acid of animal origin, namely deoxycholic acid (Lee & Whitehouse, 1965).

(2) The uncoupling activity (and solubility at pH 7) of these particular triterpenoid acids is dependent upon their chemical structure. It should therefore be distinguished from many of the other biological activities of terpenes which are structurally non-specific (Martin-Smith & Khatoon, 1963). This dependence of the uncoupling activity upon chemical structure distinguishes these triterpenoid acids from most aliphatic fatty acids, and possibly also arylacetic acid (Whitehouse, 1964b), the uncoupling activity of which appears to depend only upon solubility in a lipid phase and the presence of a carboxyl group.

Each of the functional groups of glycyrrhetic acid (3-hydroxy,11-oxo and 30-carboxy) apparently contributes to its uncoupling activity, the carboxyl group and double unsaturation in ring C (either an 11-oxo-12-ene or a 9(11),12-diene system) being apparently essential for this purpose. The much weaker uncoupling activity of the olean-28-oic isomers (oleanolic acid derivatives) demonstrates that the activity of the glycyrrhetic acid derivatives is not a structurally non-specific activity of a highly lipophilic acid; if this were so, the oleanolic acid derivatives and glycyrrhetic acid derivatives should be approximately equipotent. The difference in activity of 18α- and 18β-glycyrrhetic acid further emphasizes this.

Similar conclusions may be drawn from our rather more limited survey of the tetracyclic terpenoid acids. The presence of the double bond in polyporenic acid A and the 20-iso-configuration in fusidic acid may be of particular importance in determining uncoupling potency. It is remarkable that the ketones, derived by oxidation of the hydroxy-group in position 3 in glycyrrhetic acid and in position 12 in polyporenic acid A, should be so much less active than the parent alcohols. This parallels the observation that 3-, 12- (and 7-) oxocholanic acids are very much weaker uncoupling agents than the corresponding hydroxycholanic acids (Lee & Whitehouse, 1965). Oxidation of these hydroxy-acids to the corresponding ketones does not seem to alter the affinity for albumen amino-groups however.

The prospect of using triterpenoid acids as effective drugs in man (antibacterials or antirheumatic agents) is tempered by the fact that they may be strongly bound to plasma proteins *in vivo* and effectively rendered unavailable, just as we found to be the case with glycyrrhetic acid in the presence of serum albumen *in vitro*. Though this protein binding of the potential drug may be much diminished by increasing the water solubility of the triterpenoid, e.g. by β-carboxypropionylation, such modification of the molecule also diminishes or abolishes the uncoupling activity. Thus the highly water-soluble natural glycoside, glycyrrhizin, is virtually inactive. The relatively low uncoupling activity of the rather water-soluble fusidic acid (salts) and of 3-*O*-(β-carboxypropionyl)-18β-glycyrrhetic acid compares unfavourably with that of the less oxygenated and much less water-soluble polyporenic acid A or glycyrrhetic acids. This increase in water solubility must effectively lower the concentration

of the acid in, or across, lipid-rich phases such as the cell membranes and within the mitochondria themselves. Furthermore in view of the rapidity with which circulating cholanic acids and many steroid hormones are taken up and conjugated by the liver, to be subsequently excreted with the bile flow, it is likely that these higher polycyclic acids will suffer a similar fate. Parke, Pollack & Williams (1963) have already shown that 95% of a single dose of radioactive glycyrrhetic acid administered intraperitoneally to rats is excreted via the bile within 6 to 8 hr, mainly as three metabolites.

One of these has now been shown to be the 3-*O*-(hydrogen sulphate) ester of glycyrrhetic acid (Iveson, Parke & Williams, 1966). This particular metabolite (comp. 18) does uncouple oxidative phosphorylation, even in the presence of plasma albumen, although it is less potent than glycyrrhetic acid itself in the absence of albumen. The glycine congugate of glycyrrhetic acid (comp. 19) was synthesized and found to be much less potent in uncoupling phosphorylation than glycyrrhetic acid itself. This conjugate does not seem to be formed in the rat; it is actually less polar than any of the glycyrrhetic acid metabolites detected by Parke & others (1963) in rat bile (Dr. D. V. Parke, private communication).

The only compounds that were more potent uncoupling agents than glycyrrhetic acid and, therefore, of potential interest as novel anti-inflammatory drugs, were the conjugates with anthranilic acid (comp. 20, 21, 22). That this was largely due to the anthranyl pharmacophore rather than the glycyrrhetyl moiety was indicated by (*a*) the feeble uncoupling activity of the analogue conjugated with *p*-aminobenzoic acid (comp. 23) and (*b*) the uncoupling activity of other *N*-acylanthranilic acids (Leader & Whitehouse, 1966; Whitehouse, 1964a and unpublished observations), including *o*-(oleanolamido)benzoic acid [*N*-(oleanoyl)anthranilic acid, comp. 34].

All these terpenoid acids appear to uncouple phosphorylation by interaction with key lysyl amino-groups participating in mitochondrial phosphorylation. There was a correlation between their ability to bind to these same groups on plasma albumen and their uncoupling potency *in vitro*. Neither the hemisuccinates of 18α- or 18β-glycyrrhetic acid (comp. 7, 8) nor fusidic acid inhibited papain (a thiol enzyme), when tested at 1 mM in the presence of 0·25 mM mercaptoethanol; the uncoupling activity of these acids and polyporenic acid A was not reversed by thiols, so it is unlikely that they uncouple phosphorylation merely by interaction with essential thiol groups in mitochondria. [This is an alternative mechanism of uncoupling phosphorylation and explains the uncoupling activity of ninhydrin (Whitehouse & Leader, 1967)].

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Influence of chronic phenobarbitone treatment on uterine phosphofructokinase induction

SIR,—Administration of oestradiol-17β was shown to produce a rapid increase in phosphofructokinase activity in the uterus of the ovariectomized rat (Singhal & Valadares, 1966). A significant increase in enzyme activity was observed at 4 hr and peak levels were reached 16 hr after administration of a single intramuscular injection of oestradiol-17β (10 μg/100 g). Actinomycin, puromycin, ethionine, cycloheximide and 5-fluorouracil all blocked this hormone-induced response, suggesting that both new RNA and new protein syntheses are involved in the observed increases in uterine phosphofructokinase activity (Singhal & Ling, 1966b; Singhal & Valadares, 1967; Singhal, Valadares & Ling, 1967).

