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## SCIENCE PAPERS

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# Science Papers

# Quality control of white soft paraffin

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Samples of white soft paraffin complying with pharmacopoeial requirements vary in their physical properties. Some samples produce hard waxy lumps and permit excessive separation of liquid components (bleeding). Measurement of melting and congealing points and consistency by penetrometer failed to reveal these differences. The viscosity of samples after 20 min shear (1159$s^{-1}$) is related to lump formation as detected by usual examination of samples heated and cooled without stirring and spread on a tile after gentle mixing.

Various tests have been applied in the United States and British Pharmacopoeial monographs in attempts to control the physical properties of white soft paraffin. It is our experience that these tests are inadequate to determine the tendency of samples to form a hard waxy crust when heated and cooled without agitation, and the dispersion of the crust as hard waxy lumps on stirring the product, and also the separation of liquid components (bleeding) particularly after shearing and storage at elevated temperatures.

The chemical composition and difficulties involved in the definition of white soft paraffin have been described by Schulte & Kassem (1963) and by Franks (1964). The presence of n-, iso- and isocyclic paraffins commonly referred to as paraffin, intermediate and microcrystalline waxes respectively is recognized. The liquid paraffin fraction is believed to be held in a gel structure within a network of microcrystalline waxes. The rate of structural breakdown after shearing and the time for regeneration of structure have been related to the composition of the white soft paraffin by Schulte & Kassem (1963). Van Ooteghem (1968) has suggested that the quality of white soft paraffin may be determined by measurement of the speed of breakdown of its structure under shear at a rate of shear of approximately 250 $s^{-1}$. Boylan (1966, 1967) has investigated the thixotropic behaviour of white soft paraffin and has suggested that comparison of the hysteresis loop of samples and a standard will provide a measure of the spreadability of the product.

Whilst the definition of "quality" in white soft paraffin varies with individual formulators' ideas and the intended use of the end product, it is clearly desirable that lump formation and bleeding should be minimized. The work in this paper describes our attempts to initiate test procedures designed to select those paraffins free from such undesirable tendencies and to illustrate the inadequacy of the present pharmacopoeial standards.

## EXPERIMENTAL

Samples of white soft paraffin (A–P, Table 1), claimed to be of British Pharmacopoeial quality, were obtained from several manufacturers. Samples 1–5 (Table 2) of known composition were obtained from one manufacturer. All were subjected to the tests described below:

(1) *Tendency to form lumps.* Each sample (20 g) of white soft paraffin was heated to 80° in a 50 ml beaker, cooled to 25° over 2 h, mixed by stirring gently with a glass rod, spread on a black tile and observed for the presence of hard waxy lumps.

(2) *Bleeding tendency.* Samples (30 g) were treated as described in (1) above up to the mixing stage and then subjected to a test based on that of Van der Pol (1960). Strips of Whatmans No. 1 filter paper (2 × 25 cm), supported vertically above the beaker, were inserted in the unmixed samples to a depth of 2 cm and the height of ascent of oil after 24 h at 25° was measured. The height of ascent expressed in mm is the bleeding number.

(3) Melting and congealing points were determined as described in the British and United States pharmacopoeias respectively.

(4) *Unctuous nature.* The samples pretreated as in (1) above were assessed subjectively as described in the British Pharmacopoeia.

(5) *Consistency.* Penetration values were determined using a Stanhope penetrometer in combination with a cone shaped metal plunger weighing 7·5 g. Samples were prepared for testing by the U.S.P. method to produce an unworked sample. Certain samples were subjected to standard shear by repeated forcing through a narrow aperture (standard working conditions) and their "worked penetration" value determined immediately. The results are expressed as the average of three determinations.

(6) *Viscosity.* Samples pretreated as described under (2) above were introduced to the plate of the Rotovisko viscometer PK1 system with minimal shear. The samples were sheared at speed 9 (rate of shear 1159 $s^{-1}$) or speed 5 (193 $s^{-1}$) and the fall in viscosity with time and the final viscosity after 20 min shear were recorded. The pretreatment of samples and minimal shear involved in the introduction of samples to the cone and plate system ensured greater reproducibility of results than could be obtained using other systems.

## RESULTS AND DISCUSSION

The results presented in Tables 1 and 2 show that all samples tested comply with the B.P. and U.S.P. tests for melting and congealing points. Although samples A–P can be described as unctuous to the touch, their tendency to lump formation and bleeding vary considerably.

Consistency determinations by penetrometer methods are widely used for quality control of white soft paraffin. The U.S.P. method for the preparation of samples for such determinations involves heating and cooling of samples without stirring, to ensure homogeneity. This may result in the formation of a waxy surface which in samples 1, 2 and E, F, G, K led to low penetration values suggestive of a paraffin of high consistency. Subjection of samples 1 and 2 to standard working conditions showed the structure to be rapidly destroyed, the resulting samples being too fluid for consistency determinations. Comparison of the penetrometer values and final

Table 1. *Melting and congealing points, penetrometer values, viscosity and tendency to form lumps of samples of white soft paraffin*

| Sample | M.p. °C | Congealing point °C | Penetrometer (mm) | Viscosity cP P.K.1 system shear rate 1159 $s^{-1}$ | Lumpiness |
|---|---|---|---|---|---|
| A | 50 | 50 | 39·2 | 1390 | — |
| B | 50 | 51 | 43·6 | 1495 | — |
| C | 52 | 51 | 20·3 | 3065 | — |
| D | 52 | 53 | 27·0 | 970 | — |
| E | 47 | 51 | 30·5 | 334 | + |
| F | 51 | 50 | 17·7 | 160 | + |
| G | 50 | 51 | 22·3 | 300 | + |
| H | 56 | 56 | 24·5 | 760 | — |
| I | 51 | 55 | 36·3 | 960 | — |
| J | 51 | 52 | 29·8 | 1250 | — |
| K | 57 | 59 | 33·3 | 180 | + |
| L | 47 | 51 | 38·7 | 600 | — |
| M | 52 | 55 | 39·3 | 780 | — |
| N | 51 | 53 | 30·3 | 750 | — |
| O | 47 | 51 | 37·0 | 499 | — |
| P | 51 | 56 | 32·6 | 1320 | — |

Table 2. *The physical properties of samples of white soft paraffin of known composition*

| | Sample | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| *Composition* % by weight | | | | | |
| Paraffin wax | 15 | — | — | — | — |
| Intermediate wax | — | 14 | 10 | 7 | — |
| Microcrystalline wax | — | — | 10 | 28 | 50 |
| White oil | 85 | 86 | 80 | 65 | 50 |
| *Test* | | | | | |
| Lump formation | + | + | — | — | — |
| Bleeding No. | 41 | 32 | 30 | 10 | 4 |
| Consistency (mm) (Penetrometer) unworked | 12·3 | 26·6 | 49·9 | 28·8 | 16·7 |
| Consistency (mm) (Penetrometer) worked | Fluid | Fluid | 75·2 | 43·2 | 31·8 |
| Melting point °C | 38 | 44 | 49 | 51·5 | 53 |
| Congealing point °C | 42 | 50 | 52 | 53 | 51 |
| Viscosity (cP) (1159 $s^{-1}$) | 194 | 281 | 359 | 1748 | 2893 |

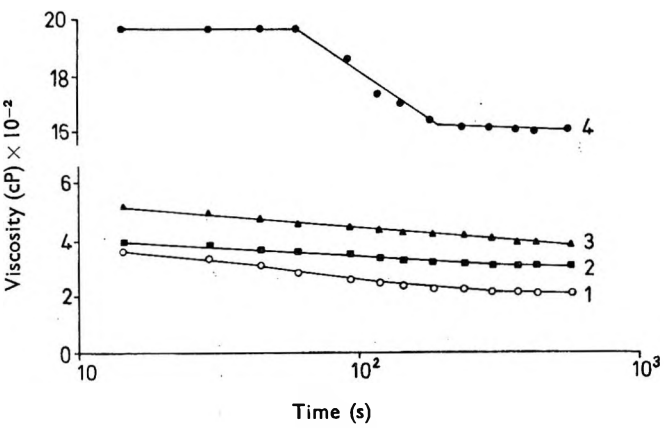


FIG. 1. Effect of time of shear (shear rate 1159 $s^{-1}$) on the viscosity of samples 1–4.

viscosities recorded in Tables 1 and 2 indicates that the penetrometer does not give a true measure of consistency when testing samples which tend to form lumps, nor do the results reflect the differences in viscosity between samples as measured by the Rotovisko.

The change in viscosity of samples 1–4 with time when sheared at a rate of 1159 $s^{-1}$ are shown in Fig. 1. Measurements made at a shear rate of 193 $s^{-1}$ produced results comparable with those shown. However, much difficulty was encountered when using this lower shear rate with samples of high viscosity and the results were not reproducible. The effect of time of shear (1159 $s^{-1}$) on the structural breakdown of samples A–P was similar to that of samples 1–3 shown in Fig. 1. In all cases structural breakdown was complete within 20 min of the application of shear. The final viscosity of the samples after 20 min shear are shown in Tables 1 and 2. One sample tested showed unusual behaviour and reproducible results were difficult to obtain. This sample tended to be thrown out from between the cone and plate of the viscometer.

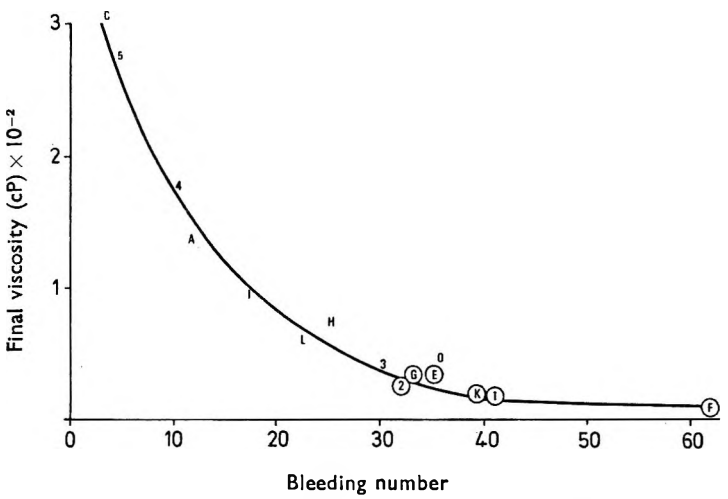


FIG. 2. The relation between final viscosity and bleeding number of samples of white soft paraffin. Those ringed samples produced lumps.

The relation between final viscosity, bleeding number and tendency to form lumps is shown in Fig. 2. The tendency of the liquid fraction of white soft paraffin to separate and for lumps to form is greater with samples of lower viscosity. Sample O, of relatively low viscosity, is an exception in that it does not form lumps.

Difficulties involved in the chemical analysis of white soft paraffin are such that it is unlikely that chemical definition of the product is practical. Attempts to use gas-liquid chromatography to determine differences in the composition of samples revealed that such differences existed but it was not possible to relate results to the physical properties of the samples. Franks (1964) believes that a strong stable homogeneous gel structure capable of withstanding repeated shear is perhaps the most important characteristic of white soft paraffin. The results in Table 2 and Fig. 3 show that an increase in microcrystalline wax content and therefore in network structure produces an increase in final viscosity with a reduction in bleeding tendency.

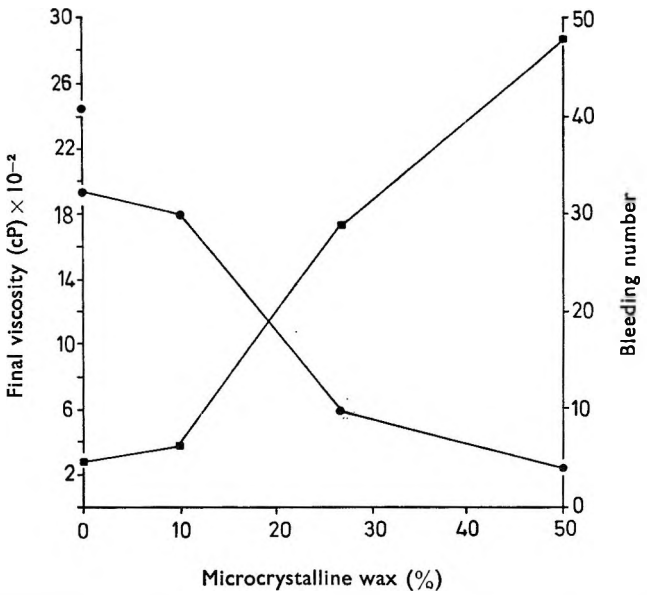


FIG. 3. The effect of microcrystalline wax content on the final viscosity and bleeding number of samples 2–5 of white soft paraffin. ■—■ Final viscosity. ●—● Bleeding number.

In our experience two extra tests must be added to those described in the B.P. These are the measurement of the final viscosity after shearing and the tendency of the sample to form lumps. With these, adequate information is available to predict the acceptability of a particular sample for use in ointment formulations. The use of rate of structural breakdown as a measure of performance is unnecessary and difficult to determine. At present the finer qualities of white soft paraffin such as fibre length and spreadability are best measured by subjective tests, but the application of the tests for lumpiness and viscosity described can minimize problems of lump formation and bleeding.

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SHORT COMMUNICATION

# The possible use of polytetrafluoroethylene (Fluon) as a tablet lubricant

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The preparation of free flowing granules usually obviates the need for a lubricant to be selected for improved glidant action alone. The reduction of wall friction during the compression and ejection of the tablet is the most important criterion for selecting a particular lubricant.

To obtain lubrication at the die wall a material of low shear strength must be incorporated in the granules. Magnesium stearate in particular possesses such qualities and acts suitably as a die wall lubricant in the tabletting process (Lewis & Shotton, 1965). However, magnesium stearate has the disadvantages that it can be converted to stearic acid and thus react with other ingredients; it may also reduce the hardness of the tablets produced by weakening the bonds between particles (Lewis & Shotton, 1964).

Polytetrafluoroethylene (Fluon) is reputedly non-toxic and chemically inert below about 250°, with a very low coefficient of friction. It possesses a low shear strength, 110 kg $cm^{-2}$ at zero applied load together with a high yield pressure resulting in an extremely low coefficient of friction (Hersey, 1960). Polytetrafluoroethylene has been successfully bonded to the tips of punches to reduce the adhesion between tablet and punches.

A patent describing the use of polyfluorocarbon type polymer as a tablet lubricant has been granted to Hotko (1967) in the U.S.A. but no experimental evidence is given.

This paper describes a limited trial with fluon L169 lubricant powder (ICI Ltd.) to compare its usefulness as a lubricant in the compression of powders.

## *Experimental*

Spray dried lactose (120–150 mesh) was compacted in an instrumented Lehman single punch reciprocating tablet machine. The instrumentation used was essentially similar to that previously described by Shotton & Ganderton (1960). Five tablets were prepared at each of four different pressure levels (machine settings, PI, PII, PIII and PIV) and the mean value of force calculated for the purpose of the figures. The tablet weight (0·6895 g) taken was sufficient to give a tablet of 0·4 cm length at zero porosity using a 1 cm diameter punch and die set.

One per cent magnesium stearate and one per cent Fluon L169 powder were separately incorporated into identical lots of the lactose by tumbling on rollers for 1 h. Tablets were then prepared from each of these two mixtures as described for spray-dried lactose above.

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The strength of the tablets was assessed immediately after preparation using the diametral crushing test (Shotton & Ganderton, 1960) and the disintegration time measured using the official B.P. method.

*Results and discussion*

These preliminary results show that 1% of Fluon L169 reduces the die wall friction and also the force needed to eject the tablet, by an amount similar to 1% magnesium stearate in lactose tablets (Fig. 1A, B).

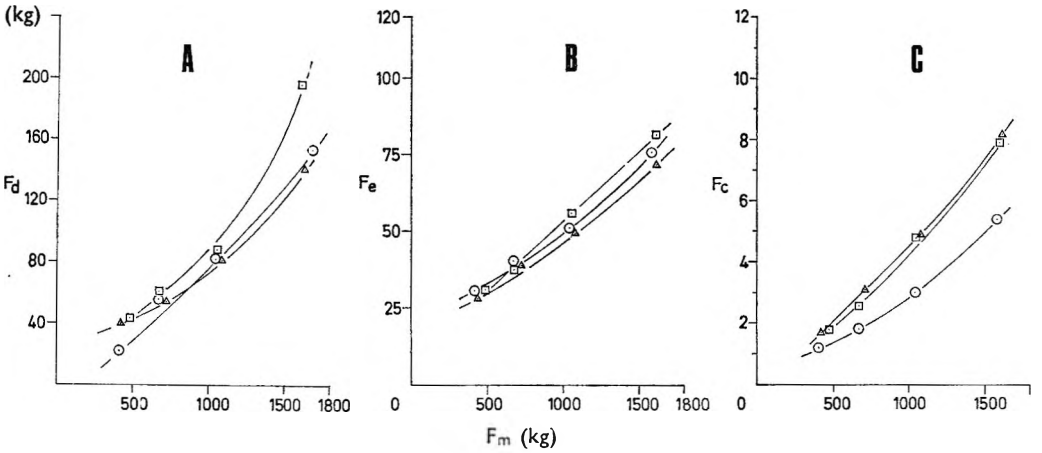


FIG. 1. The effect of compression force on (A) die wall friction, (B) the ejection force and (C) on the crushing strength of tablets for: □ Spray-dried lactose. △ Spray-dried lactose with 1% Fluon L169. ○ Spray-dried lactose with 1% magnesium stearate. ($F_m$ = the mean compression force).

Fig. 1C shows the effect of the two lubricants on the crushing strength of the tablets. The results show that the presence of the Fluon powder does not impair the crushing strength of the lactose tablets, whereas a reduction is obtained when magnesium stearate is used as a lubricant.

The results of the disintegration tests are given in Table 1. The presence of Fluon does not increase the disintegration time of the lactose tablets whereas the magnesium stearate produces a marked increase.

Table 1. *Disintegration times*

| Pressure levels | Disintegration time (s) | | |
|---|---|---|---|
| | Spray dried lactose unlubricated | Spray dried lactose 1% Fluon | Spray dried lactose 1% Mg stearate |
| PI .. .. .. .. | 7 | 6 | 420 |
| PII .. .. .. .. | 9 | 8 | 960 |
| PIII .. .. .. .. | 14 | 10 | 1080 |
| PIV .. .. .. .. | — | 20 | 1200 |

In this preliminary trial with a single tablet excipient (lactose), Fluon powder does not appear to be much better as a tablet lubricant than magnesium stearate. Fluon is chemically inert and tablets incorporating Fluon powder disintegrate much more

rapidly than those with magnesium stearate; it would also appear that the Fluon is less likely to bring about "water-proofing" of the tablet. This is probably due to the yield value of Fluon being high, the particles do not break down and spread by shear at the die wall or between the lactose particles.

The remaining problem is that because of the nature of the thermal decomposition products (above 250°) of polytetrafluoroethylene, doubts have arisen as to its toxicity. However, the polymer has been shown to be non-irritant and harmless when implanted in the peritoneal cavity of dogs over a period of 70 days (LeVeen & Barberio, 1949), it produces no reaction when implanted in human tissues (Charnley, 1960) and has been shown to be safe for use in cooking utensils (Coppock & Knight, 1957). Further chronic toxicity studies must be encouraged, with the view to deciding whether this possibly very useful lubricant may be added to the armoury of the tablet formulator.

*Acknowledgements*

We gratefully acknowledge the gift of Fluon L169 powder from ICI Limited and of spray-dried lactose from McKesson-Robbins Limited. We also gratefully acknowledge the financial support of one of us (O.A.) by the Turkish Government which enabled this work to be undertaken.

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# The effect of distribution of magnesium stearate on the penetration of a tablet by water

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Magnesium carbonate powder and granules were compressed and the tablets characterized by their permeability to air and penetration by liquids. To the same materials, magnesium stearate was added, its distribution being varied by varying its concentration, the size of the base material and the method of mixing. The inhibition of penetration by liquids is roughly proportional to the concentration of magnesium stearate and very susceptible to the method of mixing. The influence of granule size on its distribution was seen mainly as a change in the uniformity of penetration.

Magnesium stearate is a boundary lubricant widely used in tabletting and, like most boundary lubricants, it is strongly hydrophobic. If its concentration in a tablet is sufficiently high, penetration of water into the tablet is prevented. In lower concentrations, its less extreme effects are lengthening of tablet disintegration time (Strickland, Nelson & others, 1956; Kwan, Swart & Mattocks, 1957) and decrease in the rate of dissolution of tablet constituents (Levy & Gumtow, 1963). The mechanism by which this is achieved is complex. Contamination of surfaces by magnesium stearate will modify the mechanisms of bonding during compression and, therefore, the break-up of a tablet in water. Changes in the shape and size distribution of the capillaries which conduct water into the tablet may result from the volumetric contribution of a powder like magnesium stearate which compacts with ease, or the reduction of interparticulate friction which it produces. It is probable, however, that the major mechanism lies in the inhibition of penetration due to the high contact angle of magnesium stearate with water.

The force driving a liquid into a tablet is derived from the pressure difference, $\Delta P$ existing across the curved menisci of the liquid entering the capillaries of the tablet. Carman (1941) gives this pressure difference as:

$$\Delta P = \frac{\gamma \cos \theta}{m}$$

where $\gamma$ is the surface tension of the penetrating liquid and $\theta$ is the contact angle between the penetrating liquid and the capillary surface. m is the ratio of the cross-sectional area of the capillary and its perimeter. This expression of dimensions takes account of irregularity in capillary shape. If $\theta$ is less than 90°, $\Delta P$ is positive and the liquid moves through the capillary. The contact angle of water on magnesium stearate is greater than 90° so that water will not enter a tablet composed of this material. An intermediate effect will be found when magnesium stearate is mixed and compressed with a powder which is freely wetted by water. Capillaries with

surfaces composed of some proportion of magnesium stearate will not then transmit water and the proportion of capillaries so affected will depend upon the distribution of magnesium stearate through the tablet matrix. For given samples of lubricant and powder base, the three major factors which will affect this property are: (i) concentration of magnesium stearate; (ii) the processes of tablet manufacture; (iii) the intensity of mixing operations.

The effect of concentration is relatively straightforward. Higher proportions of lubricant yield an increase in the non-wetting internal surface and the number of capillaries not contributing to the transport of water.

Two aspects of the manufacture process are important. The first is the stage at which the lubricant is added. In tabletting by precompression, some lubricant is added before 'slugging' and some after. In tabletting by direct compression, all constituents are mixed as fine powders. In wet granulation and compression, all the lubricant is added to the aggregated powder. At any level of concentration these processes give a different distribution of lubricant. This may be further modified by the size of the base material. For example, differences in granule size are encountered for reasons of tablet dimensions and press coating and magnesium stearate added after granulation will be discontinuously distributed, the thickness of the regions of lubricant and the scale of discontinuity being determined by the size of the granules.

Finally, mixing will play an important part in the distribution of a lubricant. The problems of dispersing a small proportion of a highly cohesive powder through a mass which is probably of quite different particle size are obvious enough. When a limit is imposed on the mixing energy which can be expended in order to avoid the break-up of granules, the dangers of maldistribution become severe and a gross variation in the aqueous penetration of tablets subsequently prepared becomes possible.

The assessment of the role played by magnesium stearate within the tablet will be complicated if conditions at the die wall are allowed to vary. These conditions, which affect the pattern of forces within the tablet and therefore its pore structure and penetration, are themselves influenced by the concentration of magnesium stearate. A further difficulty is that the method of granulation affects pore structure and liquid penetration (Ganderton & Selkirk, 1969). In the work described below, tablets prepared from magnesium carbonate were characterized by their permeability to air and penetration by liquids. These properties were compared with those of tablets containing magnesium stearate compressed under conditions which eliminated variation in activity at the die wall.

## EXPERIMENTAL

### *Granulation*

Magnesium carbonate (5 kg) was granulated by massing with 2·7 litres of 10% w/v aqueous dextrose solution in a ribbon blender and forcing the mix through a coarse screen. The wet granules were dried, rescreened and the fractions 8–16 mesh, 16–22 mesh and 30–44 mesh collected. In addition, a sample of fines passing through a 200 mesh screen was taken. The great bulk of the material lay in the 16–22 mesh fraction and to this, quantities of magnesium stearate, which had been freed from lumps, were added to give a number of granulations containing between 0% and 5% lubricant. The components were mixed by first subdividing each component and

then blending the fractions. The fractions were then mixed by tumbling. To the other granule sizes, magnesium stearate was added to give a concentration of 1%. The initial proportionation of the components was omitted and the components mixed by tumbling.

Other materials were prepared as follows: Samples of 16–22 mesh granules containing $\frac{1}{2}$ or 1% magnesium stearate added before massing. A sample of the original magnesium carbonate powder containing 1% magnesium stearate mixed in by tumbling. A sample of the original magnesium carbonate powder containing 1% magnesium stearate mixed in by shearing in a pestle and mortar.

*Compression*

Granules or powder (2·5 g) were placed in a die 1·92 cm in diameter which was closed at the lower end by a spigot which penetrated 0·95 cm into the die. The punch was inserted and the assembly pressed between the platens of a small hydraulic press. The studied pressure range of 10–200 $MNm^{-2}$ was measured in a manner described by Shotton & Ganderton (1960) with strain gauges affixed to the shank of the punch.

Before each compression, the wall of the die was liberally coated with stearic acid which was applied as a 2% solution in carbon tetrachloride and allowed to dry.

Die wall conditions were assessed for all materials in a further experiment in which the spigot of the punch assembly was replaced by a lower punch bearing strain gauges. The apparatus was re-assembled with an independent die support and an accurately measured force of about 29 kN applied. The lower punch force was simultaneously measured, the difference between these values giving the force loss at the wall of the die.

*Measurement of porosity, permeability and liquid penetration rate*

The porosity of a tablet was calculated from its weight and volume after the density of each component had been measured with a density bottle. In this calculation, the volumetric contribution of each component was assumed to be additive.

The air permeability of the tablets was measured, while they were still in the die, with an apparatus similar to that described by Lea & Nurse (1939). The flow of air through the tablet, Q, was measured at a pressure difference of 62 $\pm$ 0·7 $kNm^{-2}$. The permeability coefficient, $B_o$, was calculated from the relation

$$B_o = \frac{2QL\eta P_1}{A\,(P_1^2 - P_2^2)}$$

where A and L are the area and thickness of the tablet and $\eta$ is the viscosity of the air. $P_1$ and $P_2$ are the upstream and downstream pressures.

The rate at which liquid entered a tablet by capillarity was measured by the method of Ganderton & Selkirk (1969). It consisted of moving the tablet to a position in which its lower surface was flush with the die. This was placed in a cup which formed one arm of a liquid-filled U-tube and the rate of uptake measured as the withdrawal of liquid from the other arm. Water and cyclohexane were used in the tests and all experiments were made on tablets compressed to a porosity of 33·4 $\pm$ 0·1%.

## RESULTS

Measurement of applied and transmitted forces at a moderate level of compaction showed that the ratio of the lower punch force to the upper punch force for all materials fell within the range 0·96–0·99. Details of these measurements for the compression of 16–22 mesh granules, to which up to 5% magnesium stearate was added, are given in Table 1.

Table 1. *Effect of concentration of magnesium stearate on the force lost at the die wall during compression of 16–22 mesh granules of magnesium carbonate*

| Magnesium stearate % | Upper punch force kN | Lower punch force kN | Force lost kN |
|---|---|---|---|
| 0 | 29·3 | 28·9 | 0·4 |
| 0·5 | 29·8 | 29·1 | 0·7 |
| 1·0 | 30·1 | 29·2 | 0·9 |
| 2·0 | 29·5 | 29·2 | 0·3 |
| 5·0 | 29·3 | 29·1 | 0·2 |

In Fig. 1, the permeability coefficient of tablets containing no lubricant is shown as a function of porosity. Similar data are presented in Fig. 2 for tablets prepared from granules in which the concentration and distribution of magnesium stearate was varied.

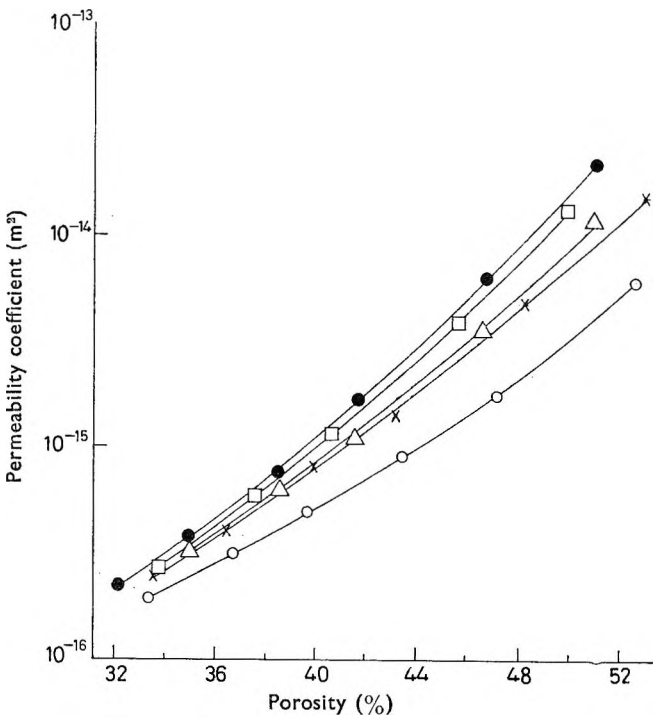


FIG. 1. The permeability of tablets containing no magnesium stearate. ● Compressed granules: 8–16 sieve, □ 16–22 sieve, △ 30–44 sieve, × −200 sieve, ○ Compressed ungranulated powder.

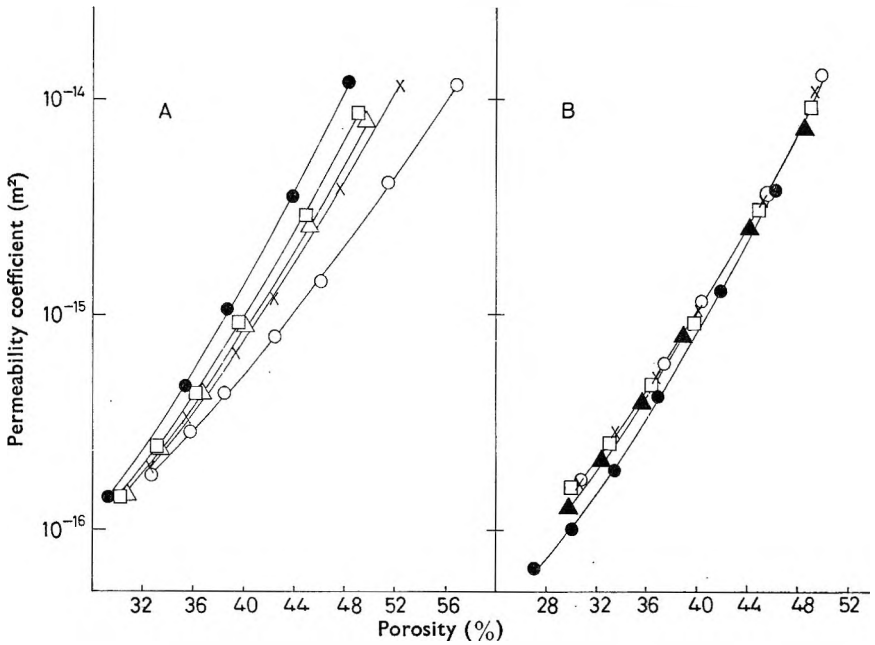


FIG. 2. The permeability of tablets containing magnesium stearate. A. Containing 1% lubricant. ○ Compressed ungranulated powder. Compressed granules: ● 8–16 sieve, □ 16–22 sieve, △ 30–44 sieve, × –200 sieve. B. Compressed 8–16 granules. Magnesium stearate: ○ 0%, × ½%, □ 1%, ▲ 2%, ● 5%.

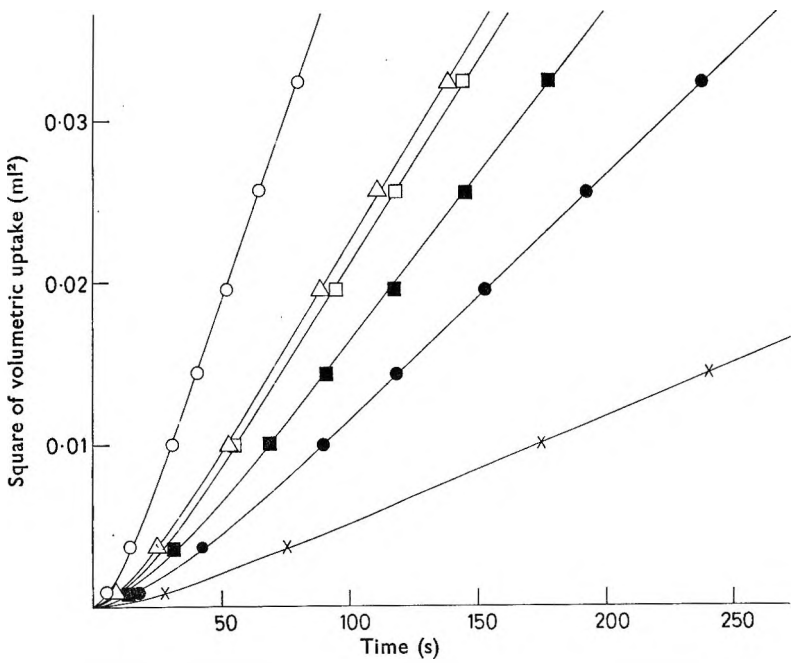


FIG. 3. Penetration of tablets by liquids. ○ Compressed ungranulated powder by water. Compressed 16–22 granules by water: △ no lubricant, ■ 1% lubricant, ● 2% lubricant, × 5% lubricant, □ Compressed 16–22 granules containing 1% lubricant by cyclohexane.

The general form of the liquid penetration tests is presented in Fig. 3. Data are plotted as the square of the volume taken up against the time. Details of these tests are given in Tables 2–4.

Fig. 4 describes the penetration of water into tablets into which magnesium stearate was incorporated by different methods.

Table 2. *Penetration of tablets by cyclohexane (figures are the mean of* 4 *results)*

| Material compressed | Uptake time for 0·1 ml (s) | |
|---|---|---|
| | No magnesium stearate | 1% magnesium stearate* |
| 8–16 mesh granules .. .. | 56·5 ± 3 | 58 ± 7·5 |
| 16–22 mesh granules .. .. | 51·5 ± 3·5 | 55 ± 4 |
| 30–44 mesh granules .. .. | 59 ± 2 | 54 ± 6·5 |
| –200 mesh granules .. .. | 52·5 ± 4·5 | 51·5 ± 4·5 |
| Original powder .. .. | 78 ± 6 | 77 ± 9 |

* Added after granulation by tumbling.

Table 3. *Effect of granule size on the penetration of a tablet by water*

| Material compressed | Uptake time of 0·1 ml (s) | | | | | |
|---|---|---|---|---|---|---|
| | No magnesium stearate | | | 1% magnesium stearate* | | |
| | Mean (8 results) | Standard deviation | Coefficient of variance % | Mean (8 results) | Standard deviation | Coefficient of variance % |
| 8–16 mesh granules | 67 | 6·5 | 9·7 | 99 | 25·0 | 25·1 |
| 16–22 mesh granules | 55·5 | 4·0 | 7·2 | 70 | 15·0 | 21·4 |
| 30–44 mesh granules | 47 | 1·7 | 3·6 | 62 | 4·7 | 7·6 |
| –200 mesh granules | 33 | 1·4 | 4·2 | 44·5 | 4·0 | 9·0 |
| Original powder | 30·5 | 1·0 | 3·3 | 51·5 | 2·7 | 5·2 |

* Added after granulation by tumbling.

Table 4. *Effect of magnesium stearate concentration on the penetration of water into tablets prepared from* 16–22 *mesh granules*

| Concentration of magnesium stearate* (%) | Uptake time for 0·1 ml (mean of eight results) (s) | Standard deviation (s) | Coefficient of variance (%) |
|---|---|---|---|
| 0 | 55·5 | 3·8 | 6·9 |
| 0·5 | 68·5 | 6·7 | 9·8 |
| 1 | 80 | 7·6 | 9·5 |
| 2 | 94 | 8·7 | 9·2 |
| 5 | 186 | 15·4 | 8·3 |

* Added after granulation by subdivision of components, blending and tumbling.

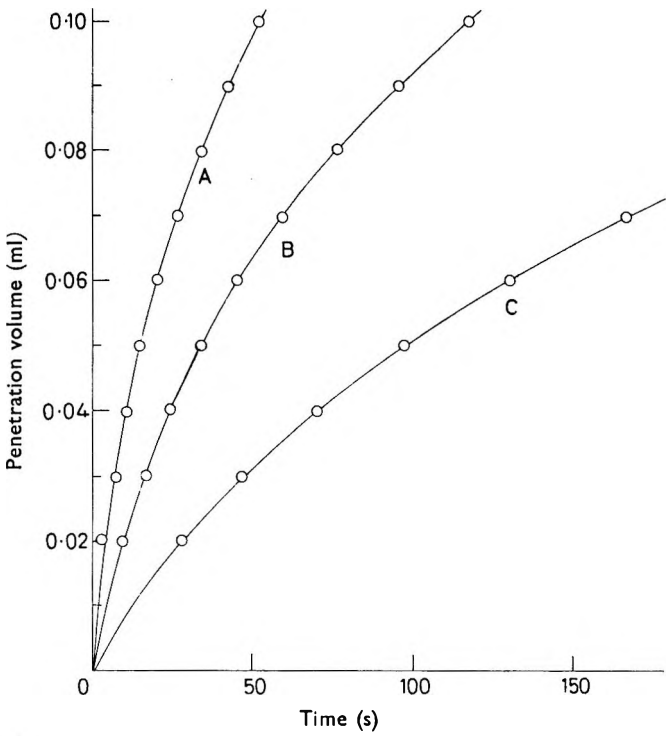


FIG. 4. The effect of method of mixing on the penetration of tablets by water. Magnesium stearate A, 1% tumbler mixed; B, 1% shear mixed; C, ½% wet mixed.

## DISCUSSION

Air permeability and liquid penetration tests reveal different aspects of the addition of excipients to a tablet. Changes in air permeability must be ascribed solely to changes in pore volume and its distribution within the tablet. Whilst these geometric effects influence liquid penetration, this test is also susceptible to any change in the wetting of the tablet. Thus, if the additive increases the contact angle, the forces promoting penetration will be decreased and the rate will fall. If on the other hand the contact angle is unchanged, changes in penetration will be similar to those found by air permeation because the pore network transporting fluid is the same.

### *Tablets containing no lubricant*

Fig. 1 shows that decrease in granule size decreases the permeability of tablets compressed to a given porosity. The effect is not large and it diminishes as the tablets become denser. All granule systems, however, were markedly more permeable than tablets prepared from the ungranulated powder.

In the absence of detailed knowledge of the pore size distribution, no conclusive statement can be made on the nature of these permeability changes. However, since the work is a comparative study based on a single powder, it is reasonable to assume that the pore structure of tablets prepared from the original powder is more uniform than that of compressed granular materials and that, in the latter, a coarse pore network originating from the spaces between the granules is increasingly sustained as the granule size increases. A high permeability results because such a network is capable of transporting a disproportionately large amount of fluid.

Similarly, the more uniform structure of tablets prepared from the original powder is more slowly penetrated by cyclohexane than a structure of granular origin. This test did not, however, reveal any effect of granule size on liquid penetration. Differences in structure to be inferred from permeability are small and it is presumed that their effect on liquid penetration is masked by the lower precision of the test.

The pattern of aqueous penetration given in Table 3 is quite different. The rate at which water enters the tablet increases as the size of the granules used decreases. It is fastest in the compressed ungranulated powder. This reversal of the behaviour expected from the permeability studies can only be explained by a change in the structure of the tablet brought about by the penetrating liquid.

The equation given in the introduction evaluates the potential ability of a capillary to draw liquid through its length. This potential increases as the capillary becomes finer but, since the same capillary must transport the liquid, the higher potential is more than offset by increased viscous resistance (Washburn, 1921). The rate of penetration, therefore, falls, which explains the low penetration rate of cyclohexane into fine-structured and relatively impermeable tablets prepared from the ungranulated powder. The high capillary potential of this structure would, however, be realized if the passage of liquid immediately opened the structure, thus reducing the viscous resistance of the wetted portion of the tablet. The coarser structure of more permeable tablets prepared from larger granules has a smaller potential for drawing liquid into a tablet. An opening of the wetted portion of these tablets to the same extent as in tablets prepared from fine powder or granules, would result in a lower penetration rate. By such a mechanism, the order of aqueous penetration rates would be the reverse of permeability.

With the materials compressed here, there are at least two mechanisms by which the wetted structure might be opened. The first is the dissolution of the soluble fraction of the tablet, namely dextrose, which constitutes about 7% by volume of the total solids. The second is a softening of the tablet, probably by the action of a liquid of high dielectric constant on a structure united mainly by secondary bonds. Softening would permit the dissipation of stresses in the tablet by structural rearrangement. Both effects will be present but the fast penetration of magnesium carbonate tablets containing no dextrose suggests that the latter is more important.

The results of the penetration test given in Fig. 3 indicates a linear relation between the square of the volume taken up and the time. This relation results from the balance of the constant capillary forces promoting penetration, and the opposing viscous forces: the latter increase as the depth of penetration increases. Some deviation occurs in the early stages of penetration, the rate being slower than expected. A possible explanation is the existence of a region of the tablet adjacent to the punch which is atypically dense in structure.

*Studies of tablets containing magnesium stearate*

A die wall previously coated with stearic acid is effective in maintaining almost constant die wall conditions even with a varying concentration of magnesium stearate in the powder being compressed. This conclusion can be drawn from the small variation in die wall losses presented in Table 1. In this study, therefore, die wall friction can be eliminated as a variable influencing the structure of tablets. Under these conditions, the addition of magnesium stearate has little effect on the permeability of tablets. The coincidence of data presented in Figs 1 and 2A shows that the change

in pore structure as the material is compressed is unaffected by the presence, in conventional quantities, of a lubricant. Such an effect might have been expected from the reduction of interparticulate friction and the facilitation of shear failure. When, as shown in Fig. 2B, the concentration is increased, the permeability is reduced, but the effect is significant only at low porosity when the volumetric contribution of the lubricant in relation to the available pore volume is large. Table 2 confirms the insignificant geometric contribution of conventional quantities of magnesium stearate in terms of the penetration of cyclohexane. This liquid freely wets the lubricant so that large effects due to a change in contact angle were not expected.

Wetting effects must therefore be dominant in the large depression of the aqueous penetration rate which occurs when the concentration of magnesium stearate is increased. As shown in Table 4, the increase in penetration time is roughly proportional to the concentration, suggesting a proportional increase in the internal surface of the tablet composed of magnesium stearate and in the number of capillaries unable to conduct water.

It is argued earlier that the size of granules will influence the distribution of a given amount of magnesium stearate throughout a tablet. Examination of Table 3 reveals no clear effect of this variable on the mean rate of penetration. The effect on the uniformity of penetration is, however, most marked. All tablets containing lubricant showed greater variation than the corresponding unlubricated tablets. Deviations in the tablets containing lubricant increased sharply with the coarser granules, the coefficient of variance reaching over 25% with tablets prepared from 8–16 mesh granules. With the smaller overall surface area presented by such granules, even distribution of an additive will be more difficult to achieve. Maldistribution will be locked into the tablet on compression to give a large variation in aqueous penetration from one tablet to another. These variations were least in tablets prepared from the ungranulated powder, the material closest in form to the additive.

There is much evidence to show that the degree of mixing affects the penetration of tablets by water. A comparison of tablets prepared from 16–22 mesh granules to which 1% magnesium stearate was added, can be made from the experimental series given in Tables 3 and 4. Because a lower concentration of lubricant was used in the experiments in Table 4 a more rigorous mixing procedure than that used in other experiments was adopted. This resulted in a slowing of the penetration. A clearer demonstration is seen in Fig. 4. Curves A and B show the effect of 1% magnesium stearate dispersed by different dry mixing actions on the penetration of the tablets subsequently prepared. The more intimate dispersion produced by the energetic shearing process is reflected in a greater inhibition of penetration. Even this process is mild compared to the dispersive capacity of wet mixing. 1% magnesium stearate subjected to this process by an addition during wet massing completely stopped penetration. At half this concentration, the greatly delayed penetration given in curve C is obtained.

In conclusion, the experimental program showed that concentration of magnesium stearate and the degree of mixing greatly affected penetration of tablets by water and the physical form of the granules or powder influenced uniformity of penetration. These factors, which together control the distribution of lubricant, are determined by the tabletting process adopted and the way it is carried out. Since a formulation may vary in both these respects penetration will vary, and so, to some extent, will the steps of disintegration and dissolution which depend upon it.

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# The hardness and elastic modulus of some crystalline pharmaceutical materials

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The surface microhardnesses of single crystals of the following materials have been measured: aspirin, hexamine, sodium chloride, potassium chloride, urea, salicylamide and sucrose. A Leitz microhardness tester was used, with indentor loads of 25 g or less, in order to give indentations of less than 50 $\mu$m diagonal length and enable measurements to be made on single crystals. The compressive elastic modulus of some of the crystals was also determined using a "Techne" microtensile tester modified to work in compression. When the materials are listed in order of magnitude of Young's modulus, this order is the same as when they are listed in order of magnitude for surface hardness.

The large amount of work done on the compaction of materials to form tablets has shown the importance in this process of the physical nature of the materials themselves. Few measurements have been made of the mechanical properties of pharmaceutical materials, and the present work was initiated to obtain values for the elasticity and surface hardness of a series of crystalline substances in order to explore any relation these might have to the compaction behaviour of the various materials.

There are difficulties in measuring the hardness and elastic modulus of small crystals. Standard hardness testers used in the metallurgical industries are of little value in determining microhardness of crystals of non-metals since the loads applied and the size of the indentations produced are much too large for the very small and (relative to metals) soft particles which comprise pharmaceutical materials. With the development of microhardness testers which need only apply loads as small as 10 g, such measurements become possible.

Many workers have attempted by mechanical, electrical and optical methods to determine the moduli of elasticity of crystalline materials. Unfortunately the value of the modulus of elasticity varies in different crystal directions and is closely linked with the degree of symmetry exhibited by the crystals. The work of Neumann (1954) has shown that for complete expression of the elasticity in different directions of a triclinic crystal, the general case, 21 elastic constants are required. This number reduces in stages, some of the constants becoming equal, as the amount of crystal symmetry increases.

This complexity provides an interesting mathematical situation, and many mathematicians have produced equations, describing the elasticity, which are often very complicated but do not help to explain practical results. There is, however, in any practical, and particularly any industrial situation, a need for a measurement which may not be very precise but which will nonetheless be useful.

With this in mind a commercial micro-tensile testing machine for testing metal whisker crystals, described originally by Marsh (1961) was modified to make

compressive tests on crystals of various materials. The results are subject to inaccuracy due to crystal defects, misorientation, slip and cracking under load, but the averaged values so obtained do enable some conclusions to be reached.

## EXPERIMENTAL

The aspirin used was B.P. grade from Laporte Industries Limited. All the other materials used were B.P. grade chemicals from B.D.H. Limited, except sucrose, which was Analar grade. They were not treated in any way beyond sieving, and orienting on the testing machines as described below.

The microhardness tester (Leitz Ltd., London) used can be considered as a microscope fitted with a movable carrier which enables a pyramidal-ended diamond to be pressed into a specimen. The size of the resultant impression is then accurately measured under high magnification. Various weights between 5 and 2000 g can be placed on the loading disc on the top of the diamond holder. The diamond for Vickers hardness tests has a square pyramidal point of 136° angle which is slowly brought into contact with the specimen at a rate controlled by a hydraulic damper. It produces an indentation which appears square in shape, with diagonals, when viewed from above. Illumination is by normal or polarized light shining onto the specimen surface through the objective itself. By measuring the lengths of the diagonals using the measuring graticule, the Vickers hardness of the specimen can be found.

Crystals were mounted by pressing lightly with another slide into heat-softened picene wax on a mounting slide, thus ensuring that the upper surfaces of the crystals were horizontal. The mounting slide and the crystals were then placed on the testing table of the hardness tester ready for indentation.

It was not possible to determine the orientation in many of the samples and a random positioning of the indentations was carried out whilst avoiding obvious irregularities of the surface.

The deformation of crystals under load was determined using a microtensile testing machine (Techne Ltd., Duxford, Cambridge). Normally the specimen is glued between two silica anvils, and a tensile force applied to it by means of a torsion wire capable of providing a load of up to 400 g. As the specimen extends, one anvil moves. It is connected to an inclined-mirror system, through which two images of a slit are projected. A micrometer allows the anvil movement to be cancelled out, as indicated by superpositioning of the two slit images. The micrometer, driving through a 100:1 reduction arm, combined with the sensitivity of the null-point mirror detecting system, is claimed to be capable of measuring length changes down to 50 Å.

The anvils, the use of tensile force, and the specimen mounting system were not suitable for three reasons: (*a*) the adhesive may affect the surface and hence the properties of the crystal; (*b*) it may form a collar and support the crystal; and (*c*) the machine measures tensile properties, whereas compressive behaviour is more relevant in tabletting work.

Reversing the direction of torsion wire twist, replacing both chuck assemblies by flat-faced compression anvils and adding a spring to maintain contact between the carriage and the coarse strain micrometer enabled the machine to be used with specimens in compression instead of in tension.

For compressive tests, crystals devoid of cracks and with flat, uniform and parallel faces were selected from a batch without subjecting them to extra stress. A manipulating device was used consisting of a hypodermic needle with its point ground flat attached by flexible plastic tube to a vacuum pump. The crystal was held onto the needle by pump suction, the vacuum pressure being controlled by covering or uncovering a small hole in the tube.

A low-power projection microscope arrangement was constructed for crystal measurement, the area over which the load was applied in the subsequent compressing experiments, as well as the length of the specimen, being found from the projection diagrams obtained.

After area measurements the crystal, still held on the hypodermic needle, was placed between the compression anvils, and a load just sufficient to deflect the mirror (0·01 g) was applied. The crystal was thus supported by the friction between it and the anvils and the holding device could be removed. The load was increased at a uniform rate in twelve increments and after each load application the strain micrometers were adjusted to return the optical detection system to the null position. The specimen was observed through a microscope during the test to detect cracks or slipping.

## RESULTS AND DISCUSSION

### *Surface microhardness*

The results of many determinations are given in Table 1. The limits of error are usually larger for the softer materials since in general the definition and quality of the indentation decline as the load which can be applied diminishes: to obtain a precise indentation under reasonable loading a hard material is needed.

Table. 1. *The Vickers hardness of crystalline materials*

| | Indentation | | | | | | |
|---|---|---|---|---|---|---|---|
| | Length ($\mu$m) | Depth ($\mu$m) | Load (g) | s.d. | No. of indentations | Hardness value (kg/mm$^2$) | Limits of error ($P$=0·05) |
| Steel .. .. | 18·0 | 2·6 | 100 | 0·23 | 25 | 565 | 0·01 |
| Sodium chloride | 49·2 | 7·0 | 25 | 1·30 | 60 | 21·2 | 0·7 |
| Aspirin .. | 46·4 | 6·6 | 10 | 3·13 | 325 | 8·7 | 0·9 |
| Sucrose.. .. | 27·0 | 3·9 | 25 | 1·63 | 30 | 63·6 | 4·8 |
| Hexamine .. | 37·3 | 5·3 | 10 | 1·32 | 25 | 13·3 | 0·9 |
| Urea .. .. | 45·1 | 6·4 | 10 | 1·43 | 25 | 9·1 | 0·7 |
| Salicylamide .. | 35·1 | 5·1 | 10 | 1·41 | 25 | 15·1 | 1·1 |
| Potassium chloride .. | 51·8 | 7·4 | 10 | 1·52 | 25 | 17·7 | 0·9 |

Previous work on the microhardness of non-metals is sparse except in two fields. In crystallography large pure or doped crystals, especially sodium chloride and other electrovalent compounds have been prepared and tested by Matkin & Caffyn (1963) and other workers. In geology the microhardness of single crystals of minerals has been studied with a view to their identification by e.g. Mitsche & Onitsch (1948), who also investigated factors which could influence mineral crystal microhardness values.

Of the materials tested, aspirin was by far the most difficult, conchoidal cracking and flaking obscuring most of the indentations. Sucrose crystals also showed

this effect, though clearer indentations were obtainable than with aspirin. Although urea and salicylamide did not show the cracking, definition was not ideal. Hexamine and potassium chloride produced clear indentations, provided a flat natural surface could be found.

For sodium chloride, the measurements were made at several loadings, since it had previously been suggested (Meyer, 1909) that hardness could be a function of applied load. Sodium chloride is the only material tested here for which literature results are available (Hofer, 1962).

This author found that with a non-symmetrical indentor there appeared to be a variation of hardness with orientation for sodium chloride, although he was unable to measure any difference, even on different faces of the crystal, using a symmetrical indentor. Our hardness results for sodium chloride agree well with Hofer's, and our value of n, the Meyer exponent, of 1·9 may be compared with his value, 1·89, obtained for loadings below 60 g. A Meyer exponent of 2 means that hardness is independent of applied load: this is very nearly true for sodium chloride. The hardness value from the present work, 21·2, agrees with Hofer's value and also with that of Matkin & Caffyn (1963) who measured the change in hardness of sodium chloride when calcium chloride was incorporated into the lattice by fusion and re-crystallization.

For the other materials it was not possible to use a series of different loads, the load used being restricted to the value producing the clearest and most convenient size of indentation relative to the size of the specimen. In these cases the indentor load and indentation depth must be stipulated for each hardness value quoted.

*Modulus of elasticity*

The determination of Young's modulus of elasticity of the materials proved difficult. Crystal inhomogeneities such as cracks or piled-up dislocations played a large part in determining the results and it was not always possible to see these defects during the selection of crystals for testing. Where cracking occurred during the tests it usually revealed itself by a sudden increase in strain without increased stress. However, four crystals of each of the substances sodium chloride, sucrose, hexamine and aspirin and one crystal of salicylamide, all free from cracks or outgrowths, were tested using the modified microtensile tester. The results were corrected as far as possible for machine deformation or specimen slip and the stress-strain curves were plotted (Fig. 1). Each graph shows an initial curved portion, due to the breaking of surface asperities and possibly a slight shifting in position of the crystal between the anvils as compressive force is applied. This initial portion is followed by a straight line, for which Hooke's law applies, the slope being the modulus of elasticity.

Further tests suggested that testing many more samples would yield little more information since the same scatter of results was produced however many tests were carried out.

Sodium chloride crystals behaved well in the machine, little specimen slip occurring. Susceptibility to deformation depends on the crystal structure; materials like sodium chloride, having a face centred cubic lattice, are easily deformed, whilst body centred crystals tend to work harden. Theile (1932) obtained a value of $1{\cdot}8 \times 10^5$ g/mm$^2$ for the modulus of elasticity of sodium chloride at 20° which compares well under these conditions with the present value of $1{\cdot}9 \times 10^5$ g/mm$^2$. No literature values were available for the other substances studied.

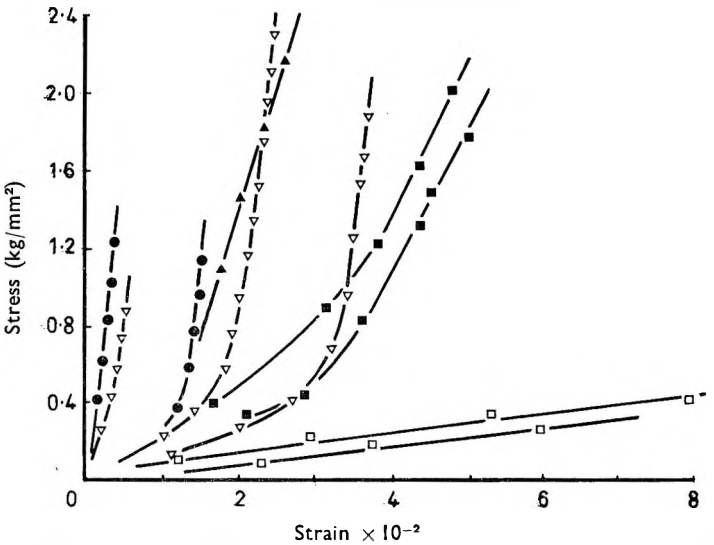


FIG. 1. Some typical stress-strain curves for crystals of five substances: ● Sodium chloride. ▽ Sucrose. ■ Hexamine. ▲ Salicylamide. □ Aspirin. The slope of the straight part of each curve is the elastic modulus. The values are in g/mm²: sodium chloride, $1{\cdot}9 \times 10^5$; sucrose, $2{\cdot}2 \times 10^5$; salicylamide, $1{\cdot}3 \times 10^5$; hexamine, $0{\cdot}9 \times 10^5$; aspirin, $0{\cdot}09 \times 10^5$.

The elastic moduli of urea and potassium chloride were not measured, because satisfactorily-shaped crystals could not be found in the available crystal batches.

