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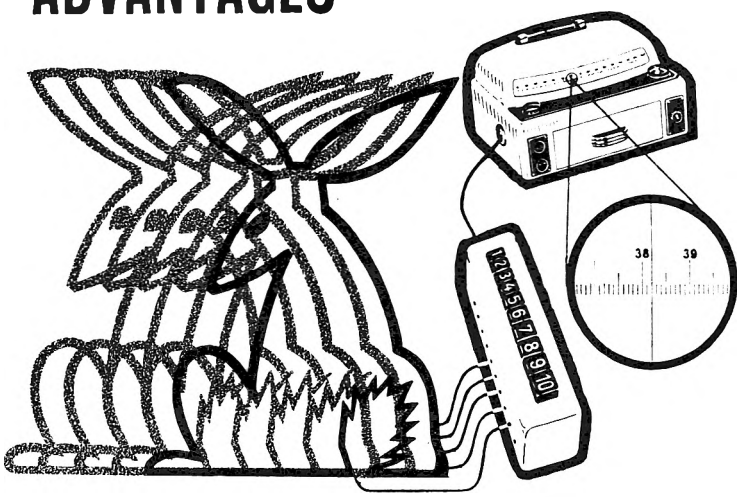

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RESEARCH PAPERS

PHYSICO-CHEMICAL EXPERIMENTS WITH PHOSPHATIDYL ETHANOLAMINE SOLS

By D. C. Robins and I. L. Thomas

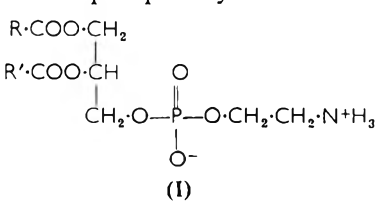
From the Welsh School of Pharmacy, The Welsh College of Advanced Technology, Cardiff

Received August 10, 1962

The solubilities of phosphatidylethanolamine (PE) in some organic solvents have been determined. The results of surface tension studies of aqueous sols of PE show that it has marked surface-active properties and surface ageing occurs. The effect of pH, concentration, mono- and divalent salts on surface activity has been investigated, and discussed in the light of previous theories. The critical micelle concentration is in the range 0·002 to 0·01 per cent w/v. The isoelectric point is at pH 3·1. The effect of mono- and divalent salts on the stability of PE sols has been studied.

Phosphatidylethanolamine is a member of the group of phosphatides called cephalins, which occur in nearly all living cells. The phosphatides form an important part of cell walls (Danielli and Stein, 1956), and a knowledge of their physical chemistry will assist in elucidating the structure of cell membranes and the physico-chemical aspects of drug action.

The structure of naturally occurring PE is shown by I which is the zwitterionic structure of L-α-phosphatidylethanolamine.



(I)

Where R and R′ are fatty acid chains

The fatty acid chains are of variable length and degree of unsaturation. The fatty acids attached to the α-carbon of the glycerol moiety contain mainly 16 or 18 carbon atoms and are predominantly saturated. The fatty acids attached to the β-carbon atom contain between 18 and 22 carbon atoms and are predominantly unsaturated.

The molecule is amphipathic since it contains long non-polar hydrocarbon chains which are lipophilic and a polar phosphate-ethanolamine grouping which is hydrophilic. It might be expected therefore, to be surface-active and its surface activity at the air-water interface has been studied.

Experimental and Results

Preparation of Phosphatidylethanolamine

The yolks of 60 eggs were separated and extracted repeatedly with acetone until a white powder was obtained. The powder was then

extracted with 5 litres (2 × 2 × 1 litre quantities) of absolute ethanol at 55°. The ethanolic solution was evaporated to dryness under vacuum and the residue dissolved in the minimum quantity of ether and reprecipitated by pouring the solution into 3 litres of acetone. The mixture of crude phosphatides obtained (105 g.) was passed down a cellulose column (4 cm. in diameter), containing 100 g. of cellulose, using a mixture of chloroform : methanol : water (800 : 200 : 25 v/v) as eluent. This procedure was necessary to remove amino-acids (Lea and Rhodes, 1953). The mixed phosphatides were then subjected to a chromatographic separation

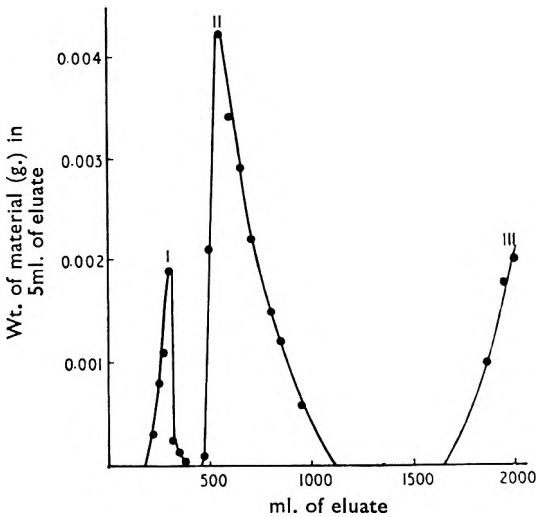


FIG. 1. The separation of mixed egg phospholipids on silicic acid. I, Yellow pigment. II, Phosphatidylethanolamine fraction. III, Lecithin fraction (commencement).

using silicic acid as the stationary phase and a mixture of chloroform: methanol (4 : 1 v/v) as eluent. A column 7·5 cm. in diameter was used into which 350 g. of silicic acid (Mallinkrodt 100 mesh) was slurried and allowed to pack under a slight pressure of nitrogen (2 lb./in.^{2}). The loading of the column was 14 g. No Celite was required as a filter aid because, with that pressure of nitrogen applied to the column, the flow-rate was satisfactory. The phosphatidylethanolamine travelled as a well-defined band immediately behind the pigment band and was well separated from the lecithin fraction (see Fig. 1). The yield of PE was 3·3 g.

Amino-containing compounds were identified by Lea and Rhodes' (1954) modification of the ninhydrin method of Moore and Stein (1948). Choline-containing compounds were identified by Lea, Rhodes and Stoll's modification (1955) of the phosphomolybdic acid test of Chargaff Levine and Green (1948).

The phosphatidylethanolamine was purified by dissolving in the minimum quantity of methyl ethyl ketone at room temperature and then adding acetone and warming the solution until the precipitate would just

dissolve at 50°. The solution was then allowed to stand at 0°, when the PE crystallised out. This recrystallisation procedure was repeated four times. The product was a very pale buff powder (yield, 3 g.). The compound had the following properties: iodine value = 75; N:P ratio = 1:1·02. The product was dissolved in chloroform, and stored under nitrogen at −20° in a desiccator.

Solubility Studies

The solubility of PE in some organic solvents was determined at 25° and 45° (Table I). Excess solute was placed in 25 ml. quickfit tubes together with 5 ml. of the solvent and the tubes shaken at an elevated temperature for 1 hr. and then allowed to cool to the required temperature. The tubes were then allowed to stand in a thermostat (±0·1°) for 8 hr. to reach

TABLE I

SOLUBILITY OF PE IN SOME ORGANIC SOLVENTS

Solvent	Solubility in g./100 ml. solution	
	at 25° C.	at 45° C.
Diethyl ether	18·88	—
Ethanol	5·05	6·06
Methanol	3·87	5·26
Methyl ethyl ketone	20·60	—
Acetone	0·43	1·06
Chloroform	36·23	—

equilibrium. 2 ml. of the supernatant solution was removed and evaporated to dryness overnight under vacuum at 50°. The residue was then weighed.

All organic solvents used were of Analar quality and were redistilled before use.

Surface Tension Studies

Preparation of aqueous sols. Aqueous sols could not be obtained by direct solution of PE in water, so the solute was dissolved initially in 5 ml. of ether. Successive small quantities (2 ml.) of distilled water were added with intermittent shaking. The sol became progressively thicker until it became a gel, but on further addition of water, it became fluid again. The ether was removed by gentle warming and finally by bubbling nitrogen through the sol. The sol was then passed down an ion-exchange column containing a mixture of 1·5 g. of Amberlite IR 120(H) and 1·3 g. of Amberlite IR 45(OH) resins to remove traces of electrolytes. The resins were washed with small successive quantities of distilled water and the sol was finally made up to volume.

It was found during preliminary studies that 5 per cent of the PE was lost on the resins, so a corresponding adjustment was made to the final volume of the sol. A 0·01 per cent w/v sol had a specific resistance of 100,000 ohm cm.

On preparing a number of sols of PE of the same concentration, small variations in the equilibrium values of the surface tension were observed,

because of inherent errors in the method of preparation. To overcome these, in a series of experiments, a large volume of sol was prepared and portions of this used as required.

Apparatus for surface tension measurements. A static method (Wilhelmy plate) was used for measuring the surface tension of the sols. The method involved using a chainomatic balance, reading to 10^{-4} g., as described by Harkins and Jordan (1930). It stood on a platform which could be raised or lowered by a screw thread mechanism so that zero contact angle could be obtained between the plate and the surface of the

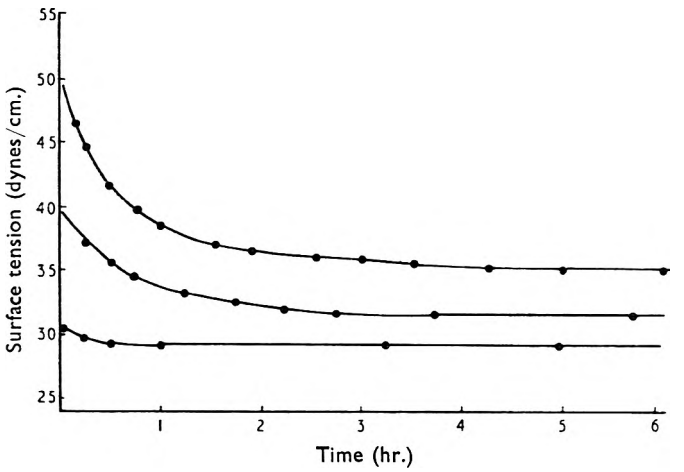


FIG. 2. Variation of surface tension of phosphatidylethanolamine sols with time. Upper curve, 0·005 per cent. Middle curve, 0·0075 per cent. Lower curve, 0·01 per cent.

sol. The platinum plate was suspended from one arm of the balance by a thin chrome-nickel wire, which had a levelling device on it to ensure that the plate hung horizontally to the surface of the sol. The sol was in a large Pyrex glass beaker immersed in a thermostat (±0·05°) which was fitted with a lid to ensure that the atmosphere above the sol was saturated with water vapour. The wire on which the platinum plate hung, passed through a small hole in the lid.

Variation of surface tension of PE *sols with time.* The surface tensions of a series of sols of PE were measured over a period of time. The results (see Fig. 2) show an ageing effect. There was an initial rapid fall of the surface tension with time, then the rate of the fall gradually decreased until an equilibrium value was reached. As the concentration of the sol increased, the time taken to reach equilibrium was reduced.

Effect of concentration of PE *on the surface tension of aqueous sols.* Since the surface tension varied with time, it was evident that sufficient time was needed for an equilibrium value to be reached. Hence, the surface tension was studied for 6 hr. for each concentration and the equilibrium values obtained plotted against concentrations (see Fig. 3).

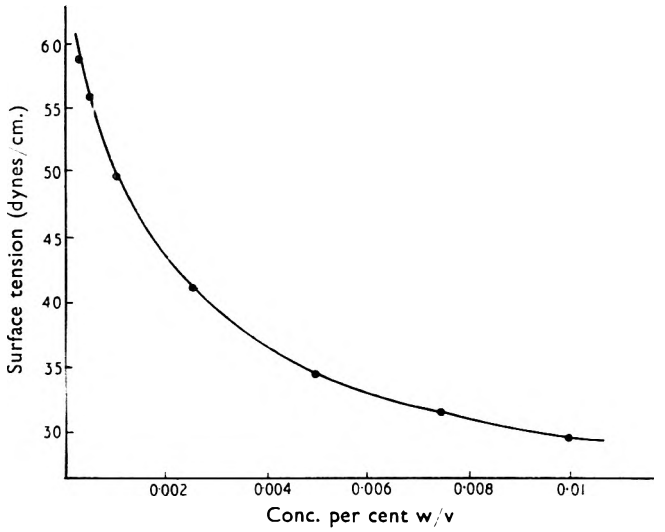


FIG. 3. Variation of surface tension of phosphatidylethanolamine sols with concentration.

Effect of pH on the surface tension of PE *sols.* The surface tensions of PE sols were investigated over the pH range of 1·65 to 6·1 which should include the isoelectric point of PE. The pH values were obtained using a Dynacap pH meter after the addition of small volumes of hydrochloric acid to the sols. The surface ageing effect was still present even in acid solution (see Table II). The equilibrium values obtained for the surface tensions at various pH values of the sols are also given in Table II.

TABLE II

VARIATION OF THE SURFACE AGEING EFFECT WITH pH (0·005 PER CENT W/V PE)

pH	Time taken to reach equilibrium value (in hr.)	Equilibrium value of S.T.
6·1	6	38·10
5·7	5	35·57
4·9	4¼	32·55
4·0	2¼	28·50
3·23	¼	27·28
2·60	¾	27·00
1·75	2¼	28·40

Effect of monovalent and divalent cations on the surface tensions of PE *sols.* The effects of potassium chloride and calcium chloride on the surface tension of the sols were investigated. The equilibrium values, and the time taken to reach them are given in Table III.

No visible precipitation was seen during the investigation in those sols containing sufficient electrolyte to cause precipitation in 24 hr.

Precipitation Studies

1 ml. of sol containing either 0·04 or 0·02 per cent of PE was placed in each of a series of sample tubes. Varying quantities of salt solution were

added to the tubes by means of an Agla micrometer syringe and the volume in each tube was adjusted so that the final concentration of phosphatide in each tube was either 0·036 per cent or 0·018 per cent. The effect of potassium and calcium chlorides on the stability of the sols was investigated and the results are given in Table IV.

TABLE III

EFFECT OF SALTS ON THE SURFACE TENSION OF A 0·005 PER CENT PE SOL

Molar conc. of potassium chloride	S.T. at equilibrium	Time taken to reach equilibrium value (in hr.)
—	33·0	7
5×10^{-5}	36·2	5
1×10^{-4}	37·30	6
5×10^{-4}	36·64	$4\frac{3}{4}$
1×10^{-3}	36·70	5
5×10^{-3}	35·10	$4\frac{1}{4}$
1×10^{-2}	28·8	3
Molar conc. of calcium chloride		
1×10^{-5}	32·1	$1\frac{1}{2}$
5×10^{-6}	32·1	2
1×10^{-4}	32·5	$3\frac{1}{2}$
5×10^{-4}	31·3	2
1×10^{-3}	30·8	$1\frac{1}{2}$
5×10^{-3}	28·9	$\frac{1}{2}$
1×10^{-2}	28·4	$\frac{1}{2}$

TABLE IV

EFFECT OF SALTS ON STABILITY OF PE SOLS

Potassium chloride moles/litre	0·036 per cent PE	0·018 per cent PE
$2{\cdot}5 \times 10^{-1}$	+ + −	+ − +
$2{\cdot}0 \times 10^{-1}$	− − −	+ − −
$1{\cdot}5 \times 10^{-1}$	−	−
$1{\cdot}0 \times 10^{-1}$	−	−
$1{\cdot}0 \times 10^{-2}$	−	−
$1{\cdot}0 \times 10^{-3}$	−	−
Calcium chloride moles/litre		
$5{\cdot}0 \times 10^{-1}$	− − −	− − −
$5{\cdot}0 \times 10^{-2}$	+ − +	− + +
$5{\cdot}0 \times 10^{-3}$	+ − −	− − −
$6{\cdot}0 \times 10^{-4}$	+ − +	+ + +
$4{\cdot}0 \times 10^{-4}$	+ +	− +
$2{\cdot}0 \times 10^{-4}$	−	−
$1{\cdot}0 \times 10^{-4}$	−	−
$1{\cdot}0 \times 10^{-6}$	−	−
$1{\cdot}0 \times 10^{-7}$	−	−
$1{\cdot}0 \times 10^{-11}$	−	−
Control	−	−

+ + + = heavy precipitate
+ = faint precipitate
− = no precipitate

DISCUSSION

Although the cephalin group of phosphatides predominates in brain tissue (Chargaff, Ziff and Rittenberg, 1942) it was not used as the source of PE because other members of the cephalin group are present in high proportions and are not easy to separate. Instead, egg-yolk was employed, where the cephalin fraction, despite being in small concentration compared with the lecithin fraction, is almost pure PE.

Hawke (1959) has reported that, using a chromatographic column containing 50 g. of silicic acid and a loading of phosphatides of 30 mg. of phosphorus for 25 g. of adsorbent, a separation of PE and lecithin was obtained. When he tried to scale up the preparation using 200 g. of adsorbent and the same loading ratio no separation of PE and lecithin was obtained. Since we required fairly large quantities of PE we scaled up the silicic acid chromatographic separation by following a suggestion made by Rhodes (personal communication) of increasing the diameter of the column rather than its length. By doing this we obtained about 3 g. of PE which was well separated from the lecithin.

We were unable to compare in detail the solubilities of PE and lecithin in various organic solvents since solubility figures for lecithin do not appear to be in the literature. However, lecithin is very soluble in ether, chloroform and methyl ethyl ketone, fairly soluble in ethanol and methanol, and slightly soluble in acetone. PE exhibits a similar solubility pattern.

The surface ageing of PE sols is an interesting phenomenon. Adam and Shute (1935), working with paraffin chain salts, noted this slow ageing

TABLE V

THE EFFECT OF TIME ON THE SURFACE TENSION OF A 0·005 PER CENT PE SOL

Time (in hr.)	Surface tension (in dynes/cm.)		
	1st day	2nd day	3rd day
1	42·7	38·5	40·3
2	37·4	36·2	37·6
3	36·2	35·8	36·6
4	35·5	35·2	35·8
5	35·1	34·9	35·5
6	35·0	34·8	35·3

The sol was agitated at the beginning of each day.

effect and also that as the concentration of the surface-active agent was increased, the time required to reach an equilibrium surface tension value was decreased. Above the critical micelle concentration these authors found an equilibrium value was attained rapidly. We have observed similar results with PE sols (see Fig. 2). This ageing phenomenon applies only to the surface and not to the solution itself, because when surface tension measurements were made on the same sol on different days after agitation, the changes in the surface tension with time were similar (see Table V).

There is at present no adequate theory to explain this slow surface ageing effect. It can not be explained by a simple diffusion theory, because the time taken for the surface tension to reach equilibrium is much longer than it would take molecules to diffuse from the bulk of the phase into the surface layer. Doss (1939), Nutting, Long and Harkins (1940) consider that the diffuse electrical double layer which is formed by the monolayer of the surface-active molecules in the surface, acts as an electrical barrier past which molecules must pass before they can be adsorbed. This view is incompatible with McBain and Perry's (1940) observation that the effect occurs in non-ionised systems. Alexander

(1941) also disagrees with the concept of an electrical barrier because he found that the ageing effect which occurs with hydrocinnamic acid, disappeared when the sodium salt was used. McBain and Perry (1939) think that in some cases the ageing effect may be due to the formation of two or more superimposed monolayers in the surface. Alexander (1941) thinks that the main factor determining the rate of adsorption in non-micellar solutions is the rate of penetration and reorientation of the molecules in the surface layer, possibly coupled with a dehydration of the hydrophobic portion of the molecule. Sutherland (1959) considered the effect to be due to the transport of very small amounts of electrolyte impurities to the surface.

There are, therefore, many possible explanations for the slow surface ageing of a solution. It is probable that the predominant factor controlling the rate of attainment of equilibrium in the surface is different for different surface-active agents.

Robinson and Saunders (1958), using a static method for measuring surface tension, have reported that a lysolecithin sol, the concentration of which was close to its critical micelle concentration, showed only a very small surface ageing effect. We found with lysophosphatidylethanolamine (LysoPE) sols, at a concentration just below the critical micelle concentration, that there was a long surface ageing effect which was even longer with PE sols. In the light of these facts we do not think that the ageing effect exhibited by PE sols can be explained by (*a*) the time taken for the molecules to diffuse into the surface, (*b*) the formation of multilayers at the surface, or (*c*) the presence of minute amounts of electrolytes, because if any of the above explanations were correct then lysolecithin would behave similarly. In our opinion one main factor causing the ageing is the existence of an electrical double layer at the surface. Evidence in support of this is firstly that the ageing effect is much greater with lysoPE, where the surface-active ion has a strong negative charge, than with lysolecithin, where the surface active ion has only a very weak negative charge. Secondly the surface tension studies on PE sols at various pH values have shown (see Table II) that as the charge on the molecule is reduced, so the surface ageing effect is reduced, reaching a minimum value at the iso-electric point. The second main factor involved is the time required for reorientation of the molecules in the surface. That PE sols take longer to reach an equilibrium value compared with lysoPE can be explained by the fact that the PE molecules are more bulky and a high proportion of the fatty acid chains present are unsaturated.

Many workers (Nutting, Long and Harkins, 1940; Hartley, 1936; Adams and Shute, 1938; Nutting and Long, 1941) have noticed that at or above the critical micelle concentration the surface ageing effect is very small or non-existent. No definite theory to explain this has yet been evolved, but some possible explanations are given here. Doss (1939) thinks that the electrical barrier which impedes the diffusion of ions to the surface would be reduced if the ionic strength of the solution were increased by the formation of micelles having a high charge. Adam and

Shute (1938) consider that micelles carrying a high charge may repel any similarly charged long chain ions so much more than such ions repel each other, that ions are driven to the surface more easily if micelles are present in the solution. Another suggestion they make is that the surface layer may be regarded as a kind of two-dimensional micelle, the conditions for its formation being much the same as those for the formation of micelles in the interior of the solution, so that it is formed most easily at similar concentrations. Nutting, Long and Harkins (1940) state that an ion in a micelle can get into the surface more easily than can a single ion because the rate of diffusion per ion is larger for the ions in a micelle than for simple ions. Also as the micelle approaches the surface it will encounter the diffuse region in which the gegenions of the surface-active ions in the surface are concentrated. The high charge density at the surface of the micelle will cause a large number of the gegenions to become attached to it. Consequently the net charge of the micelle will be reduced as it approaches the surface. Therefore, the electrostatic barrier to the approach of an ion in a micelle to the surface will be considerably less than for a simple ion. They have also suggested that the effect could be explained in terms of micelles having very low charge density (as postulated by McBain). The diffusion of such micelles would be only slightly affected by the electrical double layer.

Since there is no sharp break in the surface tension-concentration curve for PE sols (see Fig. 3) it is difficult to estimate the critical micelle concentration from the graph, but the greatest change of slope of the graph occurs at a concentration of about 0·002 per cent. However, since the surface ageing effect is very small when the concentration is 0·01 per cent we conclude that the critical micelle concentration is within the concentration range of 0·002–0·01 per cent.

The results given in Table II show that a minimum occurs in the surface tension-pH curve at a pH value of about 3·1. We conclude that this is the isoelectric point for PE, because when the resultant charge on the molecules is zero the packing of the molecules in the surface will be at an optimum and consequently the surface tension will be at a minimum.

