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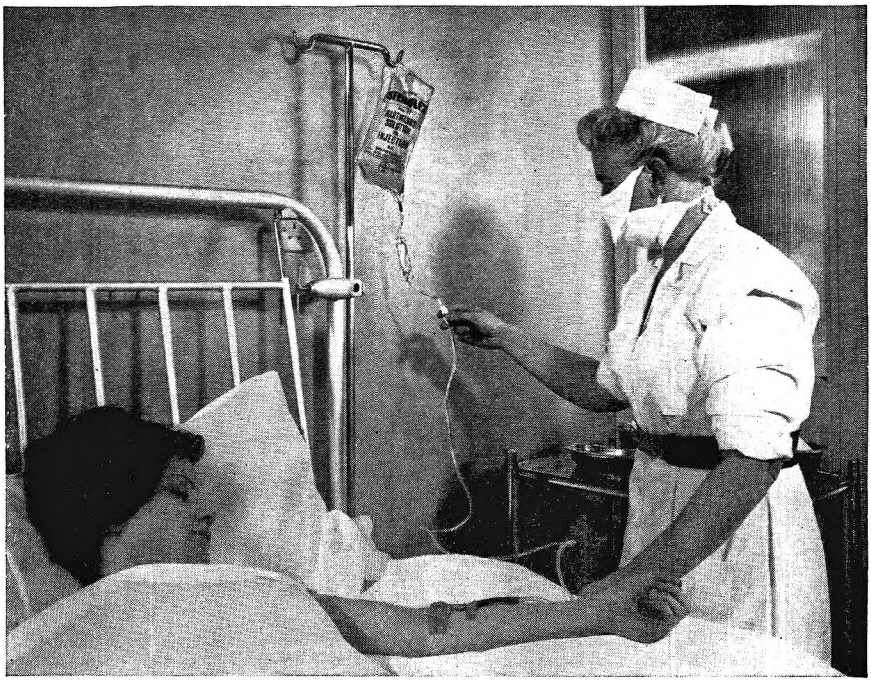

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J. Pharm. Pharmac., 1966, **18**, 65–80 Received June 17, 1965

Research Papers

The diuretic activity of clorexolone and some related phthalimides and 1-oxoisoindolines

(MRS.) E. J. CORNISH, G. E. LEE AND W. R. WRAGG

N-Substituted 4-chloro-5-sulphamoylphthalimides showed diuretic activity in the rat. Reduction of the carbonyl group in the position *para* to the 5-sulphamoyl group in the phthalimides produced even more active compounds. The relationship between potency and the nature of the *N*-substituent was examined in 32 phthalimides and 10 reduced derivatives. Maximum potency was found in the *N*-cycloalkyl and *N*-cycloalkylmethyl compounds, one of which, 5-chloro-2-cyclohexyl-1-oxo-6-sulphamoylisoindoline (clorexolone), was 300 times as active as chlorothiazide in the rat. This compound was examined in more detail in both the rat and the dog.

THE discovery of clinically useful diuretic activity amongst sulphamoylbenzothiadiazines (I; Novello & Sprague, 1957) has been followed by the synthesis of a great number of derivatives and analogues, many of which proved to be potent compounds.

We decided to investigate some new major structural modifications of the heterocyclic ring of the benzothiadiazines (Cornish, Lee & Wragg, 1963; Lee & Wragg, 1963), leaving intact the *o*-chlorobenzenesulphonamide part of the molecule apparently essential to diuretic activity. It was already known that this approach could result in new structures with diuretic activity, for example, the saccharin derivative (II; Merck & Co. Inc., 1960), and the 1,2,3,4-tetrahydro-1-oxoquinazoline (III; quinethazone; Cohen, Klarberg & Vaughan, 1959). On the other hand, the approach could lead to inactive compounds, for example, the disulphimide (IV; Logemann, Giraldi & Galimberti, 1959).

The present work concerns the 4-chloro-5-sulphamoylphthalimide (V) and 5-chloro-1-oxo-6-sulphamoylisoindoline (VI) analogues of chlorothiazide (I; R=H). Of 32 *N*-substituted derivatives of the type (V) prepared (Table 1), most had diuretic activity (Lee & Wragg, 1960). Chemical reduction of a number of these phthalimides gave the corresponding 1-oxoisoindolines (VI) which proved to be even more active diuretics (Table 2; Lee & Wragg, 1961). One of these (VI; R=cyclohexyl; compound No. 39; clorexolone) was considered of sufficient interest to justify clinical trials (Simpson, 1964).

A 5-chloro-1-oxo-6-sulphamoylisoindoline structure (VI) was assigned to the products obtained by chemical reduction of the phthalimides (V) on the basis of the following evidence:

(*a*) Theoretically, the reaction could also yield the isomeric 6-chloro-1-oxo-5-sulphamoylisoindolines (VII), but in practice only a single reduction product is formed.

(*b*) In the most interesting case, the single reduction product, clorexolone, was assigned the structure (VI; R=cyclohexyl) because the same compound was obtained by the alternative route 1 (Fig. 1) from 5-chloro-2-cyclohexyl-1-oxoisoindoline (VIII; R=H).

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(*c*) To minimise the possibility of ambiguity, the 6-chloro-1-oxo-5-sulphamoylisoindoline isomer (VII; R=cyclohexyl) of clorexolone (VI; R=cyclohexyl) has also been synthesised by the similar route 2 (Fig. 1), starting from 6-chloro-2-cyclohexyl-1-oxoisoindoline (IX; R=H). The product (VII; R=cyclohexyl) and the starting material (IX; R=H) were both shown to be different from their isomers clorexolone (VI; R=cyclohexyl) and 5-chloro-2-cyclohexyl-1-oxoisoindoline (VIII; R=H) respectively.

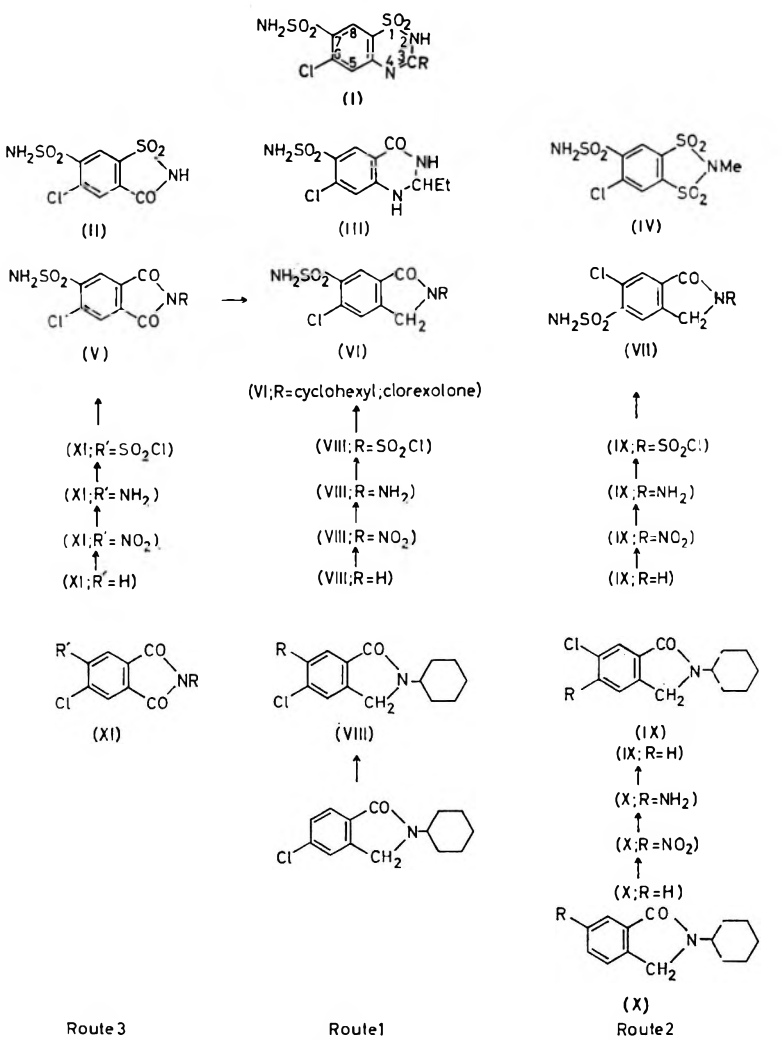


FIG. 1. (The authors' formulae have been reorientated to conform with this Journal's practice).

(*d*) The structure assigned to the starting material (IX; R=H) depended on its preparation from 2-cyclohexyl-6-nitro-1-oxoisoindoline (X; R=NO_2),

prepared in turn from 2-cyclohexyl-1-oxoisoindoline (X; R=H) under nitration conditions which, when applied to 2-methyl-1-oxoisoindoline, had been shown to give the 6-nitro-derivative (Borsche, Diacont & Hanau, 1934).

Since this work was completed certain 4-substituted 5-sulphamoylphthalimides have been described as diuretics in a patent (Novello, 1962) in which an entirely different synthetic route to that described above was used.

Experimental

SYNTHETIC METHODS

Preparation of the N-*substituted phthalimides* (V). Each of the compounds examined (Table 1) was prepared from 4-chloro-5-sulphamoylphthalimide (V; R=H) by reaction with the appropriate primary amine (Method A), or halogeno-compound in the presence of a suitable condensing agent (Method B).

The primary intermediate (V; R=H) was prepared from 4-chlorophthalimide (XI; R=R′=H) by the reaction sequence shown as route 3 (Fig. 1).

4-*Chloro*-5-*nitrophthalimide* (XI; R=H, R′=NO_2). 4-Chlorophthalimide (20 g; Levy & Stephen, 1931) was added to a mixture of 20% oleum (200 ml) and fuming nitric acid (24 ml). The solution was heated at 80° for 30 min, cooled, and poured on to ice (2 kg) to give, after recrystallisation from ethanol, 4-chloro-5-nitrophthalimide, m.p. 198–200°. Found: C, 42·4; H, 2·1; N, 12·3; $C_8H_3ClN_2O_4$ requires C, 42·4; H, 1·3; N, 12·4%.

Similarly prepared was 4-chloro-*N*-methyl-5-nitrophthalimide (XI; R=Me, R′=NO_2) m.p. 173–175°, from chloroform. Found: N, 11·6; Cl, 15·5; $C_9H_5ClN_2O_4$ requires N, 12·05; Cl, 14·8%.

5-*Amino*-4-*chlorophthalimide* (XI; R=H, R′=NH_2). 4-Chloro-5-nitrophthalimide (8 g) was added to a solution of stannous chloride (28 g) in concentrated hydrochloric acid (150 ml). The mixture was heated at 60° for 30 min. When cold, the precipitated solid was filtered off, washed well with water, and recrystallised from dimethylformamide to give 5-amino-4-chlorophthalimide, m.p. 314–315°. Found: C, 48·9; H, 3·05; N, 14·5; $C_8H_5ClN_2O_2$ requires C, 48·9; H, 2·55; N, 14·25%.

Similarly prepared was 5-amino-4-chloro-*N*-methylphthalimide (XI; R=Me, R′=NH_2), m.p. 209–211°, from ethanol. Found: N, 14·0; Cl, 17·0; $C_9H_7ClN_2O_2$ requires N, 13·3; Cl, 16·8%.

4-*Chlorophthalimide*-5-*sulphonyl chloride* (XI; R=H, R′=SO_2Cl). Preparation of this intermediate by direct chlorsulphonation of 4-chlorophthalimide failed even at 150°. The following procedure was therefore adopted:

5-Amino-4-chlorophthalimide (20 g) was diazotised at 0–5° with sodium nitrite (9 g) in concentrated hydrochloric acid (200 ml). The diazonium solution was added to a solution of sulphur dioxide (40 ml) in glacial

acetic acid (110 g) containing cuprous chloride (1 g). The reaction product was filtered off, washed with water and dried. Recrystallisation from benzene gave 4-chlorophthalimide-5-sulphonyl chloride, m.p. 169–171°. Found: C, 34·7; H, 2·5; N, 5·2; $C_8H_3Cl_2NO_4S$ requires C, 34·3; H, 1·1; N, 5·0%.

Similarly prepared was 4-chloro-*N*-methylphthalimide-5-sulphonyl chloride (XI; R=Me, R′=SO_2Cl), m.p. 161–163° Found: N, 4·9; S, 11·3; $C_9H_5Cl_2NO_4S$ requires N, 4·7; S, 10·9%.

4-*Chloro*-5-*sulphamoylphthalimide* (V; R=H). 4-Chlorophthalimide-5-sulphonyl chloride (20 g) was added to liquid ammonia (200 ml). The solution was evaporated to dryness and the solid product triturated with water. The solid was filtered off and heated with concentrated hydrochloric acid (100 ml) on a steam-bath for 30 min. The product was filtered off and washed well with water. Recrystallisation from methanol gave 4-chloro-5-sulphamoylphthalimide, m.p. 292–294°. Found: C, 36·8; H, 2·45; N, 10·6; $C_8H_5ClN_2O_4S$ requires C, 36·9; H, 1·9; N, 10·75%.

Similarly prepared was 4-chloro-*N*-methyl-5-sulphamoylphthalimide (V; R=Me), m.p. 238–240°. Found: Cl, 12·6; S, 11·5; $C_9H_7ClN_2O_4S$ requires Cl, 12·9; S, 11·65%.

Typical example of the use of preparative Method A. 4-Chloro-5-sulphamoylphthalimide (10 g) was refluxed in amyl alcohol (100 ml) with cyclohexylamine (3·8 g) for 4 hr. The reaction mixture was cooled. The crystalline solid was filtered off and recrystallised from methanol to give 4-chloro-*N*-cyclohexyl-5-sulphamoylphthalimide (V; R=cyclohexyl), m.p. 216–218°. Found: N, 8·3; Cl, 10·6; $C_{14}H_{15}ClN_2O_4S$ requires N, 8·2; Cl, 10·4%.

The success of this route was dependent, amongst other factors, on the volatility and the degree of steric hindrance of the primary amine used. For instance, t-butylamine failed to react under these conditions. Amine in excess of one mole with more stringent reaction conditions led to side reactions; this reaction with undiluted benzylamine in excess at reflux resulted in the formation of 3,*NN*-tribenzyl-4-sulphamoylphthaldiamide.

Typical example of the use of preparative Method B. 4-Chloro-5-sulphamoylphthalimide (10 g) was dissolved in dry dimethylformamide (40 ml). To this solution was added sodium hydride (50% oil dispersion; 1·81 g). The mixture was heated to 70° and methyl iodide (5·9 g) in dry dimethylformamide (10 ml) was added. The solution was stirred at 70° for 1 hr, cooled, and poured on to water. The precipitated solid was filtered off and recrystallised from methanol to give 4-chloro-*N*-methyl-5-sulphamoylphthalimide (V; R=Me), m.p. 238–240°. Found: C, 39·5; H, 2·9; N, 10·1; $C_9H_7ClN_2O_4S$ requires C, 39·35; H, 2·55; N, 10·2%.

This route could feasibly have given the isomeric derivative with the methyl group on the sulphonamide nitrogen atom. This possibility was excluded by the preparation of an authentic sample from 4-chloro-*N*-methylphthalimide (XI; R=Me; R′=H) *via* route 3 (Fig. 1), a synthesis already detailed above. Mixed melting-points and infrared

spectra showed the two samples of 4-chloro-*N*-methyl-5-sulphamoylphthalimide (V; R=Me) to be identical.

Preparation of the N-*substituted* 1-*oxoisoindolines* (VI). Each compound (Table 2) was prepared by the tin and hydrochloric acid reduction of the corresponding phthalimide (V).

Typical reduction, yielding 5-*chloro*-2-*cyclohexyl*-1-*oxo*-6-*sulphamoylisoindoline* (VI; R=cyclohexyl; compound No. 39; clorexolone). 4-Chloro-*N*-cyclohexyl-5-sulphamoylphthalimide (V; R=cyclohexyl) (1000 g) was dissolved in a mixture of dimethylformamide (5·65 litres) and methanol (5·65 litres). Granulated tin (835 g) was added, followed by concentrated hydrochloric acid (3·45 litres). Some heat was applied from a steam-bath to the stirred reaction mixture, whereupon an exothermic reaction was initiated. The reaction mixture was gently refluxed for 3 hr with stirring and the solution was decanted from the tin residue and concentrated *in vacuo* on a steam-bath until crystallisation started. Concentrated hydrochloric acid (2·5 litres) was added with stirring and the suspension cooled and filtered. The solid was washed with hydrochloric acid and finally water. The damp solid was dissolved in 2N sodium hydroxide, filtered, diluted with water and poured into rapidly stirred 2N hydrochloric acid (3 litres). After stirring for 1 hr the solid was filtered off and washed thoroughly with water. The product was dried at 80° to give a white solid which was recrystallised from a 50/50 mixture of dimethylformamide and methanol to give 5-chloro-2-cyclohexyl-1-oxo-6-sulphamoylisoindoline (548 g, 55%) as a white solid, m.p. 266–268°. Found: N, 8·4; Cl, 10·7; $C_{14}H_{17}ClN_2O_3S$ requires N, 8·5; Cl, 10·8%.

Alternative preparation of 5-*chloro*-2-*cyclohexyl*-1-*oxo*-6-*sulphamoylisoindoline* (VI; R=cyclohexyl) *via* route 1 (Fig. 1). 4-Chlorophthalimide (263 g) was reacted in amyl alcohol (2·6 litres) with cyclohexylamine (143·5 g, 1 mole) at reflux temperature for 16 hr. The cooled solution was filtered and the solid was recrystallised from ethanol to give 4-chloro-*N*-cyclohexylphthalimide (250 g, 66%) as a solid, m.p. 134–136°. Found: N, 5·5; Cl, 13·5; $C_{14}H_{14}ClNO_2$ requires N, 5·3; Cl, 13·5%.

4-Chloro-*N*-cyclohexylphthalimide (250 g) was dissolved in glacial acetic acid (2·5 litres). Concentrated hydrochloric acid (555 ml) and tin (278 g) were added and the suspension was heated on a steam-bath for 16 hr. The cooled solution was filtered and concentrated to dryness *in vacuo* to give a white solid. This solid was dissolved in water and the precipitated oil extracted with chloroform. The chloroform solution was dried and concentrated *in vacuo* to give a solid which, after recrystallisation from acetone, yielded 5-chloro-2-cyclohexyl-1-oxoisoindoline (VIII; R=H; 103 g, 43%), m.p. 140–142°. Found: N, 5·6; Cl, 14·5; $C_{14}H_{16}ClNO$ requires N, 5·5; Cl, 14·2%.

5-Chloro-2-cyclohexyl-1-oxoisoindoline (102·9 g) was dissolved in concentrated sulphuric acid (665 ml) and potassium nitrate (723 g) in concentrated sulphuric acid (166 ml) was added at 0°. The reaction mixture was allowed to warm to room temperature and stirred at 25° for

12 hr. The reaction mixture was poured on to ice to give a cream solid which, after recrystallisation from benzene, gave 5-chloro-2-cyclohexyl-6-nitro-1-oxoisoindoline (VIII; R=NO_2; 46·7 g, 44%) as a white solid, m.p. 164–168°. Found: N, 9·3; Cl, 12·2; $C_{14}H_{15}ClN_2O_3$ requires N, 9·55; Cl, 12·0%.

5-Chloro-2-cyclohexyl-6-nitro-1-oxoisoindoline (93·9 g) was reduced in concentrated hydrochloric acid (1970 ml) with stannous chloride (376 g), whereupon the reaction temperature rose to 70°. The resulting solution was cooled in ice and filtered. The product was washed well with water, filtered and dried to give 6-amino-5-chloro-2-cyclohexyl-1-oxoisoindoline (VIII; R=NH_2; 74·1 g, 87·6%) which, after recrystallisation from benzene, had m.p. 216–218°. Found: N, 10·7; Cl, 13·7; $C_{14}H_{17}ClN_2O$ requires N, 10·6; Cl, 13·4%.

6-Amino-5-chloro-2-cyclohexyl-1-oxoisoindoline (42·5 g) was dissolved in concentrated hydrochloric acid (425 ml) and the solution diazotised at 0–5° by the addition of sodium nitrite (21·25 g) in water (125 ml). The resulting diazonium salt solution was added to a solution of liquid sulphur dioxide (93 ml) in glacial acetic acid (243 ml) containing cuprous chloride (2·25 g). A yellow solid was precipitated; this was filtered off, washed, dried and recrystallised from benzene to give 5-chloro-2-cyclohexyl-1-oxoisoindoline-6-sulphonyl chloride (VIII; R=SO_2Cl; 45 g, 80%) as a cream coloured solid, m.p. 171–174°. Found: N, 4·1; Cl, 19·6; $C_{14}H_{15}Cl_2NO_3S$ requires N, 4·0; Cl, 20·4%.

This sulphonyl chloride (23·7 g) was reacted with liquid ammonia (237 ml). Crystallisation of the product from methanol gave 5-chloro-2-cyclohexyl-1-oxo-6-sulphamoylisoindoline (VI; R=cyclohexyl; 14·2 g, 53%), m.p. 259–261°, identical (mixed m.p.; infrared spectrum) with the sample prepared above by chemical reduction of the corresponding phthalimide (V; R=cyclohexyl). Found: N, 8·2; Cl, 11·0; $C_{14}H_{17}ClN_2O_3S$ requires N, 8·5; Cl, 10·8%.

Preparation of 6-chloro-2-cyclohexyl-1-oxo-5-sulphamoylisoindoline (VII; R=cyclohexyl) *via* route 2 (Fig. 1). *N*-Cyclohexylphthalimide (9 g) was dissolved in glacial acetic acid (100 ml). To this solution was added tin (10 g) followed by concentrated hydrochloric acid (50 ml). The solution was heated on a steam-bath until all the tin had dissolved. The cooled solution was filtered and poured into water (250 ml) and the oil was extracted into ether. The extract was dried and concentrated. The yellow residue was dissolved in acetone and then water was added. The crude solid thus formed was recrystallised from acetone to give 2-cyclohexyl-1-oxoisoindoline (X; R=H; 4·3 g, 51%), m.p. 92–97°.

2-Cyclohexyl-1-oxoisoindoline (5 g) was dissolved in concentrated sulphuric acid (33 ml). A solution of potassium nitrate (3·66 g) in concentrated sulphuric acid (8·3 ml) was added to the stirred solution at 0°. The solution was stirred at 0° for 6 hr and then poured on to ice/water (200 ml). The precipitated crude solid was recrystallised from ethanol to give 2-cyclohexyl-6-nitro-1-oxoisoindoline (X; R=NO_2; 4·5 g, 85%) as a white solid, m.p. 125–128°. Found: C, 64·8; H, 6·5; N, 11·2; $C_{14}H_{16}N_2O_3$ requires C, 64·6; H, 6·15; N, 10·75%.

2-Cyclohexyl-6-nitro-1-oxoisoindoline (132 g) was added to a solution of stannous chloride (454 g) in concentrated hydrochloric acid (2770 ml). The reaction mixture was warmed to 50° on a steam-bath. The solution was filtered hot and the filtrate cooled. The white solid which crystallised out was dissolved in the minimum quantity of water and was treated with a large excess of sodium hydroxide (2N). The white solid precipitated was washed with water and dried *in vacuo*. The dried solid (80 g, 70%) was 6-amino-2-cyclohexyl-1-oxoisoindoline (X; R=NH_2).

The crude amino-compound (80 g) was added to concentrated hydrochloric acid (809 ml). To this suspension was added a solution of sodium nitrite (41 g) in water (100 ml) at 0–5°. The solution of diazonium salt was poured into a solution of cuprous chloride (110 g) in concentrated hydrochloric acid (715 ml). The precipitated solid was recrystallised from acetone to give 6-chloro-2-cyclohexyl-1-oxoisoindoline (IX; R=H; 70 g, 80%), m.p. 134–137°. Found: N, 5·8; Cl, 14·3; $C_{14}H_{16}ClNO$ requires N, 5·6; Cl, 14·25%). This product was different (mixed m.p., infrared spectrum) from a sample of the isomer 5-chloro-2-cyclohexyl-1-oxoisoindoline (VIII; R=H) prepared, as described above, *via* route 1 (Fig. 1).