Sucrose showed a similar value for the modulus of elasticity to that of sodium chloride. Initially more deformation occurred either due to the breakdown of asperities on the crystal faces to which the load was applied, or to slip in the crushing anvils. Hexamine crystals and the single salicylamide crystal tested showed still greater slip. Aspirin crystals, however, seemed to show cracking throughout the crystal at very low loads, the crystal rapidly showing complete breakdown. The form of the cracks was completely different from that in the other materials: one or two straight cracks occurred parallel to the force axis between the anvils and this different breakdown effect in aspirin produced a very low modulus of elasticity.

A comparison of the surface hardness and modulus of elasticity results shows that the materials lie in the same order whichever property is considered. The hardest material has the highest modulus of elasticity and the softest, aspirin, the lowest. The surface hardness test probably reflects the properties of the bulk material.

This conclusion is supported by the suggestion of Matkin & Caffyn (1963) that surface hardness is a measure of the rate at which dislocations dissipate energy when moving through the crystal lattice. Thus both the modulus of elasticity and the surface microhardness depend on the way the crystal lattice is made up and should reflect the bulk mechanical behaviour of the crystal.

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# The effect of crystal hardness on radial pressure at the wall of a tabletting die

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The force transmitted to the die wall during the tabletting of seven crystalline pharmaceutical materials has been measured using a photoelastic technique. The Vickers hardness of these materials has also been measured. The compounds examined were aspirin, urea, hexamine, salicylamide, potassium chloride, sodium chloride and sucrose. The pressure on the die wall increases as the hardness value decreases. The Vickers hardness of the resultant tablets was also measured; aspirin and sodium chloride both showed the occurrence of work hardening in that the tablets had a greater hardness value than the parent crystalline substance.

The radial force exerted on the die-wall by a powder under compression in a tabletting die is of great interest in any study of the tabletting process. The larger the radial pressure, the greater will be the amount of friction and shear at the die-wall, and the greater the ejection force needed to remove the tablet from the die. It would be expected that the radial pressure would be greater for softer substances, since they approximate more closely to the idealized case of a liquid, which has zero hardness. Here the pressure would be hydrostatic, so that the radial pressure would be equal to the pressure exerted at the punch face. The purpose of the work reported here was to examine the relation between radial pressure and Vickers hardness by making tablets from a number of substances whose hardness was also measured, the compaction being carried out in a die of a type allowing the radial pressure to be ascertained.

The method of Ridgway (1966) was used. It involves the application of photoelastic stress analysis using the stress patterns observed by means of polarized light in the wall of a Perspex die during compression, as a method of measuring radial pressure.

This method is easy and convenient, and has the advantage that if the radial force should vary over the thickness of a tablet, its value can be found at any point. The other methods described above are only capable of giving the mean pressure over the entire tablet thickness.

Radial pressures have been measured during the application and relaxation of the punch pressure, notably by Leigh, Carless & Burt (1967), who distinguish the behaviour of an ideal elastic material, a constant yield stress material, and a Mohr body, for which the yield stress in shear is a function of the applied normal stress. They were unable to do more, however, than obtain the pressure cycle plots, since they had no data on the mechanical properties of the materials being compressed.

Jaffe & Foss (1959) tabletted a large range of substances and endeavoured to find a factor common to all those which tabletted well, but without success. Varsano & Lachman (1966) measured the compressibilities of beds of potassium chloride and potassium citrate 80 mesh crystals, and also granulations of them using various

binders, but did not relate their results to tablet formation or properties. Higuchi, Shimamoto & others (1965) determined both die wall pressure and its rate of decay when the punch pressure was relaxed, but had no mechanical property data for the substances being compressed. They were able to state, but only qualitatively, that softer substances seemed to give higher die wall pressures.

## EXPERIMENTAL

Seven crystalline substances were chosen for examination, basing the choice upon pharmaceutical interest and upon relevance to other tabletting work carried out in the Department. The substances examined were aspirin, urea, hexamine, salicylamide, potassium chloride, sodium chloride and sucrose. The sources of supply of the materials were as reported in the previous paper (Ridgway, Shotton and Glasby, 1969).

The surface microhardnesses of the crystals were determined with a microhardness tester (Leitz Ltd., London) in the same manner as reported previously (Ridgway, Shotton & Glasby, 1969). The hardness of the tablets after compression was also determined using the same instrument. The surface of the tablet, which was made using flat-faced punches, was rubbed lightly over graphite powder dusted onto a sheet of paper. This gave the surface a slightly polished appearance, and greatly enhanced the contrast and therefore the ease and accuracy of measurement of the diagonal length of the impressions made by the diamond indentor.

The apparatus for the compression of the tablets and the measurement of die-wall stress was that described by Ridgway (1966). Light passes through a polarizing filter and a quarter-wave plate to give circularly polarized light. This illuminates a Perspex die, the punches for which are compressed by a 100 ton Tangye hydraulic press. Viewing the die through a second quarter-wave plate and an analysing

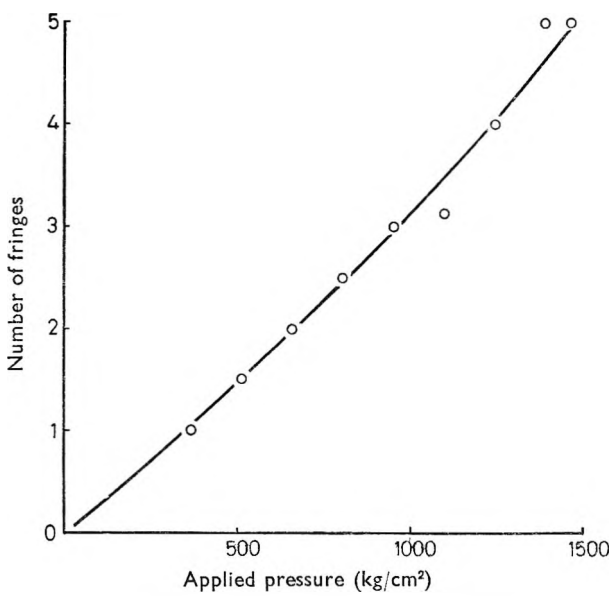


FIG. 1. The calibration curve for the die: fringe order immediately inside the die wall is almost a linear function of applied pressure for an isostatic material under compression.

polaroid filter gives a fringe system within the die which is related to the stress transmitted to the die wall by the compressed tablet. The fringe patterns were photographed at various applied pressures during the compression and relaxation cycles, and the pressed tablets removed for examination. Tablet weights of 1 g were used for all the substances, the die diameter being 12·5 mm. No lubricants were used.

The die was calibrated by compressing a rubber plug as suggested by Windheuser, Misra & others (1963). Rubber under compression gives approximately a hydrostatic pressure so that the die-wall stress is equal to the punch pressure applied. This calibration agreed with that obtained earlier by Ridgway (1966) using silicone putty enclosed in a small chamois leather bag. The fringe order at the die-wall could thus be plotted against applied pressure (Fig. 1).

## RESULTS AND DISCUSSION

### *Surface hardness and transmission ratio*

Fig. 2 shows that the force transmitted to the die wall is directly proportional to the compacting pressure exerted on the powder. This is so for all seven substances tested; the constant of proportionality depends on the material being compacted. Because we are interested in the slope of the lines, these graphs are plotted so that the all pass through the origin. The lower the surface hardness of the crystals being compacted, the larger is the force transmitted to the die wall at a given compacting pressure. The relation between the surface hardness of the crystals and the force

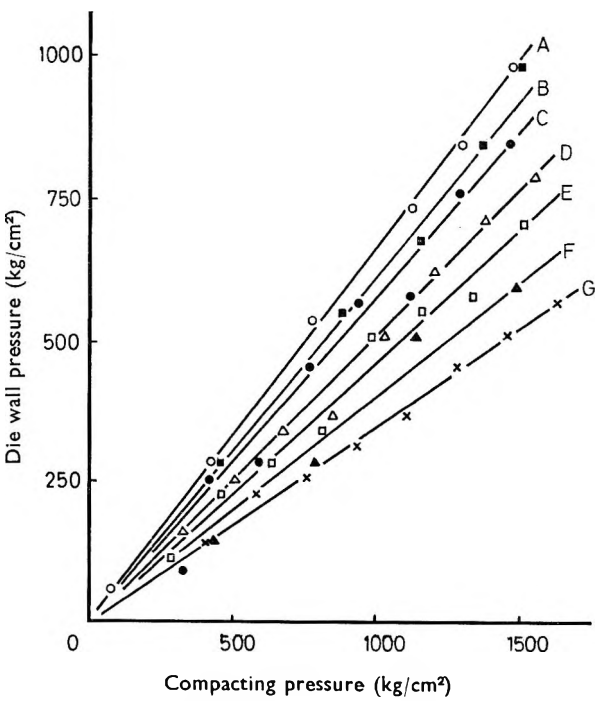


FIG. 2. Radial pressure on the die wall as a function of the pressure applied at the punch face. The lines are plotted so that they pass through the origin. The substances used are as follows: A, Aspirin ○. B, Urea ■. C, Hexamine ●. D, Salicylamide △. E. Potassium chloride □. F, Sodium chloride ▲. G, Sucrose ×.

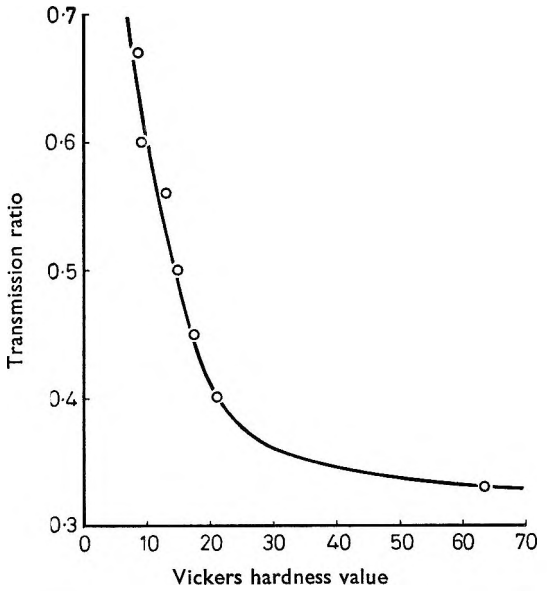


FIG. 3. Transmission ratio, defined as (radial pressure on the die wall) ÷ (axial stress applied by the punches), plotted as a function of the Vickers hardness value of the material being compressed.

they transmit to the die wall at a given compacting pressure, is shown in Fig. 3. The shape of the curve is not unexpected. For softer materials, the transmission ratio (= die wall pressure ÷ applied pressure) is high, and is of course unity at a hardness value of zero. It levels out at about 0·3 for harder materials. This fits in with Nelson's (1955) observation that about 30% of the applied force was transmitted to the die wall by sodium bicarbonate granulation and by sulphathiazole granulations. Nelson also found that lubricants added to the granules increased the transmission ratio, and an important point was made by Windheuser & others (1963) who found that when there was good transmission of forces to the die wall, good tablets were produced.

The results here are in agreement with those of Higuchi & others (1965) who found that the ratio of die wall pressure to upper punch pressure seemed to reflect the hardness of the crystals being compacted, the crystal hardness and the transmission ratio being inversely related to one another. But since these authors did not know the value of the hardness of the crystals used, no quantitative information was derived.

*Hardness of tablet in relation to compacting pressure*

The surface hardnesses of tablets of the substances tested varied with the compacting pressure used to produce them (Fig. 4). With potassium chloride, hexamine and urea the surface hardness of the tablets showed a slight decrease with increase in compacting pressure. This may be due to incipient failure of the material being compacted, which may occur as plastic deformation or fracture (Train, 1956).

Aspirin and sodium chloride tablets, however, show a tendency to increase in surface hardness as the compacting pressure increases. The increase stops at a certain stage during the compression of sodium chloride crystals and after this point the surface hardness of the tablets remains almost static. An explanation for the behaviour of these materials may be that on compaction, work hardening occurs.

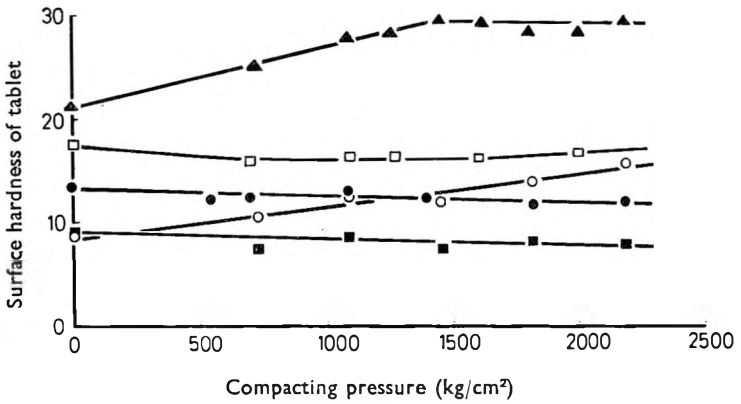


FIG. 4. The surface hardness of the tablets produced at various compacting pressures. Key: ▲ Sodium chloride. ○ Aspirin. □ Potassium chloride. ■ Urea. ● Hexamine.

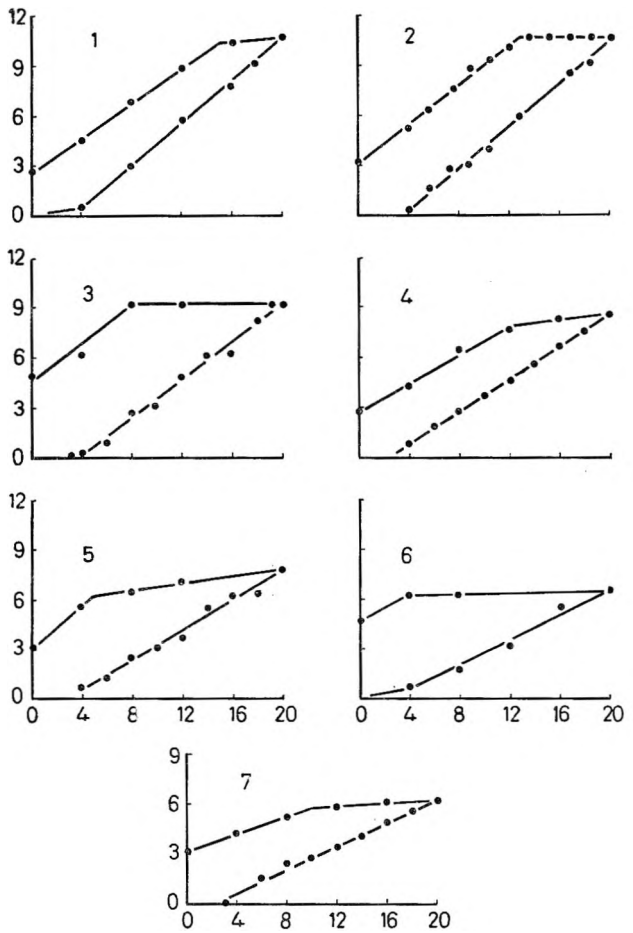


FIG. 5. Variation of radial pressure exerted on the die wall as a function of the punch pressure during the application and relaxation of the compressive force. The units on both axes are thousands of lb/inch²; radial pressure is ordinate and axial pressure the abscissa. The substances used are as follows: 1. Aspirin. 2. Urea. 3. Hexamine. 4. Salicylamide. 5. Potassium chloride. 6. Sodium chloride. 7. Sucrose.

*Compression cycles*

In Fig. 5 the "compression cycles" of the materials are plotted, following Long (1960). The scatter of the points is sufficiently large to prevent any conclusions being drawn with certainty, but the cycles have the expected shapes. Those for sodium chloride, aspirin and sucrose agree with the cycles obtained by Leigh & others (1967). In the present work, the cycles tend to be wider and the residual die-wall pressures higher. This may be due to the radial strain being greater in a Perspex die than it is in a steel die. Lateral movement of the material being compacted would allow plastic flow and a greater "permanent set" to occur. The larger lateral strain can be detected on the photographs of the fringe pattern, and work is in hand to produce a glass die which will be photoelastic but, within its limits, as rigid as a steel die.

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# The effect of particle shape on powder properties

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Sand in the size ranges 18–20 mesh, 30–36 mesh and 44–60 mesh has been separated by means of a vibratory shape-sorting table into fractions of different shape. The ratios of the surface area of the particles to their volumes—the shape coefficients—ranged from 8 to 15. For each fraction the following properties were measured: angle of repose, bulk density and rate of flow through an orifice. In all cases, with increasing departure from the spherical, the angle of repose increased, whilst the bulk density and flowability decreased. Improved methods are reported for the measurement of bulk density, angle of repose and specific surface area in the range 30–130 $cm^2/g$.

It is generally accepted that the shape of the particles of a powder or a granular solid has an effect upon such bulk properties as angle of repose, bulk density and rate of flow through an orifice, but there is little published experimental work to support this intuitively acceptable statement. This arises from the difficulty of obtaining samples of a particular material which have the same particle size but different particle shapes and because of the labour involved in measuring the shape coefficients of the samples once they have been obtained. The shape coefficient used in the present work is the ratio of the surface area of a particle to its volume (see also p. 34 S).

The effect of particle shape on bulk properties, particularly orifice flow, has been given some attention by Fowler & Glastonbury (1959), Fowler & Chodziesner (1959) and by Pilpel (1965). Variation of particle shape was achieved only by using materials as different as wheat, rice, sugar and sand. The shape range covered was relatively narrow, and the density and surface structure were extremely diverse. The angle of repose was said to depend upon particle shape, particle density and the roughness of the supporting surface.

Working with lactose and starch granules, Fonner, Banker & Swarbrick (1966) obtained a shape variation by using different granulation methods. These authors found no dependence of angle of repose upon particle shape, but again, the particles differed not only in density, due to varying amounts of entrapped air, but also in surface structure.

In the present work, closely-sized batches of sand have been separated into fractions of different shape by using a vibratory shape-sorting table of a type originally developed for the shape classification of grinding grit in the diamond industry. The surface shape coefficient and volume shape coefficient as defined on p. 4 have been determined for the various fractions; the ratio of these two parameters, the shape coefficient, is the accepted criterion for the degree of departure of particle shape from the spherical. Angles of repose have been determined by two methods in which the base of the heap is formed by a static layer of the powder itself and the

angle is measured by weighing; bulk density, orifice flow rate and specific surface have also been measured. The aim of the work is to show that for any particlar material, these properties are a single-valued function of shape, so that a measurement of any of them will serve to characterise the particle shape of a new batch. Also, the rate of flow on a chute and the rate of mixing under the same conditions are being examined using the inclined plane apparatus described last year (Ridgway & Rupp, 1968) to see whether the effective diffusion coefficient between two flowing layers is also a function of particle shape.

## EXPERIMENTAL

The material used was sand from King's Lynn, Norfolk and Leighton Buzzard, Herts. (supplied by British Industrial Sand Ltd., Reigate, and George Garside (Sand) Ltd., Leighton Buzzard). The sand was washed with water and dried in an air oven before being sieved into the required narrow size ranges. Particles with shape coefficients greater than 12 were scarce in these samples and to obtain more angular fractions some sand was mechanically crushed: this is a standard procedure for angular foundry sand manufacture. The required shape factor was obtained by the creation of flats and sharp angles rather than by the formation of particles whose principal axes were of different lengths, but no properties other than shape were affected by the crushing process.

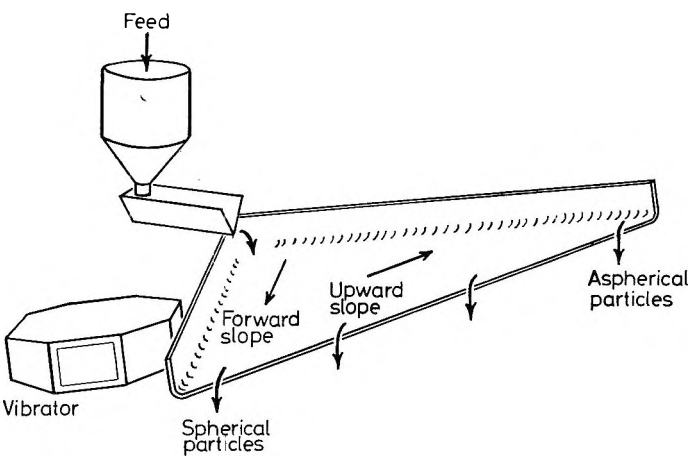


FIG. 1. The shape sorting table.

The shape-sorting table is shown diagrammatically in Fig. 1. It consists of a triangular metal deck carried on a tubular metal framework fitted with two screw-jacks so that the slope of the deck can be adjusted in two independent directions. The powder is fed onto the deck at one corner and moves across to the opposite side, fanning out as it goes. The vibration tends to make all particles ride up the slope across which they are travelling. The extent to which they ride up is governed by their area of contact with the deck and their inability to roll. The result is that near-spherical particles leave the deck at the lowest point of the exit edge, whilst block or flaky material leaves at the highest point of the same edge. The different

shape fractions are collected along this edge of the deck. Experimentation is necessary to find the best combination of table feed angles, table slope and vibration amplitude for optimum separation. The apparatus was originally developed for the sorting of industrial diamonds and is manufactured by Jeffrey-Galion Ltd., Johannesburg.

*Bulk density*

A 100 ml beaker with a known total capacity was filled under constant conditions. To do this, a funnel with a spout diameter of 0·8 cm was held centrally 2 cm above the top of the beaker. Sand was poured into the funnel so that the outlet ran full, and a 20% excess of sand was poured over and above the amount required to fill the beaker. Taking care not to shake the beaker, the sand was struck off level with a knife edge. Beaker and contents were then weighed, and the loose bulk density calculated. The reproducibility of this method was $\pm$ 0·15%, probably because no volumetric measurements of sand in a graduated cylinder is involved.

*Angle of repose*

Two methods of measuring the angle of repose were used; in one a heap of material was formed, and in the other a drained crater. In both methods, however, the actual angle was not measured except as a check: the apparatus was so arranged that the weight of powder standing on a defined base could be obtained. Using the known value of the bulk density, the volume of material could be found, and hence the cone angle required to give a heap having this volume.

Method (a): convex angle of repose. The apparatus is shown in Fig. 2A. The bottle, containing about 20% excess sand over that needed to make the heap, is inverted onto the upper plate. Sand flows through the orifice onto the lower plate, where it forms a heap. The heap is weighed and the angle of repose calculated.

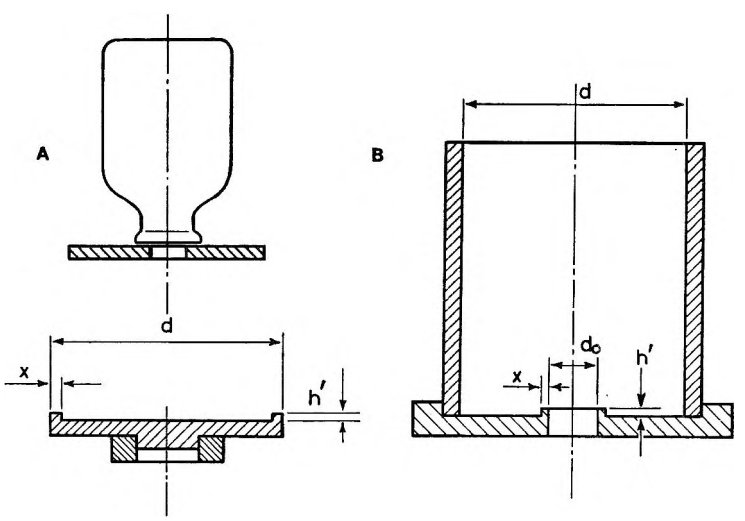


FIG. 2A. The apparatus for measuring the cone angle of repose.

FIG. 2B. The apparatus for measuring the crater angle of repose.

The volume, V, of a conical heap, height h and diameter d, is $\pi/12\ d^2h$. For a bulk density $\rho B$ and a heap weight w,

$$V = \frac{w}{\rho_B} \qquad \text{so that } h = \frac{12}{\pi} \cdot \frac{w}{\rho_B\, d^2}$$

and the angle of repose, $\alpha$, is given by

$$\tan \alpha = \frac{2h}{d} = \frac{24}{\pi} \cdot \frac{w}{\rho_B\, d^3}$$

Successive determinations of $\alpha$ did not disagree by more than 0·1°, provided a plate having a ridge round the edge was used in order to make a defined base for the heap. Under such conditions the formula must be corrected to allow for the base layer of the heap. For the apparatus used here, the weight of the lower layer is given by

$$w' = \frac{\pi}{4}(d - 2x)^2\, h'\, \rho_B = 11{\cdot}55\, \rho_B$$

where x is the width and h′ the height of the ridge at the edge of the plate. Thus,

$$\tan \alpha = \frac{24}{\pi} \cdot \frac{w - 11{\cdot}55\, \rho_B}{\rho_B\, d^3}$$

Method (b): concave or crater angle of repose. This apparatus is shown in Fig. 2B. In use, it is filled with sand in the same manner as in the determination of bulk density. The bottom closure is then removed and the crater allowed to form. The weight of sand remaining in the container is then measured.

For this case,

$$V = \frac{w}{\rho_B} = \frac{\pi}{12} h(2d^2 - d_o^2 - d\, d_o)$$

$$\tan \alpha = \frac{2h}{d - d_o}$$

$$= \frac{24}{\pi} \cdot \frac{w}{(2d^2 - d_o^2 - dd_o)(d - d_o)\, \rho_B}$$

$$= \frac{24}{\pi} \cdot \frac{w}{(2d^3 - 3d^2\, d_o + d^3_o)\, \rho_B}$$

Some experiments were done to determine the effect of the size of the hole, $d_o$, and the height h′ of the ridge round the edge of it. For values of $d_o$ less than eight particle diameters (0·95 cm), the angle of repose obtained is higher than with other methods. The height h′ had no effect over the range 0·075 to 0·32 cm.

Correction of the above formula due to the base layer of powder leads to the replacement of w by w − w′ where

$$w' = \frac{\pi}{4} h' (d^2 - d_o^2 - 4\, d_o x - 4x^2)\, \rho_B$$

*Flowrate from an orifice*

A vertical glass tube, 50 cm long and 2·5 cm internal diameter, had a flange coupling at the lower end to which could be attached one of a range of orifice plates. These

were made of thick brass sheet with the holes drilled and reamed to an accurate size and circularity. 200 g of the sand was poured carefully into the tube, and the orifice opened. Avoiding any measurement in the first or the last few centimetres of travel, the rate of flow was determined by timing with a stopwatch and weighing the quantity emerging.

*The determination of the shape coefficient*

For an arbitrarily-shaped particle with projected mean diameter $d_a$, two parameters are normally quoted, namely the surface and volume shape coefficients. Following the nomenclature of Heywood (1969), the definitions of these are that the surface area of the particle is

$$S = \alpha_{s,a} \cdot d_a^2$$

and its volume is

$$V = \alpha_{v,a} \cdot d_a^3$$

For a sphere, $\alpha_{s,a} = \pi$ and $\alpha_{v,a} = \pi/6$

The shape coefficient (without qualifying adjective) is the ratio of the above two quantities, i.e.

$$\alpha_{sv,a} = \alpha_{s,a}/\alpha_{v,a}$$

which has a numerical value of 6 for a sphere and increases as the particle shape departs from the spherical.

To determine the shape coefficient for a batch of particles, it is necessary to measure the average values of the particle volume, surface area and projected diameter.

Particle volume was determined by direct counting of a fairly large sample (between 300 and 800 particles, depending on mesh size). The counted sample was weighed and the average particle volume obtained by dividing by the true density of the sand, 2·65 g/cm$^3$.

The projected diameter $d_a$ is the diameter of a circle having the same area as the projection of a particle resting in its most stable position. The mean projected diameter was measured by photographing a sample of particles under a low powered microscope. Prints were made on which individual particles had a diameter of at least 1·5 cm. About 100 particle pictures were cut out from the photograph and weighed: this gave the projected area of the 100 particles, and hence the mean projected diameter.

The surface area of the sand was more difficult to measure. In the range 30–130 cm$^2$/g air permeability is the only method. At measurable pressure drops across beds of sand however, the volume flowrate of air is large. Accordingly, a modified Lea and Nurse apparatus was constructed which had a much greater bed depth than normal. The sand was contained in a glass tube, 2·5 cm in diameter thus giving a bed depth of from 24 to 30 cm using a sample weight of 200 g. The bed depth was determined by inserting a flat-ended cylindrical rod. The length of the capillary resistance was reduced from 200 to 5 cm so that flowrates of up to 600 cm$^3$/min could be measured. The beds were packed by means of a vibrating tool and, for any one sample, measurements were made for at least two porosities and two flowrates. Fourfold variation of the flowrate caused no consistent trend in the surface area results obtained, and overall reproducibility was usually within 1%, with occasional readings at $\pm$ 2%.

## RESULTS AND DISCUSSION

The properties of the various sand fractions which were prepared are given in Table 1, where the bulk density is also listed. Fig. 3 shows the trend of bulk density with shape coefficient. For all size ranges, the bulk density falls as the shape of the particles becomes less regular.

Table 1. *Shape coefficient, specific surface area and bulk density of the material used*

| Sample and mesh size | Sand* | Projected diameter (cm) | Volume coefficient $\alpha_{v,a}$ | Surface coefficient $\alpha_{s,a}$ | Shape coefficient $\alpha_{sv,a}$ | Specific surface area ($cm^2/g$) | Bulk density ($g/cm^3$) |
|---|---|---|---|---|---|---|---|
| 44/60–1 | n | 0·0343 | 0·345 | 3·03 | 8·78 | 97·2 | 1·443 |
| –2 | n | 0·0357 | 0·256 | 2·68 | 10·47 | 110·4 | 1·375 |
| –3 | c | 0·0386 | 0·270 | 2·91 | 10·77 | 105·5 | 1·341 |
| –4 | n | 0·0358 | 0·271 | 2·99 | 11·03 | 116·3 | 1·348 |
| –5 | c | 0·0423 | 0·209 | 2·57 | 12·30 | 109·5 | 1·294 |
| –6 | n | 0·0378 | 0·198 | 2·58 | 13·03 | 130·5 | 1·270 |
| –7 | c | 0·0451 | 0·179 | 2·47 | 13·80 | 115·3 | 1·235 |
| 30/36–1 | n | 0·0559 | 0·428 | 3·28 | 7·66 | 51·5 | 1·528 |
| –2 | n | 0·0609 | 0·375 | 2·89 | 8·70 | 54·1 | 1·488 |
| –3 | n | 0·0661 | 0·302 | 2·98 | 9·81 | 56·4 | 1·442 |
| –4 | n | 0·0694 | 0·246 | 2·84 | 11·54 | 62·8 | 1·386 |
| –5 | c | 0·0755 | 0·208 | 3·07 | 14·76 | 73·8 | 1·231 |
| 18/20–1 | n | 0·0909 | 0·433 | 3·54 | 8·17 | 34·0 | 1·552 |
| –2 | n | 0·0967 | 0·368 | 3·39 | 9·21 | 36·0 | 1·504 |
| –3 | n | 0·1024 | 0·318 | 3·14 | 9·87 | 36·5 | 1·486 |
| –4 | n | 0·1051 | 0·279 | 3·08 | 11·04 | 39·5 | 1·436 |
| –5 | c | 0·1128 | 0·187 | 2·82 | 15·08 | 50·6 | 1·286 |

* n = natural
c = crushed

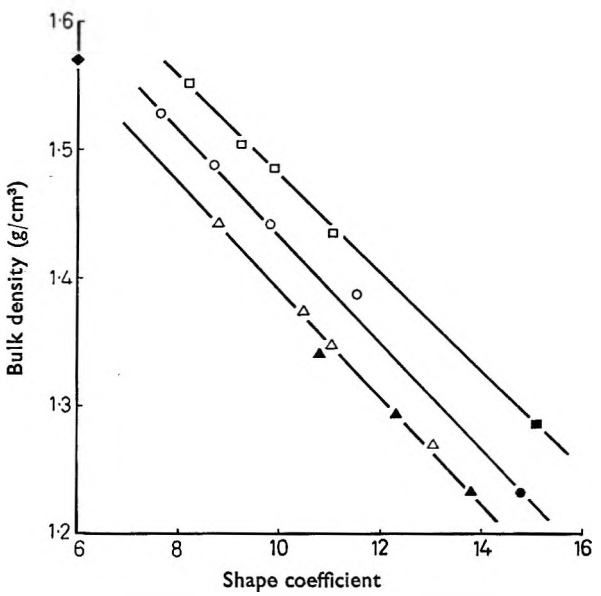


FIG. 3. Bulk density as a function of shape factor for three different sizes of sand. The following point convention applies to all graphs: open points: □ mean projected diameter 805 μm, ○ mean projected diameter 461 μm, △ mean projected diameter 302 μm; closed points, ■, ● and ▲ refer to crushed sand of the same mean diameters.

The point on the bulk density axis at 1·57 g/cm³ is the experimentally determined density for spheres of true density 2·65 g/cm³ formed into a loose-packed random array. All three lines should converge at this point, since for spherical particles, bulk density is not a function of absolute size, provided the container is large. In practice this is not the case, and the larger particles give the larger bulk densities, presumably because the impact energy as they are deposited into the container is greater. Macrae & Gray (1961) have shown that increased energy of deposition gives higher bulk density in sphere packings. Although quite a good linear relation applies between bulk density and shape factor, the linearity cannot extend much beyond the range covered by the graph. The bulk density cannot be expected to decline toward zero at a linear rate as the particles become more flaky: the bulk density extrapolates to zero at a shape factor of 45 on the graph shown.

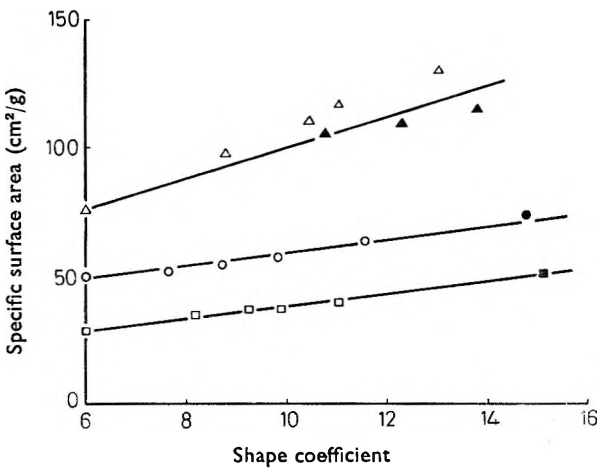


FIG. 4. Specific surface area as a function of shape factor.

Fig. 4 shows the relation between specific surface area and shape factor at constant particle size. Here also the points on the vertical axis are the calculated surface areas for spherical particles of the mean size of each of the sand fractions used. These values are listed in Table 2. Both crushed and natural sand fractions lie close to the same line. That for crushed sand there are three points lying slightly below the line for the smallest particles, is due to the differing size distributions within the fractions.

Table 2. *The mean diameters of the sand fractions used, and the properties of the equivalent sphere*

| Mesh size | Size μm | Mean diameter μm | Wt. of one sphere, g | Particles per g | Surface area of one sphere, cm² | Specific surface cm²/g |
|---|---|---|---|---|---|---|
| 18–20 | 853–758 | 805 | $7{\cdot}2 \times 10^{-4}$ | $1{\cdot}4 \times 10^{3}$ | $2{\cdot}03 \times 10^{-2}$ | 28·2 |
| 30–36 | 500–422 | 461 | $1{\cdot}35 \times 10^{-4}$ | $7{\cdot}4 \times 10^{3}$ | $6{\cdot}7 \times 10^{-3}$ | 49·3 |
| 44–60 | 353–251 | 302 | $3{\cdot}8 \times 10^{-5}$ | $2{\cdot}6 \times 10^{4}$ | $2{\cdot}9 \times 10^{-3}$ | 75·5 |

The 44–60 mesh crushed sand was the extreme fines, sieved out from a coarse batch of which only about 1% was in fact below 44 mesh size. Thus within this

fraction, 60 mesh particles will be scarce. The 44–60 mesh natural sand was sieved from a batch ranging from 30 to 150 mesh, so that 44 and 60 mesh particles are present in more nearly equal proportions. The true mean size of the crushed sand fractions is probably larger than the mean sieve aperture.

As a check on the modified air permeability method, surface areas were measured on three samples of glass ballotini. These compare well with the calculated values (Table 3). Ideally it would have been preferable to measure the surface area for spheres having the diameters of Table 2 but these were not available.

Table 3. *Experimental and calculated surface areas for spherical particles using the modified air permeability apparatus*

| Mean particle size $\mu$m | Porosity | Specific surface area, cm²/g | |
|---|---|---|---|
| | | experimental | calculated |
| 350 | 0·336 | 56·0 | 56·7 |
| 350 | 0·321 | 57·8 | 56·7 |
| 275 | 0·336 | 67·8 | 73·9 |
| 275 | 0·325 | 67·3 | 73·9 |
| 195 | 0·334 | 97·2 | 104·2 |
| 195 | 0·309 | 97·0 | 104·2 |

Table 4. *Angle of repose and orifice flow in relation to particle size shape*

| Sample and mesh size | Shape coefficient | Angle of repose in degrees | | Orifice flow (g/s) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Orifice diameter (cm) | | | |
| | $\alpha_{sv,a}$ | Concave | Convex | 0·953 | 0·793 | 0·634 | 0·476 |
| 44/60–1 | 8·78 | 35·21 | 35·1 | 18·93 | 11·28 | 6·41 | 2·75 |
| –2 | 10·47 | 36·6 | 36·9 | 18·28 | 11·14 | 6·01 | 2·56 |
| –3 | 10·77 | 37·0 | 36·7 | 17·73 | 11·59 | 6·11 | 2·58 |
| –4 | 11·03 | 37·4 | 37·5 | 18·14 | 11·00 | 5·78 | 2·49 |
| –5 | 12·03 | 38·3 | 38·3 | 17·60 | 11·06 | 5·77 | 2·44 |
| –6 | 13·03 | 38·6 | 38·7 | 17·87 | 9·94 | 5·33 | 2·30 |
| –7 | 13·80 | 39·8 | 39·6 | 17·19 | 9·83 | 5·32 | 2·20 |
| 30/36–1 | 7·66 | 35·3 | 35·2 | 18·46 | 10·49 | 5·65 | 2·34 |
| –2 | 8·70 | 37·4 | 37·2 | 18·11 | 10·38 | 5·47 | 2·24 |
| –3 | 9·81 | 39·3 | 38·8 | 17·96 | 10·24 | 5·28 | 2·14 |
| –4 | 11·54 | 40·6 | 40·5 | 17·30 | 9·69 | 4·96 | 2·01 |
| –5 | 14·76 | 43·0 | 42·1 | 13·85 | 8·24 | 4·32 | 1·63 |
| 18/20–1 | 8·17 | 37·9 | 36·8 | 15·85 | 8·70 | 4·48 | — |
| –2 | 9·21 | 40·5 | 39·2 | 15·44 | 8·44 | 4·31 | — |
| –3 | 9·87 | 41·6 | 40·8 | 15·15 | 8·24 | 4·13 | — |
| –4 | 11·04 | 43·2 | 42·1 | 14·63 | 7·91 | 3·98 | — |
| –5 | 15·08 | 45·6 | 43·1 | 12·23 | 7·06 | 3·54 | — |

Table 4 lists the experimental results for angle of repose and for flowrate through a range of orifices. Fig. 5 shows the measured angles of repose as a function of the shape coefficient. The point on the vertical axis is the angle of repose as determined by the two methods for ballotini of each of two sizes. All four measured angles agreed to 0·15°, the mean being 27·4°. Thus for spheres and for the smaller sand particles, the two methods give results which do not differ, but the influence of shape in giving a difference between the two methods is very apparent for the larger particles. The crater method is the better to use as a test of particle shape, since the rate of change of angle with shape is greater, especially at the higher shape coefficients.

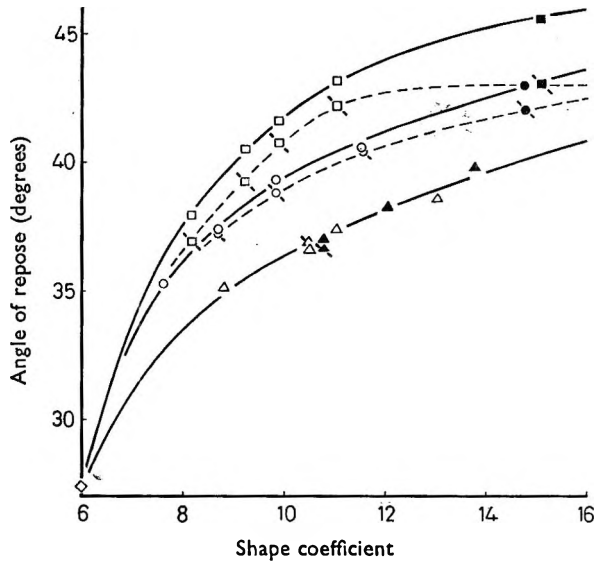


FIG. 5. Angle of repose as a function of shape coefficient for three sizes of sand. The point on the vertical axis is the angle of repose measured for glass ballotini by both methods. Points with a diagonal line, represent the convex heap method; normal points refer to the crater method.

In all cases (Fig. 6), an increase in shape factor leads to a decrease in flowrate through an orifice. This is in line with the angle of repose measurements in that the resistance to shear, or flow, of an assembly of irregular particles is greater than that of an assembly of spherical particles of the same size and density.

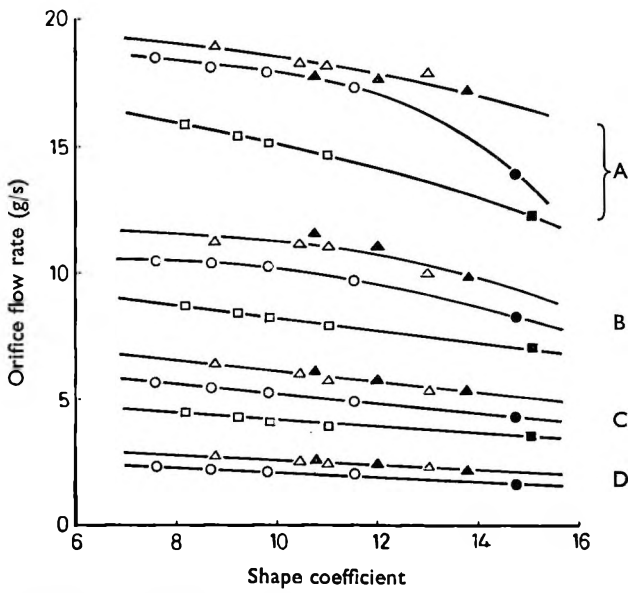


FIG. 6. Efflux rate from an orifice as a function of particle shape. Orifice diameter—A, 0·953 cm; B, 0·793 cm; C, 0·634 cm; D, 0·476 cm.

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# A quantitative chromatographic method for the determination of purity of oxytetracycline

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A chromatographic method for the determination of oxytetracycline is described, employing a hexane–ethyl acetate–pH 3·3 buffer partition system supported on Celite. Chromatographic separation is followed by measuring the absorbance of the eluate at 263 nm. The system enables the determination of oxytetracycline to be made in the presence of anhydro-oxytetracycline, chlortetracycline, α- and β-apo-oxytetracycline, and epi-oxytetracycline, the last three being the most likely contaminants of crude material.

The method currently employed for the assay of oxytetracycline (B.P. 1968) uses standard microbiological procedures and *Bacillus pumilus* as test organism. An accuracy of ± 2% is obtained only if multiple determinations are made on three successive occasions making the method expensive in its use of laboratory space and personnel. The method is also non-specific in that any impurities present which show activity against the test organism, will be estimated as oxytetracycline. Previous attempts have been made to assay oxytetracycline by methods based on the estimation of a specific functional group or physical properties of the oxytetracycline molecule, viz. by ultraviolet absorption spectroscopy, by measurement of the colour produced with ferric chloride (Monastero, Means & others, 1951), by means of fluorimetric analysis (Serembe, 1951) or polarography (Doshocil, 1954). All suffer serious interference from the structurally similar impurities likely to be present and the correlation between the results obtained by these procedures and those of the official microbiological assay, has been poor.

The physical properties of oxytetracycline and some of the likely contaminants are listed in Table 1. In view of their close similarity, preliminary separation of the oxytetracycline from other likely impurities is obviously essential in any analysis specific to this substance. Previous experience with partition chromatography on Celite suggested a possible solution to the problem.

## EXPERIMENTAL

### *Reagents*

*Prepared Celite.* Stir Celite 545 (500 g) intermittently (12 h) with concentrated hydrochloric acid (2 litres). Decant the hydrochloric acid and suspend the residue in water (1 litre). Filter through a Buchner funnel, wash the residue with water until free from acid, wash with methanol (500 ml) then methanol–ethyl acetate (1 : 1) (1 litre). Dry the residue in an oven at 100°. Store in well-stoppered jars.

*pH* 3·30 *Buffer.* Dissolve an accurately weighed quantity of Analar potassium hydroxide pellets (120 g) in water (600 ml). Dilute an accurately measured volume of Analar syrupy phosphoric acid (120 ml) to about 600 ml with water. Mix the two

solutions, allow to cool and adjust the volume to 2 litres with water. Adjust the pH of this solution accurately to 3·30 by the addition of more potassium hydroxide or syrupy phosphoric acid as required.

*Solvent system.* Shake together ethyl acetate Analar (675 ml), n-hexane (325 ml) and pH 3·3 buffer (100 ml) and allow to separate. The upper layer is the eluent phase, the lower layer the stationary phase.

Table 1. *Physical properties of oxytetracycline and some likely impurities*

| Compound | M.p. °C | Ultraviolet absorption Max nm | Log ε | Infrared absorption | Specific rotation $[\alpha]_D^{20}$ |
|---|---|---|---|---|---|
| Oxytetracycline anhydrous | 184–185 | 222 | 4·15 | KBr disc | −197° |
| | | 270 | 4·30 | 1675 $cm^{-1}$ | (0·01 HCl) |
| | | 364 | 4·16 | | |
| | | Acid | Ethanol | | |
| Anhydro-oxytetracycline .. | 180–190 | 271 | 4·56 | KBr disc | +52° |
| | | 425 | 3·80 | 1670 $cm^{-1}$ | (1 : 1 Methanol |
| | | Acid | Ethanol | | Dioxan) |
| α-Apo-oxytetracycline .. | 190–200 | 250 | 4·77 | KBr disc | −45° |
| | | 377 | 3·87 | 1720 $cm^{-1}$ | (D.M.F.) |
| | | Acid | Ethanol | | |
| β-Apo-oxytetracycline .. | 195–205 | 248 | 4·78 | KBr disc | −28° |
| | | 375 | 4·0 | 1740 $cm^{-1}$ | Ethanol |
| | | Acid | Ethanol | | |
| Chlortetracycline .. .. | 168–169 | 234 | 4·22 | KBr disc | −245° |
| | | 269 | 4·25 | 1670 $cm^{-1}$ | (0·03N HCl) |
| | | 376 | 4·10 | | |
| | | Acid | Ethanol | | |
| Epioxytetracycline .. .. | 163–164 | 215 | 4·09 | KBr disc | −253° |
| | | 253 | 4·15 | 1680 $cm^{-1}$ | (0·03N HCl) |
| | | 275 | 4·08 | | |
| | | 355 | 4·08 | | |
| | | 0·1N sulphuric acid | | | |

*Preparation of sample and standard solutions.* Dissolve about 50 mg accurately weighed in 0:1N hydrochloric acid (10 ml), adjust the pH to approximately 7 with 0·1N sodium hydroxide solution (approx. 11 ml) and immediately dilute to 50 ml in a volumetric flask with stationary phase. Prepare a standard solution of pure anhydrous oxytetracycline base in an identical manner.

*Method.* Mix prepared Celite (25 g) with stationary phase (12·5 ml) and pack into a chromatographic column (70 cm × 2·2 cm internal diameter fitted with a sinter plate) in portions of about 3 g, tamping down firmly between each addition. To a further 2 g of prepared Celite add sample solution (1·0 ml), mix thoroughly and transfer the mix quantitatively to the top of the stationary phase in the column. Carefully add eluent phase until the stationary phase is covered to a depth of about 50 cm and adjust the flow of eluate from the column to about 10 ml/75 s. Collect 50 successive 10 ml fractions of eluate in 6 × 1 inch stoppered test tubes. Measure the absorbance at 263 nm (1 cm path length) of each fraction against eluent phase in the reference cell. Repeat the chromatogram using 1·0 ml of standard oxytetracycline solution.

The per cent oxytetracycline in the sample $= \frac{\text{Ea. Wg. } 100}{\text{Eg. Wa}}$ where Ea and Eg

are the sums of the absorbance values under the sample and standard peaks respectively. Wa = weight of sample (mg), Wg = weight of standard oxytetracycline anhydrous base (mg).

## RESULTS AND DISCUSSION

Samples of anhydro-oxytetracycline, α- and β-apo-oxytetracycline, chlortetracycline, and epi-oxytetracycline were assayed by the above procedure, and the relations between absorbance at 263 nm and volume of eluate for two of these are illustrated in Fig. 1. For the other three compounds, anhydro-oxytetracycline proved

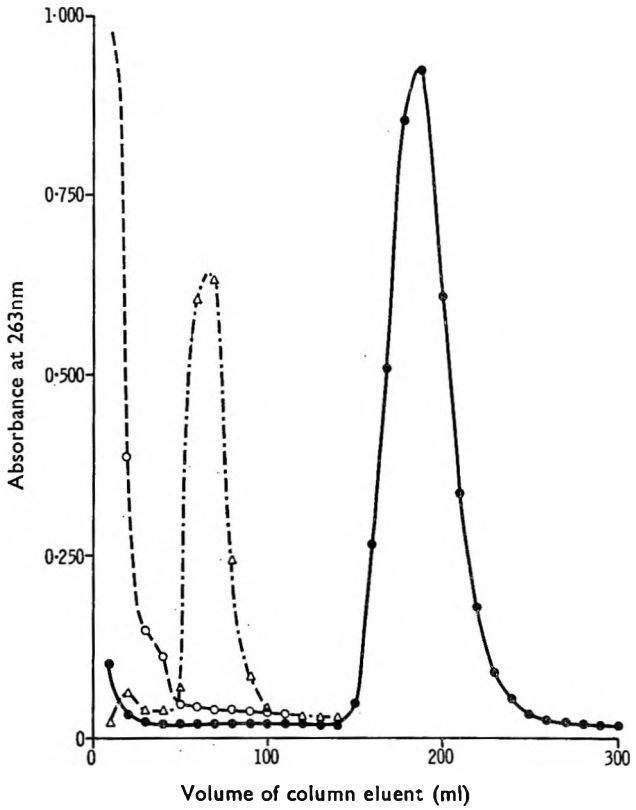


FIG. 1. Graph relating absorbance and retention volume for oxytetracycline and possible impurities. ○ — — — — ○, β-apo-oxytetracycline; △ — · — · — △, chlortetracycline and ●———●, oxytetracycline.

to be so labile in the presence of dilute acid (used in the first stage of the sample preparation) that it was converted quantitatively to a mixture of α- and β-apo-oxytetracycline. α-Apo-oxytetracycline and epi-oxytetracycline retained on the column were not eluted after 600 ml. Thus none of the above compounds interfere with the assay of oxytetracycline by the method described.

The proposed method has been applied to a series of samples of both pharmaceutical and crude grades of oxytetracycline and to material isolated from mother liquors at various stages of the purification process. The results compared with those using the microbiological assay (B.P. 1968) are shown in Table 2 and illustrate

Table 2. *Comparison of results obtained using the proposed method with those of the microbiological assay*

| Sample | Potency calculated to Oxytetracycline base: Microbiological (±2%) | Chromatographic (±1%)* |
|---|---|---|
| Laboratory prepared samples .. .. .. | 974 | 980 |
| | 972 | 980 |
| | 981 | 983 |
| | 983 | 990 |
| | 988 | 990 |
| | Potency calculated to oxytetracycline dihydrate | |
| Crude Base .. .. .. .. .. | 847 | 833 |
| | 820 | 818 |
| | 842 | 837 |
| Pharmaceutical Grade .. .. .. .. | 899 | 898 |
| | 869 | 875 |
| | 916 | 904 |
| | 912 | 892 |
| | Potency calculated to oxytetracycline hydrochloride | |
| Pharmaceutical Grade .. .. .. .. | 851 | 854 |
| | 850 | 832 |
| | 889 | 863 |
| | 903 | 895 |
| | Potency calculated as calcium salt | |
| Pharmaceutical Grade .. .. .. .. | 873 | 872 |
| | 849 | 849 |
| | 824 | 817 |
| | *International standards* | |
| | Declared microbiological potency | Chromatographic |
| 1st Int. Standard .. .. .. .. | 900 | 890 |
| | | 903 |
| 2nd Int. Standard .. .. .. .. | 880 | 880 |
| | | 878 |
| | | 876 |
| | | 880 |

* Based on 10 replicate determinations.

the value of the method for both control and pharmaceutical grade oxytetracycline and process development studies. Much information is gained about the mixture and quantity of any impurity present and although the method described is not recommended for the accurate determination of trace impurities in the pharmaceutical grade, simple adaptation of the technique makes this possible.

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SHORT COMMUNICATION

# A direct non-aqueous titration procedure for determining barbiturates in different pharmaceutical forms

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Non-aqueous titration methods for the analysis of barbiturates all involve titration of the barbiturate itself with a basic reagent (Heiz, 1952; Vespe & Fritz, 1952; Pifer, Wollish & Schmall, 1953; Swartz & Foss, 1955; Vincent & Blake, 1959; Chatten, Mainville & Pernarowski, 1961). Difficulties are caused in all those methods especially with the sodium salts by the need to liberate the barbiturate itself, and to extract it into an organic solvent. The use of an acidic reagent directly applicable to sodium salts was therefore examined.

*Apparatus and reagents*

Pye-titrimeter model 79 equipped with a combination electrode. A 10 ml burette graduated to read in 0·01 ml. 0·02N solution of perchloric acid in dioxane. 0·1% tropeolin OO in absolute ethanol. Chloroform–acetic anhydride mixture (3:2).

*Procedure*

*Pure barbiturates.* About 50 mg, accurately weighed, was dissolved in about 25·0 ml of chloroform–acetic anhydride mixture using a magnetic stirrer and warming if necessary. Ethanolic solution of tropeolin OO (3 drops) was added and the solution titrated with 0·02N perchloric acid in dioxan to the first permanent red colour.

Titrations were also followed potentiometrically when it was found that the appearance of a permanent red colour coincided with the point of maximum change in mV reading.

*Tablets and capsules.* A quantity of powdered tablets or mixed contents of capsules equivalent to 50 mg was dissolved in chloroform–acetic anhydride mixture. The titration was carried out as described above.

*Injections.* A volume of the mixed contents of ampoules equivalent to 50 mg of barbiturate was evaporated to dryness, and the residue titrated in chloroform–acetic anhydride mixture as described above.

The barbiturates, in pure form and in different pharmaceutical forms such as tablets, capsules and ampoules, were assayed by the gravimetric procedures described in the U.S.P. XVII and B.P. 1968.

The same barbiturates were also assayed using the proposed non-aqueous titration procedure. The results (Table 1) show that this method is equivalent to the official methods in accuracy and precision. The method does not involve the conversion of salts to free acid before titration. It is more rapid, and eliminates processes of extraction.

Table 1. *Analysis of barbiturates* (*mean* ± s.d. *of eight experiments*)

| Compound | Non-aqueous titration recovery % | U.S.P. XVII recovery % | B.P. 1968 recovery % |
|---|---|---|---|
| *Pure compounds* | | | |
| Phenobarbitone sodium .. .. | 100·2 ± 0·10 | 99·0 ± 0·15 | 98·6 ± 0·18 |
| Barbitone sodium .. .. .. | 100·2 ± 0·27 | — | 98·9 ± 0·22 |
| Amylobarbitone sodium .. .. | 100·1 ± 0·21 | 98·8 ± 0·14 | 98·5 ± 0·15 |
| Pentobarbitone sodium .. .. | 100·2 ± 0·18 | 98·9 ± 0·25 | 98·6 ± 0·25 |
| Quinalbarbitone sodium .. .. | 100·0 ± 0·23 | 99·1 ± 0·13 | 98·9 ± 0·19 |
| *Tablets* | | | |
| Phenobarbitone sodium .. .. | 99·3 ± 0·42 | — | 98·4 ± 0·19 |
| Barbitone sodium .. .. .. | 99·4 ± 0·22 | — | 98·4 ± 0·24 |
| Amylobarbitone sodium .. .. | 99·3 ± 0·17 | — | 98·7 ± 0·14 |
| Pentobarbitone sodium .. .. | 99·4 ± 0·22 | — | 98·0 ± 0·26 |
| Quinalbarbitone sodium .. .. | 99·3 ± 0·33 | — | 98·5 ± 0·29 |
| *Capsules* | | | |
| Phenobarbitone sodium .. .. | 99·0 ± 0·42 | — | — |
| Barbitone sodium .. .. .. | 98·9 ± 0·22 | — | — |
| Amylobarbitone sodium .. .. | 98·9 ± 0·22 | 98·8 ± 0·13 | 98·7 ± 0·14 |
| Pentobarbitone sodium .. .. | 99·0 ± 0·29 | 98·6 ± 0·17 | 98·5 ± 0·11 |
| Quinalbarbitone sodium .. .. | 98·9 ± 0·21 | 98·9 ± 0·24 | — |
| *Injections* | | | |
| Phenobarbitone sodium .. .. | 99·9 ± 0·21 | — | 98·8 ± 0·17 |
| Barbitone sodium .. .. .. | 99·2 ± 0·32 | — | — |
| Amylobarbitone sodium .. .. | 99·6 ± 0·22 | — | — |
| Pentobarbitone sodium .. .. | 99·3 ± 0·33 | 98·9 ± 0·25 | — |
| Quinalbarbitone sodium .. .. | 99·5 ± 0·21 | — | — |

Blank determinations showed that the colour change of the indicator, tropeolin OO, took place with a negligible volume of titrant. Tablet excipients such as lactose, glucose, starch, stearic acid and gelatin did not interfere with the determination.