It has been reported that the presence of electrolytes reduces the ageing effect. Generally it has been found that the higher the concentration of electrolyte the bigger the reduction. It seems that with PE sols, the ageing effect is dependent upon the equilibrium value of the surface tension rather than the electrolyte concentration. The lower the equilibrium surface tension value the shorter the ageing effect (see Table III). It is interesting to recall that this statement also applies to the sols containing hydrochloric acid (see Table II). It would seem that the greater the interaction between the added electrolyte and PE the less charged the PE molecules become and consequently they are able to pack more closely into the surface causing a lower equilibrium surface tension. The ageing effect should be shorter the higher the valency of the ions of the added electrolyte. We have found this to be so.

Calcium chloride causes a slight fall in the surface tension of the sols. Generally speaking as the concentration of calcium chloride is increased

so the surface tension is decreased. A possible explanation is that the higher the concentration the greater the interaction between the calcium ions and the PE molecules. It is possible that the calcium ion forms a linkage between the phosphate groups of two molecules of the phosphatide, causing them to pack more closely in the surface. Potassium chloride in concentrations of 5×10^{-3} M and below caused a rise of between 2 and 4 dynes/cm., whilst a concentration of 10^{-2} M caused a fall of about 4 dynes. We have no explanation to offer for these results.

In the flocculation studies it was found that concentrations of calcium chloride of 4×10^{-4} M and above caused flocculation, whilst with potassium chloride, concentrations of $1{\cdot}5 \times 10^{-1}$ M and above were required. It is interesting to note that PE sols do not exhibit a peptisation zone in the presence of divalent metal ions, as has been reported for lecithin by Malquori (1932), Rona and Deutsche (1926), Saunders and Elworthy (unpublished), and Thomas (1962).

REFERENCES

Adam, N. K. and Shute, H. (1935). *Trans. Farad. Soc.*, **31**, 204–205.
Adam, N. K. and Shute, H. (1941). *Ibid.*, **34**, 758–765.
Alexander, A. E. (1941). *Ibid.*, **37**, 15–25.
Chargaff, E., Ziff, M. and Rittenberg, D. (1942). *J. biol. Chem.*, **144**, 343–352.
Chargaff, E. Levine, C. and Green, C. (1948). *Ibid.*, **175**, 67–71.
Danielli, J. and Stein, W. (1956). *Disc. Farad. Soc.*, **21**, 238–251.
Doss, K. (1939). *Kolloid Z.*, **86**, 205–213.
Harkins, W. and Jordan, H. (1930). *J. Amer. chem. Soc.*, 1751–1772.
Hartley, G. (1936). *Aqueous Solutions of Paraffin-chain Salts*, Paris: Heimann & Cie.
Hawke, J. C. (1959). *Biochem. J.*, **71**, 588–592.
Lea, C. and Rhodes, D. N. (1953). *Ibid.*, **54**, 467–469.
Lea, C. and Rhodes, D. N. (1954). *Ibid.*, **56**, 613–618.
Lea, C. Rhodes, D. H. and Stoll, R. (1955). *Ibid.*, **60**, 353–363.
Malquori, G. (1932). *Atti IV Congr. naz. chim. pura applicata*, 752–753.
McBain, M. and Perry, L. (1939). *Industr. Engng Chem.* **31**, 35–39.
McBain, M. and Perry, L. (1940). *J. Amer. chem. Soc.*, **62**, 989–991.
Moore, S. and Stein, W. (1948). *J. biol. Chem.*, **176**, 367–388.
Nutting, C. Long, F. and Harkins, W. (1940). *J. Amer. chem. Soc.*, **62**, 1496–1504.
Nutting, C. and Long, F. (1941). *Ibid.*, **63**, 84–88.
Robinson, N. and Saunders, L. (1958). *J. Pharm. Pharmacol.*, **10**, 384–391.
Rona, D. and Deutsch, W. (1926). *Biochem. Z.*, **171**, 89–118.
Sutherland, K. (1959). *Australian J. Chem.*, **12**, 1–13.
Thomas, I. L. (1962). *J. Pharm. Pharmacol.*, **14**, 456–463.

THE PREPARATION AND MEASUREMENT OF THE SURFACE ACTIVITY OF A SERIES OF 4-ALKYL-1,1′-SPIROBIPIPERIDINIUM BROMIDES

By J. Thomas and D. Clough

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Received September 7, 1962

The preparation of a series of 4-alkyl-1,1′-spirobipiperidinium bromides is described. The measurement of the surface tension of aqueous solutions of these quaternary salts is reported. Within the concentration ranges studied none of the spiran quaternary compounds form micelles. The relationship between micelle formation of quaternary ammonium ions and antibacterial activity is discussed.

The general problems associated with structure-activity interpretations of the antibacterial actions of aqueous solutions of quaternary ammonium compounds can be divided into two main groups. The first is the factors involved in transferring a quaternary ammonium ion from the environment of the water molecules of the solution to a "receptor" area on the bacterial cell. The second is the factors involved in the mechanism by which death of the cell is brought about once the quaternary ammonium ions are adsorbed onto the "receptors". For an understanding of the first problem the nature of aqueous solutions of quaternary ammonium ions and the nature of, and factors involved in, the adsorption of these compounds onto an anionic type "receptor" require to be studied.

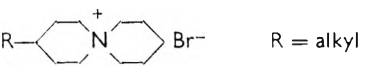
Quaternary ammonium compounds are not typical electrolytes in that the ions are not randomly distributed among the water molecules but are concentrated at interfaces and also tend to become associated in micelles. The formation of micelles is a limiting factor on the activity of quaternary ammonium salts because it limits the effective concentration of monomolecular form available for adsorption onto the bacterial "receptors" (Cella and others, 1952). The surface activity of quaternary ammonium ions is a useful property in their antibacterial actions in that higher concentrations of ions are present in the region of the bacterial "receptors" than in the bulk of the solution although it has been shown that surface activity *per se* is not necessarily accompanied by activity (Stacey and Webb, 1947). It is clear that the relationship between chemical structure and micelle formation of quaternary ammonium compounds in aqueous solution is an important part of the overall structure-activity relationships of the antibacterial action of these ions.

If micelle formation could be prevented or reduced, such bactericidal activity in an homologous series would move further along the series. Hartley (1941) showed that the critical micelle concentration of dialkyl ethers of dihydric phenols was greater than the critical micelle concentration of the monoalkyl compound containing the same number of carbon atoms. The bactericidal activities of dialkyldimethylammonium bromides have been compared with alkyltrimethylammonium bromides by Davis and others (1949). They found that in the twin-chain series beginning with dihexyldimethylammonium bromide and ending with

didodecyldimethylammonium bromide, bactericidal activity increased with increasing chain length, whereas with the single-chain compounds bactericidal activity reached a maximum when there were 18 carbon atoms in one chain. In the twin-chain series a total of 24 carbon atoms did not appear to be the limit. A limiting factor with the twin-chain compounds was their low solubility in water.

Thomas (1961 a, b) and Thomas and Starmer (1961) have used spiran quaternary compounds, in which the stereochemistry is "rigid" and known, to investigate the factors involved in the adsorption of quaternary ammonium ions onto anionic-type "receptors". Since the physicochemical properties of quaternary ammonium salts in solution play an important part in their antibacterial action, then data on the surface activity, critical micelle concentration and solubility of the spirans are needed for structure-activity studies. Because of the control over the shape of the ions which the spiran system allows it may be possible to use this type of structure for studying the relationship between critical micelle concentration, solubility and stereochemistry.

A series of 4-alkyl-1,1′-spirobipiperidinium bromides (I) has now been prepared and the surface tensions of their aqueous solutions determined.



R = alkyl

I

Experimental

Chemical

All melting points were determined on a Kofler block and are corrected.

Diethyl tetrahydropyran-4,4-dicarboxylate was prepared essentially by the method of Gibson and Johnson (1930) by condensing di(2-chloroethyl) ether (214 g., 1·5 mole) with diethyl malonate (520 g., 3·25 mole) in super-dry ethanol (1 litre) with sodium (69 g., 3 mole) dissolved in it. The product was isolated by fractionation under reduced pressure. Two fractions were collected: diethyl malonate b.p. 89° at 14 mm., yield 192 g., and diethyl tetrahydropyran-4,4-dicarboxylate b.p. 136° at 14 mm., yield 193 g. (56 per cent based on the di(2-chloroethyl) ether).

Tetrahydropyran-4,4-dicarboxylic acid was prepared by hydrolysis of diethyl tetrahydropyran-4,4-dicarboxylate (115 g., 0·5 mole) with potassium hydroxide (62 g., 1·1 mole) dissolved in ethanol (500 ml., 95 per cent). The free acid was isolated by diluting the mixture with water, removing the ethanol by distillation, acidifying the solution with hydrochloric acid and extracting with ether in a continuous extractor for 8 hr. Yield 83 g. (95 per cent).

Tetrahydropyran-4-carboxylic acid was prepared from tetrahydropyran-4,4-dicarboxylic acid (100 g.) by decarboxylation at 180°. The product was distilled under reduced pressure b.p. 152–154° at 20 mm., and crystallised to a white solid. Yield 73 g. (95 per cent). Recrystallised from ethyl methyl ketone m.p. 88°. Found: C, 55·2; H, 7·7. $C_6H_9O_3$ requires C, 55·4; H, 7·7 per cent.

Tetrahydropyran-4-carbonyl chloride. Tetrahydropyran-4-carboxylic acid (72 g.) was refluxed with thionyl chloride (120 ml.) for 1 hr. The product was isolated by distillation under reduced pressure b.p. 85° at 16 mm. Yield 80 g. (95 per cent).

Propyl tetrahydropyran-4-yl ketone. A Grignard reagent was prepared in the usual manner from propyl bromide (37 g., 0·3 mole) and transferred to a flanged flask (700 ml.) by connecting the two flasks with a glass tube and blowing the clear ethereal solution over with dry nitrogen. The flanged flask was fitted with a reflux condenser, dropping funnel and a mechanical stirrer. A Hinsberg-type stirrer made from nicrom wire

TABLE I

ALKYL TETRAHYDROPYRAN-4-YL KETONES AND THEIR 2,4-DINITROPHENYLHYDRAZONES

Alkyl radical	B.p. °C	Yield per cent	2,4-Dinitrophenylhydrazones				
			M.p. °C	Analysis (per cent)			
				Calculated		Found	
				C	H	C	H
Ethyl	100–102 at 11 mm.	60·2	148–149	52·17	5·59	51·9	5·5
Propyl	105–106 at 10 mm.	75·0	137–138	53·57	5·95	53·8	6·3
Butyl	124–125 at 15 mm.	69·0	98–99	54·85	6·28	54·9	6·4
Pentyl	130 at 10 mm.	66·0	84–85	56·04	6·59	56·3	6·8
Hexyl	146–148 at 10 mm.	53·0	61–62	57·15	6·88	56·9	7·0
Heptyl	162 at 15 mm.	56·0	61–62	58·16	7·14	57·9	7·2
Octyl	172–173 at 15 mm.	58·0	72–73	59·12	7·39	58·9	7·4
Nonyl	176–178 at 11 mm.	60·0	108	60·00	7·62	60·1	7·6
Decyl	192–193 at 13 mm.	56·0	74–75	60·82	7·83	60·5	8·1

fused to a glass rod was used to ensure efficient stirring and scraping of the vessel walls. Anhydrous cadmium chloride (28 g., 0·15 mole), which had previously been sifted through a No. 60 sieve and dried at 105° for 1 hr., was added with vigorous stirring and the mixture refluxed under nitrogen until a negative Gilman Grignard test was obtained. The ether was replaced by sodium-dried benzene (300 ml.), tetrahydropyran-4-carbonyl chloride (22·6 g., 1·6 mole) dissolved in sodium-dried benzene (100 ml.) was slowly introduced and the mixture refluxed for 30 min. with vigorous stirring to keep the insoluble material as a fine suspension in the benzene. The product was isolated by adding water followed by dilute hydrochloric acid and separating the benzene layer, which was washed in turn with water (50 ml.), sodium carbonate solution (50 ml. 10 per cent) and water (50 ml.) and then dried over anhydrous sodium sulphate. Distillation under reduced pressure gave 17·3 g. (75 per cent), b.p. 105–106° at 10 mm.

2,4-*Dinitrophenylhydrazone*, yellow plates from ethanol m.p. 137–138°. Found: C, 53·8; H, 6·3. $C_{15}H_{20}N_4O_5$ requires C, 53·6; H, 6·0 per cent. A list of n-alkyl tetrahydropyran-4-yl ketones and their 2,4-dinitrophenylhydrazone derivatives which were prepared is given in Table I.

4-*Butyltetrahydropyran.* Propyl tetrahydropyran-4-yl ketone (15 g.), potassium hydroxide (10 g.) and hydrazine hydrate (10 g.) were added to triethylene glycol (80 ml.) and the mixture heated on a steam-bath for 1 hr. A thermometer was then placed in the reaction mixture and the condenser set for distillation. The temperature of the mixture was raised to 190° and the distillate collected. The mixture was then maintained under reflux for 3 hr. and then allowed to cool. The previous distillate was added with water (100 ml.) and the mixture extracted with ether (3 × 50 ml.). The ethereal solution was dried over anhydrous

TABLE II

4-ALKYLTETRAHYDROPYRANS

Compound	Mol. formula	B.p. °C	Yield per cent
4-Propyltetrahydropyran	$C_8H_{16}O$	55–56 at 11 mm.	68·5
4-Butyltetrahydropyran	$C_9H_{18}O$	84–85 at 25 mm.	58·5
4-Pentyltetrahydropyran	$C_{10}H_{20}O$	83–84 at 15 mm.	50·0
4-Hexyltetrahydropyran	$C_{11}H_{22}O$	101–102 at 15 mm.	68·5
4-Heptyltetrahydropyran	$C_{12}H_{24}O$	122–124 at 13 mm.	64·5
4-Octyltetrahydropyran	$C_{13}H_{26}O$	130–132 at 10 mm.	57·0
4-Nonyltetrahydropyran	$C_{14}H_{28}O$	150–152 at 10 mm.	62·0
4-Decyltetrahydropyran	$C_{15}H_{30}O$	164–166 at 15 mm.	53·0
4-Undecyltetrahydropyran	$C_{16}H_{32}O$	178–180 at 13 mm.	67·0

calcium chloride and the product obtained by distillation under reduced pressure, b.p. 84–85° at 25 mm., yield 8·0 g. (58·5 per cent). A similar method was used to reduce a series of alkyl (ethyl to hexyl) tetrahydropyran-4-yl ketones but failed to reduce heptyl tetrahydropyran-4-yl ketone and higher homologues.

4-*Octyltetrahydropyran.* Heptyl tetrahydropyran-4-yl ketone (15 g.) and hydrazine hydrate (7 g.) were refluxed in absolute ethanol (100 ml.) for 1 hr. Triethylene glycol (80 ml.) and potassium hydroxide (10 g.) were then added and the ethanol removed. A thermometer was placed in the reaction mixture and the temperature raised to 190°, any distillate collected when the temperature was being raised from 120 to 190° being kept. The procedure was the same as that described for 4-butyltetrahydropyran from this point. 4-Octyltetrahydropyran b.p. 130–132° at 10 mm., yield 8·0 g. (57 per cent). A similar method was used to reduce heptyltetrahydropyran-4-yl ketone acid to nonyl tetrahydropyran-4-yl ketone but gave a low yield with the decyl homologue.

4-*n-Undecyltetrahydropyran* was prepared from decyl tetrahydropyran-4-yl ketone (10 g.) by the method described for 4-octyltetrahydropyran but substituting a mixture of benzene (10 ml.) and ethanol (90 ml.) for absolute ethanol in the first part of the reaction. 4-Undecyltetrahydropyran, b.p. 182–184° at 15 mm., yield 6·4 g. (68 per cent).

A list of 4-alkyltetrahydropyrans prepared is given in Table II.

1-*Bromo*-3-(2-*bromoethyl*)-*heptane*. 4-Butyltetrahydropyran (7 g.) was added to a mixture of hydrobromic acid (35 ml., 60 per cent solution) and concentrated sulphuric acid (2 ml.). The mixture was heated on a steam-bath for 5 hr. with occasional shaking. The cooled mixture was diluted with water (100 ml.), extracted with ether (2 × 50 ml.) and the ethereal extract washed in turn with water (50 ml.), sodium carbonate (50 ml., 10 per cent solution) and water (50 ml.) and then dried over anhydrous calcium chloride. The product was isolated by distillation under reduced pressure b.p. 148–150° at 13 mm., yield 9·8 g. (60 per cent).

Bisthiuronium dipicrate was prepared by heating the alkyl halide (0·5 g.) with thiourea (1 g.) in ethanol (20 ml.) for 20 min. on a steam-bath and then pouring the solution into an aqueous solution of picric acid (100 ml.

TABLE III

3-ALKYL-1,5-DIBROMOPENTANES AND THEIR BISTHIURONIUM DIPICRATES

n-Alkyl radical	B.p. °C	Yield per cent	Bisthiuronium dipicrate derivatives				
			M.p. °C	Analysis (per cent)			
				Calculated		Found	
				C	H	C	H
Propyl	147 at 17 mm.	64·0	135	36·66	3·89	36·5	3·8
Butyl	148 at 13 mm.	60·5	181	37·60	4·09	37·4	4·4
Pentyl	157 at 13 mm.	69·5	175	38·50	4·28	38·7	4·3
Hexyl	166 at 15 mm.	59·5	159	39·36	4·46	39·2	4·3
Heptyl	180 at 13 mm.	66·0	161	40·20	4·64	40·3	4·9
Octyl	188–190 at 10 mm.	42·0	148	41·00	4·81	40·9	5·1
Nonyl	203–205 at 16 mm.	78·0	107–108	41·80	4·97	42·1	5·3
Decyl	210 at 14 mm.	61·0	158	42·54	5·13	42·9	5·1

1 per cent). Recrystallisation of the precipitate twice from ethanol (50 per cent) gave yellow crystals m.p. 181°. Found: C, 37·4; H, 4·4. $C_{23}H_{30}N_{10}O_{14}S_2$ requires C, 37·6; H, 4·1 per cent.

A list of 3-alkyl-1,5-dibromopentanes and their bisthiuronium dipicrates prepared is given in Table III.

4-*Alkyl*-1,1′-*spirobipiperidinium bromides* were prepared by condensing 3-n-alkyl-1,5-dibromopentanes (1 mole) with piperidine (2 moles) by heating them under reflux in chloroform and then evaporating the solution to dryness. Sodium hydroxide (1 mole) solution was added and the solution again evaporated to dryness under reduced pressure on a steam-bath and the residue extracted with ethyl methyl ketone in a Soxhlet extractor, after which the product precipitated from the solvent. The salts were recrystallised from ethyl methyl ketone. A list of spiran quaternary ammonium compounds prepared is given in Table IV.

Surface Activity Measurements

The Wilhelmy plate method was used as it has been shown to be an accurate method for solutions of surface-active compounds (Padday and Russel, 1960).

Apparatus. A glass microscope cover slip was suspended from the beam of a torsion balance with wire. The balance was calibrated up to a 1,000 mg. in 2 mg. divisions and it could be adjusted so that when the blade was freely suspended in air it read zero. The width and thickness of blade were measured on the calibrated stage of a microscope. A 6 cm. crystallising dish was used to hold the solutions while measurements were taken.

Calibration of equipment. All the volumetric glassware used in the determinations were previously calibrated to N.P.L. class A standards.

Water (40 ml.) was pipetted into the clean dry basin and the height of the basin adjusted so that the blade did not touch the bottom when the torsion arm was free to move, but was not pulled out of the water when

TABLE IV

4-ALKYL-1,1′-SPIROBIPIPERIDINIUM BROMIDES

Alkyl radical	M.p.* °C	Analysis (per cent)					
		Calculated			Found		
		C	H	Br	C	H	Br
H	335–336	51·28	8·54	34·18	51·1	8·4	34·3
Propyl	287	56·50	9·42	29·00	56·35	9·7	28·9
Butyl	288	57·90	9·65	27·60	57·8	9·5	27·4
Pentyl	298	59·20	9·87	26·30	59·1	9·8	26·4
Hexyl	303	60·40	10·06	25·16	60·0	10·3	25·1
Heptyl	302	61·44	10·24	24·10	61·3	10·3	23·9
Octyl	300–301	62·43	10·40	23·12	62·5	10·4	23·0
Nonyl	298	63·33	10·54	22·22	63·2	10·4	22·1

* All the spiran quaternary compounds melt with decomposition and it was difficult to obtain consistent values. The following method was used to give reproducible values. A value was obtained by heating the block at 5°/min. The melting point of a new sample of salt was then obtained by starting from 5° below the first value and raising the temperature of the block at 2°/min. By this method it was possible to observe liquifaction before decomposition of the solid.

the torsion arm was restored to its zero position. The depth of the immersion of the blade at zero position was measured accurately. The basin was clamped in this position and provided that the same blade was used with 40 ml. of solution, the depth of immersion of the blade when the torsion arm was in its equilibrium position would be constant. Under these conditions the torsion balance reading is directly proportional to the surface tension of a solution of surface-active agent, assuming the variation in density with concentration is negligible over the range of concentrations studied.

Measurement of surface tension of pure liquids. The blade was cleaned in chromic acid, washed with distilled water and dried in an oven at 120°. Care was taken not to finger the blade at any time. The blade was suspended from the torsion arm and the balance adjusted to give a zero reading. Distilled water (40 ml.) was pipetted into the basin. From the weight now required to keep the torsion arm in its equilibrium position the surface tension of distilled water was calculated using the equation:

$$\text{S.T.} = \frac{g(W + \rho tah)}{2(t + a)}$$

g = acceleration due to gravity.
W = torsion balance reading in g.
ρ = density of liquid.
t = width of blade in cm. = 1·9 cm.
a = thickness of blade in cm. = 0·02 cm.
h = depth of immersion of blade in cm. = 0·3 cm.

The experiment was repeated substituting in turn Analar benzene and pyridine for the water. The results obtained are given below.