6-Chloro-2-cyclohexyl-1-oxoisoindoline (69 g) was added at below 10° to concentrated sulphuric acid (460 ml). To this solution was added at 0° during 30 min a solution of potassium nitrate (50 g) in concentrated sulphuric acid (115 ml). The solution was stirred at 0° for a further 5 hr and then poured on to ice/water. The precipitated solid was recrystallised from benzene to give 6-chloro-2-cyclohexyl-5-nitro-1-oxoisoindoline (IX; R=NO_2), 10·4 g, m.p. 158–162°.

6-Chloro-2-cyclohexyl-5-nitro-1-oxoisoindoline (10·4 g) was dissolved in a solution of stannous chloride (40·8 g) in concentrated hydrochloric acid (214 ml). The solution was heated at 85° for 1 hr, cooled in an ice-bath and then filtered. The solid was recrystallised from methanol to give 5-amino-6-chloro-2-cyclohexyl-1-oxoisoindoline (IX; R=NH_2; 9·0 g), m.p. 188–190°. Found: N, 10·5; Cl, 13·5; $C_{14}H_{17}ClN_2O$ requires N, 10·4; Cl, 13·45%.

5-Amino-6-chloro-2-cyclohexyl-1-oxoisoindoline (7·7 g) was diazotised in concentrated hydrochloric acid (77 ml) at 0–5° with sodium nitrite (3·85 g) in water (22 ml). The diazonium solution was added to a solution of sulphur dioxide (16·7 ml) and cuprous chloride (0·4 g) in glacial acetic acid (44 ml). The 6-chloro-2-cyclohexyl-1-oxoisoindoline-5-sulphonyl chloride (IX; R=SO_2Cl; 8·8 g, 87%), m.p. 167–169°, was used without further purification.

The crude 6-chloro-2-cyclohexyl-1-oxoisoindoline-5-sulphonyl chloride (8·8 g) was added to liquid ammonia (88 ml). After the excess liquid ammonia had evaporated the crude solid was washed with water and recrystallised from methanol to give 6-chloro-2-cyclohexyl-1-oxo-5-sulphamoylisoindoline (VII; R=cyclohexyl; compound No. 36; 5·0 g, 86%), m.p. 234–236°. Found: N, 8·4; Cl, 10·85; $C_{14}H_{17}ClN_2O_3S$ requires N, 8·5; Cl, 10·8%.

This product was different (mixed m.p., infrared spectrum) from the sample of the isomer 5-chloro-2-cyclohexyl-1-oxo-6-sulphamoylisoindoline (VI; R=cyclohexyl) prepared, as described above, *via* route 1 (Fig. 1).

PREPARATION OF THE COMPOUNDS NOS. 33–35

5-Chloro-2-cyclohexyl-3-hydroxy-1-oxo-6-sulphamoylisoindoline (*compound No.* 33). 4-Chloro-*N*-cyclohexyl-5-sulphamoylphthalimide (1·0 g) was dissolved in a mixture of methanol (50 ml) and 5N sulphuric acid (11 ml). The solution was electrolysed at room temperature for 15 min at a current density of 0·03 A cm^{-2} using a lead cathode and a carbon anode contained in a Visking sausage skin. The solvent employed above was also used as anolyte. The potential of the working cathode was −0·7 V referred to a saturated calomel electrode. After about 8 min a precipitate appeared in the catholyte and precipitation was complete after 15 min. The catholyte was filtered and the filtrates from 14 such reductions were bulked, rendered neutral with solid sodium carbonate, filtered and concentrated. The residue was recrystallised from methanol to give 5-chloro-2-cyclohexyl-3-hydroxy-1-oxo-6-sulphamoylisoindoline (2·8 g, 20%), m.p. 198–200° (decomp.). Found: C, 50·4; H, 5·6; Cl, 10·1; N, 8·2; S, 8·4; $C_{14}H_{17}ClN_2O_4S$ requires C, 48·8; H, 4·9; Cl, 10·3; N, 8·1; S, 9·3%.

That the hydroxyl group was in position 3 in the foregoing product was established by its reduction to 5-chloro-2-cyclohexyl-1-oxo-6-sulphamoylisoindoline (VI; R=cyclohexyl), as follows.

5-Chloro-2-cyclohexyl-3-hydroxy-1-oxo-6-sulphamoylisoindoline (10 g) was dissolved in a mixture of dimethylformamide (56 ml) and methanol (56 ml). Concentrated hydrochloric acid (34 ml) and tin (8·5 g) were added. The suspension was stirred and heated on a steam-bath for 18 hr, after which the hot reaction mixture was filtered and the filtrate concentrated. The residue was treated with concentrated hydrochloric acid (100 ml) and the residue filtered off. This residue was dissolved in sodium hydroxide (2N, 50 ml) and then treated with hydrochloric acid (2N, 200 ml). The precipitated solid was filtered off, dried and recrystallised from dimethylformamide/methanol to give 5-chloro-2-cyclohexyl-1-oxo-6-sulphamoylisoindoline (VI; R=cyclohexyl; 4·7 g, 50%), m.p. 259–261°, which was identical (mixed m.p. and infrared spectrum) with an authentic sample.

5-Chloro-2-cyclohexyl-6-methylsulphamoyl-1-oxoisoindoline, (*compound No.* 34). 4-Chloro-*N*-cyclohexyl-5-methylsulphamoylphthalimide (10·0 g) was reduced with tin (8·05 g) and a mixture of hydrochloric (33 ml) and acetic (100 ml) acids to give after filtration, concentration and dilution with water, a solid. This solid was recrystallised from methanol to give 5-chloro-2-cyclohexyl-6-methylsulphamoyl-1-oxoisoindoline as a white solid (1·75 g, 20%), m.p. 233–234°. Found: N, 8·15; S, 9·5; $C_{15}H_{19}ClN_2O_3S$ requires N, 8·2; S, 9·35%.

6-Acetylsulphamoyl-5-chloro-2-cyclohexyl-1-oxoisoindoline (*compound No.* 35). 5-Chloro-2-cyclohexyl-1-oxo-6-sulphamoylisoindoline (10 g)

was dissolved in acetic anhydride (100 ml). The solution was heated at reflux for 2 hr. The excess acetic anhydride was removed *in vacuo* and the sticky residue triturated to a cream coloured solid with water. Recrystallisation from 50% aqueous acetic acid gave 6-acetylsulphamoyl-5-chloro-2-cyclohexyl-1-oxoisoindoline as a white solid (8·7 g, 80%), m.p. 145° (decomp.). Found: Cl, 9·6; N, 7·5; $C_{15}H_{19}ClN_2O_4S$ requires Cl, 9·6; N, 7·55%.

Pharmacological methods

DIURETIC ACTIVITY

The method was similar to that of Lipschitz, Hadidian & Kerpscar (1943).

Eight groups of four male albino rats were starved overnight and were then given by stomach tube, isotonic saline at 37°, 25 ml/kg, in which the test compound was dispersed. The doses used were chosen after a preliminary experiment so as to produce an approximately threefold increase in urine excretion. Four randomly chosen groups received the standard compound, chlorothiazide, and the remaining groups received the test compound. Samples of urine were collected for 5 hr. Na^+ and K^+ in the urine were determined by flame photometry, and Cl^- was estimated with a potentiometric microtitration apparatus. Control experiments were made 2 days before and four days after the test experiment, the object being first to establish, and then to check, the base-line urinary excretion pattern for each group of 4 rats.

Quantitative dose-response relationships. To compare accurately the dose-response relationships, two of the *N*-substituted phthalimides and two reference compounds, chlorothiazide and hydrochlorothiazide, were assayed at 3 dose levels (in the ratio 1 : 2 : 4) selected to fall on the maximum slope portion of the dose-response curves. At weekly intervals, all 32 rats received a single dose level of one of these compounds on a random basis. Otherwise, the experimental conditions were as described above. The hydrochlorothiazide was obtained from powdered 25 mg tablets (Ciba Laboratories Ltd.).

ACUTE ORAL TOXICITY IN MICE

Three groups of 5 albino mice, each weighing 16–18 g, were used. The mice were starved overnight and then given, by stomach tube, 1, 2 and 3 g/kg, respectively, of the compound under test suspended in 0·6% tragacanth solution. Observations were made for 2 weeks after dosing.

SUPPLEMENTARY METHODS USED TO STUDY 5-CHLORO-2-CYCLOHEXYL-1-OXO-6-SULPHAMOYLISOINDOLINE (VI; R=cyclohexyl; compound No. 39; clorexolone)

A cross-over test (cf. British Pharmacopoeia, 1963, p. 1093) was performed in the rat with clorexolone and chlorothiazide.

The two compounds were also compared in the dog using a cross-over test. Perineostomised female dogs were starved overnight and given

distilled water, 40 ml/kg, by stomach tube. One hr later the bladder was emptied by catheterisation. Compounds were given orally by capsule, and the bladder emptied hourly for 5 hr. Control values were obtained the day before an experiment. Na^+, K^+ and Cl^- were estimated in the urine. When the pH was measured the urines were collected under liquid paraffin. Each dog received individual doses in a randomly selected order at weekly intervals.

In creatinine clearance estimations in dogs, 200 mg/kg creatinine hydrochloride was injected subcutaneously as a 12·5% solution. Supplementary creatinine and water were administered to achieve constant conditions. Two control clearance determinations preceded administration of clorexolone, and, thereafter, clearance determinations were made and compared with similar observations in the same animals when they had not received a diuretic. Creatinine in the urine and plasma was estimated by the alkaline picrate method of Bosnes & Taussky (1945).

The carbonic anhydrase inhibition *in vitro* was studied by the method of Philpot & Philpot (1936). The carbonic anhydrase extract was prepared from sheep or pig erythrocytes using an ethanol : chloroform mixture and was stored at −70° (Booth & Roughton, 1946).

In some experiments the urine was made acid or alkaline by the oral administration of ammonium chloride or sodium bicarbonate respectively. Rats received 0·5 g/kg/day ammonium chloride, or 1 g/kg/day sodium bicarbonate for 7 days (Farah, Bender, Kruse & Cafruny, 1959). Dogs received 100 m-equiv./dog/day of the salts for 7 days (Baer, Russo & Beyer, 1959).

Acute oral and intravenous LD50 values were determined in mice and rats.

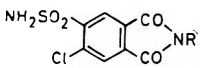
Results

The diuretic potency of the *N*-substituted 4-chloro-5-sulphamoylphthalimides (V) is summarised in Table 1. Highest activity was found when the *N*-substituent in (V) was a cyclohexyl, a methyl-substituted cyclohexyl, cycloheptyl or cyclo-octyl ring. Such compounds (Nos. 11 to 15) were about six times as potent as chlorothiazide. Reduction in size of the *N*-substituent to a cyclopentyl ring (No. 10) diminished activity, whilst the larger cyclododecyl ring eliminated activity (No. 16).

The pattern of excretion of water, Na^+, K^+ and Cl^- was similar to that produced by equipotent doses of any of these compounds or of chlorothiazide. More detailed comparisons of these excretions showed that compounds Nos. 10 and 11 (V; R=cyclopentyl and cyclohexyl respectively) produced dose-response curves which were parallel to those obtained for chlorothiazide and hydrochlorothiazide (e.g. see Fig. 2). Compounds Nos. 10 and 11 were 3·3 and 6·0 times as active as chlorothiazide, and 0·14 and 0·30 times as active as hydrochlorothiazide with respect to sodium excretion.

After the administration of compounds Nos. 1 and 2 (V; R=H and Me respectively) the urine was a bright yellow colour; tests for bile

TABLE 1. 4-CHLORO-5-SULPHAMOYL-*N*-SUBSTITUTED-PHTHALIMIDES (V): CHEMICAL CHARACTERISTICS AND DIURETIC ACTIVITY IN THE RAT

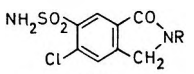


(V)

No.	R	Preparative method; m.p.; crystallisation solvent	Analytical figures			Diuretic activity (chlorothiazide = 1)
				N %	Cl %	
1	H	, 293–295°, methanol	Found $C_9H_5ClN_2O_4S$ req.	10·6 10·75		0·25
2	Methyl	B, 242–244°, methanol	Found $C_{10}H_7ClN_2O_4S$ req.	10·3 10·2		0·5
3	Allyl	A, 173–174°, ethanol	Found $C_{11}H_9ClN_2O_4S$ req.	9·2 9·3	12·0 11·8	0·25
4	Prop-2-ynyl	B, 188–191°, ethanol	Found $C_{11}H_7ClN_2O_4S$ req.	9·3 9·4	11·8 11·9	1
5	Butyl	A, 178–181°, amyl alcohol	Found $C_{12}H_{13}ClN_2O_4S$ req.	8·4 8·8	11·2 11·3	1
6	Dodecyl	A, 169–171°, ethanol	Found $C_{20}H_{29}ClN_2O_4S$ req.	6·65 6·5	8·0 8·3	0·5
7	Isobutyl	A, 177–178°, methanol	Found $C_{12}H_{13}ClN_2O_4S$ req.	8·85 8·85	11·4 11·2	1
8	2-Bromoethyl	(1), 191–193°, benzene	Found $C_{10}H_8BrClN_2O_4S$ req.	7·85 7·6		1
9	2-Hydroxyethyl	A, 193–195°, ethanol	Found $C_{10}H_9ClN_2O_5S$ req.	9·4 9·2	11·8 11·7	1
10	Cyclopentyl	A, 182–185°, ethanol	Found $C_{13}H_{13}ClN_2O_4S$ req.	8·4 8·5	10·9 10·8	3
11	Cyclohexyl	A, 216–218°, methanol	Found $C_{14}H_{15}ClN_2O_4S$ req.	8·1 8·2	10·6 10·4	6
12	4-Methylcyclohexyl	A, 206–209°, benzene	Found $C_{15}H_{17}ClN_2O_4S$ req.	7·6 7·9	10·0 9·9	6
13	3-Methylcyclohexyl	A, 170–172°, benzene	Found $C_{15}H_{17}ClN_2O_4S$ req.	7·7 7·9	9·5 9·9	6
14	Cycloheptyl	A, 206–208°, ethanol	Found $C_{15}H_{17}ClN_2O_4S$ req.	7·8 7·9	10·05 9·9	6
15	Cyclo-octyl	A, 185–187°, ethanol	Found $C_{16}H_{19}ClN_2O_4S$ req.	7·6 7·55	9·6 9·6	6
16	Cyclododecyl	A, 228–230°, glacial acetic acid	Found $C_{20}H_{27}ClN_2O_4S$ req.	6·3 6·5	8·3 8·3	0
17	Bicyclohexyl-4-yl	A, 197–200°, benzene	Found $C_{20}H_{25}ClN_2O_4S$ req.	6·9 6·6	8·45 8·4	0
18	3-Phenylnorborn-2-yl	A, 217–218°, glacial acetic acid	Found $C_{20}H_{19}ClN_2O_4S$ req.	6·4 6·5	8·1 8·2	0
19	Decahydronaphth-2-yl	A, 249–251°, n-butanol	Found $C_{14}H_{21}ClN_2O_4S$ req.	6·9 7·1	8·9 9·0	0
20	1,2,3,4-Tetrahydronaphth-2-yl	A, 210–213°, amyl alcohol	Found $C_{20}H_{15}ClN_2O_4S$ req.	6·5 6·8	8·6 8·9	0·25
21	Benzyl	A, 213–215°, amyl alcohol	Found $C_{16}H_{11}ClN_2O_4S$ req.	7·6 8·0	10·2 10·1	1
22	Phenethyl	A, 255–257°, amyl alcohol	Found $C_{16}H_{13}ClN_2O_4S$ req.	7·3 7·7	9·65 9·7	1
23	*p*-Methylbenzyl	A, 215–218°, ethanol	Found $C_{16}H_{13}ClN_2O_4S$ req.	7·7 7·7	9·5 9·7	1
24	*p*-Isopropylbenzyl	A, 190–191°, ethanol	Found $C_{18}H_{17}ClN_2O_4S$ req.	6·9 7·1	9·1 9·05	0·25
25	*p*-Chlorobenzyl	A, 219–221°, methanol	Found $C_{15}H_{10}Cl_2N_2O_4S$ req.	7·5 7·3	18·2 18·5	1
26	*m*-Trifluoromethylbenzyl	B, 200–202°, isopropanol	Found $C_{16}H_{10}ClF_3N_2O_4S$ req.	(C = 46·3) (C = 45·9)	(H = 2·2) (H = 2·4)	< 1
27	*p*-Nitrobenzyl	B, 242–244°, glacial acetic acid	Found $C_{15}H_{10}ClN_3O_6S$ req.	10·4 10·6		0·5
28	Cyclohexylmethyl	B, 161–164°, isopropanol	Found $C_{15}H_{17}ClN_2O_4S$ req.	7·7 7·85	9·8 9·95	1
29	Cyclopentylmethyl	B, 174°, methanol	Found $C_{14}H_{15}ClN_2O_4S$ req.	8·2 8·2	10·4 10·4	1
30	Norborn-2-ylmethyl	A, 182°–184°, ethanol	Found $C_{16}H_{17}ClN_2O_4S$ req.	7·6 7·8	9·6 9·5	1
31	Tetrahydrothiopyran-4-yl	A, 243–246°, n-butanol	Found $C_{13}H_{13}ClN_2O_4S_2$ req.	7·4 7·75		1
32	Tetrahydropyran-2-ylmethyl	A, 221–223°, amyl alcohol	Found $C_{14}H_{15}ClN_2O_5S$ req.	8·0 7·8	9·8 9·9	1

(1) by the action of PBr_3 on compound No. 9.

TABLE 2. 2-SUBSTITUTED 1-OXOISOINDOLINES (VI): CHEMICAL CHARACTERISTICS AND DIURETIC ACTIVITY IN THE RAT



(VI)

No.	R	M.p.; crystallisation solvent	Analytical figures	N%	Cl%	Diuretic activity (chlorothiazide = 1)
37	Isobutyl	242–243°, methanol	Found	8·9	11·9	75–100
			$C_{19}H_{15}ClN_2O_3S$ req.	9·3	11·75	
38	Cyclopentyl	281–285°, methanol	Found	8·8	11·2	100
			$C_{13}H_{15}ClN_2O_3S$ req.	8·9	11·3	
39	Cyclohexyl	266–268°, dimethylformamide/methanol	Found	8·4	10·6	300
			$C_{14}H_{17}ClN_2O_3S$ req.	8·5	10·8	
40	4-Methylcyclohexyl	265–270°, methanol	Found	8·0	10·3	100
			$C_{15}H_{19}ClN_2O_3S$ req.	8·2	10·4	
41	3-Methylcyclohexyl	245–250°, methanol	Found	8·1	10·0	100
			$C_{15}H_{19}ClN_2O_3S$ req.	8·2	10·4	
42	3,4-Dimethylcyclohexyl	274–276°, methanol	Found	7·9	10·1	200
			$C_{16}H_{21}ClN_2O_3S$ req.	7·9	10·0	
43	Cycloheptyl	261–262°, methanol	Found	8·1	10·4	50–100
			$C_{15}H_{19}ClN_2O_3S$ req.	8·2	10·6	
44	Cyclo-octyl	252–253°, methanol	Found	7·9	10·0	100
			$C_{16}H_{21}ClN_2O_3S$ req.	7·9	10·0	
45	Norborn-2-yl	274–275°, methanol	Found	8·0	9·0	100
			$C_{15}H_{17}ClN_2O_3S$ req.	8·2	9·4	
46	Cyclohexylmethyl	220–222°, methanol	Found	8·0	10·1	200
			$C_{16}H_{19}ClN_2O_3S$ req.	8·2	10·4	

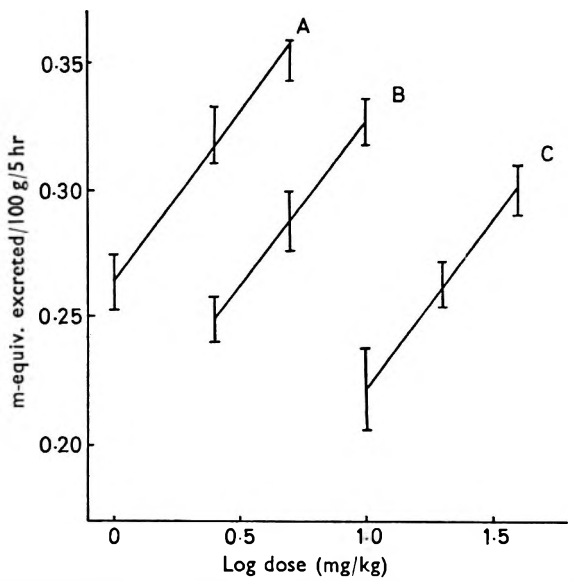


FIG. 2. Sodium ion excretion after hydrochlorothiazide (A), Compound No. 41 (B) and chlorothiazide (C). Vertical lines represent standard errors of the group mean value.

pigments were negative. Apart from this, the observation was not studied further.

With the exception of compounds Nos. 3 and 8, the *N*-substituted phthalimides (V) were not lethal to mice in oral doses of 3 g/kg. No toxic symptoms were noted. Compounds Nos. 3 (V; R=allyl) and 8 (V; R=2-bromoethyl) had LD50 values between 1-2 g/kg and 2-3 g/kg respectively.

Reduction of one of the carbonyl groups of the phthalimide (V; R=cyclohexyl) to -CH(OH)- giving the corresponding 3-hydroxy-1-oxoisoindoline (compound No. 33) produced a 10-fold increase in activity, whilst complete reduction of this carbonyl group to $-CH_2-$ to form the corresponding 1-oxoisoindoline (Table 2, compound No. 39; clorexolone), increased activity 50 fold. Table 2 illustrates the activity of 10 such 1-oxoisoindolines. Methylation or acetylation of the sulphonamido- group of 5-chloro-2-cyclohexyl-1-oxo-6-sulphamoylisoindoline produced at least a 10-fold decrease in diuretic activity (compound Nos. 34 and 35 respectively).

Reduction of the other carbonyl group of the phthalimide (V; R=cyclohexyl) to $-CH_2-$, giving the isomeric 6-chloro-5-sulphamoylisoindoline (VII; R = cyclohexyl; compound No. 36), abolished diuretic activity. The foregoing oxoisoindolines were not lethal to mice at 3 g/kg.

The most active compound in the above tests, clorexolone, was chosen for further study. In a cross-over test using 32 rats, it was 450 times as potent as chlorothiazide with respect to water excretion (fiducial limits

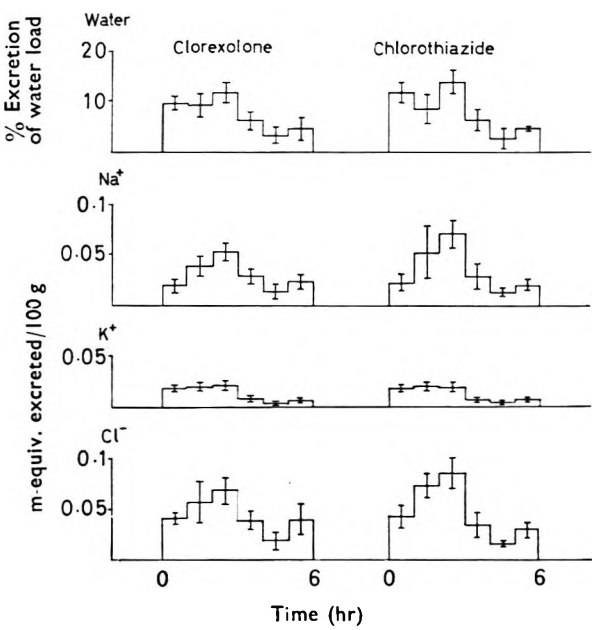


FIG. 3. Electrolyte and water excretion in rats after approximately equipotent doses of clorexolone, 0·025 mg/kg orally, and chlorothiazide, 10 mg/kg orally.

of error, P=0·05, 57–176%), and 320 times as potent with respect to sodium excretion (fiducial limits of error, P=0·05, 64–155%). The effect of approximately equipotent doses of the two compounds on hourly excretions of water, Na^+, K^+ and Cl^- are shown in Fig. 3. Both compounds produced their maximum effects in the third hr after an oral dose.