The ability of phenobarbitone to enhance drug metabolism by increasing the activity of liver microsomal enzymes is well documented (Remmer, 1962; Conney & Burns, 1962). Pretreatment of immature female rats with phenobarbitone for several days inhibits the uterotrophic response of tritiated oestradiol and decreases the concentration of the labelled oestrogen in the uterus (Conney, 1967; Levin, Welch & Conney, 1967). The stimulatory effect of chronic phenobarbitone administration on oestradiol metabolism was shown to result in an inhibition of the oestradiol-induced increase in uterine wet weight and in the incorporation of [^{14}C]glycine into uterine protein (Levin & others, 1967). We now report the effects of phenobarbitone treatment on oestrogen-induced increases in the activity of uterine phosphofructokinase.

Mature female Wistar rats, 180–200 g when killed, were ovariectomized bilaterally under light pentobarbitone anaesthesia. Two weeks later the following groups of ovariectomized rats were used: (1) control rats injected with saline solution; (2) animals injected with phenobarbitone; (3,4,5) rats administered oestradiol-17β in doses of 2·5, 5·0 or 10·0 μg/100 g respectively; (6,7,8) animals injected intraperitoneally with phenobarbitone (37·0 mg/kg) twice daily for 3 days before the administration of 2·5, 5·0 or 10·0 μg/100 g of

TABLE 1. THE EFFECT OF PHENOBARBITONE PRETREATMENT ON OESTROGEN-INDUCED ENZYME SYNTHESIS IN UTERI OF OVARIECTOMIZED RATS

Treatment	Uterine weight (mg)		Phosphofructokinase activity	
Control	110 ± 0 (100)		6·5 ± 0·1 (100)	
Phenobarbitone	126 ± 7 (115)		5·9 ± 0·1 (90)	
	Oestrogen-injected rats			
	Without phenobarbitone	With phenobarbitone	Without phenobarbitone	With phenobarbitone
Oestradiol-17β				
2·5 μg/100 g	220 ± 14 (200)*	158 ± 5 (143)*†	19·0 ± 0·5 (292)*	7·9 ± 0·3 (122)†
5·0 μg/100 g	219 ± 14 (199)*	160 ± 1 (146)*†	20·6 ± 1·2 (317)*	10·2 ± 0·4 (150)*†
10·0 μg/100 g	293 ± 47 (207)*	222 ± 17 (201)*	26·0 ± 0·5 (400)*	12·7 ± 0·9 (196)*†

Each value for PFK represents the mean ± s.e. based on 3 determinations of enzyme activity in uteri pooled from 2–3 rats. Rats were treated with 37 mg/kg of phenobarbitone intraperitoneally, twice daily for 3 days. Various doses of oestradiol-17β were administered intramuscularly to different groups of rats 16 hr before death. Enzyme activity is calculated as μmoles of alkali-labile phosphate formed per g of tissue per hr at 37°C × fresh weight of the tissue. Figures are in percentages taking the values of control rats as 100%.

* Statistically significant difference compared to the values of control rats ($P = <0{\cdot}05$).

† Statistically significant difference compared to the corresponding group of oestradiol-injected rats without phenobarbitone treatment ($P = <0{\cdot}05$).

oestradiol. All animals receiving oestradiol were injected 16 hr before death. Uteri were excised, cleaned of all adhering tissue and weighed rapidly on a Roller Smith torsion balance. The uteri were pooled, finely minced with scissors and 5% homogenates were then prepared in isotonic KCl solution of pH 7·4. The supernatant fluid was obtained and phosphofructokinase activity assayed under linear kinetic conditions as described previously (Lea & Walker, 1965; Weber & Singhal, 1965). Enzyme activity was calculated as μmoles of alkali-labile phosphate formed per hr per g of tissue at 37° times the weight of the organ (Singhal, 1967; Singhal & others, 1967). Statistical significance was calculated by Student's *t* test; a P value $<0{\cdot}05$ was considered significant.

Table 1 summarizes the effect of phenobarbitone administration on oestrogen-induced changes in uterine wet weight and phosphofructokinase activity. Treatment with phenobarbitone alone was without any significant effect on either of these two parameters, since values obtained after its administration were similar to those of saline-injected control rats. Uterine wet weights were increased to 200, 199 and 267% of the controls by 2·5, 5·0 and 10·0 μg/100 g of oestradiol respectively. In contrast, rats pretreated with phenobarbitone, but receiving the above doses of oestradiol, had increased uterine weights of only 143, 146 and 201%. Uterine phosphofructokinase activity was increased to 292, 317 and 400% of the control group in rats treated with 2·5, 5·0 and 10·0 μg/100 g of oestradiol-17β. Pretreatment with phenobarbitone blocked almost completely the oestradiol-induced enzyme increase by the 2·5 μg/100 g dose. However, the increases in phosphofructokinase activity induced by 5·0 and 10·0 μg/100 g of oestradiol were inhibited partially by phenobarbitone, and enzyme activity in these latter groups of animals increased to only 150 and 196% respectively.

Conney & others have shown that treatment of animals with phenobarbitone for several days increased the activity of liver microsomal enzymes which hydroxylate oestrogens (Kuntzman, Jacobson & others, 1964; Conney, Schneidman & others, 1965) and glucocorticoids (Conney & Schneidman, 1964; Conney, Jacobson & others, 1965). An earlier report from this laboratory has shown that pentobarbitone effectively prevented triamcinolone-induced increases in the activities of hepatic glucose 6-phosphatase and fructose 1,6-diphosphatase (Singhal & Ling, 1966). It is likely that this inhibition of glucocorticoid-induced liver gluconeogenic enzyme synthesis by pentobarbitone and the interference with oestrogen-induced biochemical responses by phenobarbitone described here may both be due to the stimulation of microsomal hydroxylases necessary for the inactivation of steroid hormones.