	W g.	g./ml.	Surface tension dynes/cm.	Literature values dynes/cm. (International Critical Tables 1928)
Water	0·272	0·997	72·14	72·75
Benzene	0·1	0·877	28·1	28·9
Pyridine	0·136	0·982	37·6	38·0

Measurement of surface tension of solutions of spiran quaternary compounds. An approximately 10^{-2} M solution of spiran quaternary compound was accurately prepared. The solution (40 ml.) was pipetted into the basin and the surface tension of the solution determined as

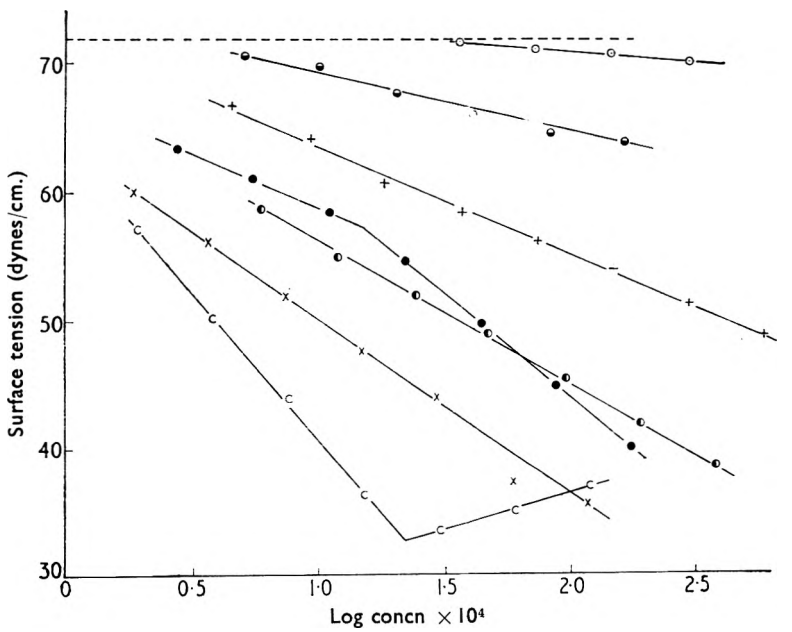


FIG. 1. Surface tension/concentration curves for 4-alkyl-1,1′-spirobipiperidinium bromides and cetrimide.

○ 1, 1′-Spirobipiperdinium bromide
◒ 4-Pentyl-1,1′-spirobipiperidinium bromide
– 4-Hexyl-1,1′-spirobipiperidinium bromide
◐ 4-Heptyl-1,1′-spirobipiperidinium bromide
× 4-Octyl-1,1′-spirobipiperidinium bromide
● 4-Nonyl-1,1′-spirobipiperidinium bromide
C Cetrimide
--- Distilled water

described above. The blade was then placed in chromic acid, washed with distilled water and dried at 120°. Some of the solution (20 ml.) was removed from the basin and distilled water (20 ml.) added using a clean pipette and the surface tension of this new solution taken after 2 min. This procedure was repeated several times so that a series of surface tension measurements at different concentrations was obtained. All the

TABLE V

SURFACE TENSION MEASUREMENTS OF 4-ALKYL-1,1′-SPIROBIPIPERIDINIUM BROMIDES AND CETRIMIDE. TEMP. 20°. DENSITY OF SOLUTIONS TAKEN TO BE 0·997 G./ML.

Alkyl radical	Conc. of soln. g. mole/litre	Torsion balance reading in mg.		Surface tension in dynes/cm. (mean)
		1st	2nd	
H	$2{\cdot}964 \times 10^{-2}$	264		70·4
	$1{\cdot}482 \times 10^{-2}$	266	266	70·9
	$7{\cdot}410 \times 10^{-3}$	268	267	71·2
	$3{\cdot}705 \times 10^{-3}$	270	269	71·8
Pentyl	$1{\cdot}650 \times 10^{-2}$	239		64·0
	$8{\cdot}250 \times 10^{-3}$	242	242	64·7
	$4{\cdot}125 \times 10^{-3}$	248	248	66·2
	$2{\cdot}062 \times 10^{-3}$	254	256	68·0
	$1{\cdot}031 \times 10^{-3}$	262	263	70·0
	$5{\cdot}155 \times 10^{-4}$	266	266	70·9
Hexyl	$5{\cdot}848 \times 10^{-2}$	180		48·9
	$2{\cdot}924 \times 10^{-2}$	190	190	51·4
	$1{\cdot}462 \times 10^{-2}$	204	202	54·2
	$7{\cdot}310 \times 10^{-3}$	210	208	56·3
	$3{\cdot}655 \times 10^{-3}$	219	218	58·7
	$1{\cdot}827 \times 10^{-3}$	226	228	60·9
	$9{\cdot}135 \times 10^{-4}$	240	240	64·2
	$4{\cdot}567 \times 10^{-4}$	250	250	66·8
Heptyl	$3{\cdot}804 \times 10^{-2}$	140		38·7
	$1{\cdot}902 \times 10^{-2}$	154	153	42·1
	$9{\cdot}510 \times 10^{-3}$	166	168	45·6
	$4{\cdot}755 \times 10^{-3}$	183	182	49·5
	$2{\cdot}377 \times 10^{-3}$	194	194	52·5
	$1{\cdot}188 \times 10^{-3}$	206	206	55·5
	$5{\cdot}940 \times 10^{-4}$	218	220	58·9
Octyl	$1{\cdot}177 \times 10^{-2}$	128		35·6
	$5{\cdot}885 \times 10^{-3}$	136	134	37·4
	$2{\cdot}942 \times 10^{-3}$	162	160	44·0
	$1{\cdot}471 \times 10^{-3}$	172	180	47·6
	$7{\cdot}355 \times 10^{-4}$	190	194	52·0
	$3{\cdot}677 \times 10^{-4}$	207	211	56·3
	$1{\cdot}838 \times 10^{-4}$	222	226	60·1
Nonyl	$1{\cdot}755 \times 10^{-2}$	146		40·2
	$8{\cdot}775 \times 10^{-3}$	165	165	45·1
	$4{\cdot}387 \times 10^{-3}$	186	182	49·9
	$2{\cdot}193 \times 10^{-3}$	204	202	54·7
	$1{\cdot}096 \times 10^{-3}$	219	217	58·6
	$5{\cdot}480 \times 10^{-4}$	228	228	61·2
	$2{\cdot}740 \times 10^{-4}$	236	238	63·5
Cetrimide	$1{\cdot}213 \times 10^{-2}$	134		37·1
	$6{\cdot}065 \times 10^{-3}$	124	128	35·1
	$3{\cdot}032 \times 10^{-3}$	121	120	33·6
	$1{\cdot}516 \times 10^{-3}$	132	130	36·4
	$7{\cdot}580 \times 10^{-4}$	162	160	44·0
	$3{\cdot}790 \times 10^{-4}$	187	184	50·3
	$1{\cdot}890 \times 10^{-4}$	214	211	57·2

glassware used was cleaned in chromic acid, washed with water and dried and then a duplicate series of results was obtained using the same original spiran quaternary solution. The temperature of the solution was 20°. After each series of determinations on a compound the cleanliness of the apparatus was checked by determining the surface tension of distilled water.

The surface tensions of solutions of the spiran quaternary compounds and cetrimide were determined in this manner. The results are given in Table V, and shown graphically in Fig. 1.

Discussion

The reactions used to prepare the 4-alkyl-1,1′-spirobipiperidinium bromides were standard ones but a few points of detail arose during the work which are worthy of mention. In the preparation of alkyl tetrahydropyran-4-yl ketones from the carbonyl chloride and alkyl cadmium salts, the stirring employed was found to have a marked influence on the yields. It is necessary to maintain the particulate matter as a fine suspension throughout the reaction with constant scraping of the walls of the flask. By using a Hinsberg stirrer and carrying out the reaction in a flanged flask yields were improved from the 35 to 40 per cent region up to 70 to 75 per cent. The replacement of ether by benzene as described by Cason and Prout (1944) and Casson (1946) was also advantageous.

All the standard methods of reducing the alkyl tetrahydropyran-4-yl ketones to the hydrocarbon were examined and the Huang-Minlon (1946) modification of the Wolff-Kishner reaction was found to be the most efficient. The method worked well up to the hexyl homologue but when used with the heptyl one the original ketone was recovered unchanged. This was clearly due to the hydrazone not being formed and it was found that this was due to the ketone being practically insoluble in the triethylene glycol. This was overcome by preparing the hydrazone in ethanol and then replacing this solvent with triethylene glycol for the subsequent stage of the reaction. This modification worked for the higher homologues, except decyl tetrahydropyran-4-yl ketone, for which only a 4 per cent yield was obtained. The reason for this low yield was that the hydrazone was insoluble in ethanol and triethylene glycol and co-distilled with the ethanol and condensed as a white solid in the condenser. It was found that the addition of a mixture of ethanol and benzene kept the hydrazone and triethylene glycol in one phase for a sufficient time to prevent co-distillation and a high yield of 4-decyltetrahydropyran resulted.

A number of methods of opening the tetrahydropyran ring were tried, including one reported by Kaluszyner and Galum (1961) in which hydrobromic acid was prepared *in situ* from sodium bromide and sulphuric acid. However, yields were not as high as desired. It was found that an important factor which governed yields was the degree of charring which occured and so the mildest conditions which would open the ring were used. A mixture of hydrobromic acid (60 per cent solution) and sulphuric acid on a steam-bath was found to produce yields of 60 per cent with little charring (Thomas, 1954).

The preparation of spiran quaternary ammonium compounds has been studied in detail by Thomas (1954, 1957). It was found that the most satisfactory method was to react a substituted 1,5-dibromopentane (1 mole) with a cyclic amine (2 moles) in a solvent such as chloroform. The reactions involved are shown in Fig. 2, from which it can be seen that there are two possible products formed either by an intramolecular cyclisation (*a*) or an intermolecular cyclisation (*b*). When the concentration of reactants is below 5 per cent of the total volume of reaction mixture an intramolecular cyclisation occurs giving the required spiran. Since, of the two possible products, one is a dimer of the other, elemental

analysis will not distinguish between them. Final proof of which type of compound was formed under conditions of high dilution was obtained by molecular weight determination (Thomas, 1957).

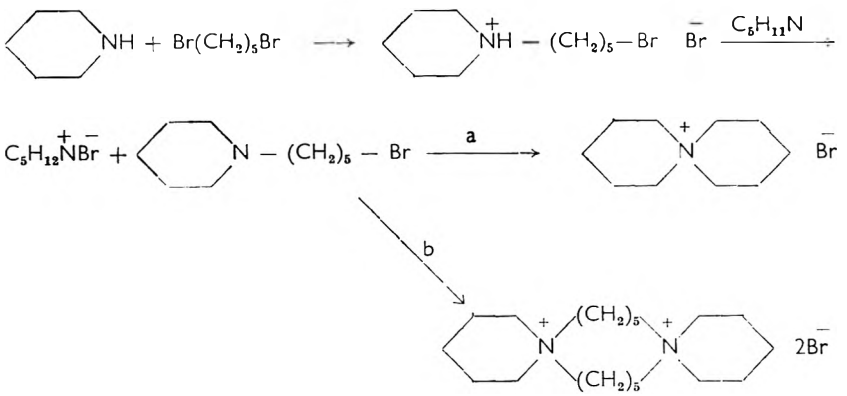
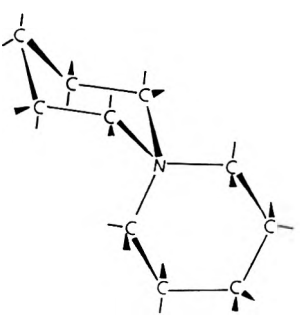


FIG. 2

The results of the surface activity measurements are given in Table V and Fig. 1. Over the concentration range studied there is a linear relationship between the log of concentration and surface tension for all the homologues except the nonyl compound. It is difficult to see why this compound was anomalous. None of the spiran quaternary compounds showed the characteristic break in its surface tension/concentration curve which occurs at the critical micelle concentration of surface-active agents. The curve for cetrimide is included for comparison. It is concluded that micelles are not formed by spiran quaternary compounds over the concentration range studied. The 1,1′-spirobipiperidinium ion consists of two piperidinium rings linked at right angles by a common nitrogen atom. Assuming that the two piperidinium rings are in the chair form, the structure of the 1,1′-spirobipiperidinium ion is as indicated in II. The fixed and "rigid" structure of the two piperidinium rings

II



results in a large bulk of the ion being situated around the positively charged nitrogen atom. It has been shown that increase in bulk around the nitrogen atom leads to an increase in the critical micelle concentration

(Cella and others, 1952), although increases in the critical micelle concentration of 4-n-alkyl-1,1′-spirobipiperidinium bromides were greater than were expected from the work of Cella. Increase in bulk around the nitrogen atom also leads to a decrease in solubility in long-chain quaternary ammonium compounds (Kuhn and Westphal, 1940; Niederl and Weingarten, 1941). This effect was seen with the spirans. The lower homologues were very soluble in water but their solubility in water at 20° fell rapidly as the series was ascended above the heptyl homologue. Hartley (1941) suggested that the lack of micelle formation could be the main factor causing low solubility and this idea is supported by the results obtained with the spiran quaternary compounds.

References

Cason, J. (1946). *J. Amer. chem. Soc.*, **68**, 2078–2081.
Cason, J. and Prout, F. S. (1944). *Ibid.*, **66**, 47–50.
Cella, J. A., Eggenberger, D. N., Noel, D. R., Harriman, L. A. and Harwood, H. J. (1952). *Ibia.*, **74**, 2061–2062.
Davis, J. G., Resuggan, J. C. L., Ive, R. and Wood, H. M. (1949). *J. gen. Microbiol.*, **3**, vii.
Gibson, C. S. and Johnson, J. D. A. (1930). *J. chem. Soc.*, 2525–2530.
Hartley, G. S. (1941). *Trans. Faraday Soc.*, **37**, 130–133.
Huang-Minlon (1946). *J. Amer. chem. Soc.*, **68**, 2487–2488.
International Critical Tables (1928). McGraw Hill Publications, New York. Vol. IV, pp. 446–463.
Kaluszyner, A. and Galum, A. B. (1961). *Bull. Research Council, Israel*, **10A**, 25.
Kuhn, R. and Westphal, O. (1940). *Ber.*, **73**, 1105–1108.
Niederl, J. B. and Weingarten, H. (1941). *J. Amer. chem. Soc.*, **63**, 3534–3535.
Padday, J. F. and Russell, D. R. (1960). *J. Colloid Sci.*, **15**, 503–511.
Stacey, M. and Webb, M. (1947). *Proc. roy. Soc.*, **134B**, 523–537.
Thomas, J. (1954). M.Sc. Thesis, Manchester University.
Thomas, J. (1957). Ph.D. Thesis, Manchester University.
Thomas, J. (1961a). *J. med. pharm. Chem.*, **3**, 45–51.
Thomas, J. (1961b). *Ibid.*, **3**, 309–321.
Thomas, J. and Starmer, G. A. (1961). *J. Pharm. Pharmacol.*, **13**, 752–758.

THE MODE OF ACTION OF TYRAMINE

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The repeated administration of tyramine to the isolated perfused guinea-pig heart gradually decreased the positive inotropic response which was paralleled by a decrease in the noradrenaline content of the heart and an increase in the noradrenaline content of the perfusate. Phenethylamine, ephedrine, amphetamine, guanethidine and bretylium were administered to the isolated heart until no further positive inotropic effect was obtained. The absence of an inotropic response with phenethylamine and guanethidine was associated with a decrease in the noradrenaline content of the heart. The absence of an inotropic effect with amphetamine, bretylium and ephedrine was not associated with a decrease in the amount of noradrenaline in the heart.

Burn and Rand (1958) proposed that tyramine exerts its sympathomimetic effect by effecting a release of noradrenaline from tissue stores, since postganglionic sympathetic denervation of a tissue greatly reduces its responsiveness to tyramine (Burn and Tainter, 1931; Burn, 1932; Fleckenstein and Burn, 1953). Prior treatment with reserpine reduces the pressor action of tyramine (Carlsson, Rosengren, Bertler and Nilsson, 1957) and also depletes endogenous stores of noradrenaline (Burn and Rand, 1957), and the pressor action of tyramine may be re-established, after treatment with reserpine, by infusion of noradrenaline (Burn and Rand, 1958).

Tyramine increases the concentration of adrenaline and noradrenaline in the aortic plasma of cats (Lockett and Eakins, 1960); and adrenalectomised dogs pretreated with phenoxybenzamine (Weiner, Draskoczy and Burack, 1962) and elevates the level of noradrenaline in venous effluents of cat spleen (Stjärne, 1961). Tyramine releases noradrenaline from isolated chromaffin and adrenergic neurone granules. (Schüman, 1960; von Euler and Lishajko, 1960). In addition tyramine causes the release of radioactivity from tissue equilibrated with (^{3}H)-noradrenaline (Hertting, Axelrod and Patrick 1961; Burn and Burn, 1961). Weiner (1962) and others observed that large subcutaneous doses of tyramine reduce the amount of noradrenaline in the heart, spleen and brown fat of the rat.

Experiments in this laboratory (Davey, Farmer and Reinert, 1962) have shown that the noradrenaline content of the isolated guinea-pig heart is markedly reduced if successive doses of tyramine are given until no positive inotropic effect is obtained. The object of the present work was to determine whether the noradrenaline content of the isolated perfused guinea-pig heart and its perfusate, as well as the magnitude of the positive inotropic effect, are related to the total amount of tyramine administered. Some observations have also been made with guanethidine, phenethylamine, amphetamine, bretylium and ephedrine. Since the completion of this work, Axelrod and his colleagues have published the observation that tyramine releases (^{3}H)-noradrenaline from isolated rat hearts previously perfused with (^{3}H)-noradrenaline and that with successive injections of

tyramine the amount of (^{3}H)-noradrenaline released is reduced progressively and there is a parallel decrease in the increment of amplitude and rate of contraction of the heart.

METHODS

Guinea-pig hearts were perfused by the method of Langendorff with a warm (36°) oxygenated (95 per cent O_2, 5 per cent CO_2) Krebs's solution which was of the following composition (g./litre NaCl, 7·1; KCl, 0·35; $CaCl_2$, 0·28; $MgSO_4 \cdot 7H_2O$, 0·28; $NaHCO_3$, 2·09; KH_2PC_4, 0·16; glucose 1·0); the perfusate was collected in containers immersed in ice; 100 μg. of the amine under investigation was injected into a cannula close to the heart every 5–10 min. until no positive inotropic effect was observed,

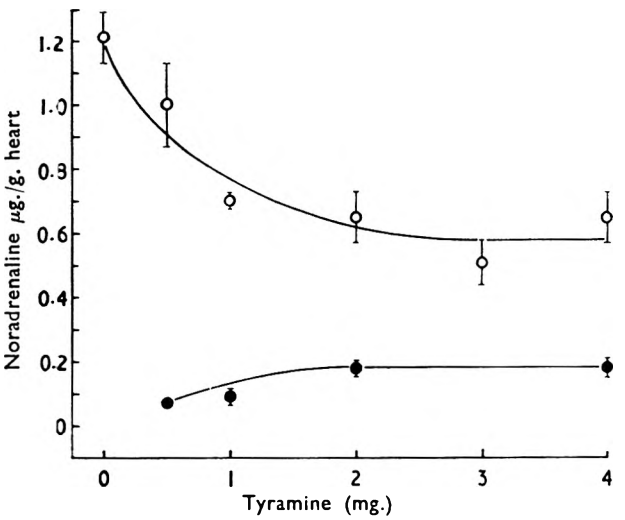

FIG. 1 The effect of tyramine (1, 2, 3 and 4 mg.) on the noradrenaline content (○) and output (●) of the isolated guinea-pig heart. The hearts were given 100 μg. every 5-10 min. Results expressed as μg. noradrenaline per g. heart.

except with guanethidine where it was found that 200 μg. was necessary to produce a positive inotropic effect. The noradrenaline content of the whole heart and perfusate was determined by the method of Merrills (1962). The method in outline involved the homogenisation of the heart in 0·3M perchloric acid, adsorption of the noradrenaline contained in a neutralised aliquot of this perchloric acid extract on alumina, elution by means of relevant pH adjustment and finally fluorimetric estimation of noradrenaline in the eluate. The perfusate from the heart was initially acidified with hydrochloric acid, and then neutralised and adsorbed onto alumina as above. The use of thioglycollic acid as a stabilising agent makes this method more specific for the estimation of noradrenaline. Tyramine, adrenaline, isoprenaline and 3,4-dihydroxyphenylalanine produced no interference with the estimation of noradrenaline and the recovery of noradrenaline added to tissues was 90 per cent.

RESULTS

The effects of graded doses of tyramine on the content and output of noradrenaline from the isolated guinea-pig heart are shown in Fig. 1. Fig. 2 shows two typical tracings obtained during the continued administration of tyramine to the isolated heart. A total of 3 mg. was needed before the tyramine failed to produce a positive inotropic effect, but the

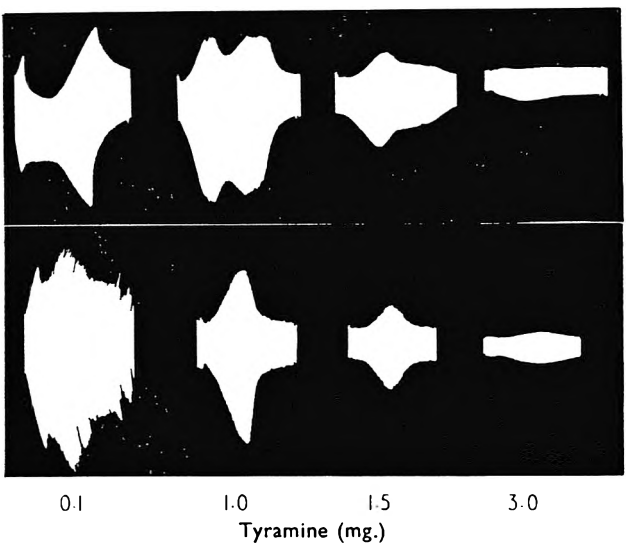

FIG. 2. The effect of successive doses of tyramine on the force of contraction of the isolated guinea-pig heart. The upper and lower records are two representative tracings. The hearts were given 100 μg. tyramine every 5-10 min. The response of the hearts at an accummulated dose of 0.1, 1.0, 1.5 and 3.0 mg. tyramine are shown. Note the marked decrease in response between 1.0 and 1.5 mg. tyramine.

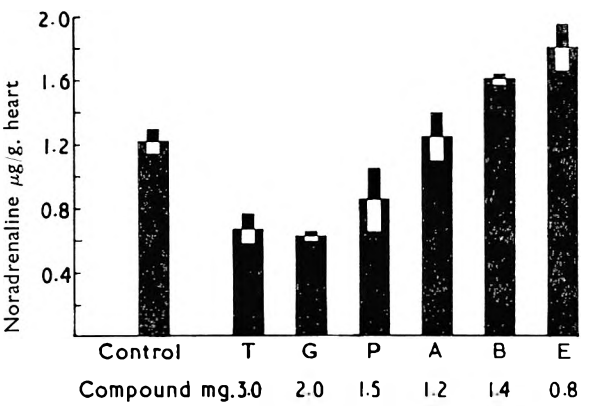

FIG. 3. The effect of tyramine (T), guanethidine (G), phenethylamine (P), amphetamine (A), bretylium (B), and ephedrine (E) on the noradrenaline content of the isolated guinea-pig heart. The columns represent the mean result for 5 hearts $\pm$ s.e.

response was much reduced after 1·5 mg. (accummulative dose) (see Fig. 2). The depletion of noradrenaline in the heart by tyramine was accompanied by the release of noradrenaline into the perfusate.