In rats, clorexolone (0·07 mg/kg), and chlorothiazide (20 mg/kg) produced a threefold increase in the total excretion of Na^+; they increased the concentration of Na^+ from a control value of 0·99 m-equiv./litre to 1·24 and 1·29 m-equiv./litre respectively.

In dogs, a comparison of clorexolone and chlorothiazide by a cross-over test is shown in Table 3. Although the doses chosen from a pre-

TABLE 3. COMPARISON OF THE DIURETIC ACTIVITIES OF CLOREXOLONE AND CHLOROTHIAZIDE IN DOGS

(The same 5 dogs, 9–19 kg, were used to obtain each result)

Compound	mg/kg orally	% Excretion water load	m-equiv. excreted/kg/5 hr		
			Na^+	K^-	Cl^-
None		67 ± 3*	0·43 ± 0·10	0·19 ± 0·03	0·37 ± 0·05
Clorexolone	0·1	81 ± 4	1·20 ± 0·16	0·45 ± 0·13	1·95 ± 0·20
"	0·3	91 ± 4	1·54 ± 0·29	0·54 ± 0·17	2·41 ± 0·30
Chlorothiazide	1·0	79 ± 3	0·90 ± 0·20	0·43 ± 0·10	1·38 ± 0·20
"	3·0	86 ± 5	1·11 ± 0·08	0·43 ± 0·09	1·57 ± 0·09

* Standard error of the mean.

liminary experiment were not equipotent, it can be shown graphically that clorexolone was about 50 times as active as chlorothiazide with respect to Na^+ excretion. The lowest dose of clorexolone (0·1 mg/kg) produced a threefold increase in Na^+ excretion, whereas at 1 mg/kg, chlorothiazide produced only a twofold increase in Na^+ excretion.

Both 0·1 and 0·3 mg/kg of clorexolone decreased the pH of the urine by 0·9 to 1·8 units. Clorexolone did not affect the clearance of exogenous creatinine in the conscious dog.

Carbonic anhydrase inhibition was studied in the presence of three doses of clorexolone and chlorothiazide, and in controls, with three observations at each concentration. The results may be summarised by stating that about 50% inhibition of carbonic anhydrase action *in vitro* was obtained by adding 1 ml of $2{\cdot}2 \times 10^{-5}$M clorexolone, or 6×10^{-5}M chlorothiazide to the reaction mixture; clorexolone was therefore about three times as active as chlorothiazide.

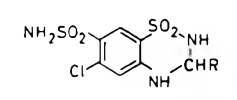
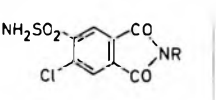
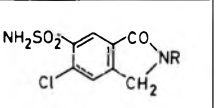
Clorexolone was diuretic in acidotic animals (8 rats, 1 dog; urine pH 0·5–0·9 units < controls) and in alkalotic animals (8 rats, 1 dog; urine pH 0·3–0·4 units > controls). The rats were given 0·07 mg/kg and the dogs 0·1 mg/kg of clorexolone. These treatments increased the excretion of water in the acidotic animals from 30 to 84% in the rats, and 49 to 85% in the dog, with corresponding increases in Na^+ excretion of 0·5 to 2·3 and 0·75 to 1·41 m-equiv./kg/5 hr. These treatments increased the excretion of water in alkalotic animals from 46 to 110% in the rats and 62 to 82% in the dogs with corresponding increases in Na^+ excretion of 1·0 to 3·1 and 0·6 to 1·49 m-equiv./kg/5 hr.

Acute oral toxicity tests showed that clorexolone, 6 g/kg, was not lethal to mice and 10 g/kg was not lethal to rats. No toxic symptoms were observed in either species. The acute intravenous LD50 in mice was 230 mg/kg (fiducial limits of error at P=0·05 were 83–120%). All deaths occurred immediately after injection; the mice convulsed and died of respiratory arrest. The acute intravenous LD50 in rats was 120 mg/kg (limits of error at P = 0·05 were 91–110%). Hypopnoea and convulsions were observed 30 to 60 min after injection and the rats died of respiratory arrest.

Discussion

Diuretic activity was as widespread amongst *N*-substituted 4-chloro-5-sulphamoylphthalimides (Table 1), as it was already known to be amongst 2-substituted and, particularly, 3-substituted derivatives of hydrochlorothiazide (Schlittler, de Stevens & Werner, 1962). Moreover there was a considerable degree of similarity in the pattern of structure-activity relationship amongst the *N*-substituted phthalimides (V) compared with the 3-substituted derivatives of hydrochlorothiazide (Table 4). There

TABLE 4. SUBSTITUENTS R ARRANGED IN ORDER OF THE DIURETIC ACTIVITY THEY CONFER IN THE 3 DIFFERENT SERIES

3-substituted derivatives of hydrochlorothiazide*	*N*-substituted derivatives of the phthalimides (V)	2-substituted 1-oxo-isoindolines (VI)	
			Order in which values of R confer increased activity
R =	R =	R =	
hydrogen methyl cyclopentyl butyl benzyl, phenethyl, isobutyl, *cyclohexyl*, cycloheptyl, cyclo-octyl cyclohexylmethyl cyclopentylmethyl	hydrogen methyl butyl, isobutyl benzyl, phenethyl cyclohexylmethyl, cyclopentylmethyl cyclopentyl *cyclohexyl*, cycloheptyl, cyclo-octyl 3-methylcyclohexyl, 4-methylcyclohexyl	isobutyl cyclopentyl, cycloheptyl, cyclo-octyl 3-methylcyclohexyl, 4-methylcyclohexyl cyclohexylmethyl *cyclohexyl*	↓

* From Schlittler, de Stevens & Werner (1962).

was, however, one notable point of difference; in the former series (V), a cycloalkyl substituted on the nitrogen atom conferred greater activity than a cycloalkylalkyl substituent, while in the latter series this relationship was reversed.

Reduction of the phthalimides (V) to the 1-oxoisoindolines (VI) gave compounds with greatly enhanced activities (Table 2), paralleling the increase in activity seen on reduction of the C=N bond in the 3,4-position in chlorothiazide (I; R=H).

The comparisons in Table 4 again show a parallel structure-activity relationship resulting from varying the substituent R in position 2 in the

1-oxoisoindolines (VI); in this instance a cyclohexyl group conferred the most activity.

The fact that the *N*-substituted 4-chloro-5-sulphamoylphthalimides (V) and the corresponding 1-oxoisoindolines (VI) produce similar excretion of Na^+, K^+ and Cl^- to chlorothiazide and to hydrochlorothiazide, suggests that they all act on the same tubular mechanism for the reabsorption of Na^+.

In vivo inhibition of carbonic anhydrase would appear to contribute little to the activity of the phthalimides (V) or the 1-oxoisoindolines (VI) in effective diuretic doses.

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The flow properties of granular magnesia

T. M. JONES AND N. PILPEL

Measurements have been made of the flow of granular magnesium oxide through circular orifices and the equation

$$D_0 = (1{\cdot}6822\ Dp_{av} + 1{\cdot}9779)\left(\frac{4W}{60\pi\ \rho_P\ \sqrt{g}}\right)^{0{\cdot}2571\ -\ 0{\cdot}0855\ \log\ Dp_{av}}$$

has been derived. This is a particular form of a more general equation

$$D_0 = A\left(\frac{4W}{60\pi\ \rho_P\ \sqrt{g}}\right)^{1/n}$$

which can be shown to apply to a wide range of materials, A and 1/n being functions of particle size and shape.
The equation has been employed for predicting the flow rates of binary and ternary mixtures of magnesia with an accuracy of 5%.

NUMEROUS equations have been published for the flow of powders through circular orifices. Some are purely empirical in nature (Bingham & Wikoff, 1931; Newton, Dunham & Simpson, 1945; Gregory, 1952; Franklin & Johanson, 1955; Luk'yanov, Gusev & Nikitina, 1960; Beverloo, Leniger & Van der Velde, 1961; Ciborowski & Badzynski, 1963); others have been derived from dimensional analysis (Deming & Mehring, 1929; Takahashi, 1933; Rausch, 1949; Brown & Richards, 1959; Fowler & Glastonbury, 1959; Rose & Tanaka, 1959) and others from consideration of the energy of a moving column of powder (Brown, 1961a; Harmens, 1963).

In most cases the equations have been derived from data pertaining to a large number of different materials, e.g. glass spheres, sand, coal dust and cereal grains, this being necessary to cover a reasonable range of particle sizes. As a result, it has been necessary to include in the equations parameters such as particle shape, density and surface roughness. But with the availability now of granular materials whose physical properties remain appreciably constant over a wide range of sizes, it has become possible to eliminate the effects of some of these variables. In principle this should enable a more accurate flow equation for a particular material to be formulated than heretofore.

The majority of the equations currently available apply primarily to systems containing monodispersed particles. But real powders invariably consist of mixtures of particles. From the purely practical point of view it would be advantageous to have a flow equation that could be equally applied to mono, binary and higher systems of a particular material.

In the present investigation, therefore, a study has been made of the flow behaviour of both single and multicomponent systems of magnesia. One objective has been to derive an equation which can be used to predict the flow rate of any mixture whose composition is known. At the same time it has been found possible to explain certain apparent discrepancies between several of the previously published flow equations.

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Experimental

PREPARATION OF MATERIALS

A batch of a hard grade of granular magnesia from the Washington Chemical Company containing 1·4% by weight of material volatile at 150° and 5·7% volatile at 800°, was sieved into 13 fractions in the size range 0·0032 to 0·2812 cm using British Standard sieves. Immediately before use, the fractions were resieved for 3 min with the Alpine Airjet sieve, using 20 g quantities, and were stored in tightly stoppered glass vessels. Some of the physical properties of the fractions are presented in Table 1, particle densities having been measured by immersion in toluene at 25° (Bauer, 1949) and bulk and tap densities by the British Standard (1948) method. Microscopic examination of particles taken from each of the sieve fractions revealed that, for practical purposes, they were all approximately spherical in shape and of similar rugosities.

TABLE 1. PROPERTIES OF DIFFERENT SIEVE FRACTIONS

B.S.S. size	Arithmetic mean sieve diameter (cm)	Density (g/ml)		
		Particle	Bulk	Tap
6/8	0·2435	3·490	0·910	0·596
8/10	0·1866	3·456	0·856	0·533
10/16	0·1340	3·460	0·860	0·541
16/22	0·0851	3·445	0·860	0·530
22/36	0·0561	3·458	0·870	0·530
36/52	0·0358	3·456	0·856	0·538
52/72	0·0253	3·458	0·887	0·570
72/150	0·0158	3·431	0·903	0·585
150/200	0·0090	3·458	0·920	0·588
200/240	0·0071	3·465	0·937	1·032
240/300	0·0059	3·443	0·994	1·063
300/350	0·0048	3·439	1·000	1·095
350/DIN 4188 32μ	0·0038	3·401	1·039	1·127

Binary and ternary mixtures of the various sieve fractions were made up by weight, employing a total of 400 g of powder. Equal amounts of the components were mixed together and the remaining material was then added incrementally, the mixing itself being effected by pouring the powder 30 times from one 1 litre beaker into another.

APPARATUS

The apparatus for the flow rate measurements consisted of a vertical copper tube, internal diameter D_c of 3·82 cm, fitted with a base plate of perspex. This held a shutter and a sliding aluminium sheet, $\frac{1}{16}$ inch thick, into which had been accurately cut with a lathe, six circular orifices with mean diameters D_o of 0·603, 0·740, 0·898, 1·140, 1·353 and 1·686 cm (see Fig. 1).

PROCEDURE

The tube was filled with 400 g of powder. For mixtures the method of filling was to introduce 20 g of the mixture; all but 20 g was then poured into the second beaker and the final 20 g was then introduced into the tube. The rejected 360 g was mixed three times by pouring

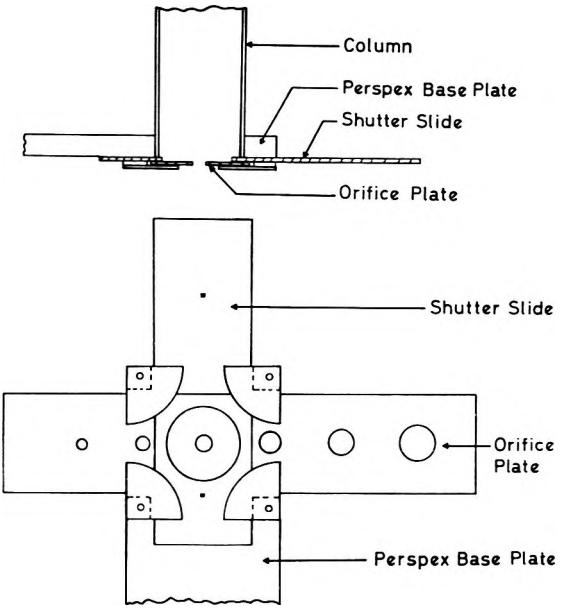

FIG. 1. Upper diagram: side elevation of flow rate apparatus. Lower diagram: plan of orifice/shutter arrangement.

between the two beakers and the process was repeated until the tube was full. Sieve analysis on each 20 g fraction before its introduction into the tube showed that the maximum variation in the compositions of mixtures throughout the length of the tube was $\pm 9\%$ (w/w) the average variation was $\pm 4{\cdot}5\%$ (w/w), and this was considered satisfactory.

Flow rates, W g/min, were determined in triplicate by collecting and weighing the powder that escaped from the tube in fixed time intervals ranging from 5 to 60 sec. Shutter manipulation and incipient failure of the powder column (Jenike, 1962) were found to affect the flow rate in the initial stages. Also, when the head of powder fell below $2 \times D_c$, the flow rate increased. For these reasons the measurements were restricted to the (approximate) central one half of the powder column, when the maximum difference between separate determinations was found to be 5%.

Measurements were made on 13 mono, about 50 binary and about 30 ternary systems.

Results

Fig. 2 shows the effect of particle size and orifice diameter on the flow rates of monodispersed sieve fractions. Provided the orifice was greater than $6 \times D_P$ (Langmaid & Rose, 1957) maximum flow occurred when the particles were 0·0253 cm in diameter.

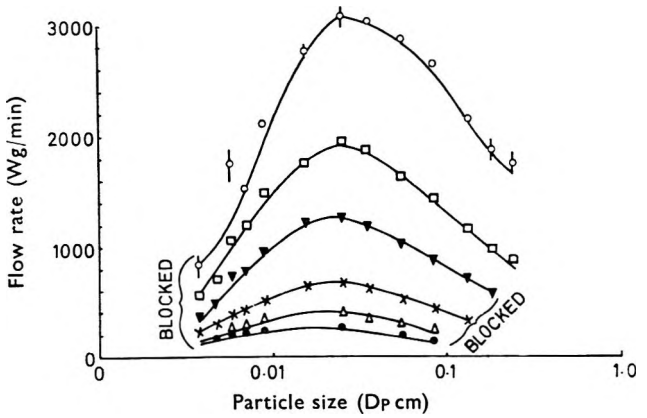

FIG. 2. Effect of particle size on flow of monodisperse systems. Orifice diameters (D_0) in cm: ○, 1·686; □, 1·353; ▼, 1·140; ×, 0·898; △, 0·740; ●, 0·603.

Fig. 3a is representative of the effect that orifice diameter had on the flow rate of a particular sized powder (>0·0253 cm) when a second component, also >0·0253 cm, was added in various amounts.

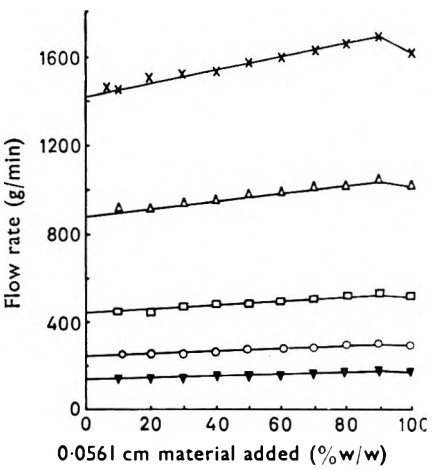

FIG. 3A. Effect of orifice diameter and composition on flow rate of binary mixtures of 0·0851 and 0·0561 cm particles. Orifice diameter in cm: ×, 1·353; △, 1·140; □, 0·898; ○, 0·740; ▼, 0·603.

Fig. 3b is representative of the effects produced by changing the size of this second component while maintaining the orifice constant.

It is seen that the addition of a smaller sized powder to a larger one increased its flow rate up to a maximum value at about 90% addition.

The flow behaviour of typical ternary systems, in which all three components were larger than 0·0253 cm, are shown in Figs 4a and 4b. The contour lines enclose the systems having similar flow rates. It is

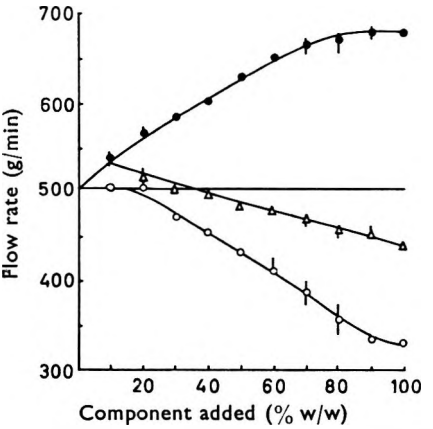

FIG. 3B. Effect of composition and size of additive on flow rate of 0·0561 cm particles in binary mixtures. Orifice diameter 0·898 cm. Additive size in cm: ●, 0·0253; △, 0·0851; ○, 0·1340.

seen that the addition of a small sized third component increases the flow rates of mixtures of two larger sizes and that, as with the mono and binary systems, the larger the value of D_o, the higher the flow rate.

χ^2 tests on the reproducibility of the results were, in all instances, in excess of 0·99 probability.

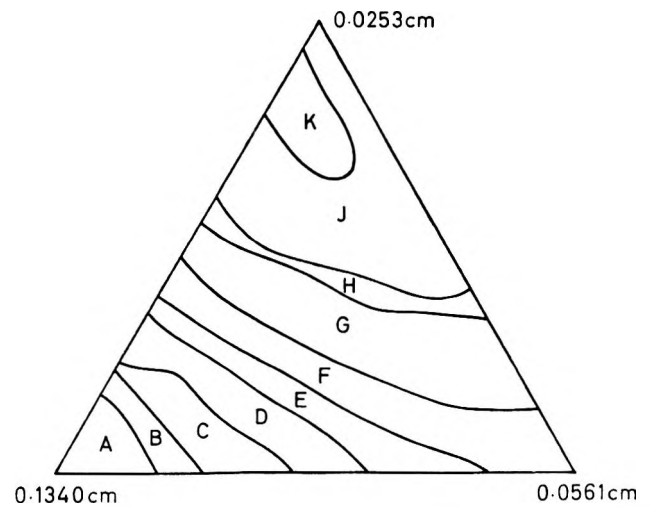

FIG. 4A. Flow rate of ternary mixtures. Orifice diameter 1·353 cm. A, 1100–1200. B, 1200–1300. C, 1300–1400. D, 1400–1500. E, 1500–1600. F, 1600–1700. G, 1700–1800. H, 1800–1900. J, 1900–2000. K, >2000. (g/min).

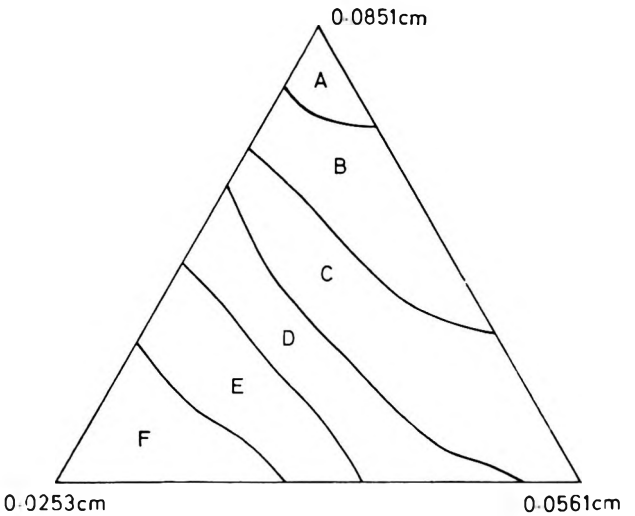

FIG. 4B. Flow rate of ternary mixtures. Orifice diameter 0·740 cm. A, <250. B, 250–280. C, 280–300. D, 300–350. E, 350–380. F, 380–400. (g/min).

Discussion

MONO SYSTEMS

The shapes of the curves in Fig. 2 are similar to those that have been obtained on other materials (Fowler & Glastonbury, 1959; Rose & Tanaka, 1959) though as far as can be ascertained, this is the first occasion on which particles of one material having a constant shape, density, surface roughness have been investigated over such a wide range of sizes.

When the particles are less than 0·0253 cm, complications in the flow arise due to mechanical factors and the operation of van der Waals', electrostatic and surface tension forces between particles (Pilpel, 1964). The present analysis has therefore been restricted to particles >0·02 cm where there is a steady decrease in flow rate with increasing particle size.

Some of the flow equations that have been proposed in the past have included a term for the bulk density, ρ_B, of the powder (Fowler & Glastonbury, 1959). But in more recent work this has been replaced by the particle density ρ_P, since at sizes greater than 0·02 cm the flow rate is independent of the tightness of the initial packing, i.e. independent of ρ_B (Brown & Richards, 1959; Harmens, 1963). It has been shown (Brown & Richards, 1960), for example, that in many monodispersed systems in which $D_P > 0{\cdot}0253$ cm, plots of $\left(\frac{4W}{60\pi\,\rho_P\sqrt{g}}\right)^{0{\cdot}4}$ versus D_o yield straight lines, whose intercepts, k, on the abscissa are some function of the empty annulus at the periphery of the orifice.

Expressing this in the form

$$4W\left(\frac{4W}{60\pi\,\rho_P\sqrt{g}}\right)^{0{\cdot}4} = m\,D_o + C \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where C is an extrapolated negative intercept on the ordinate, it follows that

$$D_0 = \frac{1}{m}\left(\frac{4W}{60\pi\,\rho_P\,\sqrt{g}}\right)^{0.4} - \frac{C}{m} \quad .. \quad .. \quad .. \quad (2)$$

Now clearly $-\frac{C}{m} \equiv k$

thus $$D_0 \equiv \frac{1}{m}\left(\frac{4W}{60\pi\,\rho_P\,\sqrt{g}}\right)^{0.4} + k \quad .. \quad .. \quad .. \quad (3)$$

and this is a general equation which applies to such varied materials as coal, glass beads, sand and tapioca (Brown & Richards, 1960).

Its applicability to the present experimental results on the mono systems of magnesia has been tested in Fig. 5, when good straight lines are obtained for the various values of D_P. The slopes, which have been obtained by regression analysis, have been plotted against particle size and obey the relationship

$$1/m = 0{\cdot}5244\ D_P + 1{\cdot}9738 \quad .. \quad .. \quad .. \quad .. \quad (4)$$

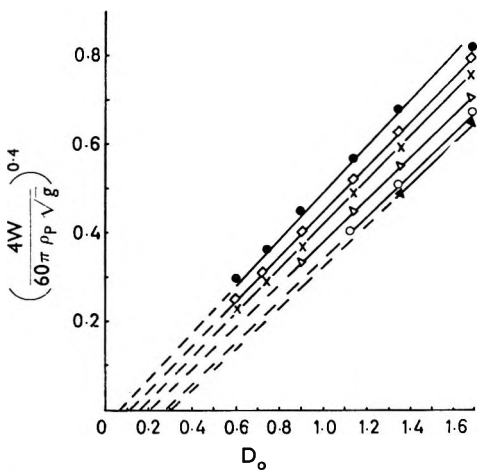

FIG. 5. Plot of $\left(\frac{4W}{60\pi\,\rho_P\,\sqrt{g}}\right)^{0.4}$ against orifice diameter D_0 cm. Particle size in cm: ●, 0·0253; □, 0·0561; ×, 0·0851; △, 0·1340; ○, 0·1866; ▼, 0·2435.