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SHORT COMMUNICATION

# Determination of 21-acyloxy corticosteroids and other steroid esters*

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Corticosteroids in which the 21-hydroxyl group is acylated form an important group of steroid drugs. The usual methods for their determination include ring A spectrophotometry or colorimetry as well as tetrazolium methods, neither of them being specific to the ester group.

Methods based on the ester group, such as the ferric hydroxamate method (Forist & Theal, 1958) and the differential kinetic variant of the tetrazolium method (Guttman, 1966), are seldom used in pharmaceutical analysis.

Although the classical method of alkaline hydrolysis followed by the back-titration of excess base can be used successfully for the determination of some steroid esters, it cannot be applied directly to 21-acyloxy corticosteroids. This is because the α-ketol side-chain produced on hydrolysis is oxidized by atmospheric oxygen under alkaline conditions, the main oxidation products being the corresponding etianic acids and formic acid (Velluz, Petit & others, 1947). Even under anaerobic conditions products such as α-hydroxy acids are formed (Guttman & Meister, 1958).

The difficulties caused by such side-effects may be overcome by reducing the 20-keto-group with sodium borohydride when the resulting glycol side-chain is insensitive to further change either by alkali or atmospheric oxygen.

## *Experimental*

*Reagents.* All chemicals were of analytical reagent grade.

Reagent for hydrolysis: an aqueous solution of sodium hydroxide (0·2N) containing 0·1 mol/litre of sodium borohydride. Stored at room temperature, this solution is usable up to 2 months. 0·1N Hydrochloric acid. Phenol red indicator, 0·1% aqueous solution containing 20% v/v ethanol. 0·5M aqueous solution of sodium acetate.

*Apparatus.* A Radelkis OP–204 pH-titrimeter was used with the usual glass and saturated calomel electrodes.

*Procedure.* Dissolve an accurately weighed sample of the steroid ester (about 0·5 m-equiv) in ethanol (15 ml, 96% v/v). Add reagent for hydrolysis (5·0 ml) and reflux (1 h) at 100°. Add methyl ethyl ketone (0·5 ml) and reflux a further 5 min. After cooling add water (20 ml) and phenol red indicator (5 drops). Titrate the solution with 0·1N hydrochloric acid to a salmon-pink colour.

Make a blank titration treating the solutions in a manner identical to the actual estimation but substituting 0·5M sodium acetate solution (1 ml) for the steroid ester. The percentage of the steroid ester can be calculated from the difference between blank and sample titrations.

* Part XII of a Series "Analysis of Steroids". For other parts *see: Acta Chim. Hung.*, 1966, **47**, 1, 7, 121; **48**, 121, 249; 1967, **51**, 221; 1969, in the press. *Steroids*, 1963, **11**, 93. *J. pharm. Sci.*, 1968, **57**, 1737. *Analyst*, 1969, **94**, in the press.

*Results*

Table 1 summarizes the results obtained using the recommended procedure on different types of steroid esters. The steroids used for this work were of the highest quality available. This was controlled by thin-layer chromatography and measuring their physical constants.

As it can be seen from the data of Table 1, the standard deviation of the method does not exceed $\pm 0{\cdot}7\%$. This precision is better than that of the colorimetric methods.

*Discussion*

By reducing the 20-keto-group of steroids, side reactions are avoided; unreacted sodium borohydride is decomposed before back-titration of excess alkali by boiling with methyl ethyl ketone.

Before the titration, solutions thus contain sodium hydroxide, sodium borate and the sodium salt of the esterifying acid. Fig. 1 shows the titration curves both of sample and blank solutions.

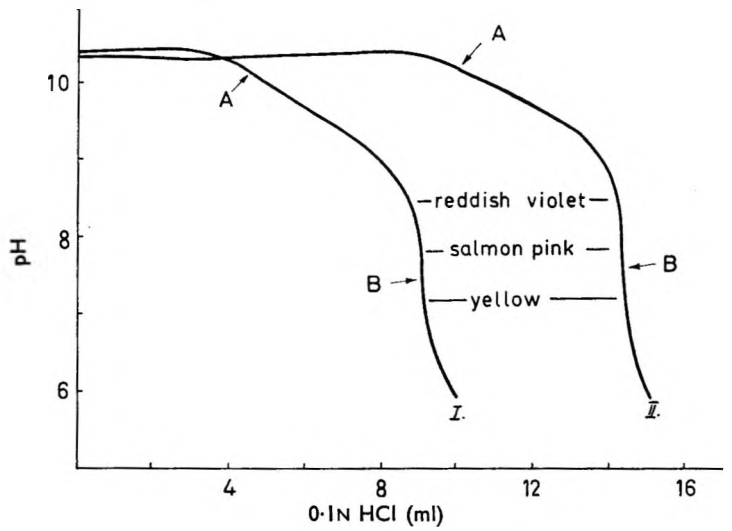


FIG. 1. I. Titration curve taken after hydrolysing 0·2276 g of pregn-5-ene-3$\beta$,17$\alpha$,21-triol-21-acetate as described in the Experimental. II. Titration curve of the blank.

The point A on the curves indicates the indistinct neutralization point of sodium hydroxide while B shows the sharp end point of the displacement titration of sodium borate. The first end point has no importance while the difference between the second end points of the blank and sample solutions is characteristic of the amount of the steroid ester. 0·5 m-equiv of sodium acetate was added to the blank to keep the conditions for the titration similar to those of the sample titration.

It was found that phenol red exhibited a sharp colour change at a point corresponding exactly with the potentiometric end point and therefore this indicator has been used routinely. Dilution with water before the titration gave a sharper end point. Occasionally, as a result of the dilution (or during the titration), precipitation of the steroid occurred. This, however, did not affect the titration.

Although some groups of the compounds mentioned in Table 1 may be determined by the classical method of saponification with boiling alcoholic alkali followed by back-titration using phenolphthalein as indicator, the application of the proposed

Table 1. *Determination of some steroid esters by the proposed method*

| | Number of determinations | Purity % | Standard deviation ±% |
|---|---|---|---|
| 21-*Acyloxy derivatives* | | | |
| Cortisone acetate | 3 | 100·6 | — |
| Hydrocortisone acetate | 5 | 100·2 | 0·7 |
| Prednisone acetate | 3 | 99·0 | — |
| Prednisolone acetate | 3 | 99·5 | — |
| Prednisolone *p*-toluenesulphonate* | 3 | 99·3 | — |
| Desoxycorticosterone acetate | 3 | 99·7 | — |
| Pregn-5-ene-3β,17α,21-triol 21-acetate | 8 | 99·7 | 0·5 |
| Pregn-4-ene-17α,21-diol-3-one 21-acetate | 5 | 100·3 | 0·3 |
| 17-*Acyloxy derivatives* | | | |
| Testosterone propionate | 3 | 100·1 | — |
| Testosterone phenylpropionate | 5 | 99·2 | 0·5 |
| 19-Nortestosterone phenylpropionate | 3 | 98·9 | — |
| Norethisterone acetate | 6 | 100·0 | 0·4 |
| 3-*Acyloxy derivatives* | | | |
| Oestrone acetate | 3 | 99·0 | — |
| Oestrone caproate | 3 | 100·4 | — |
| Oestradiol benzoate | 5 | 99·6 | 0·6 |
| Dehydroepiandrosterone acetate | 5 | 98·5 | 0·5 |
| Pregna-5,16-diene-3β-ol-20-one acetate | 3 | 98·9 | — |
| 3,17-*Diacyloxy derivatives* | | | |
| Oestradiol dipropionate | 3 | 100·5 | — |
| Ethynodiol diacetate | 6 | 99·8 | 0·4 |

* Sodium acetate in the blank omitted.

method offers the following advantages: 1. Ketosteroids as well as carbonyl impurities of ethanol may undergo polymerization, when boiled with a base. This can give rise to coloured solutions and the formation of acidic by-products. The former makes it difficult to observe the colour change of the indicator while the latter causes high results. The use of sodium borohydride obviates these sources of error. 2. Phenol red is less sensitive to atmospheric carbon dioxide than phenolphthalein. 3. The reagent for hydrolysis described above is more stable than alcoholic alkali.

From rate curves for some characteristic steroid esters, using the recommended conditions, all but one of the compounds (17α-hydroxyprogesterone caproate) examined could be determined.

The recommended method seems to be useful for the determination of steroid esters especially in conjunction with other methods such as spectrophotometry, colorimetry and thin-layer chromatography.

Any substances consuming alkali interfere with the determination. The interference of acid type substances can be overcome by preliminary titration with standard sodium hydroxide. Ester type solvents have to be removed by careful drying. Halogen-containing steroids suffer partial or complete hydrolysis under the reaction conditions described and therefore interfere with the determination.

*Acknowledgement*

The author wishes to thank Miss M. Kapás for technical assistance.

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SHORT COMMUNICATION

# The gas chromatographic determination of adrenaline in pharmaceutical products

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The determination of adrenaline in formulated products is complicated by the possibilities of decomposition, racemization and reaction with the bisulphite ion commonly incorporated in such products. Of the many available methods that of Welsh (1955) attempts to deal with all these points by a combination of acetylation and solvent extraction followed by measurement of weight and optical rotation; it has been adopted by the B.P. and the U.S.P. The procedure is rather insensitive and its specificity has been questioned by Higuchi, Sokoloski & Schroeter (1959) who suggest the addition of a liquid - liquid chromatographic purification stage.

The gas chromatography of catecholamines has been described by several workers, though not its direct application to formulated products. The potential specificity and sensitivity of the method lead to its selection for further study. Formulated products are usually aqueous and often dilute (e.g. injection of lignocaine and adrenaline 0·00125%) hence a concentrating stage must be introduced before chromatography.

*Solvent extraction of adrenaline*

Temple & Gillispie (1966) have described the solvent extraction of adrenaline with the aid of the ion-pairing compound di-(2-ethylhexyl)phosphoric acid (DEHP). We have shown that the extraction of adrenaline (2·5 mg) from aqueous buffer (pH 7·4) is essentially complete with four equal volumes of 1% DEHP in chloroform.

*Gas chromatography of adrenaline*

Derivative formation is essential and the silanization procedure of Capella & Horning (1966) with NO-bis(trimethylsilyl)acetamide was selected for this work; the reaction is said to give the tri-*O*-trimethylsilyl derivative. Rates of silanization in the absence and presence of DEHP are shown in Fig. 1. The reaction was shown to be essentially stoichiometric and complete after 120 min by the determination of the derivative content of reaction mixtures in terms of a purified specimen of the tri-*O*-trimethylsilyl derivative obtained from a preparative scale reaction. The gas chromatographic separation of a mixture of noradrenaline, adrenaline and isoprenaline as their trimethylsilyl derivatives is shown in Fig. 2.

*Proposed method*

*Materials, phosphate buffer* (pH 7·4): add 0·2M potassium dihydrogen phosphate (500 ml) to 0·2N sodium hydroxide (391 ml), dilute to 1 litre with water and mix. *Extracting solvent:* 1% v/v di-(2-ethylhexyl)phosphoric acid in chloroform. *Silanizing reagent:* mix equal volumes of dry pyridine and NO-bis-(trimethylsilyl)acetamide, add an appropriate amount of methyl myristate as an internal standard.

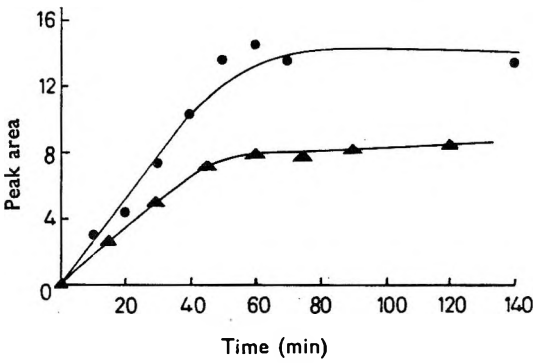


FIG. 1. Rate of silanization of adrenaline in the presence; —●—, and absence; —▲—, of DEHP.

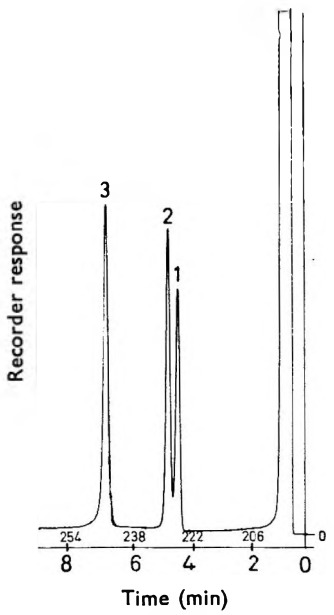


FIG. 2. Gas chromatographic separation of (1) Noradrenaline, (2) Adrenaline, (3) Isoprenaline as their silanized derivatives.

*Procedure* (suitable for concentrations down to 0·01%). To a volume of sample containing 10 mg of adrenaline add sufficient phosphate buffer to produce pH 7·4 and a minimum volume of 10 ml. Extract with 4 × 10 ml of extracting solvent, combine the extracts and evaporate them to near dryness on a water bath, removing the last 1–2 ml of chloroform in a current of air. Add 2·0 ml of silanizing reagent to the residue, stopper and set aside for 2 h with occasional shaking. Concomitantly prepare a standard by treating a suitable quantity of adrenaline similarly. Gas chromatograph suitable volumes of standard and sample under the following conditions:

Column: 5% O.V. 17 (a partially phenylated polysiloxane) 5 ft supported on acid-washed, silanized Gas Chrom P packed in a glass column, internal diameter 4 mm; column temperature, initially 190°, programming at 8°/min to 250°; injection port temperature, 210°; gas flow, 45 ml nitrogen/min; load, 2 $\mu$l.

(The instrument used for this investigation was a Pye 104 Chromatograph with flame ionization detector.)

Obtain the adrenaline content of the sample by comparison of the area of the peaks due to methyl myristate and silanized adrenaline in both the sample and standard chromatograms.

The general method is modified to deal with preparations containing less than 0·01% adrenaline. The sample aliquot may be reduced to as low as 0·25 mg adrenaline and extracted with 0·1% DEHP in chloroform. The chromatography conditions are adjusted to (i) column temperature 180°, (ii) temperature programming 4°/min.

Table 1. *Application of the proposed method to formulated products*

| Formulation | Adrenaline % w/v Declared | Found |
|---|---|---|
| Adrenaline injection B.P. | 0·1 | 0·098 |
| Adrenaline solution B.P. | 0·1 | 0·105 |
| Compound spray of adrenaline and atropine B.P.C. | 0·444 | 0·438 |
| Zinc sulphate and adrenaline eye drops B.P.C. | 0·05 | 0·0514 |
| Injection of lignocaine and adrenaline | 0·0005 | 0·00047 |
| Injection of procaine and adrenaline | 0·002 | 0·00198 |

Table 2. *Comparison of proposed method with U.S.P. XVII and biological methods on degraded preparations*

| | Adrenaline % w/v | | | |
|---|---|---|---|---|
| | Initial | GLC | U.S.P. | Biological |
| Sample 1 | 1·00 | 0·95 | 0·89 | 0·90 |
| 2 | 1·00 | 0·51 | 0·43 | 0·50 |
| 3 | 2·00 | 1·42 | — | 1·25 |
| 4 | 1·00 | 0·99 | 0·93 | — |

*Results*

The scope of the method for control purposes has been demonstrated by its application to a number of freshly prepared formulations with adrenaline contents in the range 0·5 to 0·0005%, the results are given in Table 1. Whilst the method will not detect racemization in preparations that have undergone decomposition as a result of prolonged or unsatisfactory storage, it is of value in the examination of degraded preparations which are not susceptible to racemization. The results obtained by applying the method to degraded preparations, formulated to be optically stable, are compared in Table 2 with those obtained by the U.S.P. method and a biological method (pithed rat). Thus the method is valid in preparations where up to 50% degradation has occurred.

The reproducibility of the method is estimated to be $\pm 5\%$ for preparations containing 0·05% adrenaline and above and $\pm$ 10% for lower concentrations.

*Acknowledgment*

We wish to thank N. J. P. Winsey for the biological determinations.

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SHORT COMMUNICATION

# The gas chromatographic determination of nicotinamide and thiamine* in vitamin preparations

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Methods available for the determination of thiamine and nicotinamide are generally slow and tedious. Gas chromatography alone offers the practical advantages of speed and convenience. It has already been shown (Senello & Argoudelis, 1969) that nicotinamide, ascorbic acid and pyridoxine can be converted to their BSA [N-O-bis (trimethylsilyl) acetamide] derivatives and quantitatively estimated in admixture using gas chromatography. The present communication outlines a method which avoids prior conversion to the silyl ether derivatives, thus eliminating an extra reaction stage and the need for careful monitoring of the conversion.

*Experimental*

A Varian Aerograph No. 204b gas chromatograph with a flame ionization detector was operated isothermally under the following conditions: injector 250°; oven 210° for nicotinamide and 190° for thiamine; detector 300°. The carrier gas was nitrogen flowing at 30 ml/min for nicotinamide and 9 ml/min for thiamine. The column was a 5 ft × $\frac{1}{8}$ in o.d. stainless steel coil packed with 4% silicone gum nitrile X.E.60 on acid-washed silanized chromosorb G (60/80 mesh).

*Results and discussion*

Calibration curves were constructed in the usual manner using nicotinamide over the weight ranges 1–8 mg/ml in methanol with 4 mg/ml phenacetin B.C.R.S. as an internal standard. For thiamine, the weight range included 1–10·6 mg/ml with acetyl-*o*-phenetidine (2·5 mg/ml) as internal standard. 1 $\mu$l injections of each solution were used throughout, and each estimation was made in triplicate. Peak areas were measured by the triangulation (peak height × $\frac{1}{2}$ height width) method. A plot of weight of vitamin against the ratio of peak areas (compound : internal standard) was linear over the range examined. Retention time for nicotinamide was 5 min at 210° and for thiamine 5 min at 190°. Resolution for nicotinamide/phenacetin was 3·4 and for thiamine/acetyl-*o*-phenetidine 3·6, as determined by the method of the British Pharmacopoeia 1968.

Pharmaceutical preparations were extracted as shown in Tables 1 and 2. The solutions (1 $\mu$l) were chromatographed in triplicate and the peak area ratios of the sample and internal standard determined. The vitamin content of the samples was

* With a note added in proof, on "the behaviour of thiamine under electron impact" by B. Blessington and D. W. Mathieson.

calculated by reference to the standard curves. Results are also shown in Tables 1 and 2.

Table 1. *Sample preparation and results for nicotinamide*

| Sample and nicotinamide content | Amount of powder extracted with 25 ml methanol | Dilution | Nicotinamide found mg | B.P.C. method mg |
|---|---|---|---|---|
| Tablets of nicotinamide B.P.C. (50 mg) | ≡ 5 tablets | 5 ml of extract with internal standard* (40 mg) to 10 ml with methanol | 52·1<br>53·2<br>54·1 | 52 |
| Compound thiamine tablets B.P.C. (15 mg) | ≡ 14 tablets | 5 ml of extract with internal standard* (40 mg) to 10 ml with methanol | 15·7<br>15·7<br>15·6 | 15·3 |
| Strong compound thiamine tablets (20 mg) | ≡ 10 tablets | 5 ml of extract with internal standard* (40 mg) to 10 ml with methanol | 20·1<br>20·0<br>19·8 | 20 |
| Vitamins B and C injection B.P.C. 160 mg in 2 ml (ampoule 2) | — | 1 ml of ampoule 2 with internal standard* (80 mg) to 20 ml with methanol | 159·3<br>160·6<br>162·4 | 161 |

* Phenacetin British Chemical Reference Substance.

Table 2. *Sample preparation and results for thiamine*

| Sample and thiamine content | Amount of powder extracted with 10 ml methanol | Dilution | Thiamine found mg | B.P.C. method mg |
|---|---|---|---|---|
| Compound thiamine tablets B.P.C. (1 mg) | ≡20 tablets with internal standard* (12·5 mg) | — | 0·95<br>0·97<br>0·99 | 0·99 |
| Compound thiamine tablets strong B.P.C. (5 mg) | ≡4 tablets with internal standard* (12·5 mg) | — | 4·97<br>4·91<br>5·2 | 5·2 |
| Vitamins B and C injection B.P.C. (strong for intravenous use) 250 mg in 5 ml (ampoule 1) | — | 1 ml injection with internal standard* (30 mg) to 25 ml with methanol | 257<br>253<br>249 | 252 |

* Acetyl-*o*-phenetidine.

To assay thiamine in the presence of nicotinamide (as in compound thiamine tablets B.P.C.) the two components must be determined separately at the requisite temperatures of 190° and 210° unless a temperature program is used. At the higher temperature, thiamine is only partially resolved from the solvent peak.

No interference with the method has been experienced from ascorbic acid, pyridoxine and riboflavine.

*Acknowledgement*

The authors wish to thank Mr. J. Ganley for technical assistance.

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*Note added in proof*

## The behaviour of thiamine under electron impact

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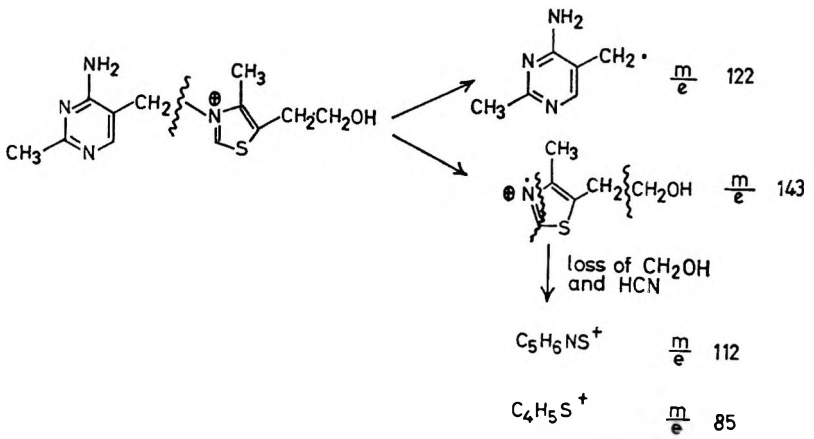
With a structure such as thiamine, which contains a quaternary nitrogen, it would appear that the effluent peak from the gas chromatograph column may represent a degradation product. Circumstantial evidence that this is so is provided by mass spectrometry.

When thiamine chloride is examined using the direct insertion probe of an MS902, the mass spectrum shown in Fig. 1 is obtained. Mass measurement of the underlined ions shows them to possess the molecular formulae:

m/e 112 ($C_5H_6NS$); m/e 122 ($C_6H_8N_3$); m/e 143 ($C_8H_9NOS$).

This is consistent with the fragmentation shown below and metastable peaks are observed at m* = 89·3 (m/e 143→113) corresponding to loss of $CH_2O$ from the alcohol side chain and at m* = 64·5 (m/e 112→85 corresponding to loss of HCN from the thiazole ring).




When the "thiamine" peak from the gas chromatography column is examined, only the fragment ions ascribed to the thiazole half of the molecule can be detected, that of *m/e* 122 from the pyrimidine ring is absent.

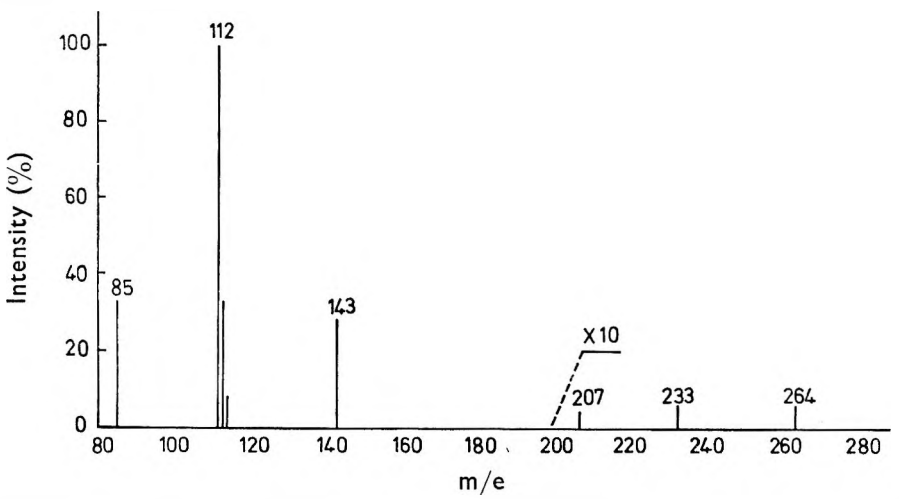



FIG. 1. Mass spectrum of thiamine chloride. Direct insertion probe: temperature 300°. Electron beam energy 70ev.

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SHORT COMMUNICATION

# A chromatographic assay for male fern extract

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There is not yet a suitable assay for male fern extract. Variation in proportions of phloroglucinol derivatives are not reflected by the gravimetric determination for filicin (B.P. 1968). Neither the paper chromatographic method of Klevstrand (1961) nor the thin-layer method of von Schantz, Ivars & others (1962) are satisfactory. The present paper describes a two-dimensional thin-layer method for the separation of the two major constituents in *Dryopteris filix-mas* (L) Schott., namely flavaspidic acid and filicic acid, followed by their ultraviolet spectrophotometric determination.

## *Experimental*

Silica gel G (Merck): washed with purified water, twice with dry ethanol and twice with dry chloroform, filtered off and dried in a current of air between washings. After final drying the material was powdered and then stored in an airtight container.

*Method.* The two-dimensional method of Fish & Kirk (1968) was used. Plates were prepared with the pre-treated silica gel (25 g) mixed with ascorbic acid (150 mg) in buffer solution, pH 6·0. Before spreading, the plates were soaked overnight in detergent solution, throughly rinsed and dried.

Commercial extract of male fern B.P. was dissolved in ether (100 mg/ml) and, by means of a Hamilton syringe fitted with a PB600 dispenser, 10 $\mu$l of the solution was spotted near one corner of a plate, using a spotting template (Brain & Hardman, 1968). Alongside each test plate, a control (blank) plate was developed two-dimensionally, then both plates were dried in the dark at 25° to remove all traces of solvent (about 2–3 h).

The male fern extract (1 g) was treated by the official assay process (B.P. 1968) and 10 $\mu$l of the chloroform solution of filicin obtained (75 ml) was spotted on to plates and chromatographed as above.

Samples of flavaspidic acid and filicic acid were obtained as described previously (Fish & Kirk, 1968) and fixed volumes (10 $\mu$l) of ethereal solutions containing known amounts of either of these compounds were also chromatographed. From the results calibration curves were constructed; they were linear over the range 0·001–0·004% w/v.

*Elution and determination.* The test plates were examined in ultraviolet light of 366 nm and the dark purple, fluorescence-quenched areas corresponding to flavaspidic acid and filicic acid were marked. The adsorbent from those areas, and from areas corresponding exactly in size and position on the blank plates, were separately removed using the glass transfer tool described by Bird, Brickley & others (1963).

Each portion of adsorbent was extracted repeatedly by mixing with dry chloroform (12 × 0·4 ml), the solutions being bulked and made up to volume (5 ml).

The absorbances (Aho, 1958) of the test solutions were measured at 290 nm for flavaspidic acid and at 283 nm for filicic acid using a path length of 1 cm. The chloroform extract of adsorbent from the control plate was used as a blank.

*Results*

The amounts of flavaspidic acid and filicic acid present in male fern extract and in the separated filicin, expressed as percentages by weight of the original extract, were: by direct determination—flavaspidic acid 19·8 (±1·7), filicic acid 10·8 (±2·1); calculated from figures obtained by assay of the filicin separated from the extract: 5·1 (±1·4) and 2·3 (±1·1) respectively. Each mean value was calculated from results of ten determinations.

*Discussion*

In the male fern used in Britain, *Dryopteris filix-mas*, the main activity can be ascribed to flavaspidic acid, and to traces of desaspidin usually present, and we suggest that assay for the former alone would give a good indication of potency in the crude drug and its extract. A similar method could be useful for the continental drug, derived from "*D. austriaca*". The method could also be extended to chemotaxonomic studies of various ferns.

The results show that the male fern extract we examined, which came from a 1968 batch of crude drug, and was of good quality, contained high proportions of the active flavaspidic acid and the inactive filicic acid, both of which must contribute largely to the weight of filicin determined in the official gravimetric assay. They also show that the alkali treatment during the isolation of filicin in that assay greatly reduces the contents of those compounds present. In the direct chromatographic assay proposed, undesirable breakdown of the labile compounds is avoided.

Use of pre-washed silica gel for chromatography is essential since impurities from adsorbents interfere with subsequent spectrophotometric determinations (Kirchner, Muller & Rice, 1954; Stanley & Vannier, 1957). The treatment described was successful in removing interfering substances.

For flavaspidic acid recoveries from chromatoplates were 72% and for filicic acid 84% of the amounts applied, and the method, applied directly to the extract, gave results which were reproducible over the range quoted and were as consistent as those given by Takki (1967) for the estimation of flavaspidic acid in filicin.

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SHORT COMMUNICATION

# Quantitative determination of emetine and cephaëline in ipecacuanha root

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Ipecacuanha was introduced into British medicine in 1672 and at the present time two species, *Cephaëlis ipecacuanha* (Brot) A. Rich. (Rio or Brazilian Ipecacuanha) and *Cephaëlis acuminata* Karsten (Cartagena, Nicaragua or Panama Ipecacuanha) are official (British Pharmacopoeia, 1968). The present Pharmacopoeia requires not less than 2% of alkaloid, calculated as emetine; formerly a standard was required for the proportion of non-phenolic alkaloids and this is still of commercial significance.

Volumetric analysis is commonly used for the estimation of the total alkaloid content (British Pharmacopoeia, 1968) but is rather unsatisfactory. It has been claimed (Beckett & Stenlake, 1962) that the phenolic alkaloids exert a buffering action. In addition the yellow colour of the extract tends to mask the end point. Ion-exchange, followed by spectrophotometry, has been applied to ipecacuanha root (Higuchi & Bodin, 1961) and tincture (Kamp, 1957). Kori & Kano (1962) used column partition chromatography for the quantitative separation of the alkaloids. Thin-layer chromatography has previously been applied as a limit test for alkaloids other than emetine (cephaëline, isoemetine, *O*-methylpsychotrine) in emetine hydrochloride (B.P. 1968). It has also been used to identify the constituents of tincture of ipecachuana (Ghosh, Data & Bose, 1968). Such methods have not however been applied to the direct assay of emetine and cephaëline.

This work describes a method for the quantitative estimation of emetine and cephaëline directly on the developed chromatoplates.

## *Experimental*

*Materials.* Commercial samples of the root of *C. ipecacuanha* and *C. acuminata* were investigated. Pure samples of emetine and cephaëline were used for the preparation of standard solutions of the alkaloids.

*Extraction of the root.* 1 g of finely powdered root was shaken with 10 ml of a mixture of 3 volumes solvent ether and 1 volume chloroform for 15 min. After standing for 10 min 0·75 ml dilute solution of ammonia was added and the mixture shaken for a further 2 h. The contents of the flask were transferred to a small percolator and the marc percolated with the ether–chloroform mixture until the eluate was free from alkaloid. The extract was evaporated to dryness under reduced pressure and the residue dissolved in absolute ethanol to give 40 ml of solution. This solution was applied to the chromatoplates.

*Chromatographic separation.* 20 × 20 cm glass plates were coated with a 250 $\mu$m layer of Kieselgel G (E. Merck) (aqueous slurry) and dried at 25° for 48 h before use.

An amount of 5 $\mu$l of the solution of ipecacuanha alkaloids was applied to the origin together with 5 $\mu$l of standard solutions of emetine and cephaëline in ethanol.

The diameter of the zones was 3–7 mm. The loaded plates were developed with a mixture of toluene–benzene–ethyl acetate–diethylamine (35:35:20:10 v/v) for a distance of 12 cm from the origin. The developed plates were dried at 80° for 5 min.

*Visualization.* Emetine and cephaëline were located by means of their fluorescence in ultraviolet light (366 nm) and by heating at 60° for 20 min after spraying with 0·5% w/v solution of iodine in carbon tetrachloride.

*Determination of spot area* produced on the chromatoplates was carried out by tracing their outline on 1 cm graph paper and counting squares.

*Calibration curves* were constructed by plotting the logarithm of the weight of alkaloid applied against the spot area produced by standard solutions of mixtures of emetine and cephaëline developed on the same chromatoplates as the extracts of ipecacuanha root. These were linear over the range 1·5–8 $\mu$g.

*Total alkaloid content* was determined by the method of the B.P. 1968.

Table 1. *Thin-layer chromatography of emetine and cephaëline in ipecacuanha root. Kieselgel G. Toluene–benzene–ethyl acetate–diethylamine* (35:35:20:10 *v/v*)

| Alkaloid | Mean Rf value | Ultraviolet light | Iodine reagent: Daylight | Iodine reagent: Ultraviolet light |
|---|---|---|---|---|
| Emetine .. .. | 0·54 | yellow | yellow | yellow |
| Cephaëline .. .. | 0·38 | blue | brown | blue |

Table 2. *Analysis of the alkaloid content of ipecacuanha root*

| Method | Alkaloid | % w/w alkaloid: Rio drug | % w/w alkaloid: Cartagena drug |
|---|---|---|---|
| B.P. 1968 .. .. | Total | 2·49 | 2·37 |
| TLC .. .. | Emetine | 1·47* ± 0·16 | 1·22* ± 0·15 |
| | Cephaëline | 0·98* ± 0·13 | 1·03* ± 0·13 |
| | Total | 2·45 | 2·25 |

* s.d. on 15 results for each determination.

*Results and discussion*

Of the various thin layers, alumina gave poor resolution and tailing of the alkaloids; activated silica gel gave good resolution but poor reproducibility. Air-dried plates showed less variation and optimum reproducibility was achieved on plates stored at 25° for 48 h. The solvent system used gave well defined, rounded zones with both alkaloids. Pipetting errors were reduced by applying all solutions with the same micropipette and the use of a constant delivery time for each application.

The results of the chromatographic separation are shown in Table 1.

The total alkaloid of the ipecacuanha samples (B.P. 1968) and the determination of emetine and cephaëline separately are shown and compared in Table 2. The results by the suggested method are in close agreement with those obtained by the official method. Thin-layer chromatography has the advantage that it is less laborious.

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# Crystal growth studies involving phase transitions in aqueous drug suspensions

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The growth of oxyclozanide crystals in quiescent suspensions has been monitored using the Coulter Counter. Increase in particle size is the result of an isothermal, solvent-mediated phase transition between two unsolvated polymorphs, one having a lower solubility than the other. Effects due to Ostwald ripening and temperature cycling are absent. Theophylline, which shows crystal growth in suspension by hydration, has been studied by photomicrography. Preliminary results indicate that the initial rate of growth in such systems may be qualitatively described in terms of steady-state diffusion theory.

The physical stability of pharmaceutical suspensions has long been a formulation problem, and the effect of particle size and fluctuating temperature on crystal growth is well known (Wagner, 1961; Carless & Foster, 1966; Varney, 1967). Recent experience of pronounced crystal growth in suspensions which were virtually unaffected by temperature has directed attention to a third mechanism of growth, that of isothermal, solvent-mediated phase transitions between different polymorphs or solvates of a substance. A prime consideration is the difference in solubility between the various crystal forms and instability may be expected whenever the production of a less soluble form of the solid phase is possible.

Some examples of the effect of solvation on the solubilities of several pharmaceutical compounds have been described by Higuchi & Shefter (1963). Carless, Moustafa & Rapson (1966) have described the morphology of cortisone acetate and more recently (1968) the effect of the various polymorphic forms on crystal growth in suspensions.

Most of the previous studies of crystal growth involving phase transitions from an existing solid phase in suspensions, have either been concerned with characterizing the solid phases involved (Eanes, Gillessen & Posner, 1965; Arakwa, Kobayashi & Suito, 1966), or have used stirred systems. Since pharmaceutical suspensions are quiescent under typical storage conditions there is scope for practical investigations into the mechanism of changes in the absence of agitation. A disadvantage is that more complicated systems have to be used, but the results are of direct relevance to many formulation problems.

Ideally the growth problem can be solved by selecting the most stable form of a drug for formulation. However, it is important to consider the kinetics of phase transitions in such systems for the following reasons: (i) Conditions of plant production, such as economy, ease of filtration, production time, may make production of a desired physical form difficult. Seeding with quantities of the desired polymorph may help but is not invariably successful. (ii) The most stable forms may not be the

most effective physiologically (e.g. slower dissolution rates). In such cases it would be desirable to formulate a suspension of a less stable form and to reduce the rate of phase transition sufficiently to produce an acceptable shelf life.

The purpose of this paper is to describe some preliminary results which have been obtained for theophylline (1,3-dimethylxanthine), which shows growth by hydration, and for oxyclozanide (2,2′-dihydroxy-3,3′5,5′,6-pentachlorobenzanilide) in which a polymorphic phase change is involved.

## THEORETICAL

Under conditions of constant temperature and pressure, the stable or equilibrium configuration for a crystal will be that for which the total (Gibbs) free energy is a minimum. The unit volume free energy is taken to be independent of shape, and the surface free energy is assumed to be constant over each face, though its value may be different for each different type of face. No free energy is assigned to the edges or to the corners of crystals. The Gibbs free energy (G) for a crystal of N faces may therefore be expressed as

$$G^{\alpha} = Vg_v^{\alpha} + \sum_{i}^{N} A_i g_i^{\alpha} \quad .. \quad .. \quad .. \quad (1)$$

$$G^{\beta} = Vg_v^{\beta} + \sum_{i}^{N} A_i g_i^{\beta} \quad .. \quad .. \quad .. \quad (2)$$

where V is the crystal volume, $g_v$ is the free energy per unit volume, $A_i$ is the area of the $i$'th face and $g_i$ is the free energy per unit area of that face. The superscripts refer to polymorphs (or solvates) $\alpha$ and $\beta$ respectively.

If $G^{\alpha} \neq G^{\beta}$ there exists a thermodynamic potential to establish equilibrium by an appropriate change of phase or crystal habit. In the dry state a solid phase change may not be possible unless favoured by suitable proximity to a transition temperature. In the presence of a suitable solvent, however, the rearrangement of molecules in the crystal can occur through selective dissolution and redeposition between crystals of different chemical potential. By this mechanism the less soluble phase grows at the expense of the more soluble phase. During growth the equilibrium crystal habit for the given environment is developed, which, in the presence of surface-active substances, often appears in an extreme form such as plates or needles.

Three stages are involved: (i) Solute molecules transfer from crystals of the more soluble phase and pass into solution. (ii) Solute migrates through solution to the surface of crystals of the less soluble phase. In the absence of fluid currents, migration takes place by diffusion along the concentration gradient between the two phases. (iii) Solute molecules deposit on the crystal lattice of the less soluble phase, adopting the same structure and thereby continuing the process until all the dissolving phase has disappeared.

With adequate wetting, the dissolution rate for a given quiescent system is not readily varied. However, stages (ii) and (iii) can be modified to control the rate of crystal growth, an obvious example being the use of surface-active agents to inhibit (iii). Consideration of diffusion kinetics will show the factors controlling the rate of stage (ii).

The steady-state laws of vapour diffusion through a membrane between two fixed vapour pressures are readily adapted to the present case of solute diffusion along an inter-particle solvent path between two fixed concentration levels. Steady-state diffusion applies since the concentration at the crystal surfaces are constant. The rate of transfer, F, of solute through the solution under these conditions is given by Fick's First Law:

$$F = -D\,dc/dx \qquad (3)$$

where D is the diffusion coefficient and dc/dx is the concentration gradient of the diffusing species. On integration (3) becomes

$$\int_0^x F dx = -\int_{C_1}^{C_2} D\,dc \qquad (4)$$

Since we are concerned with a steady state, F is the same through each section of the membrane, i.e. F is independent of x, resulting in a linear concentration gradient between dissolving and growing crystals. Hence,

$$\int_0^x F dx = F\int_0^x dx = Fx \qquad (5)$$

and therefore,

$$F = -1/x\int_{C_1}^{C_2} D\,dc \qquad (6)$$

Since at the low concentration levels considered D may be assumed constant,

$$F = -D/x\int_{C_1}^{C_2} dc = -D(C_2 - C_1)/x \qquad (7)$$

where x is the average inter-particle distance and $C_2 - C_1$ is proportional to the difference in concentration between the solution immediately adjacent to the two types of crystal. To a close approximation this is equal to the difference in solubility of the two phases.

The above equations apply to diffusion in one dimension. However, it is readily shown that the solutions of many problems in radial diffusion in three dimensions can be deduced immediately from those of the corresponding linear problems (Crank, 1967).

Since the rate of crystal growth is proportional to F, it can be seen from equation (7) that stability problems are increased as the solubility difference is increased or the inter-particle distance is decreased. Concentrated suspensions should therefore be less stable than dilute ones and the stability should decrease further on addition of materials which enhance the solubility difference.

## EXPERIMENTAL

### *Preliminary considerations*

The requirements for an ideal suspending medium for investigating diffusion-controlled growth presented several practical difficulties. Eventually, aqueous

systems gelled with Carbopol 940* were found to be suitable for the following reasons: (i) The Carbopol systems ensured permanent suspension of particles with the absence of fluid currents. (ii) The open network of dilute gels permits free diffusion. There is an increasing volume of literature on the growth of crystals in such media. Halberstadt & Henisch (1968) have given a status report on various aspects of the technique, and Heyrovsky, Kratochvil & Sprusil (1968) have stated that the method is particularly suitable for growing crystals isothermally. (iii) Carobopol gels can tolerate large quantities of organic solvents, enabling the solubility of the solid phases to be varied within wide limits. (iv) The viscosity is only slightly affected by temperature. (v) Since the gelling properties of Carbopol are obtained by neutralization with alkali, dispersion of the solid can be produced without difficulty in a mobile system and the pH adjusted subsequently.

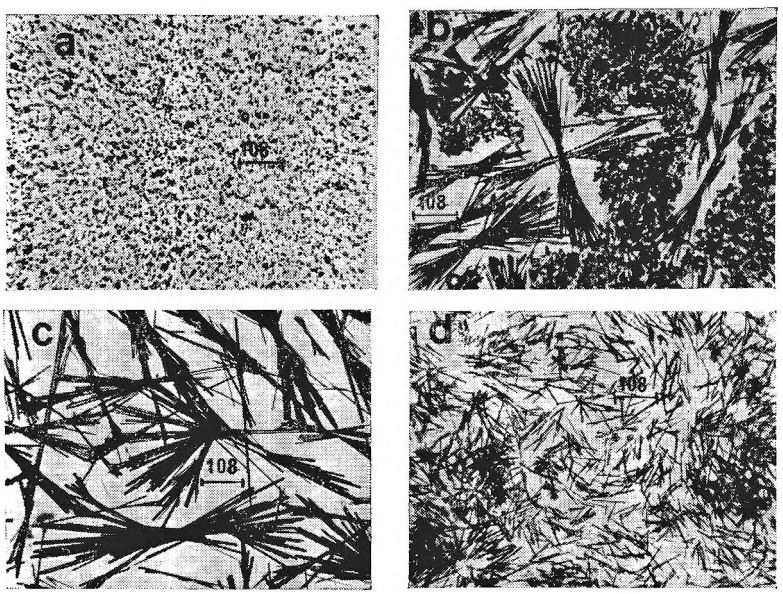


FIG. 1. (a) Micronized theophylline, anhydrous. Initial aqueous suspension. (b) Crystal growth after 5 min. (c) Crystal growth after 15 min. (d) Aqueous theophylline suspension, solid phase "seeded" with 5% w/w hydrated form. Crystal growth after 15 min. Measurements are in $\mu$m.

*Studies using theophylline*

As a demonstration of crystal growth by solvation, an aqueous suspension of anhydrous micronized theophylline was prepared and microphotographs taken at several intervals (Fig. 1a–d). The system provided striking visual evidence of the isothermal growth of one phase at the expense of another. Fig. 1a shows the initial suspension; Fig. 1b shows growth in progress after 5 min. Trace particles of hydrate served as nuclei, and the subsequent growth of these apparently held the supersaturation below the level required for further nuclei generation. Therefore, only a small number of very large crystals were formed. Anhydrous crystals could be seen to dissolve in the neighbourhood of the hydrated ones while the latter grew out as long needles into the voids formed. After 15 min the transformation was

* A polycarboxylic acid resin producing a gel in solution on neutralization with alkali (Goodrich Ltd.).

complete (Fig. 1c) and the final photograph (Fig. 1d) shows the effect of including 5% w/w of the hydrated phase in the initial suspension. A large number of smaller crystals were formed due to the higher nuclei concentration.

*Studies using oxyclozanide*

Certain production batches of bead-milled oxyclozanide concentrates, 25% w/w, exhibited pronounced crystal growth (Fig. 2a, b). Long, interlocking needle crystals were formed giving a viscous and lumpy suspension. The rate of growth varied with the batch of concentrate, the most extreme case showing virtually complete transformation after several days, while some batches appeared stable indefinitely. In no case was it possible to accelerate growth by temperature cycling. Examination by X-ray, infrared and elemental analysis showed that the crystal growth was produced by a phase transition between two unsolvated polymorphs, the final form "B" having a lower solubility than the initial form "A".

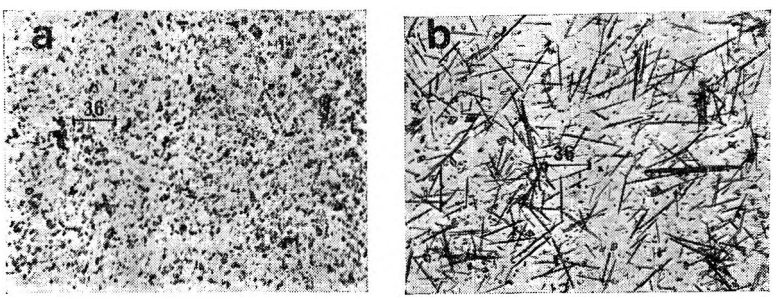


FIG. 2. (a) Oxyclozanide 25% w/w concentrate, selected batch, before polymorphic change. (b) Same batch after polymorphic change. Pronounced growth of needle crystals is shown. Measurements are in $\mu$m.

*Measurement of crystal growth*

A Coulter Counter "Model A", fitted with a 50 $\mu$m orifice tube and a 0·05 ml manometer (Coulter Electronics Limited, Dunstable) was used. The instrument was calibrated using polyvinyltoluene latex spheres, diameter 2·68 $\mu$m (Dow Chemical Company, Michigan). The technique for rapid monitoring of growth was as described by Carless, Moustafa & Rapson (1968). The Coulter Counter had the considerable advantage for the present work of being a volume sensing device, so that variation of crystal habit during growth would not seriously affect the results.

The counting electrolyte was a 0·9% w/v solution of sodium chloride in distilled water containing 0·02% w/v Perminal BXN (sodium alkylnaphthalene sulphonate, ICI Ltd.) as wetting agent and saturated with oxyclozanide. The solution was clarified through filter paper and then through 0·45 $\mu$m pore size Millipore filters under light vacuum.

Samples of the test suspensions were transferred to the stirred counting electrolyte by means of a glass rod. The Carbopol gel rapidly dissolved from the rod, releasing the particles into the electrolyte for counting. Measurements were then taken at only four threshold levels for speed of operation. Since all counts were related to a total count for a given series of measurements, it was not necessary to measure an exact quantity of gel into the electrolyte solution. The time taken to complete a size analysis rarely exceeded 10 min. Background and coincidence corrections were made as usual.

*Effect of different polymorphs on counting technique*

It is conceivable that some dissolution or growth of the test suspension could take place in the counting electrolyte, depending on which polymorph of oxyclozanide was used for saturation. Batches of counting electrolyte saturated with forms A and B were therefore prepared and size distributions of micronized samples of each polymorph obtained. In both cases there was no noted effect with change of electrolyte. It was therefore concluded that for the present system the selection of specific batches of oxyclozanide to saturate the counting electrolyte was not necessary.

*The disperse phase*

The micronized oxyclozanide used in the growth trials was a 10% w/w mixture of the less-soluble polymorph B in the more-soluble form A, the former serving as nuclei for the growing phase. Too low an initial nuclei concentration produced overlarge crystals with resultant blockage of the Coulter orifice, whereas at higher concentrations growth was insufficient before the phase change was complete. 10% was about the optimum, producing needle crystals of about 20 $\mu$m maximum length.

*Solubility*

Oxyclozanide has a very low solubility in water, less than 5 ppm at room temperature. Addition of methanol increased the solubility over a wide range, enabling the period of phase transition to be adjusted to a convenient value for measuring. The advantages of using methanol were that complete miscibility at all concentrations was assured, and the gelling properties of Carbopol were retained. From several screening tests a final concentration of 25% w/w methanol in a solution gelled with 0·3% w/w Carbopol 940 and containing 0·1% w/w Lissapol NX (a nonylphenol ethylene oxide condensate, ICI Ltd.) as wetting agent, was found to produce complete oxyclozanide conversion within about 8 h. It has however, been possible to vary the period of phase change from seconds to several days by increasing or decreasing the methanol concentration respectively.

The solubility of oxyclozanide has been determined in an aqueous solution containing 25% methanol and 0·1% w/w Lissapol NX by the method of Higuchi and Shefter (1963). Carbopol was omitted due to experimental difficulties. The values determined were 4·8 and 2·4 mg/100 ml for the "A" and "B" polymorphs respectively. The assumption was made that the presence of 0·3% w/w Carbopol did not materially alter the solubilities. Evidently solute transfer can occur under very small concentration gradients.

*Preparation of suspension*

The order of mixing the constituents is critical. In the presence of large amounts of methanol any attempt to disperse the solid phase by rapid agitation caused initial growth, presumably by accelerating solute diffusion. Dispersion by ultrasonics degraded the Carbopol and prevented gel-formation. It was finally found most convenient to disperse the solid mechanically in a simple aqueous Lissapol solution since no change in particle size was detected after several hours of such treatment. Only gentle mixing was then necessary to ensure a homogeneous blending with the remaining constituents. It was essential to ensure complete dispersion since any residual aggregates tended to break down as the more soluble phase dissolved, producing an initial dip in the growth curves.

The dispersion of micronized oxyclozanide in aqueous Lissapol was prepared by mechanical agitation using a stainless steel perforated piston moving inside a 25 ml measuring cylinder. The piston head had a total diameter of 11/16 inch and 12 individual holes of 1/16 inch diameter, equidistant from each other, drilled in it. Spot checks with the Coulter Counter indicated complete dispersion after about 15 min, provided the Lissapol concentration was not lower than 0·1% w/w. Microscopic examination confirmed the absence of residual aggregates. A weighed quantity of a solution of Carbopol 940 in aqueous methanol was added and the system mixed by several gentle strokes of the piston. Finally a standard solution of sodium hydroxide was added to gel the system at pH 7. The addition of the methanolic solution caused weak flocculation, possibly due to some desorption of the Lissapol. Complete dispersion was obtained however with a few seconds agitation of the gelled suspension. The amounts of the various constituents used in the preparation were chosen to give a final suspension of the following composition (% w/w): oxyclozanide, 4; Lissapol NX, 0·1; Carbopol 940, 0·3; methanol, 25; distilled water up to 100.

A further two suspensions were prepared to the above specification but containing 2 and 1% w/w oxyclozanide respectively. In each of these suspensions the solid phase was a mixture of 10% w/w polymorph B in A, but a 4% w/w suspension was prepared containing exclusively polymorph A. All tests were made at room temperature (23 $\pm$ 1°). Crystal growth in the suspensions was taken to begin on addition of the methanol.

*Infra-red measurements*

At intervals during the growth process samples of oxyclozanide were extracted from the suspensions and examined for polymorphic change by infrared spectroscopy. A portion of the suspension was diluted with water to thin the gel and arrest further crystal growth. The system was then centrifuged to precipitate the solid phase and the liquid decanted off. The precipitate was washed by stirring in water and centrifuging again. After washing three times the precipitate was dried over silica gel under vacuum for 48 h.

## RESULTS AND DISCUSSION

The Coulter plots at four different threshold levels for the 4% w/w suspension are given in Fig. 3. Taking the 4 $\mu$m plots for reference, the growth of the 4, 2 and 1% w/w suspensions are compared in Fig. 4. The results require some caution in their interpretation, but a definite increase in the rate of growth with suspension concentration is shown. The infrared spectra (Fig. 5) confirmed that the crystal growth was accompanied by a progressive polymorphic transformation from A to B. There was no change in the infrared spectra, or detectable growth, in the suspension prepared without nuclei, confirming that the phase transition was effected by interparticle migration of solute rather than by molecular rearrangement within the solid phase. The unseeded suspension also showed that "Ostwald ripening", i.e. growth of the larger particles at the expense of the small ones was not responsible for the change observed.

It is unlikely that interaction with the solvent had any net effect on the energy balance in favour of the transformation from form A to B since no solvation product

was formed. Conceivably the production of crystals of higher surface energy than the disappearing ones could retard the phase change, and vice versa, but much more data will be required to produce evidence of this effect in the present system.

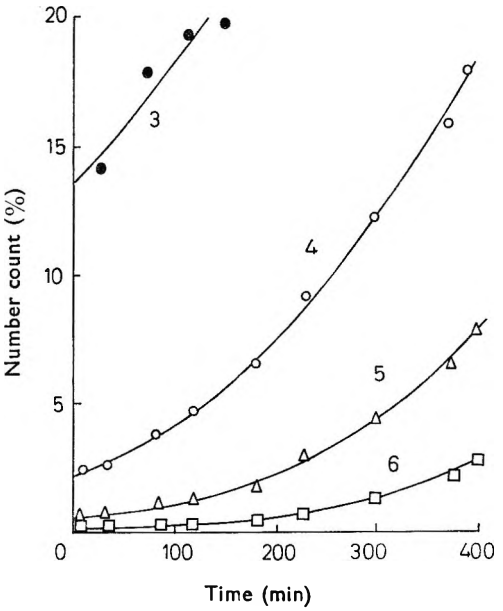


FIG. 3. Change in cumulative counts with time, representing crystal growth in a 4% w/w suspension of oxyclozanide. Counts taken at 3, 4, 5 and 6 $\mu$m threshold levels. Numbers on the curves are $\mu$m.

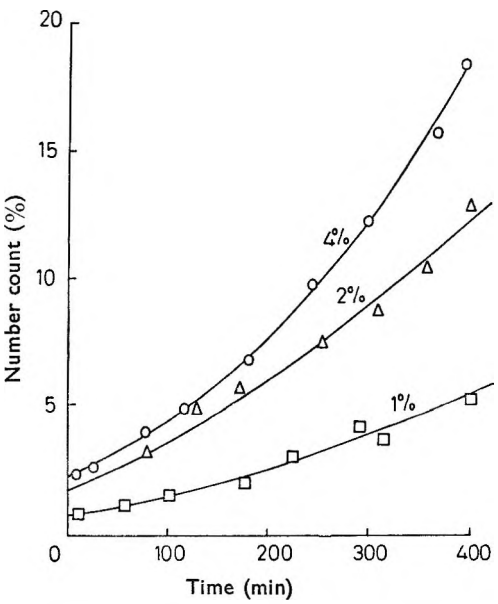


FIG. 4. Change in cumulative counts with time, representing crystal growth in 4, 2 and 1% w/w suspensions of oxyclozanide. All counts taken at 4 $\mu$m threshold level.

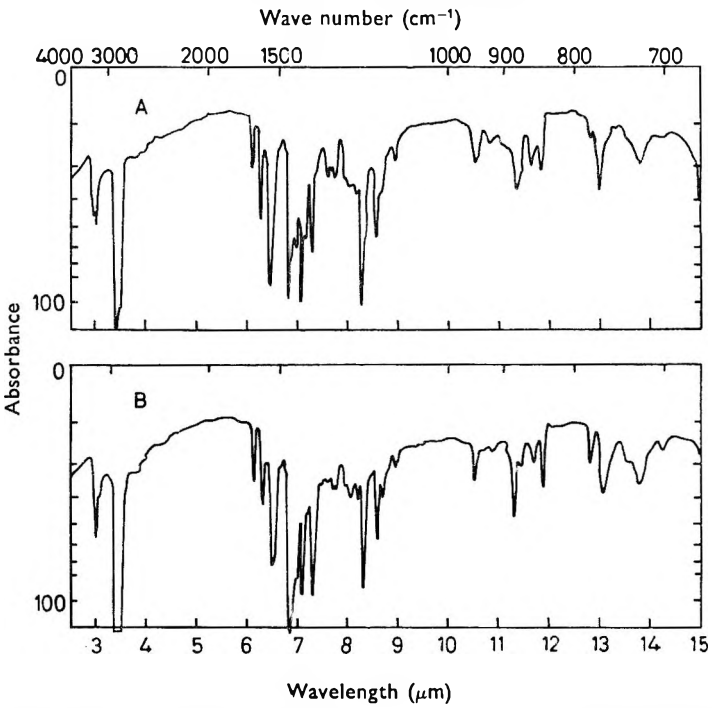


FIG. 5. A. Infrared spectrum of oxyclozanide used in crystal growth trials: 10% w/w mixture of the less soluble polymorph B in polymorph A.
B. Infrared spectrum of solid phase from 2% w/w oxyclozanide suspension extracted three days after preparation. No further growth apparent, spectrum corresponding to 100% polymorph B. Both spectra were obtained from micronized samples mulled in Nujol and read on a Perkin Elmer Infracord.