Guanethidine, phenethylamine, amphetamine, bretylium and ephedrine produced sympathomimetic effects on the isolated guinea-pig heart. The repeated administration of these agents caused successively smaller responses and finally no response. With guanethidine and phenethylamine there was a decrease in the amount of noradrenaline in the heart. Amphetamine produced no change in the heart noradrenaline concentration; with ephedrine and bretylium there was an increase in heart noradrenaline concentration (Fig. 3).

Discussion

Our results support the hypothesis of Burn and Rand (1958) that tyramine mediates its sympathomimetic effects by the release of catecholamines. The evidence for this is that the reduction of the noradrenaline level in the isolated guinea-pig heart is proportional to the amount of tyramine administered to the heart and that the depletion is associated with the appearance of noradrenaline in the perfusate. When tyramine no longer produces an inotropic effect there is a depletion of noradrenaline; further injections of tyramine do not produce any greater decrease in the noradrenaline levels of the heart. These results are in agreement with those of Axelrod and others (1962) who studied the effect of tyramine on the content and release of (^{3}H)-noradrenaline from isolated rat hearts equilibrated with (^{3}H)-noradrenaline.

The response to tyramine disappears at a time when the noradrenaline stores of the heart are approximately 45 per cent. Von Euler and Lishajko (1960) observed that tyramine reduced the noradrenaline content of isolated adrenergic granules to a maximum of 40–50 per cent of that of the controls. This indicated that noradrenaline in the heart is stored in two forms, one, a "bound" form and the other, a "free" form. The amine in the "free" form appears to be readily available to tyramine.

There was a depression of the spontaneous heart rate when tyramine no longer produced a positive inotropic effect. This may indicate that the noradrenaline stores through which tyramine acts are those concerned with controlling heart rate by spontaneous liberation of noradrenaline and possibly are those acted upon by impulses from postganglionic sympathetic nerve fibres.

Lockett and Eakins (1960) observed an increase in plasma adrenaline as well as noradrenaline in the aorta of cats injected with tyramine. Adrenaline was not determined in experiments as we found it to constitute less than 5 per cent of the total catecholamines in the guinea-pig heart. Therefore, our results do not preclude the possibility that tyramine liberates adrenaline as well as noradrenaline.

Burn and Rand (1958) proposed that certain sympathomimetic amines in addition to tyramine, namely, phenethylamine, amphetamine, and ephedrine depended upon the presence of stores of noradrenaline in the tissues to exert their sympathomimetic action. Similar observations have

been made with guanethidine and bretylium (Bein, 1960; Furchgott, 1960). The continued administration of single doses of these compounds to the isolated guinea-pig heart produced successively smaller positive inotropic effects and finally no response. The estimation of the noradrenaline content of the whole heart showed that the loss of a positive inotropic effect after phenethylamine or guanethidine could be ascribed to a decrease of the heart noradrenaline concentration, that is, the positive inotropic effect was limited by the amount of noradrenaline available in the heart itself. The responses to amphetamine, ephedrine and bretylium disappeared when the heart amine levels were either normal as with amphetamine or raised as for ephedrine and bretylium. Therefore, failure of amphetamine, ephedrine and bretylium to elicit an inotropic effect is not due to lack of noradrenaline *per se*. The increase in noradrenaline concentration produced by ephedrine and bretylium indicates some interference with the release or destruction of noradrenaline.

REFERENCES

Axelrod, J., Gordon, E., Hertting, G., Kopin, I. J. and Potter, L. T. (1962). *Brit. J. Pharmacol.*, **19**, 56–63.

Bein H. J. (1960). *Adrenergic Mechanisms*, pp. 162–170. Editors Vane, J. R., Wolstenholme, G. E. W. and O'Connor, M. O. London: Churchill.

Burn, J. H. (1932). *J. Pharmacol.*, **46**, 75–95.

Burn, G. P. and Burn, J. H. (1961). *Brit. J. Pharmacol.*, **16**, 344–351.

Burn, J. H. and Rand, M. J. (1957). *Lancet*, **2**, 1097.

Burn, J. H. and Rand, M. J. (1958). *J. Physiol.*, **144**, 314–336.

Burn, J. H. and Tainter, M. L. (1931). *Ibid.*, **71**, 169–193.

Carlsson, A., Rosengren, E., Bertler, A. and Nilsson, J. (1957). *Psychotropic Drugs*, pp. 363–372. Editors Garattini, S. and Ghetti, V., Amsterdam: Elsevier.

Davey, M. J., Farmer, J. B. and Reinert, H. (1962). Communication to the Winter Meeting of the British Pharmacological Society, Jan. 1962.

von Euler, U. S. and Lishajko, F. (1960). *Experientia*, **16**, 376–377.

Fleckenstein, A. and Burn, J. H. (1953). *Brit. J. Pharmacol.*, **8**, 69–78.

Furchgott, R. F. (1960). *Adrenergic Mechanisms*, p. 172. Editors Vane, J. R., Wolstenholme, G. E. W. and O'Connor, M. O., London: Churchill.

Hertting, G., Axelrod, J. and Patrick, R. W. (1961). *Biochem. Pharmacol.*, **8**, 246–247.

Merrills, R. J. (1962). *Nature, Lond.*, **193**, 988.

Lockett, M. F. and Eakins, K. E. (1960). *J. Pharm. Pharmacol.*, **12**, 513–517.

Schüman, H. J. (1960). *Arch. exp. Path. Pharmak.*, **238**, 41–43.

Stjärne, L. (1961). *Acta physiol. scand.*, **51**, 224–229.

Weiner, N., Draskoczy, P. R. and Burack, W. R. (1962), *J. Pharmacol.*, **137**, 47–55.

THE ABSORPTION AND EXCRETION OF CASTOR OIL IN MAN

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Contrary to popular belief castor oil can be absorbed by human subjects. The absorption is inversely related to the dose and in small (4 g.) doses absorption is virtually complete. The fat balance studies were made both by the radio-iodine labelling of castor oil and by a new chemical technique, which utilises the rapid detection of ricinoleic acid by gas-liquid chromatography on a silicone gum column and incorporates an improved method for the collection, preparation and extraction of faecal lipid.

Hans Meyer demonstrated in 1890 that the purgative action of castor oil was due to ricinoleic acid. Despite the later work of Valette and Salvanet (1936) and Stewart and Sinclair (1945) which demonstrated the absorption of ricinoleic acid in man and in animals, the belief has persisted that castor oil is not absorbed. This belief partly underlies its widespread use as a safe and non-toxic purgative. Elsewhere (Watson and Gordon, 1962) we have described the general metabolism of ricinoleic acid in the rat, showing that in many respects it is digested, absorbed and metabolised like other fatty acids.

This is an account of a series of balance experiments with castor oil in man which show that the absorption is inversely related to the size of the dose.

Methods

Eighteen of the subjects were under investigation for hypertension, but were otherwise metabolically normal. One of these patients participated twice. Three others were young, normal, male volunteers. Castor oil† of medicinal purity and containing a trace amount of oil labelled with ^{131}I prepared by the method of Rutenberg, Seligman and Fine (1949) was administered in a range of doses from 4–60 g. containing about 6 μc of radioactivity. The dose of castor oil administered was determined by weight. A paper cup was weighed, and the desired dose of castor oil, with an average of about 5 g., was placed in it. The full cup was weighed, and the cup plus residual oil was finally weighed again after the patient had drunk as much oil as would easily flow out. Thus the quantity of oil consumed could be determined, and a known amount remained in the cup, to be used as a standard for comparison with excreta in the subsequent analytical procedures.

A 120 mg. dose of potassium iodide was given each subject to block thyroid uptake of radioactive iodine. The hypertensive patients were

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† Kindly supplied by the Baker Castor Oil Company, Bayonne, New Jersey.

given castor oil at 5 p.m. on the day before a scheduled intravenous pyelogram, and allowed only a light meal of coffee and toast thereafter. Stool collections, uncontaminated by urine, were made in two parts: for the first 24 hr. after the dose (I) and during the subsequent 72 hr. (II). Urine was collected in 24 hr. samples. The normal volunteers were given small doses of oil and allowed free diet. Their stools were collected in 3 samples of 2, 2 and 3 days after the oil.

Stools were collected in 1 gallon paint cans and mixed as suggested by Gordon (1959). In the course of the investigation the method was modified to give a more homogeneous and stable emulsion as follows. To each can were added approximately 50 g. coarse granular silica,* water to bring the wet weight of faeces to 970 g., 100 ml. of 0·4 per cent cellulose gum solution,† and 1,000 ml. 95 per cent ethanol. The can was then tightly closed and agitated for 15 min. in a commercial paint shaking machine. The paper cup containing the weighed residue of castor oil was treated in the same way as the stools. Radioactivity was measured by placing the paint can on a scintillation counter, taking care to match the position of each can. Radioactivity in urine was measured by adding the urine to a paint can and making up to 2 kilograms with water.

At first, total fatty acids were estimated by the method of van de Kamer, Ten Bokkel Huinink and Weyers (1949), but early in the study it was suspected that the results were too low. This led to a reinvestigation of the method for extraction of faecal fatty acids, and a modification of the procedure, using toluene as a solvent, to effect better extraction of hydroxy fatty acids. The details of the modified method have been published elsewhere (Jover and Gordon, 1962).

Total lipid for the preparation of methyl esters was extracted from the faecal homogenate by the method of Bragdon (1960) and the chloroform extract so obtained was dried over anhydrous sodium sulphate. A suitable aliquot of this water-free lipid extract was evaporated under nitrogen, and methyl esters for gas-liquid chromatography prepared by heating the lipid at 60° for 16 hr., in a sealed tube containing 1 ml. of the following mixture; anhydrous methanol, benzene and concentrated sulphuric acid (100:10:2 v/v). After the mixture had cooled, the seal was broken, 1 ml. water was added and the esters were extracted in 3 × 1 ml. light petroleum (b.p. 40–60°). The light petroleum extract was evaporated under nitrogen and the esters were re-dissolved in a suitable volume of iso-octane.

Gas-liquid chromatography was carried out in a 6 foot column packed with 3 per cent SE-30 silicone gum on Chromosorb W (80–100 mesh) (Vanden Heuvel, Sweeley and Horning, 1960). Analyses were made at 196° and 23 p.s.i. argon pressure using a modified argon ionisation detector (Lovelock, 1958) containing 80 μc of radium D as a source of ionising radiation. The detector was calibrated by injecting the same volume of a series of dilutions of the methyl esters of corn and castor oils.

* Silica, coarse granular, about 4 mesh. Fisher Scientific Co., Fair Lawn, N.J.

† Carboxymethylcellulose sodium salt, type 70 High, Hercules Powder Co., Wilmington, Delaware.

The detector voltage chosen was one at which the areas of the peaks were linearly related to the quantity of ester injected. The system used for designating the major fatty acids is that of Dole, James, Webb, Rizack and Sturman (1959). Fatty acid methyl esters were identified by comparison of their retention times with those of known standards, particular care being taken to distinguish between methyl arachidonate (20:4), methyl ricinoleate (OH—18:1), methyl hydroxystearate (OH—18:0), and methyl nonadecanoate (19:0) which had similar retention times but were nevertheless separated clearly when the silicone gum column was used. In practice, however, ricinoleic acid was the predominant fatty acid in this region, because of the special conditions of the study. The retention time of ricinoleic acid on silicone gum under the stated conditions is about 18 min. One minor disadvantage of the column is that it does not separate the unsaturated 18 carbon fatty acids. For the discriminatory analysis of these acids a column packed with 22 per cent ethylene glycol adipate on Chromosorb W (80–100 mesh) at 192° and argon pressure 15 p.s.i. was used. The percentage amounts of fatty acid were calculated from the peak areas measured by triangulation.

Calculation

I. Faecal ricinoleic acid = Total titratable faecal fatty acid × per cent ricinoleic acid obtained by gas-liquid chromatography (m-equiv.).

II. Ricinoleic acid administered =

$$\text{dose of castor oil (in g.)} \times \frac{9}{10} \times \frac{896}{988} \times \frac{1000}{298} \text{ (m-equiv.)}$$

$$= \text{dose of castor oil} \times 2{\cdot}7 \text{ (m-equiv.)}$$

where $\frac{9}{10}$ is a factor accounting for the fact that ricinoleic acid is 90 per cent of castor oil fatty acids.

$\frac{896}{988}$ is the factor accounting for the glycerol component of castor oil.

298 is the molecular weight of ricinoleic acid.

Then the recovery of ricinoleic acid $= \frac{\text{I}}{\text{II}} \times 100$ (per cent).

Results

The fatty acid composition of the castor oil under study is given in Table I. Allowing for the presence of the doubly unsaturated linoleic acid (4·7 per cent) and the mono-unsaturated oleic acid (3·2 per cent) one might expect that at least 13 per cent of the radioactivity in the ^{131}I labelled oil would be due to radioactivity incorporated in these fatty acids.

The results of the 22 ^{131}I-castor oil balance studies are shown in Table II. Clearly ^{131}I-labelled castor oil can be absorbed, and the degree of absorption is approximately inversely related to the dose. With a small dose of oil, less than 4 g., absorption is at least 99 per cent, whereas with large purgative doses faecal excretion approaches 90 per cent. Thus even in

the presence of gross purgation there probably still is some absorption of the oil. This could, however, be due entirely to absorption of label in the oleic and linoleic acids. 4 g. is not the maximum amount of oil that may be absorbed since 50 per cent of 20 to 30 g. doses may be absorbed.

TABLE I

FATTY ACID COMPOSITION OF CASTOR OIL DETERMINED BY GAS-LIQUID CHROMATOGRAPHY ON SILICONE GUM AND ETHYLENE GLYCOL ADIPATE COLUMNS

Fatty acid		Per cent
Palmitic	(16:0)	1·0
Palmitoleic	(16:1)	0·1
Stearic	(18:0)	1·0
Oleic	(18:1)	3·2
Linoleic	(18:2)	4·7
Ricinoleic	(OH—18:1)	90·0

TABLE II

RECOVERY OF ^{131}I-RICINOLEIC ACID FOLLOWING THE ORAL ADMINISTRATION OF VARIOUS DOSES OF CASTOR OIL. RESULTS OF 22 EXPERIMENTS ON 21 SUBJECTS—18 HYPERTENSIVE PATIENTS AND 3 NORMAL VOLUNTEERS

	Dose castor oil (g.)	Per cent dose of ^{131}I in stools
Normal volunteers	3·8	0·5
	3·9	0·3
	3·9	0·9
Hypertensives	10·0	11·4
	18·3	42·9
	32·2	31·1
	33·4	60·7
	37·4	53·5
	42·2	72·7
	42·9	71·5
	43·6	75·4
	43·9	61·5
	44·4	86·0
	46·3	72·2
	46·4	59·6
	46·4	64·9
	47·9	81·0
	49·3	54·6
	50·2	64·5
	53·7	84·0
	57·5	82·5
	60·6	89·7

TABLE III

COMPARISON OF PER CENT FAECAL RECOVERY OF CASTOR OIL IN 5 SUBJECTS USING ^{131}I-LABELLING AND CHEMICAL TECHNIQUES

No.	Dose of castor oil (g.)	Faecal recovery of ricinoleic acid, per cent (chemical method)	Faecal recovery of ^{131}I, per cent
1	10·0	12·7	11·4
2	18·3	55·0	42·9
3	33·4	61·9	60·7
4	37·4	57·1	53·5
5	44·4	90·0	86·0

In 17 of the 18 patients who received doses of 10 g. or more, almost all of the radioactivity recovered was present in the first 24 hr. faecal collection, and each of these individuals had either frank purgation or mild laxation. In one subject a dose of 43·9 g. was not noticeably effective,

and of the 61·5 per cent recovery of radioactivity 31·5 per cent was present in the first 24 hr. collection, and the remainder in the second collection. In a general way the degree of recovery correlated with the purgative effect. The volunteers receiving a 4 g. dose had no alteration of normal bowel function.

During the initial phase of this study, poor correlation between the results obtained by the ^{131}I tracer and chemical techniques led to a critical scrutiny of the latter. Serious errors were discovered in the methods for preparing the faecal homogenate and extracting the lipids and these were modified (Jover and Gordon, 1962). Results obtained by the revised procedure are compared in Table III with radioisotopic data obtained simultaneously in 5 subjects. The comparison shows substantial agreement between the techniques in 4 cases. In subject No. 2, however, there is a difference between the results which we believe to be greater than should be accountable on the basis of analytic errors alone.

Discussion

The appearance of ricinoleic acid (12-hydroxy-9-octadecenoic acid) in faeces uniquely follows the administration of castor oil. James, Webb and Kellock (1961) have reported the presence of hydroxystearic acid, but not ricinoleic acid, in the faeces of individuals on normal diet. It is this exclusive occurrence of ricinoleic acid and its rapid resolution by gas-liquid chromatography on a silicone gum column which make possible the technique for accurate and specific fat balance reported here.

The labelling of unsaturated fatty acids by radioactive iodine has two main disadvantages. Firstly, the exact fate of the ^{131}I label in the gut is not known. Cox (1961) has discussed at length the reasons for the unreliability of ^{131}I-triolein, mentioning in particular the evidence for the instability of the ^{131}I label in the gut. Dissociation of the label from the oil will lead to absorption and lower faecal recovery. A chemical method avoids this fallacy. Secondly, iodine labelling alters the chemistry of a fatty acid. The pharmacology of ricinoleic acid may be different from that of hydroxyiodochlorostearic acid, the product of its iodination by the method of Rutenberg and others (1949) where iodine monochloride is the iodinating agent. In this respect it is reassuring that in the cases studied by the improved chemical technique and by iodine labelling there is a close relationship between the results. However differences do exist, and they are substantial in case 2. While this is probably due to the imperfections of the isotope method there are two possible sources of error in the chemical technique. Firstly, if absorbed ricinoleic acid is re-excreted into the gut this will raise the faecal recovery of ricinoleic acid and lead to underestimation of the real degree of absorption. We have no evidence about this in man, but experimental studies in rats have shown that the intestinal excretion of unaltered ricinoleic acid is not a mechanism in its overall metabolism (Watson and Gordon, 1962), and we believe that the same is likely in man.

Secondly, and of more practical importance, the result will be affected by any chemical modification of ricinoleic acid in its passage through the

gut. Both our animal and human studies have shown that this is not a problem when castor oil is administered in purgative or even mildly laxative doses. But in 2 of the 3 normal subjects given small non-purgative doses of castor oil, and in rats in chronic castor oil feeding, hydroxystearic acid appeared in the faeces, although it had not been detectable in pre-castor oil faecal collections. This finding is consistent with the belief that intestinal hydrogenation of fatty acids occurs. It also indicates that difficulties will arise in the interpretation of balance studies made with non-purgative doses of castor oil, unless it can be shown that hydroxystearic acid is not present in faeces collected before the administration of castor oil. Absence of hydroxystearic acid was demonstrated in those faecal samples listed in Table III.

We have described our studies on the experimental pharmacology of castor oil in animals elsewhere (Watson and Gordon, 1962). Its purgative action appears to depend on rapid hydrolysis of ricinolein and retarded activation of free ricinoleic acid to ricinoleyl-CoA, thus leading to the accumulation of free ricinoleic acid and its soaps. In this connection, the emetic effect of soap solutions, the purgation that results from a soap enema, and the diarrhoea that accompanies steatorrhoea may be related phenomena. Ricinoleic acid differs from oleic only in possessing one hydroxyl group, but this appreciably increases its solubility in polar solvents, and would be expected to affect its behaviour at lipid-water interfaces. Precisely how this affects its rates of hydrolysis and activation cannot now be specified, but it would seem reasonable that such effects should exist, and be the chemical basis for the observed purgative effect.

References

Bragdon, J. H. (1960). *Lipids and the Steroid Hormones in Clinical Medicine.* P.7, Philadelphia: J. B. Lipincott Company.
Cox, A. G. (1961). *Brit. med. J.*, **2**, 933–938.
Dole, V. P., James, A. T., Webb, J. P. W., Rizack, M. A. and Sturman, M. F. (1959). *J. clin. Invest.*, **38**, 1544–1554.
Gordon, R. S. Jr. (1959). *Lancet*, **1**, 325–326.
James, A. T., Webb, J. P. W. and Kellock, T. D. (1961). *Biochem. J.*, **78**, 333–339.
Jover, A. and Gordon, R. S. Jr. (1962). *J. Lab. clin. Med.*, **59**, 878–884.
Lovelock, J. E. (1958). *J. Chromatog.*, **1**, 35–46.
Meyer, H. (1890). *Arch. exp. Path. Pharmak.*, **28**, 145–152.
Rutenberg, A. M., Seligman, A. M. and Fine, J. (1949). *J. clin. Invest.*, **28**, 1105–1109.
Stewart, W. C. and Sinclair, R. G. (1945). *Arch. Biochem.*, **8**, 7–11.
Valette, G. and Salvanet, R. (1936). *Bull. Soc. Chem. biol.*, **18**, 911–917.
van de Kamer, J. H., Ten Bokkel Huinink, H. and Weyers, H. A. (1949). *J. biol. Chem.*, **177**, 347–355.
Vanden Heuvel, W. J. A., Sweeley, C. C. and Horning, E. C. (1960). *J. Amer. chem. Soc.*, **82**, 3481.
Watson, W. C. and Gordon, R. S. Jr. (1962). *Biochem. Pharmacol.*, **11**, 229–236.

THE INHIBITION OF MALIC DEHYDROGENASE BY SALICYLATE AND RELATED COMPOUNDS

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Salicylate and γ-resorcylate affected the distribution of radioactivity incorporated from labelled succinate into the soluble intermediates of rat liver mitochondria in a manner consistent with an inhibitory action of the drugs on malic dehydrogenase. The addition of nicotinamide adenosine dinucleotide to the mitochondrial preparations reversed the effects of salicylate and γ-resorcylate. A general structural requirement for inhibitory activity against malic dehydrogenase *in vitro* in salicylate congeners appears to be a phenolic hydroxyl group in the *ortho* position to a carboxyl group except that 2-hydroxyphenylacetate also inhibited the enzyme.

Salicylate and γ-resorcylate have been reported to inhibit pig heart malic dehydrogenase activity *in vitro*; the mechanism of the inhibition involving competition with the coenzyme, nicotinamide adenosine dinucleotide (NAD) (Bryant, Smith and Hines, 1963). The present paper is concerned with the reversal by NAD of the changes caused by the drugs in the distribution of radioactivity incorporated from [1,4-^{14}C] succinate into the soluble intermediates of rat liver mitochondria. In addition a study has been made of the relation between chemical structure and the inhibitory action against malic dehydrogenase activity in congeners of salicylate.

Experimental

Materials

[1,4-^{14}C] succinate was obtained from the Radiochemical Centre, Amersham, Bucks, and pig heart malic dehydrogenase and NAD from C. F. Boehringer and Soehne. The salicylate congeners were obtained commercially and recrystallised until their melting-points remained constant. They were dissolved in glycine buffer at pH 9·6 (Gomori, 1955) to give solutions, which after admixture with the other constituents of the reaction mixtures used for the measurement of malic dehydrogenase activity, produced final concentrations of the drugs ranging from 5 to 30 mM.