It had previously been shown (Brown, 1961b) that for the materials already mentioned, k is qualitatively related to D_P; this can be seen in a limited way from the data in Table 2. But with the present grade of magnesia in which the densities, shapes and rugosities of the particles are similar, a quantitative relationship between k and D_P may be expected. Log k has been plotted versus log D_P when a good straight line graph is obtained in which the maximum deviation is ±3%. From this

$$k = 0{\cdot}8375\ D_P^{0{\cdot}7} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (5)$$

TABLE 2. VARIATION OF k WITH PARTICLE SIZE

Material	Size (cm)	k	Reference
Magnesia	0·0158 0·0253 0·0358 0·0561 0·0851 0·1340 0·1866	0·005 0·065 0·07 0·11 0·15 0·19 0·28	Present work
Sand	0·0170 0·0380 0·0540	0·036 0·079 0·12	Brown 1961(a)
Glass beads ..	0·0230 0·0960	0·041 0·13	
Rounded sand ..	0·0620	0·10	
Coal	0·0610	0·14	
Tapioca	0·1450	0·22	

Now we can eliminate k, which is a function of D_P from equation (3) by writing it in the form

$$D_0 = A\left(\frac{4W}{60\pi\,\rho_P\,\sqrt{g}}\right)^{1/N} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (6)$$

where 1/N and A are also functions of D_P. This in turn can be written

$$D_0 \propto \left(\frac{4W}{60\pi\,\rho_P\,\sqrt{g}}\right)^{1/N} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (7)$$

and it is seen that the expression (7) is formally similar to the well known relationship

$$D_0 \propto W^{1/n} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (8)$$

which has been shown to apply to a very wide range of different materials (references already cited). It may be inferred, therefore, that the exponents 1/n and 1/N should, in fact, be identical.

Many workers, using a wide variety of materials (Ketchum, 1911; Franklin & Johanson, 1955; Beverloo & others, 1961), have reported n as lying between 1/2·5 and 1/3·1. It is implicit in their data that a functional relationship should exist between 1/n and D_P. But to establish this and thereby explain the several anomalous results that have been reported by Beverloo & others (1961) it is necessary to eliminate the complicating effects of particle density, shape and rugosity. This has been done with the present grade of magnesia, and we now find that from a plot of 1/n against log D_P

$$1/n = 0{\cdot}2571 - 0{\cdot}0855 \log D_P \quad .. \quad .. \quad .. \quad (9)$$

which is a linear relationship. (Clearly, having eliminated the effect of ρ_P, it is not now pertinent to use the present data for testing the validity of other equations in which ρ_P appears as a parameter.) The exponent, 1/n, varies from 1/2·0 at 0·0038 cm to 1/3·2 at 0·2435 cm. The mean value of this range is 1/2·6 and this approximates to the value of 0·4 obtained by dimensional analysis (Deming & Mehring, 1929; Takahashi,

1933; Rose & Tanaka, 1959; Beverloo & others, 1961), the same value, in fact, that appears in equation (3). Furthermore, when $10 > D_P > 1$ cm, 1/n is approximately 1/3, which leads to the relationship $W \propto D_o^3$ originally established by Ketchum (1911).

To make use of equation (6) which, as we have seen, is simply another form of equation (3), we employ equation (9) to obtain $1/n$ ($\equiv 1/N$). Then plotting $\left(\frac{4W}{60\pi \rho_P \sqrt{g}}\right)^{1/n}$ versus D_o for each particle size, should yield a series of straight lines passing through the origin. The result is shown in Fig. 6 and satisfactorily agrees with expectation. The slopes, A, of the lines have been obtained, as before, by regressional analysis; when plotted against D_P they yield the relationship

$$A = 1{\cdot}6822\, D_P + 1{\cdot}9779 \qquad \ldots\ \ldots\ \ldots\ \ldots\ (10)$$

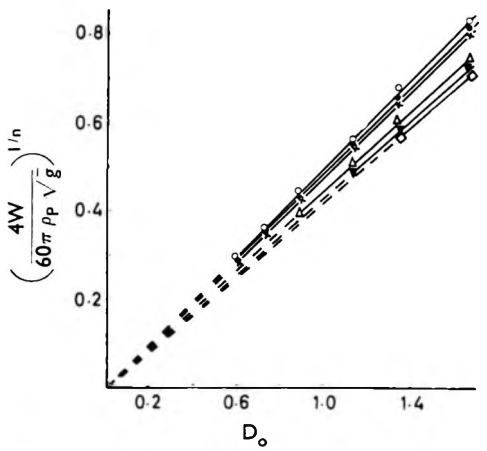

FIG. 6. Plot of $\left(\frac{4W}{60\pi \rho_P \sqrt{g}}\right)^{1/n}$ against orifice diameter D_o cm. D_P in cm: ○, 0·0253; ●, 0·0561; ×, 0·0851; △, 0·1340; ▼, 0·1866; □, 0·2435.

Hence the particular form of equation (6) that is applicable to the grade of magnesia now employed is

$$D_o = (1{\cdot}6822\, D_P + 1{\cdot}9779)\left(\frac{4W}{60\pi \rho_P \sqrt{g}}\right)^{0{\cdot}2571 - 0{\cdot}0855 \log D_P} \qquad \ldots\ (11)$$

There will, however, be occasions when it may be preferable to use equation (6) in its alternative form of equation (3), since this includes the empty annulus term k and this has physical significance. Substituting for 1/m and k from equations (4) and (5) into equation (3) we obtain, for magnesia

$$D_o = (0{\cdot}5244\, D_P + 1{\cdot}9738)\left(\frac{4W}{60\pi \rho_P \sqrt{g}}\right)^{0{\cdot}4} + 0{\cdot}8375\, D_P^{0{\cdot}7} \qquad \ldots\ (12)$$

which gives equivalent results to equation (11).

These equations have been used to predict the flow rates of the monodispersed systems and the results are given in Table 3. It is seen that the mean error is $\pm 3\%$, the maximum error is $\pm 4{\cdot}2\%$ and this is considered very satisfactory.

TABLE 3. FLOW RATES OF MONODISPERSED SYSTEMS

Particle size (cm)	$D_{o(cm)}$	W_{calc} (g/min)	W_{obs} (g/min)	Error
0·0253	0·74 1·353	398 1842	397–399 1923–1945	0% −4·2%
0·0561	0·74 0·898	302 513	291–293 514–517	+3% −0·13%
0·0851	0·898 1·353	432 1400	435–437 1401–1413	−1% −0·35%
0·1340	0·898 1·353	341 1173	322–338 1128–1158	−0·9% −2·5%
0·1866	1·353 1·686	980 1952	936–953 1788–1942	−3·5% −0·5%

The fact that both k and the exponent $1/n$ ($\equiv 1/N$) are functions of the particle size shows that it is inadvisable to employ equations which include only average values of k or of $1/N$ (Leva, 1959; Beverloo & others, 1961; Zenz, 1962), for predicting flow rates over a wide range of particle sizes. Clearly the same treatment which has been employed in the present work for obtaining equations (4), (5), (9) and (10) in the particular case of magnesia, should also yield the values of k, $1/m$, $1/n$ and A that have to be substituted into equations (3) or (6) for predicting the flow rates of other materials.

MIXTURES

So far we have been concerned only with the flow behaviour of mono sieve fractions. But by means of a simple procedure equation (11) can also be employed to predict the flow behaviour of the binary and of the ternary systems. The quantity D_P in equations (9), (10) and (11) is replaced by a geometrical mean diameter $D_{P_{av}}$. Being a geometrical mean, weighting is automatically given to the smaller sized particles which are known to influence flow rates most.*

For a binary system

$$D_{P_{av}} = \sqrt{D_{P_1}^{M_1 0{\cdot}5} \times D_{P_2}^{M_2 0{\cdot}5}} \quad .. \quad .. \quad .. \quad (9)$$

For a ternary system

$$D_{P_{av}} = \sqrt[3]{D_{P_1}^{M_1 0{\cdot}33} \times D_{P_2}^{M_2 0{\cdot}33} \times D_{P_3}^{M_3 0{\cdot}33}} \quad .. \quad .. \quad (10$$

* While an arithmetic mean is normally used to characterise real powders where the particle size distribution is a continuous function, in the present mixtures where the histogram is discontinuous, it is unrealistic to employ an arithmetic scale, e.g. a modal, median, mean weight or mean surface diameter. A χ^2 test showed a much closer fit between observed and predicted values of flow rate using a geometric mean than could be obtained with any conventional arithmetic mean.

where M_1 is the mass fraction of particles of diameter D_{P_1}

M_2 ,, ,, ,, ,, ,, ,, ,, ,, D_{P_2}

M_3 ,, ,, ,, ,, ,, ,, ,, ,, D_{P_3}

D_{Pav} for quaternary and higher systems will be obtained similarly.

Values of D_{Pav} have been calculated for several of the binary and ternary systems employed in the present investigation and then substituted into equation (11). Tables 4 and 5 show that the resulting calculated values of flow rate W_{calc}, agree with the observed values on average to within $\pm 5\%$ and at worst to within $\pm 13\%$.

TABLE 4. FLOW RATES OF BINARY MIXTURES

Mixture				D_o (cm)	W_{calc} (g/min)	W_{obs} (g/min)	Error
D_{P_1}	Conc (% w/w)	D_{P_2}	Conc (% w/w)				
0·0561	20	0·1340	80	0·898 1·353	377 1267	341–372 1242–1290	+1·3% 0%
	50		50	0·898 1·353	430 1397	431–435 1405–1429	+0·025% −0·6%
	80		20	0·898 1·353	481 1512	501–510 1566–1607	−4% −3·4%
0·0561	20	0·0851	80	0·898 1·353	450 1444	448–462 1490–1510	0% −3%
	50		50	0·898 1·353	474 1498	482–489 1553–1591	−1·2% −3·5%
	80		20	0·898 1·353	497 1550	515–529 1645–1675	−3·5% −5·8%
0·0561	20	0·0253	80	0·74 1·14 1·353	379 1155 1797	391–396 1215–1233 1931–1941	−2·8% −4·9% −6·9%
	50		50	0·74 1·353	350 1724	361–365 1879–1900	−3·0% −8·2%
	80		20	0·603 1·353	185 1645	185–197 1725–1740	0% −5·6%
0·1340	80	0·0253	20	0·898 1·353	409 1348	324–352 1254–1274	+13·9% +5·6%
	50		50	0·898 1·353	507 1569	489–533 1689–1740	0% −7·1%
	20		80	1·353	1745	1932–2105	−9·7%
0·0851	20	0·0253	80	0·603 1·353	217 1772	238–242 2016–2039	−8·8% −12·1%
	40		60	0·74 1·14	340 1074	356–367 1180–1200	−4·5% −8·9%
	80		20	0·898 1·353	481 1514	466–482 1505–1548	0% −2·19%

By employing the procedure in reverse, it is possible to calculate the composition of any binary mixture to within about $\pm 12\%$ if its flow rate is measured and the sizes of the constituent particles are known. Both procedures might be useful in certain circumstances for quality control of magnesia in industry.

TABLE 5. FLOW RATES OF TERNARY MIXTURES

Mixture (% w/w) D_P			D_o (cm)	W_{calc} (g/min)	W_{obs} (g/min)	Error
0·1340	0·0253	0·0561				
20	40	40	0·74	320	322–340	−0·6%
			1·14	1029	1144–1174	−10%
60	20	20	0·898	444	378–425	+4·5%
			1·14	877	844–910	0%
	D_P		D_o (cm)	W_{calc} (g/min)	W_{obs} (g/min)	Error
0·0851	0·0561	0·0253				
20	40	40	0·898	556	591–608	−9·3%
			1·14	1054	1122–1188	−6·1%
50	10	40	0·603	181	179–187	0%
			1·14	978	1076–1102	−5·2%

CONCLUSION

The principle involved in the derivation of equations (11) or (12), namely the employment of an exponent 1/n and an annulus term k, both of which are functions of particle size, should be of general application to other granular materials.

k, 1/n, A and 1/m are obtained as functions of particle shape and size, it should become possible to predict the flow rates of other sizes of the same materials through any sized circular orifice.

The equations that have been developed for granular magnesia in the present work provide, for the first time, a means of predicting its flow rate when particles of many different sizes are present.

NOTATION

A An empirical function of D_P: equation (10).

D_c Tube diameter (cm).

D_o Orifice diameter (cm).

D_P Mean arithmetic sieve diameter (cm).

D_{Pav} Geometric mean diameter of particles in a mixture (cm).

g Acceleration due to gravity (cm/sec^2).

k A function of the empty annulus around the orifice (cm) dependent on D_P: equation (5).

1/m An empirical function of D_P: equation (4).

M The mass fraction of particles at size D_P.

1/n An empirical function of D_P: equation (9).

ρ_P Particle density (g/ml).

ρ_B Bulk density (g/ml).

W Flow rate (g/min).

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The determination of liothyronine and thyroxine in thyroid preparations

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A procedure is described for determining liothyronine and thyroxine in thyroid substances. This involves stepwise hydrolysis with barium hydroxide, extraction of combined iodinated amino-acids by n-butanol, separation and isolation of liothyronine and thyroxine by paper chromatography, subsequent ignition of their separated chromatograms by oxygen flask combustion and estimation of evolved iodine in benzene solution by spectrophotometric absorption at 295 mμ. The method is suitable for routine quality control of thyroid products.

THE United States Pharmacopeia and British Pharmacopoeia both contain monographs on thyroid preparations which specify a total organic iodine or thyronine iodine assay, the inadequacies of which are generally recognised. The advisability of analysing thyroid preparations for liothyronine and thyroxine content has been noted by Mandl & Block (1959), Devlin & Stephenson (1962), and Backer (1964). Meister, Williams & Florsheim (1963) have developed a method for detecting clinically ineffective thyroid preparations which gives a good correlation between chemical assay and biological potency although the liothyronine and thyroxine contents are not individually determined. Meister found Blau's (1935) method of measuring thyronine iodine to be inadequate for the detection of defective thyroid preparations.

Devlin & Stephenson (1962) determine the two amino-acids separately. Their method, which uses enzymatic hydrolysis, appears to liberate maximum amounts of liothyronine and thyroxine from the proteinaceous gland extracts and we have found it to be an excellent research tool. However, the method has some disadvantages which precluded its adoption by us for routine control in the manufacture of thyroid preparations. Five days are required for complete enzymatic hydrolysis and a uniformly potent and stable enzyme preparation must be assured if reproducible hydrolysis conditions are to be achieved. Also the ceric sulphate-arsenious acid reaction used gives variable results (Custer & Natelson, 1949). Backer's method (1964) uses enzymatic hydrolysis, paper chromatography, oxygen flask ignition of the amino-acid areas of the papergrams and determination of the liberated iodine by the ceric sulphate-arsenious acid reaction. While this method has the disadvantages already described, the oxygen flask ignition was considered to be superior to elution. We sought a procedure having the precision, accuracy and ease of handling required for the routine quality control of thyroid preparations.

In the method we describe, Blau's procedure (1935) for the hydrolysis and extraction of the thyroid substance is subsequently coupled with paper chromatography. The procedure uses 80 μg of total iodine on each paper and triplicate papers are combined for the assay thus allowing direct observation of developed chromatograms by viewing in ultraviolet

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light, or by conventional spraying techniques. Oxygen flask ignition of the relevant areas of developed chromatograms is similar to that described by Backer (1964), and direct determination of the resulting iodine is made in a total inorganic environment.

Experimental

REAGENTS

All chemicals used for the preparation of solutions were analytical grade, except where specified. Common laboratory solutions not listed were prepared from analytical grade reagents.

Barium hydroxide solution. Prepared carbonate-free to contain 7·8–8·0% $Ba(OH)_2.8H_2O$. *Ammoniacal methanol solution.* To 2,6-di-t-butyl *p*-cresol (60 mg) add concentrated ammonia (1 ml) and anhydrous methanol to 100 ml. *t-Amyl alcohol.* Use the freshly distilled fraction, boiling between 100–103°. *4-Aminoantipyrine solution*, 2%. Dissolve 4-aminoantipyrine (m.p. 107–108°) (2 g) and sodium bicarbonate (1·7 g) in water to 100 ml. *Potassium ferricyanide solution*, 2%. Dissolve $K_3Fe(CN)_6$ (5 g) in water to 250 ml. Prepare fresh daily. *Benzene.* Use thiophene-free benzene.

PREPARATION OF SAMPLE

Tablets. Transfer the equivalent of 60 15 mg U.S.P. thyroid tablets, finely ground and accurately weighed, to a 50 ml glass stoppered centrifuge tube with water (25 ml). Shake for 10 min, centrifuge at 2,000 rpm for 5 min, decant and discard the supernatant. Wash the residue with water (25 ml) and with shaking to ensure complete dispersal. Heat for 5 min on a steam-bath, centrifuge for 5 min and discard the supernatant. Repeat this wash procedure. Add 50% v/v methanol in chloroform (25 ml) to the centrifugate, stopper, and shake for 2 min. Centrifuge and discard the supernatant. Repeat this and dry the residue with nitrogen, while warming on a steam-bath.

Bulk powders. Transfer an accurately weighed quantity of thyroid powder equivalent to 2 mg of iodine to a 50 ml glass stoppered centrifuge tube.

HYDROLYSIS

Pipette exactly 10 ml of barium hydroxide solution into the centrifuge tube and add n-butanol (0·5 ml). Reflux using an air condenser over a steam-bath for 6 hr, shaking vigorously at 15 min intervals, cool to room temperature and adjust to pH 0·8 with 6 N hydrochloric acid.

EXTRACTION

Extract the hydrolysate with n-butanol (8 ml), shake for 2 min, and centrifuge for 1 min. Repeat twice using 5 ml of butanol each time. Transfer the butanol top layers to a 50 ml glass stoppered centrifuge tube using a 10 ml syringe equipped with a 3½ inch No. 17 gauge needle with a

filed or flat ground tip. Wash the combined extracts with 0·1 N sulphuric acid (1 ml), centrifuge, remove and discard the bottom layer with a syringe equipped with the same needle as above. In the same manner, wash with two 1 ml portions of water. Transfer the washed butanol extract to a 50 ml round bottom boiling flask, through a medium sintered glass filter under vacuum. Rinse the centrifuge tube with 3 × 2 ml n-butanol, and add via the filter to the butanol extract. Add concentrated ammonia (0·5 ml) and evaporate to dryness using a rotary film evaporator connected to a vacuum pump through a safety trap cooled with a mixture of dry ice and acetone. Transfer the residue with repeated 1 ml portions of ammoniacal methanol solution to a 5 ml volumetric flask through a fine sintered glass filter and dilute to volume with the ammoniacal methanol solution.

CHROMATOGRAPHY

Equilibrate t-amyl alcohol (150 ml) with 6 N ammonia (150 ml) by shaking for 5 min. Transfer the ammonia layer in a container to the bottom of a developing tank lined with Whatman No. 1 paper. Pour half of the t-amyl alcohol phase around the walls of the tank, cover and allow to equilibrate for 2 hr. Wet Whatman No. 1 chromatographic paper 5 × 1½ inch by 18 inch strips with 0·05% sodium sulphite solution and air dry. Along a starting line located 3 inch from one end, apply 200 μl aliquots of the sample solution in streaks 1 inch wide and ¼ inch to ⅜ inch high. Allow to equilibrate for 2 hr in a tank saturated with water vapour. Transfer the papers to the developing tank, fill the trough with the t-amyl alcohol phase and cover. Develop for 18 hr or until the solvent front is about 1–2 inch from the bottom of the papers. Mark the solvent fronts, and allow to air dry. View the chromatograms in ultraviolet light using an apparatus such as that described in the U.S.P. XVI, p. 923. The thyroxine can be seen as an ultraviolet absorbing zone at Rf 0·6, while the liothyronine can sometimes be seen at Rf 0·7. Confirm by spraying the two end chromatograms lightly with 4-aminoantipyrine solution, allow to dry, then spray with the potassium ferricyanide solution. The pink band occurring at about Rf 0·7 is liothyronine. The band at about Rf 0·6 is thyroxine. Mark and cut out the two bands on the three unsprayed assay papers and prepare for oxygen flask ignition.

OXYGEN FLASK IGNITION

For thyroxine, pipette 20 ml of N sodium hydroxide into a 500 ml Schoeniger ignition flask, add water (10 ml) and flush the flask with oxygen. Ignite one paper by means of a wick of Whatman No. 1 paper (1½ × ⅛ inch). Stopper and invert the flask and shake for 2 min. Using the same sodium hydroxide solution, treat the remaining thyroxine papers in the same manner. The liothyronine papers are similarly treated except that 10 ml of sodium hydroxide and 20 ml of water are used. Pipette 3 ml of 0·1 N potassium permanganate into both flasks, and also into a third flask containing 10 ml of N sodium hydroxide, together with

sufficient water to approximately equal the volume in the other flasks. Mix and allow to stand for 5 min. To the flasks add 5 N sulphuric acid—3 ml to the blank and liothyronine, and 5 ml to the thyroxine—and 1% sodium nitrite solution (2 ml). Two min after the sodium nitrite add 5% ammonium sulphamate (1 ml). Transfer the contents of each flask to 125 ml separating funnels and wash the contents with benzene 2 × 25 ml. Discard the benzene and add 3 ml of a 7·5% solution of potassium iodide to each funnel. Mix, and extract the liberated iodine with 1 × 25, 1 × 15, and 1 × 10 ml of benzene. Pass each benzene extract through anhydrous sodium sulphate, collect in a 50 ml graduated cylinder, and dilute to volume with benzene. Determine the absorbance of the liothyronine solution and of the reagent blank solution against benzene at 295 mμ, using 10 cm silica cells. Subtract the reagent blank absorbance. Dilute the thyroxine sample (20 ml) to 50 ml with benzene, and determine its absorbance at 295 mμ. Dilute the reagent blank solution similarly.

Standard iodine calibration curve in benzene. This is prepared by measuring the absorbance at 295 mμ of solutions containing 80, 120, 160 and 200 μg/100 ml reagent grade iodine prepared accurately by dilution.

CALCULATION

From the standard calibration curve, determine the μg of iodine per 50 ml of solution, corresponding to the corrected absorbances found for the amino-acids.

Bulk powder:

$$\text{Liothyronine in mg/g} = \frac{\mu\text{g of iodine} \times 5}{3{\cdot}6 \times 0{\cdot}585 \times 1{,}000 \times \text{sample weight in g}}$$

$$\text{Thyroxine in mg/g} = \frac{\mu\text{g of iodine} \times 50 \times 5}{3{\cdot}6 \times 0{\cdot}655 \times 1{,}000 \times 20 \times \text{sample weight in g}}$$

Tablets:

$$\text{Liothyronine in } \mu\text{g/tablet} = \frac{\mu\text{g of iodine} \times 5}{3{\cdot}6 \times 0{\cdot}585 \times \text{number of tablets}}$$

$$\text{Thyroxine in } \mu\text{g/tablet} = \frac{\mu\text{g of iodine} \times 5 \times 50}{3{\cdot}6 \times 0{\cdot}655 \times 20 \times \text{number of tablets}}$$

Results

Table 1 presents values obtained for the liothyronine and thyroxine contents of various batches of commercial thyroid powders of porcine origin. Information permitting a statistical evaluation is also given in the form of standard deviations. The pooled standard deviation of the 40 analyses of Table 1 is 0·17 for thyroxine and 0·07 for liothyronine. All of the thyroid powders have total iodine contents between 0·8% and 1·07%. Results from the analysis of tablets containing proprietary thyroid powders are given in Table 2.