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Haemorrhagic, traumatic and tourniquet shock in the rat

SIR,—Gecse, Karady & West reported in 1964 that one colony of Wistar rats (termed non-reactors) was genetically more resistant to tourniquet and traumatic shock than were other colonies (termed reactors). We have now considered whether these differences can be accounted for by differences in the reactivity of their plasma kinin systems, especially as bradykinin is known to be involved in some forms of shock (Rocha e Silva & Antonio, 1960; Brocklehurst & Lahiri, 1962).

Groups of 10 non-reactor Wistar rats, weighing 150–200 g, obtained from the Agricultural Research Council's Field Station at Compton, and groups of 10 reactor Wistar rats from Fison's Ltd., Holmes Chapel, were subjected to haemorrhagic shock by the withdrawal of 15 ml blood/kg, traumatic shock (Noble & Collip, 1942), or tourniquet shock (Wilson & Roome, 1936). At different times after these procedures, the circulating levels of free kinin, kininogen, kininase and kinin-forming enzymes were measured (Dawson, Starr & West, 1966). Plasma kininogen level was the only parameter to show consistent changes and these occurred within 10 min of each type of shock; for example, the levels of the kinin precursor in reactor rats increased about three-fold but these were not sustained and returned to control values by 30–60 min. These changes in kininogen are similar to those reported by Diniz & Carvalho (1963) during haemorrhagic shock in the dog. Non-reactor rats showed similar changes in haemorrhagic and traumatic shock (25 min at 40 rev/min) but not in tourniquet shock (4 hr duration) where the kininogen levels were not raised during the experimental period. The plasma kinin systems in liver, heart, lung and small intestine were also unchanged after each type of shock in both types of rat.

Rats dying after severe shock always showed intestinal haemorrhage and experiments were therefore made to study kinin release into the peritoneal cavity, where it may arise from activation of its precursor by the action of kinin-releasing enzymes originating from pro-enzymes in the stagnating blood or from stores in the walls of the intestine. Immediately after subjecting other groups of rats to the different shock procedures, therefore, the peritoneal cavity of each rat was washed with 5 ml of 0·9% (w/v) saline and the washings were assayed for kinin-like activity. Whereas at all times after haemorrhagic shock, the bradykinin levels in the peritoneal fluid did not increase above the basal values (about 10 ng), the levels after the other two types of shock increased, the extent depending upon the intensity of the shock applied (Table 1).

TABLE 1. EFFECT OF TOURNIQUET SHOCK AND TRAUMATIC SHOCK ON THE AMOUNT OF BRADYKININ (ng) DETECTED IN THE PERITONEAL FLUID AND ON THE MORTALITY RATE (%) OF RATS OVER 24 HR

Type of shock	Conditions of shock	Bradykinin		Mortality rate	
		R	NR	R	NR
Tourniquet	2 hr	12	10	45	0
	3 hr	17*	10	100	45
	4 hr	40*	24	100	85
	6 hr	73*	32*	100	100
Traumatic	10 min	75	28	0	0
	15 min	101	65	0	0
	25 min	31*	35*	25	10
	40 min	40*	41*	100	75

R = Reactor animals. NR = Non-reactor animals.
* These samples also contained significant amounts of histamine.

Kinin levels in reactor animals were nearly always higher after tourniquet and traumatic shock than those in non-reactor rats, and when the mortality rates were recorded over 24 hr non-reactor rats were found to be more resistant. Histamine also occurred in the peritoneal fluid in relatively large amounts (over 50 ng per rat) when the stimulus was large enough to be lethal in 24 hr, but only small amounts of 5-hydroxytryptamine (about 10 ng) were present in all samples.

No differences were found between the plasma kinin systems *in vitro* of reactor and non-reactor rats and it may be that kinins are only of secondary importance in the shock states studied. But the high kininase activity of the blood, liver, lung and intestine may account for the rapid disappearance of the free peptide. The ease with which kinin release is achieved, especially in rat plasma (Jacobsen & Waaler, 1966), indicates that when conditions are favourable (as in congested intestine), secondary kinin formation may occur and this then aggravates the existing state of shock.

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Development of resistance to the lowering of plasma free fatty acids induced by pyrazole derivatives

SIR,—Several pyrazole derivatives, including 3,5-dimethylpyrazole (Gerritsen & Dulin, 1965a; Bizzi, Jori & others, 1964), 5-carboxy-3-methylpyrazole (Gerritsen & Dulin, 1965b) and 5-carboxamide 3-methylpyrazole (5CA3MP) (Bizzi, Codegoni & Garattini, 1967) are powerful inhibitors of free fatty acid (FFA) mobilization from adipose tissue. However, the blockade of lipolysis is frequently followed by an increase of plasma FFA above the normal level (Bizzi, 1966). Repeated daily treatments with 3,5-dimethylpyrazole or 5CA3MP tend to decrease the duration of the fall of plasma FFA. The onset of the resistance is, however, shortened if treatment with 5CA3MP in several doses far exceeding the amount necessary to produce a full effect are followed by a dose sufficient to give this effect.

In the experiments reported in Table 1 two groups of male Sprague Dawley rats, of 150 g average body weight, received saline or 5CA3MP, 50 mg/kg orally, twice daily for 3 days. The day after, a dose of 5CA3MP (7·5 mg/kg, orally) was markedly effective in the control group and completely ineffective in the treated group. When 5CA3MP was ineffective on plasma FFA, it did not show any capacity to lower plasma and liver triglycerides.