The difference between the respective bulk or lattice free energies is at present regarded as the fundamental driving force for the phase transition. The opportunity for molecules to re-orientate themselves in a crystal lattice by dissolution from one type and deposition on another evidently results in a lower energy barrier to the change than exists for re-orientation entirely within the solid phase. It was in fact impossible to induce changes in either polymorph of oxyclozanide in the dry state at temperatures between −80° and 150°. Such facilitation of phase changes by solvent mediation would account for many precipitation and filtration problems, particularly in the dyestuffs industry when unwanted colour changes may occur.

Although many stirred systems are said to be diffusion-controlled owing to a stationary layer of solution surrounding each particle, the thickness of the layer and hence the rate of dissolution, varies with the speed of stirring. In some cases the term "surface-controlled" has been applied to crystals growing in fast-streaming solutions, and there is some loose terminology in the literature on this point. In quiescent suspensions the "diffusion layer" extends throughout the disperse medium, and in the absence of surface inhibitors the system may truly be termed "diffusion controlled".

*Acknowledgements*

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# Stabilization of oil-in-water emulsions by non-ionic detergents: the effect of polyoxyethylene chain length

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The stability of emulsions of anisole and chlorobenzene in water has been estimated by following microscopically the size distribution of emulsions as a function of time. The effect of increasing the length of the polyoxyethylene glycol chain of the hexadecyl ether non-ionic stabilizers from three units to nine units was determined. Electrophoretic measurements indicated that the higher stability obtained on increasing the glycol chain length is not due to an increasing surface potential: it is ascribed to an entropic effect. Stability data are tabulated as rates of coalescence ($s^{-1}$) and results are presented relating these rates to zeta-potential, surface concentration and polyoxyethylene chain length. The results are discussed qualitatively and compared with the results obtained with cetomacrogol 1000 (Elworthy & Florence, *J. Pharm. Pharmac.*, 1967, **19**, 140S).

Non-ionic detergents are used widely in the pharmaceutical and food industries as emulsifiers and stabilizers because of their relatively low toxicity. The mechanism by which they exert their stabilizing action has not been elucidated, although some studies have made progress towards fuller understanding. Mathai & Ottewill (1966a, b) in investigations into the stability of some inorganic sols obtained evidence of steric stabilization resulting from the interaction of adsorbed polyoxyethylene glycol (PEG) chains on neighbouring particles.

Steric or entropic stabilization should be a function of polyoxyethylene chain length (Heller & Pugh, 1960). The present paper attempts to clarify some of the unknown factors concerning non-ionic-stabilized dispersions. It is concerned with the stability of emulsions of anisole and chlorobenzene emulsified with the cetyl ethers of tri-, hexa- and nona-oxyethylene glycol whose behaviour at the anisole–water and chlorobenzene–water interfaces we have already discussed (Elworthy & Florence, 1969a).

By varying the polyoxyethylene chain length we have altered the characteristics of the stabilizer molecules. With a knowledge of the effect of these changes on the interfacial film and on the charge on the particles, it should be possible to assess the mode of stabilization or at least to determine the contribution of electrostatic, entropic (steric) or solvational forces. A qualitative discussion of the results is included. A more rigorous scrutiny of the results in the light of current theories of colloid stability is made by Elworthy & Florence (1969b).

## EXPERIMENTAL

The detergents used were synthetic cetyl polyoxyethylene glycol ethers, $C_{16}H_{33}[OCH_2CH_2]_xOH$ with x = 3, 6 and 9 (Elworthy & Florence, 1969a). The

oils used were anisole and chlorobenzene, whose characteristics have been described (Elworthy & Florence, 1967). They were chosen because their densities are close to unity.

The emulsions were generally prepared by ultrasonic dispersion. Measurement of particle size was made using a projection microscope immediately after their preparation. The emulsions were stored in sealed glass containers at room temperature (23° ± 1°) and particle size was measured at intervals until oil-separation was noticeable.

Particle-size data were treated according to Elworthy & Florence (1967), rates of coalescence $k(^{-1})$ being calculated from the slope of log *n versus* time plots. Here, *n* is the number of particles in a certain arbitrary volume, taken as the volume of particles observed in the initial sizing, and proportional to $\Sigma n_i d_i^3$, $d_i$ being the diameter of the particle.

Measurements of globule mobility were made using a Zeta-Meter (Zeta Meter Inc.) by dilution of a quantity of emulsion (generally 0·1 ml) into water saturated with oil. As the partitition coefficients of the detergents always favoured the oil, dilution with water rather than detergent solution was valid. (Saturation of the water with the anisole or chlorobenzene had considerable effect on the electrophoretic mobility.) The electrophoretic mobility ($u$) was calculated from v/x, where v is the mean velocity of at least ten particles and x is the field strength. The calculation of zeta-potential, $\xi$, from mobility measurements is simple only when $\kappa a$ is very large ($\geqslant 100$) or very small ($\leqslant 0{\cdot}1$). $\kappa$ is the reciprocal Debye-Hückel length in $cm^{-1}$ and $a$ is the radius of the particle in cm. For intermediate values of $\kappa a$ a variable $\kappa$-dependent correction must be applied. The value of $\kappa a$ for the anisole–water and chlorobenzene–water systems being about 6·5 these corrections must be applied. Henry's (1931) equation was employed in the form:

$$u = f(\kappa a)\frac{\epsilon\xi}{4\pi\eta}$$

where $f(\kappa a)$ was obtained from Fig. 1, curve 1 of Henry's paper.

For $\log \kappa a = 0{\cdot}812$, $f(\kappa a) = 0{\cdot}762$. Thus

$$u = \frac{0{\cdot}762}{4\pi\eta}\,\epsilon\,\xi$$

where $\eta = 0{\cdot}01005$ poise and $\epsilon = 80$. We have quoted the values of $u$ in $\mu m\ s^{-1}\ V^{-1}\ cm^{-1}$. For conversion of these results into zeta-potentials (mV) the relation is

$$\xi = 18{\cdot}66\,u$$

Consideration of the paper of Wiersema, Loeb & Overbeek (1966) shows there is only a small error, of 1 to 2·5 mV, in using Henry's equation in calculations of $\xi$ for such values of $\kappa a$.

## RESULTS AND DISCUSSION

Some typical globule size distribution plots and how they change with time are shown in Fig. 1. The greater stability as the detergent series is ascended and the increased stability as the concentration of the $C_{16}n_3$ stabilizer is increased, is immediately apparent.

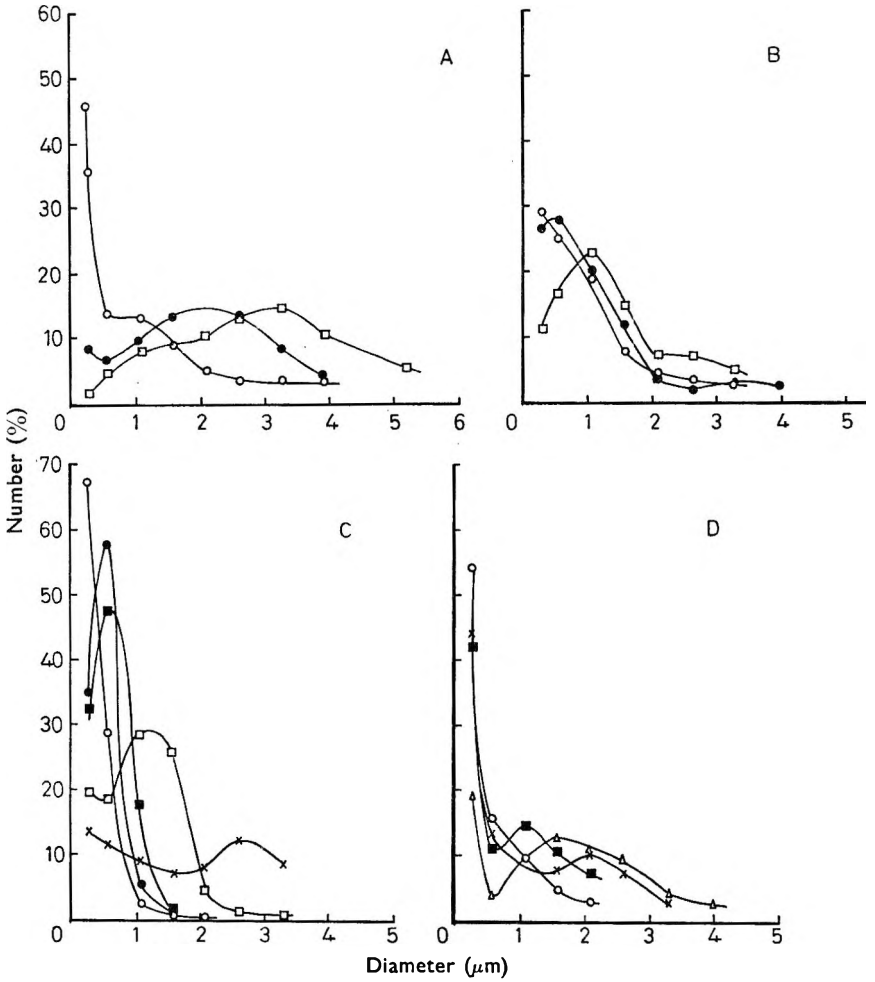


FIG. 1. Globule size distribution plots for typical emulsions of anisole and chlorobenzene showing ageing of the emulsions by changes in the number percentage–diameter distributions. A. Anisole emulsion, $\phi = 0{\cdot}20$, 0·1% $C_{16}n_2$. B. Anisole emulsion, $\phi = 0{\cdot}20$, 9·99% $C_{16}n_3$. C. Chlorobenzene emulsion, $\phi = 0{\cdot}14$, 0·1% $C_{16}n_6$. D. Chlorobenzene emulsion, $\phi = 0{\cdot}20$, 0·11% $C_{16}n_9$.
A, O 0h, ● 1h, □ 4·5h. B, O 0h, ● 3·5h, □ 3 days. C, O 0h, ● 40 min, ■ 2·5h, □ 44h, × 234h. D, O 0h, ■ 3·5h; × 6h; △ 24 days.

In this form the results are difficult to compare. The rates of coalescence ($s^{-1}$) calculated from the change in the logarithm of the number of globules $n$ in a given volume with time (Van den Tempel, 1957) are useful for comparison and are collected in Table 1. As was evident with the cetomacrogol emulsions (Elworthy & Florence, 1967), an initial fast rate of coalescence is followed by a slower rate which continues for a longer period of time. The time over which each rate holds is obviously a factor determining the state of the emulsion at some point in time. If the faster rate is due to equilibration or distribution of the detergent, the more quickly this is achieved the better the emulsion will be when the second rate begins.

$k_1$ for chlorobenzene emulsions stabilized with 1·0, 5·0 and 7·8% $C_{16}n_3$ holds over 6, $5\frac{3}{4}$ and 2 h respectively while $k_2$ was followed up to 180 h. In the cetomacrogol emulsions $k_1$ held up to about 4 days and the particle sizes of many of these emulsions were measured for ~100 days. The general trend is that $k_1$ persists longer as the poly-

oxyethylene chain length is increased, and, for any one detergent, the duration of $k_1$ decreases as the concentration of detergent is increased.

Table 1. *Rates of coalescence ($k_2$ $s^{-1}$) of emulsions of anisole and chlorobenzene in water stabilized with polyoxyethylene glycol ethers*

| | Anisole | | | Chlorobenzene | | |
|---|---|---|---|---|---|---|
| Detergent | Detergent (%) | Phase volume ($\phi$) | Rate ($10^6$ $k_2$) | Detergent (%) | Phase volume ($\phi$) | Rate ($10^6$ $k_2$) |
| $C_{16}n_3$ | 0·1* | 0·20 | 7·2 | 0·1* | 0·20 | 27·4 |
| | 1·0* | 0·20 | 17·2 | 1·0* | 0·10 | 1·44 |
| | 5·0* | 0·20 | 2·7 | 1·0* | 0·20 | 1·11 |
| | 10·0* | 0·20 | 0·31 | 1·0* | 0·50 | 1·67 |
| | 10·0* | 0·10 | 0·12 | 4·9* | 0·10 | 0·52 |
| | 10·0* | 0·20 | 0·42 | 4·9* | 0·20 | 0·53 |
| | — | — | — | 7·8* | 0·20 | 1·67 |
| | — | — | — | 10·0* | 0·02 | 0·26 |
| | — | — | — | 10·0* | 0·05 | 0·06 |
| | — | — | — | 10·0* | 0·20 | 0·11 |
| $C_{16}n_6$ | 0·11 | 0·20 | 4·72 | 0·1 | 0·14 | 3·61 |
| | 0·11* | 0·05 | 2·81 | 0·5 | 0·20 | 2·67 |
| | 1·0* | 0·20 | 0·29 | 1·0* | 0·20 | 0·44 |
| | 1·0 | 0·02 | 6·21 (20°) | 5·0* | 0·20 | 0·14 |
| | 1·0 | 0·02 | 22·8 (30°) | 10·0* | 0·10 | 0·44 |
| | 1·0 | 0·05 | 32·5 | — | — | — |
| | 1·0 | 0·20 | 5·7 | — | — | — |
| | 5·0* | 0·20 | 0·24 | — | — | — |
| $C_{16}n_9$ | — | — | — | 0·11 | 0·20 | 2·7 |
| | — | — | — | 0·1* | 0·10 | 1·04 |
| | 0·1 | 0·20 | 2·78 | 0·1* | 0·02 | 0·24 |
| | 0·1* | 0·20 | 0·46 | 1·0* | 0·20 | 0·70 |
| | 1·0 | 0·20 | 0·21 | 1·0 | 0·20 | 0·27 |
| | — | — | — | 1·0 | 0·20 | 0·19 |
| | 5·0* | 0·20 | 0·24 | 5·0 | 0·20 | 0·05 |
| | — | — | — | 5·0* | 0·20 | 0·23 |
| | — | — | — | 10·0* | 0·20 | inverts |

* Surfactant dissolved in oil.

Table 1 shows that the values of $k_2$ (the second rate) of some of the emulsions prepared with high concentrations of short chain detergents approach the earlier reported values obtained for emulsions stabilized with cetomacrogol (Elworthy & Florence, 1967), but the second rate of coalescence of the latter holds over longer periods.

The results will be discussed under two main headings.

*Effect of detergent concentration.* The critical micelle concentration determined in simple aqueous solution bears no relation to the apparent critical micelle concentration (CMC) obtained in the presence of polar oil phase (Elworthy & Florence, 1969a). Increasing the surfactant concentration above that apparent CMC in emulsions stabilized by $C_{16}n_3$, $C_{16}n_6$ and $C_{16}n_9$ resulted, in general, in increased stability. This is in contrast to the behaviour of $C_{16}n_{25}$ (cetomacrogol 1000) which when present in high concentrations caused a slight increase in the rate of breakdown.

Fig. 2 shows the slower rate of growth in the presence of increasing concentrations of $C_{16}n_3$. Here the mean number diameter ($dn_m$) is plotted against time, where

$$dn_m = \left(\frac{\Sigma n_i d_i^2}{\Sigma n_i}\right)^{\frac{1}{2}}$$

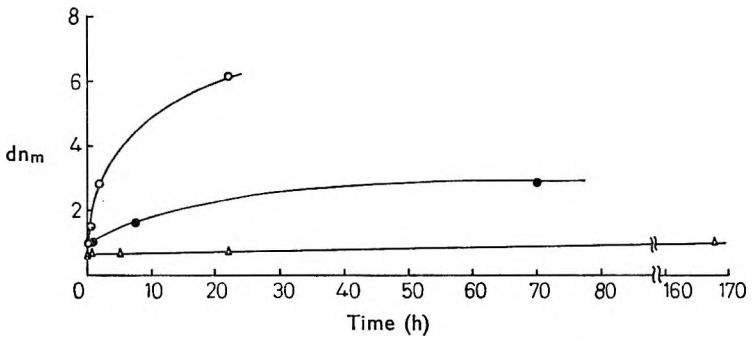


FIG. 2. Plot of $dn_m$ *vs* time (h) showing the rate of growth of anisole emulsions, $\phi = 0{\cdot}20$ as a function of detergent concentration. ○ 1·0% $C_{16}n_3$. ● 5·0% $C_{16}n_3$. △ 9·99% $C_{16}n_3$.

In emulsions stabilized by cetomacrogol 1000 the interfacial tension is virtually constant above the CMC; in the present series, however, there is a fall in interfacial tension above the CMC.

Fig. 3 indicates that there is an empirical relation between interfacial tension ($\sigma_1$) and log $k_2$ at 0·1% surfactant levels for both anisole and chlorobenzene emulsions, but at higher concentrations results for anisole and chlorobenzene do not fall on the same line. Hence other factors influencing stability are coming into play at higher concentrations.

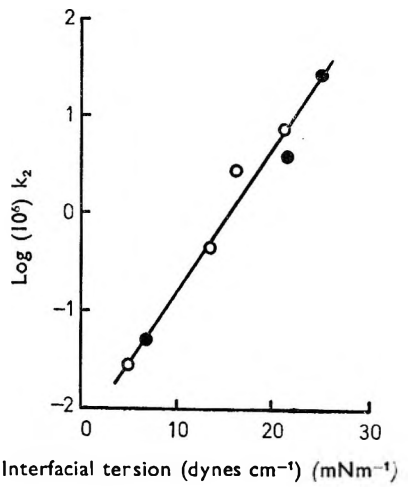


FIG. 3. Plot of log $k_2$ *vs* interfacial tension indicating an empirical relation between the stability of emulsions stabilized with 0·1% $C_{16}n_3$, $C_{16}n_6$, $C_{16}n_9$ and cetomacrogol 1000. ○ Anisole emulsions. ● Chlorobenzene emulsions.

An emulsion can never be thermodynamically stable. Stability is therefore relative and whatever the barriers to coalescence these can be overcome. Interfacial energy must be the driving force for coalescence, hence a decreased interfacial tension will inevitably result in increased stability, but at a particular interfacial tension other stabilizing factors can alter the lifetime, i.e., "stability" of the emulsion.

Table 2 lists the electrophoretic mobilities ($u$) and zeta potentials ($\xi$) of globules of anisole and chlorobenzene with varying concentrations of detergents. It is apparent that the increasing detergent concentration lowers the zeta potential of the

Table 2. *Electrophoretic mobilities and zeta-potentials*

| | Anisole | | | Chlorobenzene | | |
|---|---|---|---|---|---|---|
| Detergent | Detergent (%) | $u$ ($\mu$m $s^{-1}$ $V^{-1}$ $cm^{-1}$) | $\xi$ (mV) | Detergent (%) | $u$ ($\mu$m $s^{-1}$ $V^{-1}$ $cm^{-1}$) | $\xi$ (mV) |
| $C_{16}n_3$ | 0·1 | −4·1 | −76·5 | 0·1 | −4·2 | −78·4 |
| | 1·0 | −3·55 | −66·2 | 1·0 | −4·05 | −75·6 |
| | 5·0 | −2·35 | −43·9 | 5·0 | −3·65 | −68·1 |
| | 10·0 | −1·65 | −30·8 | 7·8 | −3·5 | −65·3 |
| | — | — | — | 10·0 | −3·3 | −61·6 |
| $C_{16}n_6$ | 0·11 | −3·7 | −69·0 | 0·1 | −4·0 | −74·6 |
| | 1·0 | −3·45 | −64·4 | 1·0 | −3·95 | −73·7 |
| | 5·0 | −3·30 | −61·6 | 5·0 | −3·75 | −69·9 |
| | — | — | — | 10·0 | −3·35 | −62·5 |
| $C_{16}n_9$ | 0·1 | −3·95 | −73·7 | 0·1 | −3·4 | −63·4 |
| | 1·0 | −3·7 | −69·0 | 1·0 | −4·0 | −74·6 |
| | 5·0 | −2·88 | −53·7 | 5·0 | −2·8 | −52·2 |
| | — | — | — | 10·0 | −2·1 | −39·2 |
| $C_{18}n_{25}$* | 0·1 | −2·0 | −37·3 | 0·1 | −1·7 | −31·7 |
| | 1·0 | −1·40 | −26·1 | 1·0 | −1·3 | −24·3 |
| | 5·0 | −0·40 | −7·5 | 5·0 | −0·35 | −6·5 |
| | 10·0 | 0 | 0 | 10·0 | 0 | 0 |

* Values for cetomacrogol mobilities are those published previously (Elworthy & Florence, 1967); the zeta-potentials are larger than the previously quoted values because of the use of the more exact Henry's equation.

globules and hence the increasing stability must be due to some source other than the interaction of double layers, although the electrical contribution is not unimportant.

*Effect of polyoxyethylene glycol chain length.* As was seen from Fig. 1, increasing PEG chain length increased stability at a given detergent concentration. Fig. 4 shows for one representative detergent concentration the variation of stability (as log $10^6k_2$) and electrophoretic mobility as a function of polyoxyethylene chain length; results for cetomacrogol are also included. The experimental quantity of electrophoretic

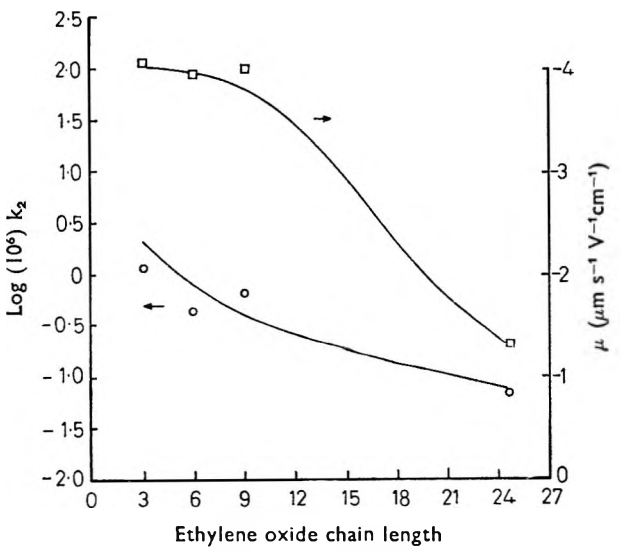


FIG. 4. Log $k_2$ and electrophoretic mobility of chlorobenzene emulsions with 1·0% detergent as a function of ethylene oxide chain length. The lower $k_2$ the greater the stability.

mobility ($u$) rather than calculated zeta-potential has been used because of doubt about the exact relation between the two (Elworthy & Florence, 1969b). It is again obvious that the electrical contribution to stability decreases as the series is ascended and hence other factors must come increasingly into play.

At 0·1% detergent levels there is a noticeable difference in stability between the $C_{16}n_3$-stabilized emulsions and those stabilized with higher members of the series, but at higher concentrations the differences are small. For example, 10% $C_{16}n_3$ and 10% $C_{16}n_{25}$ give similar rates of coalescence in emulsions of anisole of phase volume ($\phi$) 0·2.

Stryker, Helin & Mantell (1966) observed little difference in the stability of polyethylene latexes in the presence of 9·5 and 16 unit octyl phenyl polyoxyethylene condensates and at longer chain lengths a decrease in stability was observed.

The particle size distribution plots in Fig. 1 show that for $C_{16}n_3$ emulsions, the peak of the distribution moves rapidly to higher diameters; with $C_{16}n_6$ the increase is not so rapid and the decrease in the number percent of particles below 0·5 $\mu$m is slower. Emulsions stabilized with $C_{16}n_9$ sometimes showed a bimodal distribution (see Fig. 1D) in which the smallest particles which could be observed microscopically ($\sim$0·5 $\mu$m) appear to be more stable than those of the 0·5 $\mu$m–1·5 $\mu$m range. The result is that the numbers of particles in the 0·5 $\mu$m–1·0 $\mu$m range decrease more rapidly than those in the smallest range, and two maxima appear.

*Kinetics of breakdown*

Emulsions stabilized with cetomacrogol exhibited two phase breakdown, a rapid initial change in particle number ($n$) followed by a slower rate during which log $n$ varied linearly with time (Elworthy & Florence, 1967); in addition, a function of globule surface area $\Sigma^{-1}$ defined previously (see Hill & Knight, 1965) was linear with time and the Smoluchowski equation, which is essentially $\Sigma^{-3} = ct + d$, was found not to hold. The same applies to the present series, in spite of these emulsions being less stable. They must still be regarded as "slowly coagulating systems" in terms of the kinetics of breakdown. For the more common examples of rapid coagulation, the time for coagulation is of the order of seconds (Verwey & Overbeek, 1948). In Fig. 5 plots of log $n$ and $\Sigma^{-1}$ and $\Sigma^{-3}$ versus time are given, showing the non-linear variation of $\Sigma^{-3}$ for an emulsion of chlorobenzene ($\phi = 0{\cdot}14$) stabilized by 0·1% $C_{16}n_6$.

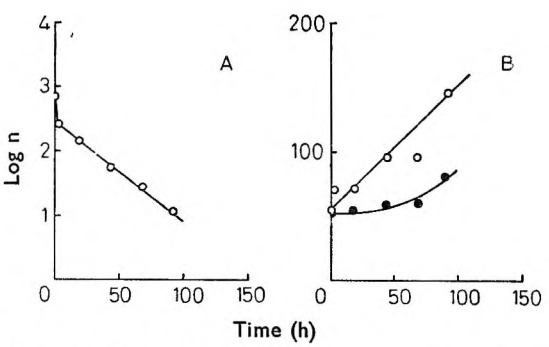


FIG. 5. A. Plot of log n *vs* time (h) for a chlorobenzene emulsion, $\phi = 0{\cdot}14$, stabilized with 0·1% $C_{16}n_6$ from which $k_1$ and $k_2$ can be calculated. Ordinate: log n. B. Hill & Knight plot for the same emulsions of $\Sigma^{-1}$ (⊙) and $\Sigma^{-3}$ (●) *vs* time indicating the linearity of the former within experimental error. Ordinate in arbitrary units. ○ = 104 (interfacial area, cm²)/$\pi$ and ● = $\Sigma^{-3} \times 10^4 + 50$.

*Phase volume* ($\phi$)

In the less stable emulsions the effect of varying the phase volume of the disperse phase is pronounced. Fig. 6 illustrates the effect of phase volume on the rate of breakdown of an anisole emulsion.

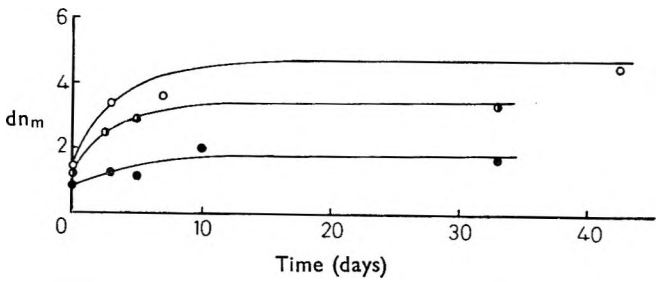


FIG. 6. Plot of $dn_m$ (mean number diameter) *vs* age of emulsion (days) for an anisole emulsion stabilized with 9·99% $C_{16}n_3$ showing the effect of phase volume. ● $\phi = 0{\cdot}02$, ◑ $\phi = 0{\cdot}10$ ○ $\phi = 0{\cdot}20$.

The ratio of the rate of coalescence of the 0·20 and 0·02 phase volume anisole emulsions (9·99% $C_{16}n_3$, interfacial tension ~5 $mNm^{-1}$) is 1·4, while for the 0·1% $C_{16}n_9$ chlorobenzene emulsions (interfacial tension ~13 $mNm^{-1}$) the ratio is about 4. The increase in interfacial energy in the latter case is greater and even if the stabilizing forces remained the same at both $\phi = 0{\cdot}02$ and 0·20, the driving force for coalescence would be much greater in the latter emulsion.

*Zeta-potentials*

The reduction in effective electrical repulsion on increasing phase volume due to the overlap of broad double layers, is discussed by Albers & Overbeek (1959). This might be a factor which should be taken into account with the present systems. For practical reasons, zeta-potentials are determined in extremely dilute emulsions and the extrapolation to real systems is not always straighforward. However, the percentage reduction in the repulsive energy which results from the initial overlap of double layers may be calculated, using Albers & Overbeek's (1959) derivation, and this indicates a 6·7% correction to be applicable. This is deemed unimportant in view of the other approximations used.

When the detergent is dissolved in the oil phase initially, slower rates of coalescence are observed. As an example, when a chlorobenzene emulsion was stabilized with 0·1% detergent $C_{16}n_9$ in the *oil* $\phi = 0{\cdot}2$, $k_2$ had a value of $10{\cdot}4 \times 10^{-7}$ $s^{-1}$. With 0·11% detergent in the *water*, a value of $26{\cdot}9 \times 10^{-7}$ $s^{-1}$ was observed. For the equivalent anisole emulsion with detergent in the aqueous phase, $k_2$ was $27{\cdot}8 \times 10^{-7}$ $s^{-1}$. Prigorodov, Nikitina & Taubman (1965) noted a similar effect in emulsions of xylene in water with the emulsifier octyl phenyl nonaoxyethylene glycol ether ($OPn_{10}$). With 0·25% $OPn_{10}$ in the xylene there resulted a more rapid and more effective stabilization than the same concentration in the aqueous phase. This is perhaps due to re-equilibration in the system. As $K_w^o$ for the aqueous $C_{16}n_9$-anisole and chlorobenzene system is high,* at equilibrium most of the detergent will be in the oil. It can be envisaged that the higher interfacial tension of the

* Rough $K_w^o$ values have been determined for chlorobenzene. These are: $C_{16}n_8$ 100 (0·1%), 300 (1%), $C_{16}n_9$ 50 (0·1%), 120 (1·0%), cetomacrogol, 8·8 (0·2%), 4·9 (1%). These are expected to be approximate because of the lack of sensitivity of existing assay procedures at low concentrations of non-ionics.

non-equilibrated system will lead to a more rapid breakdown, hence when the second rate of coalescence comes into play the mean particle size is already greater and the particles exhibit a faster rate of coalescence. The effect of initial particle size has been noted in anisole emulsions stabilized with 1·0% $C_{16}n_9$ (in the oil phase), that emulsion having the larger initial mean number diameter grew more rapidly than a finer emulsion.

Emulsions stabilized with $C_{16}n_3$, $C_{16}n_6$ and $C_{16}n_9$ are sensitive to increased temperature. An emulsion stabilized with 1% $C_{16}n_9$ broke after 2 days at 30° and after 1 day at 37°. At 20° the rate of coalescence of 1% $C_{16}n_6$-anisole emulsion was $6{\cdot}2 \times 10^{-6}$ $s^{-1}$ and at 30°, $22{\cdot}8 \times 10^{-6}$ $s^{-1}$. Using standard equations the enthalpy of coalescence was found to be 20 Kcal, which might suggest that desolvation is involved in the process of coalescence.

*Summary of results*

Broad trends have been illustrated in this paper: the effect of detergent concentration, the effect of surfactant chain length, of phase volume and temperature. The electrophoretic properties of the emulsion globules indicate the magnitude of the surface charge of the particles and give some indication of the contribution of electrical double-layer repulsion to the stabilizing forces. But it is apparent that a reduction in zeta-potential with increasing detergent concentration and chain length is accompanied by an increase in stability. Therefore, as with cetomacrogol, an electrical component must be taken into account, but it is a secondary stabilizing factor; the "entropic" mechanism must then be involved.

In qualitative terms, an increase in polyoxyethylene chain length will increase the closest distance of approach of two particles and, other things being equal, minimize the attractive forces between them. But the magnitude of the entropic stabilizing force is not known and the effect of the adsorbed layer on the attraction between two globules is not known.

*Acknowledgements*

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# Stabilization of oil-in-water emulsions by non-ionic detergents: electrical and entropic contributions to stability

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Calculations of the attractive and repulsive forces between globules of anisole and chlorobenzene in the presence of non-ionic detergents of varying polyoxyethylene chain length are presented. The equations of the Derjaguin–Landau–Verwey–Overbeek theory are used and the effect on the attractive potential of the adsorption of stabilizer onto the particle is taken into account. The adsorbed film was found to *increase* the attractive forces between the particles, $V_A$. In the previous paper (Elworthy & Florence, *J. Pharm. Pharmac.*, 1969, **21**, 70) it was shown that the electrical contribution to stability is a secondary stabilizing factor. More quantitative estimates of this contribution have been made. The entropic repulsion has been calculated using an equation of Ottewill & Walker. The energy-distance diagrams are discussed in the light of the experimental results presented in the previous paper.

Previous papers (Elworthy & Florence, 1967, 1969a, b) have dealt with experimental results on the stabilization of emulsions of anisole and chlorobenzene dispersed in water in the presence of non-ionic detergents of the polyoxyethylene ether class. In those papers the results were discussed qualitatively. The present paper attempts to correlate the experimental findings with the theoretical predictions for stability as given by the theory of stability of lyophobic colloids derived by Derjaguin, Landau, Verwey and Overbeek (D.L.V.O.); it includes a discussion of the contribution to stability from the interaction of the adsorbed polyoxyethylene chains on neighbouring particles, the so-called "entropic" contribution.

Modern theories of colloid stability agree that it is the balance between various attractive and repulsive forces operating between the dispersed particles that governs stability. The D.L.V.O. theory (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948) has been reasonably successful in interpreting the flocculation of suspensions, but according to Vold & Vold (1965) the behaviour of emulsion systems deviates widely from its predictions.

Two main differences between emulsion droplets and solid dispersion particles can be listed: (i) the non-rigidity of the emulsion particles which can result in dispersal of energy and change in shape on the close approach of two particles, and (ii) the different criterion of stability which is coalescence and not flocculation. In emulsions, coalescence (i.e., the irreversible union of two or more globules to form one) may occur after flocculation but does not necessarily follow from it.

It was found (Elworthy & Florence, 1967) that electrical contributions to stability in emulsions of anisole and chlorobenzene stabilized with cetomacrogol 1000 are of secondary importance; reduction in the zeta-potential of the particles with increasing

concentration of cetomacrogol resulted in a slight increase in the rate of coalescence. However, salts at low concentration levels markedly affect the stability of non-ionic emulsions (Elworthy, Florence & Rogers, unpublished) and hence an electrical contribution to the repulsive forces must be included in any formulation of the forces between the particles. This, of course, does not preclude the possibility that salts dehydrate or alter the conformation of the polyoxyethylene chain, rendering in the first instance the particle more hydrophobic, or in the second the stabilizing layer less obtrusive.

Vold (1961) has calculated that the adsorbed detergent layer at the globule surface influences the value of the Hamaker constant, but Derjaguin (1965) has doubted whether attentuation of van der Waals' forces of attraction is the main reason for the stabilizing properties of solvated polymeric chains.

In this paper calculations have been made of the forces of attraction and repulsion operative in the present o/w emulsion systems in order to assess the role of the adsorbed layer in contributing to stability. Unfortunately, the entropic contribution to stability—the decrease of entropy caused by the intermingling of the polyoxyethylene chains on approaching particles and the consequent energy rise—has not been satisfactorily formulated. As a working basis, calculations have been made on the assumption that the particles were not deformed and that the detergent is not desorbed on close approach of the particles. The results of these calculations are presented in the first part of the paper. In the second part some possible reasons for deviation from behaviour as predicted by these calculations are given.

*Attractive forces*

Attractive forces between colloidal particles are due to van der Waals' forces. Between atoms, these forces are of short range, but when the London contribution (London, 1937) is summed over all the atoms on two colloidal particles, the attractive forces are long range. For two spherical particles, both of radius a, at a distance of separation between their surfaces, H, under the condition that $H/2a \ll 1$

$$V_A = \frac{-Aa}{12H} \qquad (1)$$

where $V_A$ is the mutual energy of attraction, and A is the Hamaker constant (Hamaker, 1937) defined by

$$A = \pi^2 q^2 \mu$$

where q is the number of molecules per $cm^3$ of material and

$$\mu = 3hv_o\alpha_c^2$$

in which h = Planck's constant, $v_o$ is the characteristic frequency of the molecules, and $\alpha_o$ their polarizability.

Since $v_o$ can be derived from refractive index data, the refractive indices of the four detergents were measured at the blue, green, and yellow lines of a mercury arc lamp using a Pulfrich refractometer. A temperature of 45° was used to ensure that all detergents were liquid; the difference in polarizabilities between 45° and 20° (the temperature of the stability work) was ignored. Refractive index data for anisole, chlorobenzene and water were obtained from Timmermans (1950) and Kaye & Laby (1957). Using a dispersion equation of the form (Bauer & Lewin, 1960)

$$\frac{1}{\dot{\psi}} = \frac{v_0^2}{c} - \frac{v^2}{c} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where $\dot{\psi} = (n^2 - 1)/(n^2 + 2)$, c is a constant, and v is the frequency of light at which the refractive index, n, is measured, $v_0$ was determined. As the value of $\dot{\psi}$ at zero frequency $(\dot{\psi})_v{}^2 = 0$ can be obtained by a graphical treatment of equation (2),

$$\alpha_0 = \frac{3\, V_m(\dot{\psi})_{v^2=0}}{4\pi N}$$

where $V_m$ is the molar volume. The values of the relevant quantities are given in Table 1.

Table 1. *Values of characteristic frequencies, polarizabilities and Hamaker constants of water, oils and detergents*

| | $v_0 \times 10^{-15}$ $s^{-1}$ | $\alpha_0 \times 10^{24}$ $cm^3\ mol^{-1}$ | $\mu \times 10^{57}$ erg $cm^6\ mol^{-2}$ | $A \times 10^{13}$ erg |
|---|---|---|---|---|
| Water .. .. | 3·27 | 1·45 | 0·0344 | 3·78 |
| Anisole .. .. | 2·54 | 12·52 | 1·98 | 6·05 |
| Chlorobenzene .. | 2·57 | 11·86 | 1·80 | 6·29 |
| $C_{16}n_3$ .. .. | 3·52 | 43·48 | 33·4 | 6·57 |
| $C_{16}n_6$ .. .. | 3·46 | 57·00 | 55·8 | 6·65 |
| $C_{16}n_9$ .. .. | 3·46 | 69·25 | 82·5 | 6·71 |
| $C_{16}n_{25}$ .. .. | 3·77 | 124·9 | 291·9 | 7·46 |

From Table 1 it is apparent that the Hamaker constants of the detergents are greater than those of the oils used; hence the effect of coating an oil particle with surfactant will be to increase the attractive force between particles.

*Influence of medium and adsorbed films.* The above Hamaker constants are for interactions between particles in a vacuum. If $A_{11}$ is the constant for the oils *in vacuo* and $A_{22}$ the constant for interaction between water molecules, then the Hamaker constant for oil particles in water is given by

$$A = (A_{11}^{\frac{1}{2}} - A_{22}^{\frac{1}{2}})^2$$

For anisole–water $A = 2{\cdot}6 \times 10^{-14}$ and for chlorobenzene–water $A = 3.1 \times 10^{-14}$ erg; the variation of the energy of attraction with distance can be calculated from equation (1).

A further modification is required when the particles are coated with a homogeneous adsorbed film (Vold, 1961). If the thickness of the film is $\delta$ and the surfaces of the coated particles are a distance $\Delta$ apart, Ottewill (1967) gives the equation

$$V_A = -\frac{1}{12}\left[(A_{22}^{\frac{1}{2}} - A_{33}^{\frac{1}{2}})^2\left(\frac{a+\delta}{\Delta}\right) + (A_{33}^{\frac{1}{2}} - A_{11}^{\frac{1}{2}})^2\left(\frac{a}{\Delta + 2\delta}\right)\right.$$
$$\left. + \frac{4a\,(A_{22}^{\frac{1}{2}} - A_{33}^{\frac{1}{2}})\,(A_{33}^{\frac{1}{2}} - A_{11}^{\frac{1}{2}})\,(a+\delta)}{(\Delta+\delta)\,(2a+\delta)}\right] \quad .. \quad .. \quad .. \quad (3)$$

in which $A_{33}$ is the Hamaker constant of the adsorbed film. Equation (3) permits evaluation of the attraction between particles with adsorbed layers of different thickness as a function of their distance apart. We assume in using equation (3) that the particles are monodisperse, that the adsorbed film is composed of detergent only, that it is homogeneous. Although none of these assumptions is entirely correct,

we are more concerned with the general picture emerging from the calculations than with details.

The values of $V_A$ for anisole and chlorobenzene, calculated from equation (3), are very similar (1–2%) when coated particles are considered. In choosing a value for the film thickness, $\delta$, two limits can be used. The first is the fully extended length of the surfactant monomers, measured from atomic models, which are 36, 50, 64 and 138Å for $C_{16}n_3$, $C_{16}n_6$, $C_{16}n_9$ and $C_{16}n_{25}$ respectively. As the molecules are known to be contracted when present in a micelle, with total lengths of *ca* 32, 40, 46 and 48 Å respectively (Elworthy, 1960; Elworthy & Macfarlane, 1962, 1963; Elworthy & McDonald, 1964), these figures provide a more realistic set of values for film thickness than the fully extended lengths. In Fig. 1a, the effect on $V_A$ of adsorbed films of

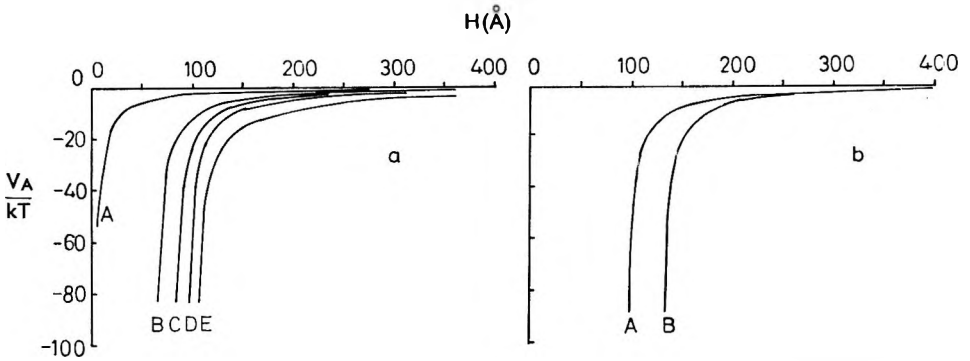


FIG. 1 a. The effect of an adsorbed layer of thickness δÅ on $V_A$, as a function of the distance between the surfaces of the particles, H. A. No adsorbed film, B. $C_{16}n_3$, $\delta$ = 32Å, C. $C_{16}n_6$, $\delta$ = 36Å, D. $C_{16}n_9$, $\delta$ = 40Å, E. $C_{16}n_{25}$, $\delta$ = 43Å. Values of A (Hamaker constant) as recorded in Table 1.

b. The effect of altering $\delta$ on $V_A$ for the detergent $C_{16}n_9$. A. $\delta$ = 45·4Å, B. $\delta$ = 64Å. Value of A (Hamaker constant) as recorded in Table 1.

thickness equal to the micellar radius is given. The attraction between the particles is increased considerably when the film is present. Fig. 1b shows how increasing film thickness increases attraction between particles for a film of $C_{16}n_9$. In all subsequent calculations $\delta$ is taken as the length of the surfactant monomer in a micelle. This choice is made after consideration of the areas/molecule found at the relevant oil–water interfaces (Elworthy & Florence, 1969a) which are much greater than those which would be expected for fully extended molecules.

*Repulsive forces*

(i) *Entropic.* A number of workers have calculated the repulsive energy between two particles arising from the interaction of adsorbed polymer chains (Clayfield & Lumb, 1966; Meier, 1967; Fischer, 1958; Ottewill & Walker, 1968). Meier (1967) considers the loss of possible chain configurations on close approach of the particles and the change in free energy of mixing of polymer and solvent as the chains interact. Fischer (1958) obtained the following equation on consideration of interactions in the volume of overlap of the adsorbed layer, thickness $\delta$ on particles of radius *a*

$$V_{RS} = \frac{4\pi}{3} B'c^2 \left[\delta - \frac{H}{2}\right]^2 \left[3a + 2\delta + \frac{H}{2}\right] \quad .. \quad .. \quad (4)$$

B′, the second virial coefficient, takes account of solvent-solute interactions, c is the concentration (g $ml^{-1}$) of the surfactant in the stabilizing layer, and H is the distance between the surfaces of the uncoated particle. A modified form of this equation has been used by Ottewill & Walker (1968) in which B′ is replaced by the term $(\psi_1 - \chi_1)/V_1\rho_2^2$. $\psi_1$ is an entropy term equal to 0·5 and $V_1$ is the solvent molecular volume. $\chi_1$ is a solvent-polymer interaction parameter which Ottewill & Walker take to be from 0·25 to 0·40 for $C_{12}n_6$, Malcolm & Rowlinson (1957) having obtained a value of ~0·35 for a polyoxyethylene glycol in water. The entropic repulsive force, $V_{RS}$, was calculated using the modified equation

$$V_{RS} = \frac{4\pi c^2}{3V_1\rho_2^2}\left[\psi_1 - \chi_1\right]\left[\delta - \frac{H}{2}\right]^2\left[3a + 2\delta + \frac{H}{2}\right] \quad .. \quad .. \quad (5)$$

c was calculated from the surface excess concentrations of the detergents obtained by Elworthy & Florence (1969a).

The choice of $\chi_1$, on which the magnitude of the calculated $V_{RS}$ largely depends, is a more difficult problem. As $\chi_1$ is a function of phase volume it is reasonable to expect that $\chi_1$ will vary with $\Gamma$, that is, with c. Variation of $\chi_1$ with c may be estimated in a very approximate manner by using values of $\chi_1$ *vs* volume fraction obtained by Malcolm & Rowlinson (1957). In this the variation of $\chi_1$ with c along a homologous series was obtained (Table 2).

The values of $\delta$ chosen from micellar radii gives values of c which appear to be reasonable if one calculates the hydration of the detergents in the film. For cetomacrogol c = 0·302 g $ml^{-1}$ gives approximately a hydration of 2·3 g water per g detergent, which agrees closely with the micellar hydration obtained by Elworthy (1960). If $\delta$ is chosen as the extended length of the molecule, i.e., 138Å, then c = 0·088 g $ml^{-1}$ and the apparent hydration rises to 8·2 g $g^{-1}$ which, although it might include the unbound water separating the detergent chains, does not agree with experimental values of hydration as does the former value.

Fig. 2a and b shows $V_{RS}$ as a function of H for 1 $\mu$m globules stabilized with the non-ionic detergents and shows the effect of polyoxyethylene chain length using the values of $\delta$ and $\chi_1$ listed in Table 2. When equal values of $\chi_1$ are used for all detergents $V_{RS}$ does not increase in the way shown for small values of H (<30Å) but at greater distances the same trends apply.

Table 2. *Surface excess concentrations* ($\Gamma$), *concentrations* (c) *and solvent interaction parameters* ($\chi_1$)

| Detergent | Oil | $\Gamma$* mol $cm^{-2}$ | $\delta$ (Å)† | c g $ml^{-1}$ | $\chi_1$ | $(\psi - \chi_1)$ |
|---|---|---|---|---|---|---|
| $C_{16}n_3$ | Anisole | $3{\cdot}69 \times 10^{-10}$ | 32 | 0·43 | 0·44 | 0·06 |
| $C_{16}n_3$ | Chlorobenzene | $3{\cdot}46 \times 10^{-10}$ | 32 | 0·41 | 0·41 | 0·09 |
| $C_{16}n_6$ | Anisole | $2{\cdot}97 \times 10^{-10}$ | 40 | 0·38 | 0·37 | 0·13 |
| $C_{16}n_6$ | Chlorobenzene | $2{\cdot}08 \times 10^{-10}$ | 40 | 0·26 | 0·24 | 0·26 |
| $C_{16}n_9$ | Anisole | $2{\cdot}19 \times 10^{-10}$ | 46 | 0·31 | 0·30 | 0·20 |
| $C_{16}n_9$ | Chlorobenzene | $1{\cdot}66 \times 10^{-10}$ | 46 | 0·23 | 0·22 | 0·28 |
| $C_{16}n_{25}$ | Anisole | $1{\cdot}08 \times 10^{-10}$ | 48 | 0·30 | 0·28 | 0·22 |
| $C_{16}n_{25}$ | Chlorobenzene | $0{\cdot}90 \times 10^{-10}$ | 48 | 0·25 | 0·23 | 0·27 |

* From interfacial tension data (Elworthy & Florence, 1969a).
† $\delta$ = micellar radius, i.e., non-extended length of molecule.
*Note.*—When the effect of alteration in $\delta$ is considered c is correspondingly altered; the values of c quoted have been adjusted in proportion, e.g., for $C_{16}n_{25}$ when $\delta$ = 138Å, c for the chlorobenzene system is 0·088 g $ml^{-1}$.

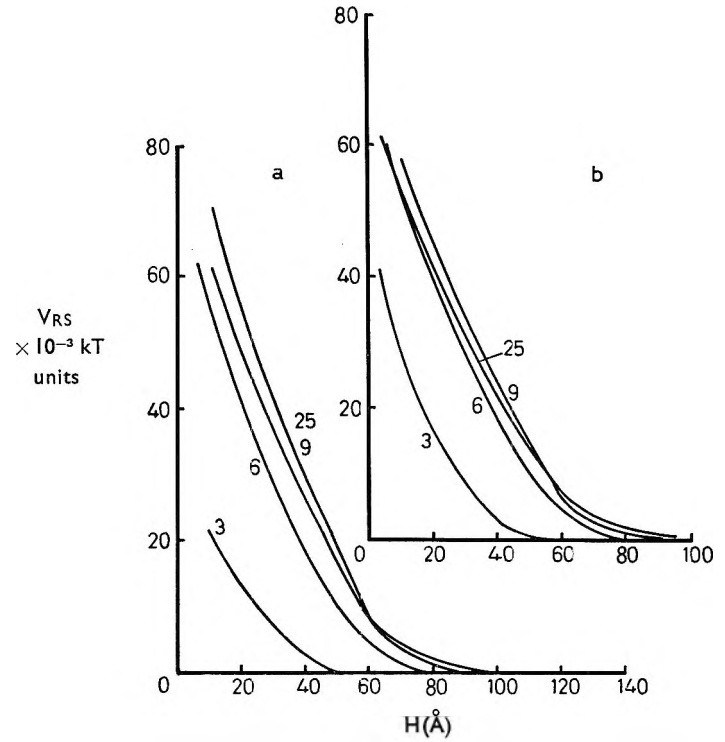


FIG. 2. The entropic stabilizing force $V_{RS}$ as a function of distance of approach of the particles, H. (a) for anisole, (b) for chlorobenzene emulsions for four non-ionic detergents $C_{16}n_3$, $C_{16}n_6$, $C_{16}n_9$, $C_{16}n_{25}$. Ethylene oxide chain length marked. δ as in Fig. 1.

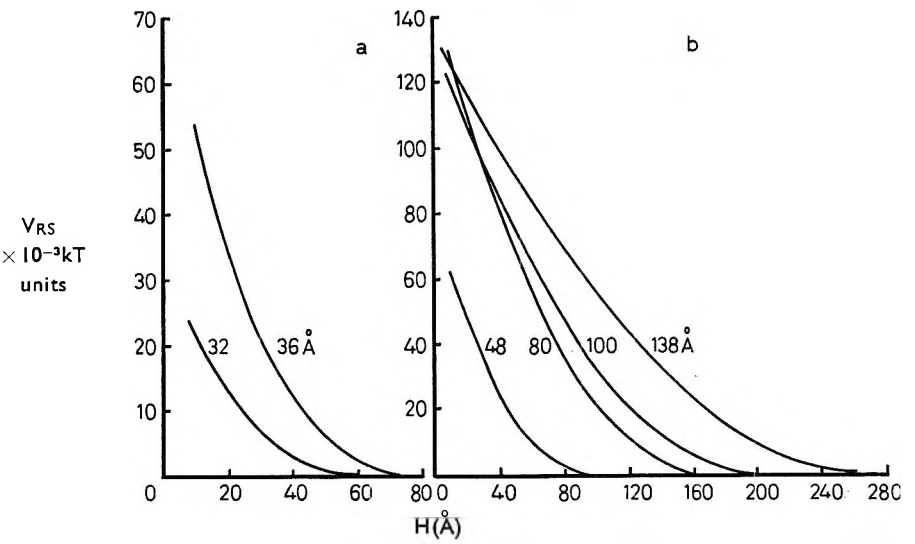


FIG. 3. The effect of $V_{RS}$ *vs* H plots of altering δ for (a) $C_{16}n_3$ and (b) $C_{16}n_{25}$. δ(Å) as marked on diagram.

The effect of altering δ for $C_{16}n_3$ and cetomacrogol 1000 ($C_{16}n_{25}$) is illustrated in Fig. 3, giving the expected increase in $V_{RS}$ with increasing δ. Obviously some choice has to be made in the absence of experimental data of a value of δ and for bulk of the calculations of total interaction curves the micellar radius δ has been chosen.

Whilst the equation of Ottewill & Walker refers to a homogeneous adsorbed layer, this is not the situation with the non-ionic detergent layer. This can be overcome to some extent by consideration of the polyoxyethylene glycol layer only. Some calculations of $V_{RS}$ on this basis are summarized in Fig. 4. These show the same trend of an increasing entropic repulsion as the ethylene oxide chain length is increased, but there is a greater variation up the series and in two cases negative values of $V_{RS}$ are obtained for anisole systems. The relative merits of considering the adsorbed layer to be detergent or glycol are discussed later.

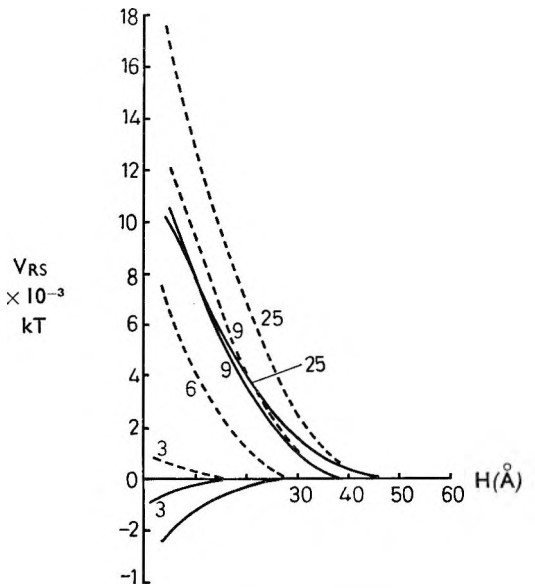


FIG. 4. Entropic stabilizing force, $V_{RS}$, as a function of distance of approach of particles, H, taking only the polyoxyethylene glycol region into account. Ethylene oxide chain length marked on plots. Solid lines anisole, broken lines chlorobenzene emulsions.

(ii) *Electrical forces.* The electrical contribution to stability $V_R$ was calculated using the equation

$$V_R = \tfrac{1}{2}\epsilon a \dot{\psi}_0^2 \ln [1 + \exp(-\kappa H)] \quad .. \quad .. \quad .. \quad (6)$$

where $\epsilon$ = dielectric constant, a is the particle radius, $\kappa$ the reciprocal Debye-Hückel length and H the distance between the surfaces of the particles. The zeta-potential, $\xi$, was substituted for $\dot{\psi}_0$, the values from Elworthy & Florence (1969; Table 2) being used. The equation is valid for systems in which $\kappa a \geqslant 1$ and for separations, H, large compared with $1/\kappa$. We have used equation (5) for smaller values of H, and when $V_R$ is compared with the energy of repulsion taken from the tables* of Verwey & Overbeek (1948), the error is not large when the other uncertainties are taken into account, namely the substitution of zeta-potential for $\dot{\psi}_0$. In the present system, where the ionic concentration is extremely low, the error in equating $\xi$ and $\dot{\psi}_0$ is a small one because of the slow decay of the double layer potential. In any case, the error will be uniform throughout the series.

* Tables XV–XX, on pp. 152–155 extended by extrapolation to the value of $\kappa a$ used in the present system.

At close distances of approach the distortion of the globules or the formation of a flat lamella between them will increase the repulsive forces as the area of contact will be increased. This refinement has been neglected.

The effect of detergent concentration above the CMC is not reflected by the calculated values of $V_{RS}$ or $V_A$. It appears only in $V_R$, due to the lowering of $\psi_0$ with increasing detergent concentration (Fig. 5), but according to this the total repulsive energy decreases. The experimental results indicate increasing stability with increasing concentration. If the surface concentration c increases above the CMC, $V_{RS}$ will also increase and this could explain the increased stability. Closer packing at the interface could conceivably increase the depth of the adsorbed layer through the extension of the polyethyleneglycol (PEG) chain.