Radioactive Experiments

Rat liver mitochondria, separated from 12 g. wet weight of liver by the method of Schneider and Hogeboom (1950) were finally suspended in 2 ml. of 0·25M sucrose. 50 μl. samples of the suspension were mixed with 25 μl. of a solution containing 0·5 μc of [1,4-^{14}C] succinate, 0·03 mM cytochrome C, 0·1 mM adenosine triphosphate, 1·0 mM adenosine diphosphate, 2·0 mM $MgSO_4.7H_2O$ and 10·0 mM KCl dissolved in 0·1 M potassium phosphate buffer, pH 7·4 (Gomori, 1955) and incubated for 30 min. at 37°. Salicylate and γ-resorcylate, when present, were added in the phosphate buffer to give final concentrations of 5 mM and NAD was also added in some experiments to give a final concentration of 3·25 mM. At the end of the incubation period the mitochondria were killed by the

addition of 300 μl. of boiling ethanol and the radioactive substances in the ethanolic extract were separated by two-dimensional paper chromatography, visualised by radioautography, and the ^{14}C measured by the techniques described by Smith and Moses (1960).

TABLE I

EFFECTS OF 5 mM SALICYLATE AND 5 mM γ-RESORCYLATE ON THE METABOLISM OF [1,4-^{14}C] SUCCINATE BY RAT LIVER MITOCHONDRIA IN THE PRESENCE OR THE ABSENCE OF 3·25 mM NAD

Results expressed as 10^{-3} × counts/min.

Soluble intermediate	Control	Control + NAD	Salicylate	Salicylate + NAD	Resorcylate	Resorcylate + NAD
Succinate	0·9	1·8	1·0	1·5	1·4	1·1
Fumarate	9·7	5·4	12·0	5·0	14·4	6·2
Malate	20·6	15·0	36·4	16·4	43·1	13·5
Citrate	0·4	0·2	0·9	0·3	0·6	0·2
Aspartate	2·1	2·1	2·0	2·3	1·3	3·3
Glutamate	2·1	1·7	0·8	1·3	0·5	1·0
Alanine	0·5	0·6	0·2	0·7	0·2	0·4

TABLE II

INHIBITION OF PIG HEART MALIC DEHYDROGENASE ACTIVITY BY SALICYLATE CONGENERS

The results, expressed as mean percentage inhibitions, have been analysed by the *t*-test and values of P are included. The minimum acceptable level of significance has been taken as P = 0·02.

Congener	No. of observations	Conc. (mM)	Per cent inhibition after 30 sec.	P
Salicylate	6	10	34·6	0·001
γ-Resorcylate	6	5	54·3	0·001
Benzoate	6	30	5·0	0·2
Phthalate	5	10	3·7	0·1
2-Methoxybenzoate	4	10	2·5	0·4
2-Hydroxyphenylacetate	6	10	29·6	0·001
3-Hydroxybenzoate	4	10	2·5	0·7
4-Hydroxybenzoate	6	10	0	—
Phenol	6	30	5·0	0·1
trans-Hexahydrosalicylate	4	10	4·1	0·3

Measurement of Malic Dehydrogenase Activity

The commercial enzyme was dialysed against phosphate buffer, pH 7·4, during which procedure it was diluted twenty times, and the dialysed enzyme was then diluted 1 to 100 with glycine buffer, pH 9·6 before use. The reaction mixtures contained 1 ml. of 30 mM sodium malate, 1 ml. of 0·75 mM NAD, 0·01 ml. of the enzyme preparation and 1 ml. of either glycine buffer or a solution of the salicylate congener in glycine buffer. The enzyme activity was estimated by measuring the change in optical density at 340 mμ in a Hilger Uvispek spectrophotometer at 15 sec. intervals over a period of 2 min. (Burton and Wilson, 1953).

RESULTS

The results given in Table I show the amounts of radioactivity from the labelled succinate incorporated into the soluble intermediates of rat liver mitochondria. In the control experiments radiocarbon was found in fumarate, malate, citrate, aspartate, glutamate and alanine and the

addition of NAD decreased the incorporation of the isotope into these intermediates and presumably increased the conversion of succinate carbon to CO_2. The most prominent action of salicylate and γ-resorcylate was to increase the accumulation of ^{14}C into fumarate and malate but the addition of NAD reversed this effect.

Table II gives the results of the effects of the salicylate congeners on malic dehydrogenase activity *in vitro*. Salicylate, γ-resorcylate and 2-hydroxyphenylacetate significantly inhibited the enzyme activity but the other substances had no effect.

Discussion

The present results confirm the work of Huggins, Bryant and Smith (1961) and Bryant, Smith and Hines (1963) in that salicylate and γ-resorcylate increase the incorporation of ^{14}C into fumarate and malate of rat liver mitochondria incubated with labelled succinate. It has been reported that both drugs inhibit malic dehydrogenase activity *in vitro* and that the mechanism of inhibition involves competition with the coenzyme, NAD (Bryant, Smith and Hines, 1963). The results in Table I show that the presence of a large excess (3·25 mM) of NAD in the mitochondrial experiments reverses the most prominent effect of salicylate and γ-resorcylate, that is the increased formation of radio-active malate. Thus the addition of an adequate amount of the coenzyme appears to counteract the inhibitory action of the drugs on the mitochondrial malic dehydrogenase and prevent the increased accumulation of the labelled malate.

Only salicylate, γ-resorcylate and 2-hydroxyphenylacetate significantly inhibited malic dehydrogenase activity *in vitro*. If salicylate represents the parent molecule then absence of the hydroxyl group (benzoate), alteration of its position relative to the carboxyl group (3- and 4-hydroxybenzoates), substitution of it by a carboxyl group (phthalate) or its methylation (2-methoxybenzoate), all caused a loss of activity. The absence of the carboxyl group (phenol) and hydrogenation of the benzene ring (hexahydrosalicylate) also produced inactive compounds. A general structural requirement for inhibitory activity against the dehydrogenase in congeners of salicylate therefore appeared to be a phenolic hydroxyl group in the *ortho* position to a carboxyl group except that the introduction of a methylene group between the benzene ring and the carboxyl group (2-hydroxylphenylacetate) did not remove the activity.

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References

Bryant, C., Smith, M. J. H. and Hines, W. J. W. (1963). *Biochem. J.*, in the press.
Burton, K. and Wilson, T. H. (1953). *Ibid.*, **54**, 86–94.
Gomori, G. (1955). In *Methods of Enzymology*, vol. 1, p. 138. Edited by Colowick, S. P. and Kaplan, N. O. New York: Academic Press Inc.
Huggins, A. K., Bryant, C. and Smith, M. J. H. (1961). *J. Pharm. Pharmacol.*, **13**, 654–662.
Schneider, W. C. and Hogeboom, G. H. (1950). *J. biol. Chem.*, **13**, 123–128.
Smith, M. J. H. and Moses, V. (1960). *Biochem. J.*, **76**, 579–585.

THE STABILITY OF LIQUID MULTIVITAMIN PREPARATIONS DURING USE

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The stability during normal use of vitamin A, vitamin C and thiamine in 6 liquid multivitamin preparations has been examined. Three samples were below label claim when purchased but no further losses occurred during storage of unopened bottles. Destruction of the vitamins was increased by daily removal of a portion of the contents and the stability of most samples stored at room temperature was unsatisfactory. The study suggests that the results of stability tests should be examined more critically to ensure not only that the products meet label claim when purchased, but also maintain a satisfactory potency during normal use. From the consumer's point of view the use of expiration dates which take account of loss during normal use would be most valuable. The finding of retrovitamin A in one preparation suggests the need for more detailed study of the complex changes which may take place in these preparations during storage and of methods to evaluate such changes.

In 1955, Campbell and McLeod reported that many market samples of oral multivitamin preparations, particularly those over one year old, did not meet the label claim for one or more vitamins. They suggested that the situation could be improved by the use of expiration dates based on actual shelf life. Since that time legislation has been enacted (F.D.A.R., 1954) requiring manufacturers of vitamin preparations to determine the period during which the drug will maintain its labelled potency and to indicate on the label the coded date of manufacture or an expiration date after which the drug is not recommended for use. Although these regulations afford the consumer some protection against purchasing products which have passed their normal shelf life the possibility exists that a product may meet labelled claim when purchased but may fall in potency during normal use. There appears to be no published date on stability during this period.

This report concerns the stability of representative vitamin products during the time and under conditions encountered in normal use in the home. Liquid preparations were used because they were the most likely preparations to be adversely affected by the conditions of the experiment.

Experimental

Three bottles each of the largest available size of six liquid multivitamin preparations were purchased from a local wholesale outlet. The three bottles of each product were of the same batch (lot) number but no attempt was made to choose products of a particular age. Two bottles of each product were opened and assayed for vitamin A, vitamin C and thiamine, while the unopened bottles were refrigerated at about 4° until the end of the experiment. One of the opened bottles was refrigerated,

the other kept at room temperature, and from both a recommended daily dose was removed each day except Saturday and Sunday. Vitamin A, vitamin C and thiamine were determined at intervals until the bottles were emptied. Vitamin A was determined by the U.S.P. XVI method (1960) where possibly, by the antimony trichloride method, by a chromatographic method (Murray, 1962) and, biologically by the liver storage assay (Ames and Harris, 1956). Vitamin C was determined by 2,6-dichlorophenolindophenol titration and thiamine by conversion to thiachrome (Methods of Vitamin Assay, 1951).

Results and Discussion

The initial and final vitamin contents of the samples are shown in Table I and all analyses are plotted in Fig. 1. Samples containing less

TABLE I

Potency of liquid multivitamin preparations during use (as per cent of label claim)

Product	Vitamin	Refrigerated			Room temperature	
		Initial	Final		Initial	Final
			Opened	Unopened		
1	A	84	103	84	104	98
	C	100	92	98	100	69
	B_1	104	100	106	106	79
2	A	111	107	111	107	95
	C	97	97	99	96	89
	B_1	130	137	135	133	118
3	A	90	85	93	93	94
	C	124	114	117	126	88
	B_1	111	110	114	116	90
4	A	90	89	92	92	77
	C	93	93	93	94	86
	B_1	103	101	103	107	91
5	A	108	116	135	154	98
	C	109	82	110	108	67
	B_1	124	97	126	127	75
6	A	111	103	103	105	95
	C	85	64	86	86	27
	B_1	100	88	98	104	69

than 95 per cent of the labelled amount of the vitamins were arbitrarily considered not to meet the label claim. Initially, one product (No. 4) contained less than the labelled amount of vitamins A and C, one, less than the labelled amount of vitamin A (No. 3) and one was deficient in vitamin C alone (No. 6). In addition, one of two bottles of another product (No. 1) did not meet label claim for vitamin A but this might be because of inadequate mixing. The very high vitamin A content of product 5 was verified by repeated analyses. At least half of the products, therefore, did not meet label claim initially for one or more of the vitamins.

All products exhibited good stability, as they should, when stored unopened in the refrigerator for periods of from 8 to 24 weeks. When, under the same storage conditions, daily doses were removed, two

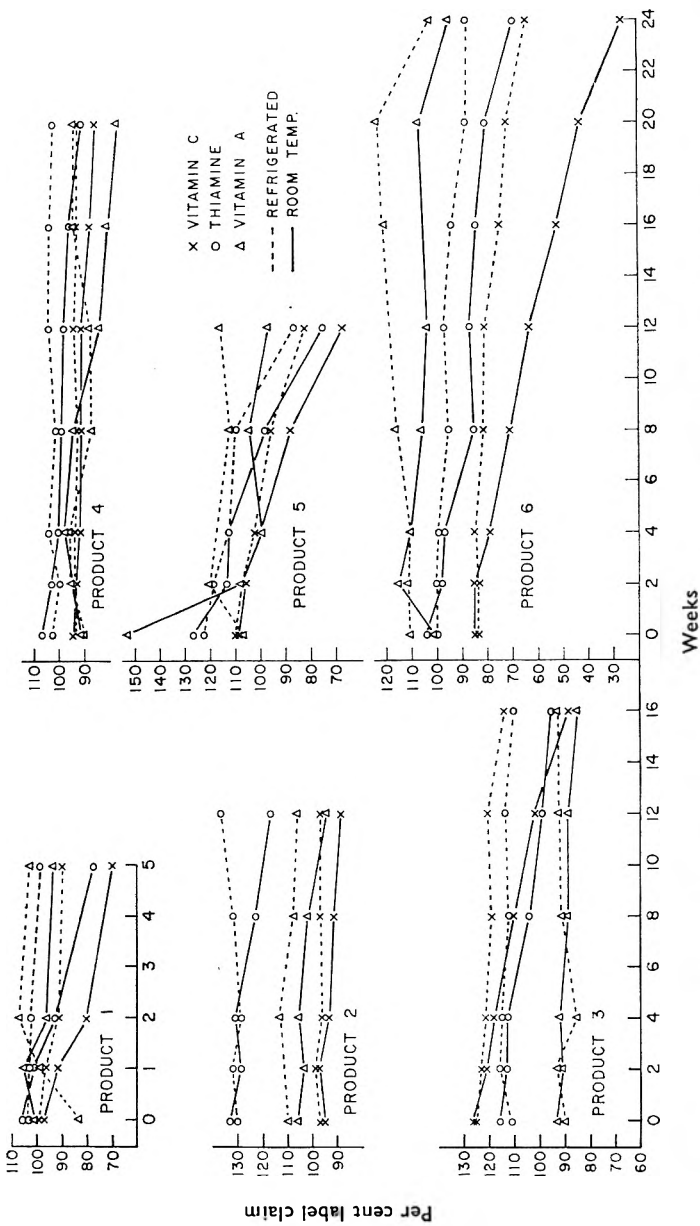

FIG. 1. Changes in potency of vitamin A, vitamin C and thiamine during storage of liquid multivitamin preparations.

products (Nos. 5 and 6) lost more than 10 per cent of vitamin C and thiamine but vitamin A proved relatively stable under these conditions. At the end of this storage period only one product met the label claim for all three vitamins.

The decrease in potency was more marked in samples held at room temperature. Losses of more than 10 per cent were found in all samples

for thiamine, in samples 1, 3, 5 and 6 for vitamin C, and in samples 2, 4 and 5 for vitamin A. Under these conditions no product met the label claim for the three vitamins at the end of the test period.

The least satisfactory aspect of these products was in their low potency when purchased. Had the products as purchased contained the amount indicated on the label plus a 10 per cent overage, all but two of the refrigerated samples (Products 5 and 6) would have met the label claim at the end of the experiment. Vitamin C and thiamine were markedly unstable in products 5 and 6. Samples held at room temperature, on the other hand, lost up to 59 per cent of their vitamin content and a 10 per cent overage would not have permitted any to meet the label claim at the end of the experiment. A 15 per cent overage would have permitted two products to do so. Although refrigeration was very important in the stability of these products only one product's label had a statement to this effect.

Most of the products examined were available in smaller sizes in which stability could have been studied over a shorter period. Had the samples been half the size used, and had all met claim initially, three would have been deficient at the end of the experiment. Obviously, the stability of liquid multivitamin preparations during use requires further study.

It is obvious that the use of expiration dates is much more valuable to the consumer than is the coded date of manufacture and such dates should allow for losses incurred during normal use.

An interesting analytical problem arose in these studies. The U.S.P. XVI method for vitamin A could not be applied to Product 6 since the absorbance curve was distorted. The values shown in Table I were obtained by the antimony trichloride method because it was thought this would measure any change in potency. At the beginning and end of the experiment, Product 6 (unrefrigerated sample) was assayed by the U.S.P. spectrophotometric procedure after purification by chromatography (Murray, 1962) and by the liver storage assay. The results as per cent of label claim are:

	Method		
Week	$SbCl_3$	Chromatographic	Biological
0	105	58	67
24	95	10	43

The values found by the antimony trichloride method greatly overestimated biological potency and did not measure the decrease in potency which occurred during the experiment. It is obvious that this method is inadequate for estimating the potency of certain samples. Chromatographic purification gave a fair estimate of the initial potency but grossly underestimated it at the end.

The absorbance curve of the interfering substance removed by chromatography at the end of the experiment is shown in Fig. 2. The peaks at 330 350 and 370 mμ identify it as retrovitamin A (Beutel, Hinkley and Pollak,

1955), a compound which produces a blue colour with antimony trichloride but which has been reported to have a low biological potency (Shantz, 1950). It has not been previously reported to occur in pharmaceuticals although it is recognised (Miguchi and Reinstein 1959) that anhydrovitamin may do so on occasion. Since both retro and anhydrovitamin A interfere with the estimation of vitamin A activity, their occurrence in pharmaceuticals is being further investigated.

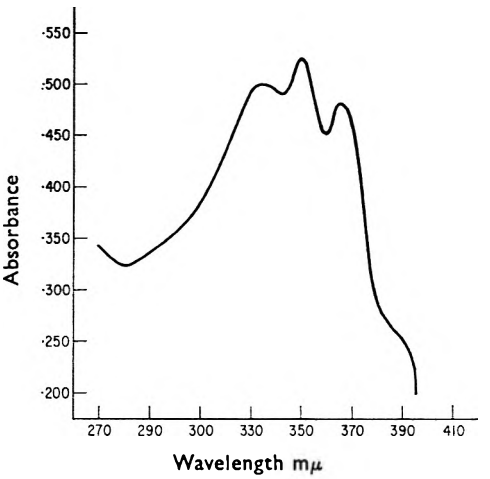

FIG. 2. Absorbance curve of retrovitamin A isolated from a liquid multivitamin preparation.

It may be concluded that both overages and adjustment of formulation are necessary to ensure that some liquid multivitamin preparations meet the label claim when purchased and maintain it during normal use. The stability of all the products examined was improved by refrigeration.

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REFERENCES

Ames, S. R. and Harris, J. L. (1956). *Analyt. Chem.*, **28**, 874–878.
Beutel, R. H., Hinkley, D. F. and Pollak, P. I. (1955). *J. Amer. chem. Soc.*, **77**, 5166–5167.
Campbell, J. A. and McLeod, H. J. (1955). *J. Amer. pharm. Ass., Sci. Ed.*, **44**, 263–269.
Food and Drugs Act and Regulations (1954). D.02.029, p. 132 (and amendments). Ottawa: Queen's Printer and Controller of Stationery.
Higuchi, T. and Reinstein, J. A. (1959). *J. Amer. pharm. Ass., Sci. Ed.*, **48**, 155–161.
Murray, T. K. (1962). *Analyt. Chem.*, **34**, 1241–1243.
Methods of Vitamin Assay (1951). 2nd ed. New York: Interscience Publishers, Inc.
Shantz, E. M. (1950). *J. biol. Chem.*, **182**, 515–524.
United States Pharmacopeia (1960). 16th rev. Easton, Pa.: Mack Printing Co. p. 938.

THE PREPARATION OF PORPHYROXINE FROM OPIUM*

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Porphyroxine, a minor alkaloid from opium, is isolated by an extraction procedure, passed through Florisil and alumina (basic and neutral) columns, and eluted with solvents of varying polarity. Porphyroxine from the chloroform and acetone fractions recrystallised from methanol/chloroform in ribbed plates, darkens at 218° and melts at 234–236° (decomp.) corresponding to $C_{22}H_{24}NO_7$, or $C_{23}H_{24}NO_7$. Spectral, and paper chromatographic data of porphyroxine and derivatives are reported.

Porphyroxine, a minor alkaloid of opium was first mentioned by Merck (1837). It is distinguished from other alkaloids of the opium poppy (*Papaver somiferum*) by its reactivity with dilute mineral acids which produces a red solution. Dey (1881), Bamford (1930), Fulton (1950, 1952) and Farmilo and Kennett (1950) used this property for the determination of the origin of opium. Hesse (1870), Rakshit (1919) and Machiguchi (1926) claimed to have isolated the alkaloid, but, according to Fulton (1952) had only succeeded in obtaining impure preparations. In Rakshit's claim, it is considered by Rajahgopolan (1945) and Fulton 1952) that codeine, with thebaine, papaverine and a small portion of the red-turning alkaloid were isolated. Fulton (1952) described a method, based mainly on liquid-liquid extraction by which Clair (1956) was able to produce a brown syrupy concentrate of porphyroxine, which did not crystallise. More recently Klayman (1956) reported the isolation of crystalline porphyroxine by liquid-liquid extraction with non-polar solvents and column chromatography. Klayman's method is very time-consuming, for example, a light petroleum (b.p. 30–60°) extraction-step required approximately two months. By combining parts of Fulton's and Klayman's procedures a method has been developed by which a purer porphyroxine may be made in a shorter time.

Experimental

Test for porphyroxine. 2 ml. of extracts were mixed with 5 drops of 2N hydrochloric acid in a small crucible and evaporated to dryness on the boiling water bath; a red colour indicates porphyroxine.

Paper chromatography. All stages of the purification process were checked in isobutanol : acetic acid : water (System 2) (Genest and Farmilo, 1960). The final product was tested in six paper chromatographic systems (Genest and Farmilo, 1961).

Spectra. Ultra-violet spectra of porphyroxine and porphyr (Genest and Farmilo, 1962) were measured with a Beckman DK 2-spectrophotometer and used for assay purposes.

Procedure. The starting material was the residue of an ether extraction prepared by T. & H. Smith, Ltd., Edinburgh, by the K/14-method (Farmilo

* The preceding paper of this series was published in *Analyt. Chem.*, 1962, **34**, 1464–1468.

and Kennett, 1953), from 40 lb. of Indian Export opium. This was extracted in light petroleum (b.p. 30–60°), 97 g. of this extracted material was taken up in hot ethanol. The solvent was evaporated until a precipitate was formed. After cooling overnight and filtration, the crystalline precipitate—mostly narcotine—was discarded and the filtrate evaporated to dryness. The residue was taken up in about 140 ml. of dilute acetic acid (3·5 per cent). Sodium acetate (25 g.) were added and the solution extracted continuously with carbon tetrachloride in a liquid-liquid extractor. After about 4 hr. the porphyroxine test was negative. The carbon tetrachloride extract (about 180 ml.) was now shaken out with sodium hydroxide solutions of decreasing concentration; (50 ml. each of 10, 5 and 2 per cent sodium hydroxide) until all porphyroxine was removed. Each alkaline extract was washed with carbon tetrachloride (4 × 50 ml.); and when it became saturated with impurities was refreshed. All sodium hydroxide extracts were pooled, made slightly acidic with acetic acid, then made strongly ammoniacal. The porphyroxine was now exhaustively extracted with chloroform in a liquid-liquid extractor (about 4 hr.). The chloroform extract was evaporated to dryness (1·57 g.). A Florisil (44 g. 60/100 mesh) column (24 × 300 mm.) was packed with the aid of benzene. The resinous chloroform residue was dissolved in benzene: chloroform (4 : 1), transferred to the column and eluted at 2 ml./min. with the following solvents: 5 × 20 ml. benzene, 1 × 20 ml. benzene : chloroform (1 : 1), 5 × 20 ml. chloroform, 1 × 20 ml. chloroform : acetone (1 : 1), 5 × 20 ml. acetone, 1 × 20 ml. acetone : methanol (1 : 1) and 5 × 20 ml. methanol. Twenty-four fractions of 20 ml. each were collected. Fractions which showed the highest amount of porphyroxine and the least amount of impurities (chloroform and acetone fractions) were combined and the solvent evaporated. An alumina (Woelm, basic) column (12 × 200 mm.) was prepared. Material containing up to 150 mg. porphyroxine can be eluted from this column without too much tailing. For the elution in 10 ml. fractions the same set of solvents as described above was used. Most porphyroxine is eluted in the methanol fractions. The best fractions were rechromatographed several times on alumina (basic or neutral) columns until no more impurities of the now crystalline material could be discovered by paper chromatography. Porphyroxine recrystallised from methanol/chloroform in ribbed plates (73 mg.).