One of the possible reasons for explaining the onset of resistance relates to the observation that adipose tissue of animals pretreated with 5CA3MP shows *in vitro* a supersensitivity to lipolytic agents such as noradrenaline, ACTH or

TABLE 1. RESISTANCE TO THE LIPOLYTIC INHIBITOR EFFECT EXERTED BY 5-CARBOXAMIDE-3-METHYLPYRAZOLE (5CA3MP). Rats received saline or 5CA3MP for 3 days twice a day. On the 4th day they received saline or 5CA3MP 7·5 mg/kg orally

Treatment mg/kg orally		Plasma			Liver	Adipose tissue
from the 1st to the third day (twice a day)	4th day	FFA** μ-equiv./litre	Glycerol μM/litre	Triglycerides mg/100 ml	triglycerides mg/100 ml	FFA μ-equiv./g
Saline	Saline	613 ± 27	153 ± 7	55 ± 3	419 ± 30	5·0 ± 0·2
,,	5CA3MP 7·5 (15)*	199 ± 20	61 ± 4	36 ± 3	257 ± 59	3·5 ± 0·1
,,	,, (30)	214 ± 14	64 ± 3	42 ± 3	439 ± 72	2·9 ± 0·1
,,	,, (60)	135 ± 7	54 ± 7	36 ± 2	359 ± 17	2·9 ± 0·1
,,	,, (120)	214 ± 8	50 ± 2	29 ± 3	224 ± 34	2·9 ± 0·1
5CA3MP	Saline	728 ± 94	183 ± 2	77 ± 7	804 ± 71	5·8 ± 0·1
,,	5CA3MP 7·5 (15)	749 ± 18	169 ± 8	64 ± 11	927 ± 132	7·4 ± 0·6
,,	,, (30)	654 ± 82	140 ± 12	68 ± 12	1061 ± 356	5·8 ± 0·5
,,	,, (60)	664 ± 41	131 ± 6	58 ± 5	717 ± 106	5·8 ± 0·2
,,	,, (120)	721 ± 89	141 ± 10	55 ± 8	1140 ± 193	6·1 ± 0·4

* The time (min) between the last dose and killing the animals is in parentheses.
** Plasma FFA determinations were made according to Trout, Estes & Friedberg (1960) with minor modifications; glycerol according to Wieland (1957) and triglycerides according to Van Handel & Zilversmit with minor modifications (1957).

TABLE 2. EFFECT OF LIPOLYTIC AGENTS ON ADIPOSE TISSUE OBTAINED FROM NORMAL and 5-CARBOXAMIDE-3-METHYLPYRAZOLE (5CA3MP) RESISTANT RATS

Additions μg/ml	FFA μ-equiv./g/hr		Glycerol μM/g/hr	
	controls	5CA3MP	controls	5CA3MP
—	8·6 ± 0·3	8·99 ± 0·3	3·57 ± 0·4	3·8 ± 0·3
Noradrenaline[1] 0·25	21·6 ± 0·6	30·9 ± 1·0	8·4 ± 0·2	13·6 ± 0·5
Theophylline[2] 75	32·0 ± 0·9	37·0 ± 0·8	12·2 ± 0·4	15·0 ± 0·7
ACTH[3] 0·05	23·0 ± 1·0	32·0 ± 1·0	9·2 ± 0·6	14·0 ± 0·7

200 ± 10 mg of pooled and minced epididymal adipose tissue were incubated in 4 ml of Krebs Ringer bicarbonate medium pH 7·4, containing albumine 3%. Preincubation time 30 min at room temperature. Incubation time 60 min at 37° in air.
[1] As bitartrate monohydrate. [2] Monohydrate. [3] Synacthen.

theophylline (see Table 2). Since these agents are known to act by increasing the level of 3′,5′-cyclic AMP in the adipose tissue although not by the same means (Butcher, 1966), it may be possible that an elevation of 3,5-cyclic AMP in the adipose tissue is the cause of the resistance.

One of us (A.B.) has found that 3′,5′-cyclic AMP (dibutyryl ester) also antagonizes *in vitro* the effect of 5CA3MP and other pyrazole derivatives.

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Interaction of aspirin with urea in water

SIR,—Previously it has been shown that urea increases the aqueous solubility of benzoic and salicylic acids (Bolton, 1963). As an extension of this, we have observed the effect of urea on aspirin solubility and stability.

Excess aspirin in water was shaken at 30° in the presence of varying amounts of urea for 5 hr. Clear aliquots were then analysed spectrophotometrically for aspirin content (Bolton, 1960). At pH 2·0, hydrochloric acid was used as a buffer and, at pH 3·5, formic acid-sodium formate. The pH of all solutions was carefully checked before and after equilibration.

Kinetic studies were made at pH values of 2·0, 2·5, 2·75, 3·0 and 3·5 at 30° $\pm 0{\cdot}2°$. Formate buffers were used at pH values above 2·0 and hydrochloric acid was the buffer at pH 2·0.

The effect of urea on aspirin solubility at pH 2·0 and 3·5 is shown in Fig. 1. Although the increased solubility observed may be due to other than complexing effects, e.g. solvent effects, the solubility curve may well be described by two constants, K_1 and K_2, corresponding to the formation of 1:1 and 2:1 urea-aspirin complex species.

K_1 and K_2 can be determined graphically (Higuchi & Bolton, 1959). The values of the constants are in Table 1.

It is surprising that the values of K_1 and K_2 for the unionized and ionized complexes are of the same order of magnitude. However the calculations involved approximations as well as the neglect of other factors which may be responsible for the solubilization. The stronger solubilization of the unionized species is to be expected because of the weak basic nature of the urea molecule.

The first order rate constants from the kinetic studies are in Table 2.

Urea increases the rate of hydrolysis below pH 2·75 and decreases the rate at pH values greater than 2·75. It is interesting to note that this "crossover"

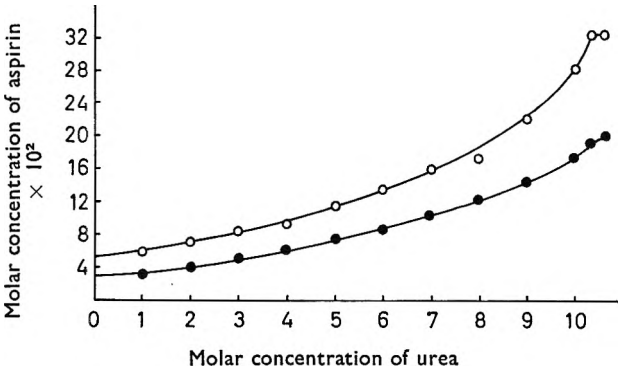

FIG. 1. Effect of urea on aspirin solubility in water at 3·0°. ○, pH 3·5; ●, pH 2·0.