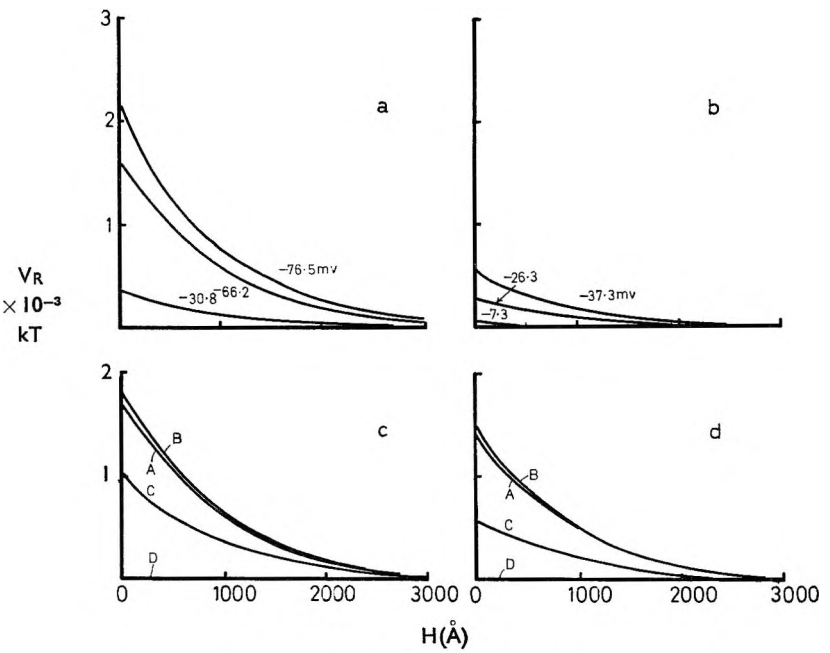


FIG. 5. $V_R$ as function of H at three detergent concentrations of (a) $C_{16}n_3$-anisole and (b) $C_{16}n_{25}$-anisole. Concentration dependence of $V_R$ is reflected by a change in $\psi_0$. Values in mV shown on graph are experimental values from electrophoresis at 0·1, 1·0 and 10% levels for $C_{16}n_3$ and 0·1, 1·0 and 5% levels for $C_{16}n_{25}$. (c) $V_R$ as a function of detergent at 5% concentration levels. PEG chain length A : 3, B : 6, C : 9, D : 25; (d) same but 10% surfactant. Oil in (c) and (d) is chlorobenzene.

## CORRELATION OF EXPERIMENT AND THEORY

*Electrical factors.* Experimental facts are that as the series is ascended the emulsions become more stable. At the same time the electrical contribution to stability becomes smaller, so it must be concluded that there is some movement of charge on approach of the particles stabilized by the short chain detergents, that is, those with the highest apparent $V_R$.

The conclusion drawn, therefore, is that the primary stabilizing force must be the entropic one but the inability of reasonably high zeta-potentials (~70 mV) to stabilize the particles must be explained.

The origin of the charge on these particles is not, as in the case of ionic detergents, due to the presence of large charged molecules but to the presence on the globule

surface of adsorbed hydroxyl ions. These ions have a high mobility and the movement of the ions may or may not be directly related to the mobility of the detergent films. Fig. 6 shows the effect of neglecting the electrical contribution to stability on the energy/distance relation of $C_{16}n_3$ and $C_{16}n_{25}$ emulsions. The minimum is deeper in the more stable ($C_{16}n_{25}$) system hence one might conclude that some charge remains effective in this system—which has a more coherent surface film.

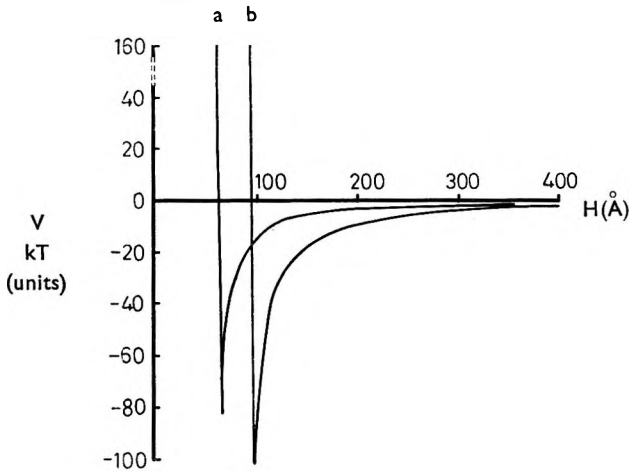


FIG. 6. Plot of $V_{RS} + V_A$ *vs* H for $C_{16}n_3$ (a) and $C_{16}n_{25}$ (b), neglecting $V_R$ the electrical repulsive force. The two plots are not sufficiently different to explain the totally different behaviour of $C_{16}n_3$ and $C_{16}n_{25}$ stabilized emulsions.

As $V_A$ increases with increasing polyoxyethylene chain length, consideration of $V_A + V_R$ alone results in a decreasing barrier to coalescence as the series is ascended. This leads to the conclusion of the primary importance of $V_{RS}$ or some other physical property of the surfactant film.

*Properties of the surface film and entropic stabilization.* The barrier to coalescence must be a final barrier at close approach. The only force which we have calculated which has a direct relation to stability as assessed by rates of coalescence ($K_2$) is $V_{RS}$. Fig. 7 shows this relation for the $C_{16}n_3$, $C_{16}n_6$ and $C_{16}n_9$ compounds. The

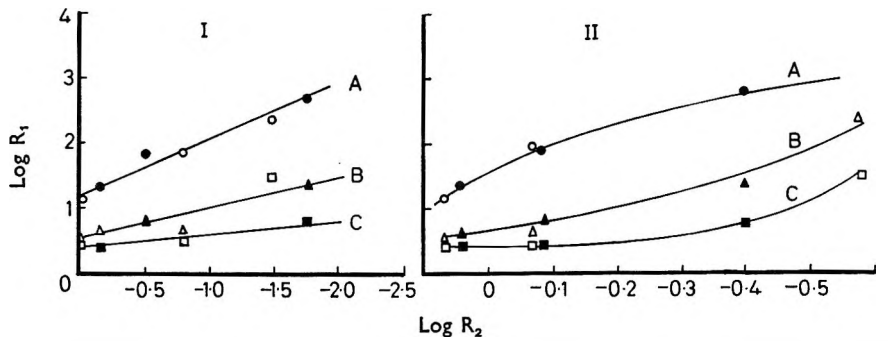


FIG. 7. Relation between stability and $V_{RS}$ expressed as ratios. I showing entropic repulsion taking into account PEG layer only ($V_{RS}$ values chosen at H = 10Å), II takes whole molecule into account ($V_{RS}$ being taken as H = 20Å). In both, results for three different concentrations of detergent are shown: A 0·1, B 1 and C 5%. $R_1 = (K_2)_{n=x}/(K_2)_{n=25}$, $R_2 = (V_{RS})_{n=x}/(V_{RS})_{n=25}$ where $(K_2)_{n=x}$ and $(V_{RS})_{n=x}$ refer to experimental rates of coalescence and entropic stabilization for emulsions stabilized with $C_{16}n_x$. Open symbols, anisole, solid symbols, chlorobenzene emulsions.

fact that the theory predicts large entropic barriers for all the emulsions studied suggests that when the emulsions are found experimentally to be less stable, the entropic force calculated is an over-estimate. One possible reason for this is the movement of the stabilizing molecules away from the point of contact. This was the explanation given by Albers & Overbeek (1959a) for the anticorrelation which existed between the zeta-potential of water-in-oil emulsions stabilized by oleates and stability.

It is possible, therefore, that the experimental stability values can be correlated with the compressibility of the film or its surface viscosity, $\eta_S$. Results published earlier (Elworthy & Florence, 1969a) indicate that the ease of compression varies in the order

$$C_{16}n_3 > C_{16}n_6 > C_{16}n_9 > C_{16}n_{25}$$

which is the order in which rates of coalescence vary with chain length. The quantity, $\pi_R$, calculated previously (Elworthy & Florence, 1969a) bears an empirical relation to $V_{RS}$ (Fig. 8).

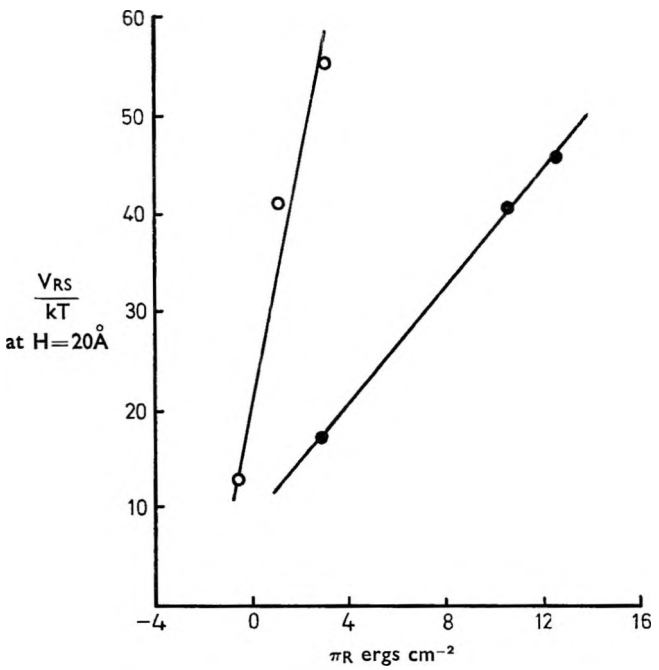


Fig. 8. Plot of $V_{RS}/kT$ *vs* $\pi_R$ showing the linear relation between them for detergents with x = 3, 6 and 9 units. ⊙ Anisole, ● Chlorobenzene.

Neither $\pi_R$ or $V_{RS}$ appear to be significantly concentration-dependent so the increase in stability with increase in surfactant concentration above the CMC is still not completely explained. One experimental quantity which is known to vary with concentration is $\eta_S$. If we write

$$\Delta G_{\text{coalescence}} \propto A'\eta_S$$

where $\eta_S$ is in surface poises (sP) and A′ in cm² then for two 1 $\mu$m particles with $\eta_S = 1 \times 10^{-4}$ sP, $\Delta G$ is of the order of 1 or 2 kT for a contact area, A′, of $4 \times 10^{-10}$

$cm^2$. For a high surface areal viscosity of $1 \times 10^{-2}$ sP, $\Delta G$ is 100–200 kT. These are crude estimates but would seem to show that the magnitudes of $\eta_s$ found by Carless & Hallworth (1967) for non-ionic detergents, i.e., around $10^{-4}$ sP are not sufficient in themselves to account for stability.

If the film is mobile on close approach of the globules, the adsorbed molecules may move to the far side of the globule leaving an exposed surface ($V_{RS} = 0$). If $V_{RS} = 0$ the resultant energy-distance diagrams still show a decreasing barrier to coalescence and flocculation with increasing detergent concentration. It might be concluded, therefore, that as the concentration of detergent is increased above 0·1% the surface viscosity increases to prevent movement of the molecules. The surface viscosity does increase with increasing concentration of cetomacrogol 1000 (Elworthy, Florence & Rogers, unpublished). Hence this might explain the increase in stability with increase in detergent concentration.

If the film cannot be compressed it is possible that the detergent can diffuse into the disperse phase and again produce the situation where $V_{RS} = 0$ (MacRitchie, 1967). The more water-soluble the detergent, in the series considered here, the more likely this is to happen. This may further explain the trend of increasing stability as the non-ionic chain length is increased. Whether the time of encounter of two globules undergoing a collision due to Brownian motion is long enough to allow the surfactant molecules to desorb is a factor in this argument. If two particles, radius a, are separated by a distance $2x$ the time, t, for collision as a result of normal Brownian motion is

$$t = \frac{x^2}{2D} \text{ where } D = \frac{kT}{6\pi\eta a}$$

For a particle of radius 0·5 $\mu$m, and $D = 5{\cdot}0 \times 10^{-9}$ $cm^2$ $s^{-1}$, t is of the order of $10^{-6}$ s. It is doubtful whether the molecule can desorb in this time. Studies of the behaviour of detergent films during rupture are likely to provide answers to this question to some extent (Mysels, Florence & Frens, 1969).

The effect of concentration of surfactant is not satisfactorily formulated in any of the equations discussed. Neither is the effect of emulsion phase volume ($\phi$). Albers & Overbeek (1959b) calculated the percentage reduction in $V_R$ from the simple equation due to the overlap of extended double layers in concentrated emulsions. In the present systems with $\kappa = 1{\cdot}42 \times 10^5$ $cm^{-1}$, $a = 5 \times 10^{-5}$ cm and $\phi = 0{\cdot}20$ the lowering of $V_R$ based on the two particle equation is 6·7%, which is not deemed significant. Decrease in stability with increasing phase volume is partly due to the increased probability of two particles colliding. In addition, the greater the value of $\phi$, the greater the surface free energy of the system.

*HLB and stabilizing forces.* The concept of HLB (hydrophile-lipophile balance) is often used practically in determining the detergent to employ for optimum stability of a given oil-in-water emulsion. It was therefore of interest to determine whether any of the calculated stabilizing forces could be correlated with HLB. Chlorobenzene has been stated to require for optimum stability a surfactant with HLB of $13 \pm 1$ (Atlas Chemical Industries, 1967). This corresponds to $C_{16}n_9$, whereas $C_{16}n_{25}$ emulsions are more stable. The HLB of $C_{16}n_{25}$ is 16·9. It must be considered that HLB is not an entirely satisfactory parameter. It neglects the concentration-dependence of stability. The optimum HLB is said to hold whatever the non-ionic detergent type used, but this probably neglects subtle differences in rates of breakdown.

## CONCLUSIONS

The application of theories of colloid stability to the present systems has enabled the contributions of the entropic and electrical barriers to be assessed, and has led to the following conclusions:

1. Electrical forces appear to be not significant due to the possibility of charge movement from the point of contact of the globules.

2. Entropic stabilization is of major importance with the longer PEG chain compounds.

3. Attractive forces can be increased by the presence of an adsorbed film which means that the term "protective colloid" can have an ambiguous meaning.

4. Where the surface film is not desorbed or mobile, electrical repulsive forces will contribute to stability and the reduction in zeta-potential will result in a slight decrease in stability. Where the mobility of the film is high the potential will bear no relation to stability and stability will increase with an increase in the compact nature of the film.

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# The use of a thermoelectric osmometer to measure the osmotic coefficients of aqueous solutions of sodium salicylate at 25°

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The use of a thermoelectric osmometer to measure osmotic coefficients for the binary system water–sodium salicylate at low concentrations (0·0 to 0·3 molal) is described. The accuracy and reproducibility of the instrument have been enhanced by employing a microscope to measure sample and reference drop sizes. Activity coefficients, calculated from the osmotic coefficients obtained in these experiments, have been combined with density and diffusional measurements to calculate frictional coefficients for the system.

Diffusional measurements made on the system water-sodium salicylate have been combined with data received from measurements using a vapour pressure osmometer, to evaluate frictional coefficients. Unlike diffusional coefficients these last are independent of the reference frame used for measurement. The technique required to obtain meaningful and reproducible values for osmotic coefficients is discussed in detail.

## THEORY

### *Expression for frictional coefficients*

In a system of q + 1 components in which the solvent is designated by 0, and the solutes by (1, 2 . . . q), the isothermal diffusional flow $(J_i)_s$ of component i produced by the chemical potential gradients $X_k$ can be represented by equation (1) (Kirkwood, Baldwin & others, 1960)—

$$(J_i)_s = \sum_{k=0}^{q} (L_{ik})_s X_k \quad (i = 0, 1, 2 \ldots q) \ldots \quad \ldots \quad \ldots \quad (1)$$

in which the flux $J_i$ and the phenomenological constants $L_{ik}$ are measured with respect to reference frame S.

For one dimensional flow at constant temperature and pressure,

$$X_k = -\left(\frac{\partial \mu_k}{\partial x}\right)_{T,P} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

where

$$\mu_k = \mu^0{}_k + RT \ln C_k y_k \quad \ldots \quad \ldots \quad \ldots \quad (3)$$

in which $\mu_k$ and $\mu^0{}_k$ are the chemical and standard chemical potentials of component k, R is the gas constant, T the absolute temperature, $C_k$ the concentration in mol $dm^{-3}$ and $y_k$ is the corresponding activity coefficient.

By considering the relative velocities $(V_i)_s$ and $(V_k)_s$ of components i and $k$ according to reference frame S, it is possible to write a restricted phenomenological equation (4), which in the form due to Dunlop (1964) is,

$$X_i = \sum_{k=0}^{q} R_{ik} C_k [(V_i)_s - (V_k)_s] \quad .. \quad .. \quad .. \quad (4)$$

and by using activity coefficient, density and diffusion data to calculate a frictional coefficient $R_{ik}$. For a two-component system—

$$R_{10} = \frac{\overline{V}_0 RT}{D_A}\left[1 + \frac{C_1 \partial \ln y_1}{\partial C_1}\right] \quad .. \quad .. \quad .. \quad (5)$$

where $\overline{V}_0$ is the partial molar volume of the solvent, $D_A$ the mutual diffusion coefficient, $C_1$ and $y_1$ are the concentration (mol $dm^{-3}$) and activity coefficient of the solute.

## EXPERIMENTAL

### *Materials*

Sodium salicylate and Analar sodium chloride were purchased from British Drug Houses Ltd. The molecular weights were taken as 160·11 and 58·454 respectively.

### *Solutions*

All solutions were prepared by weighing in air and were subsequently corrected for weight *in vacuo*. Before each diffusion measurement the densities of the initial solutions were determined in triplicate at $25 \pm 0{\cdot}01°$ using matched single-stem pycnometers each having a volume of about 30 cc. These were weighed against a calibrated sealed tare filled with air-free double distilled water, whose density was taken as 0·997048 g $cc^{-1}$.

By a least squares analysis it was found that the density in g $cc^{-1}$ of the sodium salicylate solution could be represented by the equation

$$d_4^{25} = 0{\cdot}997048 + 0{\cdot}41378\rho - 0{\cdot}0506\rho^2 \quad 0 \leqslant \rho \leqslant 0{\cdot}16 \quad .. \quad .. \quad (6)$$

with an average deviation of $\pm 0{\cdot}0004\%$, where $\rho$ is the concentration expressed in g $cc^{-1}$.

The osmotic coefficients of the solutions were measured on a Mechrolab Vapour Pressure Osmometer Model 302, an instrument which measures the depression in vapour pressure of a solvent when small quantities of solute are added to it.

The two principal units of the osmometer connected electronically by shielded cables are a thermally insulated "detector" module and a control module which also contains an amplifier and a D.C. Wheatstone Bridge.

A thermostat and heater are inserted into the body of the thermal block and maintain the centrally situated vapour chamber at a constant temperature of $25 \pm 0{\cdot}001°$. Further holes machined into the block and opening into the vapour chamber accommodate the thermistor probe and six microsyringes and provide a horizontal viewing tube at the end of which an angled plane mirror is attached. By looking into the mirror, an image of the two carefully matched thermistor beads may be seen within the vapour-chamber.

To set up an experiment, a short glass cylinder was fitted with an absorbent wick and approximately half-filled with a sample of the same water used in preparing the solutions. This reservoir was placed at the base of the vapour chamber by raising and replacing the upper portion of the detector block; and the chamber was left overnight to attain temperature equilibrium.

Four syringes were filled in pairs with freshly prepared solutions of sodium chloride and sodium salicylate, the molalities of reference and unknown solutions being

approximately equal. The remaining syringes were filled with water; all were inserted in the block and allowed to equilibrate for 20 min before any measurement was attempted.

Using the microsyringe, one droplet of water was placed on the tip of each of the thermistor beads, and a stop-watch was started. Exactly 50 s later the bridge was brought to zero, this process taking exactly 8 s. After 2 min the galvanometer was brought to a point of balance. Further drops of water were added to the "sample" thermistor bead and the above process was repeated until no further adjustment of the bridge was necessary to maintain a state of balance. The drop of solvent on the "reference" bead remained unchanged throughout the course of any one experiment.

The sample drop was washed off with 5 drops of a salicylate solution, readings were taken using the same time schedule as above, until the resistance increment to balance the bridge remained constant to within $\pm 0{\cdot}01$ ohm for five successive drops of sample.

After washing the salicylate off with three drops of solvent when the bridge should show a dekastat reading of zero ohms, sodium chloride solution of the same molality as the salicylate most recently measured was placed on to the sample thermistor bead and the sample measurement was repeated. Finally, the sample bead was washed off with three drops of water and the zero was rechecked with solvent on both beads.

Using literature values (Robinson & Stokes, 1959) for the osmotic coefficient of the NaCl solution and knowing the molalities of solutes measured and their respective ohmic increments, a value for the osmotic coefficient of the salicylate solution could be calculated from the expression

$$\phi_X = \frac{\Delta R_X \, m_R \, \phi_R}{\Delta R_R \, m_X} \qquad (7)$$

where $\Delta R$ is the resistance increment; $m$ is the molality of the solute; and $\phi$ is the osmotic coefficient, the subscripts $_R$ and $_X$, referring to reference (NaCl) and unknown (Na salicylate) solutions respectively.

To obtain meaningful results, the drops on the reference and sample beads must be equal in size and the finite size of the reference bead is critical, moreover, estimates of drop size made with the unaided eye proved inadequate.

The viewing mirror was therefore soldered in a retaining clip at a suitable angle and the thermistor bead image was brought to a focus on the eyepiece of a compound microscope fitted with a ruled graticule. The substage lens of the microscope, the mirror and the sighting tube were enclosed in a light-tight cylinder made of black card to improve the definition of the image.

Diffusion measurements were made using a Gouy diffusiometer. The apparatus and the method of obtaining the diffusion measurements have been described previously (Deshmukh & Fleming, 1968) and only a brief description will be given here.

The light source is a water-cooled vapour pressure lamp used in conjunction with a Wratten 77A filter to isolate the mercury green line ($\lambda = 5460{\cdot}7$Å) and a Hilger and Watts source slit. Light from the illuminated slit is focussed by an achromatic doublet lens through the water-bath containing the Tiselius cell on to a Kodak Ortho 800 photographic plate.

Measurements of the fringe minima were measured to an accuracy of 0·001 mm using a Gaertner M2001P toolmakers' microscope. Details of calculating the mutual diffusion coefficient $D_A$, the refractive index increments $\Delta n/\Delta\rho$ and Q, the area of the fringe deviation graphs, have already been published by Woolf, Miller & Gosting (1962) and Deshmukh & Fleming (1968).

## RESULTS

The results of the diffusional measurements are shown in Table 1. Columns 1, 2 and 3 show the values of the mean concentration $\bar{\rho}$ of the solutions of sodium salicylate which are listed in g $cc^{-1}$, and in terms of the molar and molal scales respectively, where

$$\bar{\rho} = \frac{(\rho_A + \rho_B)}{2} \quad .. \quad .. \quad .. \quad .. \quad (8)$$

and $\rho_A$ and $\rho_B$ are the initial concentrations of the solutions above and below the boundary of the Tiselius cell.

Table 1. *Diffusional data for system water–sodium salicylate at* 25°

| $\bar{\rho}$ | $\bar{\rho}_r$ | $\bar{\rho}_1$ | $\Delta\rho$ | $\Delta\rho_r$ | $10^5 D_A$ | $10^6 \frac{\Delta n}{\Delta\rho_r}$ | Jm |
|---|---|---|---|---|---|---|---|
| 0·005012 | 0·031304 | 0·031490 | 0·010025 | 0·062615 | $1·009_3$ | 3291·8 | 94·27 |
| 0·024980 | 0·156022 | 0·158822 | 0·009988 | 0·062384 | $0·960_8$ | 3270·9 | 93·35 |
| 0·049909 | 0·311725 | 0·322143 | 0·009939 | 0·062078 | $0·913_9$ | 3256·5 | 92·54 |
| 0·099627 | 0·622258 | 0·663289 | 0·009920 | 0·061980 | $0·834_1$ | 3219·7 | 91·23 |
| 0·149068 | 0·931060 | 1·024789 | 0·010055 | 0·062802 | $0·760_0$ | 3200·5 | 91·94 |

Units: Concentrations: $\bar{\rho}$, g $cc^{-1}$; $\bar{\rho}_r$ mol $litre^{-1}$; $\bar{\rho}_1$ mol/1000 g.
Concentration difference: $\Delta\rho$, g $cc^{-1}$; $\Delta\rho_r$, mol $litre^{-1}$.
Diffusion coefficient: $D_A$, $cm^2\ s^{-1}$.
Refractive index increment: $\frac{\Delta n}{\Delta\rho_r}$, litre $mol^{-1}$.

Columns 4 and 5 list the concentration differences in g $cc^{-1}$ and mol $litre^{-1}$ of solution, where

$$\Delta\rho = \rho_B - \rho_A \quad .. \quad .. \quad .. \quad .. \quad (9)$$

whilst the refractive index increments in litre $mol^{-1}$ appear in column 6 and were computed using the relation

$$\frac{\Delta n}{\Delta\rho_r} = \frac{J_M\lambda}{a\Delta\rho_r} \quad .. \quad .. \quad .. \quad .. \quad (10)$$

where $J_M$ is the total number of fringes in the diffusion pattern.

Values of the area of the fringe deviation graph were computed and show that within the range of concentration the system is not concentration dependent.

The variation of the mutual diffusion coefficient $D_A$ with $\bar{\rho}$ (g $cc^{-1}$) is summarized by the expression

$$D_A = 1·022_0 - 2·661_3\bar{\rho} + 11·332_0\bar{\rho}^{-2} - 35·34\bar{\rho}^3 \quad 0 \leqslant \bar{\rho} \leqslant 0·15 \quad .. \quad (11)$$

which was obtained by a least squares analysis of the data in columns 1 and 6.

Table 2. *Osmotic coefficient measurements for the system water–sodium salicylate at* 25°

| $m$ | $\phi$ | $m$ | $\phi$ |
|---|---|---|---|
| 0·02 | $0·944_4$ | 0·10 | $0·918_2$ |
| 0·03 | $0·940_2$ | 0·15 | $0·909_6$ |
| 0·04 | $0·932_9$ | 0·20 | $0·901_6$ |
| 0·05 | $0·929_1$ | 0·30 | $0·895_6$ |
| 0·08 | $0·922_0$ | — | — |

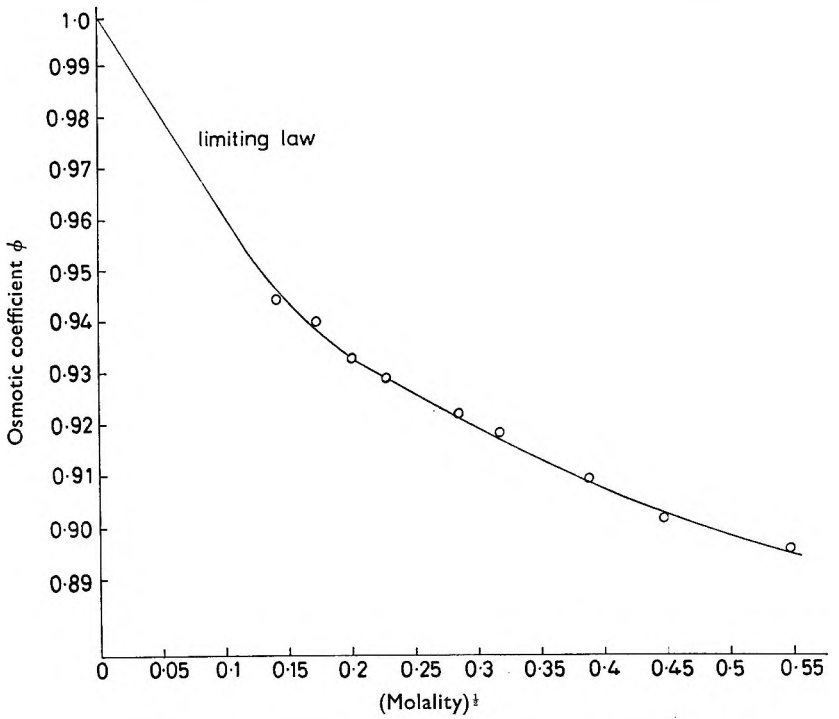


FIG. 1. Plot of osmotic coefficient against (molality)$^{\frac{1}{2}}$ for water-sodium salicylate solution at 25°.

Table 2 summarizes the osmotic coefficient data, whilst Fig. 1 is a plot of osmotic coefficient $\phi$ against $\sqrt{m}$ for sodium salicylate in water varying in molality $m$ from 0 to 0·3. The values of the activity and frictional coefficients derived from these measurements are listed in Table 3.

Table 3. *Activity coefficient data derived from vapour pressure measurements on system water–sodium salicylate at 25°*

| C | $\frac{\partial \ln y_{\pm}}{\partial C}$ | $\bar{V}_0$ | $10^5$ $D_A$ | $10^{-16}$ $R_{10}$ |
|---|---|---|---|---|
| 0·05 | —1·291 | 18·0679 | $1·001_4$ | $4·183_9$ |
| 0·10 | —1·069 | 18·0678 | $0·982_2$ | $4·072_9$ |
| 0·15 | —0·846 | 18·0675 | $0·964_1$ | $4·055_7$ |
| 0·20 | —0·624 | 18·0671 | $0·947_2$ | $4·138_1$ |
| 0·25 | —0·402 | 18·0665 | $0·931_4$ | $4·325_9$ |
| 0·30 | —0·179 | 18·0659 | $0·916_4$ | $4·624_7$ |

Units: Concentration C, mol dm$^{-3}$.
Partial molar volume $\bar{V}_0$, cm$^3$ mol$^{-1}$.
Diffusion coefficient $D_A$, cm$^2$ s$^{-1}$.
Frictional coefficient $R_{10}$, erg cm s mol$^{-1}$.
$R = 8·3144 \times 10^7$ erg deg$^{-1}$ mol$^{-1}$.
$T = 298·15°$ K.

The partial molar volume of the solvent $\bar{V}_0$ was calculated using the equation due to Geffcken (1931),

$$\bar{V}_0 = \frac{1000\,\bar{V}_0^0}{1000 + C^2\left(\frac{\partial\theta}{\partial C}\right)} \quad .. \quad .. \quad .. \quad .. \quad (12)$$

where $\bar{V}_o^o$ is the partial molar volume of pure solvent (18·0681 cc), $\theta$ is the apparent molar volume of the solute and C is the concentration of solute expressed in moles $dm^{-3}$.

To use this equation it was necessary to evaluate values of $\theta$ using the expression

$$\theta = \frac{1000}{C}\left(\frac{d_o - d}{d_o}\right) + \frac{B}{d_o} \qquad (13)$$

where $d_o$ is the density of water at 25° (0·997048 g $cc^{-1}$), d is the density of the solution at concentration C (mol $dm^{-3}$) and B is the molecular weight of the solute. Smoothed values of d were computed with the aid of equation (6).

Further regression analysis produced the relation

$$\theta = 94{\cdot}14108 + 1{\cdot}24667C + 0{\cdot}23792C^2 - 0{\cdot}30909C^3 \qquad (14)$$

which after differentiation and evaluation for various rounded values of C was substituted into equation (10) to give a series of values for $\bar{V}_o$ which appear in column 3 of Table 3.

Using a form of the Gibbs–Duhem equation

$$-\ln \gamma_{\pm} = 1 - \phi + 2\int_0^{\sqrt{m}} \frac{(1-\phi)}{\sqrt{m}} \cdot d\sqrt{m} \qquad (15)$$

mean molal activity coefficients $\gamma_{\pm}$ were calculated from the osmotic coefficients $\phi$, and the variation of $\gamma_{\pm}$ with molality m is shown in Fig. 2. The integral in equation (15) was evaluated using Simpsons' $\frac{1}{3}$ Rule, taking into account that

$$\lim_{m \to 0} (1-\phi)/\sqrt{m} = 0{\cdot}3903 \qquad (16)$$

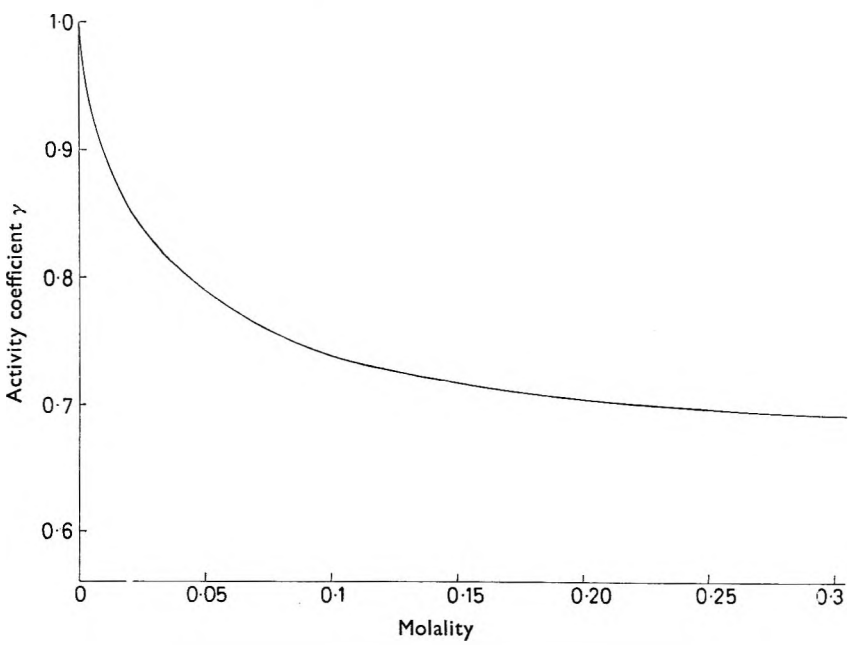


FIG. 2. Plot of activity coefficient against molality for water-sodium salicylate solutions at 25°.

The polynomial expression

$$\ln y_{\pm} = -0{\cdot}15758 - 1{\cdot}51386C + 2{\cdot}22475C^2 \quad .. \quad .. \quad (17)$$

was obtained by least squaring and values of $\partial \ln y_{\pm}/\partial C$ were then obtained by differentiation and appear in column 2. The values of $\partial \ln y_{\pm}/\partial C$ were checked graphically by drawing tangents to a large scale graph of $\ln y_{\pm}$ against C.

The values of the mutual diffusion coefficient $D_A$ of salicylate (column 4) at the relevant molarities were calculated using equation (11). The values for the frictional coefficient $R_{10}$ could now be computed and are enumerated in column 5.

## DISCUSSION

The calculations demonstrate how diffusion measurements allied to vapour pressure measurements may be used to evaluate thermodynamic parameters. The advantages of computing frictional coefficients in preference to other phenomenological coefficients are that they are independent of the measurement frames of reference. Thus the values of frictional coefficient may be compared directly with those measured on a diaphragm cell of the Stokes type.

With the advent of this type of thermoelectric osmometer sensitive to temperature changes of the order of 0·0001°, it is now possible to make direct measurements leading to a calculation of activity coefficients for a wide range of dilute solutions.

The technique of using the osmometer has been described in some detail, as considerable difficulty was experienced in obtaining reproducible results of satisfactory accuracy using water as a solvent.

In this series of experiments, it was found necessary to leave the osmometer for at least 12 h to equilibrate before attempting to take any readings.

Drop size and the timing sequence for each measurement are also critical. Furthermore, the readings for any one pair of solutions must be completed within 1 h since, the bridge fails to return to a balance point with solvent if left much longer than this.

However, by observing such precautions and by calculating the mean of several results for any one solution, the osmotic coefficient values are reproducible to within $\pm 0{\cdot}5\%$ of a mean value.

*Acknowledgements*

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SHORT COMMUNICATION

# A punched card system for the rapid identification of powdered crude drugs and spices

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Multiple-entry, edge-punched card systems have been introduced as aids in the microscopical analysis of hardwoods (Clarke, 1938; Brazier & Franklin, 1961) and of softwoods (Phillips, 1948). Systems have also been developed for synthetic fibres (Culliford, 1963) and solid dosage forms (McArdle & Skew, 1965). The usefulness of such schemes prompted us to apply a general system to the identification of vegetable materials including powdered crude drugs and spices, with a view to eliminating the tedium of searching the usual identification tables or keys (Claus, 1956; Anon., 1963; Trease & Evans, 1966; Wallis, 1967).

*Preparation of the system*

The microscopical characters of over a hundred powdered crude drugs were systematically tabulated and then grouped according to types of tissues, cells, cell contents, etc., as follows: I Epidermis, II Covering Trichomes, III Glandular Trichomes, IV Cork, V Parenchyma, VI Sclereids/Lignified Parenchyma, VII Fibres, VIII Vessels/Tracheids, IX Sieve Tubes, X Pollen, XI Fibrous Layer of Anther, XII Lamina, XIII Medullary Rays, XIV Oil Glands/Vittae, XV Epiphytes, XVI Lignified Rods, XVII Starch, XVIII Calcium Oxalate, XIX Other Cell Contents.

For most of the groups several appropriate descriptive characters were selected so that a total of 106 characters was available for the scheme and they were arranged as shown on the key card (Fig. 1).

Each character was strictly defined so that all users of the system adhere to the same interpretation of the various parameters described. For example, in this system we define parenchyma (V) as thin-walled, non-lignified, unspecialized cells and thus all kinds of so-called lignified parenchyma are not included here, but under VI. This is necessary because in some powdered vegetable materials it is virtually impossible to decide whether particular cells are lignified parenchyma or thin-walled sclereids. A definite delineation is required between isodiametric sclereids (VI, 44) and elongated sclereids (VI, 45), and between the latter and fibres (VII). Here a strict numerical criterion is laid down, namely that an elongated sclereid has a length equal to at least twice its maximum diameter and a fibre has a length equal to at least ten times its maximum diameter.

Table 1 lists the characters present in individual crude drugs and spices. This was compiled after microscopical examination of authentic powdered materials and by consulting published literature (Winton & Winton, 1932–1939; Trease & Evans, 1966; Wallis, 1967; B.P., 1968; B.P.C., 1968; Jackson & Snowdon, 1968). An individual key-card for each drug or spice was then prepared. Each character

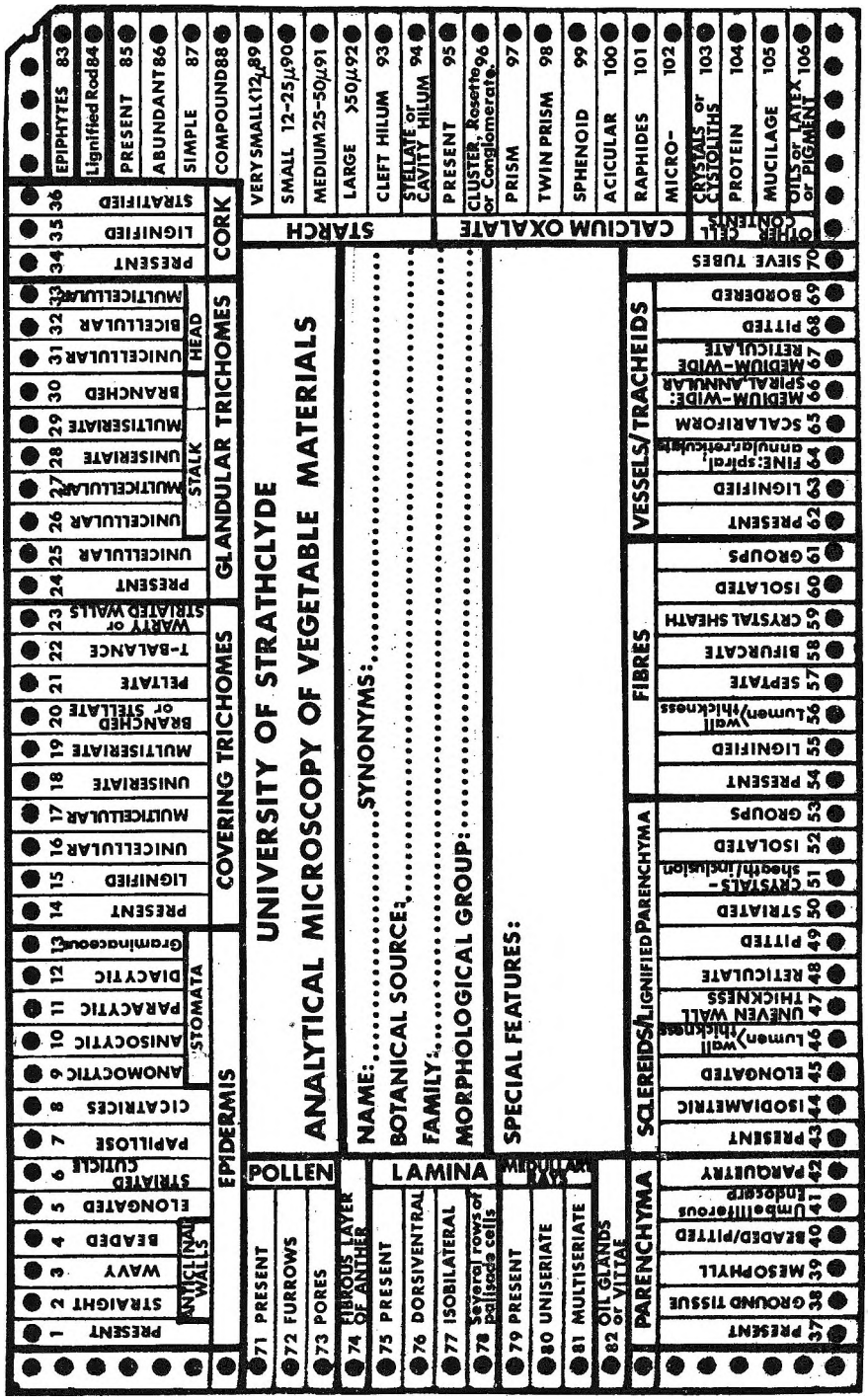


FIG. 1. Multiple entry card.

invariably present in the powder was clipped (Fig. 2a) and where a character is normally present in but small amount, or is only sometimes present, it was marked with an inked notch (Fig. 2b). On each card the botanical source, family, common

Table 1. *Keys for selected crude drugs*

| Morphological group | Drug | Characters |
|---|---|---|
| Barks | Cascara | 34, 37, 38, 40, 43, 44, 45, 49, 50, 51, 53, 54, 55, 59, 61, 70, 79, 81, 83, 85*, 87, 89, 95, 96, 97, 106 |
| | Frangula | 34, 37, 38, 40, 54, 55, 59, 61, 70, 79, 81, 83, 85*, 87, 89, 90*, 95, 96, 97, 106 |
| | Wild cherry | 34, 37, 38, 43, 44, 45*, 49, 50, 52*, 53, 54*, 55, 56, 60, 62*, 63, 68, 69, 79, 80, 81, 83, 85, 87, 88*, 89, 90, 95, 96, 97, 106 |
| Fruits | Anise | 1, 2, 4, 6, 9, 14, 16, 23, 37, 38, 41, 43, 44, 46, 49, 53, 54, 55, 56, 61, 62, 63, 64, 82, 95, 96, 102, 104, 106 |
| | Coriander | 1, 2, 9*, 37, 38, 41, 42, 43, 44, 45, 46*, 49, 53, 54, 55, 56, 61, 62, 63, 64, 82, 95, 96, 97, 102, 104, 106 |
| | Capsicum (pedicals and calyces present) | 1, 2, 3*, 4,* 6*, 9*, 10, 14, 16, 17, 18, 23, 24, 26, 27*, 33, 37, 38, 43, 44, 45*, 46, 47*, 49, 50*, 53, 62*, 63, 64, 95, 99, 102, 104, 106 |
| | Capsicum (pedicels and calyces absent) | 1, 2, 3*, 4*, 6*, 37, 38, 43, 44, 45*, 46, 47*, 49, 50*, 53, 62*, 63, 64, 95, 99, 102, 104, 106 |
| Seeds | Cardamom | 1, 2, 5, 37, 38, 40*, 43, 45, 47, 53, 62*, 63, 64, 85, 86, 87, 88, 89, 90, 95, 97, 102, 103, 104, 106 |
| | Nux-vomica | 1, 2, 14, 15, 16, 37, 38, 43, 45, 53, 84, 106 |
| | Strophanthus | 1, 2, 6*, 8, 14, 15, 16, 37, 38, 43, 44, 46, 53, 62*, 63, 64, 84, 95*, 96, 97, 102, 104, 106 |
| Underground organs | Ginger | 37, 38, 54, 56, 57, 61, 62, 64*, 67, 85, 86, 87, 89, 90, 91, 106 |
| | Liquorice (unpeeled) | 34, 37, 38, 43, 44, 45, 46, 49, 53, 54, 59, 61, 62, 63, 68, 69, 70, 79, 80, 81, 85, 86, 87, 88*, 89, 90, 95, 97 |
| | Liquorice (peeled) | 37, 38, 43, 44, 45, 46, 49, 53, 54, 55, 59, 61, 62, 63, 68, 69, 70, 79, 80, 81, 85, 86, 87, 88*, 89, 90, 95, 97 |
| | Rauwolfia | 34, 35, 36, 37*, 38, 43, 44, 46, 49, 53, 54, 55, 56, 58, 60*, 61, 62, 63, 68, 69, 79, 80, 81, 85, 86, 87, 88*, 89, 90, 91*, 93*, 94*, 95, 96, 97 |
| Leaves and herbs | Digitalis | 1, 2, 3, 4*, 8, 9, 14, 17, 18, 23, 24, 26, 27*, 28, 31*, 32, 37, 38*, 39, 62, 63, 64, 75, 76 |
| | Belladonna herb | 1, 3, 6, 10, 14*, 17, 18, 24, 26, 27, 28, 30, 31, 33, 37, 38*, 39, 43*, 45, 46, 49, 54*, 55, 56, 60, 61, 62, 63, 64, 67, 71, 72, 73, 74*, 75, 76, 95, 97*, 99, 102 |
| | Stramonium | 1, 2, 3, 10, 14, 17, 18, 23, 24, 26, 33, 37, 38*, 39, 62, 63, 64, 71, 73, 74*, 75, 76, 95, 96, 97*, 99*, 102* |
| Inflorescences and flowers | Clove | 1, 2, 3*, 6*, 9, 37, 38, 39, 40, 43*, 44, 49, 50, 52, 53, 54, 55, 60, 61*, 62, 63, 64, 71, 72, 73, 74, 75, 82, 95, 96, 106. |
| | Chamomile | 1, 2*, 3*, 5, 6*, 7*, 9, 14, 17, 18, 23, 24, 29, 32, 33, 37, 39, 43, 45, 46, 49, 53, 62, 63, 64, 71, 72, 73, 74, 75, 95, 96, 102 |
| | Pyrethrum | 1, 3, 4*, 5, 6*, 7*, 9, 14, 17, 22, 24, 27, 29, 32, 33, 37, 39, 43, 44, 45, 46, 49, 50*, 51, 53, 62, 63, 64, 71, 72, 73, 74, 75, 95, 96, 97, 102, 106 |

* Characters of rare occurrence.

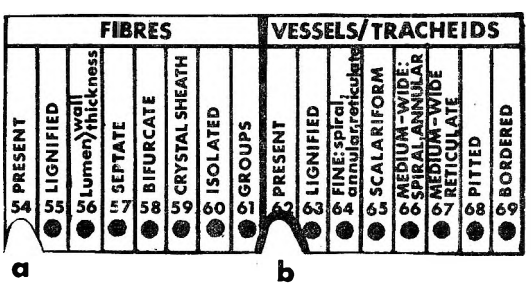


FIG. 2. Part of card showing method of recording characters, a, character invariably present: b, character normally present in small amount or only sometimes present (notch with inked edge).

names and synonyms for the drug were recorded along with the morphological group to which it belongs and any special characters not listed elsewhere on the key-card.

*Use of the system in analysis*

During the microscopical examination of an unknown powder the characters found are listed, those of rare occurrence being marked with an asterisk (see Table 1) and any about which there is doubt, queried.

The cards are then aligned and a needle inserted through an appropriate perforation. Cards positive for that character fall out. The process is then repeated until no more can be selected in this way. Further selection may be possible by the use of characters which are absent.

With most single powders it is possible to select the one correct card; where more than one card is left, however, it is usually possible to make a positive identification by further examination of the powder. In all cases it is advisable to check identification by comparison with authenticated material and published descriptions.

Where a mixture of drugs is examined, the system is used in a similar way. In recording the observations, those characters found in the same piece, or in obviously related pieces of tissue, are listed together while characters such as starch, which could occur in any of the components, are listed as *possibly* present. Making use of the list of characters known to be related, a selection of cards is then made and those characters on the second list are used both positively and negatively until a definite diagnosis can be achieved. This is repeated for each component. Comparisons with authentic materials, etc., are again made and it may be advantageous to prepare and examine a known mixture of the relevant drugs as a final check.

*Discussion*

With this key-card system there is a fundamentally different approach to the analysis of powdered vegetable materials, in that the analyst does not need to know in detail the characters of any individual powder but rather has to be able to identify the basic anatomical characters recorded in the system. Time can be saved both in training the analyst and in his handling of any unknown vegetable powder.

Experience in using the system with students has shown that the identification of single powders can be effected quickly and that with mixtures of two or three components the system is also useful. With more experienced workers it is anticipated that more complex mixtures will also be readily analysed.

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# The morphology and anatomy of the flowers of *Mitragyna inermis* (Willd.) O.Kuntze

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The morphology and anatomy of the flowers of *Mitragyna inermis* have been described and a comparison made with the flowers of *Mitragyna ciliata* and *Mitragyna stipulosa*.

The alkaloidal contents of the leaves of the three species of *Mitragyna* growing in Ghana have now been fully reported (Beckett, Shellard & Tackie, 1963a, b; Shellard & Sarpong, 1969). The morphology and anatomy of the leaves of the three species have also been described (Shellard & Shadan, 1963; Pillay, 1964). Leaves of *Mitragyna stipulosa* and *M. ciliata* are almost identical and these species may be differentiated only by an investigation of the alkaloids present (Beckett & others, 1963a, b), or by an examination of the flowers (Shellard & Wade, 1967).

Although the alkaloidal patterns of *M. stipulosa* and *M. ciliata* differ, those of *M. ciliata* and *M. inermis* shows few differences. Major differences obtain between the leaves of the two species; nevertheless, for Rubiaceae and particularly the subfamily Naucleoideae, a study of the flowers is indispensable for determining the genus and species (Aubreville, 1959). The present work reports a detailed study of the flowers of *M. inermis*.

*Materials.* The flowering tops were obtained from trees growing in the coastal plains of the Sogakofe district of the Volta region, Ghana. The identity of the species was confirmed by Mr. A. Enti, Government Silviculturist and by comparison with specimens in the Herbarium at the Royal Botanic Gardens, Kew.

## *Macroscopical features*

The *inflorescence* consists of a number of globose flower heads, each growing to about 20 mm in diameter, arranged in a dichasial cyme (Fig. 1A). The 100 to 120 individual florets of each flowerhead are closely packed on a spherical receptacle, each floret in the mature flower head being surrounded by 10 to 15 closely packed overlapping paleaceous bracteoles (Fig. 1B). In the flower bud stage the bracteoles completely cover and protect the developing florets which, as they develop to maturity, gradually push their way through the centre of the group of bracteoles. The first part of the floret to become visible during this development is the corolla, the tube of which is closed by the infolding of the petal lobes so that the margins of each lobe are adjacent in a valvate arrangement. When the floret is mature, the combined length of the calyx and ovary is much shorter than the length of the bracteoles so that the calyx is not readily visible on the fully grown flower head. The long corollas with their recurving lobes also tend to mask the bracteoles (Fig. 1C).

In the mature flower the calyx with ovary is 1·08–**1·70**–2·03 mm long and the bracteoles 2·47–**3·22**–4·0 mm long, the ratio of the average dimensions being 0·53.

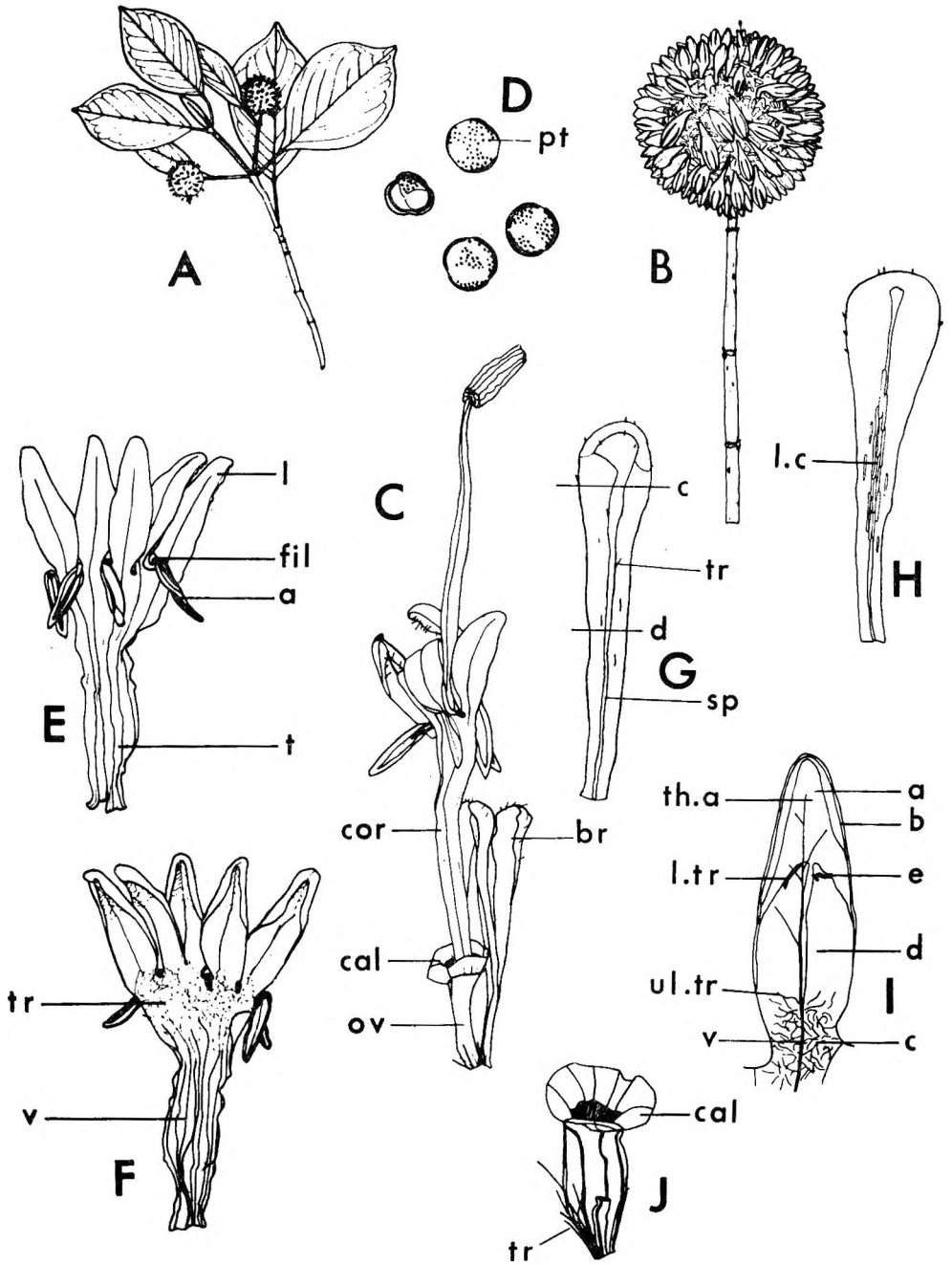


FIG. 1.

FIG. 1. *Mitragyna inermis*, A, inflorescence × ¼; B, flower head and peduncle × 1½; C, floret × 9; D, pollen × 400; E, corolla, outer epidermis and anthers × 9; F, corolla, inner epidermis × 9; G, bracteole, outer epidermis, surface view × 15; H, bracteole, inner epidermis, surface view × 15; I, corolla, inner epidermis of lobe, surface view × 15; J, calyx and ovary × 15.
a, anther; br, bracteole; cal, calyx; cor, corolla; fil, filament; l, lobe; l.c, lignified cell; l.tr, lignified trichome; ov, ovary; pt, pit; sp, spine; t, tube; th.a, thickened apex; tr, trichome; ul.tr, unlignified trichome; v, vein.