TABLE 1. RESULTS OBTAINED FOR THE LIOTHYRONINE AND THYROXINE CONTENTS OF VARIOUS BATCHES OF COMMERCIAL THYROID POWDERS OF PORCINE ORIGIN

Sample No.	Number of analyses	$\bar{x}$ Thyronine mg/g	s	v	$\bar{x}$ Liothyronine mg/g	s	v
9449	6	2·31	0·12	5·2	0·85	0·06	7·1
1744EM3	4	1·95	0·18	9·2	0·74	0·02	2·7
3557082	4	1·11	0·09	8·1	1·03	0·16	16·0
1386	4	2·34	0·12	5·1	0·77	0·03	3·9
3832	7	2·30	0·21	9·1	0·82	0·05	6·1
6829	5	2·34	0·25	10·6	0·81	0·05	6·2
6375	4	2·11	0·19	9·0	0·81	0·08	9·9
1507	6	1·45	0·06	4·1	1·20	0·08	6·7

s = standard deviation

v = coefficient of variance = $\frac{s}{\bar{x}} \times 100$.

TABLE 2. RESULTS OF ANALYSIS OF TABLETS CONTAINING PROPRIETARY THYROID POWDERS

Sample	Analysis	Thyroxine µg/tab	Liothyronine µg/tab
15 mg tablets. Lot 01063–21..	1	6·20	2·07
	2	6·45	2·18
	3	6·28	2·50
Average		6·31	2·25
s		± 0·13	± 0·23
v		± 2·1%	±10·0%
60 mg tablet. Lot 6832 ..	1	20·7	9·60
	2	20·6	10·90
	3	21·2	10·60
Average		20·8	10·37
s		± 0·3	± 0·7
v		± 1·4%	± 6·8%

s = standard deviation

v = coefficient of variance = $\frac{s}{\bar{x}} \times 100$.

Discussion

Since the analysis of thyroid preparations for liothyronine and thyroxine consists of several analytical procedures, it is convenient to discuss it in terms of the following critical parameters.

Sample preparation. Bulk thyroid powder requires no sample preparation. Thyroid tablet formulations usually contain excipients such as lactose, gelatin or magnesium stearate, which frequently comprise the major fraction of the total mass. It is therefore desirable to remove these ingredients before analysis. No appreciable loss of iodine occurs in the wash procedure described. Of the samples studied, the thyroid component of tablets was sufficiently denatured to be completely insoluble in cold or hot water. The results of the analysis of tablets, when correlated with results for the bulk powders from which they were prepared, showed no loss of liothyronine or thyroxine.

Hydrolysis. To determine if the Blau method of hydrolysis liberated maximum amounts of the two amino-acids, samples were analysed by using a modification of Devlin and Stephenson's procedure for hydrolysis

and extraction. This modification consisted of increasing by a factor of 5 their quantities of sample and reagents for hydrolysis and extraction. The extracted hydrolysate was then analysed for liothyronine and thyroxine by the method previously given. A comparison of enzyme hydrolysis with barium hydroxide hydrolysis using one sample of material gave 2·16 and 2·31 mg/g of thyroxine for the enzyme hydrolysis and the hydroxide hydrolysis respectively. For liothyronine, the figures were 0·73 and 0·85 mg/g. The Blau method of hydrolysis is retained for analysis of the two amino-acids because good agreement with enzyme hydrolysis exists, and because the hydrolysis time is only 6 hr compared with 5 days for enzymes.

Extraction. In recovery experiments, with barium hydroxide hydrolysis, n-butanol would not quantitatively extract the amino-acids at pH 3·5, as given in Blau's procedure. To obtain quantitative extraction, it was necessary to reduce the pH to 0·8. Ammoniacal methanol would not quantitatively dissolve the amino-acids if barium salts were present; these were removed from hydrolysis extracts by washing the butanol extracts with 0·1 N sulphuric acid. By using the above modifications to Blau's procedure, it was possible to recover 97–100% of the standards from the 6 hr barium hydroxide hydrolysis and n-butanol extraction.

Chromatography. Morreale de Escobar, Llorente Rodriquez, Jolin & Escobar del Ray (1963) indicated that liothyronine and thyroxine undergo deiodination during paper chromatography and subsequent handling of paper chromatograms. To minimise this effect, chromatographic papers are treated with sodium sulphite, and the solutions to be chromatographed contain 0·06% of 2,6-di-t-butyl *p*-cresol. This combination of anti-oxidants gave the best recovery values. The sulphite also caused the amino-acids to migrate much farther from the iodine band on the paper chromatogram and acts as a combustion accelerator in the oxygen flask ignition.

The solvent system 6 N ammonia:t-amyl alcohol is used because it not only separates the amino-acids from each other, but also removes them from other iodine-containing species. It also has the advantage of confining liothyronine and thyroxine to small areas. Moisture equilibration of the chromatographic papers is necessary because separation is dependent on the amount of moisture in the papers before development. If the sample spots are not saturated with water before development, developing solvent accumulates and appears held in the sample areas until the excessive amount spills down the chromatogram.

Location of the amino-acids on paper chromatograms. The method of locating liothyronine and thyroxine on paper chromatograms is described in the test for sodium thyroxine in sodium liothyronine of the sodium liothyronine monograph of U.S.P. XVI. Sufficient liothyronine is present in most thyroid preparations to allow visualisation. However, if this proves difficult, 5–10 μg of the pure amino-acid can be spotted *with* the sample solution on papers which are used for spot detection. This is because in the presence of the large amount of sample material the amino-acids do not migrate with the same Rf values as they do when pure.

Determination of liothyronine and thyroxine from developed chromatograms. The oxygen flask technique was found to be rapid, convenient, accurate and more sensitive than other methods. After ignition, all the iodine present is oxidised to iodate by potassium permanganate. Excess permanganate is quickly and easily removed with sodium nitrite which is itself destroyed by ammonium sulphamate. Sodium nitrite immediately reduces permanganate to manganous ion in acid solution but since some manganic ion may be present, a 2 min reaction period is required to ensure complete conversion. No loss of iodate has been observed during the 2 min that nitrite is present.

This procedure for determining the iodine content is sufficiently sensitive to permit the measurement of the low levels of both amino-acids found in thyroid preparations. With normal precautions, the reagent blanks are low and a precision of $\pm 2\%$ is obtained when standard solutions are spotted on paper, ignited by the oxygen flask method and the iodine determined as described. A recovery of 97·9% is obtained for 2-iodobenzoic acid (National Bureau of Standard reference sample), with a standard deviation of 1·0% for five determinations.

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A critical assessment of an accelerated storage test

B. R. COLE AND L. LEADBEATER

The accelerated storage test proposed by Rogers enables the rate constant (at a known temperature) and the energy of activation of a reaction occurring in solution to be calculated from a single experiment. This test has been applied to a number of first and second order reactions, including the heat inactivation of horse serum cholinesterase. The theoretical and practical limitations of the technique are discussed. It is concluded that the test has an important role in determining the stability of pharmaceutical and related compounds in solution and in comparing the stabilities of different batches of the same preparation.

AN accelerated storage test, which enables the rate constant of a reaction at a known temperature, and the energy of activation of a reaction, to be calculated from data obtained in a single experiment, has been described by Rogers (1963). The novelty of the technique is that the temperature is increased with time in the following way

$$\frac{1}{T_o} - \frac{1}{T_t} = 2{\cdot}303\ B \log (1 + t)$$

Where T_o is the temperature (°K) at the start of the experiment, T_t is the temperature (°K) at time t, and B is a constant which determines the rate of rise of the temperature.

By substituting this equation into the Arrhenius equation and assuming an order of reaction it may be shown that

$$\log f(c) = \log k_o - \log\left(1 + \frac{E_A B}{R}\right) + \left(1 + \frac{E_A B}{R}\right) \log (1 + t) + \log\left[1 - \left(\frac{k_o}{k_t}\right)^{1 + \frac{R}{E_A B}}\right]$$

where f(c) is a function of the concentration of the reactants; k_o is the rate constant at temperature T_o; k_t is the rate constant at temperature T_t; E_A is the energy of activation; and R is the gas constant (1·987 cal. degree^{-1} mole^{-1}).

For first order reactions $f(c) = 2{\cdot}303 \log \frac{c_o}{c_t}$

for second order reactions $f(c) = \frac{1}{c_t} - \frac{1}{c_o}$

or $\frac{2{\cdot}303}{a_o - b_o} \log \frac{a_t}{b_t} + \frac{2{\cdot}303}{a_o - b_o} \log \frac{b_o}{a_o}$

where a_o and b_o are the concentrations of the reactants at the beginning of the experiment and a_t and b_t their concentrations at time t.

The value of the final term of the equation rapidly tends to zero as k_t becomes greater than k_o. Thus, a graph of log f(c) against log (1 + t) will

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be a straight line. From the slope of the line the energy of activation may be calculated and the rate constant (k_0) may then be calculated from the intercept when log $(1 + t) = 0$.

Rogers suggested that this accelerated storage test has advantages over the conventional methods for determining stabilities of pharmaceutical preparations (Garrett, 1962). These were: (1) the data required to calculate the stability of the compound are obtained in a single experiment lasting one day rather than in a series of experiments which may last for several weeks; (2) no preliminary experiments are required to determine the optimum temperatures for the accelerated storage test; (3) the linearity of the plot of log f(c) against log $(1 + t)$ confirms that the correct order of reaction for the decomposition has been assumed. Deviations from linearity may indicate that the wrong order of reaction has been chosen or that the mechanism of the reaction changes as the temperature is raised.

Since this accelerated storage test could be of great use in rapidly providing data for the stabilities of compounds in solution, the technique has been investigated both theoretically and experimentally.

Experimental

TEMPERATURE CONTROL

The temperature of a bath containing 17·5 litres of oil was raised, at the rate required by the programme, by the continuous manual adjustment of a variable transformer connected to a 2 kW heater. At any given instant the temperature of the bath was within less than 1° of that required by the programme. Typical programmes are shown in Fig. 1.

EXPERIMENTAL TECHNIQUE

The solution to be tested was divided amongst an appropriate number of tubes with ground glass stoppers. The tubes were placed in the bath and brought to the initial temperature before the temperature programme was commenced. At appropriate times during the experiments tubes were withdrawn and chilled in an ice-water bath.

METHODS OF ASSAY

Hydrolysis of sucrose. The reaction was followed polarimetrically as described by Rogers (1963).

*Solvolysis of methyl toluene-*p*-sulphonate.* A modification of the method of Robertson (1953) was used. The ester concentration was 10^{-2}M and the sulphonic acid produced was titrated with $7{\cdot}5 \times 10^{-3}$M sodium hydroxide using B.D.H. "4·5" indicator in the case of the 50% (v/v) ethanol-water solutions and lacmoid indicator for the 50% (w/w) dioxane-water solutions.

Hydrolysis of ethyl benzoate. The reaction was followed by a modification of the method described by Ingold & Nathan (1936). Equal volumes of 0·05M ethyl benzoate and 0·05M sodium hydroxide were mixed together and 10 ml samples pipetted into the stoppered tubes. Immediately after cooling the samples, excess hydrochloric acid (5 ml, 0·06M) was added.

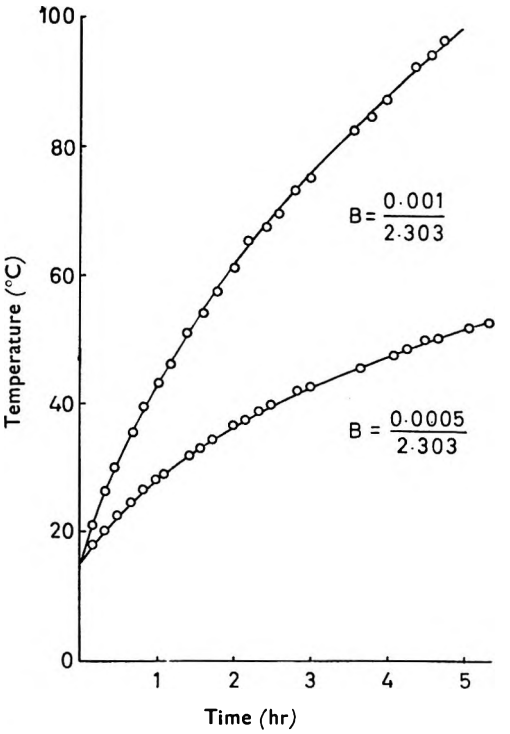

FIG. 1. Temperature programmes. The temperature was raised so that $\left(0{\cdot}00347 - \frac{1}{T_t}\right) = 2{\cdot}303.B.\log(1 + t)$. The points represent the temperature of the sample at the times indicated and the lines the required programme.

Carbon tetrachloride (1 ml) was added to remove the unhydrolysed ester from the aqueous phase and the solution titrated, under nitrogen, with 0·01M sodium hydroxide using bromothymol blue indicator.

Decomposition of N-*methyl pyridinium-2-aldoxime methane sulphonate* (*P2S*). The disappearance of the oxime group was determined by measuring the decrease in the extinction at 335 mμ of the P2S in alkaline solution (Creasey & Green, 1959).

Cholinesterase activity. 15 ml of 0·9% (w/v) saline and 1 ml of acetylcholine chloride (100 mg/ml) were pipetted into a titration cell. The pH was adjusted to 7·40 and the temperature was maintained at 25·00 $\pm$0·05°. 1 ml of the cholinesterase (from horse serum) solution was added and the acetic acid produced by the enzymic hydrolysis of the acetylcholine was titrated with 0·0125M sodium hydroxide, under nitrogen, at pH 7·40, using a Radiometer automatic titrimeter.

The standard deviations of the data are presented in Tables 1 and 2 to present an estimate of the reproducibility of the accelerated storage test.

Results

VALIDITY OF THE TECHNIQUE

Rate constants and energies of activation for the following reactions were determined by the accelerated storage technique and compared with data published in the literature: (*a*) the hydrolysis of sucrose, 40% (w/v), by 0·167N hydrochloric acid; (*b*) the solvolysis of methyl toluene-*p*-sulphonate (10^{-2}M) in 50% (v/v) ethanol-water and in 50% (w/w) dioxane-water; (*c*) the hydrolysis of ethyl benzoate ($2{\cdot}5 \times 10^{-2}$M) by sodium hydroxide ($2{\cdot}5 \times 10^{-2}$M) in 50% (v/v) and in 85% (w/w) ethanol-water.

The data are summarised in Table 1. The rate constants for the solvolysis of methyl toluene-*p*-sulphonate were determined at 40° and were calculated for 50° by means of the Arrhenius equation. Similarly the rate constants for the hydrolysis of ethyl benzoate were determined at 20° and calculated for 25°.

THE STABILITY OF P2S IN SOLUTION

The decomposition of P2S in dilute aqueous solution (3×10^{-5} M) was shown to follow first order kinetics, since log c_o/c_t varied linearly with time when the solution was maintained at 60° and 71° and the first order equation for Rogers' test gave a straight line (Fig. 2).

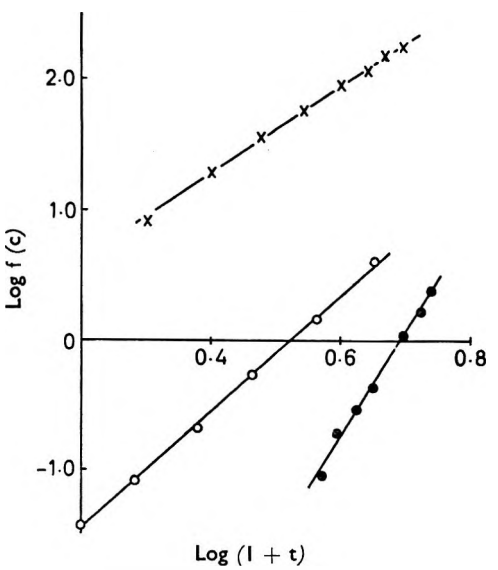

FIG. 2. Plots derived from Rogers' equation for the alkaline hydrolysis of ethyl benzoate (×), the decomposition of P2S in phosphate buffer (○) and the thermal inactivation of horse serum cholinesterase (●).

The stability of P2S was determined both in 0·0485M phosphate buffer, pH 7·38, and in unbuffered aqueous solution. The pH of the latter solution was 5·80 and was unchanged at the end of the reaction. The results are shown in Table 2.

TABLE 1. THE HYDROLYSIS OF SUCROSE, METHYL TOLUENE-*p*-SULPHONATE AND ETHYL BENZOATE

Reaction	No. Expts.	Rate constant	Energy of activation kcal mole^{-1}	Method of determination	Reference
Hydrolysis of sucrose in 0·167N HCl	3	$k_{30} = 6{\cdot}90 \pm 2{\cdot}82 \times 10^{-5}$	26·9 ± 1·6	Accelerated storage	—
Hydrolysis of sucrose in 0·167N HCl	1	$k_{30} = 3{\cdot}49 \times 10^{-5}$	25·8	Accelerated storage	Rogers (1963)
Hydrolysis of sucrose in 0·00167N HCl	1	$k_{30} = 3{\cdot}66 \times 10^{-5}$	27·6	Accelerated storage	Rogers (1963)
Hydrolysis of sucrose in 0·19N HCl	—	$k_{20} = 7{\cdot}11 \times 10^{-5}$ (litre mole^{-1} sec^{-1})	25·4	Isothermal storage	Moelwyn-Hughes (1934)
Solvolysis of methyl toluene-*p*-sulphonate in 50% (v/v) ethanol-water	4	$k_{50} = 4{\cdot}22 \pm 0{\cdot}21 \times 10^{-5}$	20·1 ± 1·6	Accelerated storage	—
	—	$k_{50} = 4{\cdot}41 \times 10^{-5}$ (sec^{-1})	—	Isothermal storage	Robertson (1953)
Solvolysis of methyl toluene-*p*-sulphonate in 50% (w/w) dioxane-water	3	$k_{50} = 1{\cdot}91 \pm 0{\cdot}12 \times 10^{-5}$	19·7 ± 0·2	Accelerated storage	—
	—	$k_{50} = 2{\cdot}29 \times 10^{-5}$ (sec^{-1})	22·1	Isothermal storage	Robertson (1953)
Alkaline hydrolysis of ethyl benzoate in 50% (v/v) ethanol-water	3	$k_{25} = 1{\cdot}27 \pm 0{\cdot}08 \times 10^{-3}$	14·8 ± 0·4	Accelerated storage	—
	—	$k_{25} = 1{\cdot}33 \times 10^{-3}$ (litre mole^{-1} sec—^{1})	—	Isothermal storage	Ingold & Nathan (1936)
Alkaline hydrolysis of ethyl benzoate in 85% (w/w) ethanol-water	3	$k_{25} = 5{\cdot}90 \pm 0{\cdot}66 \times 10^{-4}$	17·0 ± 1·1	Accelerated storage	—
	—	$k_{25} = 5{\cdot}5 \times 10^{-4}$ (litre mole^{-1} sec^{-1})	17·7	Isothermal storage	Ingold & Nathan (1936)

TABLE 2. THE STABILITY OF P2S IN DILUTE AQUEOUS SOLUTION

Solution		No. Expts.	k_{15} (sec^{-1})	Energy of activation (kcal mole^{-1})	Half-life at 15° (years)	Method
pH 7·38	0·0485M phosphate	4	$7{\cdot}55 \pm 2{\cdot}75 \times 10^{-9}$	34·15 ± 1·14	2·9 ± 0·8	Accelerated storage
		3	$9{\cdot}61 \pm 4{\cdot}07 \times 10^{-9}$	33·51 ± 2·35	2·3 ± 1·0	Isothermal storage at 60° and 71°
	0·01M phosphate	—	$6{\cdot}8 \times 10^{-9}$	32	3·2	Isothermal storage*
pH 5·8	Water unbuffered	4	$5{\cdot}28 \pm 2{\cdot}55 \times 10^{-11}$	43·34 ± 2·38	416 ± 163	Accelerated storage

* Data interpolated from the data of Fan, Nairn & Walker (1964).

THE THERMAL STABILITY OF CHOLINESTERASE

Horse serum cholinesterase which had been purified eighteenfold by chromatography on diethylaminoethylcellulose and by gel filtration through Sephadex was employed. The enzyme was dissolved either in carbon dioxide-free water, when the final pH was 7·35, or in 0·0056M veronal-acetate buffer, pH 7·40 (Michaelis, 1931) at a concentration of 2 mg protein per ml. The inactivation was assumed to be first order and good straight lines were obtained (Fig. 2). The data obtained from two preparations of cholinesterase are shown in Table 3.

TABLE 3. THERMAL STABILITY OF HORSE SERUM CHOLINESTERASE IN AQUEOUS SOLUTION

Preparation number	No. assays	Solution	E kcal mole^{-1}	k_{25} sec^{-1}
A	2	H_2O	84·4	$2·6 \times 10^{-10}$
X12	2	H_2O	66·9	$8·1 \times 10^{-9}$
X12	2	Veronal-acetate	75·8	$4·8 \times 10^{-9}$

The half-life of preparation X12, dissolved in water, was calculated for various temperatures, from the data obtained by Rogers' technique, and the results compared with direct observations made on the enzyme solution stored at those temperatures (Table 4).

TABLE 4. A COMPARISON OF THE STABILITY OF HORSE SERUM CHOLINESTERASE AT VARIOUS TEMPERATURES DETERMINED BY ISOTHERMAL STORAGE AND CALCULATED FROM DATA OBTAINED IN THE ROGERS ACCELERATED STORAGE TEST

Half life $t_{\frac{1}{2}}$	Temperature °C			
	30	50	60	71
Determined	> 70 hr	3·5 hr	4·6 min	21 sec
Calculated	76 days	3·75 hr	9·8 min	19 sec

The half-life of the preparation was not determined at 30° since no special precautions were taken to ensure that the solution was aseptic. No loss of activity was detected over 70 hr but inactivation after that length of time could have been produced by bacterial invasion of the solution.

Discussion

LIMITATIONS OF THE ARRHENIUS EQUATION

The accelerated storage test is based on the Arrhenius equation which relates the rate constant of a reaction with temperature, thus:

$$\log k_t = \log k_o + \frac{E_A}{2·303\ R}\left(\frac{1}{T_o} - \frac{1}{T_t}\right)$$

This equation is an approximation but nevertheless it permits the calculation of rate constants, over a range of temperatures, which are in reasonable agreement with those determined experimentally (Moelwyn-Hughes,

1947) and it has been widely used as a basis for accelerated storage tests (Garrett, 1962).

In Rogers' equation the slope of the line obtained, which is equal to $(1 + E_AB/R)$, is not constant but changes as the energy of activation varies. However, this change in slope has not been detected in any of the experiments reported in this paper (see Figs 2 and 3), even though they were made over a temperature range of at least 40°. The value of the activation energy determined by this method is an average value for the temperature range employed.

There are a number of reactions to which the Arrhenius equation does not apply (Moelwyn-Hughes, 1947). Thus accelerated storage tests based on the equation, either the test devised by Rogers or the conventional method of a series of isothermal experiments, are not of universal application. However, in Rogers' method, deviations from the Arrhenius equation would be detected as a change in slope of the line.

The mechanism of a decomposition may change as the temperature is raised. The change can be detected by Rogers' technique provided it involves a change in the energy of activation or order of reaction. Even slight changes in the energy of activation could be detected since Rogers' equation gives good linear plots (see Figs 2 and 3).

LIMITATION OF ROGERS' EQUATION

In the accelerated storage test it must be assumed that the final term in Rogers' equation, namely,

$$\log\left[1 - \left(\frac{k_0}{k_t}\right)^{1 + \frac{R}{E_AB}}\right]$$

rapidly tends to zero and can be ignored. It is essential to establish the conditions when this assumption is valid.