TABLE 1. APPARENT EQUILIBRIUM CONSTANTS FOR UNIONIZED ASPIRIN-UREA AND IONIZED ASPIRIN-UREA INTERACTIONS

Complex	K
Unionized aspirin urea (1:1)	0·186
,, ,, ,, (1:2)	0·188
Ionized aspirin urea (1:1)	0·072
,, ,, ,, (1:2)	0·114

TABLE 2. RATE CONSTANTS FOR HYDROLYSIS OF ASPIRIN IN THE PRESENCE OF UREA AT 30° ($k = hr^{-1} \times 10^{-3}$)

Urea concentration M	pH				
	2·0	2·5	2·75	3·0	3·5
0	3·2	4·8	6·4	8·8	14·5
2	3·6	4·8	5·6	7·9	13·3
4	3·7	5·2	5·6	7·0	11·9
8	4·1	6·0	5·8	6·6	10·0

occurs at a pH corresponding to the pH of maximum stability as reported by Edwards (Edwards, 1950). This pH may thus represent a point where the hydrolysis mechanism changes, and could provide an explanation for the change in the effect of urea. Since the sites and mechanism of the interaction are not defined, any presentation of possible reasons for this effect on the basis of the present data would be highly speculative.

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Influence of cannabis, tetrahydrocannabinol and pyrahexyl on the linguomandibular reflex of the dog

SIR,—Reflex jaw opening to faradic stimulation of oral structures, particularly the tongue, was described by Cardot & Laugier (1922) among others. As the central pathway of this reflex includes the trigeminal system (Harrison & Corbin, 1942) which, in the rabbit, is sensitive to cannabis, tetrahydrocannabinol and pyrahexyl (Valle, Souza & Hyppolito, 1966), the influence of these agents upon the dog jaw jerk reflex was investigated.

Adult mongrel dogs of either sex weighing between 4·5 and 15 kg, were anaesthetized with pentobarbitone sodium (30 mg/kg i.p.). Jaw opening, arterial blood pressure and either respiration or foot movements after stimulation of the peroneus nerve, were registered on smoked kymographic paper. A glass cannula in the carotid or femoral artery was connected to a mercury manometer through rubber tubing filled with an 8% sodium citrate solution. Openings of the mandible and dorsal flexions of the hindpaw were recorded by means of a pneumatic device consisting of a large tambour as receptor and an adjustable tambour provided with a stylus. Respiration was recorded through a rubber pneumograph around the chest and connected to a tambour. The cut, central end of the lingual nerve at the level of the salivary duct was stimulated by monophasic shocks of 0·5 to 5 V, 2 msec duration at a frequency of 12/min. Drying of the nerve was prevented by immersing it in mineral oil. Water insoluble drug preparations were injected through a catheter inserted into a femoral vein as a fine suspension in polysorbate 80 plus saline or, preferably, as an emulsion in homologous blood plasma. The polysorbate control solution, even if slowly injected, produced a decrease of the arterial blood pressure accompanied by a slight improvement of the jaw jerk reflectivity. In practice it was only after tachyphylaxis to this vehicle had developed that the analysis of the drug preparations was made.

Cannabis crude resin (5 mg/kg), a fraction obtained from it by chromatography on alumina column (0·2–0·4 mg/kg), tetrahydrocannabinol (THC, 0·5 mg/kg) or pyrahexyl (1 mg/kg), all induced a prolonged fall of the arterial blood

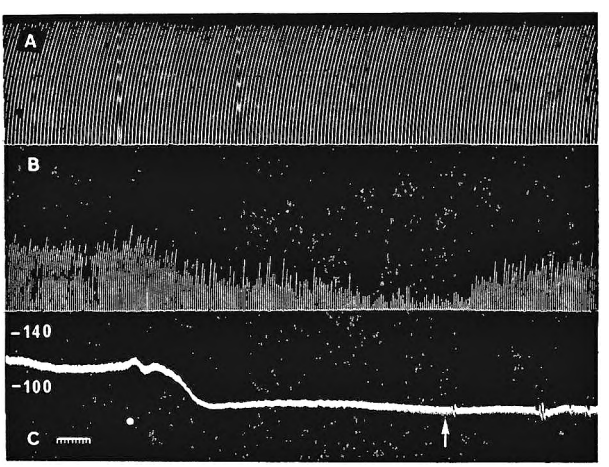

FIG. 1. Dog, 9 kg. Simultaneous recordings of dorsal flexion of the hindpaw (A), of mandibular opening (B), and of carotid blood pressure (C) in mm Hg. The linguomandibular reflex before complete abolition by cannabis crude resin was enhanced by strychnine. At (●) cannabis 5 mg/kg; at (↑) strychnine 50 μg/kg. Time marker: 10 sec.

TABLE 1. CHANGES (MEAN AND LIMITS %) OF DOG JAW-JERK REFLEX AFTER TETRAHYDROCANNABINOL (THC)*

Agent	Number of animals	Time after intravenous injection (min)					
		5	10	15	20	25	30
Vehicle	8	97·8 (125·7–64·7)	104·6 (121·7–93·7)	114·4 (153·2–83·7)	106·4 (139·9–82·2)	96·8 (118·5–79·8)	95·3 (118·7–59·4)
0·1 mg/kg THC	3	92·6 (125·3–64·7)	107·7 (123·9–91·0)	104·4 (117·8–58·5)	83·0 (149·2–80·4)	70·3 (87·6–50·7)	75·1 (118·6–38·7)
0·2 ,, ,,	4	82·9 (106·1–52·4)	57·8 (79·4–32·5)	38·7 (58·9–18·7)	29·9 (53·1–5·8)	24·0 (40·5–2·0)	19·3 (35·0–0·4)
0·4 ,, ,,	4	61·0 (67·8–51·5)	35·2 (53·5–25·7)	19·6 (38·8–6·3)	9·0 (25·2–0·9)	5·2 (18·1–0·0)	4·2 (16·4–0·0)

* Mean amplitude in mm of jaw openings, during 20 min before injection, taken as 100%.

pressure, depressed the respiration, and decreased or abolished the linguomandibular reflex. No impairment of neuromuscular transmission was seen (Fig. 1). These results were observed even after atropine (0·1 mg/kg) or pyrilamine maleate (5 mg/kg). Chlorpromazine in saline, taken as a reference compound, provoked the same effects as THC at a comparable dose level. Mephenesin (20 mg/kg) also decreased the mandibular reflex without influencing the hindpaw flexions. It showed a low potency compared with that of THC.