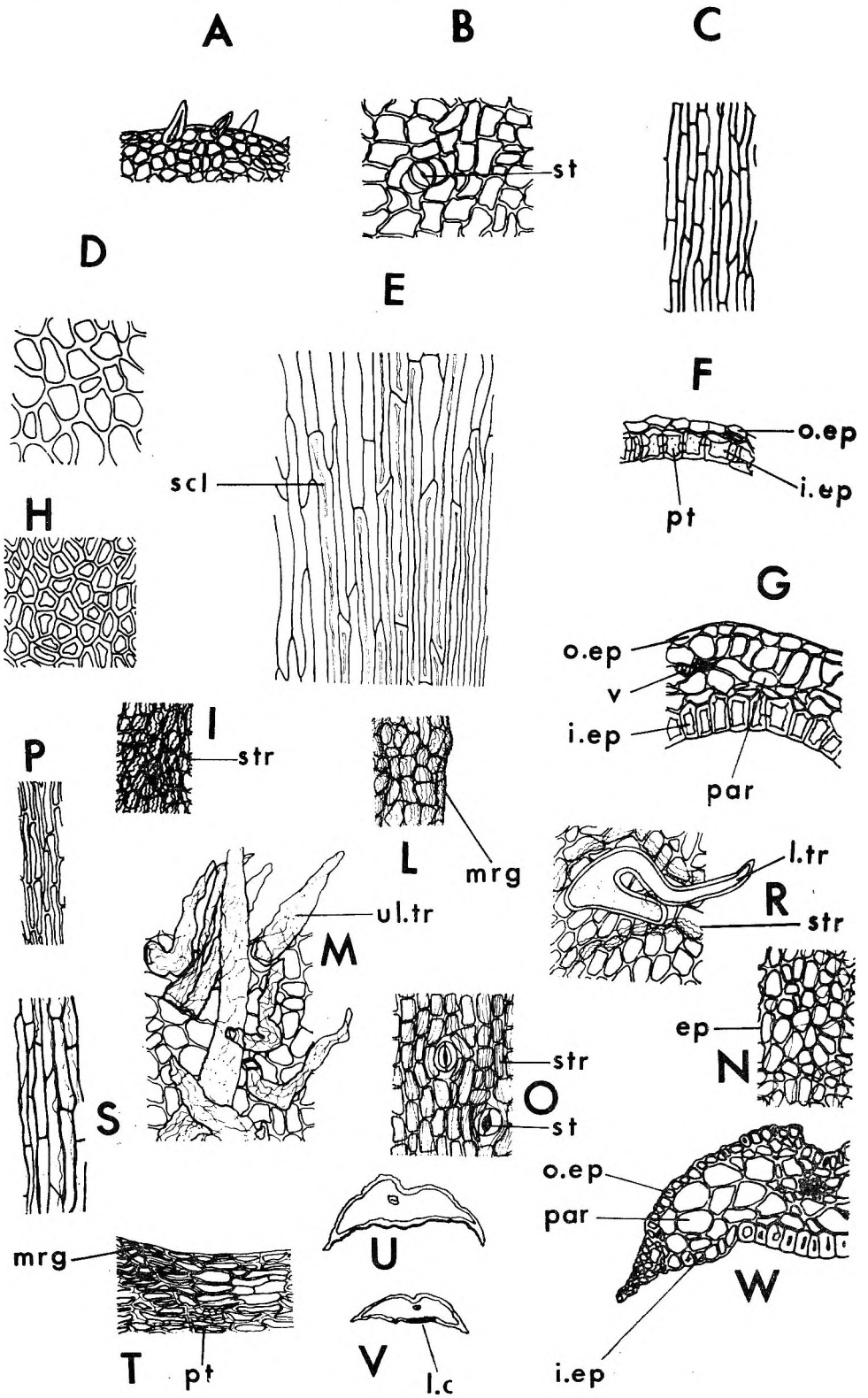
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FIG. 2.

Each floret is pentamerous, complete, perfect, actinomorphic and epigynous. The peduncle is 20 to 65 mm long, up to 1·5 mm in diameter, and woody with a thin brown cork; it bears longitudinal ridges and alternate pairs of leaf scars. The receptacle is spherical and is about 4 to 5 mm in diameter, the individual florets being attached over its entire surface. It is brown and covered with long stiff trichomes, the surface being rugose because the base of each ovary is attached to a raised portion of the receptacle. The transversely cut surface is white and exhibits numerous vascular strands.

The bracteoles are clavate to spathulate and are 2·4 to 4·0 mm long. They are thickened along a central spine which, on the outer surface, extends over most of the apical region, so that they have a planoconvex transverse section near the apex (Fig. 1G and H).

The calyx is gamosepalous (Fig 1J) consisting of five sepals united into a truncate cone about 300 $\mu$m deep and 0·9–1·05 mm in diameter at the open end. The veins on the outer surface are inconspicuous. The calyx is thicker near the base where it is fused to the ovary wall.

The corolla is gamopetalous consisting of five petals which are joined for over half their length. It is from 4·0 to 5·5 mm long of which the lobes comprise 1·0 to 1·9 mm, the diameter at the open end being 0·9 to 1·3 mm (Fig. 1E and F).

The individual petals are linear, but widen slightly near the lobes, which taper to a blunt incurving point at the apex. Closure is valvate. The tips of the lobes are wrinkled but otherwise the outer surface of the corolla is matt and glabrous The inner surface is wrinkled and the top half raised into a thickened pad. Occasional trichomes occur in the middle region of the lobe but there are many white tangled trichomes where the lobes unite to form the tube. Towards the base of the tube the inner surface is ridged (Fig. 1 I).

The androecium consists of five epipetalous stamens which alternate with the corolla lobes. Each anther is borne on a short filament attached to the corolla just below the lobes. The bilobed anthers are exserted and pendulous between the lobes of the corolla, dehiscence being through a longitudinal slit in each lobe. The spindle-shaped lobes of the anthers are joined for most of their length and are 1·0–1·4 mm long and 0·3 to 0·6 mm wide, the filaments being about 300 $\mu$m long and 50 $\mu$m wide. Pale yellow pollen is found on the stigma and entangled in the trichomes of the corolla lobes (Fig. 1D).

The gynaecium consists of a bicarpellary, syncarpous, inferior ovary and a reddish-brown cylindrical style surmounted by a lobed and grooved mitriform fleshy stigma. The ovary, which is 0·8 to 1·7 mm long and up to 1·0 mm in diameter at the top, is conical but flattened, with longitudinal ridges. It is inserted on a small mound on the receptacle and surrounded by the bracteoles. Long, stiff pale yellow trichomes

FIG. 2. *M. inermis*, A, bracteole, margin of head, after epidermis, surface view; B, bracteole, centre of head, outer epidermis, surface view; C, bracteole, shaft, outer epidermis, surface view; D, bracteole, head, inner epidermis, surface view; E, bracteole, shaft, inner epidermis, surface view; F, calyx, margin, transverse section; G, calyx, base, transverse section; H, calyx, base, inner epidermis, surface view; I, L, M and N, corolla, inner epidermis, surface view at a, b, c and d respectively on Fig. 1, I; O, corolla, lobe, outer epidermis, surface view; P, corolla, tube, outer epidermis, surface view; R, corolla, inner epidermis, surface view at e on Fig. 1, I; S, corolla, tube, inner epidermis, surface view; T, calyx, margin, inner epidermis, surface view; all × 150. U and V, bracteole, transverse sections at c and d respectively on Fig. 1 G × 35; W, bracteole, shaft, transverse section × 150.
o.ep, outer epidermis; i.ep, inner epidermis; l.c, lignified cell; l.tr, lignified trichome; mrg, margin; par, parenchyma; pt, pit; scl, sclerenchyma; st, stoma; str, striation.

arise from the receptacle and pack the space between the ovaries and bracteoles. The ovary is bilocular with axile placentation, numerous minute ovules arranged in an overlapping pattern filling each loculus. The ovules are roughly triangular in section and each is joined to the placenta by a short funicle. In the opened, mature flower the style is 8·0 to 11·0 mm in length, thus extending about 4 mm beyond the corolla (Fig. 1C).

*Microscopical features*

*Peduncle.* The general anatomy is similar to that of the young stem described by Pillay (1964). The outer layers of the peduncle consist of 1 to 2 rows of thick-walled, flattened cork cells with dark brown contents. The outer cortex varies in thickness from 1 to 3 rows of isodiametric thin-walled parenchymatous cells while the inner part of the cortex is collenchymatous and consists of up to 5 rows of cells with thickened walls, although the innermost cell walls are thinner. Occasional cells are slightly lignified and pitted like the pith cells.

Within the cortex is a ring of thickened unlignified phloem fibres. The zone is 1 to 3 cells deep; individual fibres are 1–5 mm long and 10 to **18·3** to 27 $\mu$m in diameter. The phloem is made up of groups of 1 to 4 lignified and thick-walled phloem cells and parenchyma, sieve cells and some latex cells with yellow to brown contents. No medullary rays can be distinguished in the phloem at the mature flower stage.

There is a thin yellowish zone of tightly-packed cambial cells with thin walls, inside which is a rectangular zone of xylem. The xylem has vessels 12 to 30 $\mu$m in diameter, with lignified walls bearing reticulated or spiral thickening. On two opposite sides of the rectangle the large vessels are outermost, but on the other two sides they are usually nearer the axis. In each case the remainder of the xylem consists of fibres with narrow lumens, occasional xylem parenchyma and prominent medullary rays. The rays are mainly uniseriate, but some are biseriate, and the cells often have dark brown contents.

The central pith consists of a narrow outer zone of small yellow pith cells and an innermost zone of large polygonal cells with lignified, pitted and thickened walls. No crystals of calcium oxalate are present.

*Receptacle.* The epidermis consists of small polygonal cells from which the bracteoles arise together with numerous straight, unicellular trichomes with thick lignified walls, and measuring 160 to **400** to 720 $\mu$m in length and about 20 $\mu$m in width.

The internal structure is somewhat similar to that of a young stem but with numerous groups of spirally thickened and lignified vessels, each running to the base of one of the 120 or so ovaries. In consequence, these vascular strands may appear in any one section to have been cut obliquely, transversely or longitudinally. There is usually a pale band of phloem cells on the outer side of the strand. The cortex consists of cells with thickened cellulosic walls. Nearer the centre there are several groups or a continuous ring of lignified vessels arranged radially round the centre of the receptacle. No scattered lignified fibres or calcium oxalate crystals are present.

*Bracteoles*

The *outer epidermis* of the unthickened margin of the apical region consists, in surface view, of polygonal cells with somewhat sinuous slightly thickened walls (Fig. 1G and 2B). They are 13 to **30·6** to 44 $\mu$m long, 10 to **18·3** to 33·3 $\mu$m wide and 8 to 22 $\mu$m deep. A thin, slightly wrinkled cuticle is visible. There are several

unicellular conical trichomes along the margin (Fig. 2A) with thickened, lignified walls, and measuring from 30 to 50 μm in length. On the thickened central spine there are also several trichomes, but these are about 80 to 120 μm in length. Both types have thickened, pitted and lignified bases. There are numerous anisocytic and paracytic stomata, about 30 μm in diameter, particularly on the thicker centre of the bracteole head.

Where the head begins to merge with the shaft the polygonal cells become narrower and more elongated. Along the shaft the cells have thickened cellulosic walls and are not lignified. Calcium oxalate crystals are absent and cuticle is not visible. The cells are 35 to 155 μm long and 6 to 12 μm wide. There is an occasional long trichome near the base of the shaft similar to those on the receptacle. Along the central thickened spine the cells have thin brown walls with dark brown contents.

The *inner epidermis* (Fig. 1H, Fig. 2D) has cells similar to those of the outer surface except that on the head of the bracteole the walls are somewhat thicker. On the shaft the cells become elongated and some groups of cells near the centre of the shaft have lignified walls and narrow lumens. The non-lignified cells alongside, similar in size and shape but longer than the outer epidermal cells, measure 130 to 320 μm in length and 6 to 18 μm in width (Fig. 2E). Lignified cells are absent from the base of the shaft and there are no stomata or trichomes present (Fig. 2W).

The *mesophyll* is composed of parenchymatous cells and there is a single vascular strand running up the centre of the shaft (Fig. 2U, V and W). Cluster crystals of calcium oxalate occur but are uncommon.

*Calyx*

The cells of the *outer epidermis* are polygonal with fairly straight, thin walls which become a little thicker towards the base of the calyx. Occasional isolated cells have cellulosic thickening and are 15 to 35 μm long, 6 to 22 μm wide, and 8 to 14 μm deep.

The *inner epidermis* shows two zones. In the marginal region the cells are elongated transversely and have thickened lignified walls apart from a few small patches of similar unlignified cells. They are 15 to 45 μm long, 5 to 15 μm wide and 20 to 30 μm deep. Small pits are visible on the walls of some groups of lignified cells and the middle lamella is very prominent (Fig. 2T). This marginal region extends for 10 to 12 cells towards the base, i.e. $\frac{1}{4}$ to $\frac{1}{3}$ of the length of the calyx.

In the basal region the cell walls become more heavily thickened and lignified, the cells are wider, and pits are absent. These cells are 18 to 45 μm long, 11 to 24 μm wide and 20 to 40 μm deep, the longest axis being generally aligned towards the ovary. This zone is about 12 to 14 cells deep (Fig. 2H).

The mesophyll consists of thin-walled parenchyma increasing in thickness from one row of flattened cells near the margin, to 4 rows of larger cells near the ovary (Fig. 2F and G). Stomata, trichomes, cuticle and calcium oxalate are absent from the calyx.

*Corolla*

On the lobes the cells of the *outer epidermis* are generally rectangular with straight walls. Near the tube, the cells are narrower and more elongated. The cells, which are 16 to **25·5** to 42 μm long and 6 to **13·6** to 22·0 μm wide, are covered by a thick cuticle bearing parallel slightly sinuous striations. Near the edge of the lobe the striations are very sinuous. There are numerous paracytic stomata measuring about

30 μm in diameter which are raised above the level of the surrounding cells. There are no trichomes on the outer surface (Fig. 2 O).

On the corolla tube, the cells of the outer epidermis are narrower and more elongated along the corolla axis than on the lobes; they are 43 to **58·2** to 70 μm long and 5 to **7·1** to 12 μm wide. The surface is ridged over the vascular bundles and has a faint, thin, slightly striated cuticle (Fig. 2S).

The *inner epidermis* of the corolla lobes consists of polygonal to rectangular cells with thin, straight walls measuring 11 to **21·7** to 40 μm long, 6 to **14·2** to 27 μm wide, and 3·3 to **7·1** to 12 μm deep. The surface of the fleshy pad which covers the top half of the lobes is heavily ridged and covered by a thick cuticle with prominent sinuous striations. These striations become more patchy and less prominent towards the middle of the lobe. There are a few lignified trichomes near the middle of the lobes. They are generally bent over near their lignified bases and appear to lie between the ridges on the surface. They measure 200 to 300 μm in length and about 25 μm at the base. There are occasional lignified cells on or just below the epidermis near these trichomes (Fig. 2I, L and R). The lower half of the lobes is very thin. In the region where the lobes join to form the tube, in the throat of the corolla, the surface is covered with thin-walled sinuous twisted trichomes measuring 100 to **175** to 400 μm long and up to 50 μm in diameter at the base (Fig. 2M).

The inner surface is heavily indented with ridges which follow the main vascular bundles (Fig. 3E). The cells are rectangular and elongated; they measure 70 to **100** to 120 μm long, 8 to **11** to 15 μm wide, and 5 to **9·7** to 17 μm deep, and are covered by a thin cuticle with faint longitudinal striations (Fig. 2S).

The *mesophyll* consists of two main tissues. In the lobes there is a variable region of cellulosic collenchymatous cells immediately below each epidermis. The main part of the mesophyll is composed of large, yellow, thin-walled parenchymatous cells (Fig. 3A and F). Lower down in the ridged corolla tube the cells are mainly collenchymatous with thickened cellulosic walls, but there are also very large parenchymatous thin-walled cells, measuring 20 to 50 μm in diameter in transverse section, which appear to be lacunae in the mesophyll, (Fig. 3E and G). Cluster crystals of calcium oxalate occur mainly in the lobes and are 6 to **10** to 17 μm in diameter. The vascular tissue consists of lignified spiral vessels with associated phloem cells.

*Androecium*

The characteristic tissue of the anther lobe is the "fibrous layer," the cells of which are isodiametric, with a contorted shape in surface view. The cell walls bear spiral thickening which appears to be linked with that of adjacent cells in an intricate pattern. The thickening is only slightly lignified. The cells are 6 to **13** to 19 μm in diameter and 11 to **20·5** to 37 μm deep. Epidermal cells were not visible in the material examined (Fig. 3H).

The connective consists of small yellowish cells, some containing cluster crystals of calcium oxalate. Crystals are, however, more common in the larger paler cells next to the fibrous layer and measure 5 to **11·1** to 17 μm in diameter. There is a vascular bundle consisting of small lignified vessels (Fig. 3H).

The *pollen* grains are subspherical and measure 14 to **16·7** to 18 μm in diameter. The exine is covered with minute pits and there are three germinal furrows and pores (Fig. 1D). There are occasional immature pollen grains that appear more triangular in polar view.

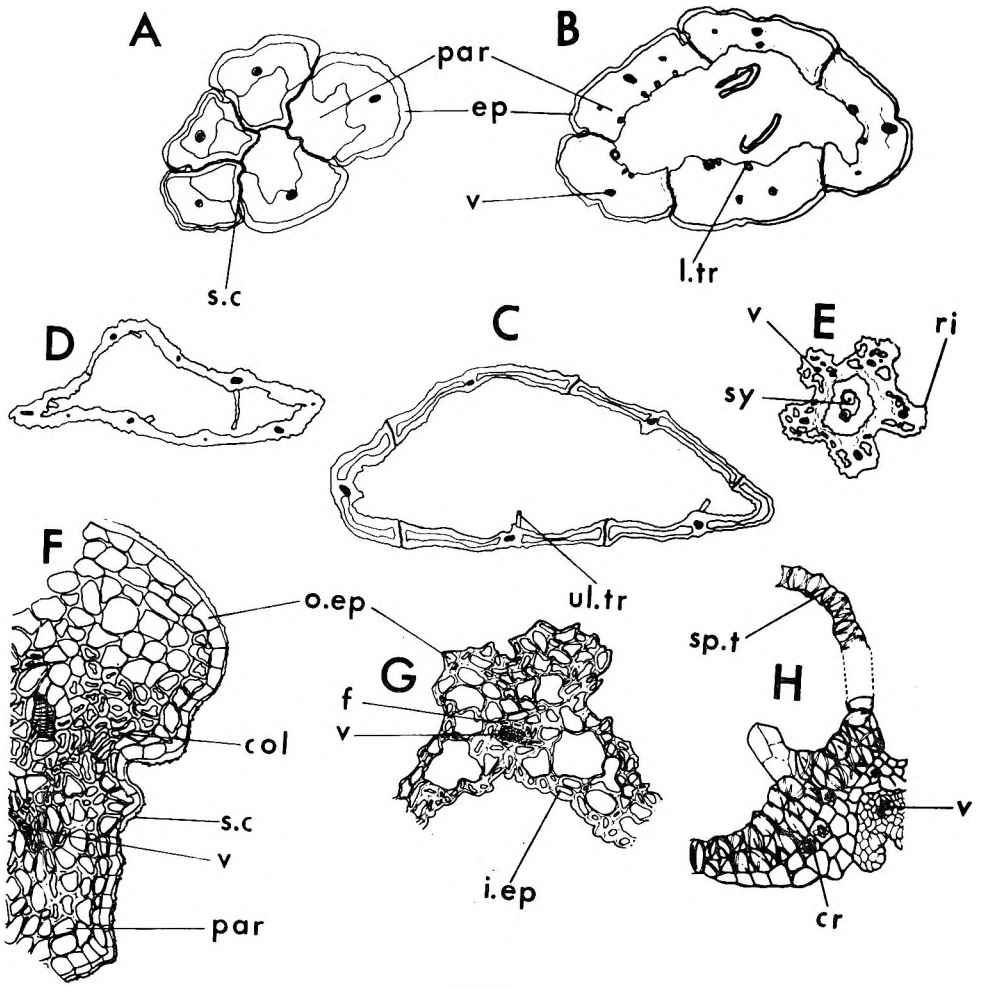


FIG. 3.

FIG. 3. *M. inermis*, A, B and C, corolla, transverse sections at a, e and c respectively on Fig. 1, I; D, corolla, just below lobes, transverse section; E, corolla, near base, transverse section; all × 35. F, corolla, apex of lobes, transverse section; G, corolla, tube, transverse section; H, anther lobe and connective, transverse section; all × 150.
cr, cluster crystal of calcium oxalate; col, collenchyma; ep, epidermis; f, fibre; i.ep, inner epidermis; l.tr, lignified trichome; o.ep, outer epidermis; par, parenchyma; ri, ridge; s.c, striated cuticle; sp.t, spiral thickening; sy, style; ul.tr, unlignified trichome; v, vein.

*Gynaecium*

*Stigma.* The epidermal cells of the stigma are elongated radially and have thin suberized anticlinal walls. Small globules of fixed oil are present in these cells. The cortex has an outer parenchymatous region of larger thin-walled cells and an inner layer of thick-walled collenchymatous cells. The centre of the stigma consists of parenchyma and some of the cells contain cluster crystals of calcium oxalate. There are also two major groups of lignified spiral vessels orientated in various directions (Fig. 4E, F and H).

*Style.* The epidermal cells of the style are small and polygonal with thick cellulosic walls and a thin striated cuticle. The cortex is composed of larger collenchymatous

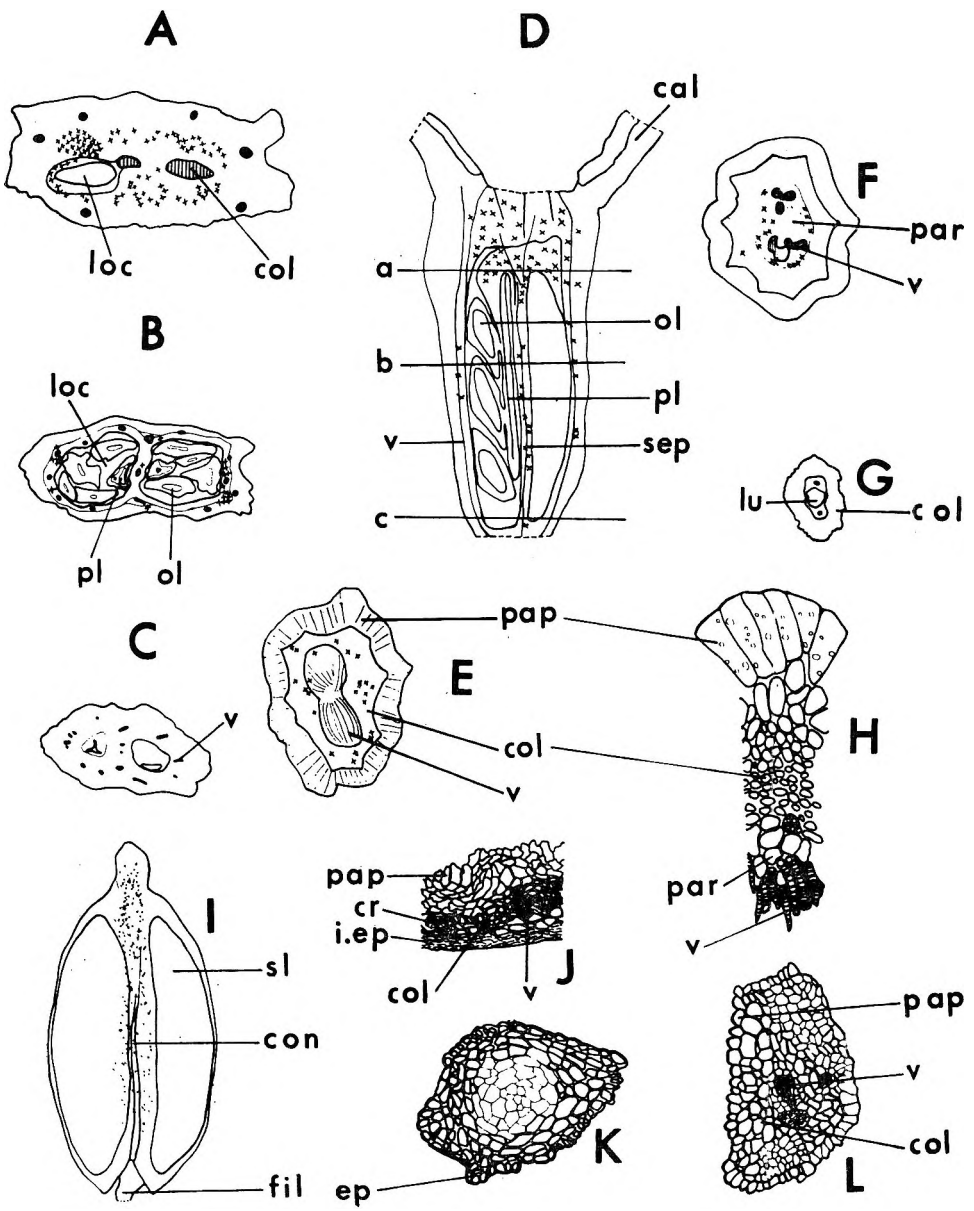


FIG. 4.

FIG. 4. *M. inermis*, A, B and C, ovary, transverse sections at a, b and c respectively on Fig. 4, D; all × 45; D, ovary and calyx, medium vertical section × 35; E, stigma, upper part, transverse section; F, stigma, middle part, transverse section; G, style, transverse section; all × 35. H, stigma, upper part, transverse section × 150; I, anther, adaxial surface, × 35; J, ovary, wall, transverse section; K, ovule, transverse section; L, placenta, transverse section; all × 150.
col, collenchyma; con, connective; cr, cluster crystal of calcium oxalate; ep, epidermis; fil, filament; i.ep, inner epidermis; loc, loculus; lu, lumen; o.ep, outer epidermis; ol, ovule; pap, papillose epidermis; par, parenchyma; pl, placenta; sep, septum; sl, slit; v, vein.

cells. There is an inner region of thick-walled cells, each with a narrow reddish lumen, surrounding two distinct groups of lignified spiral vessels. In the lower half of the style there is a central lumen but this becomes filled with parenchyma in the upper part of the style (Fig. 4G).

*Ovary.* The *outer epidermis* is of small subrectangular papillose cells with thin walls. The cells are 13 to **23·8** to 43 $\mu$m long and 6 to **11·1** to 17 $\mu$m wide. There are occasional conical lignified trichomes near the top of the ovary, similar to those growing on the receptacle. They are 180 to 350 $\mu$m long and about 15 $\mu$m wide.

The *inner epidermis* is not readily visible in surface view, but in section is seen to consist of 4 to 5 rows of similar radially flattened thin-walled cells (Fig. 4B and J).

The *mesophyll* consists of a layer of parenchymatous cells beneath each epidermis. Below the inner epidermis some of these cells contain cluster crystals of calcium oxalate, particularly towards the top of the ovary. The crystals measure 3·5 to **9·4** to 19 $\mu$m in diameter. In each half of the ovary there are 4 to 6 groups of spirally thickened and lignified vessels, each group being surrounded by small dark brown cells. The septum dividing the two loculi has a structure similar to that of the inner region of the ovary wall (Fig. 4A, B and C), with a central vascular bundle. The placenta is only attached to the septum at the top and bottom of the ovary (Fig. 4B and D). It has an epidermis of clear cells, those facing towards the septum being papillose. There is a yellow layer of large thin-walled cells with yellow contents below the papillose cells and a central vascular bundle of lignified spirally thickened vessels. The remainder of the tissue is composed of small parenchymatous cells, some of which contain cluster crystals of calcium oxalate (Fig. 4L).

When immature the ovules consist of an outer epidermis of thin-walled yellowish cells and an inner region of small undifferentiated cells. In transverse section these cells appear to radiate from the centre of the ovule, with the cell walls decreasing in thickness towards the centre (Fig. 4K).

## DISCUSSION

Although at first sight the globose flower-heads of *M. inermis*, *M. ciliata* and *M. stipulosa* look similar, the flowers of *M. inermis* differ markedly from those of the other two species. Neither the calyx of *M. inermis* nor of *M. ciliata* are visible above the surface of the globular receptacle, as is the case with *M. stipulosa*, but dissection of the flower-heads shows that the calyx of *M. inermis* is small with an entire, glabrous margin while that of *M. ciliata* is large with a lobed, ciliate margin. The ratio of the average length of the calyx plus ovary to the average length of the bracteole in mature flowers is—*M. inermis*, 0·53; *M. ciliata*, 0·95; and *M. stipulosa*, 1·42. This clearly distinguishes each of the three species.

Other macroscopical differences are that the corolla of *M. inermis* does not have a hood in the corolla lobe, the top half being fleshy, and further, there are no trichomes on the outer epidermis. With *M. ciliata* and *M. stipulosa*, on the other hand, there are many golden trichomes present. The anthers of *M. inermis* are exserted and pendulous on a short filament, while those of *M. ciliata* and *M. stipulosa* are sessile with introrse dehiscence.

*M. inermis* also differs by having an ovary in which the placentas hang freely from the septum and a style with a distinct central lumen.

Microscopically, the three species differ in many details, though only a few are of major diagnostic significance, for example, the absence of crystals of calcium

oxalate from the peduncle, receptacle and calyx of *M. inermis*. This species also differs in having lignified cells only down the centre of the shaft of the bract, whereas in the other two species almost the entire shaft is lignified. Further, stomata are absent from the calyx of *M. inermis* but are present in the other species, while they are numerous on the lobes of the petals of *M. inermis* and absent or rare on the lobes of the petals of *M. ciliata* and *M. stipulosa*.

The differences described above should be sufficient to distinguish between the leaves and flowering tops of *M. inermis* and *M. ciliata* in the coarsely powdered condition.

*Acknowledgements*

Our thanks are due to Mr. E. Allman, former Head of the Faculty of Pharmacy, and to Professor A. N. Tackie, the present Head of the Faculty of Pharmacy, University of Science and Technology, Kumasi, Ghana, for their invaluble assistance in collecting the flowering tops. We also thank Mr. A. Enti for identifying the trees from which the flowers were collected

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# The alkaloids of the leaves of *Mitragyna inermis* (Willd.) O.Kuntze

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The leaves of *Mitragyna inermis* (Willd.) O. Kuntze have been examined for alkaloids and shown to contain routundifoline, isorotundifoline, rhynchophylline, isorhynchophylline, ciliaphylline, rhynchociline, speciophylline, a small amount of uncarine F, mitraciliatine and traces of a second indole alkaloid which appears to be of the corynanthine type. There is also a polar compound present, Dragendorff positive, which remains on the base line when subjected to thin-layer chromatography with the usual solvent systems used for the mitragyna alkaloids.

The alkaloidal content of the leaves of *Mitragyna stipulosa* (D.C.) O. Kuntze and of *Mitragyna ciliata* Aubr. et Pellagr. has been reported by Beckett, Shellard & Tackie (1963a,b). Tackie (1963) made a preliminary study of the alkaloids of the leaves of *Mitragyna inermis* (Willd.) O. Kuntze and found rotundifoline, isorotundifoline and isorhynchophylline in addition to rhynchophylline previously reported by Raymond-Hamet & Millat (1934).

A detailed examination of the leaves of *M. inermis* now confirms the presence of rotundifoline, isorotundifoline, rhynchophylline, isorhynchophylline, ciliaphylline, rhynchociline, speciophylline, a small amount of uncarine F, mitraciliantine and traces of a second indole alkaloid. The presence of a strongly polar substance which gives an orange colour with Dragendorff's reagents (cf. Tackie, 1963) is confirmed; it is identical with a "base line" alkaloid reported by Shellard & Phillipson (1964) as occurring in the leaves of *Mitragyna rotundifolia* (Roxb.) O. Kuntze from Burma.

## EXPERIMENTAL

*Column chromatography.* The alumina used was Spence-type H and the silica gel was silica gel H (Merck).

The hRf values were obtained using three systems (layer thickness, 250 $\mu$m): (*a*) alumina and chloroform, (*b*) silica gel and ether, (*c*) silica gel and chloroform–acetone (5:4).

For preparative thin-layer chromatography, silica gel HF 254 (Merck) 1 mm thick was used, the solvent system being either (i) chloroform–acetone (1:1) or (ii) chloroform–methanol (95:5). All melting points are uncorrected.

### *Materials*

Leaves from trees growing in the Sogakofe district of the Volta Region, Ghana, were collected during various periods up to June, 1966. Details of authentication are given by Pillay (1964) and Shellard & Wade (1969).

*Isolation of alkaloids*

Coarsely powdered leaves (6·5 kg) were extracted by maceration with 96% ethanol and the extract evaporated under reduced pressure to a thin syrup. After acidifying with glacial acetic acid and diluting with a large volume of water, the precipitated non-alkaloidal matter was filtered off. The filtrate was made alkaline with ammonia and extracted with chloroform. The extract was washed, dried and evaporated to yield crude alkaloidal extract (12·5 g). Thin-layer chromatography showed the presence of at least six alkaloids.

The crude alkaloids were dissolved in dilute sulphuric acid (100 ml) and after extracting with ether, the solution was made alkaline with ammonia and re-extracted with ether. The ethereal extract was washed, dried and evaporated to dryness to yield a purified alkaloidal extract (8·1 g). The alkaline solution was further extracted with chloroform to yield a pale brown residue (0·3 g) which on crystallization from absolute ethanol gave colourless crystals (3 mg) of "base-line alkaloid."

The purified alkaloidal residue was divided into two parts and each treated as follows: approximately 4·0 g was dissolved in chloroform (5 ml) and added to a column of alumina (15 × 2·5 cm) packed in chloroform. The alkaloids were eluted with chloroform (750 ml) and then chloroform–methanol (4:1) (250 ml), 25 ml portions of eluate being collected. Thin-layer monitoring allowed the bulking of like portions to give the following fractions: (1) Chloroform (50 ml), containing a trace of an oxindole alkaloid; the yield was too small to allow characterization. (2) Chloroform (450 ml) containing eight alkaloids—"Fraction A" (5·65 g). (3) Chloroform (150 ml) containing one major alkaloid which crystallized from acetone to give colourless needles of speciophylline, m.p. 183° (223 mg). (4) Chloroform–methanol (250 ml) containing four alkaloids (one of which gave an immediate blue colour with vanillin and hydrochloric acid)—"Fraction B" (162 mg).

Attempts to separate the alkaloids in Fraction A by column chromatography using benzene, ether and chloroform were not successful. However, the last portion of the chloroform eluate gave a pale yellow residue which contained traces of alkaloids, one of which was an indole alkaloid. This was isolated by preparative thin-layer chromatography system (i) and, on recrystallization from ether–light petroleum (b.p. 40–60°) (1:1) gave fine colourless crystals of mitraciliatine, m.p. 140° (85 mg). The remaining combined eluates after concentration were dissolved in 5% acetic acid and extracted with chloroform. The residue, after evaporation to dryness was designated "Fraction C" (4·85 g). The acid solution was made alkaline with ammonia and extracted with chloroform. The residue on recrystallization from dry ether yielded colourless needles of rhynchociline, m.p. 178° (365 mg).

Fraction C was dissolved in ether and added to a silica gel column (12·5 × 2 cm) packed in ether. The alkaloids were eluted with ether (800 ml) and ether–chloroform (4:1) (250 ml), 20 ml portions being collected. Thin-layer monitoring allowed bulking of like portions to give the following fractions: (1) Ether (60 ml) containing one alkaloid which on recrystallization from absolute ethanol gave colourless needles of rotundifoline, m.p. 239° (215 mg). (2) Ether (180 ml) containing chiefly a mixture of rotundifoline and isorhynchophylline—"Fraction D" (2·5 g). (3) Ether (80 ml), which gave a colourless amorphous powder, recrystallization of which from ether–n-hexane gave with some difficulty isorhynchophylline m.p. 144° (650 mg). (4) Ether (200 ml) containing four alkaloids. Recrystallization from absolute ethanol yielded prismatic crystals of rhynchophylline, m.p. 213° (638 mg).

The mother liquors contained one main alkaloid corresponding to that present in fraction 5. (5) Ether (100 ml). This fraction was bulked with the residue from fraction (4) and recrystallized from acetone to give ciliaphylline m.p. 222° (147 mg).

The mother liquors from which rhynchophylline and ciliaphylline had been isolated contained an alkaloid having identical hRf values to isorotundifoline. The dried residue from these mother liquors was therefore dissolved in ether and extracted with 5% sodium hydroxide. This extract was then acidified with hydrochloric acid and made alkaline again with ammonia. Extraction with chloroform yielded a material which was recrystallized three times from acetone to yield colourless rosettes of isorotundifoline, m.p. 132° (97 mg).

The residue from Fraction D was dissolved in chloroform (10 ml) and extracted with 1% citric acid (3 × 10 ml). After washing with water the chloroform was evaporated to dryness and the residue recrystallized from absolute ethanol to yield further crystals of rotundifoline, m.p. 239° (816 mg). The citric acid solution was made alkaline with ammonia, extracted with chloroform and the residue after removal of the solvent was recrystallized from ether–n-hexane to yield crystals of isorhynchophylline, m.p. 145° (1·2 g). The mother liquors from which the isorhynchophylline had crystallized contained a further alkaloid which was isolated by preparative thin-layer chromatography system (ii). Elution of the appropriate zone gave amorphous uncarine F (17 mg).

Fraction B contained traces of oxindole alkaloids previously isolated together with traces of an indole alkaloid (Sp 4). This was isolated by preparative thin-layer chromatography system (i) in a quantity insufficient for identification.

*Characterization of alkaloids*

The alkaloids were characterized by comparison of the m.p., mixed m.p., ultraviolet and infrared spectra and hRf values in thin-layer systems a, b and c by comparison with authentic specimens of each alkaloid (Table 1).

Table 1. *hRf values of alkaloids in system a, b and c*

| | System a | System b | System c |
|---|---|---|---|
| Rotundifoline[1] .. .. | 52 | 35 | 70 |
| Isorotundifoline[1] .. .. | 43 | 10 | 44 |
| Rhynchophylline[2] .. .. | 13 | 4 | 27 |
| Ciliaphylline[3] .. .. .. | 12 | 3 | 26 |
| Rhynchociline[3] .. .. | 26 | 4 | 8 |
| Speciophylline[4,5] .. .. | 8 | 0–1 | 22 |
| Uncarine F[4] .. .. .. | 43 | 23 | 61 |
| Mitraciliatine[6] .. .. | 28 | 2 | 6 |
| Indole alkaloid Sp4* .. .. | 23 | 0 | 4 |
| "Base line" alkaloid .. | 0 | 0 | 0 |

Authenticated by comparison with sample from:
[1] *M. stipulosa* (Beckett, Shellard & Tackie, 1963a). [2] *M. rotundifolia* (Shellard & Phillipson, 1964). [3] *M. ciliata* (Beckett & others, 1963b). [4] *M. parvifolia* (Shellard, Phillipson & Gupta, 1968) and [5] with picrate from *M. speciosa* (Beckett, Shellard & others, 1966). [6] Synthetic material (Trager, Phillipson & Beckett, 1968).

* $\lambda_{max}$ (nm) 226, 291, 278 shoulder; $\lambda_{min}$ (nm) 249, 288. $\nu_{max}$ (Nujol), 3300, 1700, 1580 (weak), 1460, 1380, 1330, 1250, 1100, 760, 740 $cm^{-1}$. Absence of band between 1600 and 1650 (double band).

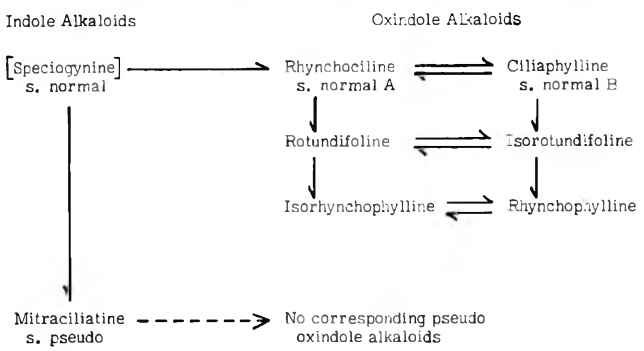
## DISCUSSION

Seven of the alkaloids present (six oxindole and one indole alkaloid) are the same as those present in the leaves of *M. ciliata*; all are E *seco* type alkaloids. However, whereas the oxindole alkaloids have the normal configuration [C(3)-H$\alpha$, C(20)-H$\beta$],

the indole alkaloid, mitraciliatine has the pseudo configuration [C(3)-H$\beta$, C(20)-H$\beta$]. On the basis of the hypothesis for oxindole alkaloid biogenesis suggested by Shellard, Phillipson & Gupta (1969) it might have been anticipated that the other indole alkaloid (Sp 4) present in traces would have been speciogynine since this is the corresponding alkaloid in the normal series. The hRf values are not consistent with this and the limited spectral data suggest an alkaloid of the corynanthine type. Should this be confirmed it would be the first alkaloid having a carbocyclic E ring to be isolated from species of *Mitragyna*, though they occur in other Rubiaceous plants.

Failure to detect speciogynine may be due to several reasons, similar to those suggested for the absence of tetrahydroalstonine in the leaves of *M. parvifolia* (Shellard, Phillipson & Gupta, 1969).

The suggested biogenetic route for the oxindole alkaloids would be:




Demethylation of the oxindole alkaloids, rhynchociline and ciliaphylline would give rotundifoline and isorotundifoline while removal of the C(9)-OH from these alkaloids would give isorhynchophylline and rhynchophylline respectively. This would be just the opposite of the route envisaged in *M. parvifolia* where the same oxindole alkaloids occur but the corresponding indole alkaloids are dihydrocorynantheine (s. normal) and hirsutine (s. pseudo), both of which are unsubstituted in the C(9) position. It is interesting to note that a (C)9-OH indole alkaloid has not been isolated from any *Mitragyna* species. The two remaining oxindole alkaloids, speciophylline and uncarine F are *closed* E ring alkaloids with epiallo configuration. Unlike *M. parvifolia* there seem to be no corresponding indole alkaloids or *closed* E ring oxindole alkaloids of other configurations present in the leaves of *M. inermis*. However, investigation of the alkaloidal pattern in the leaves, roots and bark of the plant collected at regular intervals throughout the year may reveal the presence of corresponding alkaloids or their precursors, and this is being currently undertaken.

*Acknowledgements*

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# The absolute configurations of the alkaloids of *Physostigma venenosum* seeds*

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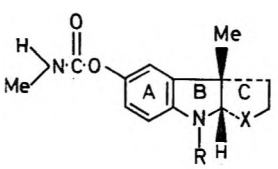
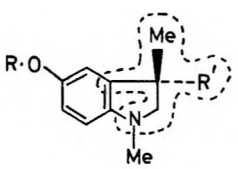
The absolute configuration of physostigmine has been established by correlating the configuration of its C-3a atom with that of the asymmetric carbon atom in (+)-3-ethyl-3-methoxycarbonyl-3-methylpropionic acid. Comparison of the optical rotatory dispersion spectra of physostigmine, $N_a$-norphysostigmine, geneserine, physovenine and eseramine have shown that all five alkaloids have the same absolute configurations.

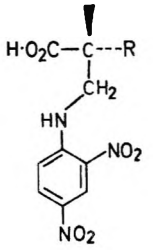
In physostigmine, the major alkaloid of *Physostigma venenosum* seeds, and in the four minor alkaloids physovenine, $N_a$-norphysostigmine, geneserine and eseramine (Robinson, 1968), the aromatic ring A and the ring B form a near-planar system. It may therefore be deduced that the B/C ring junction is represented by a *cis* fusion (Jackson, 1954, see also Witkop & Hill, 1955; McFarland, Inoue & Nakanishi, 1969; Spande, Wilchek & Witkop, 1968). This deduction is supported by the X-ray crystallographic determination of the structures and absolute configurations of the alkaloids echitamine (Hamilton, Hamor, & others, 1962; Manohar & Ramaseshan, 1961), chimonanthine (Grant, Hamor & others, 1965) and hodgkinsine (Fridrichsons, Mackay & Mathieson, 1967), all of which contain the physostigmine ring system with the B/C rings *cis*-fused, and it has recently been verified by the detection of an internal nuclear Overhauser effect between the protons of the 3a-methyl group and the 8a-proton (Newkome & Bhacca, 1969). Thus physostigmine and its above-mentioned related minor alkaloids are represented either by I [R = Me, X = N—Me; R = Me, X = O; R = H, X = N—Me; R = Me, X = (N→O) Me and R = Me, X = N—CONHMe, respectively] or the mirror images.



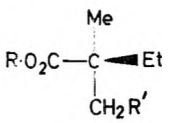

I




II




III




IV

With a view to obtaining C-3a of physostigmine in a form ($\beta$-amino-acid) suitable for comparison with a compound of known absolute configuration, the following preliminary oxidative experiments were made on model compounds. 5-Methoxy-1,3,3-trimethylindoline (II, R = R′ = Me) was prepared by sodium borohydride

* Alkaloids of *Physostigma venenosum*. Part VIII; for Part VII see Robinson & Robinson (1968): for a preliminary communication of this work see Longmore & Robinson (1969).

reduction of 5-methoxy-1,3,3-trimethyl-3*H*-indolium iodide (Ahmed & Robinson, 1967). Ozonolysis of this indoline followed by treatment with hydrogen peroxide afforded a low yield of the desired β-amino-acid, containing the moiety outlined in (II), which was isolated by the method of Rao & Sober (1954) as its 2,4-dinitrophenyl derivative (III, R = Me). (±)-5-Ethoxy-3-ethyl-1,3-dimethylindoline (±)-(II, R = R′= Et) was prepared from (±)-5-hydroxy-1,3-dimethyloxindole (Robinson, 1965; Longmore & Robinson, 1967) by *O*-ethylation, 3-ethylation and lithium aluminium hydride reduction respectively. Ozonolysis of the indoline as before gave (±)-III (R = Et), the structure of which was verified by the following synthesis. (±)-3-Ethyl-3-methoxycarbonyl-3-methylpropionic acid, (±)-IV (R = Me, R′ = COOH) (Ställberg-Stenhagen, 1951) was converted into its amide (±)-IV (R = Me, R′ = $CONH_2$) via the anhydride. Treatment of the amide with sodium hypobromite, conditions which also effected hydrolysis of the ester group, yielded (±)-IV (R = H, R′ = $NH_2$) which was isolated by the method of Rao & Sober (1954) as its 2,4-dinitrophenyl derivative (±)-III (R = Et). This oxidative route was then applied to the problem of the establishment of the absolute configuration of physostigmine as follows.

Physostigmine (I, R = Me, X = N — Me) was converted, via eserethole and eserethole methiodide, into eserethole methine (Hoshino & Kobayashi, 1934) which upon catalytic hydrogenation in acid solution followed by quaternization with methyl iodide afforded dihydroeserethole methine methiodide [II, R = Et, R′ = $(CH_2)_2N^+Me_3I^-$] (Polonovski, 1918). This was subjected to Hofmann degradation to give II (R = Et, R′ = CH = $CH_2$) which on hydrogenation yielded

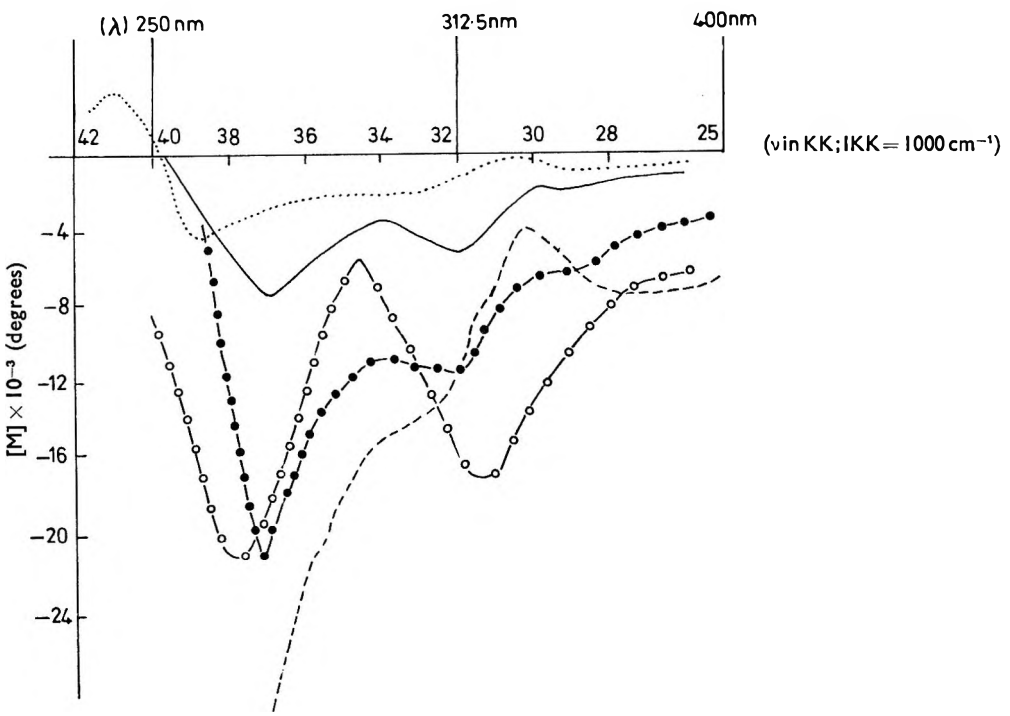



FIG. 1. Optical rotatory dispersion spectra of the alkaloids of *Physostigma venenosum* seeds. ———, Physostigmine (rotations ×4); ·····, Na-norphysostigmine; ●—●—●—●. eseramine; ○—○—○—○, geneserine and — — — — —, physovenine.

II (R = R′ = Et). When the oxidative procedure described above was applied to this indoline the 2,4-dinitrophenyl derivative III (R = Et) was obtained.

The absolute configuration of this compound was established by the synthesis of its enantiomer from (−)-3-ethyl-3-methoxycarbonyl-3-methylpropionic acid (IV, R = Me, R′ = COOH) (Ställberg-Stenhagen, 1951), by the route described above for the racemic compound. In turn the absolute configuration of IV (R = Me, R′ = COOH) follows from its hydrolysis to (−)-2-ethyl-2-methylsuccinic acid (IV, R = H, R′ = COOH), the absolute configuration of which has been established using the quasi-racemate technique (Porath, 1951; Fredga, 1960; see also Cox, Koch & others, 1967). These results have been consolidated by relating (Harris, Robertson & Whalley, 1958; Cox, Ellestad & others, 1965) the configuration of (+)-2-ethyl-2-methylsuccinic acid to the asymmetric C-13 atom of rosenonolactone, the absolute configuration of which has been determined by X-ray crystallography (Scott, Sutherland & others, 1964). The absolute configuration of physostigmine is therefore established as being as shown in I, (R = Me, X = N—Me).

The absolute configurations of the other four alkaloids follows from comparison of their optical rotatory dispersion spectra with that of physostigmine. Fig. 1 shows that all five alkaloids have closely similar spectra, comprising a negative Cotton effect for the absorption band at *ca* 300 nm, followed by a second, stronger negative Cotton effect, centred at *ca* 250 nm. Further details of the spectra are given in Table 1.

Table 1. *Salient features of the optical rotatory dispersion spectra of the alkaloids of* Physostigma venenosum *seeds*

| | 1st Trough | | 1st Peak | | 2nd Trough | | 2nd Peak | | 3rd Trough | | Crossover | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Alkaloid | λ nm | [M]° | λ nm | [M]° | λ nm | [M]° | λ nm | [M]° | λ nm | [M]° | λ nm | [M]° |
| Physostigmine[a] .. | 344 | −520 | 336 | −480 | 313 | −1560 | 294 | −940 | 272 | −2180 | 250 | 0 |
| $N_a$-Norphysostigmine | 350 | −400 | 328 | −100 | 303 | −1100[b] | 280 | −1900[b] | 258 | −3700 | 252 | 0[c] |
| Physovenine .. .. | 355 | −7600 | 332 | −4100 | 313 | −14000[b] | 294 | −15800[b] | 270 | −28000[d] | — | — |
| Geneserine .. .. | [e] | [e] | [e] | [e] | 318 | −17200 | 288 | −5700 | 263 | −21000 | — | — |
| Eseramine[f] .. .. | 333 | −6400[b] | 333 | −6400[b] | 313 | −11300 | 297 | −10700 | 270 | −21300 | 255 | 0 |

[a] Rotations for physostigmine have been multiplied by 4. [b] Inflection. [c] Further data; 242 nm, [M] = +3650° (peak); 230 nm, [M] = 0°; 222 nm, [M] = −1500° (lowest point observed). [d] Lowest wavelength of observation. [e] The spectrum for geneserine does not show a peak, trough or inflection in this region, though a marked change of slope occurs—typical values are 357 nm, [M] = −7600°; 335 nm, [M] = −13000°. [f] An inflection occurs in this spectrum at 279 nm, [M] = −15000° (see Fig. 1).

For eseramine and physovenine, the Cotton effect at longer wavelength is observed only as inflections on the steeply falling background dispersion curve, but the close similarity of all five spectra is clearly established. Only for $N_a$-norphysostigmine have both extrema of the second Cotton effect been observed (these arise from the absorption band at *ca* 250 nm), but the negative extrema at *ca* 265 nm is clear in the other cases. The observed Cotton effects, which show a correspondence of sign for corresponding transitions throughout the series, show that all five alkaloids have the same absolute configurations. This conclusion can also be independently reached for physostigmine, geneserine and physovenine since the former alkaloid has been chemically converted into the latter two by reactions which cannot cause optical inversion at the asymmetric centres (Robinson, 1963; Longmore & Robinson, 1966, respectively). The absolute configurations of the four minor alkaloids are therefore identical with that of physostigmine about their B/C ring junctions.

## EXPERIMENTAL

Melting-points were recorded on a Kofler hot-stage apparatus and are uncorrected. Ultraviolet spectra were measured in ethanolic solution on a Perkin-Elmer model 137

spectrophotometer, infrared spectra were recorded as Nujol mulls or liquid films on a Perkin-Elmer model 237 spectrophotometer and the mass spectrum was recorded on an A.E.I. MS.9 spectrometer. Optical rotatory dispersion spectra were obtained in 95% ethanol using a Bendix-N.P.L. "Polarmatic" spectropolarimeter; concentrations were varied from $4 \times 10^{-4}$ to $0{\cdot}8 \times 10^{-4}$M, all spectra were checked at several different concentrations and results are reproducible within 3%. Solutions were dried with anhydrous magnesium sulphate and solvents were removed on a steam-bath (unless otherwise stated) under reduced pressure (water pump). Solid analytical samples were dried ( 6 h) at room temperature/0·1 mm over phosphorus pentoxide.

5-*Methoxy*-1,3,3-*trimethylindoline* (II, R = R′ = Me). To an ice-cold solution of 5-methoxy-3,3-dimethyl-3-*H*-indole methiodide (Ahmed & Robinson, 1967) (16·0 g) in methanol (300 ml) sodium borohydride (10·0 g) was added in portions with occasional swirling. The solution was then kept at room temperature overnight, water (150 ml) added, the methanol evaporated and the liberated oil extracted into ether (3 × 150 ml). Evaporation of the combined dried ethereal extracts afforded an oil (8·8 g) which upon distillation gave a colourless oil (6·44 g; 67%), b.p. 80°/0·4 mm (Millson & Robinson, 1955, b.p. 118°/0·5 mm). The picrate crystallized from ethanol in yellow leaflets, m.p. 147–149° (with sweating at 135°). Found: C, 51·4; H, 4·95. $C_{18}H_{20}N_4O_8$ requires C, 51·4; H, 4·8%.

*Ozonolysis of* 5-*Methoxy*-1,3,3-*trimethylindoline* (II, R = R′ = Me). Ozone-enriched oxygen was bubbled (24 h) through a solution of 5-methoxy-1,3,3-trimethylindoline (502 mg) in glacial acetic acid (50 ml). Hydrogen peroxide solution (6% v/v, 50 ml) was then added and the mixture stood at room temperature for 4 h. The excess hydrogen peroxide was decomposed by the addition of finely-divided platinum (50 mg) and after filtration the solution was concentrated on a rotary evaporator. Ethanol (25 ml) was added and evaporated again to remove all traces of acetic acid. The residue, in 0·88 ammonia (40 ml), was treated with hydrogen peroxide (6% v/v, 40 ml) and the effervescing solution was kept at room temperature overnight. Finely-divided platinum (50 mg) was added to the solution which was heated (5 min) on a steam-bath, filtered and evaporated on a rotary evaporator. The residue was dissolved in ethanol and the solution evaporated to remove the last traces of water. The resulting semicrystalline residue was triturated with 95% ethanol (10 ml) and the colourless crystalline ammonium oxalate (159 mg) filtered off. Evaporation of the filtrate afforded a gum (324 mg) which was dissolved in 50% aqueous ethanol (40 ml), sodium bicarbonate (1·0 g) and 2,4-dinitrofluorobenzene (1·0 g) were added and the mixture was shaken (3 h) at room temperature. The ethanol was removed at room temperature on a rotary evaporator and the excess 2,4-dinitrofluorobenzene was removed by extraction into ether (3 × 25 ml). The aqueous solution was then acidified to approximately pH 1·3 with 6N hydrochloric acid, and the insoluble material was extracted into chloroform (3 × 15 ml). Evaporation of the dried chloroform extracts gave III (R = Me) (26 mg; 3·5%) which was recrystallized three times from ether-light petroleum (b.p. < 40°) to give yellow needles, m.p. 189–191° (with sweating from 160°). The high resolution mass spectrum had a molecular ion at m/e = 283·0807 ($C_{11}H_{13}N_3O_6$ requires 283·0804) and a base peak at m/e = 196 [2,4-(di$NO_2$) — $C_6H_3$ — $N^+H$ = $CH_2$ produced by cleavage of the methylene group-quaternary carbon C–C bond in III (R = Me)].