The values of the expression have been calculated for a hypothetical first order reaction. The rate constant for the reaction at 15° was assumed to be 10^{-4} hr^{-1} and the energy of activation 20·0 kcal $mole^{-1}$. The temperature was raised from 15° according to the following programme:

$$\frac{1}{288{\cdot}2} - \frac{1}{T_t} = 0{\cdot}001 \log (1 + t).$$

The values of the expression are recorded in Table 5. It may be seen that the expression rapidly tends to zero and within a temperature rise of 10° it is less than 2% of the value of

$$\left[\log 2{\cdot}303 \log \frac{c_0}{c_t}\right]$$

Thus it is safe to neglect this term provided that data are not used which were obtained within 10° of the starting temperature. For reactions with activation energies less than 20 kcal $mole^{-1}$ the value of the factor would be slightly higher and a slightly larger temperature interval would be required before data could be used. This gap between the initial

TABLE 5. THE RATE OF DECREASE OF $\log \left[1 - \left(\frac{k_0}{k_t}\right)^{1 + \frac{R}{E_A B}}\right]$ WITH RISE IN TEMPERATURE

Equation	Temperature °C						
	17·5	20·0	22·5	25·0	30·0	40·0	60·0
$\log \left[1 - \left(\frac{k_0}{k_t}\right)^{1 + \frac{R}{E_A B}}\right]$	−0·509	−0·284	−0·177	−0·067	−0·055	−0·012	−0·001
$\log \left[2{\cdot}303 . \log \frac{c_0}{c_t}\right]$	−4·570	−4·412	−4·257	−4·105	−3·808	−3·244	−2·212

temperature of the first observation which can be employed is normally unavoidable for relatively stable compounds where no detectable decomposition occurs until the temperature has been raised to 40–60°.

It is inherent in the method of Rogers that the error in the estimate of the rate constant (k_0) must be large since it is calculated from the following expression:

$$\log k_0 - \log (\text{Slope}) = \text{Intercept} \qquad [\log (1 + t) = 0]$$

Thus the integer of the intercept determines the characteristic of log k_0 and all the error in the estimate of the intercept, together with the error in the estimate of the slope, appears in the mantissa of log k. For example, if the intercept is −5·80 and the slope is 2·00 and the error in the estimates of these values is ±1% then the error in the estimate of k_0 is not ±1% but either ±15% ($k_0 = 3{\cdot}17 \pm 0{\cdot}47 \times 10^{-6}$) or ±13% ($k_0 = 3{\cdot}17 \pm 0{\cdot}41 \times 10^{-6}$), depending on whether or not the errors in the estimates of the slope and intercept are additive.

REQUIREMENTS FOR THE APPLICATION OF ROGERS' ACCELERATED STORAGE TECHNIQUE

(*a*) The reaction to be studied must occur in a homogeneous liquid or gaseous phase since the theory on which the method is based is only applicable to reactions occurring under these conditions.

(*b*) A method of assay specific either to the original compound or to one of its decomposition products must be available in order to follow the decomposition. In the latter instance the decomposition product must be stable under the conditions of the accelerated storage test.

(*c*) Before the energy of activation and the rate constant can be calculated by Rogers' equation the order of the reaction must be established. This may be found by substituting the experimental data into the zero, first, second, etc., forms of the equation. The equation which gives the best straight line allows the order of the reaction to be determined. This approach is illustrated in Fig. 3 where the data for the acid catalysed hydrolysis of sucrose are plotted as zero, first and second order reactions with respect to the sucrose concentration. If the decomposition is followed to completion, as in Fig. 3, there is no difficulty in deciding that

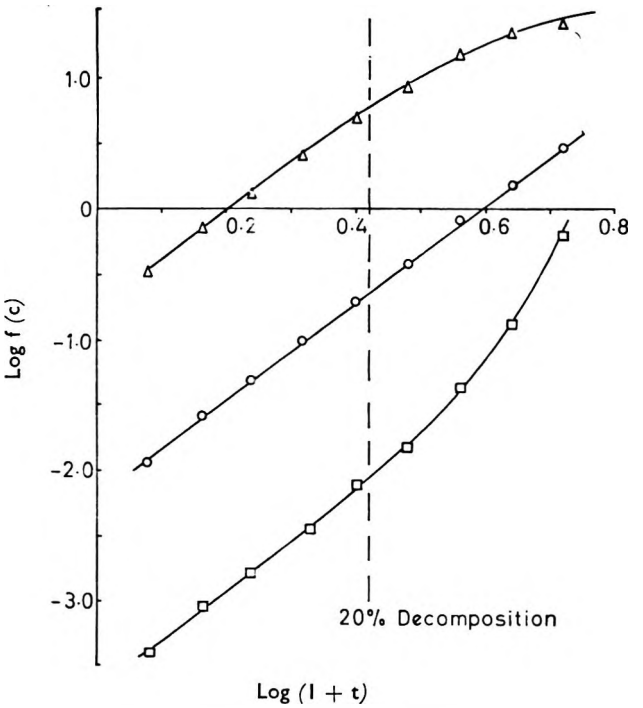

FIG. 3. The data for the acid hydrolysis of sucrose plotted in the zero (Δ), first (○) and second (□) order forms of Rogers' equation.

the hydrolysis of sucrose is a first order reaction with respect to sucrose, as was demonstrated by Moelwyn-Hughes (1934). However, if only the initial portion of the decomposition is followed, say 20%, then it is not possible to determine the order of the reaction (see Fig. 3). In studying the decomposition of more stable compounds it may not be possible to continue the experiment to 100% decomposition and in such cases the order of the reaction must be established by isothermal studies (Moelwyn-Hughes, 1947). This experiment can be performed easily in most instances and would not delay the production of data by Rogers' technique by more than one or two days.

EXPERIMENTAL ACCURACY

The values for the energies of activation and rate constants, determined by Rogers' technique, for the acid catalysed hydrolysis of sucrose, the solvolysis of methyl toluene-*p*-sulphonate and the hydrolysis of ethyl benzoate agreed very well with those reported in the literature (Table 1). Thus, in these cases, Rogers' test yielded accurate and reliable data.

As was suggested above, the data for the values of the rate constants were found to be more scattered than the data for the energies of activation; the mean of the standard deviations for the rate constant data

quoted in Table 1 was $\pm 14\%$ whereas that for the energy of activation data was $\pm 5\%$. While a large error in the value of the rate constant is an inherent feature of Rogers' technique some of the scatter in the data reported in Table 1 may be attributed to the relatively crude temperature control used for those experiments. While the temperature was within 1° of that required by the programme, the temperature programmes were not exactly reproducible in different runs—differences of up to 2° might be expected in two different runs. A more sophisticated method of temperature control which would give more reproducible temperature programmes would reduce the scatter in the data.

THE STABILITY OF P2S IN DILUTE AQUEOUS SOLUTION

The data obtained for the energy of activation and rate constant for the decomposition of P2S in dilute solution in phosphate buffer at pH 7·38 (Table 2) were in good agreement with the values reported by Fan, Nairn & Walker (1964). The study on P2S confirmed its greater stability in acid solution than in neutral or alkaline solution (Fan & others, 1964; Creasey & Green, 1959). There appear to be no other published data for the stability of P2S in dilute solution but Ellin, Carlese & Kondritzer (1962) have studied the stability of PAM (*N*-methyl pyridinium-2-aldoxime methiodide) which is likely to decompose in a similar way to P2S, *N*-methyl pyridinium-2-aldoxime methanesulphonate. Using the conventional method of storage at elevated temperatures they predicted that at 15° a solution of PAM in phosphate buffer at pH 7 would have a half-life of three years. This value is in good agreement with that found for P2S in phosphate buffer at pH 7·38 (Table 2, 2·9 $\pm$ 0·8 years).

THERMAL STABILITY OF CHOLINESTERASE

The data reported in Tables 4 and 5 demonstrate that Rogers' test may be used to determine the thermal inactivation of enzyme since the half-life of the activity of the enzyme solution at various temperatures, determined isothermally, agreed reasonably well with the values calculated from the Rogers' data. These experiments suggest an important role for this accelerated storage test in comparing the stabilities of different batches of the same preparations.

Rogers' test has thus been demonstrated to be extremely useful in determining the stabilities of compounds in the liquid phase. It may be used to determine the stability of any compound which can be investigated by isothermal studies and can produce data for the rate constant and energy of activation of the decomposition reaction very much more rapidly than the conventional methods. The results obtained by the technique are more scattered than those obtained isothermally. However, in many instances an estimate of the order of the stability is satisfactory. Thus the test may have its greatest use in preliminary studies of the storage stability of pharmaceutical and related compounds.

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Determination of micro amounts of 3-(dithien-2-ylmethylene)-1-methylpiperidine, a non-narcotic antitussive

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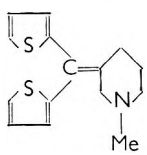
A specific colorimetric method for the determination of micro amounts of 3-(dithien-2-ylmethylene)-1-methylpiperidine, a new antitussive and expectorant, is described. Depending on amount, a characteristic colour varying from rose pink to deep bluish purple appears when 2 volumes of a 0·01% solution of ninhydrin in concentrated sulphuric acid are added to 1 volume of an aqueous solution of the drug. After 24 hr at room temperature the colour produced has an absorption maximum at 590 mμ and follows Beer's Law over the range 1–25 μg/ml. The colour is specific for the drug and is sensitive at the μg/ml level. Recoveries from aqueous solution and human urine were 97·4 ± 2·1 and 97·4 ± 1·7% respectively.

A NEW kind of potent and non-narcotic antitussive drug [3-(dithien-2-ylmethylene)-1-methylpiperidine; AT-327, Asverin; I] with expectorant activity has been described (Kasé, Yuizono, Yamasaki, Yamada, Io, Tamiya & Kondo, 1959; Sugimoto, Kowa, Higaki, Nakamura & Yasaka, 1960). In the absence of a method suitable for quantitative determination of the drug in pharmaceutical preparations and biological materials, a sensitive colorimetric method has been devised by us.*

The drug as citrate and hydrochloride, exhibits two ultraviolet absorption peaks in an aqueous solution, the higher at 208 and the other at 247 mμ. The absorption at 247 mμ follows Beer's Law and is suitable for



(I)

quantitative determination of the drug over the range of 1 to 25 μg/ml. However, it is unsuitable for the determination of the drug in biological materials such as urine, because many substances show similar absorption maxima in the neighbourhood of this wavelength.

According to Feigl (1960), thiophen, and its derivatives unsubstituted in the α-position, condense with 1,2-diketones such as benzil, isatin, and ninhydrin in concentrated sulphuric acid to yield coloured quinoidal compounds. Of these, 0·01% ninhydrin solution in concentrated sulphuric acid provides the most sensitive test for thiophen itself (limit of identification: 0·2 μg).

The reaction just described has been adapted to the determination of the new drug, and recoveries from aqueous solution and human urine have been investigated.

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* Abstract of a paper was read at the Meeting of Japanese Pharmacological Society at Kyushu University on November 2, 1960.

Experimental

METHOD

This incorporates the optimum reaction conditions, selected after examining the effects of the ratio of mixing of reagent and drug, concentration of acid and ninhydrin in the acid, and time and temperature as applied to the citrate. No significant differences were observed with other salts examined.

Reagent. 0·01% ninhydrin-sulphuric acid solution. Dissolve 10 mg of ninhydrin in 100 ml of pure concentrated sulphuric acid, thiophen-free. The reagent must be freshly prepared.

Standard solution of drug. Dissolve 169·0 mg of 3-(dithien-2-ylmethylene)-1-methylpiperidine citrate [white needles, m.p. 135° (decomp.)] in 1 litre of distilled water to give a 0·1 mg/ml of drug calculated as base. Dilute the solution further as required.

Procedure. Pipette 1·5 ml of the test solution into a small test tube, and add ninhydrin reagent (3·0 ml) slowly with ice-cooling and thorough mixing. Develop the colour by allowing the mixture to stand at 20–25° for 24 hr in the dark. Read the absorbance at 590 mμ using a spectrophotometer. For a blank, use a mixture of one part of distilled water and two parts of ninhydrin reagent.

SPECIFICITY

In testing for the specificity of the reaction, both the colour and absorption maximum of the solution yielded by the drug and those given by reaction solutions of similar compounds possessing either one thiophen or two thiophen nuclei were investigated under the experimental conditions described. 25 compounds were examined. These were: thiophen; four derivatives closely related to AT-327; thiambutene and five derivatives; two tertiary amine derivatives of ethyl 2-aminomethyl-3,3-di-thien-2′-yl-acrylates; 1,2,5,6-tetrahydro-1-methyl-4-thien-2′-yl-pyridine; 1-methyl-3-α-thiophenoyl piperidine and two derivatives; seven tertiary amine derivatives of 2-(*N*-aminoalkylacylamido)thiophen; 2-[*N*-ethyl-*N*-(2-piperidinopropyl)carbamoyl]thiophen.

Each compound yielded with the ninhydrin reagent a solution having a characteristic colour and absorption maximum different from those of the solution given by the new drug.

CALIBRATION CURVE

The colours obtained under the optimum experimental conditions follow Beer's Law over the range of 1 to 25 μg/ml of the drug as base.

RECOVERIES

(A) *From aqueous solutions.* Sodium carbonate solution (2 ml; 1%) and benzene (20 ml) were added to the drug in solution (10 ml) of various concentrations to be tested, such as 1·0, 2·0, 5·0, 10·0 and 20·0 μg/ml, in a separating funnel. The mixture was shaken 300 times/min and after 15 min the benzene layer was removed. The aqueous layer was extracted

twice with benzene, 2 × 15 ml. To the pooled benzene extracts (50 ml) was added 0·002N sulphuric acid (10 ml) and the mixture then shaken for 15 min, the acidic aqueous layer being removed. The ninhydrin reagent (4·0 ml) was added to 2·0 ml of the aqueous solution, the mixture allowed to stand for 24 hr at room temperature to develop the colour, and this was measured in a spectrophotometer. Results in Table 1 show the recovery to be about 97·4 ± 2·1% over the range of 1·0–20·0 μg/ml of drug.

TABLE 1. RECOVERIES OF 3-(DITHIEN-2-YLMETHYLENE)-1-METHYLPIPERIDINE FROM AQUEOUS SOLUTION AND URINE

Aqueous solution				Human urine			
Conc. μg/ml	No. of symples	Found average μg/ml	Found average %	Conc. μg/ml	No. of samples	Found average μg/ml	Found average %
1·0	12	1·00 ± 0·05	100·0 ± 4·9	1·0	5	0·98 ± 0·06	98·2 ± 5·6
2·0	10	1·89 ± 0·16	94·5 ± 8·2	2·0	10	1·96 ± 0·08	98·0 ± 4·0
5·0	13	4·70 ± 0·17	94·0 ± 3·4	5·0	11	4·84 ± 0·31	96·8 ± 6·2
10·0	14	10·1 ± 0·43	100·6 ± 4·3	10·0	7	10·1 ± 0·36	101·1 ± 3·6
20·0	9	19·5 ± 0·90	97·7 ± 4·5	20·0	10	18·9 ± 0·70	94·5 ± 3·5
	Average 97·4 ± 2·1%				Average 97·4 ± 1·7%		

(B) *From human urine.* Sodium carbonate solution (2 ml) was added to urine (10 ml) in which 1·0, 2·0, 5·0, 10·0 or 20·0 μg of drug/ml had been dissolved. Each alkaline urine sample was extracted with benzene as described in A. The recovery was about 97·4 ± 1·7% over the range of 1·0–20·0 μg/ml (Table 1).

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The interaction of benzoic acid and chloroxylenol with cetomacrogol

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The interaction of benzoic acid with the non-ionic surfactant cetomacrogol 1000 has been examined by means of solubility and pH measurements, potentiometric titration and equilibrium dialysis. Solubility measurements and equilibrium dialysis have also been used to assess the interaction of chloroxylenol with cetomacrogol. Following earlier proposals, the interactions have been expressed both in terms of a distribution coefficient for the partition of preservative between the micelles and aqueous phase, and as a ratio of the total preservative concentration to the amount free in the aqueous phase. It is suggested that a more generally useful method is to express the degree of saturation of the aqueous phase with preservative as a function of the degree of saturation of the total system.

THE inactivation of preservatives in the presence of non-ionic surfactants has been the subject of many investigations and it is established that the inactivation arises from an interaction between molecules of the surfactant and the preservative. Different theories have been advanced to account for the nature and extent of this interaction but it is generally agreed that antimicrobial activity depends on the concentration of unbound or free preservative (Wedderburn, 1964).

Higuchi & Lach (1954) reported the formation of hydrogen bonded complexes between polyethylene glycols and phenols and between polyethylene glycols and organic acids. Since most non-ionic surfactants have polyethylene glycol chains, many authors have attributed both the solvent properties and inactivation of preservatives to complex formation. Evans (1964) has shown that complex formation between surfactant monomer and preservative is unlikely and has suggested that the inactivation arises from solubilisation of preservative within the surfactant micelles. Moreover, Mulley (1964) has collected evidence from a number of sources which indicates that the solubilisation of a wide range of solutes in non-ionic surfactants can be treated as a solution process within the hydrocarbon-like interior of the micelle. He considers the data do not support suggestions that solubilisation is controlled by more specific factors such as complex formation. Kostenbauder (1962) maintains that it is unnecessary to distinguish between the mechanisms of complex formation and micellar solubilisation and considers that solubilisation and micelle formation itself fall within the broad scope of the complex formation described by Higuchi & Lach (1954).

Although the exact nature of the interaction is in doubt, aqueous solutions of a non-ionic surfactant probably provide ideal conditions for association to occur between the preservative and the surfactant. The possibilities exist for hydrogen bonding, both in the monomer and in the micellar states, and also for partitioning into the deeper regions of the micelle.

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Methods used to study the interaction between preservatives and non-ionic surfactants have included partition (Allawala & Riegelman, 1953), solubility (Blaug & Ahsan, 1961; Goodhart & Martin, 1962; Matsumoto & Aoki, 1962), dialysis (Patel & Kostenbauder, 1958; Deluca & Kostenbauder, 1960; Bahal & Kostenbauder 1964; Blaug & Ebersman, 1964; Patel & Foss, 1964; Blaug & Rich, 1965), and potentiometric titration (Evans 1964; Donbrow & Rhodes 1965).

Donbrow & Rhodes (1963) have shown that the pH of acid buffer solutions is increased in the presence of cetomacrogol presumably due to preferential solubilisation of the acid within surfactant micelles. Hence a simple pH measurement should also provide a convenient method of estimating the amount of free solute.

Using benzoic acid and chloroxylenol solubilised in cetomacrogol solutions, various techniques for assessing the interaction between preservatives and non-ionic surfactants have been examined, and methods of expressing this interaction are compared.

Experimental

Materials. Benzoic acid recrystallised from water, m.p. 122°, chloroxylenol recrystallised from light pertoleum, m.p. 115–116°, and cetomacrogol 1000 B.P.C. (Texofor A1P, Glovers Chemicals Ltd.). Cetomacrogol 1000 has the general formula $Me{\cdot}[CH_2]_m\ [O{\cdot}CH_2{\cdot}CH_2]_n{\cdot}OH$ where m may be 15 or 17 and n may be 19 to 23. The molecular weight was taken as 1300. Following the method of Ginn & Church (1959) solutions of cetomacrogol were passed through a column of mixed bed ion-exchange resins to remove alkaline impurities. The refractive index of the effluent was measured at 25° and the concentration of cetomacrogol determined from a calibration curve. All solutions were made using freshly boiled and cooled glass distilled water.

Determination of water-solubility. Excess benzoic acid was equilibrated with water by rotation in a sealed cylinder in a water-bath thermostatically controlled at 25° ± 0·1°. The amount of benzoic acid in an aliquot of filtrate measured potentiometrically was 0·33 g/100 ml. The water solubility of chloroxylenol at 20° is 0·031 g/100 ml (Mitchell, 1964).

Determination of solubility in solutions of cetomacrogol. The solubility of benzoic acid in varying concentrations of cetomacrogol solution was found using the method given for water-solubility. Excess chloroxylenol separates as a liquid crystalline phase which cannot be separated by filtration. The solubility of chloroxylenol in solutions of cetomacrogol at 20° was therefore determined by the method of Mulley & Metcalf (1956).

Measurement of pH and potentiometric titration. pH measurements were made using a glass-saturated calomel electrode system and a Beckman Research pH meter (relative accuracy ± 0·001 under optimum conditions). The electrode system was standardised using 0·05 M potassium hydrogen phthalate solution and the electrode response was checked on this buffer after each experiment. The pH/e.m.f. relationship

of the electrode was checked periodically using 0·05 M sodium borate. Potentiometric titrations were made using potassium hydroxide freed from carbon dioxide by the method of Albert & Serjeant (1962a). The sample volume was 20 ml and the strength of potassium hydroxide was such that 2 ml was required for neutralisation. pH measurements and potentiometric titrations were made in an atmosphere of nitrogen.

Equilibrium dialysis. Perspex dialysis cells were made to the design of Bahal & Kostenbauder (1964). The dialysis membrane was rubber latex refluxed with several changes of water until the washings were clear. The membrane was permeable to benzoic acid and chloroxylenol but not to cetomacrogol. A solution of preservative in cetomacrogol was placed in one side of the cell and water, or water plus preservative, was placed in the other. The cells were rotated in the water-bath at 25° for benzoic acid and 20° for chloroxylenol until equilibrium was reached. The amount of benzoic acid on each side of the membrane was found by potentiometric titration. The amount of chloroxylenol was assayed spectrophotometrically by the ΔE method of Elvidge & Peutrell (1961) (sample buffer C, blank buffer A, λ_{max} 301 mμ). The differential method avoids probable errors due to the release of absorbing impurities from the rubber membrane and interference from the presence of surfactant.

Results and discussion

It has been shown that the bactericidal activity of chloroxylenol in aqueous solutions of cetomacrogol is related to the degree of saturation of the system with chloroxylenol (Mitchell, 1964). The degree of saturation was given by

$$R = C/C_s \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where R is the saturation ratio, C is the chloroxylenol concentration and C_s its solubility. Bactericidal activity was shown to be due to the concentration of chloroxylenol in the free aqueous phase and not to the total amount present in the system. It was suggested that a better index of bactericidal activity would be the degree of saturation of the free aqueous phase rather than the total solution as defined in (1). Treating solubilisation in surfactant solutions as a distribution phenomenon, R can be subdivided into R_m, the degree of saturation of the micelles and R_w, the degree of saturation of the aqueous phase. Hence,

$$R_m = C_m/C_{sm} \quad \ldots\ldots\ldots\ldots \quad (2)$$

$$R_w = C_w/C_{sw} \quad \ldots\ldots\ldots\ldots \quad (3)$$

and $$C = C_m + C_w \quad \ldots\ldots\ldots\ldots \quad (4)$$

where C_m and C_w are the concentrations in the micellar and aqueous phases respectively and C_{sm} and C_{sw} are the saturation solubilities of the micellar and aqueous phases respectively. At saturation, $C_s = C_{sm} + C_{sw}$ and R, R_m and $R_w = 1{\cdot}0$.

Following the method of McBain & Hutchinson (1955) it is possible to calculate a distribution coefficient, K_m, for the partition of unionised solute between the micelles and aqueous phase where

$$K_m = \frac{[\text{unionised preservative}]\ \text{micelle}}{[\text{unionised preservative}]\text{water}} = \frac{C'_m/[\text{cetomacrogol}]}{C'_w/[\text{water}]} \quad (5)$$

Solute molecules within the micelle are apparently unionised (Evans 1964; Donbrow & Rhodes 1965), hence $C'_m = C_m$.

Values of the distribution coefficient calculated from the solubility curve (Table 1) are reasonably constant for different concentrations of

TABLE 1. DISTRIBUTION COEFFICIENTS FOR THE PARTITION OF UNIONISED BENZOIC ACID AND CHLOROXYLENOL BETWEEN THE MICELLES AND AQUEOUS PHASE OF CETOMACROGOL SOLUTIONS CALCULATED FROM SOLUBILITY CURVE

Benzoic acid (25°)			Chloroxylenol (20°)		
Cetomacrogol	C_s	$K_{sm} \times 10^{-3}$	Cetomacrogol	C_s	$K_{sm} \times 10^{-3}$
0·000	0·0273		0·000	0·00197	
0·010	0·0437	3·5	0·005	0·0106	49
0·040	0·0929	3·5	0·010	0·0196	50
0·060	0·1257	3·5	0·049	0·0958	54
0·100	0·1913	3·5	0·096	0·1916	55

C_s = Solubility of preservative.

K_{sm} = Distribution coefficient of unionised preservative in saturated surfactant solutions.

$$= \frac{C_{sm}/[\text{cetomacrogol}]}{C'_{sw}/[\text{water}]}$$

C_{sm} = Solubility of preservative in micelles = $C_s - C_{sw}$

C_{sw} = Solubility of preservative in aqueous phase. (Assumed to be the same as the water-solubility.