Results

Porporphyroxine has a m.p. 234–236° (decomp.), darkening at 218° (Kofler, 4°/min.).

Found: C, 63·70, 63·90; H, 5·79, 5·60; N, 3·26; CH_3O, 14·60; m.w. 402 (Rast). Calc. for: $C_{22}H_{24}NO_7$: C, 63·76; H, 5·82; N, 3·38; $2CH_3O$, 14·97 per cent; m.w. 414: or $C_{23}H_{24}NO_7$: C, 64·79; H, 5·67; N, 3·29; $2CH_3O$, 14·57 per cent; m.w. 426.

Results of spectral and chromatographic studies were: porphyroxine in ethanol λ_{max} 286·9, 233·9 mμ (ϵ_{max} 8,470, 11,190), λ_{min} 258·1, 227·5 mμ (ϵ_{min} 1,150 and 11,000).

Porphyr hydrochloride, λ_{max} 525, 381, 321·5, 282 mμ (ϵ_{max} 29,040, 7,520, 10,830, and 14,880), λ_{min} 411, 363·5, 302·5, 269·5 mμ (ϵ_{min} 5,450, 7,250, 8,620 and 13,750).

Porphyr phosphate, λ_{max} 535, 378, 323·9, 273·6 mμ (ϵ_{max} 26,850, 8,330, 12,470, and 16,660), λ_{min} at 415, 365, 303, 269·5 mμ (ϵ_{min} 4,890, 6,090, 10,510 and 16,580, respectively). The porphyr compounds were found to be mixtures with $R_{mophine}$ values of 0·60 (violet), 1·17 (red) and 2·76 (colourless). The R_F values of porphyroxine in 6 paper chromatographic systems are 0·69 (isobutanol : acetic acid : water/PO_4) 0·52 (isobutanol : acetic acid : water/SO_4) 0·25 (butylacetate : acetic acid : water/PO_4), 0·83 (propanol : water : diethylamine : paraffin), 0·11 ($HCOONH_4$/s-octanol), 0·02 (paraffin : diethylamine : formamide).

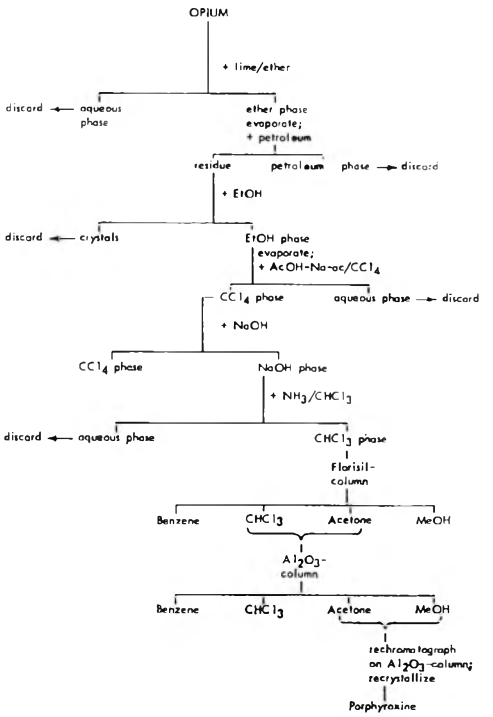

FIG. 1. Flow sheet of procedure for preparation of porphyroxine.

DISCUSSION

The method is outlined in Fig. 1. The starting material contains resins and pigments, a large amount of codeine and thebaine, and much narcotine, papaverine and morphine, some minor alkaloids of phenolic and non-phenolic character, and 0·61 per cent of porphyroxine. In the extraction from buffered acetic acid solution with carbon tetrachloride most of the morphine is left behind in the aqueous phase (Fulton, 1952). The sodium hydroxide extraction, on the other hand, removes only

phenolic alkaloids, leaving behind most of the non-phenolic alkaloids, codeine, thebaine, papaverine and narcotine. According to Fulton (1952) the sodium hydroxide extract contains only porphyroxine, laudanine, a weak base (possibly lanthopine or narcotoline) and a base called alkaloid 7. We found, by paper chromatography, small amounts of all the major alkaloids of opium, in particular codeine. The residue before

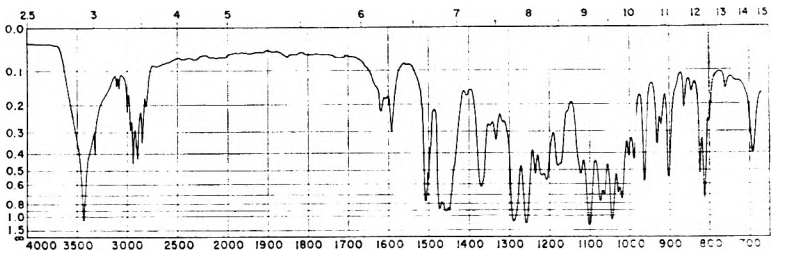

FIG. 2. Infra-red absorption spectrum of porphyroxine (0·5 per cent) in KBr.

application to the Florisil column contained 12·3 per cent porphyroxine, and afterwards, in the best fractions increased to 39 per cent. The final product showed only one spot in five chromatographic systems (Genest and Farmilo, 1961), and gave the R_F values mentioned above. The limit of detection was 0·2 μg. for both the hydrochloric acid (after heating 3 min./100°) and Kiefer's reagents (Farmilo and Genest, 1961), and 20 μg. for the potassium iodoplatinate reagent (Genest and Farmilo, 1960).

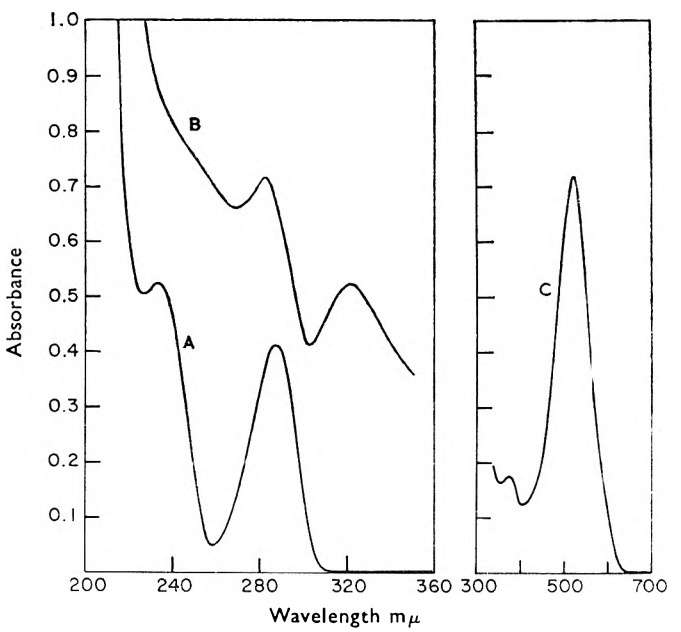

FIG. 3. Ultra-violet absorption spectrum of (A) porphyroxine (0·02 g./litre) in ethanol and of porphyr hydrochloride (B, 0·02; C, 0·01 g./litre) in 67 per cent methanol.

The structure of porphyroxine is still unknown. Klayman (1956) reported the elementary composition to be $C_{21}H_{23}NO_6$ with secondary possibilities of $C_{20}H_{23}NO_6$ and $C_{21}H_{21}NO_6$. Even though the melting point of our preparation is close to that of Klayman's, the elementary analyses of our porphyroxine corresponds to a formula of either $C_{22}H_{24}NO_7$ or $C_{23}H_{24}NO_7$. Analysis shows the presence of two methoxyl groups. Based on its behaviour in alkali, and considering its positive reactions with spray reagents such as *p*-nitroaniline and Kiefer's (Farmilo and Genest, 1961) on the paper chromatogram, porphyroxine has phenolic properties. Spectral results indicate a fair qualitative agreement of absorption maxima in the infra-red, visible and ultra-violet regions (Figs. 2, 3) with those reported by Klayman. The ultra-violet absorptivities reported here are somewhat lower ($\log \epsilon = 3{\cdot}93$ vs. 3·98), while the absorptivities in the visible for the reaction product of porphyroxine with mineral acid, are considerably higher than Klayman's ($\log \epsilon = 4{\cdot}46$ vs. 4·20). The latter's preparation may contain material which absorbs in the ultra-violet but does not produce the characteristic red colour. In a recent paper on the red-turning alkaloids of the papaver family, Pfeifer (1962) also mentioned that Klayman's product probably contained some impurities. Further study of the conditions of the red-colour-reaction is required.

Klayman found that the reaction product of porphyroxine with mineral acid, porphyr hydrochloride, consisted of three components, two coloured and one colourless. He was able to separate these by partition chromatography, but the paper chromatographic separation was incomplete. In the system isobutanol : acetic acid : water (10 : 1 : 2·4) on paper impregnated with sulphate the compounds can be separated from each other by the descending "durchlauf" technique to give the R_{morphine} values cited above. The colourless component gives an elongated spot producing a strong blue fluorescence at 3,660 and 2,537 Å.

References

Bamford, F. (1930). *Analyst*, **55**, 445–446.
Clair, E. C. (1956–57). Monthly Reports. Food and Drug Directorate, Ottawa.
Dey, K. L. (1881). *Pharm. J. and Transactions*, **12**, 397.
Farmilo, C. G. and Kennett, P. M. L. (1950). United Nations Document, E/CN7/207, October 19.
Farmilo, C. G. and Kennett, P. M. L. (1953). United Nations Document, ST/SOA/Ser.K/14, January 26.
Farmilo, C. G. and Genest, K. (1961). *Toxicology, Mechanisms and Analytical Methods*, Editors, Stewart and Stolman, Vol. II, pp. 573, 577. New York and London: Academic Press.
Fulton, C. C. (1950). United Nations Document, E/CN7/195, March 26.
Fulton, C. C. (1952). *Bull. Narcotics UN Dept. Social Affairs*, **4**, No. 1, 15–25.
Genest, K. and Farmilo, C. G. (1960). *Ibid.*, **12**, No. 1, 17–26.
Genest, K. and Farmilo, C. G. (1961). *J. Chromatography*, **6**, 343–349.
Genest, K. and Farmilo, C. G. (1962). *Analyt. Chem.*, **34**, 1464–1468.
Hesse, O. (1873). *Ann. Chem. Pharm.*, **153**, 47–83.
Klayman, D. L. (1956). Ph.D. thesis, Rutgers University.
Machiguchi, E. (1926). *J. pharm. Soc. Japan*, **529**, 185–228.
Merck, E. (1887). *Ann. Pharm.*, **21**, 201–205.
Pfeifer, S. (1962). *Pharmazie*, **17**, 298–301.
Rajagopolan, S. (1945). *J. org. Chem.*, **10**, 175–178.
Rakshit, J. N. (1919). *J. chem. Soc.*, **115**, 455–461.

STUDIES IN THE FIELD OF DIURETIC AGENTS

PART VIII. SOME MISCELLANEOUS DERIVATIVES

BY G. B. JACKMAN, V. PETROW, O. STEPHENSON AND A. M. WILD

From The British Drug Houses, Ltd., Graham Street, London, N.1

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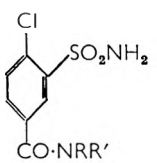
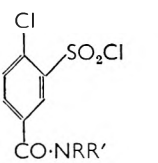
New derivatives of 4-chloro-3-sulphamoylbenzoic acid and of 4-chloro-3-sulphamoylaniline are described along with some formally related quinazolones.

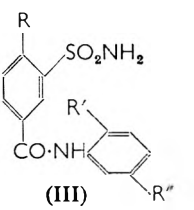
THE preparation of alkyl, dialkyl and heterocyclic amides of 4-chloro-3-sulphamoylbenzoic acid (I; R = H or alkyl, R′ = alkyl or aralkyl or NRR′ = heterocyclic radical) (compare Petrow, Stephenson and Wild, 1963) is extended herein. It was found that 4-chloro-3-chloro-sulphonylbenzoyl chloride reacted readily with the hydrochlorides of alkyl, dialkyl and heterocyclic amines in chlorobenzene at reflux temperature to give high yields of the intermediate 4-chloro-3-chlorosulphonylbenzamides (II). Even when up to 3 moles of the amine hydrochloride was employed and the heating period extended to 5 hr. no attack upon the sulphonochloride group was found to occur. Subsequent reaction of the sulphonchloride with ammonia furnished the sulphonamides listed in Table I.



(I)

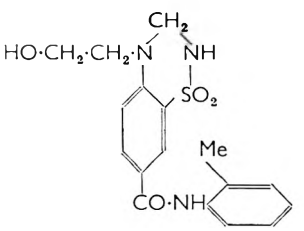


(II)

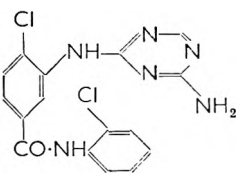


(III)

We had previously found that replacement of the chlorine atom in 5-chlorotoluene-2,4-disulphonamide by the 2-hydroxyethylamino-group yielded a water-soluble compound of equal diuretic potency. We consequently treated 4-chloro-2′-methyl-3-sulphamoylbenzanilide (III; R = Cl, R′ = Me, R″ = H) (Petrow and others, 1963) with 2-aminoethanol when smooth reaction occurred to give 4-(2-hydroxyethylamino)-2′-methyl-3-sulphamoylbenzanilide (III; R = NH·CH$_2$·CH$_2$·OH, R′ = Me, R″ = H) in high yield. This compound condensed with formaldehyde, as described earlier (Jackman, Petrow, Stephenson and Wild, 1960), to yield the 3,4-dihydrobenzothiadiazine derivative (IV).

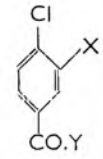


(IV)



(V)

TABLE I

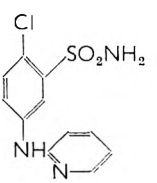


X	Y	m.p. °C	Formula	Found					Required				
				C	H	Cl	N	S	C	H	Cl	N	S
SO_2NH_2	C_4H_9·NH	171–172	$C_{11}H_{16}ClN_3O_3S$	45·2	4·7	12·1	9·7	11·2	45·4	5·2	12·2	9·6	11·0
SO_2Cl	Ph.CH_2.NH	150–152	$C_{14}H_{11}Cl_2NO_3S$	—	—	20·9	4·1	9·0	—	—	20·6	4·1	9·3
SO_2NH_2	Ph.CH_2.NH	185–187	$C_{14}H_{14}ClN_3O_3S$	51·7	4·2	10·9	8·8	10·1	51·8	4·0	10·9	8·6	9·9
SO_2NH_2	Me_4N	134–136	$C_9H_{14}ClN_3O_3S$	41·0	4·2	13·6	10·8	12·3	41·1	4·2	13·5	10·7	12·2
SO_2NH_2	Et_2N	174–176	$C_{11}H_{16}ClN_3O_3S$	45·5	5·3	12·3	9·6	10·7	45·4	5·2	12·2	9·6	11·0
SO_2Cl	Pyrrolidin-1-yl	153–155	$C_{11}H_{11}Cl_2NO_3S$	42·5	3·6	—	4·8	—	42·9	3·6	—	4·5	—
SO_2NH_2	Pyrrolidin-1-yl	191–192	$C_{11}H_{13}ClN_2O_3S$	45·6	4·5	—	9·7	11·3	45·8	4·5	—	9·7	11·1
SO_2Cl	1,2,3,6-Tetrahydropyrid-1-yl	115	$C_{12}H_{11}Cl_2NO_3S$	—	—	21·7	4·4	10·0	—	—	22·2	4·4	10·0
SO_2NH_2	1,2,3,6-Tetrahydropyrid-1-yl	157–159	$C_{12}H_{13}ClN_2O_3S$	47·6	4·3	—	8·8	—	47·9	4·4	—	9·3	—
SO_2Cl	Morpholino	131–133	$C_{11}H_{11}Cl_2NO_4S$	40·9	3·1	—	4·6	—	40·8	3·4	—	4·3	—
SO_2NH_2	Morpholino	230–231	$C_{11}H_{13}ClN_2O_4S$	43·1	4·2	11·2	—	—	43·4	4·3	11·6	—	—
SO_2Cl	Hexamethyleneimino-(Perhydroazepin-1-yl)	100	$C_{13}H_{16}Cl_2NO_3S$	—	—	—	4·5	—	—	—	—	4·2	—
SO_2NH_2	Hexamethyleneimino (Perhydroazepin-1-yl)	160–162	$C_{13}H_{17}ClN_2O_3S$	48·8	5·5	11·4	8·7	10·2	49·3	5·4	11·2	8·8	10·1

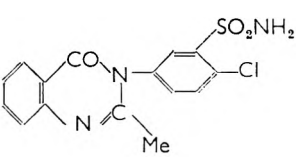
The effect of replacing the sulphamoyl group in the benzanilides (I; R = H, R′ = *o*-tolyl, *o*-chlorophenyl or 2,5-dichlorophenyl) by basic residues was next examined. The starting material in each case was the appropriate 3-amino-4-chlorobenzanilide (Petrow and others, 1963). The amino-group was replaced by an alkyl-ureido-group or by a biguanido-group, and in one case the biguanide was converted into the triazine (V) by reaction with formic acid.

The noteworthy diuretic activity possessed by 4-chloro-3-sulphamoylbenzoic acid (Jackman, Petrow, Stephenson and Wild, 1962) prompted further study of the effect of replacing the carboxyl group in this compound by other groups. With this object in view, 4-chloro-3-sulphamoylaniline (Petrow and others, 1960) was converted into acyl, aroyl [including 2-substituted aroyl derivatives which bear strong formal resemblance to the active benzanilide diuretics (III; R = Cl, R′ = Cl or Me and R″ = H or Cl)], succinoyl, phthaloyl, arylsulphonyl and ethoxycarbonyl derivatives. In addition the amino-group in 4-chloro-3-sulphamoylaniline was replaced by ureido-, substituted ureido-carbamoylureido-, and biguanido-groups. The latter derivative was converted into an amino-*s*-triazine by reaction with formic acid. Reaction of the amine with 2-bromo- or 2-chloro-pyridine furnished the 4-(2-aminopyridyl) derivative (VI).

Extending our earlier work on quinazolones (Jackman, Petrow and Stephenson, 1960) the synthesis of 2-methyl-3-(4-chloro-3-sulphamoylphenyl)-3*H*-4-quinazolone (VII) was carried out by reaction of acetanthranil with 4-chloro-3-sulphamoylaniline. The related 2-methyl-3-(4-sulphamoylphenyl)-3*H*-4-quinazolone was also prepared by reaction of *o*-acetamidobenzoic acid with 4-aminobenzenesulphonamide in pyridine, using benzenesulphonyl chloride as condensing agent (Jackman, Petrow and Stephenson, 1960).



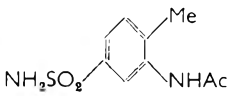
(VI)



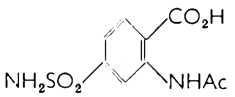
(VII)

Quinazolone derivatives containing a sulphamoyl group in the aromatic nucleus were next studied. 2-Methyl-7-sulphamoyl-3*H*-4-quinazolone (VIII) was prepared *via* acet-*o*-toluidide. This was chlorosulphonated and thence converted into the 4-sulphonamide (IX). The structure of the latter was proved by (*a*) hydrolysis and deamination to toluene-*p*-sulphonamide and (*b*) identity with the compound prepared by an unambiguous route (Petrow, Stephenson and Wild, 1960). Oxidation of the sulphonamide with alkaline potassium permanganate gave 2-acetamido-4-sulphamoylbenzoic acid (X), converted by acetic anhydride-acetic acid into 2-methyl-7-sulphamoyl-4*H*-3,1-benzoxaz-4-one (XI), which yielded the required quinazolone (VIII) on treatment with ammonia.

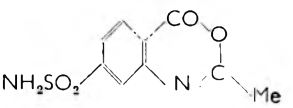
Inter alia 7-sulphamoylquinazol-2,4-dione (XII) was prepared by reaction of 2-amino-4-sulphamoylbenzoic acid with potassium cyanate in acid medium.



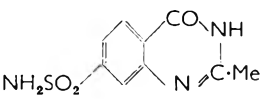
(IX)



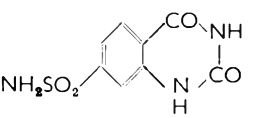
(X)



(XI)

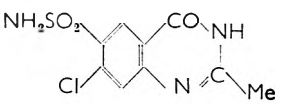


(VIII)

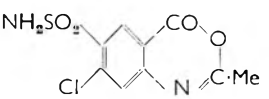


(XII)

Finally, we synthesised 7-chloro-2-methyl-6-sulphamoyl-3*H*-4-quinazolone (XIII), a compound more closely related to chlorothiazide. The synthesis employed 2-acetamido-4-chloro-5-sulphamoylbenzoic acid as starting material and proceeded *via* 7-chloro-2-methyl-6-sulphamoyl-4*H*-3,1-benzoxaz-4-one (XIV). Whilst this work was in progress the preparation of the quinazolone (XIII) by a similar process was reported by Cohen, Klarberg and Vaughan (1959, 1960).



(XIII)



(XIV)

Biological study of the above compounds by Dr. A. David and his colleagues failed to reveal a product superior to 4-chloro-2′-methyl-3-sulphamoylbenzanilide in diuretic potency.

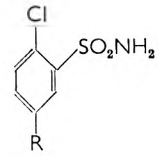
Experimental

A few of the compounds described below are included in the Tables (I and II) and in these cases analytical data is omitted.

4-*Chloro*-3-*sulphamoylbenzdiethylamide*. (*a*) A solution of 4-chloro-3-chlorosulphonylbenzoyl chloride (18·2 g.) in chlorobenzene (60 ml.) was treated with diethylamine hydrochloride (7·3 g.) and the mixture boiled under reflux for 2·5 hr.; the excess of chlorobenzene was then distilled off at reduced pressure. The residual oil was dissolved in chloroform (50 ml.) and the solution added with stirring to liquid ammonia (200 ml.). The ammonia and chloroform were boiled off, the residue dissolved in water (200 ml.) and the solution acidified with hydrochloric acid. The *product* (16·5 g.) had m.p. 174–176° (from ethanol).

(*b*) A solution of 4-chloro-3-chlorosulphonylbenzoyl chloride (18·2 g.) in chlorobenzene (60 ml.) was treated with diethylamine hydrochloride (21·9 g. = 3 mole. equivs.) and the mixture boiled under reflux for 5 hr. The reaction was then completed as in (*a*) to yield the same *product* (16·7 g.), m.p. 174 to 176° (from ethanol).