(±)-5-*Ethoxy*-3-*ethyl*-1,3-*dimethyloxindole*. (±)-5-Ethoxy-1,3-dimethyloxindole (Julian & Pikl, 1935) (42·9 g) was dissolved in a solution of sodium (7·22 g) in dry ethanol (500 ml). Ethyl iodide (83·2 g) was then added dropwise over 30 min, with stirring, at room temperature. After a further 1 h the solution was boiled under reflux (2 h). The ethanol was removed, water (100 ml) was added to the residue, and the resulting oil was extracted into chloroform (3 × 100 ml). The dried combined chloroform extracts were evaporated to leave a light-brown oil which upon distillation (b.p. 130–135°/0·5 mm) gave a pale yellow oil which soon crystallized. Recrystallization from light petroleum (b.p. < 40°) afforded pale yellow plates (38·7 g; 79%), m.p. 35·5–37°. Found: C, 71·55; H, 8·1. $C_{14}H_{19}NO_2$ requires C, 72·05; H, 8·2%.

(±)-5-*Ethoxy*-3-*ethyl*-1,3-*dimethylindoline* [(±)-II, R = R′ = Et]. Lithium aluminium hydride (650 mg) was added in small portions with stirring to a solution of (±)-5-ethoxy-3-ethyl-1,3-dimethyloxindole (2·0 g) in sodium-dried tetrahydrofuran (30 ml) at room temperature. The stirred mixture was boiled under reflux (3 h), water added to decompose excess lithium aluminium hydride and the resulting granular white precipitate removed by filtration and washed with ether. The combined filtrate and ether-washings were dried and evaporated to give a pale brown oil (1·9 g; 99%) which upon distillation afforded a pale yellow oil (1·3 g; 67%), b.p. 106°/0·2 mm. Found: C, 76·1; H, 9·4; N, 6·7. $C_{14}H_{21}NO$ requires C, 76·65; H, 9·65; N, 6·4%. The picrate crystallized from 95% ethanol in yellow prisms, m.p. 148–150° (with sweating from 135°). Found: C, 53·6; H, 5·2. $C_{20}H_{24}N_4O_8$ requires C, 53·55; H, 5·4%.

*Ozonolysis of* (±)-5-*Ethoxy*-3-*ethyl*-1,3-*dimethylindoline* [(±)-II R = R′ = Et]. Ozone-enriched oxygen was bubbled through a solution of (±)-5-ethoxy-3-ethyl-1,3-dimethylindoline (2·0 g) in glacial acetic acid (150 ml) until the colour of the solution, which became very dark during the initial stages of the reaction, was bleached (about 4½ h). Hydrogen peroxide solution (30% v/v, 50 ml) was added and the solution kept at room temperature overnight, after which it was boiled under reflux for 30 min; platinum black (50 mg) was added and the boiling under reflux continued until oxygen-evolution ceased. After filtration and removal of the acetic acid on a rotary evaporator, the residue was dissolved in 4N hydrochloric acid (20 ml), the solution was again boiled under reflux (2 h) and again evaporated. The residue was dissolved in aqueous ethanol (50%, 40 ml), sodium bicarbonate (2·0 g) and 2,4-dinitrofluorobenzene (1·0 g) were added and the mixture was stirred (2 h) at room temperature. After pouring into water (50 ml), 2,4-dinitrophenol and excess 2,4-dinitrofluorobenzene were extracted into chloroform (6 × 20 ml) (Extract 1). The aqueous phase was acidified to approximately pH 1·3 with concentrated hydrochloric acid and the insoluble material extracted into chloroform (2 × 20 ml) (Extract 2). Evaporation of Extract 2 after drying gave an orange oil (150 mg) which was shown by thin-layer chromatography [on Eastman Chromagram silica gel sheets, type 6060 using methanol–chloroform (1 : 5 v/v) as developing solvent] to be a mixture of 2,4-*dinitrophenol* and (±)-2-*methyl*-2-(2,4-*dinitrophenylaminomethyl*)*butyric acid*, (±)-III (R = Et). Authentic samples used as markers gave Rf = 0·37 and 0·70, respectively. The two components were separated by column chromatography on silica gel using ether–chloroform (1 : 10 v/v) as eluant and continuously monitoring (15 ml fractions) by thin-layer chromatography (as above).

A further yield of almost pure (±)-III (R = Et) was obtained by washing Extract 1 with saturated sodium bicarbonate solution (2 × 20 ml), washing the aqueous solution with chloroform (10 ml), acidifying with 4N hydrochloric acid and extracting the required product into chloroform (2 × 10 ml) (Extract 3).

The required eluates from the column chromatogram were combined with Extract 3 and evaporated to dryness to afford (±)-2-*methyl*-2-(2,4-*dinitrophenylaminomethyl*)-*butyric acid* as an oil (91 mg; 3·3%) which completely crystallized on trituration with ether. Recrystallization from ether containing a trace of chloroform afforded yellow needles (30 mg; 1%), m.p. 153–155° (with sweating from 141°), giving no depression on admixture with the authentic sample prepared below. Their behaviour on thin layers and their infrared spectra were likewise identical.

(±)-3-*Ethyl*-3-*methoxycarbonyl*-3-*methylpropionic anhydride*. A solution of (±)-3 ethyl-3-methoxycarbonyl-3-methylpropionic acid (Ställberg-Stenhagen, 1951) (1·0 g) in acetic anhydride (5 ml) was boiled under reflux (1½ h): acetic acid and excess acetic anhydride were then removed and the oily residue distilled to give the *anhydride* as a colourless viscous oil (720 mg; 76%), b.p. 204°/0·5 cm. Found: C, 58·15; H, 8·05. $C_{16}H_{26}O_7$ requires C, 58·15; H, 7·95%.

(−)-3-*Ethyl*-3-*methoxycarbonyl*-3-*methylpropionic anhydride* was likewise obtained from the (−) acid (Ställberg-Stenhagen, 1951) in an identical manner (86% yield), b.p. 190–195°/0·5 cm. The infrared spectrum was identical with that of the racemate prepared above.

(±) - 3 - *Ethyl* - 3 - *methoxycarbonyl* - 3 - *methylpropionamide* [(±) - IV, R = Me, R′ = $CONH_2$]. Ammonia, dried by passage over sodium hydroxide pellets, was bubbled through a solution of (±)-3-ethyl-3-methoxycarbonyl-3-methylpropionic anhydride (4·7 g) in dry ether (50 ml) (30 min) when a colourless crystalline deposit of ammonium (±)-3-ethyl-3-methoxycarbonyl-3-methylpropionate gradually formed. Water (25 ml) was then added, the aqueous layer was extracted with ether (20 ml) and the combined ether extracts were washed with water (2 × 25 ml). Evaporation of the dried ether extracts afforded the *amide* as a colourless oil (485 mg; 20%), b.p. 110° (bath temperature)/0·7 mm. Found: C, 55·45; H, 8·6. $C_8H_{15}NO_3$ requires C, 55·45; H, 8·75%.

(−)-3-*Ethyl*-3-*methoxycarbonyl*-3-*methylpropionamide* (IV, R = Me, R′ = $CONH_2$). The laevorotatory anhydride (1·6 g) was similarly converted into the (−)-*amide* (IV, R = Me, R′ = $CONH_2$) (807 mg; 97%), a colourless oil, b.p. 180°/0·5 cm, $[\alpha]_D^{23} = -7{\cdot}83°$, $[M]_D^{23} = -13{\cdot}57°$ (95% EtOH). The infrared spectrum was identical with that of the racemate prepared above.

(±)-2-*Methyl*-2-(2,4-*dinitrophenylaminomethyl*)*butyric acid* [(±)-III, R = Et]. To a mixture of (±)-3-ethyl-3-methoxycarbonyl-3-methylpropionamide (200 mg) and bromine (185 mg) 10% aqueous sodium hydroxide was added until the colour of the mixture was pale yellow. Aqueous sodium hydroxide (5 ml, containing 2·25 g of sodium hydroxide) was then added and the mixture was warmed to 70° on a steam-bath for 30 min. After cooling, the solution was made weakly acidic by the careful addition of 4N-hydrochloric acid, excess sodium bicarbonate was added to neutralize the acid, the volume of the solution was doubled by the addition of absolute ethanol and 2,4-dinitrofluorobenzene (500 mg) was added. The mixture was then shaken vigorously (3 h); the ethanol was removed on a rotary evaporator at room temperature and unreacted excess 2,4-dinitrofluorobenzene was removed by extraction into ether (3 × 25 ml). The aqueous layer was acidified to approximately pH 1·3

with 6N hydrochloric acid and the solution extracted with chloroform (3 × 15 ml). Drying and evaporation of the combined chloroform extracts gave a yellow oil which crystallized and which was recrystallized from ether containing a trace of chloroform to give (±)-2-*methyl*-2-(2,4-*dinitrophenylaminomethyl*)*butyric acid* in yellow needles (6·7 mg; 2%), m.p. 151–152° (with sweating from 142°). Found: C, 48·7; H, 5·1; N, 14·1. $C_{12}H_{15}N_3O_6$ requires C, 48·5; H, 5·1; N, 14·15%.

(+)-2-*Methyl*-2-(2,4-*dinitrophenylaminomethyl*)*butyric acid* [IV, R = H, R′ = 2,4-(di$NO_2$)-$C_6H_3$-NH]. To a stirred solution of (−)-3-ethyl-3-methoxycarbonyl-3-methylpropionamide (410 mg) in methanol (10 ml) bromine (500 mg) was added followed by a solution of sodium (0·50 g) in methanol (10 ml). The mixture was allowed to stand at room temperature for 30 min, sodium hydroxide solution [5·4 g in water (12 ml)] was added and the mixture boiled under reflux (1 h). The solution was then acidified to pH 1·3 with 4N hydrochloric acid, excess sodium bicarbonate (1·5 g) and 2,4-dinitrofluorobenzene (1·0 g) added, the volume of the mixture was doubled by the addition of ethanol and the solution stirred (2 h) at room temperature. A further quantity of sodium bicarbonate (1·5 g) and 2,4-dinitrofluorobenzene (1·0 g) were added and the stirring continued (1 h). The product was isolated in an identical manner to the racemic compound above. (+)-2-*Methyl*-2-(2,4-*dinitrophenylaminomethyl*)*butyric acid* (60 mg; 8·5%) was recrystallized from ether-light petroleum (b.p. < 40°) in yellow needles, m.p. 134–137° (with sweating from 128°). Found: C, 48·4; H, 5·2; N, 14·0. $C_{12}H_{15}N_3O_6$ requires C, 48·5; H, 5·1; N, 14·15%. Circular dichroism maxima at 199 nm ($\triangle\epsilon = +1{\cdot}0$).

(−)-5-*Ethoxy*-3-*ethyl*-1,3-*dimethylindoline* (II, R = R′ = Et). Dihydroeserethole methine methiodide (Polonovski, 1918; Hoshino & Kobayashi, 1934) (3·50 g) in a mixture of water (50 ml) and 95% ethanol (10 ml) was shaken (2 h) with freshly-prepared moist silver oxide (≡ 5 g silver nitrate). The mixture was filtered, the residue washed with 95% ethanol (20 ml) and the combined filtrate and washing evaporated to afford the *quaternary hydroxide* as a brown oil.

The oil was heated under reflux (3 h) at 120–130° (bath temperature)/10 mm and then partitioned between water (30 ml) and ether (3 × 50 ml). The combined ether extracts were dried and evaporated to give *dihydroeserethole methine methine* (II, R = Et, R′ = CH = $CH_2$) as a greenish-yellow oil (913 mg; 48%).

This was hydrogenated in ethanol (50 ml) at room temperature and atmospheric pressure over Adams' platinum oxide (50 mg). After the absorption of one mole equivalent of hydrogen, the platinum was removed by filtration and washed with ethanol (10 ml). The combined filtrate and washing were evaporated and the residue distilled to afford (−)-5-*ethoxy*-3-*ethyl*-1,3-*dimethylindoline* (II, R = R′ = Et) as a yellow oil (743 mg; 39%), b.p. 166° (bath temperature/2 mm. The picrate crystallized from ethanol in yellow needles, m.p. 147–150° (with sweating from 139°). Found: C, 52·9; H, 5·45. $C_{20}H_{24}N_4O_8$ requires C, 53·55; H, 5·4%. The free base recovered from the picrate had infrared spectrum and b.p. identical with those of the product obtained after distillation of the total reaction product and had $[\alpha]_D^{23} = -3{\cdot}45$, $[M]_D^{23} = -7{\cdot}58$ (95% EtOH). Found: C, 76·9; H, 9·6. $C_{14}H_{21}NO$ requires C, 76·65; H, 9·65%.

*Ozonolysis of* (−)-5-*ethoxy*-3-*ethyl*-1,3-*dimethylindoline* (II, R = R′ = Et) (2 g) was carried out by the method already described for the ozonolysis of the racemic indoline to give (−)-2-*methyl*-2-(2,4-*dinitrophenylaminomethyl*)*butyric acid* (III, R = Et) as yellow needles (107 mg; 4%) from ether-light petroleum (b.p. < 40°), m.p. 133–135°

(with sweating at 126°), m.p. (on admixture with an equal weight of the synthetic enantiomer prepared above), 152–154° (with sweating at 132°) [*cf.* the racemate, m.p. 151–152° (synthetic) and 153–155° (from degradation) (with sweating at 142° and 141°, respectively)]. Found: C, 48·5; H, 4·9; N, 13·8. $C_{12}H_{15}N_3O_6$ requires C, 48·5; H, 5·1; N, 14·15%. Circular dichroism maxima at 202·5 nm ($\Delta\epsilon = -1{\cdot}4$). The infrared spectra of the enantiomers and their behaviour on thin-layer chromatograms were identical.

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SHORT COMMUNICATION

# Thin-layer chromatography and high voltage electrophoresis of quaternary alkaloids from *Fagara* species

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Because of their physical properties quaternary alkaloids can be separated chromatographically by partitioning between immiscible solvents on a cellulose support using either paper (Kuck, Abonico & others, 1967) or cellulose thin-layers (Calderwood & Fish, 1966). However, the minimum time needed is some 3–4 h including a 1 h equilibration period.

Electrophoresis at low voltages was suggested for the separation of quaternary alkaloids (Marini-Bettolo & Coch Frugoni, 1958) but the time required was also about 3 h. With the introduction of high voltage electrophoresis, the possibility of shortening the separation time became apparent, for example, the separation of tertiary ergot alkaloids was reduced to 45 min (Agurell, 1965). We wished to effect rapid separation of the quaternary bases of the genus *Fagara* (Rutaceae) and have compared the results of thin-layer chromatographic separations with those achieved by electrophoresis.

## *Experimental*

*Alkaloids.* Solutions of salts (iodide or chloride) 1% in ethanol of the following alkaloids were used: candicine, coryneine, tembetarine, magnoflorine, *N*-methylcorydine, *N*-methylisocorydine, laurifoline, xanthoplanine, palmatine, berberine, chelerythrine, nitidine.

*Thin-layer chromatography.* Plates with cellulose (Whatman Chromedia CC41), spread to 0·25 mm and dried at 40°, were developed with (1) 0·1N hydrochloric acid, (2) n-butanol saturated with 2N hydrochloric acid, or (3) n-butanol–pyridine–water (6:4:3). The plates were equilibrated for 1 h before development for which times were 35, 150 and 120 min respectively.

*High voltage electrophoresis.* Of the several buffer solutions tried, the Britton–Robinson barbitone buffer, pH 6·8–8·0, gave best results with the alkaloids examined.

A Camag high voltage electrophoresis cell was used and the potential gradient was varied between 75 and 125 V/cm, the cell being cooled by water at a flow rate of 2 litres/min. Electrophoresis was for 20 min. Platinum electrodes were necessary since the alkaline barbitone buffer corrodes the usual electrodes. Chromatography papers, Whatman No. 1, 40 × 20 cm, were saturated with the buffer solution and the excess removed by pressing between layers of absorbent paper.

*Alkaloid detection.* The thin-layer chromatograms and the electropherograms were examined under ultraviolet light (366 nm), after exposure to ammonia vapour; they were then sprayed with iodoplatinate reagent.

Table 1. *Thin-layer chromatographic characteristics of the quaternary alkaloids from* Fagara *species*

| Alkaloids | Rf values* Solvents 1 | 2 | 3 | Fluorescence after $NH_3$ | Colour with iodoplatinate reagent |
|---|---|---|---|---|---|
| Candicine.. .. .. | 0·92 | 0·45 | 0·47 | — | Purple |
| Coryneine .. .. | 0·88 | 0·28 | 0·64 | — | Pale blue |
| Tembetarine .. .. | 0·85 | 0·55 | 0·57 | — | Green |
| Magnoflorine .. .. | 0·24 | 0·30 | 0·24 | Blue | Purple |
| *N*-Methylisocorydine .. | 0·77 | 0·53 | 0·49 | Blue | Purple |
| *N*-Methylcorydine .. | 0·74 | 0·57 | 0·65 | Blue | Purple |
| Laurifoline .. .. | 0·11 | 0·19 | 0·42 | Blue | Purple |
| Xanthoplanine .. .. | 0·34 | 0·42 | 0·63 | Blue | Purple |
| Palmatine.. .. .. | 0·15 | 0·28 | 0·45 | Yellow | Brown |
| Berberine .. .. | 0·13 | 0·36 | 0·49 | Lime green | Brown |
| Chelerythrine .. .. | 0·07 | 0·21 | 0·92 | Yellow | Brown |
| Nitidine .. .. .. | 0·00 | 0·00 | 0·40 | Green | Brown |

* Mean values of between 10–20 runs.

*Results and discussion*

The separation of the twelve alkaloids obtained by thin-layer chromatography using solvents 1–3 is shown in Table 1. By using these systems together with fluorescence (after exposure to ammonia vapour), and also the colours produced with iodo-

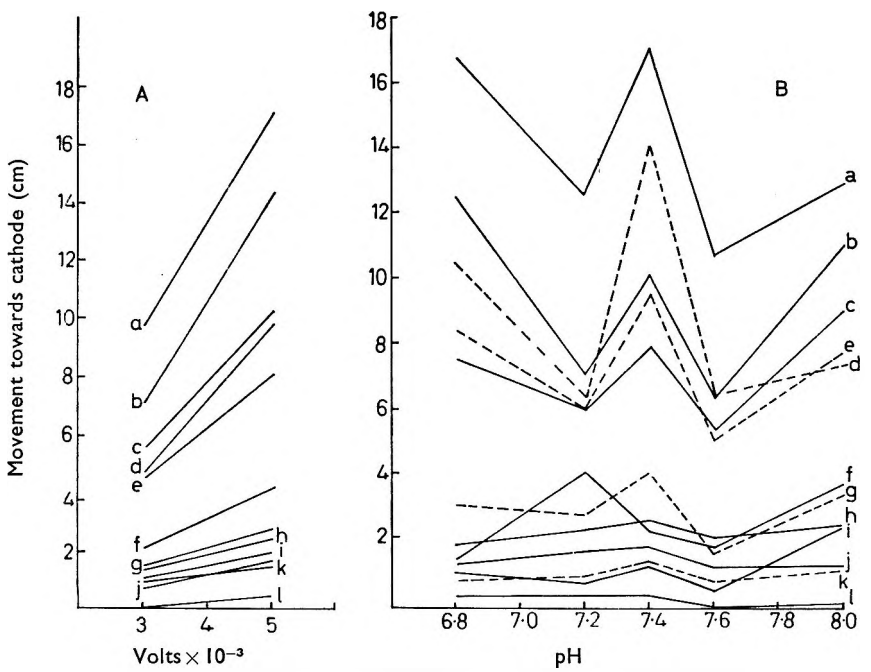


FIG. 1. A. Electrophoretic movement of alkaloids at different voltages; 20 min; pH 7·4. The alkaloids are: a; candicine; b, coryneine; c, tembetarine; d, *N*-methylisocorydine; e, *N*-methylcorydine; f, xanthoplanine; g, palmatine; h, berberine; i, chelerythrine; j, laurifoline; k, magnoflorine and l, nitidine.

B. Electrophoretic movement of alkaloids in buffered conditions at different pH values; 20 min; 5000 V. The alkaloids are: a, candicine; b, tembetarine; c, *N*-methylisocorydine; d, *N*-methylcorydine; e, coryneine; f, berberine; g, xanthoplanine; h, palmatine; i, magnoflorine; j, chelerythrine; k, laurifoline; l, nitidine.

platinate reagent, it is possible to identify all twelve alkaloids. A disadvantage is the time taken for separation; with solvent 2 this was 210 min. However, compared with paper chromatography (Kuck & others, 1967), the use of cellulose thin-layers, using solvent 3, is advantageous since candicine, tembetarine, *N*-methylisocorydine and nitidine were more easily identified.

The separation of the twelve alkaloids by high voltage electrophoresis is shown in Fig. 1A and B. Fig. 1A indicates the advantage of using the highest possible voltage, the alkaloids moving greatest distances at 5,000 V. All the alkaloids were reasonably well separated at pH 7·4 (Fig. 1B), but laurifoline, magnoflorine, berberine, palmatine, and the benzophenanthridines, chelerythrine and nitidine were best separated at pH 8·0. The principal advantage of this method is the rapidity (20 min), with which the separations can be achieved.

The influence of hydroxyl groups on mobility of the alkaloids is shown by the fact that coryneine with two hydroxyl groups, has a lower mobility than candicine with one hydroxyl group. In the aporphine series, also, the mobility of magnoflorine (two hydroxyl groups) is very much less than that of either *N*-methylcorydine (one hydroxyl) or *N*-methylisocorydine (one hydroxyl) and the mobility of laurifoline (two hydroxyl groups) less than that of xanthoplanine, and this alkaloid, with one hydroxyl group at position 9, has a lower mobility than either *N*-methylcorydine or *N*-methylisocorydine, each with one hydroxyl group at carbons 1 and 11 respectively.

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# Dissolution and absorption of ICI 49,455

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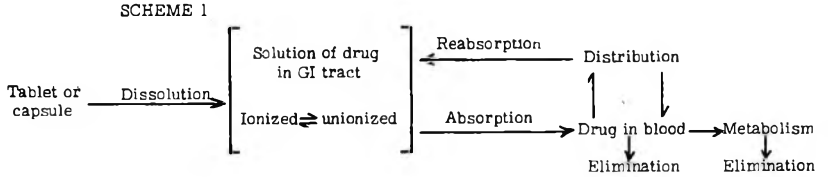
The dissolution rate of ICI 49,455 (1-isopropyl-1,2,3,4-tetrahydro-β-carboline) from tablet and capsule formulations cannot be related to *in vivo* absorption in dogs. The rate of partitioning of the drug into organic solvents and its absorption in goldfish and through human buccal membrane depend on the pH of the solution. Absorption increases with decrease in the degree of ionization. The dissociation constant and the elimination rate of the drug in man are such that high blood levels after oral administration are unlikely to occur.

Most drugs are absorbed from the gastrointestinal tract by a process of passive diffusion of the unionized moeity from solution across a lipid barrier. The amount of drug in the blood after oral administration is controlled by the factors in the following scheme.

SCHEME 1




In view of large variations in the blood levels of a potential analgesic, ICI 49,455 (1-isopropyl-1,2,3,4-tetrahydro-β-carboline) after its administration to volunteers as either plain or film-coated tablets, it was considered desirable to determine which factors in Scheme 1 were responsible.

Although most reports of dissolution rate-limited absorption relate to sparingly soluble drugs, Frostad (1961) and Levy (1964), respectively, have shown that the dissolution rate can control the absorption of the relatively water soluble sodium-*p*-aminosalicylate and acetylsalicylic acid. Levy, Leonards & Procknal (1965) have shown for aspirin formulations that *in vitro* dissolution can be correlated with rate of absorption. Rate of stirring and the pH of the dissolution medium must be carefully controlled (Levy, Leonards & Procknal, 1967).

The derivation of rate of absorption constants from blood level/time data is also the subject of much controversy (Loo & Riegelman, 1968). Intersubject variations in absorption, distribution, metabolism and excretion rates of drugs, all of which can produce variable blood levels of drug, are known to occur. Therefore, the factors affecting absorption of drug from solution should also be examined.

The work now described was an attempt to evaluate the relative contribution to the overall rate of absorption of dissolution rate of drug from formulations, the degree of ionization and the absorption of unionized drug.

For this purpose the *in vitro* dissolution rates of various formulations of the drug were determined using a modification of the method of Levy & Hayes (1960). The effect of pH on the partition rate of the drug between aqueous and organic solvent

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was examined using the method of Perrin (1967). *In vivo* absorption of the drug was examined using goldfish as described by Levy & Gucinski (1964), blood levels after oral administration of the drug to dogs, and the human buccal membrane method of Beckett & Triggs (1967).

## EXPERIMENTAL

ICI 49,455 as the hydrochloride salt has a $pK_a$ of 9·0; the equilibrium solubility at 37° in 0·1N hydrochloric acid is 1294 mg%.

The experimental formulations examined were (1) film-coated tablets, (2) uncoated tablets, (3) capsules and (4) an aqueous solution. Solid formulations contained 50 mg of the drug calculated as base.

*In vitro* dissolution rates were determined using the method of Levy & Hayes (1960) modified as follows. Formulations were enclosed within a rectangular basket (2 × 2 × 4 cm) constructed from 30 mesh stainless steel screen. The basket was centred on the base of a 400 ml water jacketed beaker, internal diameter 7 cm, containing 300 ml of simulated gastric juice, pH 1·4 (2·0 g sodium chloride, 7 ml conc. hydrochloric acid to 1 litre with water) at 37°. The dissolution medium was stirred by a single, angle bladed, glass Quickfit stirrer immersed 3 cm below the surface and driven by a geared, variable speed electric motor. Samples were removed periodically, filtered through a 0·45 μm Millipore filter and assayed spectrophotometrically at 278 nm by reference to a standard calibration curve. The percentage dissolution, corrected for cumulative sampling losses, was plotted as a function of time for each individual tablet. Dissolution times were derived from these plots and are presented as an average of six determinations for each formulation.

The effect of pH on the rate of partitioning of ICI 49,455 was determined using the three phase model described by Perrin (1967). Buffer solutions containing approximately 2 mg % of the drug were placed in compartment A, connected to compartment B containing pH 7·4 phosphate buffer via compartment C containing cyclohexane. The rate of loss of drug from compartment A was measured by spectrophotometric assay of the residual concentration.

Goldfish (*Carassius aurantus*) approximately 5 cm long and 7 g in weight were shown to survive for 48 h without visible distress in 0·05M tris buffer from pH 6·0 to 9·5. The time of death was taken as the time when gill and mouth movements ceased and fish were unable to return to the normal swimming position when overturned by means of a glass rod. To eliminate subjective bias all determinations were made by the same individual and solutions were coded. The code was broken only after completion of the experiment. The average of the death times of six individual fish for each concentration or pH was recorded.

The absorption in dogs of the four formulations given by mouth was examined is a cross-over experiment. The dogs were dosed with ICI 49,455 (approx. 20 mg/kg) and blood samples taken over 7 h. Each dog received a standard meal before the drug to minimise the risk of vomiting.

The concentration of the drug in blood was determined by extracting alkaline samples with ethanolic heptane followed by back extraction into hydrochloric acid. The fluorescence of the acid layer was then measured using an activation wavelength of 280 nm (uncorrected) and an emission wavelength of 352 nm (uncorrected).

The effect of pH on the buccal absorption of the drug in volunteers was determined by the method of Beckett & Triggs (1967). The solutions used contained 0·25 mg

in 25 ml of Sorensens phosphate buffer pH 6·0 and 7·0, Borax HCl buffer pH 9·0, 0·05M, or Tris buffer pH 10·0, 0·05M. The unabsorbed drug, remaining in the expelled solution after 5 min contact with buccal mucosa, was assayed spectrophotometrically after solvent extraction. The results are expressed as the average of the results obtained from four volunteers.

## RESULTS AND DISCUSSION

The times for 80 and 50% dissolution of ICI 49,455 from the formulations were derived from the percentage dissolved/time curves and are plotted as a function of stirring speed in Fig. 1a and b respectively. Uncoated tablets and capsules dissolved

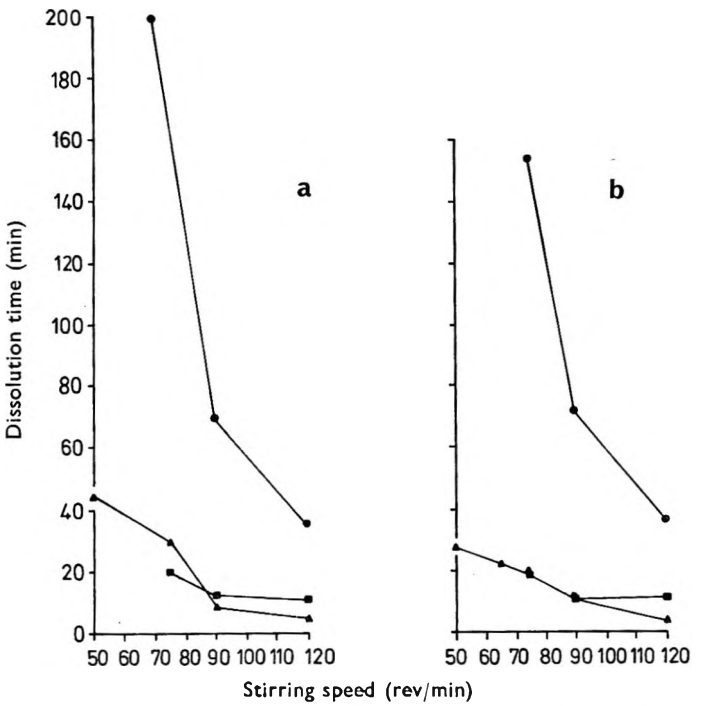


FIG. 1. Effect of stirring speed on the dissolution times of formulations of ICI 49,455. (*a*) 80% dissoln. (*b*) 50% dissoln. ●—● Film-coated tablets. ■—■ Capsules. ▲—▲ Plain tablets.

at approximately the same rate at the given stirring speed. Dissolution of film coated tablets showed a more marked dependence on stirring speed. The 50 and 80% dissolution times of film-coated tablets varied from approximately four times those of uncoated tablets at 120 rev/min to nine times at 75 rev/min. Such ninefold differences in dissolution rate might be expected to influence the rate of absorption and hence plasma level of a drug.

The effect of pH on the rate of loss of drug from compartment A of the three-phase model is shown in Fig. 2. At pH 6 no transfer from compartment A into B and C was evident after 24 h. The results agree with those from the goldfish and buccal mucosa studies and show an increase in partition rate with increase in pH of solution.

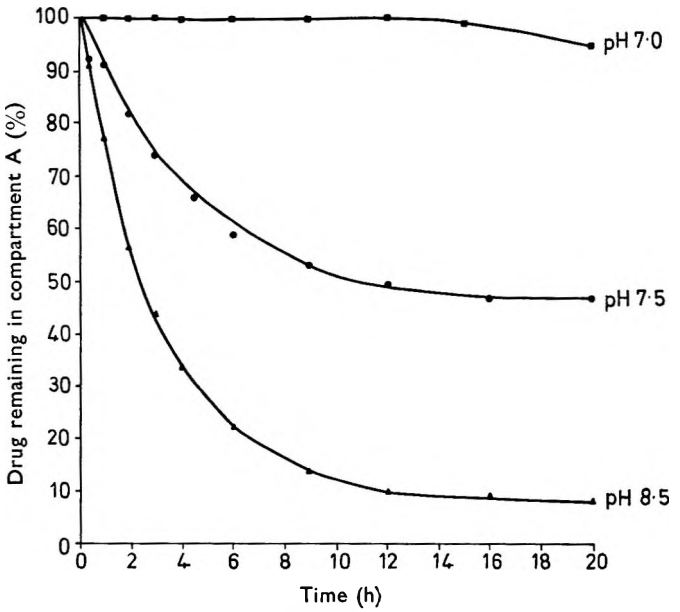


FIG. 2. Effect of pH on the loss of ICI 49,455 from compartment A of a three phase system.

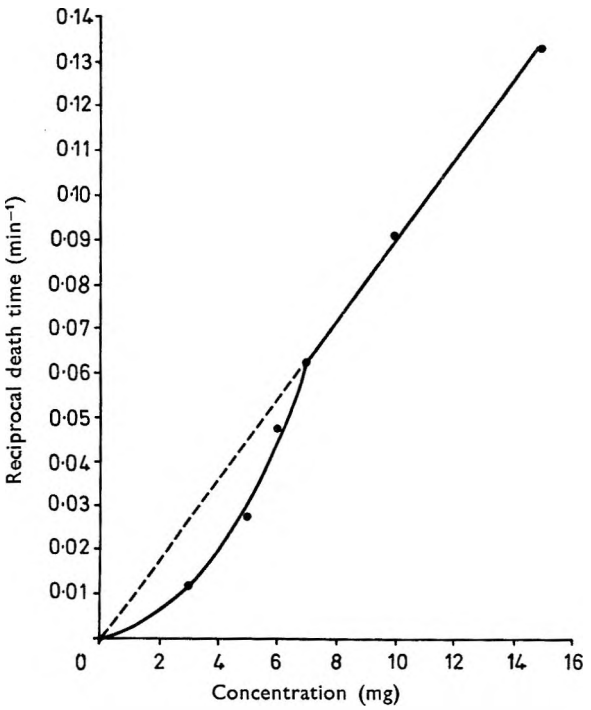


FIG. 3. Effect of concentration of ICI 49,455 on the death time of goldfish in pH 8·5 tris buffer.

The effect of concentration of ICI 49,455 on the death time of goldfish is shown in Fig. 3. According to the model proposed by Levy & Gucinski (1964) the time required for a pharmacological response (T) under constant experimental conditions is related to the drug concentration (C) in the following manner:

$$\frac{1}{T} = \frac{KC}{L} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

Where K is the relative absorption rate constant and incorporates membrane surface area and L is the amount of drug required in the fish to elicit the pharmacological response. The assumptions made in the derivation of equation 1 have been discussed by Levy & Gucinski (1964).

The value of L need not be known for studies of the effect of pH on the absorption rate provided that the lethal dose is not modified by these environmental factors. Preconditioning of fish for 2 h in buffers or water before transfer to buffered solutions of the drug had no effect on the death time. Intraperitoneal injection of 1 mg of the drug in goldfish, preconditioned in water and transferred to buffer solutions or water after injection, showed that the buffer had no effect on the subsequent death time. Thin-layer chromatographic analysis of homogenized extracts of fish exposed to lethal and sublethal concentrations of the drug failed to reveal the presence of metabolites. The departure of Fig. 3 from linearity at low concentrations of drug suggests that the assumptions made by Levy & Gucinski (1964) are not applicable to the present drug.

The effect of pH on the death time of fish immersed in 10 mg% solutions of the drug was examined using Levy & Gucinski's (1964) modified equation:

$$\frac{1}{T} = \frac{K\ F.C}{L} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where F is the fraction of unionized drug.

The results should yield a straight line plot of Fx 1/T passing through the origin (where F = 0) if the drug is absorbed only in the unionized form. Such a plot could not be obtained. Nevertheless, the drug was found to be lethal at pH 9·0 in 1 min whereas the same concentration of drug at pH 6 failed to kill within 24 h. Thus unionized drug is rapidly absorbed but a decrease in pH of the medium with a consequent increase in the degree of ionization reduces the absorption rate.

Table 1. ICI 49,455 μg/ml *of blood after oral administration of* 20 mg/kg *as* (1) *film coated tablets* (2) *uncoated tablets* (3) *capsules* (4) *an aqueous solution*

| Dog No. | Formulation | ICI 49,455 μg/ml of blood | | | | Time after dose (h) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ¼ | ½ | 1 | 1½ | 2 | 3 | 5 | 7 |
| I | 1 | — | 0·08 | 0·40 | 1·04 | 1·12 | 0·81 | 0·26 | 0·1 |
| | 2 | — | 1·04 | 1·13 | 1·01 | 0·62 | 0·42 | 0·15 | 0·18 |
| | 3 | — | 0·41 | 1·31 | 1·48 | 1·14 | 0·96 | 0·38 | 0·16 |
| | 4 | 0·26 | 0·88 | 0·84 | — | 1·12 | 0·53 | 0·25 | 0·14 |
| II | 1 | — | 0·83 | 1·98 | 1·55 | 1·15 | 0·74 | 0·31 | 0·07 |
| | 2 | — | 0·10 | 1·54 | 1·70 | 1·37 | 1·15 | 0·34 | 0·10 |
| | 3 | — | 1·62 | 1·57 | 1·10 | 0·94 | 0·56 | 0·18 | 0·08 |
| | 4 | 1·14 | 1·86 | 1·79 | — | 1·61 | 0·78 | 0·28 | 0·05 |
| III | 1 | — | 0·86 | 1·06 | 1·12 | 0·95 | 0·69 | 0·48 | 0 16 |
| | 2 | — | 0·71 | 1·14 | 1·13 | 1·13 | 1·11 | 0·46 | 0·22 |
| | 3 | — | 1·80 | 2·19 | 1·80 | 1·37 | 0·85 | 0·32 | 0·125 |
| | 4 | 0·33 | 0·62 | 0·75 | — | 0·93 | 0·72 | 0·30 | 0·10 |

The blood level/time data obtained from oral dosage in dogs are shown in Table 1. No significant advantage can be claimed for any of the formulations. There is no evidence to suggest that ICI 49,455 was more rapidly or efficiently absorbed from

solution than the solid formulations. The peak blood levels occurred 30 to 120 min after dosage and on a dose/kg body weight basis are similar to those achieved in man.

Dissolution is thus not the limiting factor in the absorption of the drug in dogs; hence the variations in blood levels obtained in human trials are unlikely to be caused by the variable dissolution rates of formulations.

The effect of pH on the absorption of the drug from the buccal cavity gave a linear response, 15% of the dose was absorbed at pH 6 whereas 75% was absorbed at pH 10. Although the method used included a rinse technique it is possible that binding of drug to the buccal mucosa or swallowing may occur, producing apparently high levels of absorption. The results show that the rate of absorption increases with increase in pH and the fraction of unionized drug.

Variable absorption of the drug cannot be related to *in vitro* dissolution rates of formulations, although large variations in dissolution rates were detected. The variations in blood levels are probably caused by variations in pH at the site of absorption and by inter-subject variations in elimination rate. Although unionized drug rapidly penetrates biological membranes, the ionic environment encountered in the upper part of the gastrointestinal tract is such that the drug will be almost completely ionized. The rapid absorption, required to balance the high elimination rate and produce adequate plasma levels of the drug, is not achieved. Formulations designed to increase the dissolution rate of drug will therefore have no effect on the absorption rate of the drug.

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SHORT COMMUNICATION

# Excretion of cocaine and its metabolites in man

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Little information exists on the metabolism, distribution and excretion of cocaine in persons dependent upon it. We now present results showing the proportions of cocaine excreted unchanged and as its metabolite benzoylecgonine.

## *Experimental*

*Regimen.* The subject was a 23-year-old male who usually administered to himself, by intravenous injection, 120 mg cocaine hydrochloride and 180 mg diacetylmorphine (as hydrochloride), in divided doses as desired. During the study period the drugs were given intramuscularly by nursing staff and a strict check was kept on drug dosage, times of administration, times of micturition, and volumes of urine voided. The pH of each urine sample was measured with a pH meter and the samples stored (−20°) pending analysis.

*Urine analysis.* Analysis for cocaine was made on 1–5 ml aliquots from each urine sample using the method of Fish & Wilson (1969). Cocaine was removed from similar aliquots of the same samples by extraction with diethyl ether (3 × 5 ml) and benzoylecgonine was then extracted by continuous liquid–liquid extraction with chloroform (12 ml) to which internal standard solution (1 ml, 5α-cholestane 1·55 mg % in chloroform) had been added. The extract was concentrated (100 μl) and treated with ethereal diazomethane (0·5 ml): excess reagent was removed (40°) after 5 min. The residue was suspended in saturated sodium bicarbonate solution (1 ml) from which cocaine was extracted with diethyl ether (2 × 1 ml). The extract was concentrated (50 μl) and 1–2 μl analysed by gas chromatography (Fish & Wilson, 1969). Calibration curves were constructed by adding known weights of benzoylecgonine to urine samples and processing these as described above. The curves were linear over the range 1–14 μg/ml.

Confirmation of the identity of benzoylecgonine in the test samples was obtained by extracting larger volumes (50 ml), concentrating the extract and separating by preparative thin-layer chromatography adapted from the system of Noirfalise & Mees (1967). The appropriate band was removed, the compound eluted and its ultraviolet spectrum obtained: this was indicative of benzoylecgonine (Orosoco, 1956).

The system of Noirfalise & Mees (1967) and that of Majlat & Bayer (1965) gave a faint band of characteristic colour with Dragendorff's reagent, and of correct position for ecgonine. The partition characteristics of ecgonine are such (Weast, 1964) that it cannot be recovered from urine by the extraction procedure described, in amounts sufficient for quantitation.

The subject was also taking orally glutethimide, orphenadrine and methadone, but none of these, or their metabolites, interfered with the determinations.

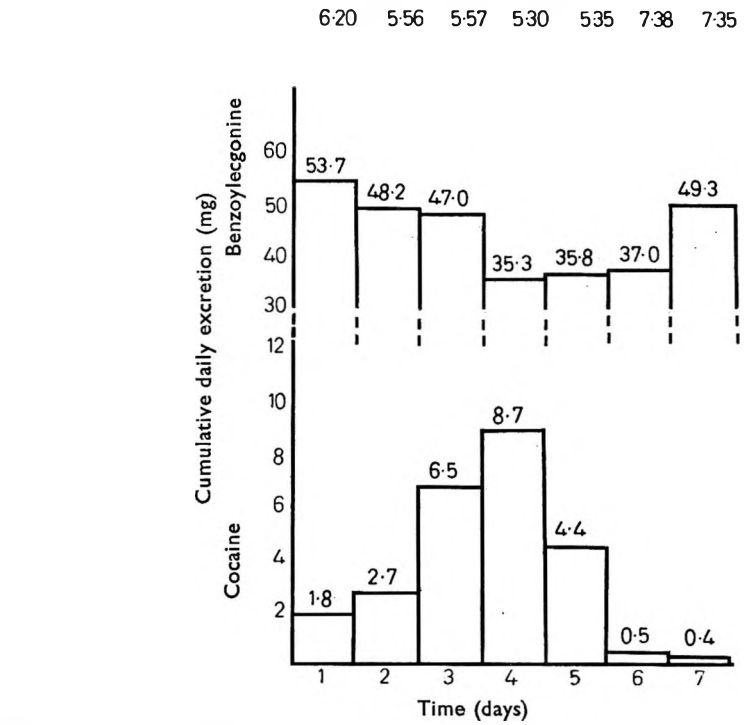


FIG. 1. Excretion (%) of cocaine and benzoylecgonine in urine of different pH values.

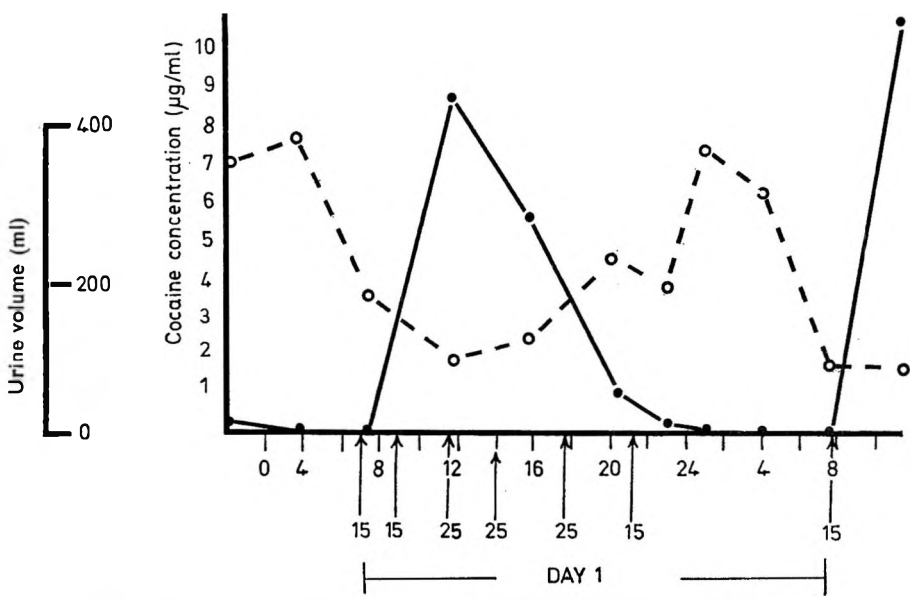


FIG. 2. Plots of total urine volumes and cocained concentration in urine against time of day (B.S.T.). The doses of cocaine hydrochloride (mg) and the times of administration are at the arrows. ●-● Cocaine concn. ○-○ Total urine volume.

*Results and discussion*

Results of analyses for cocaine and benzoylecgonine are summarized in Figs 1 and 2.

Amounts of unchanged cocaine (1–9%) excreted in the addict's urine agree with those previously reported (Woods, McMahon & Seevers, 1951) after submucosal administration (1–21%). The one report (McIntyre, 1936) of a 54% excretion in 12 h following cocaine overdosage is not strictly comparable.

As expected with an amphoteric compound, the cumulative excretion of benzoylecgonine was not pH dependent. Cumulative excretion of cocaine depended on urinary pH (Fig. 1) and this conclusion was supported by our unpublished observations on the buccal absorption of cocaine measured by the method of Beckett & Triggs (1967).

A regime to induce acid urine begun on the fourth day of the investigation was not maintained after one dose of ammonium chloride (4 g) because this caused the patient to become irritable, cold and drowsy; this despite indications of normal liver function as determined immediately before commencing the regime. Moreover, the patient's urine was normally acidic with pH values lower than the average literature values. No side-effects were experienced during the administration of sodium bicarbonate (21 g/day), to induce alkalinuria, during the sixth and seventh days and at no time did the subject describe any quantitative or qualitative variation in drug effect.

Most available data on cocaine metabolism and excretion have come from South American studies and relate to the chewing of coca leaf, which presents fundamental differences due to the rate of administration and time-course of absorption. Elimination of unchanged cocaine from habituées has been conflictingly reported as 6–20% by Ortiz (1952) and negligible by Sanchez (1957) who regarded ecgonine as the final and only urinary excretion product of cocaine. However, figures quoted for ecgonine accounted for only 2–8% of the total cocaine available. Sanchez & Guillen (1949) reported 10–20% urinary excretion of unchanged cocaine, the amount rising to 21–34% when the leaf was chewed with alkalis. In no case was benzoylecgonine considered as an important metabolite and urinary constituent.

Comparisons with data from animal studies must be made with caution because of inter-species variations in detoxification mechanisms (Langecker, 1938), especially in serum esterase activity (Glick & Glaubach, 1942), but it is of interest to note that Ortiz (1966) described cocaine, benzoylecgonine and ecgonine as urinary products in the rat. Greatest excretion occurred within 6 h of a single intraperitoneal injection and significant differences by sex were noted, there being higher urinary benzoylecgonine levels in males.

Of possible forensic significance are the low urinary concentrations encountered at certain times of the day even at the high dosage levels reported in this work. This is due to the antidiuretic side-effect of heroin on micturition, resulting in wide diurnal variations in urine volumes (Fig. 2).

*Acknowledgements*

We are grateful to Dr. Patrick Mullin and nursing staff at the Southern General Hospital, Glasgow, S.W.1, for their interest and help, and the patient for his co-operation. We thank May & Baker Ltd. for a sample of ecgonine. One of us (W.D.C.W.) thanks the Science Research Council for a research studentship.

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# The influence of substitution in phenylacetic acids on their performance in the buccal absorption test

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In the buccal test there is a linear relation between percentage absorption and alkyl chain length for a series of *p*-n-alkyl phenylacetic acids. Branching and cyclizing the alkyl chain reduces the percentage absorption. With the *p*-halogeno-acids, absorption increases as the atomic weight of the halogen atom increases; a chlorine atom is approximately equivalent to a methyl group in its effect on absorption. An oxygen atom between an alkyl group and ring is equivalent to reducing the chain length by one methylene group. The absorptions of α-methyl-substituted acids are greater than those of the unsubstituted acids.

The buccal absorption test of Beckett & Triggs (1967) has been used to explore the relative importance, in buccal absorption, of $pK_a$ and alkyl chain length in a closely related series of acids (Beckett & Moffat, 1968). The test is now used to demonstrate the relative effect of various substituents on the membrane penetrating power of phenylacetic acids.

## EXPERIMENTAL

Buffer solutions in the range pH 3·0 to 9·1 (37°) were prepared using McIlvaine citric acid-phosphate buffer for pH values between 3·0 and 8·0 (Documenta Geigy, 1962a) and borax (0·05M) for pH 9·1. Sörensen phosphate buffer and borax buffer were used for propylphenylacetic acid since citric acid interfered with the analysis. All pH values were measured at room temperature with a Pye Dynacap pH meter.

The general method of Beckett & Moffat (1968) was used: gas-liquid chromatographic conditions and the internal standards used for analyses are summarized in Table 1.

## RESULTS AND DISCUSSION

Two groups of acids were used, viz. (*a*) *p*-substituted phenylacetic acids which differ among themselves in their lipid solubility but not in their $pK_a$ values and (*b*) positional isomers of some mono-substituted phenylacetic acids and some *p*-alkyl-α-methylphenylacetic acids, differing from acids of group (*a*) in both lipid solubilities and $pK_a$ values.

The percentage buccal absorptions at pH 6·0 of the various *p*-substituted acids were found to be: hydrogen 1, nitro 1, fluoro 1·5, methoxy 3, methyl 7, chloro 7, bromo 8, n-propoxy 10, ethyl 10, iodo 10, n-propyl 25, t-butyl 25, n-butyl 34, t-pentyl 30, cyclopentyl 30, n-pentyl 49, cyclohexyl 44, n-hexyl 61.

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Table 1. *Gas-liquid chromatography conditions for the analysis of some phenylacetic acids*

| Methylester of acid | Retention time (min) | Operating temp (° C) | Methylester of internal standard | Retention time (min) |
|---|---|---|---|---|
| Phenylacetic | 6·1 | 100 | *p*-Chlorobenzoic | 9·6 |
| *o*-Tolylacetic | 4·7 | 115 | Cinnamic | 8·7 |
| *m*-Tolylacetic | 4·6 | 115 | *p*-Toluic | 3·9 |
| *p*-Tolylacetic | 4·6 | 115 | *p*-Toluic | 3·9 |
| *p*-Ethylphenylacetic | 5·7 | 130 | *p*-Chlorobenzoic | 3·1 |
| *p*-n-Propylphenylacetic | 15·6 | 125 | *p*-t-Butylphenylacetic | 20·3 |
| *p*-n-Butylphenylacetic | 4·1 | 160 | *p*-t-Butylphenylacetic | 3·2 |
| *p*-t-Butylphenylacetic | 20·3 | 125 | *p*-Chlorobenzoic | 3·9 |
| *p*-n-Pentylphenylacetic | 6·4 | 160 | *p*-t-Butylphenylacetic | 3·2 |
| *p*-t-Pentylphenylacetic | 48·8 | 125 | *p*-t-Butylphenylacetic | 20·3 |
| *p*-Cyclopentylphenylacetic | 7·9 | 165 | *p*-t-Butylphenylacetic | 3·0 |
| *p*-n-Hexylphenylacetic | 10·2 | 160 | *p*-t-Butylphenylacetic | 3·2 |
| *p*-Cyclohexylphenylacetic | 12·4 | 165 | *p*-t-Butylphenylacetic | 3·0 |
| *p*-Methoxyphenylacetic | 12·7 | 125 | *p*-t-Butylphenylacetic | 20·3 |
| *p*-n-Propoxyphenylacetic | 19·0 | 130 | *p*-Chlorobenzoic | 3·1 |
| *p*-Fluorophenylacetic | 2·6 | 125 | *p*-Chlorobenzoic | 3·9 |
| *o*-Chlorophenylacetic | 6·9 | 115 | Cinnamic | 8·7 |
| *m*-Chlorophenylacetic | 6·2 | 125 | *p*-Chlorobenzoic | 3·9 |
| *p*-Chlorophenylacetic | 7·0 | 125 | *p*-Chlorobenzoic | 3·9 |
| *p*-Bromophenylacetic | 11·4 | 125 | *p*-Chlorobenzoic | 3·9 |
| *p*-Iodophenylacetic | 20·4 | 125 | *p*-Chlorobenzoic | 3·9 |
| *p*-Nitrophenylacetic | 4·3 | 165 | *p*-t-Butylphenylacetic | 3·0 |
| *p*-Ethyl-α-methylphenylacetic | 8·0 | 128 | *p*-Chlorobenzoic | 3·4 |
| *p*-n-Propyl-α-methylphenylacetic | 13·6 | 128 | *p*-Chlorobenzoic | 3·4 |
| *p*-iso-Butyl-α-methylphenylacetic | 17·8 | 128 | *p*-Chlorobenzoic | 3·4 |
| *p*-t-Pentyl-α-methylphenylacetic | 27·4 | 128 | *p*-Chlorobenzoic | 3·4 |

Typical of the first group are the *p*-n-alkyl phenylacetic acids whose absorption increases, over the pH range 3 to 9, as the chain length increases (Fig. 1A) even though their $pK_a$ values are approximately the same (4·31–4·37; Kortum, Vogel & Andrussow, 1961). At a single pH, e.g. 6·0 which is the mean pH of saliva (Documenta Geigy,

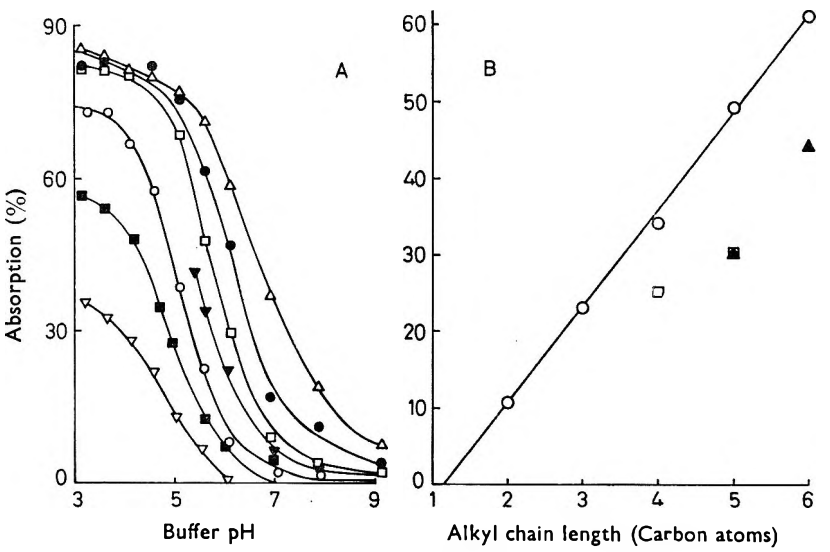


FIG. 1. A. The buccal absorption of *p*-n-alkyl phenylacetic acids (Subject I). △, hexyl; ●, pentyl; □, butyl; ▼, propyl; ○, ethyl; ■, methyl; ▽, H. B. The effect of chain length in *p*-alkyl phenylacetic acids on their buccal absorption at pH 6·0. ○, normal, □, tertiary; ▲, cyclic.

1962b), a linear relation obtains between buccal absorption and alkyl chain length of the *p*-n-alkyl phenylacetic acids (Fig. 1B). This indicates that buccal absorption is an additive property of a drug molecule. Each additional methylene group in a chain causes an increment of 12·5% in the buccal absorption. The increase resulting from the addition of a methylene group agrees with previously reported data using straight-chain fatty acids (Beckett & Moffat, 1968); the increased lipid-water partition coefficients of their unionized forms explain the results. For example, the n-heptane-0·1N hydrochloric acid partition coefficients of phenylacetic and *p*-n-hexylphenylacetic acids at 25°, assuming complete dimerization in the n-heptane phase, are 0·01 and 29·8 $ml^{1/2}\mu g^{-1/2}$ respectively (Beckett & Moffat, unpublished observations).

t-Pentyl- and cyclopentyl-phenylacetic acids are absorbed to a similar extent, but much less than the n-pentyl compound (Fig. 2A). Comparison of these results with those obtained using t-butyl-, n-butyl-, cyclohexyl-, and n-hexyl-phenylacetic acids (above and Fig. 1B) indicates that the tertiary branching or the cyclizing of an alkyl chain with the same number of carbon atoms produces a fall in absorption to the level of the next lower straight-chain homologue.

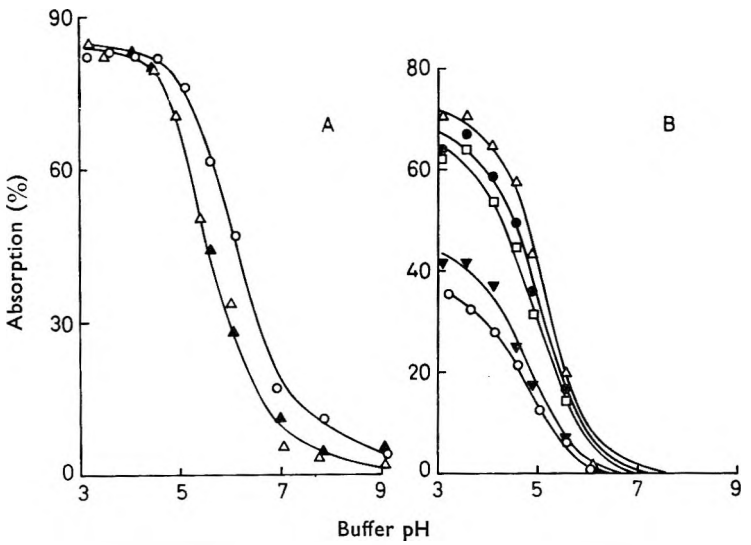


FIG. 2. A. The buccal absorption of *p*-alkyl phenylacetic acids (Subject I). ○, n-pentyl; ▲, cyclopentyl; △, t-pentyl. B. The buccal absorption of phenylacetic acid and *p*-halogen phenylacetic acids (Subject I). △, iodo; ●, bromo; □, chloro; ▼, fluoro; ,○ H.