A transient return of the reflex was seen by increasing the electric stimuli or through intravenous injection of strychnine (0·05–0·1 mg/kg).

The prolonged effect on the blood pressure and the jaw reflectivity precluded, as a rule, repeated injections of the active preparations in the same animal. This inconvenience was surmounted by injecting a single dose of 0·1, 0·2 or 0·4 mg/kg of THC in each dog of a group of at least 3 animals. Our results are in Table 1.

One may argue whether the decrease or disappearance of the mandibular response would really mean a direct action of cannabis derivatives upon the central nervous structures related to the reflex. The similarity of the action to that of chlorpromazine (0·5 mg/kg), with its depression of respiration and a prolonged fall of the blood pressure, the similar muscular relaxation to that induced by mephenesin (20 mg/kg), with increased depth of anaesthesia, and also, the return of the reflex after strychnine may be taken as signs of a central action of cannabis, THC and pyrahexyl. However, since chlorpromazine, besides having a central action, also blocks nerve conduction, as for example, shown with the afferent fibres to the spinal cord of the cat (Xavier & Timo-Iaria, 1964), the possibility must be entertained that those agents could derange the jaw jerk reflexly, either through a blocking action on a presynaptic level or directly via the synaptic transmission.

Eventual interaction of these agents with brain catecholamines was also investigated. No conclusive results upon the reflex were obtained after injecting adrenaline, noradrenaline and isoprenaline (1 μg/kg). Disappearance of the reflex still occurred, although delayed in some instances, when the animals were treated with reserpine (0·5 mg/kg s.c.) 24 hr previously.

Two synthetic derivatives of THC were studied by Dagirmanjian & Boyd (1962) who found dimethylheptylpyran (DMHP, 0·1 mg/kg) and octylmethylpyran (0·2–0·4 mg/kg) to abolish the cat linguomandibular reflex. We have also made some experiments on cats and noted that under our conditions no major difference between the two species was deduced about the level of doses of THC necessary to extinguish the jaw jerk reflex. If we admit that dogs and cats have comparable sensitivity of the linguomandibular reflex to these agents,

then the relation of potency of THC and DMHP would not differ much from unity. This is interesting because, according to Adams, Harfenist & Loewe (1949), by the approximation method on dogs, DMHP would have 70 times the potency of natural THC. Comparative assays with DMHP and THC in our experimental conditions are needed to check this.

In conclusion, abolition of the dog linguomandibular reflex as well as that of the rabbit corneo-palpebral reflex after cannabis, THC or pyrahexyl seem to indicate a marked depressant action of these agents upon the trigeminal nuclei or related structures, or both, through an unknown mechanism.

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The influence of 5-hydroxytryptamine on the actions of adrenaline

SIR,—Intravenous injection of adrenaline produces acute pulmonary oedema in several species of laboratory animals (Visscher, Haddy and Stephens, 1956). It has recently been shown that the simultaneous injection of 5-hydroxytryptamine (5-HT) and adrenaline in the rabbit and in the mouse reduces the intensity of the pulmonary oedema and significantly lowers the mortality rate (Uppal, Sen & Sanyal, 1967). We have now examined the effect of the simultaneous administration of 5-HT and adrenaline on the blood pressure of the rabbit and the dog, on the frog perfused heart, on the rabbit isolated ileum and on the blood sugar level of the rabbit.

The actions of adrenaline and 5-HT were additive except on the blood sugar level of the rabbit. Here the injections of adrenaline (1 mg per animal) caused a rise in the blood sugar level of 88–270 mg/100 ml, over fasting levels. Similar injections of 5-HT caused a rise of 5–25 mg/100 ml, only. When both the substances were administered together, the rise in the blood sugar level was 8–30 mg/100 ml.

The mechanism of the blockade by 5-HT of the hyperglycaemia induced by adrenaline is obscure. It has been suggested that a specific blocking action may be involved in interactions of 5-HT and catecholamines (Gyermek, 1961).

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Hexamethonium potentiation of noradrenaline-induced contractions in the dilatator pupillae of the cat

SIR,—The dilatator pupillae has hitherto been thought to be supplied by adrenergic nerves only. Selective denervations and staining methods, however, have now conclusively proved that cholinergic, parasympathetic fibres form a well-developed network in the dilatator of mice (Ehinger & Sporrong, 1966), rats (Ehinger & Falck, 1965, 1966; Ehinger & Sporrong, 1967) and cats (Ehinger, 1967). The acetylcholinesterase-containing nerve fibres to the dilatator of a number of other species (Laties & Jacobowitz, 1964, 1966; Lukáš, 1964; Ehinger, 1966) most probably also represent a cholinergic, parasympathetic supply. In rats and mice, it has further been shown that the adrenergic and cholinergic fibres run closely intertwined in the vegetative nerve network (Ehinger & Falck, 1965, 1966; Ehinger & Sporrong, 1967). In a recent investigation of the function of these cholinergic nerve fibres of the dilatator (Ehinger, Falck & Persson, 1967) it was found that atropine potentiates contractions induced by noradrenaline or high frequency (50 c/sec) electrical stimulation in the cat dilatator. The phenomenon could readily be explained on the basis of the newly-detected cholinergic fibres, which relax the muscle. As the cholinergic and adrenergic fibres presumably run very close to each other and as structures of synaptic character between nerve fibres have been observed in electron microscopical work on the colon (Hagen, 1966; Dr. E. van der Zypen, personal communication), and moreover, as no ganglia occur in the cat iris, it was of interest to study the effect of the pure ganglionic inhibitor (see Nádor, 1960) hexamethonium. Cat iris dilatators were mounted and tested in an organ bath as described previously (Ehinger & others, 1967). The noradrenaline concentration was usually 5 μg/ml. The electrical stimulation was 50 c/sec, 1–3 V. The fact that this stimulation produces no contractions in sympathetically denervated muscle shows that it acts via the adrenergic nerves (Schaeppi & Koella, 1964; Ehinger & others, 1967). The contractions induced were always markedly submaximal.

As is seen from Tables 1 and 2, hexamethonium (10 μg/ml) enhanced the noradrenaline-induced contractions of the dilatator, but not contractions resulting from the electrical stimulation. Selective parasympathectomy (Ehinger, 1967) abolished the enhancing effect of hexamethonium.