C'_{sw} = Concentration of unionised preservative in aqueous phase at saturation, = $C_{sw} - \bar{C}_{sw}$

$\bar{C}_{sw}$ = Concentration ionised preservative in aqueous phase at saturation calculated from the Henderson equation pH = pKa + log $[\bar{C}_{sw}/C_{sw}]$

For benzoic acid solutions pH at saturation = 2·910, pKa at 25° = 4·18, C_{sw} = 0·0273.

For chloroxylenol solutions, $C'_{sw} = C_{sw}$ = 0·00197.

All concentrations in moles/litre total solution.

cetomacrogol, but the solubility method is in effect a 'one-point' method of estimating the degree of interaction. In each case a saturated solution is being considered and it cannot be assumed that the extent of interaction will be the same in unsaturated solutions (cf. chloroxylenol). Moreover in the calculation of K_m, it is assumed that solubility in the aqueous phase at saturation, C_{sw}, is the same as the water-solubility. This assumption is open to question in certain cases (Evans, 1964) but seems valid for benzoic acid and chloroxylenol in cetomacrogol, since the solubilities are directly proportional to the concentration of cetomacrogol over the concentration range studied when corrections have been made for the solubilities in water.

The amount of benzoic acid in the aqueous phase was also determined potentiometrically as described by Evans (1964) and some of the calculated distribution coefficients are given in Table 2. Values of K_m increased slightly up to half-neutralisation and thence more markedly with further additions of base. Values of K_m also depended upon the initial concentration of acid and the degree of saturation. In the potentiometric method,

TABLE 2. DISTRIBUTION COEFFICIENTS FOR THE PARTITION OF UNIONISED BENZOIC ACID BETWEEN THE MICELLES AND AQUEOUS PHASE OF CETOMACROGOL SOLUTIONS AT 25° CALCULATED FROM POTENTIOMETRIC TITRATION

Initial R	C	Cetomacrogol	pH	C_w^-	$K_m \times 10^{-3}$
0·910	0·1000	0·0500			
	0·0990	0·0495	3·84	0·00990	3·5
	0·0952	0·0476	4·80	0·0476	3·7
0·523	0·1000	0·1000			
	0·0990	0·0990	4·09	0·00990	3·5
	0·0981	0·0981	4·44	0·0196	3·6
	0·0971	0·0971	4·67	0·0291	3·6
	0·0961	0·0961	4·87	0·0385	3·7
	0·0952	0·0952	5·07	0·0476	4·0
	0·0944	0·0944	5·26	0·0566	4·1
	0·0935	0·0935	5·46	0·0654	4·3
0·455	0·0500	0·0500			
	0·0495	0·0495	3·90	0·00495	4·1
	0·0476	0·0476	4·84	0·0238	4·2
0·266	0·0500	0·1000			
	0·0495	0·0991	4·20	0·00495	4·7
	0·0476	0·0952	5·14	0·0239	4·7
0·232	0·01000	0·01000			
	0·00971	0·00971	4·08	0·00291	4·4
	0·00952	0·00952	4·43	0·00477	4·6

R = Saturation ratio.
C = Total concentration benzoic acic.
$K_m = \frac{C_m/[\text{cetomacrogol}]}{C'_w/[\text{water}]}$
C_m = Concentration benzoic acid in micelles = $C - C_w$
C_w = Total concentration benzoic acic in aqueous phase = $C_w^- + C'_w$
C_w^- = Concentration ionised benzoic acid in aqueous phase calculated from the amount of added base.
C'_w = Concentration unionised benzoic acid in aqueous phase calculated from the Henderson equation (pKa benzoic acid at 25° is 4·18). All concentrations in moles/litres total solution.

free acid is titrated to salt and throughout a titration the ionic strength will vary continuously. Moreover there will be large variations in ionic strength in titrations where the initial concentration of acid is different. Donbrow & Rhodes (1965) have criticised Evans' method of calculation on the grounds that the Henderson equation is used without correction for ionic strength. Therefore activity coefficients were estimated and amounts of unionised acid in the aqueous phase were recalculated using the Henderson equation in the form given by Albert & Serjeant (1962b) where pK_a^T for benzoic acid at 25° was taken as 4·18 (Ives, 1933). The recalculated values of K_m however, still increased throughout an individual titration and as the degree of saturation of the initial solution with benzoic acid was reduced, despite corrections for the change in ionic strength. Hence the variations in distribution coefficients are probably due to the effect of the change in salt concentration on micelle formation, solubilisation and the distribution coefficients of semipolar solutes. Donbrow & Rhodes (1965) recommend back-titration of the acid from the salt form since the ionic strength of the solution and hence the value of the activity coefficient correction will remain relatively constant provided the volume of titrant is small. Compared with other methods of studying the interaction between solute and surfactant however, the potentiometric method suffers from the disadvantage that the titration itself disturbs the system.

The potentiometric method depends on pH measurements and the calculations are based on the assumption that the unionised acid is

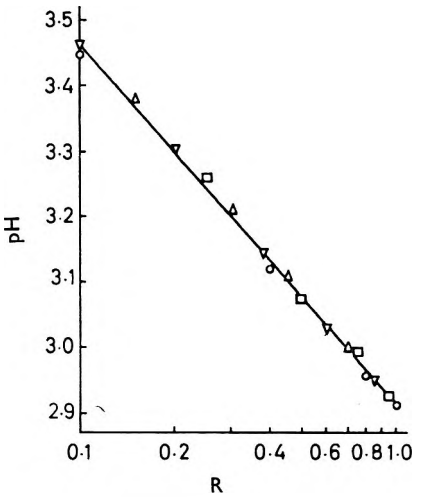

FIG. 1. Variation of pH with log saturation ratio (R) for solutions of benzoic acid in water and in cetomacrogol at 25°. ○, water; □, 0·05 M benzoic acid; ▽, 0·01 M cetomacrogol; △, 0·06 M cetomacrogol.

partitioned between the micelles and the free aqueous phase. Hence it should be possible to estimate this partition by a simple pH measurement without disturbing the system by adding base. Readings of pH at various degrees of saturation for solutions of benzoic acid are shown in Fig. 1. The pH values both in water and cetomacrogol are in close agreement for any value of R, irrespective of the actual amounts of benozic acid or cetomacrogol. This shows that the pH of the solution depends only on the concentration of benzoic acid in the aqueous phase. Values of K_m calculated from pH measurements are given in Table 3.

TABLE 3. DISTRIBUTION COEFFICIENTS FOR THE PARTITION OF UNIONISED BENZOIC ACID BETWEEN THE MICELLES AND AQUEOUS PHASE OF CETOMACROGOL SOLUTIONS AT 25° CALCULATED FROM pH MEASUREMENTS

R	Cetomacrogol	C	pH	$K_m \times 10^{-3}$
0·95	0·0152	0·0500	2·922	3·6
0·85	0·0100	0·0372	2·948	3·5
0·70	0·0600	0·0880	2·995	3·6
0·50	0·0446	0·0500	3·076	3·6
0·30	0·0600	0·0377	3·200	3·7
0·10	0·0100	0·00437	3·467	4·0

R = Saturation ratio.
C = Total concentration benzoic acid.
$K_m = \frac{C_m/[\text{cetomacrogol}]}{C'_w/[\text{water}]}$
C_m = Concentration benzoic acid in micelles = $C - C_w$
C_w = Total concentration benzoic acid in aqueous phase, from Fig. 1 and equn. 3
C'_w = Concentration unionised benzoic acid in aqueous phase = $C_w - \bar{C_w}$
$\bar{C_w}$ = Concentration ionised benzoic acid in aqueous phase calculated from the Henderson equation.
All concentrations in moles/litre total solution.

Dialysis studies were made using benzoic acid and chloroxylenol in cetomacrogol solutions. At equilibrium, the activity of the preservatives is identical on both sides of the membrane and, for the solutions used, it was assumed that the concentration of free preservative on both sides of the membrane is equal. By analysis of each of these solutions the concentration of preservative in the free aqueous phase and micelles is readily found, and the distribution coefficient calculated (Fig. 2).

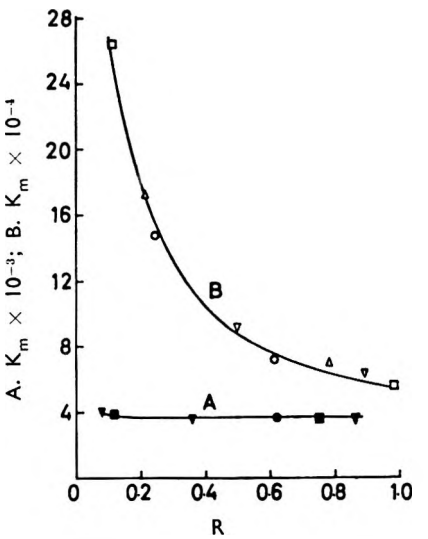

FIG. 2. Variation of distribution coefficient with saturation ratio (R) for the partition of benzoic acid and chloroxylenol between the micelles and aqueous phase of cetomacrogol solutions calculated from equilibrium dialysis. A. Benzoic acid: cetomacrogol solutions at 25°; cetomacrogol concentration (moles/litre); ●, 0·01; ■, 0·04; ▼, 0·10. B. Chloroxylenol: cetomacrogol solutions at 20°; cetomacrogol concentration (moles/litre); △, 0·005; ○, 0·01; □, 0·049; ▽, 0·096.

For solutions of benzoic acid in cetomacrogol there is close agreement between values of K_m determined by solubility, pH measurement and dialysis. K_m is constant over a wide range of benzoic acid and cetomacrogol concentrations but increases slightly at low degrees of saturation. In contrast, K_m for chloroxylenol-cetomacrogol solutions increases markedly as the saturation ratio is reduced, and is constant for different concentrations of solute and surfactant only at a given value of R (Fig. 2). Under these circumstances a distribution coefficient has little meaning.

Many authors (e.g. Higuchi & Lach, 1954; Patel & Kostenbauder, 1958; Blaug & Ahsan, 1961; Storz, DeKay & Banker, 1965) have expressed the ratio of the total solute concentration, C, to the concentration of the free form, C_w, as a function of the macromolecule concentration. Fig. 3 shows results for benzoic acid and chloroxylenol plotted in this manner. The ratio C/C_w is a function of cetomacrogol concentration for all concentrations of benzoic acid but for chloroxylenol it is a function

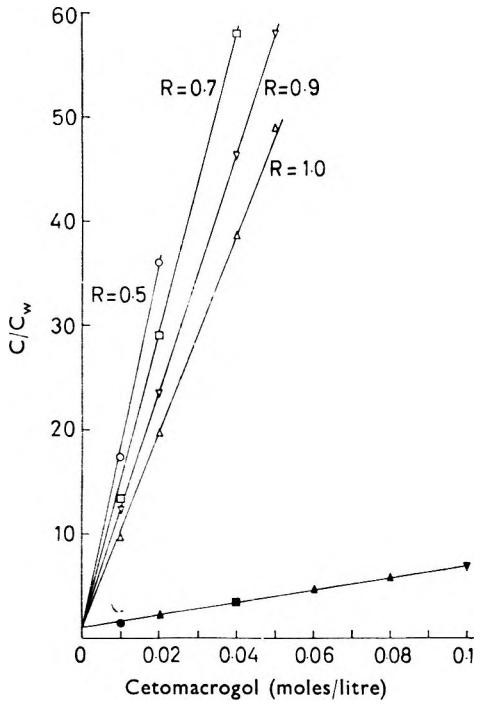

FIG. 3. Ratio of total preservative to free preservative as a function of cetomacrogol concentration. Closed symbols: benzoic acid (25°). Concentration of free benzoic acid (moles/litre): ●, 0·0164; ■, 0·0200; ▼, 0·0234; ▲, 0·0273. Open symbols: chloroxylenol (20°). Concentration of free chloroxylenol (moles/litre): ○, $5{\cdot}5 \times 10^{-4}$; □, $9{\cdot}9 \times 10^{-4}$; ▽, $15{\cdot}3 \times 10^{-4}$; △, $19{\cdot}7 \times 10^{-4}$.

both of cetomacrogol concentration and of chloroxylenol concentration and depends on the saturation ratio.

The solubility method has been used by a number of workers either to estimate K_m or the ratio C/C_w. It is apparent from Figs 2 and 3 that while this method may be satisfactory for certain preservatives, with others such as chloroxylenol it will lead to erroneous conclusions for unsaturated systems.

A more useful method of presenting the results is to plot a partition isotherm showing the % saturation of the micelles (or R_m) as a function of the % saturation of the aqueous phase (or R_w). Alternatively the % saturation of the aqueous phase (or R_w) may be plotted as a function of the % saturation of the total system (or R). The latter is more appropriate in the present study since the important consideration is the amount of preservative available in the aqueous phase as a function of the total amount in the system (Fig. 4). For benzoic acid the plot of R_w against R has a slope of 1·0 indicating that benzoic acid is partitioned between the aqueous phase and the micelles according to simple distribution theory. For chloroxylenol the plot of R_w as a function of R shows a marked

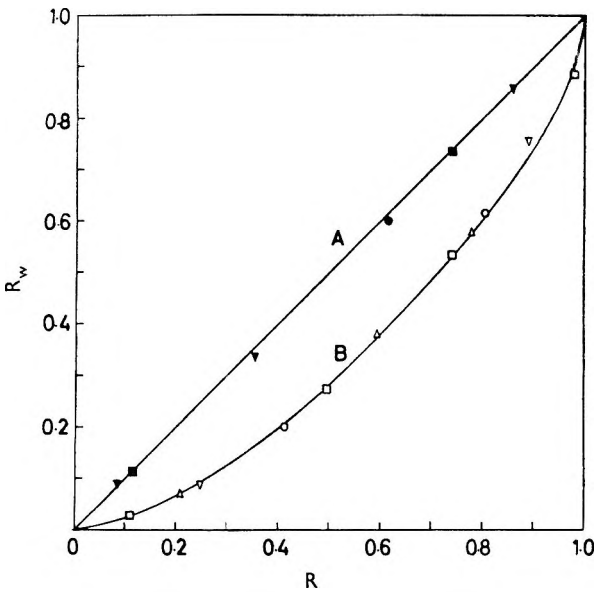

FIG. 4. Dependence of the degree of saturation of the aqueous phase (R_w) on the degree of saturation of the total solution (R) for solutions of benzoic acid and chloroxylenol in cetomacrogol. A. Benzoic acid: cetomacrogol solutions at 25°; cetomacrogol concentration (moles/litre): ●, 0·01; ■, 0·04; ▼, 0·1. B. Chloroxylenol: cetomacrogol solutions at 20°; cetomacrogol concentration (moles/litre): △, 0·005; ○, 0·01; □, 0·049; ▽, 0·096.

deviation from a slope of 1·0 showing that, for unsaturated solutions, chloroxylenol is preferentially soluble in the micelles. Allawala & Riegelman (1953) using solutions of iodine in Antarox A-400 (nonylphenol/10 moles ethylene oxide) reported similar results to those found for chloroxylenol in cetomacrogol but also found that the partition in favour of the micelles increased with surfactant concentration. From such plots it is possible to determine the amount of preservative free in the aqueous phase for any concentration of solute and surfactant, or alternatively if the amount of preservative needed in the aqueous phase to prevent microbial growth is known, the amount required in the system as a whole to achieve this concentration can be determined. It has been shown previously (Mitchell 1964) that when $R = 1{\cdot}0$, solutions of chloroxylenol in cetomacrogol have the same antibacterial activity as a saturated solution of chloroxylenol in water. However, when $R < 1{\cdot}0$, chloroxylenol-cetomacrogol solutions were less effective than corresponding chloroxylenol-water solutions. This was attributed to a change in distribution of chloroxylenol in favour of the micelles on reducing R. This is confirmed by the results in Figs 2 and 4. Thus from Fig. 4, in cetomacrogol solutions 50% saturated with chloroxylenol ($R = 0{\cdot}50$), the aqueous phase is only 28% saturated ($R_w = 0{\cdot}28$).

At a given value of R, chloroxylenol-cetomacrogol solutions were found to be equitoxic over a wide concentration range of chloroxylenol.

Fig. 4 shows that for any value of R the degree of saturation of the aqueous phase, R_w, is a constant independent of the concentrations of chloroxylenol or cetomacrogol except in so far as these control R. These findings support the views of other workers that preservative activity depends on the degree of saturation, or thermodynamic activity of the aqueous phase, and show that this is controlled by the degree of saturation of the system as a whole.

Fig. 5 shows equilibrium dialysis data for other preservatives in non-ionic surfactants plotted as R_w against R. Methyl *p*-hydroxybenzoate (Patel & Kostenbauder, 1958) and hexachlorophane (Morgan, 1965), like chloroxylenol, show a deviation from a slope of 1, while chlorbutol (Slade, 1965) behaves in the same manner as benzoic acid. It is apparent that the interaction of different preservatives with non-ionic surfactants does not take place by the same mechanism. However, the results indicate that in each case it is possible to express the interaction in terms of partition between an aqueous and a micellar phase.

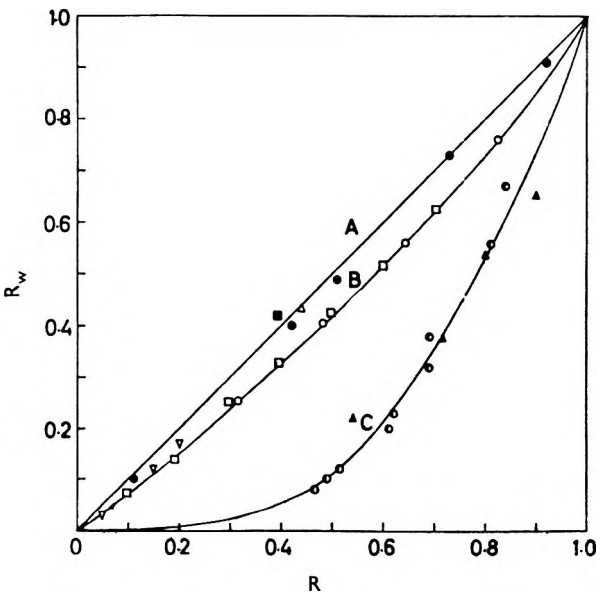

FIG. 5. Dependence of the degree of saturation of the aqueous phase (R_w) on the degree of saturation of the total solution (R) for solutions of various preservatives in non-ionic surfactants. A. Chlorbutol: Brij 35 solutions at 25° (Slade, 1965); Brij 35 concentration (%): △, 0·5; ●, 1·0; ■, 1·5. B. Methyl *p*-hydroxybenzoate: polysorbate 80 solutions at 30° (Patel & Kostenbauder, 1958); polysorbate 80 concentration (%): ○, 3·0; □, 5·0; ▽, 10·0. C. Hexachlorophane: Brij 35 solutions at 25° (Morgan, 1965); Brij 35 concentration (%): ▲, 0·1; ◐, 1·0.

In complex disperse systems the presence of an oil phase, pH and the presence of other materials will all influence preservative effectiveness, but a knowledge of the extent of interaction for a preservative: non-ionic combination will indicate whether the preservative is likely to be of value.

Where a plot of R_w against R shows a marked deviation from a slope of 1·0 then a relatively large concentration of preservative will be required in the total system to ensure an adequate concentration in the aqueous phase. From the viewpoint of overall concentration relative to the concentration in the aqueous phase a suitable preservative should have a slope approaching 1·0.

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Letters to the Editor

Distribution of some phenothiazines in red blood cells and platelets

SIR,—Chlorpromazine and some other phenothiazines are known to cause haemolysis of red blood cells *in vitro* and *in vivo* (Chaplin, Crawford, Cutbush & Mollison, 1952; Freeman & Spirtes, 1962). They also liberate 5-hydroxytryptamine (5-HT) from platelets *in vitro* (Bartholini, Pletscher & Gey, 1961; Paasonen, 1964). We have shown previously (Ahtee & Paasonen, 1965) that chlorpromazine is active in causing haemolysis but that its sulphoxide and a quaternary phenothiazine *N*-hydroxyethylpromethazine (NHP) are not. On the other hand, both chlorpromazine and its sulphoxide release 5-HT from platelets while NHP does not. The purpose of this work was to study to what extent these three phenothiazines are absorbed by the red blood cells and platelets.

Male rabbits under ether anaesthesia were bled from the carotid artery by means of a polyethylene cannula. The blood was mixed with 1/9 volume of 1·5% ethylenediaminetetra-acetic acid in 0·7% sodium chloride. In *in vitro* experiments, samples of whole blood were incubated in air with or without chlorpromazine hydrochloride (May & Baker), the sulphoxide (Rhône-Poulenc) or NHP (Orion), with gentle shaking at 37°. After incubation, the plasma, platelets and red cells were separated at 4° and the phenothiazines estimated spectrophotometrically according to Salzman & Brodie (1956). NHP was extracted from sodium carbonate-bromothymol blue solution into chloroform, transferred into 1·25N sulphuric acid and measured at 250 mμ. No recovery corrections have been made. As a value for platelet volume, 1 $\mu l/10^8$ platelet was used. The standard error of mean is given to indicate the distribution.

The distribution of chlorpromazine was estimated 0, 15, 30, 60 and 180 min after incubation with blood. In 6 experiments, immediately after the addition of 10^{-4}M/litre (31·9 μg/ml) of chlorpromazine, both the platelets and the red cells took up more of the drug per volume than was present in the plasma. The concentration of the drug in the platelets was 287 ± 42 μg/ml, which was about 10 times as high as that in the red cells (29·9 ± 3·7 μg/ml). The content of chlorpromazine in the platelets increased to 488 ± 84 μg/ml during the first hr of incubation. It then decreased to, or below, the starting level. The amount of chlorpromazine in the red cells decreased, while that in the plasma increased, during the first hr, to about 22 μg/ml. Both concentrations remained about the same during the remainder of the incubation. The total amount of chlorpromazine did not change.

Fig. 1 shows the distribution of the three phenothiazine derivatives after incubation of whole blood for 1 hr with the corresponding compound in 3 or 4 experiments. Platelets took up 5 to 22 times more chlorpromazine and its sulphoxide than was present in plasma. The uptake of NHP by platelets, on the other hand, remained relatively low. Although the concentration of chlorpromazine in the red cells was slightly higher than in the plasma, the concentration of sulphoxide and especially that of NHP remained far below the plasma levels.

In *in vivo* experiments three conscious rabbits received, 2 hr after the arterial cannulation, 10 mg/kg of chlorpromazine i.v. within 1 min. Blood samples were collected 2, 5, 10 and 15 min after the injection was started. The first sample contained 2·71 ± 0·45 μg/ml of the drug in the red cells and 1·46 ± 0·24 μg/ml in the plasma. This means that 1 min after the injection only about 1/100th of the injected phenothiazine is present in the red cells and plasma.

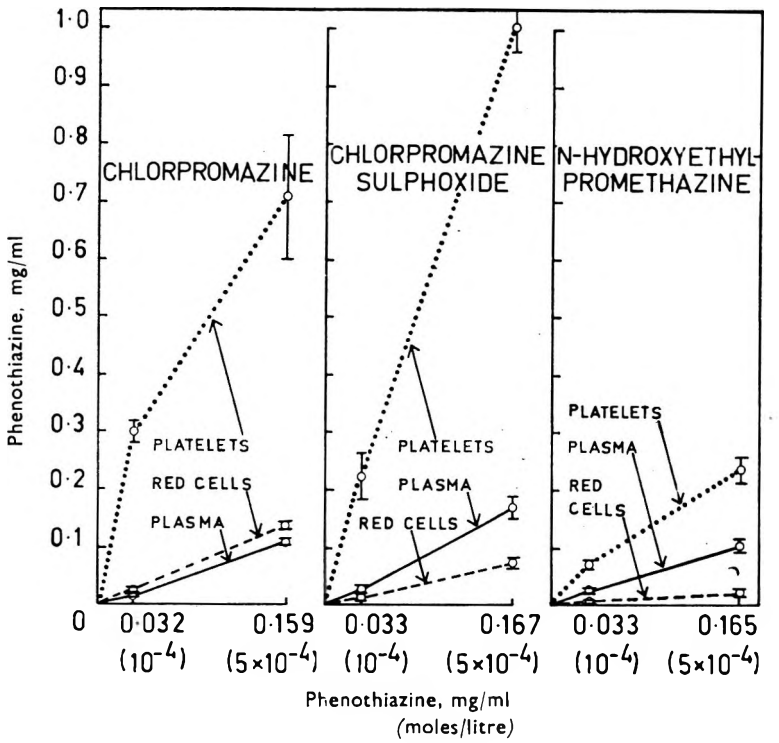

Fig. 1. The amount of phenothiazines in platelets, red blood cells and plasma after 1-hr incubation of whole blood with phenothiazines.