TABLE II



R	m.p. ° C	Formula	Found					Required				
			C	H	Cl	N	S	C	H	Cl	N	S
NH_2·HCl	236 (*d*)	$C_6H_6Cl_2N_2O_2S$	29·7	3·5	—	—	—	29·6	3·3	—	—	—
N:CH·Ph	146–148	$C_{13}H_{11}ClN_2O_2S$	53·0	3·9	12·2	9·6	—	52·9	3·8	12·0	9·5	—
NH·Ac	252–253	$C_8H_9ClN_2O_3S$	38·6	3·4	—	11·4	12·9	38·6	3·7	—	11·3	12·9
NH·CO·$CHCl_2$	199–200	$C_9H_7Cl_3N_2O_3S$	30·6	2·4	33·6	9·1	—	30·2	2·2	33·5	8·8	—
NH·CO·Ph	281(*d*)	$C_{13}H_{11}ClN_2O_3S$	50·5	3·6	11·6	9·1	10·6	50·2	3·6	11·4	8·8	10·3
NH·CO·C_6H_4·Me(*o*)	181–182	$C_{14}H_{13}ClN_2O_3S$	51·6	4·2	—	9·0	10·3	51·8	4·0	—	8·6	9·9
NH·CO·C_6H_4Cl(*o*)	210	$C_{13}H_{10}Cl_2N_2O_3S$	45·2	2·7	—	8·1	9·4	45·2	2·9	—	8·1	9·3
NH·CO·C_6H_4·Cl(*p*)	288–289	$C_{13}H_{10}Cl_2N_2O_3S$	45·0	2·6	20·2	7·8	—	45·2	2·9	20·6	8·1	—
NH·CO·CH_2·CH_2·COOH	214(*d*)	$C_{10}H_{11}ClN_2O_5S$	39·4	3·9	11·7	9·2	—	39·1	3·6	11·6	9·1	—
Phthalimido	264–265	$C_{14}H_9ClN_2O_4S$	49·8	3·1	10·7	8·3	—	49·9	2·7	10·5	8·3	—
NH·SO_2·Ph	184–185	$C_{12}H_{11}ClN_2O_4S_2$	41·6	3·0	10·5	8·1	18·1	41·5	3·2	10·2	8·1	18·5
NH·CO·OEt	199–201	$C_9H_{11}ClN_2O_4S$	39·2	4·3	—	10·3	11·5	38·8	4·0	—	10·1	11·5
NH·CO·NH_2	195–196(*d*)	$C_7H_8ClN_3O_3S$	33·4	3·3	—	16·8	13·1	33·7	3·2	—	16·8	12·8
NH·CO·NH·Pr(n)	218	$C_{10}H_{14}ClN_3O_3S$	41·3	4·9	12·2	14·1	—	41·2	4·8	12·2	14·4	—
NH·CO·NH·Bu(n)	189–190	$C_{11}H_{16}ClN_3O_3S$	43·4	5·3	11·4	13·7	—	43·2	5·3	11·6	13·7	—
NH·CO·NH·Bu(s)	181–182	$C_{11}H_{16}ClN_3O_3S$	43·1	4·9	11·6	13·9	—	43·2	5·3	11·6	13·7	—
NH·CO·NH·C_6H_{11}	203–204	$C_{13}H_{18}ClN_3O_3S$*	46·7	5·6	10·6	12·4	—	47·0	5·5	10·7	12·7	—
NH·CO·NH·Ph	222–223	$C_{13}H_{12}ClN_3O_3S$	48·0	3·6	—	12·8	—	47·9	3·7	—	12·9	—
NH·CO·NH·CO·NH_2	232–233(*d*)	$C_8H_8ClN_4O_4S$	32·7	2·8	12·0	19·4	—	32·8	3·1	12·1	19·1	—
NH·C(:NH)·NH·C(:NH)·NH_2·HCl	226	$C_8H_{12}Cl_2N_6O_2S$	29·2	4·0	21·6	25·8	—	29·4	3·7	21·7	25·7	—
4-Amino-1,3,5-triazin-2-yl hydrochloride	297(*d*)	$C_9H_{10}Cl_2N_6O_4S$	32·0	2·9	20·9	—	9·6	32·0	3·0	21·1	—	9·5
Pyrid-2-ylamino	252–253	$C_{11}H_{10}ClN_3O_2S$	46·4	3·3	12·8	14·9	11·5	46·5	3·6	12·5	14·8	11·3

* Compound readily forms a monohydrate.

4-*Chloro*-3-*chlorosulphonylbenzmorpholide*. A solution of 4-chloro-3-chlorosulphonylbenzoyl chloride (18·2 g.) in chlorobenzene (60 ml.) was treated with morpholine hydrochloride (8·25 g.) and the mixture heated under reflux for 2 hr.; the excess of chlorobenzene was then distilled off at reduced pressure. Crystallisation of the residue from 1,2-dichloroethane-light petroleum (b.p. 60 to 80°) yielded the *product* (19·0 g.), m.p. 131–133°.

4-*Chloro*-3-*sulphamoylbenzmorpholide*. The foregoing sulphonchloride (17 g.) was added with stirring to liquid ammonia (200 ml.) and the excess of ammonia was boiled off. The residue was dissolved in water and the solution acidified with hydrochloric acid to yield the *product* (15·3 g.), m.p. 230–231° (from aqueous ethanol).

4-*Chloro*-3-*dimethylsulphamoylbenzdimethylamide*. 4-Chloro-3-chlorosulphonylbenzoyl chloride (9·1 g.) was added in portions to aqueous dimethylamine (36 ml., 25 per cent w/v) and, after the addition was complete, excess of amine was boiled off. Crystallisation of the residue from acetone–light petroleum (b.p. 60–80°) yielded the *product* (7·25 g.), m.p. 92–93°. Found: C, 45·4; H, 5·1; Cl, 12·3; N, 9·6; S, 10·6. $C_{11}H_{15}ClN_2O_3S$ requires C, 45·4; H, 5·2; Cl, 12·2; N, 9·6; S, 11·0 per cent.

4-(2-*Hydroxyethylamino*)-2′-*methyl*-3-*sulphamoylbenzanilide*. A solution of 4-chloro-2′-methyl-3-sulphamoylbenzanilide (20 g.) in 2-hydroxyethylamine (20 ml.) was heated at 120° for 4 hr. and the excess of amine was then distilled off at reduced pressure. The residue was dissolved in aqueous ethanol and acidified with dilute hydrochloric acid. The *product* (19·35 g.) had m.p. 171–172° (from aqueous ethanol). Found: C, 54·6; H, 5·1; N, 12·0; S, 8·8. $C_{16}H_{19}N_3O_4S$ requires C, 55·0; H, 5·5; N, 12·0; S, 9·2 per cent.

3,4-*Dihydro*-4-(2-*hydroxyethyl*)-7-o-*tolylcarbamoyl*-1,2,4-*benzothiadiazine* 1,1-*dioxide*. A solution of the foregoing compound (7 g.) in 2-ethoxyethanol (50 ml.) was treated with 40 per cent formaldehyde solution (2 ml.) and a saturated solution of hydrogen chloride in 2-ethoxyethanol (1 ml.) added as catalyst. The mixture was heated under reflux for 1 hr., and the excess of solvent was then distilled off at reduced pressure. The residual solid was crystallised from 25 per cent ethanol to yield the *product*, m.p. 242–243°. Found: C, 56·5; H, 5·3; N, 11·6; S, 8·9. $C_{17}H_{19}N_3O_4S$ requires C, 56·4; H, 5·3; N, 11·6; S, 8·9 per cent.

4,2′-*Dichloro*-3-*nitrobenzanilide*. A solution of 4-chloro-3-nitrobenzoyl chloride (40 g.) in chloroform (250 ml.) was stirred and treated with a solution of *o*-chloroaniline (51 g.) in chloroform (100 ml.) during 20 min. with cooling below 15°. After 1 hr. water was added to dissolve the *o*-chloroaniline hydrochloride and the *product* (43·6 g.) was collected. Concentration of the chloroform layer yielded a further crop of 16·2 g. It had m.p. 140° (from aqueous ethanol). Found: C, 50·4; H, 2·4; Cl, 22·8; N, 8·9. $C_{13}H_8Cl_2N_2O_3$ requires C, 50·2; H, 2·6; Cl, 22·8; N, 9·0 per cent.

3-*Amino*-4,2′-*dichlorobenzanilide*. A solution of the foregoing nitrocompound (54 g.) in ethanol (100 ml.), water (400 ml.) and acetic acid

(3 ml.) was heated with stirring under reflux and iron powder (50 g.) added in portions during 30 min. The mixture was heated for 5 hr. and filtered hot. The product (29·4 g.) separated on cooling and had m.p. 144° (from aqueous ethanol). Found: C, 55·3; H, 3·8; N, 9·9. $C_{13}H_{10}Cl_2N_2O$ requires C, 55·5; H, 3·6; N, 10·0 per cent.

4,2′,5′-*Trichloro*-3-*nitrobenzanilide* was obtained in 90 per cent yield by reaction of 4-chloro-3-nitrobenzoyl chloride with 2,5-dichloroaniline in chloroformic solution. It had m.p. 177–178° (from aqueous ethanol). Found: C, 45·3; H, 1·9; Cl, 30·8; N, 8·1. $C_{13}H_7Cl_3N_2O_3$ requires C, 45·2; H, 2·0; Cl, 30·8; N, 8·1 per cent.

3-*Amino*-4,2′,5′-*trichlorobenzanilide*, obtained by reduction of the foregoing nitro-compound, had m.p. 167–168° (from aqueous ethanol). Found: Cl, 33·5; N, 8·6. $C_{13}H_9Cl_3N_2O$ requires Cl, 33·7; N, 8·9 per cent.

3-(3-*Butylureido*)-4-*chloro*-2′-*methylbenzanilide*. A mixture of 3-amino-4-chloro-2′-methylbenzanilide (5·2 g.) and butyl *iso*cyanate (5 g.) in dioxan (10 ml.) was heated on the steam-bath for 10 hr. The dioxan was distilled off under reduced pressure and the residual solid crystallised from aqueous methanol to yield the *product*, m.p. 192–194°. Found: C, 63·1; H, 5·8; Cl, 10·3; N, 11·7. $C_{19}H_{22}ClN_3O_2$ requires C, 63·4; H, 6·2; Cl, 9·9; N, 11·7 per cent.

3-(3-*s*-*Butylureido*)-4-*chloro*-2′-*methylbenzanilide* had m.p. 215° (from aqueous ethanol). Found: C, 63·2; H, 6·1; N, 11·9 per cent.

3-(3-*Butylureido*)-4,2′-*dichlorobenzanilide*, obtained by reaction of 3-amino-4,2′-dichlorobenzanilide with butyl *iso*cyanate in dioxan, had m.p. 171–172° (from aqueous methanol). Found: C, 56·4; H, 5·0; Cl, 18·4; N, 11·0. $C_{18}H_{19}Cl_2N_3O_2$ requires C, 56·9; H, 5·0; Cl, 18·6; N, 11·1 per cent.

3-*Biguanido*-4,2′-*dichlorobenzanilide hydrochloride*. A solution of 3-amino-4,2′-dichlorobenzanilide (28·1 g.) in ethanol (50 ml.) was treated with dicyandiamide (8·4 g.), followed by a solution of hydrogen chloride (4 g.) in ethanol (20 ml.). The mixture was heated under reflux for 90 min. After cooling, the *product* (22 g.) was collected. It had m.p. 245–247° (from ethanol). Found: C, 45·3; H, 3·7; Cl, 26·7; N, 20·9. $C_{15}H_{15}Cl_3N_6O$ requires C, 44·8; H, 3·8; Cl, 26·5; N, 20·9 per cent.

3-(4-*Amino*-1,3,5-*triazin*-2-*ylamino*)-4,2′-*dichlorobenzanilide*. A solution of the foregoing biguanide hydrochloride (10·05 g.) in formic acid (40 ml.) was heated under reflux for 2 hr., and the excess of formic acid was then distilled off at reduced pressure. The *product* (9·8 g.) had m.p 214–215° (from aqueous methanol). Found: C, 51·3; H, 3·0; N, 22·2. $C_{16}H_{12}Cl_2N_6O$ requires C, 51·2; H, 3·2; N, 22·4 per cent.

N-(4-*Chloro*-3-*sulphamoylphenyl*)*phthalimide*. Phthalic anhydride (7·4 g.) was added during 10 min. to a stirred suspension of 4-chloro-3-sulphamoylaniline (10·3 g.) in water (200 ml.) at 80–90°, and the heating was continued for 6 hr. After cooling, the *product* (12·5 g.) was collected. It had m.p. 264–265° (from aqueous acetone).

4′-*Chloro*-3′-*sulphamoylbenzenesulphonanilide*. Benzenesulphonyl chloride (7·1 g.) was added in portions with cooling to a solution of 4-chloro-3-sulphamoylaniline (8·3 g.) in pyridine (30 ml.). The mixture was

warmed for 15 min. then cooled, diluted with ice-water and acidified with hydrochloric acid. The *product* (10·6 g.) had m.p. 184–185° (from ethanol-benzene).

1-(4-*Chloro*-3-*sulphamoylphenyl*)*biuret*. A solution of 4-chloro-3-sulphamoylaniline (10·3 g.) in water (100 ml.) and ethanol (70 ml.) was heated to 90° and treated with nitrobiuret (10 g.), added during 5 min. Heating was continued for 2 hr. and most of the ethanol was allowed to evaporate. The *product* (10 g.) separated out on cooling. It had m.p. 232–233° (decomp.) (from aqueous ethanol).

2-(4-*Chloro*-3-*sulphamoylanilino*)*pyridine*. A mixture of 4-chloro-3-sulphamoylaniline (10·3 g.) and 2-chloropyridine (5·7 g.) was heated at 165° for 2 hr. The dark residue was dissolved in 80 per cent ethanol and buffered with sodium acetate. The *product* (12 g.) had m.p. 252–253° (from ethanol containing a trace of acetone). Use of 2-bromopyridine in place of 2-chloropyridine gave a somewhat lower yield of product.

3-(4-*Chloro*-3-*sulphamoylphenyl*)-2-*methyl*-3H-4-*quinazolone*. A mixture of 4-chloro-3-sulphamoylaniline (5·17 g.) and acetanthranil (4 g.) was heated at 130° for 3 hr. The resinous residue solidified on boiling with ethanol. The *product* had m.p. 295–296° (from aqueous ethoxyethanol). Found: C, 51·5; H, 3·4; Cl, 10·4; N, 12·2; S, 9·5. $C_{15}H_{12}ClN_3O_3S$ requires C, 51·5; H, 3·5; Cl, 10·1; N, 12·0; S, 9·2 per cent.

2-*Acetamido*-4-*sulphamoyltoluene*. Acet-*o*-toluidide (292 g.) was added slowly with stirring to chlorosulphonic acid (1165 g.) with cooling to keep reaction temperature below 80° and the mixture was kept at 70–75° for 3 hr. It was then cooled and poured into ice water. The crude sulphonchloride was washed with water, drained and added to liquid ammonia (2 litres). After the ammonia had evaporated the residue was dissolved in water (1 litre) and the solution acidified to pH 4 with hydrochloric acid. The *product* (124 g.) was crystallised from water and then from glacial acetic acid; it had m.p. 230–232° (decomp.).

The foregoing compound (4·6 g.) was refluxed with acetic anhydride (14 ml.) for 1 hr. The solid *triacetyl* derivative (4·9 g.) had m.p. 223–225° after crystallisation from a large volume of water. Found: C, 49·6; H, 5·0; N, 9·1; S, 10·0. $C_{13}H_{16}N_2O_5S$ requires C, 50·0; H, 5·2; N, 9·0; S, 10·3 per cent. The *diacetyl* derivative, m.p. 231–233° (from water) was obtained by refluxing the *monoacetyl* derivative (4·6 g.) with acetic acid (22 ml.) and acetic anhydride (5·5 ml.) for 30 min. or by boiling the triacetyl derivative (1·5 g.) with water (450 ml.) for 4 hr. Found: C, 48·5; H, 5·0; N, 10·5; S, 12·2. $C_{11}H_{14}N_2O_4S$ requires C, 48·9; H, 5·2; N, 10·3; S, 11·9 per cent. Hydrolysis of 2-acetamidotoluene-4-sulphonamide (46 g.) with 5N sodium hydroxide solution (500 ml.) for 2½ hr. yielded 2-aminotoluene-4-sulphonamide (26 g.), m.p. 177–179° (from water). A solution of the amine (4·6 g.) in water (50 ml.) and concentrated hydrochloric acid (7 ml.) was diazotised at 5° by the addition of a solution of sodium nitrite (1·8 g.) in water (10 ml.). The diazo-solution was cooled to 0–5° and treated with 40–50 per cent hypophosphorous acid (44 ml.). After several hours the solid (3·4 g.) which had separated was

collected. It had m.p. 135–137°, not depressed on admixture with *toluene*-p-*sulphonamide*.

2-*Acetamido*-4-*sulphamoylbenzoic acid*, prepared in 45 per cent yield by oxidation of 2-acetamidotoluene-4-sulphonamide with potassium permanganate (6·7 mole) in aqueous alkaline solution at below 80°, had m.p. 230° (decomp.) (from water). Found: N, 10·9; S, 12·5. Calc. for $C_9H_{10}N_2O_5S$: N, 10·8; S, 12·4 per cent.

2-*Methyl*-7-*sulphamoyl*-4H-3,1-*benzoxaz*-4-*one*. The foregoing acid (2·6 g.) was heated under reflux with acetic acid (8 ml.) and acetic anhydride (1·9 ml.) for 20 min. The *product* had m.p. 218° (decomp.) (from acetic acid-acetic anhydride [4:1]). Found: C, 44·8; H, 3·6; S, 13·0. $C_9H_8N_2O_4S$ requires C, 45·0; H, 3·4; S, 13·3 per cent.

2-*Methyl*-7-*sulphamoyl*-3H-4-*quinazolone*. The foregoing compound (8 g.) was added to 10N ammonia (64 ml.) and the mixture warmed on the steam-bath until the solid was almost dissolved. A solution of sodium hydroxide (10 g.) in water (136 ml.) was then added and the mixture heated under reflux for 30 min. The mixture was cooled slightly, saturated with carbon dioxide, and the *product* (5·6 g.) collected; it had m.p. 315° (decomp.) (from a large volume of water). It was soluble in sodium carbonate solution but insoluble in sodium bicarbonate solution. Found: C, 45·3; H, 3·6; N, 18·2; S, 13·3. $C_9H_9N_3O_3S$ requires C, 45·2; H, 3·8; N, 17·6; S, 13·4 per cent.

2-*Amino*-4-*sulphamoylbenzoic acid*, obtained by hydrolysis of the acetamido-compound with concentrated hydrochloric acid, had m.p. 233° (decomp.) (from water). Found: C, 38·5; H, 3·9; N, 12·7. Calc. for $C_7H_8N_2O_4S$: C, 38·9; H, 3·7; N, 13·0 per cent.

7-*Sulphamoylquinazol*-2,4-*dione*. The foregoing compound (21·6 g.) was dissolved in a mixture of water (400 ml.) and acetic acid (30 ml.) at 60° and treated with a solution of potassium cyanate (10 g.) in water (50 ml.). Concentrated sulphuric acid (50 ml.) was then added and the mixture heated under reflux for 30 min. After cooling, the product (15 g.) was collected. It had m.p. *ca.* 400° (decomp.) after crystallisation from a large volume of water. Found: C, 40·2; H, 3·2; N, 17·1; S, 13·2. $C_8H_7N_3O_4S$ requires C, 39·8; H, 2·9; N, 17·4; S, 13·3 per cent.

2-*Acetamido*-4-*chlorotoluene*-5-*sulphonamide*. 2-Acetamido-4-chlorotoluene (61 g.) was added with stirring to chlorosulphonic acid (195 g.) at 75–80° and the mixture was heated at 95–100° for 3 hr. After cooling the mixture was poured on to crushed ice and the solid sulphonchloride collected, washed with cold water and added to liquid ammonia (1 litre). The *product* (58 g.), isolated in the usual way, had m.p. 250° (decomp.) (from water). Found: C, 41·5; H, 4·2; Cl, 13·1; N, 10·5; S, 12·1. Calc. for $C_9H_{11}ClN_2O_3S$: C, 41·2; H, 4·2; Cl, 13·5; N, 10·7; S, 12·2 per cent.

2-*Acetamido*-4-*chloro*-5-*sulphamoylbenzoic acid* was obtained in 45 per cent yield by oxidation of the foregoing compound with potassium permanganate (6·7 mole.) in aqueous alkaline solution at below 70°. It had m.p. 257° (decomp.) (from water). Found: C, 37·1; H, 3·3; Cl, 12·0; S, 10·8. $C_9H_9ClN_2O_5S$ requires C, 36·9; H, 3·1; Cl, 12·1; S, 10·9 per cent.

7-*Chloro*-2-*methyl*-6-*sulphamoyl*-4H-3,1-*benzoxaz*-4-*one*. The foregoing acid (8·8 g.) was heated under reflux for 20 min. with a mixture of acetic acid (24 ml.) and acetic anhydride (5·7 ml.), and after cooling the *product* (5·4 g.) was collected. It had m.p. 240° (decomp.) from acetic acid-acetic anhydride (4:1). Found: C, 39·2; H, 2·3; Cl, 12·7; S, 11·9. $C_9H_7ClN_2O_4S$ requires C, 39·4; H, 2·6; Cl, 12·9; S, 11·7 per cent.

7-*Chloro*-2-*methyl*-6-*sulphamoyl*-3H-4-*quinazolone*. The foregoing benzoxazone (5·4 g.) was added to 10N ammonia (48 ml.) and the mixture warmed until most of the solid was dissolved. A solution of sodium hydroxide (10 g.) in water (136 ml.) was then added and the mixture heated under reflux for 30 min. It was then cooled slightly and saturated with carbon dioxide. The *product* (3·7 g.) was collected. It had m.p. 345° (decomp.) after crystallisation from a large volume of water. Found: C, 40·0; H, 2·7; Cl, 13·2; S, 11·9. Calc. for $C_9H_8ClN_3O_3S$: C, 39·5; H, 2·9; Cl, 13·0; S, 11·7 per cent.

2-*Methyl*-3-p-*sulphamoylphenyl*-3H-4-*quinazolone*. Benzenesulphonyl chloride (17·8 g.) was added during 5 min. to a solution of *o*-acetamidobenzoic acid (17·9 g.) in pyridine (35 ml.) without cooling. A suspension of *p*-aminobenzenesulphonamide (17·1 g.) in pyridine (15 ml.) was then added to the mixture, which was heated on the steam-bath for 1 hr. It was then cooled, poured into ice water and the *product* (14·5 g.) crystallised from ethanol. It had m.p. 256°. Found: C, 57·2; H, 4·1; N, 13·0; S, 10·3. $C_{15}H_{13}N_3O_3S$ requires C, 57·1; H, 4·2; N, 13·3; S, 10·2 per cent.