TABLE 1. EFFECT OF HEXAMETHONIUM (10 μg/ml) ON THE RESPONSE TO STIMULATION WITH NORADRENALINE

Normal			Ciliary ganglionectomy		
Contraction force, mg		Difference %	Contraction force, mg		Difference %
Before drug	After drug		Before drug	After drug	
32	47	+47	41	44	+ 7
24	41	+71	58	40	−31
18	38	+111	10	9	−10
24	34	+42	26	25	− 4
23	28	+22	21	20	− 5
34	64	+88	—	—	—
22	28	+27	—	—	Mean − 8·6
13	25	+92	—	—	s.e.m. ± 6·3
15	16	+ 7	—	—	—
41	58	+41	—	—	—
28	32	+14	—	—	—
—	—	Mean +51·0	—	—	—
—	—	s.e.m. ±10·4	—	—	—
Significance of difference from zero		P < 0·001			Not significant

Significance of difference between normal and denervated muscle: P < 0·01.

TABLE 2. EFFECT OF HEXAMETHONIUM (10 μg/ml) ON THE RESPONSE TO ELECTRICAL STIMULATION

Normal muscles		
Contraction force, mg		
Before drug	After drug	Difference %
22	24	+ 9
23	28	+ 8
28	29	+ 4
31	33	+ 7
58	58	0
38	26	−32
52	37	−29
16	21	+31
16	21	+31
35	42	+20
60	54	−10
37	36	− 3
26	28	+ 8
64	66	+ 3
44	43	− 2
46	44	− 4
54	48	−11
		Mean + 1·8
		s.e.m. ± 4·2

Hexamethonium is known to enhance the response of blood vessels to adrenaline and noradrenaline (see Hilton, 1962; Vitolina & Melzobs, 1964). It has been claimed that the effect is not a result of changes on the adrenergic receptor in the muscle (Vitolina & Melzobs, 1964). This is supported by the failure to record any enhancement of electrically-induced contractions; such an enhancement could be expected if the receptors had become sensitized to the release of noradrenaline from the nerve terminals. Hilton (1962) suggested that the effect was due to a shift in the position of the dose-response curve. Such a shift could well be due to the disappearance of an inhibitory system. In the dilatator, the cholinergic parasympathetic fibres have an inhibitory function (Ehinger & others, 1967) and it seems probable that hexamethonium decreases the function of these fibres. This could be effected either by lowering the transmitter release from the cholinergic fibres, or by lowering their sensitivity to stimulation with noradrenaline. There are reasons for presuming that cholinergic neurons possess receptors sensitive to noradrenaline both at the perikarya (see review by Norberg & Sjöqvist, 1966) and at the terminals (Leaders, 1963). If the effect should be due to a decrease in transmitter release, the response to electrical stimulation could be expected to be potentiated in the same way as the response to noradrenaline. This was not so; therefore it seems possible that hexamethonium exerts its effect by decreasing the sensitivity of the nerve fibre to stimulation with noradrenaline.

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Effect of tyramine on the atrium and the papillary muscle of the immunosympathectomized rat

SIR,—Burn & Rand (1958) proposed that tyramine exerted its sympathomimetic effects through the release of endogenous noradrenaline. This hypothesis is now generally accepted (Trendelenburg, 1963; Muscholl, 1966), although evidence for a direct action of tyramine has also been reported (Luduena, 1963; Varma & Benfey, 1963; Varma, Gillis & Benfey, 1964; Zaimis, 1965; Krzanowski & Woodbury, 1966). Most experiments on the mode of action of tyramine have been made after depleting noradrenaline stores by reserpine or by surgical denervation. Since immunosympathectomy can produce almost complete destruction of the peripheral sympathetic nervous system (Levi-Montalcini & Angeletti, 1962; Zaimis, 1965; Iversen, Glowinski & Axelrod, 1966), we examined the effect of tyramine on the myocardium of immunosympathectomized rats.

Immunosympathectomy was produced by subcutaneous injection of 0·2 ml of 61,000 anti-units/ml of bovine anti-serum to nerve-growth factor (kindly supplied by Dr. R. K. Richards, Abbot Laboratories, Chicago) in Sprague-Dawley rats within 24 hr of birth. The effectiveness of this treatment in producing immunosympathectomy was described by Iversen & others (1966) and confirmed by us (Varma, 1967) and also during the present experiments. Rats were used approximately 3 months after birth. A group of normal and immunosympathectomized rats were also injected subcutaneously with 1 mg/kg of reserpine one day before the experiment. Each rat was killed by a blow on the head and the heart rapidly excised. Atria were removed, freed of ventricular tissue and set up in a 100 ml organ bath containing Krebs-Henseleit solution at 37° and aerated with a mixture of oxygen 95% and carbon dioxide 5%. Spontaneous contractions were recorded by a Grass force-displacement transducer on a Gilson polygraph. Tension on the atria was adjusted to give maximum contraction. This was approximately 0·5 g. Papillary muscle was removed from the left ventricle and set up in a separate 100 ml organ bath under identical conditions. The muscle was stimulated by square wave pulses of 5 msec duration at 1 c/sec and supramaximal voltage. A Tektronix stimulator was used. Both preparations were allowed to stabilize for at least 1 hr during which period the bath fluid was changed several times. Cumulative concentration-response curves to tyramine were determined. Preparations were then washed repeatedly for 1 hr after which cumulative concentration-response curves to noradrenaline were determined. Initial concentration of tyramine hydrochloride was 0·01 μg/ml and that of noradrenaline bitartrate monohydrate 0·001 μg/ml. Concentrations were increased by a factor of about 3 and the next highest concentration was

added after the effect of the preceding concentration had reached a plateau. Significance of the difference between the responses of the experimental and control preparations was calculated according to Dunnett's procedure (Dunnett, 1955). Significance of difference between two means was tested by Student's *t* test. Doses of tyramine and noradrenaline refer to the salts used.