The amount of chlorpromazine in both components fell in 10 min to about 1/4th of the values mentioned. Due to the small amount of platelets in each sample there was not enough chlorpromazine for an accurate quantitative analysis.

The above results show that the red cells, and especially the platelets, absorb phenothiazines from plasma *in vitro* and *in vivo*. In the same volume the total surface of platelets is about 5 times as great as in the red cells. The 10 times higher concentration of chlorpromazine in platelets cannot therefore be explained solely by a difference in the surface area of these two cells. The fall of chlorpromazine in platelets and red cells during the incubation is probably due to the cellular damage it caused (Telkkä, Nyholm & Paasonen, 1964; Paasonen, 1965). The haemolysis may also mask a possible time dependent uptake of the drug in some of the red cells.

The uptake of chlorpromazine, its sulphoxide and NHP by platelets and red cells is in some way related to the ability of the drugs to liberate 5-HT from platelets and to cause haemolysis of red cells. However, haemolysis is not caused by the sulphoxide or NHP even when their content in red cells is higher than a haemolysing concentration of chlorpromazine. The uptake of these three phenothiazines by the red cells is better related to their tranquillising action.

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The effect of (+)-amphetamine on various central and peripheral catecholamine-containing neurones

SIR,—In previous experiments with rat brain (Carlsson, Lindqvist, Dahlström, Fuxe & Masuoka, 1965), support was obtained for the view that substances of the amphetamine group are capable of causing the release of extragranular catecholamines, that is, of catecholamines located intraneuronally outside the storage granules. Furthermore, it was found that in large doses these drugs may also cause the release of catecholamines from the granules. In the present work the effect of (+)-amphetamine has been further examined for its effect on extragranular amines. Special attention has been paid to the sensitivity of different catecholamine neurone systems to this drug.

Adult, male Sprague-Dawley rats, 200–300 g, were used. Since the extragranular amine fraction normally seems to be very small, the experiments were made on animals whose amine stores had been emptied by reserpine. Loading of the extragranular space was then brought about by means of the monoamine oxidase inhibitor nialamide, followed by the catecholamine precursor L-3,4-dihydroxyphenylalanine (L-dopa).

In vivo *experiments*. The animals were treated with reserpine, 10 mg/kg, i.p., 20–22 hr before being killed, nialamide, 100 mg/kg, i.p., 4 hr before death, and dopa, 25–50 mg/kg, s.c., 30 min before death. (+)-Amphetamine was administered in various doses (calculated as the base) 15 min before the dopa.

Dopamine, noradrenaline and 3-methoxytyramine were measured fluorimetrically (Bertler, Carlsson & Rosengren, 1958; Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962; Carlsson & Lindqvist, 1953; Carlsson & Waldeck, 1964).

Fifteen animals were taken for the cellular localization of monoamines in the brain, heart and vas deferens (Falck, Hillarp, Thieme & Torp, 1962; Falck, 1962; see review by Hillarp, Fuxe & Dahlström, 1965), one control group (4 animals) and two groups receiving (+)-amphetamine (0·75 mg/kg, 5 animals, and 0·4 mg/kg, 6 animals).

In vitro *experiments*. Brain slices of the neostriatum, hypothalamus, neocortex and the vas deferens of reserpine-treated rats (10 mg/kg, 12–18 hr before killing) were incubated for 30 min (Hamberger & Masuoka, 1965) with α-methylnoradrenaline, 1 or 0·03 μg/ml. In the test experiments the slices were preincubated for 15 min with (+)-amphetamine (0·0075–0·75 μg/ml), whereupon the α-methylnoradrenaline was added to the medium.

Results in vivo. Table 1 shows the effects of (+)-amphetamine on the dopa-induced noradrenaline and dopamine accumulation in different parts of the brain. Doses of (+)-amphetamine down to 0·1 mg/kg partially blocked the noradrenaline accumulation in the brain compared with the controls, the hemispheres being more sensitive to the drug than the brain stem. The dopamine levels in brain were not affected by the lower doses of (+)-amphetamine. In the higher dose range a decrease in the dopamine : methoxytyramine ratio is evident (Table 2), suggesting increased release of dopamine into the extra-neuronal space. In the heart a decrease in noradrenaline was seen after the higher doses of (+)-amphetamine; no change in dopamine was detected.

TABLE 1. EFFECT OF VARIOUS DOSES OF (+)-AMPHETAMINE ON L-DOPA-INDUCED CATECHOLAMINE ACCUMULATION IN DIFFERENT PARTS OF RAT BRAIN

(+)-Amphetamine mg/kg i.p.	Noradrenaline			Dopamine			Number of experiments
	Stem	Hemispheres	Striatum	Stem	Hemispheres	Striatum	
0·50	73 ±6·9	25 ±3·5	26 ±7·5	128 ±12·8	106 ±17·0	124 ±17·2	3
0·15	88 ±6·1	60 ±2·9	53 ±8·6	115 ±8·2	108 ±18·0	104 ±16·6	3
0·10	76 ±1·6	55 ±1·3	85 ±15·0	100 ±7·1	101 ±20·7	86 ±12·0	2
0	0·29 ±0·02	0·12 ±0·01	0·09 ±0·01	1·12 ±0·08	0·95 ±0·08	1·73 ±0·14	9

(+)-Amphetamine was given 45 min, dopa (25 mg/kg s.c.) 30 min before killing. The rats were pre-treated with reserpine (10 mg/kg i.p.) and nialamide (100 mg/kg i.p.) 22 and 4 hr before killing, respectively. Controls in which no (+)-amphetamine was given were run in parallel.

The values are means ± s.e. of the means. The single values were expressed as % of parallel controls. The bottom row gives control values in μg/g tissue. Each experiment was on pooled tissue parts of 3 rats.

TABLE 2. EFFECT OF VARIOUS DOSES OF (+)-AMPHETAMINE ON L-DOPA-INDUCED AMINE ACCUMULATION IN RAT BRAIN AND HEART

(+)-Amphetamine mg/kg i.p.	Heart		Brain		
	Noradrenaline	Dopamine	Noradrenaline	Dopamine	3-Methoxy-tyramine
2·5	34 (2) ±6·5	77 (2) ±7·4	31 (2) ±2·4	70 (2) ±10·8	167 (2) ±8·1
0·5	57 (6) ±9·9	91 (5) ±15·8	40 (3) ±7·7	108 (3) ±23·6	140 (3) ±18·7
0·25	56 (2) ±18·8	88 (2) ±24·9	54 (2) ±3·6	84 (2) ±4·1	136 (2) ±45·6
0·15	71 (3) ±21·6	137 (3) ±1·7	—	—	—
0·10	93 (3) ±11·2	112 (3) ±19·8	75 (1)	93 (1)	83 (1)
0	0·10 (16) ±0·01	1·86 (15) ±0·13	0·13 (8) ±0·01	1·29 (8) ±0·10	0·87 (8) ±0·09

The details are as described in Table 1.

With the histochemical fluorescence technique, effects were seen only after the larger dose (0·75 mg/kg) of (+)-amphetamine and changes were then only observed in the noradrenaline terminals of the neocortex, compared with the controls, whereas the noradrenaline terminals of, for example, the hypothalamus, the preoptic, septal and vagus areas as well as peripheral organs appeared to be unaffected. The fluorescence intensity of areas rich in dopamine terminals was also unaffected. This preferential sensitivity of the noradrenaline terminals

of the neopallium is in good agreement with the biochemical results. The decrease in noradrenaline obtained with the smaller dose was probably too low to be detected by the histochemical fluorescence technique. The catecholamine nerve cell bodies were not affected by the (+)-amphetamine treatment.

Results in vitro. (+)-Amphetamine in concentrations down to 0·2 μg/ml inhibited almost completely the accumulation of α-methylnoradrenaline in the noradrenaline nerve terminals of the hypothalamus, the neocortex and the vas deferens as well as the dopamine nerve terminals of the neostriatum. In a concentration of 0·075 μg/ml, the drug still had a clearcut effect on the neocortex but no definite effect on the other tissues, which is in general agreement with the *in vivo* experiments.

The present results support the view (Carlsson & others, 1965) that (−)-amphetamine may act by releasing extragranular catecholamines. It should be noted that effects are obtained with doses as low as 0·1 mg/kg *in vivo*, which appear to be threshold doses with respect to gross behaviour, and with low concentrations (0·075 μg/ml) *in vitro*. The particularly high sensitivity of the noradrenaline neocortical systems suggests that these terminal systems are of importance for the psychomotor stimulation induced by amphetamine. However, these suggestions should be considered tentative until the effect of (+)-amphetamine described above has been demonstrated under less artificial conditions.

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Effects of cocaine on brain noradrenaline in relation to toxicity and convulsions in mice

SIR,—Decreases in the concentration of noradrenaline in the brain have been reported when stress is induced by drugs (Vogt, 1954), electric shock (Maynert & Levi, 1964), or by audiogenic seizures (Breitner, Picchioni & Chin, 1963). Sensory stimulation also increases the toxicity of amphetamine (Cohen & Lal, 1964) and hence, presumably, stress. Sensory stimulation, including aggregation in mice, also enhances the effect of amphetamine on the brain concentration of noradrenaline (Moore, 1963; Lal & Chessick, 1964). Recently, it was reported that the toxicity of cocaine in mice is heightened by several types of sensory stimulation, including aggregation (Lal & Chessick, 1965). We now report the reaction between the toxicity of cocaine during sensory stimulation and the effect of cocaine on the brain concentration of noradrenaline.

Adult male HA/ICR albino mice were housed under conditions almost identical to "aggregation". Cocaine hydrochloride, 10 ml/kg, in physiological saline was injected intramuscularly. Immediately after injection, the animals were isolated one per cage, or aggregated, 10 per cage (Cohen & Lal, 1964). The surviving mice were decapitated, but mice found dead were not used. The brains of three mice were pooled and the concentration of noradrenaline measured fluorimetrically by the method of Crout, Creveling, & Underfriend (1961) modified by Harvey (1965).

A small but significant decrease in the concentration of brain noradrenaline was found at 0·5 and 1 hr after the administration of 100 mg/kg cocaine (Table 1). The animals recovered from the central stimulant effects of cocaine within 2 hr. The effects of isolation and aggregation on brain noradrenaline, on toxicity, and on convulsions are compared in Table 2. The data confirm the earlier report (Lal & Chessick, 1965) that aggregation increases the toxicity in mice treated

TABLE 1. EFFECT OF 100 mg/kg COCAINE ON THE CONCENTRATION OF NORADRENALINE IN THE BRAINS OF MICE

Hr after injection	Noradrenaline ± s.e. mean		Change %	P*
	Control	Treated		
0·5	0·36 ± 0·006 (18)†	0·33 ± 0·012 (18)	−8	0·05
1·0	0·32 ± 0·010 (18)	0·26 ± 0·023 (24)	−19	0·01
2·0	0·29 ± 0·015 (9)	0·31 ± 0·011 (15)	−6	n.s.
4·0	0·25 ± 0·013 (12)	0·26 ± 0·009 (9)	+4	n.s.

* Significance calculated on the basis of "Student's t", two-tailed.
† No. of animals in parentheses.

TABLE 2. EFFECTS OF ISOLATION AND AGGREGATION ON BRAIN NORADRENALINE, TOXICITY, AND CONVULSIONS IN MICE TREATED WITH COCAINE (100 mg/kg)*

Experimental condition	Noradrenaline† µg/g wet weight ± s.e. mean	Change %	P§	Dead‖ total	Convulsed total
Isolation—					
Control	0·30 ± 0·018 (18)‡			0/20	0/10
Treated	0·23 ± 0·023 (24)	−23	0·05	4/20	9/10
Aggregated—					
Control	0·32 ± 0·010 (30)			0/20	0/20
Treated	0·26 ± 0·017 (33)	−19	0·01	21/30	9/10

* The estimations of noradrenaline, toxicity, and convulsion figures are based on three different groups of animals. The toxicity and convulsion data presented are typical of the effect.
† 1 hr after injection.
‡ No. of animals in parentheses.
§ Significance calculated on the basis of "Student's t", two-tailed.
‖ 4 hr after injection.

with cocaine. The effect on brain noradrenaline does not seem to be related to the toxicity of the drug, but rather to the convulsive state. The convulsions were violent and clonic in nature, and appeared within 30 min of injection.

Thus cocaine can cause a small but significant decrease in the concentration of noradrenaline in the brain. Although the change is small compared with the total amount of noradrenaline in the brain, it is important when compared with the size of the noradrenaline pool proposed to be involved in physiological nerve activity (Kopin, 1964). In contrast to amphetamine, the effect of cocaine on brain noradrenaline seems to be identical under conditions of aggregation or isolation. Thus, there is either a different mechanism for the increased lethality of amphetamine and cocaine under sensory stimulation, or the noradrenaline-depleting action of convulsions obscured the differences between isolated and aggregated mice.

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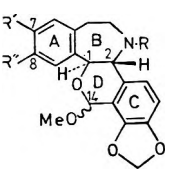
The structure of porphyroxine (papaverrubine D)

SIR,—A few years ago we reported on the isolation and characterisation of porphyroxine (m.p. 192°) from Indian opium (Pfeifer & Teige, 1962). A short time later the alkaloid was also described by Genest & Farmilo (1963), with a differing m.p. (234–236°) and with a molecular formula which we know today to be incorrect.

During our investigations of further alkaloids with the properties of porphyroxine (formation of intensely red coloured solutions when heated with dilute acids) in the genus *Papaver*, we detected a total of six such compounds, which were named papaverrubines A–F (Pfeifer & Banerjee, 1964). Porphyroxine is papaverrubine D. It could be detected in all *Papaver* spp. hitherto investigated.

Later the papaverrubines A and E were isolated from *Papaver rhoeas* L. (Pfeifer & Banerjee, 1965) and B from *Papaver glaucum* Boiss. et Hauskr. (Pfeifer, 1964a). The latter also occurs in opium (Pfeifer, 1965). By mass spectrometry and *N*-methylation it could be shown (Pfeifer, Banerjee, Dolejš & Hanuš, 1965) that A and E are desmethyl bases of the stereoisomeric alkaloids isorhoeadine and rhoeadine, while papaverrubine B is the *N*-desmethyl compound of glaudine, a new papaver alkaloid recently isolated by us (Pfeifer, 1964b; Pfeifer & Mann, 1965). Porphyroxine (papaverrubine D) differs from papaverrubine B only in having a phenolic OH group instead of a methoxyl group and thus represents an *O*-desmethylpapaverrubine B. However, it is not yet clear whether it has the same configuration as papaverrubine B, since the basic structure contains three asymmetric carbon atoms.

We therefore *O*-methylated the porphyroxine with diazomethane in methanol-ether, in which papaverrubine B, m.p. 202–203°, was formed in high yield. Ultraviolet data (methanol): λ_{max} 236, 286 mμ (log ϵ 4·06, 3·86). Infrared data (KBr, cm^{-1}): 3305 (–NH). The identity was proved by thin-layer chromatography with authentic papaverrubine B (silica gel G Merck; solvent system, benzene : acetone : methanol 7 : 2 : 1; Rf 0·64; aluminium oxide G Merck; solvent system, heptane : chloroform : ether 4 : 5 : 1; Rf 0·31). This ensures that porphyroxine (I) has the same configuration as papaverrubine B (II) at the asymmetric carbon atoms 1,2 and 14. Papaverrubine B could also be methylated to glaudine (III) with equimolecular amounts of methyl iodide in tetrahydrofuran.* m.p. 103–105°. Methiodide: m.p. 174–176°. Ultraviolet data (methanol): λ_{max} 237, 287 mμ (log ϵ 4·03, 3·92). Thin-layer chromatography (see above): Rf 0·73, 0·50. Since glaudine has B/D *trans*-configuration (Cross, Mann & Pfeifer, 1966), porphyroxine also belongs to the *trans*-series.



I; R = H; R′ = OH; R″ = OMe or R′ = OMe; R″ = OH
porphyroxine (papaverrubine D)

II; R = H; R′ = R″ = OMe
papaverrubine B

III; R = Me; R′ = R″ = OMe
glaudine

* Only partial conversion.

By means of acid hydrolysis glaudine can also be transformed into glaucamine (C-14-lactol; B/D *trans*), which can be isomerised to oreogenine (B/D *cis*); oreodine (B/D *cis*) is formed by etherifying this C-14-lactol with methanol (Pfeifer & others, 1965). Both the last-named alkaloids were recently isolated from *Papaver oreophilum* Rupr. (Pfeifer & Mann, 1964). This indicates the following affinity:

$$\text{Porphyroxine (papaverrubine D)} \xrightarrow{O\text{-methylation}} \text{papaverrubine B} \xrightarrow{N\text{-methylation}}$$

$$\text{glaudine} \xrightarrow{\text{hydrolysis}} \text{glaucamine} \xrightarrow{\text{isomerisation}} \text{oreogenine} \xrightarrow{\text{etherification}} \text{oreodine.}$$

The exact position of the OH group of porphyroxine in ring A (C-7 or C-8) has not yet been determined.

Recently, Hughes & Farmilo (1965) also reported on the methylation of porphyroxine. The product obtained by them (m.p. 202–204°) is probably identical with papaverrubine B. The authors remark that we had given no experimental details on papaverrubine B, but they have obviously overlooked my earlier publication (Pfeifer, 1964a). Hence they compare the *O*-methylporphyroxine obtained with a product (m.p. 241–243°) which we had earlier obtained from porphyroxine during methylation experiments (Pfeifer & Teige, 1962). At that time we ourselves already doubted the formation of *O*-methylporphyroxine. After repeated examination by thin-layer chromatography it can now be considered definite that, as rightly presumed by Hughes & Farmilo (1965), only another modification of porphyroxine was formed in this experiment (for this, cf. Klayman, 1956). The m.p. of this form is close to that stated by Genest & Farmilo (1963).

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The use of bradykinin-induced effects in rats as an assay for analgesic drugs

SIR,—Guzman, Braun, Lim, Potter & Rogers (1964) and Lim, Guzman, Rogers, Goto, Braun, Dickerson & Engle (1964) used the responses of dogs to intra-arterial injection of badykinin as a means of assessing analgesics. We have adapted this method to rats.

Bradykinin was injected into the right common carotid artery of unanaesthetised Wistar male rats, weighing from 280 to 320 g. The injections were made through a polyethylene catheter (internal diameter, 0·5 mm), inserted centripetally under light ether anaesthesia into the carotid artery. The catheter passed through the subcutaneous tissues to protrude from the back of the animal. After recovery from anaesthesia, the rats behaved normally. Bradykinin, tested 1 hr after recovery, produced dextro-rotation of the head, flexion of the right forelimb and occasionally squeaking. For each rat, we measured the minimum dose of bradykinin required to provoke these effects. This ranged from 0·125 to 0·500 μg/rat and was always dissolved in normal saline, 0·2 ml. Of 162 animals, the threshold of bradykinin was found to be higher than 0·500 μg in two rats only and these were discarded.

If the injection was made into the left carotid artery, the same symptoms occurred at the corresponding side. However, doses of bradykinin up to 1·5 μg were less active than those described above. We have tested codeine phosphate, phenacetin, acetylsalicylic acid, aminopyrine, phenylbutazone sodium and methadone bitartrate against the bradykinin effects. The compounds, suspended in 5% gum acacia solution or dissolved in normal saline, were injected (0·250 ml/100 g) intraperitoneally or subcutaneously. The threshold dose of bradykinin was injected 15 min after and subsequently at 5 min intervals, until the bradykinin effect reappeared. The criterion chosen for evaluating protection was the disappearance of the bradykinin effect after at least two consecutive doses of the polypeptide. Rats which did not recover the normal sensitivity were discarded. Each rat received one drug at one dose level. The ED50 values are given in Table 1.

TABLE 1. ED50 VALUES OF SIX ANALGESICS IN RATS INJECTED WITH BRADYKININ

Drug	ED50 mg/kg	Route	Number of rats
Methadone bitartrate ..	1·45 (0·97–2·18)	s.c.	23
Phenylbutazone sodium ..	16·8 (9·3–30·4)	i.p.	25
Aminopyrine	17·8 (8·1–39·2)	i.p.	26
Codeine phosphate	32·0 (10·7–96·0)	s.c.	28
Acetylsalicylic acid	38·0 (66·7–100·9)	i.p.	20
Phenacetin	108·0 (96·4–121·0)	i.p.	20

The ED50 and its 19/20 confidence limits (in parentheses) were estimated by the method of Litchfield & Wilcoxon (1949).

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Book Review

SPECTRAL DATA AND PHYSICAL CONSTANTS OF ALKALOIDS. By J. Holubek and O. Strouf. Issue 1: 300 data cards, including two binders to take Issues 1 and 2, with index and bibliography. Heyden & Sons Ltd., Spectrum House, Alderton Crescent, Hendon, London, N.W.4, 1965 £23; (U.S.A. $69.00); post free.

In natural products as in every other field, the increasing tempo of research creates problems of information retrieval. Thus identification of a known compound isolated from natural sources not infrequently may entail considerable search of the literature; the process is seldom as simple as it would appear in retrospect. As an aid towards this end in the field of alkaloids, the issue of a compilation of spectral data and physical constants is to be welcomed. The collection, published in English, comes from the Research Institute for Natural Drugs in Prague. It consists of 300 cards assembled in book form, each card being devoted to a single alkaloid. For each compound the structural formula is shown, where appropriate, with its absolute configuration. Molecular formulae, melting-points, specific rotations and pK values in 80% methyl cellosolve are also listed. Spectroscopic data include the ultraviolet and the infrared absorption spectra.

These parameters suffice to identify individual alkaloids and it is the authors' contribution to correlate and collate such information from scattered literature sources. For the majority of the alkaloids listed, melting-points and specific rotations are quoted direct from the original literature unless modified by private communication from the author concerned. Apparent dissociation constants in methyl cellosolve were measured afresh under standard conditions by Dr. Holubek and Dr. Strouf. Of the spectra, those in the ultraviolet region were redetermined in methanol solution; for the infrared region, samples were presented in two forms (*a*) as a 30% suspension in mineral oil and (*b*) in chloroform solution. Rock salt optics were used except for the region 3 300 to 2,000 cm^{-1} where a lithium fluoride prism was fitted. The quality of spectra is good, although in a few cases a higher concentration of solute could have been used with advantage. Because of a chloroform band just above 3,000 cm^{-1}, the authors rightly state that the CH stretching region in their spectra is "not of great diagnostic value". One wonders if it was solubility, or rather the lack of it, which ruled out carbon tetrachloride or carbon disulphide. Both are preferable to chloroform in the 3,000 cm^{-1} region. Such an unfortunate choice of solvent tends to vitiate the advantage gained from the higher resolution of the lithium fluoride prism.

References, which number upwards of 1,100, include the botanical sources from which the isolation of each particular alkaloid has been reported, whilst in terms of structure, papers are cited only where they do not appear in H. G. Boit, "Ergebnisse der Alkaloid Chemie bis 1960", Academie Verlag, Berlin, 1961.

This compilation is to be recommended to those engaged in alkaloid research; it would be especially helpful to workers in the chemotaxonomic field. It is to be hoped that the range of alkaloids will be extended promptly by future issues. These are promised in batches of 100 cards, and in view of the obvious care taken in checking the physical data, such issues will form a valuable extension to the present collection.

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Letters to the Editor

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