References

Cohen, E., Klarberg, B. and Vaughan, J. R. (1959). *J. Amer. chem. Soc.*, **81**, 5508–5509.
Cohen, E., Klarberg, B. and Vaughan, J. R. (1960). *Ibid.*, **82**, 2731–2735.
Jackman, G. B., Petrow, V. and Stephenson, O. (1960). *J. Pharm. Pharmacol.*, **12**, 529–538.
Jackman, G. B., Petrow, V., Stephenson, O. and Wild, A. M. (1960). *Ibid.*, **12**, 648–655.
Jackman, G. B., Petrow, V., Stephenson, O. and Wild, A. M. (1962). *Ibid.*, **14**, 679–686.
Petrow, V., Stephenson, O. and Wild, A. M. (1960). *Ibid.*, **12**, 705–719.
Petrow, V., Stephenson, O. and Wild, A. M. (1963). *Ibid.*, **15**, 138–148.

A NOTE ON THE METABOLISM OF *o*-(2-ETHOXYETHOXY)-BENZAMIDE TO *o*-(CARBAMOYL)PHENOXYACETIC ACID IN MAN

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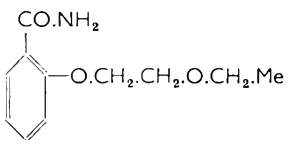
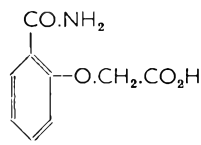
After the oral administration of *o*-(2-ethoxyethoxy)benzamide to man, the only metabolic product detected in the urine was *o*-carbamoylphenoxyacetic acid.

IN a study of the metabolism of derivatives of salicylamide in man, *o*-(2-ethoxyethoxy)benzamide (I) was investigated. Following the oral administration of this compound, *o*-carbamoylphenoxyacetic acid (II) was isolated from the urine.



I



II

This type of metabolic conversion, involving the rupture of an aliphatic ether linkage, does not appear to have been previously described in man.

EXPERIMENTAL

o-(2-Ethoxyethoxy)benzamide (0·5–1·0 g.) was administered orally in gelatin capsules to two male human subjects and a total urine collection was then made for 12 hr., most of the drug being eliminated within this time. The urine, acidified to approximately pH 2 with concentrated hydrochloric acid, was continuously extracted with ether for 12 hr. The white solid which accumulated in the ether reservoir was collected by filtration. The ether extract was evaporated to dryness under reduced pressure and yielded a pale brown powder. Approximately 0·7 g of solid material, from filtration and after drying, was isolated from the urine of one subject who had received 1 g. of *o*-(2-ethoxyethoxy)benzamide.

The material isolated was dissolved in 30–50 ml. of 0·1M phosphate buffer (pH 7·4) and filtered. The metabolite was precipitated from the filtrate by acidifying with 5N hydrochloric acid, and was recrystallised twice from water after decolorising with activated charcoal. The white crystalline product melted at 210–214°.

The extracted urine was hydrolysed with sulphuric acid (final concentration, 4N) by boiling under a reflux condenser for 3 hr. The hydrolysed urine was then re-extracted with ether as above.

Glucuronic acid and conjugated glycine in urine were determined by the methods of Fishman and others (1951) and of Tompsett (1961) respectively.

Chromatograms of a methanol solution of the residue of ether extracts of the urines were run on Whatman No. 1 paper using as a solvent system, butanol saturated with 1·5N aqueous ammonia solution. The chromatograms were examined in ultra-violet light after drying at room temperature.

Results

The metabolite isolated from the urine was an acid, but no free phenolic group could be detected. The infra-red spectrum indicated the presence of an amido-group and a carboxyl group. Found: C. 54·9, H. 4·6 and N. 6·9 per cent. Equivalent wt. (titration) $\simeq$200. $C_9H_9ON_4$ requires C. 55·3, H. 4·6 and N. 7·2 per cent.

The compound with properties most consistent with the results and which could reasonably be expected to be derived from *o*-(2-ethoxyethoxy)-benzamide was considered to be *o*-carbamoylphenoxyacetic acid: mixed m.p. with an authentic sample ("salicylamide-*O*-acetic acid", Aldrich Chemical Co. Inc., m.p. 213–215°), was 210–214°. The infra-red spectrum of the metabolite was identical with that of the authentic compound.

A precise method for the determination of *o*-carbamoylphenoxyacetic acid in urine was not available, and the proportion of the administered *o*-(2-ethoxyethoxy)benzamide excreted in this form could not be accurately determined. However, some idea was obtained by extracting the urine (acidified to pH 2) with ether, then shaking the ether extracts with 0·1M phosphate (pH 7·4) and measuring the extinction of the buffer at 292 mμ.

The results of an excretion study in one individual indicated that about half the dose of *o*-(2-ethoxyethoxy)benzamide was excreted in 4 hr.

Chromatography of the ether extracts of the original urines, before and after hydrolysis, failed to detect any salicylic acid, salicylamide or gentisic acid. There was no significant increase in the amount of glucuronic acid or conjugated glycine excreted in the urine during the period of maximum excretion of *o*-carbamoylphenoxyacetic acid. No other metabolites or unchanged drug have so far been detected in the urine.

Discussion

The metabolic conversion of *o*-(2-ethoxyethoxy)benzamide to *o*-carbamoylphenoxyacetic acid is of interest since it indicates the presence in man of a mechanism capable of breaking an aliphatic ether linkage. While many examples of the *in vivo* dealkylation of methyl and ethyl aromatic ethers are known (Brodie and Axelrod, 1949; Smith and Williams, 1949; Bray, Craddock and Thorpe, 1955) little has been reported on the metabolic fate of aliphatic ethers. Buckle and Saunders (1949) have obtained some indirect evidence that fluorinated aliphatic ethers of the type $F\cdot[CH_2]_m\cdot O\cdot[CH_2]_n\cdot CO_2H$ undergo rupture of the ether linkage *in vivo*. Patterson (1949) has stated that the toxicity results obtained with ω-fluoroalkyl ethers provide circumstantial evidence of the rupture of these ethers in the animal body, and suggested that it is not unreasonable to extend these conclusions to their unfluorinated analogues.

Axelrod (1956) studied the enzymic systems, found in the liver of some mammalian species, which cleave alkyl-aromatic ethers to a phenol and

an aldehyde. Attempts to cleave aliphatic ethers such as 3-methoxypropylamine, methoxyacetic acid and dimethoxyethane with the same systems were unsuccessful.

Duncan and Scales (1961) have shown that 2-(2-methoxyethyl)pyridine is metabolised almost exclusively to pyrid-2-ylacetic acid in five animal species. This metabolic conversion is remarkably similar to the conversion of *o*-(2-ethoxyethoxy)benzamide to *o*-carbamoylphenoxyacetic acid. There is little evidence at present to indicate the metabolic pathway by which this latter conversion is accomplished. It is possible that the first step is a dealkylation, by a mechanism similar to that which cleaves alkyl-aromatic ethers, to yield a primary alcohol and an aldehyde. (It is noteworthy that (ω-1) hydroxylation would give a hemi-acetal, which would be expected to yield readily an alcohol and an aldehyde.) The primary alcohol so formed would then be expected to be rapidly oxidised to the corresponding acid, since it is known that 2-phenylethanol is largely converted to phenaceturic acid (Smith, Smithies and Williams, 1954).

The conversion of *o*-(2-ethoxyethoxy)benzamide to *o*-carbamoylphenoxyacetic acid is also of interest in providing evidence of the *in vivo* stability of the amido-group of salicylamide derivatives in man.

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REFERENCES

Axelrod, J. (1956). *Biochem. J.*, **63**, 634–639.
Bray, H. G., Craddock, V. M. and Thorpe, W. V. (1955). *Biochem. J.*, **60**, 225–232.
Brodie, B. B. and Axelrod J. (1949). *J. Pharmacol.*, **97**, 58–67.
Buckle, F. J. and Saunders, R. C. (1949). *J. chem. Soc.*, 2774–2778.
Duncan, W. A. M. and Scales, B. (1961). *Biochem. J.*, **80**, 1 P.
Fishman, W. H., Smith, M., Thompson, D. B., Bonner, C. D., Kasdon, S. C. and Homburger, F. (1951) *J. clin. Invest.*, **30**, 685–696.
Paterson, F. L. M. (1959). *Toxic Aliphatic Fluorine Compounds*, p. 138, Elsevier Monograph.
Smith, J. N., Smithies, R. H. and Williams, R.T. (1954). *Biochem. J.* **56**, 320–324.
Smith, J. N. and Williams, R. T. (1949). *Biochem. J.*, **44**, 239–242.
Tompsett, S. L. (1961). *J. Pharm. Pharmacol.*, **13**, 115–120.

LETTERS TO THE EDITOR

Detection of Tryptophan Synthesising Enzyme in *Rauwolfia serpentina* Benth

SIR,—The presence of tryptophan synthase, which catalyses the condensation of serine and indole to form tryptophan is mostly demonstrated in micro-organisms like neurospora (Tatum, 1944). The observation by Tyler and Schwarting (1954) on the availability of such a system in *Claviceps purpurea*, which forms indole alkaloids, is interesting. During a detailed study of the biosynthesis of indole alkaloids in *Rauwolfia serpentina* plants, tryptophan synthesising enzyme was detected.

Fresh homogenates of roots and leaves were prepared in M/15, phosphate buffer, pH 7·8, and the ratio of homogenate to buffer adjusted to 1 : 2. The activity of the enzyme was determined by measuring the rate of disappearance of indole and subsequent formation of tryptophan. Samples containing indole, serine, glutathione, pyridoxal phosphate, enzyme extract and the buffer, were incubated for 4 hr. and the unreacted free indole was extracted with toluene and estimated colorimetrically (Yanofsky, 1955). Tryptophan concentrations were measured in the remaining aqueous liquid by the method of Nason, Kaplan and Colowick (1951).

The content of tryptophan formed, corresponded to the quantity of indole disappearing during the reaction (Table I).

TABLE I

THE UTILISATION OF INDOLE AND THE FORMATION OF TRYPTOPHAN IN ROOTS AND LEAVES OF *R. serpentina*. THE FIGURES ARE THE AVERAGES OF FIVE EXPERIMENTS

	Indole added µg.	Indole recovery µg.	Tryptophan formed µg.	Indole disappeared per cent
Leaves	40	14	34	65
"	40	20	26	50
"	40	22	24	45
Root	40	28	16	30
"	40	32	10	20
"	40	28	14	30

Further confirmation of the presence of the enzyme in the plant and the specific utilisation of indole in the synthesis of tryptophan was obtained by the omission of serine, one of the substrates, and the addition of sodium cyanide—an enzyme inhibitor. In the absence of the serine, indole was utilised to the extent of 5 per cent compared with 50 per cent in control experiments. There was practically no change in the indole content in the presence of sodium cyanide. The observations clearly indicate the presence of tryptophan synthase in a higher plant, not hitherto reported in literature.

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REFERENCES

Nason, A., Kaplan, N. O. and Colowick, S. P. (1951). *J. biol. Chem.*, **188**, 397–406.
Tatum, E. L. and Bonner, D. (1944). *Proc. Natl. Acad. Sci.*, **30**, 30–37.
Tyler, V. E., Jr. and Schwarting, A. E. (1954). *J. Amer. pharm., Ass. Sci. Ed.*, **43**, 207–211.
Yanofsky, C. (1955). *Methods in Enzymology*, edited by Colowick, S. P. and Kaplan, N. O. 2nd. ed. p. 233. New York: Academic Press.

Determination of Polyoxyethylene Glycol in Non-Ionic Detergents

SIR,—When ethylene oxide is polymerised in the presence of a long chain alcohol, ROH, to give a non-ionic detergent, $R(OCH_2CH_2)_mOH$, in which m is the designated number of ethylene oxide units, traces of water in the reaction mixture can lead to the formation of polyoxyethylene glycols as by-products (Ginn, Church and Harris, 1961; Drew and Schaeffer, 1958). Several methods have been devised for estimating the amount of glycol formed (Ginn and others, 1961; Weibull, 1961), but these involve separating the glycol from the detergent. A means of estimating the glycol content without separation, based on two assay methods, is reported here.

The first method is that of Siggia, Starke, Garis and Stahl (1958) and determines the total ethylene oxide content of the mixture of glycol and detergent. In a mixture of weight, W_t, containing W_1 g. glycol of mean molecular weight M_1, and W_2 g. detergent of mean molecular weight M_2, the weight of ethylene oxide, z g. is

$$z = \frac{W_1(M_1 - 18{\cdot}01)}{M_1} + \frac{W_2(M_2 - y)}{M_2} \quad .. \quad .. \quad .. \quad (1)$$

where y = molecular weight of the long chain alcohol. In the present instance the detergents are based on hexadecyl alcohol, m.w. 242·45. The second assay method is the pharmacopoeial method for the assay of hydroxy groups in benzyl alcohol (B.P. 1958) but in which the time of heating is increased until the results are constant (6–8 hr.). This is used to determine the percentage of hydroxy groups present in the mixture. If W_t g. of mixture contains x m-equiv. of hydroxyl then:

$$x = 2000W_1/M_1 + 1000W_2/M_2 \quad .. \quad .. \quad .. \quad (2)$$

Remembering

$$W_t = W_1 + W_2 \quad .. \quad .. \quad .. \quad (3)$$

and substituting (2) in (1), either W_1 and M_1, or W_2 and M_2 can be eliminated, giving:

$$n_1 = \frac{W_1}{M_1} = \frac{z - W_t + 0{\cdot}2424x}{466{\cdot}89} \quad .. \quad .. \quad .. \quad (4)$$

$$n_2 = \frac{W_2}{M_2} = \frac{2W_t - 0{\cdot}018x - 2z}{466{\cdot}89} \quad .. \quad .. \quad .. \quad (5)$$

where n_1 and n_2 are the number of moles of glycol and detergent respectively, and the denominator of the right hand side arises by subtracting the molecular weight of water from twice the alcohol molecular weight. Equations (4) and (5) give a means of determining the number of moles of each component in a chosen weight of mixture.

Prepared mixtures of triethylene glycol and hexaoxyethylene monohexadecyl ether were subjected to the proposed procedure with the following results.

Mixture number	1	2	3
Mole per cent glycol added	7·1	10·1	15·0
Mole per cent glycol found	7·0	10·1	15·3

The results were satisfactory considering assay errors of ±1 per cent in the OH determination, and ±0·3 per cent in the ethylene oxide assay.

A series of commercially produced detergents based on hexadecyl alcohol (H), and containing m ethylene oxide units, was assayed.

Detergent	Hm_4	Hm_6	Hm_{10}	Hm_{16}	Hm_{24}
Mole per cent glycol found ..	0·5	1·2	2·5	5·1	7·0

The mole percentage of glycol increased as the hexadecyl alcohol was reacted with increasing amounts of ethylene oxide. This increase may be due to the presence of traces of water in the ethylene oxide.

Hm_{23} corresponds to Cetomacrogol 1000, and the procedure may be used to determine the polyoxyethylene glycol present in this material.

I thank Miss M. Buchanan for technical assistance.

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REFERENCES

Drew, H. F. and Shaeffer, J. R. (1958). *Indust. Engng Chem.*, **50**, 1253–1254.
Ginn, M. E., Church, C. L. and Harris, J. C. (1961). *Analyt. Chem.*, **33**, 143–145.
Siggia, S., Starke, A. C., Garis, J. J. and Stahl, C. R. (1958). *Ibid.*, **30**, 115–116.
Weibull, B. (1961). Third International Congress on Surface Activity, Vol. III, p. 121–124. Mainz: University Press.

Analeptic Activity of Tremor-producing Amino-Alcohols

SIR,—Ahmed, Marshall and Shepherd (1958) described a series of amino-alcohols capable of inducing tremor when injected into mice. In higher doses, these compounds produced convulsions similar to those of picrotoxin, and consequently two of the compounds have now been tested for analeptic activity against pentobarbitone anaesthesia by the mouse-awakening test of Goodwin and Marshall (1945).

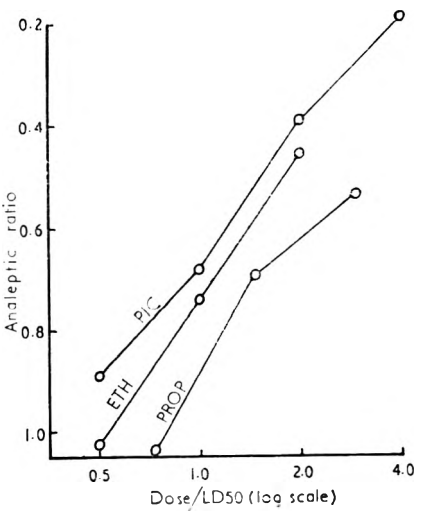

Fig. 1. Awakening time for mice under pentobarbitone anaesthesia.
Analeptic ratio = median awakening time for mice receiving analeptic/median awakening time for controls.
PIC = picrotoxin (from Goodwin and Marshall, 1945);
ETH = 1,1,2-triphenyl-2-amino-ethanol;
PROP = 1,1,3-triphenyl-3-amino-propan-1-ol.

The compounds chosen were 1,1,3-triphenyl-3-amino-propan-1-ol (PROP), the most active of the series in producing tremor, and 1,1,2-triphenyl-2-amino-ethanol (ETH), which had about one half the tremor producing activity of PROP. The median lethal doses (LD50) of ETH and PROP when injected intravenously into mice were respectively 24·5 and 17·5 mg./kg., compared with 4·5 mg./kg. for picrotoxin (Goodwin and Marshall, 1945). In Fig. 1 the analeptic potency, expressed as the analeptic ratio is plotted against log dose as a ratio of the LD50, and the curves for the two amino-alcohols are compared with that obtained for picrotoxin by Goodwin and Marshall (1945).

The analeptic potency of ETH and PROP was of the opposite order to their tremor producing potencies, ETH having about 80 per cent and PROP about 50 per cent of the activity of picrotoxin. The close parallelism between the curves for the amino-alcohols and for picrotoxin suggests a similar mechanism of action in antagonising barbiturate anaesthesia. The amino-alcohols also resemble picrotoxin in that the analeptic effect appears only in doses equal to or exceeding the intravenous median lethal dose.

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REFERENCES

Ahmed, A., Marshall, P. B. and Shepherd, D. M. (1958). *J. Pharm. Pharmacol.*, **10**, 672–682.
Goodwin, L. G. and Marshall, P. B. (1945). *J. Pharmacol.*, **84**, 12–15.

BOOK REVIEWS

METHODEN DER ORGANISCHEN CHEMIE (Houben-Weyl). Fourth Edition. Edited by Eugen Müller. Volume V, Part 3. Halogenverbindungen. Fluorverbindungen (Herstellung, Reaktivität und Umwandlung), Chlorverbindugen (Herstellung). Pp. lxv — 1217 (including 30 illustrations, 184 Tables, and Index). Georg Thieme Verlag, Stuttgart, 1962. Moleskin, DM. 262.00.

The present work (Volume 5/3), one of four books comprising volume five of the series, is the companion work to volume 5/4 which was published in 1960. Taken together, these two books provide a comprehensive survey of the chemistry of organic halogen derivatives. The need for this subdivision has arisen because of the enormous growth of fluorocarbon chemistry in the last decade, and almost half the present work is devoted to the preparation and properties of fluorine compounds. Significantly, the reactions of organic fluorine compounds are given a separate treatment from those of the other organo-halogen compounds which are discussed as a related group in the companion volume (5/4). This division reflects the atypical properties of organo-fluorine compounds which distinguish them from other organo-halogen derivatives, and which stem from the special position of fluorine in the first short period of the periodic table. In common with all the earlier volumes of this remarkable series, the treatment is extremely comprehensive and extensively referenced, though sufficient detail of preparative methods is given to enable all the more important preparations to be undertaken without reference elsewhere. Preparative methods are broadly classified into those based on the use of elementary and electrolytically-released fluorine, and inorganic fluorine compounds, on the one hand, and, on the other hand, on the use of organo-fluorine reagents. The latter group includes addition and elimination reactions, cleavage of carbon-carbon, carbon-nitrogen, nitrogen-oxygen and oxygen-oxygen bonds, isomerisation, disproportionation, and oxidation.

The remainder of the book is devoted to a similarly detailed and comprehensive review of methods for the preparation of organic chlorine compounds, the broad basis of classification being similar to that used for fluorine compounds. Despite the inconvenience of a German text to the English reader, the essential information is clearly set out, and easy to find through detailed contents pages and extensive author and subject indexes. The cost of these works almost certainly places them beyond the reach of most individual purchasers, but there can be no doubt of their value as works of reference to those engaged in preparative organic chemistry. They would make an invaluable addition to any library.

J. B. STENLAKE.

MICROORGANISMS INDIGENOUS TO MAN. By Theodor Rosebury. Pp. xiv + 435 (including Indices). McGraw-Hill Publishing Company Limited, London, 1962. £5 16s. 6d.

Man usually regards himself as a germ-free individual except when he is a harbinger of an infection in the form of a recognisable disease. It therefore comes as a surprise—indeed as a mild shock—to learn that the literature on man's indigenous microorganisms or "biota" is so vast and scattered that Prof. Rosebury has spent almost 35 years collecting, sifting and collating the material for his book. He develops the thesis that the biota shows an element of order rather than haphazardness and is the result of continual interaction between invader and host, the balance between the biota and disease being precarious. The normally inhabited regions of the body include the skin and mucous surfaces contiguous with it, conjunctiva, respiratory tract down to and including the oropharynx, mouth, lower intestines and external urogenital passages. Other regions are normally sterile and are those for which the pharmacist would produce sterile applications. The metabolic activity of the indigenous biota must affect the host and is beneficial rather than detrimental. The intestinal flora may synthesise vitamins in excess of their requirements and which are therefore available to the host. Animals bred to be germ-free have a low leucocyte count and low antibody titre, both of which are strong defence mechanisms in normal man. One species of microorganism may have an antibiotic effect against another species and a harmless commensal may aid the exclusion of a more potentially dangerous species.

The clinical use of antibiotics may alter the normal flora of an individual and it has become increasingly important to have a knowledge of that flora. Prof. Rosebury has arranged his material in chapters each dealing with a particular group of microorganisms. These are not just catalogues of immense value to the pathologist; the bacteriologist too will find a vast amount of information not normally available in one volume. There is, for example, an excellent discussion of the pseudomonads including methods of differentiation between species, cultivation, pigment production, biochemical reactions, typing and sensitivity to antibiotics. Of particular merit are the chapters on indigenous fungi and protozoa. They include a survey of the enhancement of the pathogenicity of the mould *Candida albicans* by certain antibiotics and of the relation between incidence of intestinal protozoa and standards of living. Contrary to the commonly held view the author finds no evidence that a high infection rate by protozoa is associated with poor hygiene or primitive living conditions.

The book is well produced and free from errors; it contains an extensive bibliography and an index running to nearly 50 pages.

H. S. BEAN.

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