Control atrial rates of normal rats and reserpine-treated rats were 231 and 200 beats/min, respectively. This difference was not significant. The control rates of the atria of immunosympathectomized rats and immunosympathectomized reserpine-treated rats were 234 and 180 beats/min, respectively. This difference was significant ($P > 0.01$). There was no significant difference in the control contractile force of the atria and the papillary muscles in these four groups. The inotropic and the chronotropic effects of tyramine and noradrenaline are presented in Fig. 1. The chronotropic effect of tyramine on the atria of immunosympathectomized rats was not significantly different from that on the atria of control rats. However, the chronotropic effect of tyramine on the atria of reserpine-treated rats was significantly reduced ($P > 0.05$). Both reserpine and immunosympathectomy produced significant reduction in the positive inotropic effect of tyramine on the atria and the papillary muscles ($P > 0.01$). A reduction in the inotropic effect of tyramine on the atria of immunosympathectomized rats has been reported earlier (Varma, 1967). The chronotropic effects of noradrenaline were generally greater in preparations from reserpine-treated or immunosympathectomized animals but the differences were not significant.

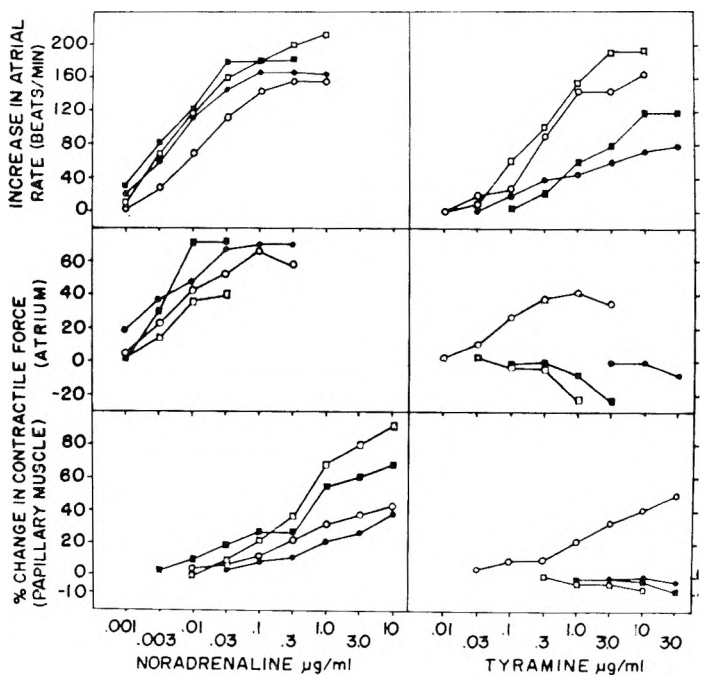

FIG. 1. Response of the isolated atria and papillary muscle of rats to noradrenaline and tyramine. ○—controls; ●—reserpine treated; □—immunosympathectomized; ■—immunosympathectomized + reserpine treated. Reserpine, 1 mg/kg, was injected (subcutaneously) one day before the experiment. Three to 6 animals in each group.

These experiments clearly show that depletion of myocardial noradrenaline by immunosympathectomy does not reduce the effect of tyramine on the atrial pacemaker. Zaimis (1965) reported that immunosympathectomy produced marked depletion of cardiac noradrenaline but did not inhibit the responses to tyramine and concluded that tyramine has a direct sympathomimetic action. Our results support this conclusion. It is interesting to note that although the chronotropic effect of tyramine is not inhibited by immunosympathectomy, the inotropic effect is significantly reduced. It seems that the inotropic and chronotropic effects are governed by different processes. It is not unlikely that these two effects are produced by activation of different receptors.

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Protamine-induced hypocalcaemia in rats

SIR,—During a clinical trial of the antitumour agent, Prolothan G (an aqueous solution of protamine standardized to contain nitrogen 2·5% w/v with dextrose 40%), some patients developed tetany and almost all became hypocalcaemic (Anderson, Tomlinson & Wright, 1967). The known neutralizing effect of protamine sulphate on heparin was therefore suspected. Heparin enhances the action of parathyroid hormone on bone resorption *in vitro* (Goldhaber, 1965) and may cause osteoporosis in man (Griffith, Nichols & others, 1965). We have now examined the effect of Prolothan G and compared it with clupeine sulphate and thyrocalcitonin in rats.

Male albino Wistar rats, 150 g, were anaesthetized with ether and a polythene cannula was inserted in the right femoral vein. Solutions were infused over an 80 min period at a rate of 0·5 ml/hr. Blood samples were taken from the tail vein (Sandiford, 1965) before, and at 20 min intervals throughout the infusion. The plasma calcium concentration was measured in 0·05 ml of plasma (MacIntyre, 1957) with the Optica CF4 spectrophotometer and flame attachment. The solutions of Prolothan G (Duncan, Flockhart & Evans), clupeine sulphate (B.D.H.) and thyrocalcitonin (M.R.C. calcitonin standard A) were prepared in physiologically normal dextrose saline. The protein estimation of the solutions was by the method of Lowry, Rosebrough & others (1951). Four animals were used for each infusion of Prolothan G, clupeine sulphate, thyrocalcitonin and dextrose saline.

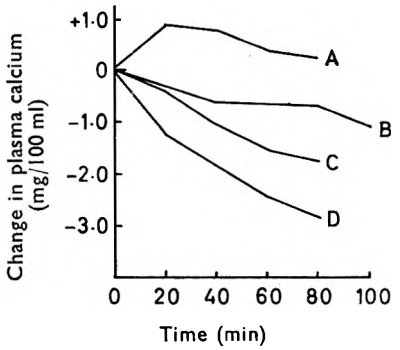

FIG. 1. The effect of intravenous infusion of dextrose-saline (A), Prolothan G (B) (1·4 mg protein), clupeine sulphate (C) (1·8 mg protein) and thyrocalcitonin (D) (0·4 mg protein) on the fall of plasma calcium concentration in young rats.

The infusion of each of the three test solutions produced hypocalcaemia whereas the infusion of dextrose saline occasioned a rise in plasma calcium (Fig. 1).

The hypocalcaemia produced by the protamines (Prolothan G and clupeine sulphate) does not seem to be merely binding of the ionic calcium to the protamine, because incubation of plasma with Prolothan G at 37° for 3 hr *in vitro* did not affect the total plasma calcium concentration on subsequent determination.

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Contents

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