That compounds with straight alkyl chains are absorbed to a greater extent than branched chain compounds with the same number of carbon atoms is to be expected since, in acids with chains larger than $C_3$, the paraffinic side-chain becomes a dominant feature (Albert, 1965); it cannot be accommodated in any interstices, it cannot force the water molecules apart and thus be solubilized, hence it tends to be "squeezed" out of the water into the mucosa, dragging the whole molecule with it. The effect is lessened by reducing the length of the chain, by branching, or forming a ring.

The percentage of *p*-halogenated phenylacetic acids absorbed increases as the atomic weight of the halogen increases (Fig. 2B). When compared with the *p*-alkyl substituted phenylacetic acids (Fig. 1A), chlorine increases buccal absorption to

approximately the same extent as a methyl group, whilst iodine is equivalent to an ethyl group. Fluoro- and bromo-groups are about half as effective in increasing absorption as the chloro- and iodo-groups respectively. This is in agreement with Brookes (1968) who found the buccal absorption of *p*-chlorophentermine to be much greater than that of phentermine.

*p*-Methoxyphenylacetic acid shows approximately the same percentage absorption as phenylacetic acid, and *p*-n-propoxyphenylacetic acid shows approximately the same absorption as *p*-ethylphenylacetic acid (Fig. 3A). The acids in each pair also have similar $pK_a$ values and lipid-water partition coefficients (Beckett and Moffat, unpublished observations). Thus, insertion of an oxygen atom between the alkyl chain and the phenyl ring produces an effect approximately equal to reducing the chain length by one methylene group.

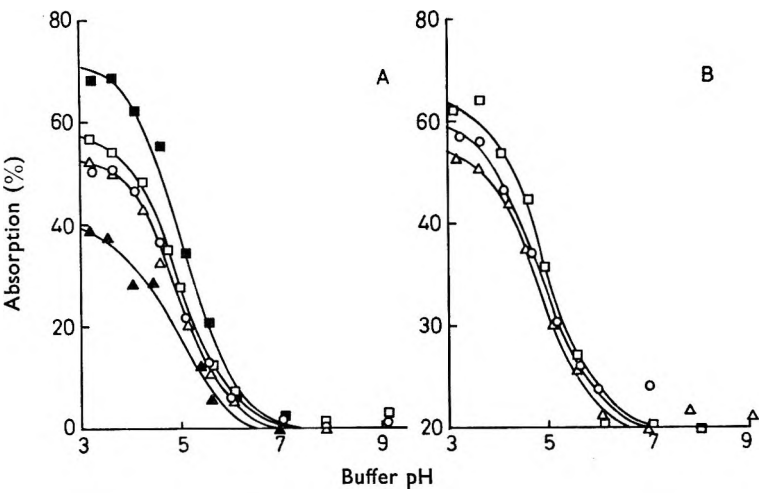


FIG. 3. A. The buccal absorption of some *p*-alkoxy and *p*-alkyl phenylacetic acids (Subject I). ■, propoxy; ▲, methoxy; □, *p*-methyl ($pK_a$ 4·37); ○, *m*-methyl; △, *o*-methyl ($pK_a$ 4·35). B. The buccal absorption of the monochlorophenylacetic acids (Subject I). □, *para* ($pK_a$ 4·19); ○, *meta* ($pK_a$ 4·14); △, *ortho* ($pK_a$ 4·07).

With the three tolylacetic and three monochlorophenylacetic acids (Fig. 3A, B) the shifts in the curves indicate that the differences in buccal absorption are primarily due to their $pK_a$ differences.

The *p*-alkyl-α-methylphenylacetic acids are absorbed more than their *p*-alkyl-phenylacetic acid homologues (cf. Figs 1A and 4); the relative percentage absorptions for the ethyl-, n-propyl- and t-pentyl-α-methyl acids and their homologues being 33·5/10, 38/23 and 40/30 respectively at pH 6·0. The absorption of *p*-isobutyl-α-methylphenylacetic acid, for which no comparable non-α-methyl compound was available, was 44% at pH 6·0. Although the $pK_a$ values of phenylacetic acid and α-methyl-phenylacetic acid differ (4·28 and 4·64 respectively: Handbook of Chemistry and Physics, 1967), and this would account for some of the absorption differences between the respective homologues (Beckett & Moffat, 1968), it is possible that the absorption differences are also due to differences in lipid solubility of the unionized forms.

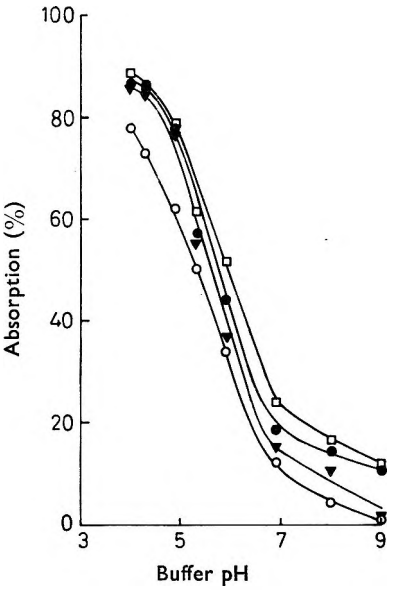


FIG. 4. The buccal absorption of *p*-alkyl-α-methylphenylacetic acids (Subject I). ○, ethyl; ▼, n-propyl; □, isobutyl; ●, t-pentyl.

The absorptions of phenylacetic acids therefore alter by changing either the subst tuent or its position in the molecule. The addition of alkyl chains to the acid gives the greatest increase in absorption, although straight alkyl chains on aromatic rings of drug molecules are susceptible to β-oxidation by enzymes, whereas branched chains are relatively much more stable. Thus, to make a drug more lipid soluble, so that it is absorbed quickly and reaches its site of action in the shortest time, the addition of a multi-branched or cyclic alkyl chain may give the best result. As an example, *p*-isobutyl-α-methylphenylacetic acid is used as an anti-inflammatory agent whilst phenylacetic acid is inactive.

*Acknowledgments*

We wish to thank Boots Pure Drug Company Limited for supplying all the compounds. One of us (A.C.M.) also thanks the Science Research Council for a research studentship.

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# Correlation of partition coefficients in n-heptane-aqueous systems with buccal absorption data for a series of amines and acids

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Partition coefficients in n-heptane-0·1N sodium hydroxide or 0·1N hydrochloric acid have been determined for a series of amines and acids; linear relations between chain-lengths and the logs of their partition coefficients were found. The plot of alkyl chain-length against buccal absorption of some amphetamines and fenfluramines, when they were 1% unionized, was linear. There was also a linear relation between the logs of the partition coefficients and buccal absorption of the amines and acids when these were 1 and 10% unionized. Those amines and acids having similar partition coefficients, when equally unionized, were absorbed to the same extent in the buccal test over the pH range 4 to 9. During the test the pH at the surface of the buccal membrane was shown to be the same as that of the solution in the mouth. n-Heptane is considered to be equivalent in solvent properties to the buccal lipid membrane for the compounds used in the present test.

For a variety of drugs, positive correlations have been noted between partition coefficients and (*a*) *in vivo* absorption (Walton, 1935; Mayer, Maichel & Brodie, 1959; Schanker, 1959; and others), (*b*) renal tubular reabsorption (Knoefel, Huang & Jarboe, 1961, 1962; Weiner & Mudge, 1964; Wilkinson, 1966; and others) and (*c*) hypnotic activity (Hansch, Maloney & others, 1968). In none of these cases were the permeability characteristics of the membranes examined, whilst the choice of partition systems was made arbitrarily.

After examining a number of solvent systems, we chose n-heptane-water since this solvent pair has negligible mutual solubility and, in general, n-heptane dissolves unionized but not ionized molecules. It was hoped that this system would give partition coefficients that could be related to previously determined buccal absorption results for both basic and acidic drugs.

A further object was to relate partition coefficients and the buccal absorptions of amines and acids when they were unionized to the same extent and from this to establish whether the pH of the buccal mucosal surface was the same as that of the bulk solution.

## EXPERIMENTAL

### *Partition coefficients*

*Apparatus.* Glass tubes (100 ml capacity), closed at both ends, with central

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sampling ports with glass stoppers were used in a rocking device similar to that of Reese, Irwin & others (1964).

*Compounds.* The compounds examined included those used previously (Beckett & Moffat, 1968, 1969) and also those amines and acids listed in Tables 1 and 2.

*Methods.* A sample of organic compound (1 mg) in 25 ml aqueous solution (0·1N hydrochloric acid for acids or 0·1N sodium hydroxide for amines, both saturated with n-heptane) was placed in each tube together with 25 ml n-heptane (saturated with the acid or alkali). The ports were stoppered and the rocking machine run for 16 h at 25° ± 1°. Tubes containing the aqueous solutions of the organic compounds were used as controls.

The final concentration in the aqueous phase was determined by gas-liquid chromatographic analysis of 5 ml aliquots of the aqueous layer (Beckett & Moffat, 1968, 1969; Tables 1 and 2). Duplicate analyses were made and the mean taken.

The partition coefficients (K) were then calculated according to the appropriate relation:

$$K = \frac{\text{(Initial-Final) concentration in the aqueous phase}}{\text{Final concentration in the aqueous phase}}$$ for the amines

$$K = \frac{\sqrt{\text{(Initial-Final) concentration in the aqueous phase}}}{\text{Final concentration in the aqueous phase}}$$ for the acids since they dimerize in the heptane phase (units: $ml^{1/2}\,\mu g^{-1/2}$)

*Buccal absorption measurements*

Aqueous solutions of the amine hydrochlorides and sodium salts of the acids were used with the method of Beckett & Moffat (1968).

For analysis, the method of Beckett & Moffat (1968) was used, except that the amines were extracted from alkaline solution and the following gas-liquid chromatography conditions used: a 2 m, ¼ inch O.D. glass tube packed with Chromosorb G (acid washed, DMCS treated, 80–100 mesh) coated with 2·0% Apiezon L/5% KOH; nitrogen pressure 20 lb/inch², hydrogen pressure 24 lb/inch², and air pressure 30 lb/inch²; injection block temperature approximately 50° above the oven temperature. The oven temperature and internal standard used for each acid and amine not already reported are summarized in Tables 1 and 2.

Table 1. *Gas-liquid chromatography conditions for the analysis of some amines on a 2% Apiezon L/5% KOH column at* 110°

| Amine | Retention time (min) | Internal standard |
|---|---|---|
| Amphetamine | 3·4 | *N*-n-Butylnorfenfluramine |
| *N*-Methylamphetamine | 4·8 | ,, |
| *N*-Ethylamphetamine | 6·5 | ,, |
| *N*-n-Propylamphetamine | 11·4 | ,, |
| *N*-n-Butylamphetamine | 21·2 | ,, |
| Norfenfluramine | 2·7 | *N*-n-Propylamphetamine |
| *N*-Methylnorfenfluramine | 3·7 | ,, |
| Fenfluramine (*N*-ethylnorfenfluramine) | 4·9 | ,, |
| *N*-n-Propylnorfenfluramine | 8·4 | ,, |
| *N*-n-Butylnorfenfluramine | 15·3 | ,, |

Table 2. *Gas-liquid chromatography conditions for the analysis of some acids on a 2·5% SE-30 column*

| Methyl ester of acid | Retention time (min) | Oven temp. (° C) | Methyl ester of internal standard | Retention time (min) |
|---|---|---|---|---|
| Isovaleric .. .. .. | 4·0 | 40 | Hexanoic | 18·0 |
| 2,6-Dimethylbenzoic .. .. | 11·0 | 100 | *p*-Toluic | 7·6 |
| *o*-Chlorobenzoic .. .. | 10·2 | 100 | *p*-Toluic | 7·6 |
| *m*-Chlorobenzoic .. .. | 9·8 | 100 | *p*-Toluic | 7·6 |
| Cinnamic .. .. .. | 7·2 | 125 | *p*-Chlorobenzoic | 3·9 |
| *p*-Phenoxyphenylacetic .. | 16·6 | 165 | *p*-t-Butylphenylacetic | 3·0 |

## RESULTS AND DISCUSSION

### *Partition coefficients*

Partition coefficients and $pK_a$ values for all the amines and carboxylic acids used are shown in Tables 3 and 4.

The values of the partition coefficients for members of both the long-chain fatty acid and *p*-n-alkylphenylacetic acid series increase in a regular geometric manner as the alkyl chain lengths increase (Fig. 1A). This phenomenon is also shown by the amphetatamines and fenfluramines (Fig. 1B). Values of K vary within a group of isomers, e.g. the chlorobenzoic acids: *o*, 0·05; *m*, 0·18; *p*, 0·17 $ml^{1/2}\mu g^{-1/2}$, which is due partly to their differences in water solubility, viz. 1/900; 1/2850; 1/5290 respectively (*Merck Index*, 1960).

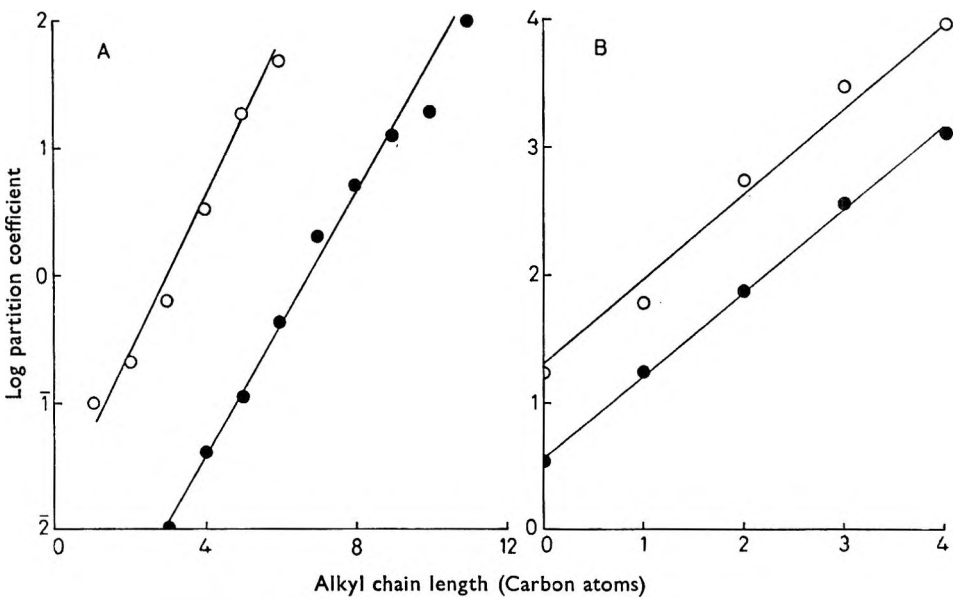


FIG. 1. A. The influence of chain length on the n-heptane-0·1N HCl partition coefficients of two series of acids. ○, *p*-n-alkylphenylacetic acids; ●, straight-chain fatty acids. B. The influence of chain length on the n-heptane-0·1N NaOH partition coefficients of two series of *N*-n-alkyl amines. ○, fenfluramines; ●, amphetamines.

### *Buccal absorption measurements*

The buccal absorptions of the amphetamines and fenfluramines, when 1% unionized, are linearly related to the alkyl chain length. This finding is similar to that for the

Table 3. *n-Heptane*-0·1N *sodium hydroxide partition coefficients and* $pK_a$ *values for some amines*

| Amine | Partition coefficient* 25° C | $pK_a$ 25° C |
|---|---|---|
| Amphetamine | 3·4 | 9·70[1] |
| *N*-Methylamphetamine | 17·3 | 10·00[1] |
| *N*-Ethylamphetamine | 75 | 10·05[1] |
| *N*-n-Propylamphetamine | 360 | 10·18[2] |
| *N*-n-Butylamphetamine | 1270 | 10·20[2] |
| Norfenfluramine | 17·0 | 9·53[1] |
| *N*-Methylnorfenfluramine | 60·3 | 9·67[1] |
| Fenfluramine (*N*-ethylnorfenfluramine) | 550 | 9·88[1] |
| *N*-n-Propylnorfenfluramine | 2800 | 10·00[3] |
| *N*-n-Butylnorfenfluramine | 9000 | 10·00[3] |

* Present results, calculated assuming no association of molecules in either phase.

1. Brookes (1968); at 22° C.
2. Present studies.
3. Approximate value, calculated from the amphetamine results.

Table 4. *n-Heptane*-0·1N *hydrochloric acid partition coefficients and* $pK_a$ *values for some carboxylic acids*

| Acid | Partition coefficient[1] 25° C | $pK_a$ 25° C | Acid | Partition coefficient[1] 25° C | $pK_a$ 25° C |
|---|---|---|---|---|---|
| n-Butyric | <0·01 | 4·82[3] | Phenylacetic | <0·01 | 4·31[3] |
| iso-Valeric | 0·02 | 4·77[3] | *o*-Tolylacetic | 0·04 | 4·35[4] |
| n-Valeric | 0·04 | 4·81[3] | *m*-Tolylacetic | 0·11 | 4·36[6] |
| n-Hexanoic | 0·11 | 4·85[3] | *p*-Tolylacetic | 0·10 | 4·37[4] |
| n-Heptanoic | 0·43 | 4·89[3] | *p*-Ethylphenylacetic | 0·21 | 4·37[4] |
| n-Octanoic | 1·94 | 4·85[3] | *p*-n-Propylphenylacetic | 0·62 | 4·36[6] |
| n-Nonanoic | 5·02 | 4·85[6] | *p*-n-Butylphenylacetic | 3·30 | 4·36[6] |
| n-Decanoic | 12·2 | 4·85[6] | *p*-t-Butylphenylacetic | 3·21 | 4·36[6] |
| n-Undecanoic | 18·5 | 4·85[6] | *p*-n-Pentylphenylacetic | 18·0 | 4·36[6] |
| n-Dodecanoic | 95·8 | 4·85[6] | *p*-t-Pentylphenylacetic | 3·4 | 4·36[6] |
| Benzoic | 0·11 | 4·20[2] | *p*-Cyclopentylphenylacetic | 2·6 | 4·36[6] |
| *o*-Toluic | 0·25 | 3·90[2] | *p*-n-Hexylphenylacetic | 29·8 | 4·36[6] |
| *m*-Toluic | 0·31 | 4·27[2] | *p*-Cyclohexylphenylacetic | 7·4 | 4·36[6] |
| *p*-Toluic | 0·23 | 4·37[2] | *p*-Methoxyphenylacetic | <0·01 | 4·36[4] |
| 2,4-Dimethylbenzoic | 0·88 | 4·22[2] | *p*-n-Propoxyphenylacetic | 0·21 | 4·36[6] |
| 2,6-Dimethylbenzoic | 0·10 | 3·35[2] | *p*-Phenoxyphenylacetic | 0·82 | 4·36[6] |
| 2,4,6-Trimethylbenzoic | 0·35 | 3·45[2] | *p*-Fluorophenylacetic | 0·01 | 4·25[4] |
| 2,3,5,6-Tetramethylbenzoic | 0·37 | 3·42[2] | *o*-Chlorophenylacetic | 0·03 | 4·07[5] |
| *o*-Chlorobenzoic | 0·05 | 2·89[3] | *m*-Chlorophenylacetic | 0·11 | 4·14[5] |
| *m*-Chlorobenzoic | 0·18 | 3·82[3] | *p*-Chlorophenylacetic | 0·06 | 4·19[4] |
| *p*-Chlorobenzoic | 0·17 | 4·03[3] | *p*-Bromophenylacetic | 0·09 | 4·19[4] |
| | | | *p*-Iodophenylacetic | 0·12 | 4·18[4] |
| Cinnamic | 0·22 | 4·41[3] | *p*-Nitrophenylacetic | 0·02 | 3·85[4] |

1. Present results, calculated assuming total dimerization in the organic phase. Units $ml^{\frac{1}{2}}$ $\mu g^{-\frac{1}{2}}$.
2. Wilson, Gore & others (1967).
3. Fieser & Fieser (1956).
4. Kortum, Vogel & Andrussow (1961).
5. Handbook of Chemistry and Physics (1967).
6. Calculated values.

percentage absorptions (at pH 6·0) of the *p*-n-alkyl-phenylacetic and straight chain fatty acids (Beckett and Moffat, 1968, 1969). Since the log of the n-heptane-aqueous phase partition coefficients of the straight chain fatty acids, *p*-n-alkyl-phenylacetic acids, amphetamines and fenfluramines and their buccal absorption values are each

related to alkyl chain length, the buccal absorption values must also be directly related to the log of the partition coefficients (Fig. 2A, B). Although a good fit to straight lines is obtained, it is obvious that the results cannot be extrapolated for higher or lower members of the homologous series of amines or acids since more than 100 or less than 0% absorption is unattainable.

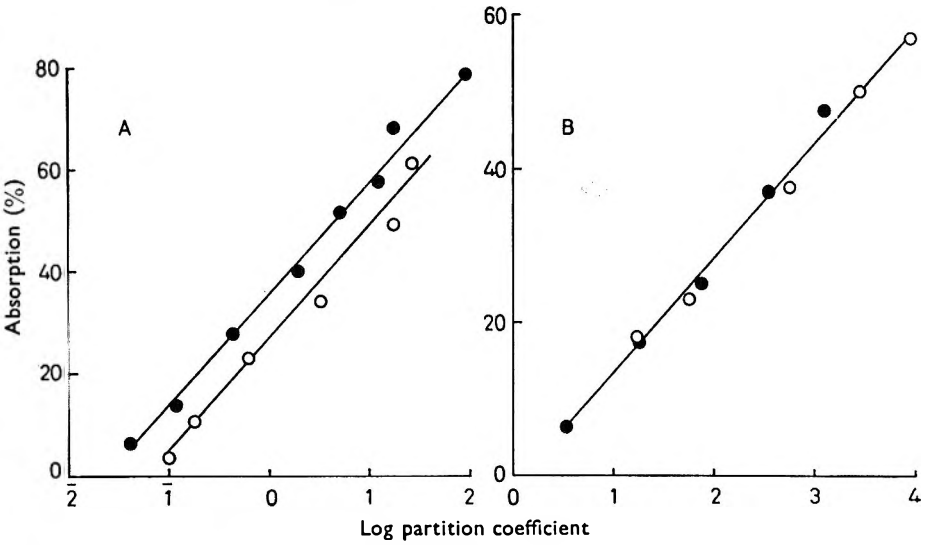


FIG. 2. A. The relation of the buccal absorption at pH 6·0 of two series of acids to their n-heptane-0·1N HCl partition coefficients. ○, *p*-n-alkylphenylacetic acids; ●, straight chain fatty acids. B. The relation of the buccal absorption of two series of *N*-n-alkyl amines (when 1% unionized) to their n-heptane-0·1N NaOH partition coefficients. ○, fenfluramines; ●, amphetamines.

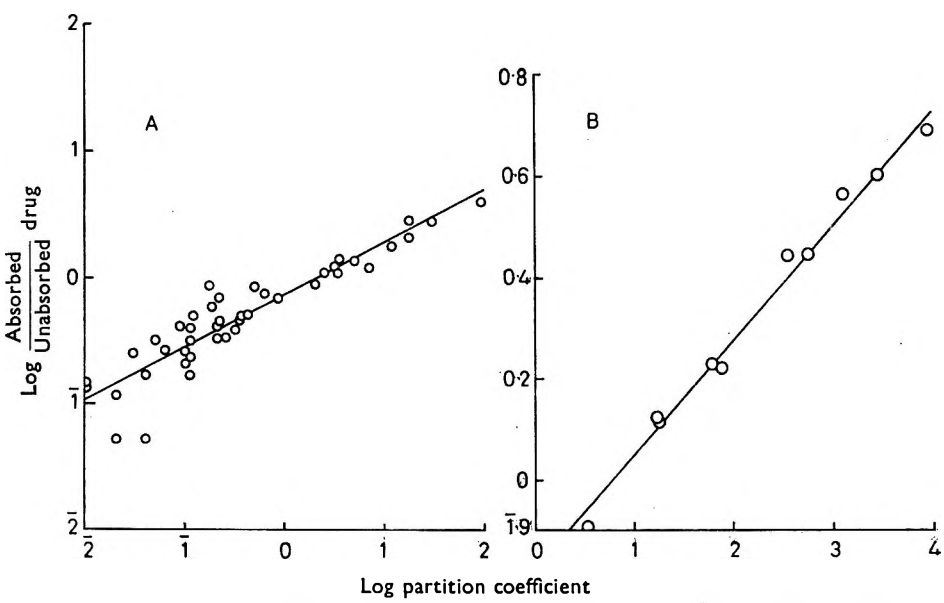


FIG. 3. A. The relation of the buccal absorption of carboxylic acids (when 10% unionized) to their n-heptane-0·1N HCl partition coefficients (correlation coefficient 0·93). B. The relation of the buccal absorption of *N*-n-alkyl substituted amines (when 10% unionized) to their n-heptane-0·1N NaOH partition coefficient (correlation coefficient 0·98).

When buccal absorptions, represented by the log of the proportion of compound absorbed, is plotted against the log of the partition coefficient a good positive correlation is obtained for the acids at 1 and 10% (correlation coefficients of 0·91 and 0·93 respectively), e.g. Fig. 3A. The lines of best fit were:

log (absorbed acid/unabsorbed acid) = 0·455 log K-0·77 (1%)

log (absorbed acid/unabsorbed acid) = 0·416 log K-0·14 (10%)

Better correlations were obtained with the amines (correlation coefficients, 0·98 and 0·98) than with the acids because only two homologous series were used, e.g. Fig. 3B. The lines of best fit were:

log (absorbed amine/unabsorbed amine) = 0·33 log K-1·12 (1%)

log (absorbed amine/unabsorbed amine) = 0·225 log K-0·174 (10%)

This indicates that the ability of any of the studied unionized amine or acid molecules to pass into the membrane is governed by their lipid solubility.

Thus n-heptane has similar partition properties to the lipid of the buccal mucosal membrane in the absorption of the acids and amines used. This is in agreement with Bickel & Weder (1969) who found that n-hexane displayed partition properties closer to the buccal mucosa, with imipramine and its metabolites, than did other less lipophilic solvents—ether, dichloroethane and chloroform. The n-heptane-aqueous system (or n-hexane-aqueous system) partition coefficient may therefore be taken to give a good indication of the degree of *in vivo* absorption of the unionized form of an acid or amine.

The pH-partition hypothesis is thus applicable to the passage of acids and bases into the buccal mucosa since when their unionized forms have the same partition coefficient, they penetrate the membrane with equal ease. For example, the unionized forms of amphetamine and *p*-n-propylphenylacetic acid have approximately the same n-heptane-aqueous phase partition coefficients (i.e. the same amount enters the n-heptane phase although the acid dimerizes in the organic solvent) and when they are 1% unionized they are absorbed by the mucosa to the extent of 6 and 15·5% respectively (a non-significant difference); methylamphetamine and *p*-cyclopentylphenylacetic acid have approximately the same K values and have similar buccal absorptions (56% and 52·5% respectively) when 10% is in the non-ionized form.

The amount of acid or amine in the unionized form is calculated from the pH of the buffer solution and the $pK_a$ of the compound. For example, an acid $pK_a$ 5 and an amine $pK_a$ 9 would be 1% unionized in a buffer solution of pH 7. If they had the same partition coefficient they would be expected to be absorbed to the same extent by the buccal mucosa. However, if the pH at the site of absorption was not the same as the pH of the buffer solution, different absorptions would be observed. For instance, using the above examples, if the pH at the site of absorption was 6 whilst that of the bulk solution was 7 the acid would be 10% unionized whereas the amine would only be 0·1% unionized at the mucosa surface. Thus, since the experimental evidence quoted above indicates that acids and bases with similar partition coefficients are absorbed to the same extent when equally unionized over the pH range 9–4, the pH at the site of absorption must be the same as the measured pH of the buffer solution.

It has been shown (Beckett & Moffat, 1968) that the absorptions of 2,4,6-trimethylbenzoic and 2,3,5,6-tetramethylbenzoic acids are anomalously low when compared with benzoic acid itself. The explanation advanced was that the buffer solution pH

was not that at the buffer-buccal membrane interface where absorption took place. This is no longer tenable, and the phenomenon is due to these acids being less lipid soluble, relative to benzoic acid, than would be expected from the presence of three or four methyl groups in the aromatic ring; experimental results in confirmation are presented in Table 4.

For many organic solvents there is a direct relation between the logs of partition coefficients (organic solvent-water) of compounds in an homologous series in one solvent-water system to the logs of the partition coefficients in another solvent-water system (Collander, 1947). The partition properties of a wide variety of organic solvents would then be expected to simulate those of the buccal mucosa for members of homologous series. However, the relative magnitudes of partition coefficients for compounds of widely differing structures change when different solvents are used, especially when solvent-solute interaction takes place (Wilkinson, 1966). The use of partition coefficients in predicting absorptions of drugs may therefore give erroneous results if an unsuitable solvent is used as the lipid substitute. Even when partition experiments are made using lipid extracted from animal buccal mucosa, the K values are not necessarily valid for the living tissue (Brändström, 1964). This is because the lipid layers bind to protein and this modifies their solvent properties. The present study suggests that the buccal membrane may for all practical purposes be considered as an homogeneous lipid phase with solvent properties similar to those of n-heptane and that buccal absorption of many carboxylic acids and amines may be predicted from their $pK_a$ values and n-heptane-aqueous phase partition coefficients.

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# Urinary excretion of the drug and its main metabolite in man, after the administration of (±)-, (+)- and (−)-ethylamphetamine

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The urinary excretion of ethylamphetamine and its metabolite amphetamine was studied in man after oral administration of (±)-, (+)-, and (−)-ethylamphetamine hydrochloride. The rates of excretion of these amines are dependant on the pH of the urine. At acid values, the (+)-isomer is metabolized faster and to a greater extent than the (−)-isomer, which is excreted mostly unchanged. The effect of alkyl chain length on stereoselective metabolism of *N*-alkylamphetamines is discussed.

The urinary excretion of amphetamine (Beckett & Rowland, 1964; Asatoor, Galman & others, 1965) and methylamphetamine (Beckett & Rowland, 1965 a,b) is influenced by urinary pH, and differences in the metabolism and excretion of the enantiomorphic forms have been investigated (Beckett & Rowland, 1965; Gunne, 1967). The metabolism of ethylamphetamine and its enantiomorphs in man has not been reported previously.

The purpose of the present work is not only to study the metabolism and excretion of ethylamphetamine in man but also to provide information to help evaluate the importance of alkyl chain length in stereoselective metabolism of *N*-alkylamphetamines.

## EXPERIMENTAL

### *Urinary excretion trials*

The five male subjects who participated in these trials were given oral doses of ethylamphetamine hydrochloride ranging from 12 to 36 mg in a total of 23 experiments.

All five were given 20 mg of the (±)-form (equivalent to 16·34 mg of the base) in water (50–100 ml) the urinary pH being maintained at acid values. On another occasion three subjects were given the same dose of the drug but the urinary pH was maintained at alkaline values; in a third experiment, the pH of the urine was not controlled.

Five subjects were given an oral dose of (+)-ethylamphetamine hydrochloride and three of (−)-ethylamphetamine hydrochloride (20 mg), the urinary pH being maintained acidic.

The administration of drug, the collection of urine specimens and measurement of urinary pH were as described by Beckett & Rowland (1965a). The dosage regimen of ammonium chloride and sodium bicarbonate for maintaining acidic and alkaline urine respectively was as described by Beckett & Brookes (1967). The urine was collected over 24 h, extracted by the method of Beckett & Rowland (1965a) and analysed by gas liquid chromatography.

Ethylamphetamine and amphetamine (0·1–10 μg/ml) were added to blank urine from some subjects. The two amines (1 μg/ml) were added to acidic and alkaline urines and stored at 4°. The drug content was determined every third day for two weeks.

In addition, some of the ether extracts of the urines from the experiments were treated as follows:—

(a) About 2 μl acetone was added to 5 μl of the concentrate and the solution heated at about 60° for 1 h and injected on the two columns described below.

(b) Two portions of the concentrate were treated separately with acetic and propionic anhydrides, and injected on the two columns.

*Gas-liquid chromatography*

Analysis using column 1 (below) was as described for amphetamine and methylamphetamine by Beckett & Rowland, (1965a,b) but aletamine hydrochloride (10 μg/ml in water) was added as an internal standard to the urine before extraction.

A Perkin Elmer F11 gas chromatograph with a flame ionization detector was employed, using the following conditions: both columns were of stainless steel 1 m × ⅛ in o.d.

*Column* 1. Acid washed DMCS treated Chromosorb G (80–100 mesh) coated with 10% w/w potassium hydroxide and 10% w/w Apiezon L. Column temperature 160°, injection block temperature ca 250°. Nitrogen flow rate 27 ml/min at room temperature. Hydrogen pressure 14 lb/in², air pressure 26 lb/in². Stream split ratio 1 : 5.

*Column* 2. Acid washed DMCS treated Chromosorb G (80–100 mesh) coated with 2% w/w Carbowax 20M and 5% w/w potassium hydroxide. Column temperature 165°, injection block temperature *ca* 250°. Nitrogen flow rate 33 ml/min at room temperature. Hydrogen pressure 15 lb/in². Air pressure 20 lb/in². Stream split ratio 1 : 5.

*Thin-layer chromatography*

Preparative thin-layer chromatography (TLC) was used to obtain samples of ethylamphetamine and amphetamine for infra-red analysis.

Glass plates 20 × 20 cm, coated with adsorbant 0·25 mm thick were developed with the following solvent systems. (a) Methanol–acetone (1 : 1); (b) methanol–chloroform (20 : 80); (c) methanol–chloroform (50 : 50).

Silica gel G (Merck) plates were used for systems (a) and (c) and aluminium oxide G (Merck) for System (b). Dragendorff's reagent and solution of bromothymol blue in ethanol were used to visualize the spots.

## RESULTS AND DISCUSSION

Linear calibration curves were obtained for ethylamphetamine and amphetamine. No substance interfering with the determination of the amines was found in urine. Both the amines were stable in urine at 4° for at least two weeks.

*Structure of the metabolite*

Gas-liquid chromatographic analysis of ethereal extracts of urines from the subjects who had taken the drug, gave two peaks representing unchanged drug and its metabolite, amphetamine. This was shown by the identity of retention times of the compounds themselves and of several derivatives with authentic samples. These

were (min): column 1—amphetamine 2·6, ethylamphetamine 4·4, amphetamine + acetone 4·5, amphetamine-acetyl derivative 11·5, amphetamine-propionyl derivative 16·0, ethylamphetamine-acetyl derivative 21·5, ethylamphetamine-propionyl derivative 28·1; column 2—amphetamine-acetyl derivative 7·2, ethylamphetamine-acetyl derivative 5·0.

Identity of Rf values of the drug and the metabolite with authentic samples was demonstrated in systems (a) 0·25/0·47, (b) 0·87/0·50, and (c) 0·43/0·33. Preparative TLC of the two amines from urine gave two products whose infrared spectra were indistinguishable from ethylamphetamine and amphetamine.

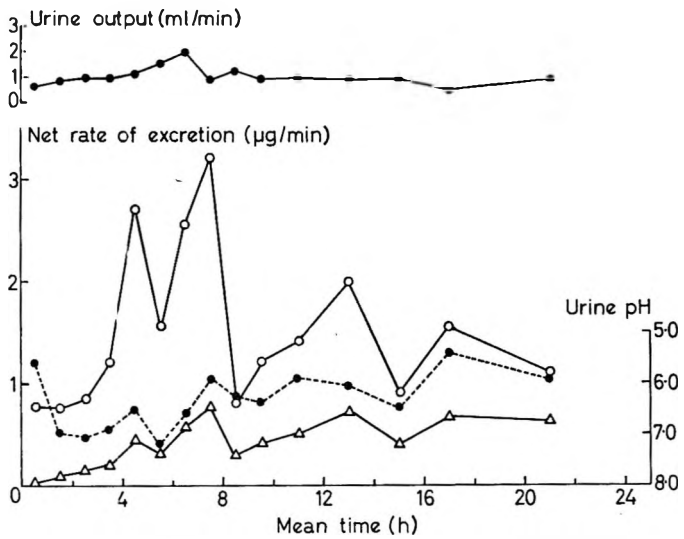


FIG. 1. Urinary excretion of ethylamphetamine and amphetamine over 24 h from subject (4) with no urine pH contrcl, who had taken an oral dose of 20 mg (±)-ethylamphetamine HCl. —○— ethylamphetamine, —△— amphetamine, —●— urine output (ml/min), - -●- - urine pH.

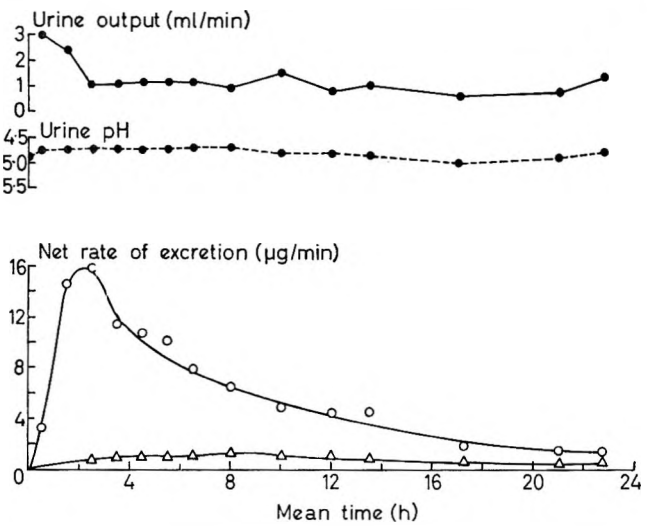


FIG. 2. Urinary excretion of ethylamphetamine and amphetamine over 24 h from a Subject (4) under conditions of acidic urine, who had taken an oral dose of 20 mg (±)-ethylamphetamine HCl. —○— ethylamphetamine, —△— amphetamine, —●— urine output (ml/min), - -●- - urine pH.

*Excretion and metabolism*

The fluctuations observed in the rate of excretion of (±)-ethylamphetamine and of its main metabolite amphetamine (Fig. 1) were abolished by maintaining the urine at an acidic pH (Fig. 2). When the urinary pH was maintained at alkaline values there was little excretion of the amines (Table 1). The excretion profile of the two

Table 1. *Urinary excretion of ethylamphetamine (EA) and amphetamine (A) over a period of 24 h in subjects receiving an oral dose of 20 mg of (±)-ethylamphetamine hydrochloride under conditions of alkaline and uncontrolled urinary pH*

| | Uncontrolled pH | | | | Alkaline pH | | | |
|---|---|---|---|---|---|---|---|---|
| | % dose excreted | | | | % dose excreted | | | |
| Subject | EA | A | Total | Ratio EA/A | EA | A | Total | Ratio EA/A |
| 4 | 12·6 | 4·4 | 17·0 | 2·9 : 1 | 0·9 | 1·2 | 2·1 | 0·8 : 1 |
| 2 | 24·2 | 10·4 | 34·6 | 2·3 : 1 | 5·9 | 3·3 | 9·2 | 1·8 : 1 |
| *3 | 13·6 | 6·0 | 19·6 | 2·3 : 1 | 0·7 | 0·4 | 1·1 | 1·8 : 1 |

* Dose 36 mg.

enantiomorphs was similar when the urine was acid but the (−)-isomer was excreted at a faster rate, mostly as unchanged drug. Of the two optical forms the (+)-isomer is metabolized to a greater extent suggesting a stereospecific metabolism (Table 2).

Table 2. *Urinary excretion of ethylamphetamine (EA) and amphetamine (A) over a period of 24 h in subjects receiving an oral dose of (+)-, (−)- or (±)-ethylamphetamine hydrochloride under acidic conditions*

| | | *Dextro* | | | | *Racemic* | | | | *Laevo* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % Dose excreted | | | | % Dose excreted | | | | % Dose excreted | | | |
| Subject | (mg) Dose | EA | A | Total | Ratio EA/A | EA | A | Total | Ratio EA/A | EA | A | Total | Ratio EA/A |
| 1 | 20 | 18·6 | 15·0 | 33·6 | 1·2 : 1 | 47·3 | 14·2 | 61·5 | 3·3 : 1 | | | | |
| 5 | 20 | 22·6 | 17·2 | 39·8 | 1·3 : 1 | 42·3 | 8·4 | 50·7 | 5·0 : 1 | 66·0 | 6·5 | 72·5 | 10·2 : 1 |
| 4 | 20 | 16·7 | 12·3 | 29·0 | 1·4 : 1 | 45·5 | 7·3 | 52·8 | 6·2 : 1 | 73·3 | 5·1 | 78·4 | 14·4 : 1 |
| 3 | 20 | 22·5 | 17·5 | 40·0 | 1·3 : 1 | 39·5 | 6·0 | 45·5 | 6·6 : 1 | | | | |
| 2 | 20 | | | | | 45·9 | 12·7 | 58·6 | 3·6 : 1 | 78·9 | 7·1 | 86·0 | 11·1 : 1 |
| 4 | 12 | | | | | 41·7 | 8·1 | 49·8 | 5·1 : 1 | | | | |
| 3 | 24·5 | | | | | 45·8 | 8·3 | 54·1 | 5·5 : 1 | | | | |
| 3 | 12 | | | | | 41·3 | 10·0 | 51·3 | 4·1 : 1 | | | | |
| 3 | 12·3 | | | | | 42·0 | 9·5 | 51·5 | 4·4 : 1 | | | | |
| 2 | 12·7 | 31·7 | 14·6 | 46·3 | 2·2 : 1 | | | | | | | | |

As with methylamphetamine (Beckett & Rowland, 1965d) the excretion of the unchanged drug is the major route of elimination for ethylamphetamine when the pH of the urine is maintained at acid values. The biological half-life of the (−)-isomer is about 5·2 h and 2·9 h for the (+)-isomer. This last value is significantly different from that of (+)-amphetamine (4·9 h) and (+)-methylamphetamine (4·3 h) (Beckett & Rowland, 1965a,b). The ratio of the metabolite, amphetamine, to the unchanged drug, indicates that (+)-ethylamphetamine is de-ethylated more than its (−)-isomer (Fig. 3) and the sum of drug and amphetamine excreted also shows that there is more metabolism of (+)-ethylamphetamine than of the (−)-isomer by a route or routes other than de-ethylation.

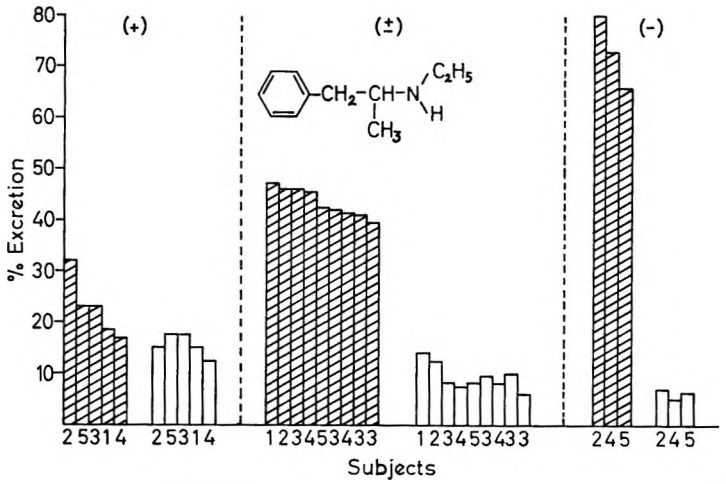


FIG. 3. The importance of stereochemistry in the metabolism and urinary excretion of ethylamphetamine in man (Acidic urine, oral dose, 24 h urine collection). Hatched columns—ethylamphetamine. Open columns—the metabolite-amphetamine.

Although there are only small differences in the excretion and thus metabolism of the isomers of amphetamine, *N*-alkyl substitution increases the total amount of metabolism (see Fig. 4) of the (+)- but not the (−)-isomer, i.e. *N*-alkyl substitution of amphetamine increases the susceptibility to stereoselective metabolism in man. Also, increase in the size of the *N*-alkyl substituent from methyl to ethyl produces relatively more *N*-dealkylation in the metabolically more susceptible isomer (Fig. 4).

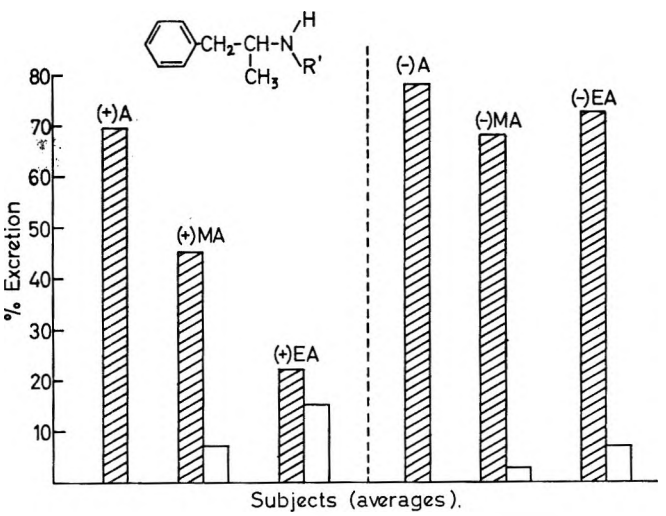


FIG. 4. The importance of stereochemistry and effect of *N*-alkyl substitution of amphetamine in the metabolism and excretion of the drug and the metabolite in man. (Acidic urine, oral dose, 24 h urine collection). Hatched columns—unchanged drug Open columns—the metabolite-amphetamine. A = amphetamine. MA = methylamphetamine. EA = ethylamphetamine.

*Acknowledgements*

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# Metabolism, excretion and biological availability of 4′-chloro-2-ethylaminopropiophenone

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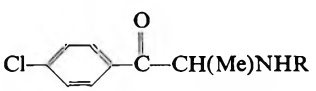
After oral administration of 4′-chloro-2-ethylaminopropiophenone to man, unchanged drug and the de-ethylated, reduced and reduced de-ethylated compounds were found in urine. Comparative urinary excretion studies after the oral administration of 4′-chloro-2-ethylaminopropiophenone in a sustained release form and in single or divided doses indicates that, when kidney tubular reabsorption is minimized, the biological availability of a drug can be followed by examining the excretion of either unchanged drug or metabolites formed quickly and directly from the administered drug.

4′-Chloro-2-ethylaminopropiophenone (compound I, Table 1) is currently being investigated as an anorectic agent. It is structurally related to the known anorectic diethylpropion (2-diethylaminopropiophenone) which in man, is excreted both unchanged and in metabolized forms (Schreiber, Min & others, 1968). These authors determined quantitatively the excretion of the metabolites over the period 8–12 h without controlling the pH of the urine or without taking into consideration that α-aminoketone-type compounds could undergo chemical transformation before and during the analysis. Similar α-aminoketones are known to be unstable in alkaline solution (Beckett & Hossie, 1969).

Table 1. *Structures of the compounds investigated*




Aminoketone


Aminoalcohol

| *Compound* | *Structure* | *Chemical Names* |
|---|---|---|
| I | ketone R = Et | 4′-chloro-2-ethylaminopropiophenone |
| II | alcohol R = Et | 1-(4-chlorophenyl)-1-hydroxy-2-ethylaminopropane |
| III | ketone R = H | 4′-chloro-2-aminopropiophenone |
| IV | alcohol R = H | 1-(4-chlorophenyl)-1-hydroxy-2-aminopropane |

The *in vivo* evaluation of the biological availability of amines from sustained release preparations relative to their availability from doses of free drug after administration at a stated interval of time to man, has been evaluated for amphetamine (Beckett & Tucker, 1966), and ephedrine (Beckett & Njikam, unpublished observations) by determining the amount of unchanged drug excreted in urine when kidney tubular reabsorption was minimized.

In the present work the excretion pattern of metabolites as well as unchanged drug, under acidic urinary pH with diuresis, has been used to establish whether the metabolites could be used to indicate biological availability from different drug forms. A further objective was to see whether the determination of drug and metabolite levels would help to explain preliminary unpublished observations that the sustained release preparation of compound I, gave less side effects than the equivalent dose of free drug in single and divided doses.

## EXPERIMENTAL

### *Reagents and apparatus*

These have previously been described (Beckett & Hossie, 1969). The sustained release preparation (150 mg of the HCl in Spansule form) was designed to give body levels of drug equivalent to that obtained by three 50 mg doses of the salt given at 4 h intervals.

### *General method*

Two healthy male subjects, who had not taken any drugs for several days previous to the experiment, excepting caffeine (beverages) and nicotine (1 smoker), took the drug orally on an empty stomach as 1 dose of 150 mg, 3 doses of 50 mg every 4 h or as the sustained release capsule. All doses, except the capsule, were taken in 50–100 ml of water. Urine was collected every $\frac{1}{2}$ h for 12 h, except for the single dose (4 h), then hourly until the 16th h and then every 4 h until the 58th h. In all cases the exact time of micturition was noted, the pH of the urine was determined shortly after collection, and the urine was stored at 4°. A "blank" urine sample was collected when the drug was administered and the compounds were quantitatively determined by the method of Beckett & Hossie (1969).

### *Acidic urine with water loading*

Acidic urine was induced and maintained by ammonium chloride or by ammonium chloride and methionine. A typical regimen for ammonium chloride was 1 g (enteric coated) every 3 h and 3 g at bedtime starting the day before the experiment. A typical regimen for methionine and ammonium chloride was 0·5 g of methionine every 2 h and 0·5 g of ammonium chloride every 4 h starting the day before the experiment. To water load, the subjects drank more than their normal intake (approx. 50–100 ml/h) of fluid the evening before the experiment and 300–600 ml of water/h the day of the experiment.

Table 2. *The recovery of 4′-chloro-2-ethylaminopropiophenone and its metabolites in two subjects after administration of 150 mg of the salt as a sustained release capsule (S.R.) or as single or divided doses*

| Subject | Dose | Urine | Time | % of compound I excreted as compounds | | | | % Recovered |
|---|---|---|---|---|---|---|---|---|
| | | | | I* | II* | III* | IV* | |
| 1 | 3 × 50** | A.D. | 30 | 22·5 | 15·1 | 7·1 | 4·9 | 49·6 |
| 2 | 3 × 50** | A.D. | 58 | 17·0 | 18·4 | 6·3 | 6·4 | 48·1 |
| 2 | 1 × 150 | A.D. | 58 | 25·8 | 15·5 | 4·2 | 4·2 | 49·7 |
| 1 | S.R. | A.D. | 54·8 | 16·3 | 18·4 | 4·6 | 9·2 | 48·5 |
| 2 | S.R. | A.D. | 58 | 17·8 | 18·0 | 6·2 | 7·3 | 49·3 |
| 1 | 3 × 50** | Uncont. | 58 | 5·9 | 14·6 | 2·0 | 2·7 | 25·2 |

A.D.—acidic urine with diuresis; Uncont.—uncontrolled urinary pH and volume;
* calculated as the equivalent amount of compound I;
** 3 doses of 50 mg of the salt given at 4 h intervals.

## RESULTS AND DISCUSSION

When the urine is maintained acidic, kidney tubular reabsorption of amines is minimized and the amount of drug recovered in the urine is increased (Beckett & Wilkinson, 1965; Beckett & Rowland, 1965; Beckett, Boyes & Appleton, 1966; Beckett, 1966). When compound I was administered, diuresis as well as acidic pH was required to maintain the smooth excretion profile necessary for the evaluation of the relative performance of the various dosage forms.

The urinary excretion pattern during acid diuresis showed a similar pattern in both subjects, comparable recoveries (Table 2) and maximum excretion of unchanged drug, from a single dose of approximately $1\frac{1}{4}$ h after drug administration (Figs 1A and 2a).

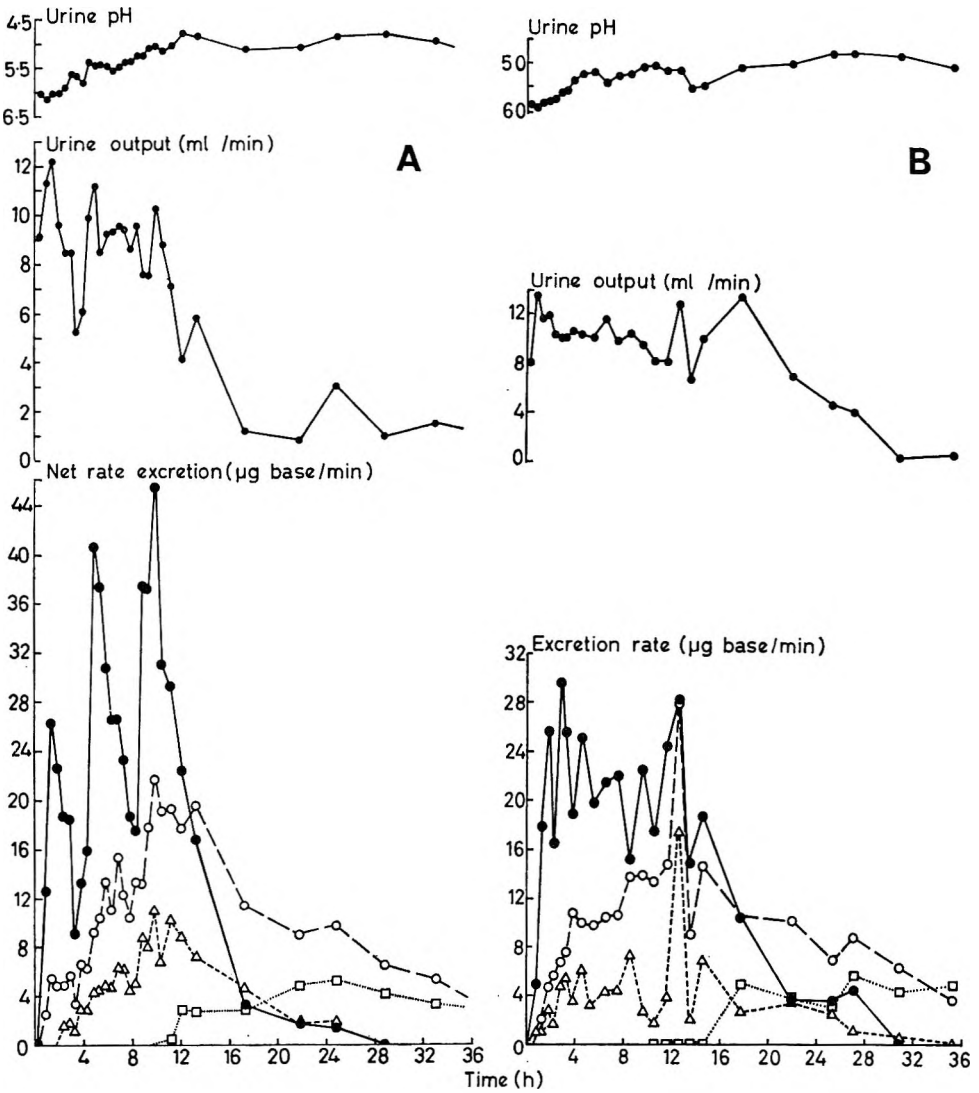



FIG. 1. Urinary excretion of 4′-chloro-2-ethylaminoprophenone (●) and its metabolites, compounds II (○), III (△) and IV (□), after the oral administration of (A) 3 × 50 mg of compound I HCl at 4 h intervals and of (B) a sustained release capsule of compound I HCl (150 mg). Acidic urine and diuresis were maintained. Subject 2.

A semi-log plot of the excretion of unchanged drug during constant urinary pH and flow rate (single 150 mg dose) gave a half-life of approximately 2 h.

The overall percentage excretion of compounds I-IV after the sustained release capsule or after 3 × 50 mg in solution at 4 h intervals was the same (Table 2), thus the drug in the sustained release form was as available for absorption as that in the solution. The "staircase" effect produced by administering 50 mg three times a day (Fig. 1A) was similar to that produced by dexamphetamine (Beckett & Tucker, 1966). The sustained release preparation (Fig. 1B) eliminated both the "staircase" effect (Fig. 1A) and the marked "peaking" effect of the unchanged drug produced by the single 150 mg dose (Fig. 2a). The metabolites do not, however, show these wide fluctuations in the rate of excretion (Figs 1A, B, 2b, c). Instead they show a gradual increase to a maximum and then a slow decrease in the rate of excretion.

Although the unchanged drug excretion rates vary in their profiles for the different dosage forms, those for metabolites II and III are very similar (Fig. 1A, B) for the 3 × 50 mg dose at 4 h intervals and the 150 mg sustained release preparation, but both differ greatly from the profile of compound II and III of the single 150 mg dose of 'free' drug (Fig. 2b, c). Compound IV is not quickly, nor directly, formed from

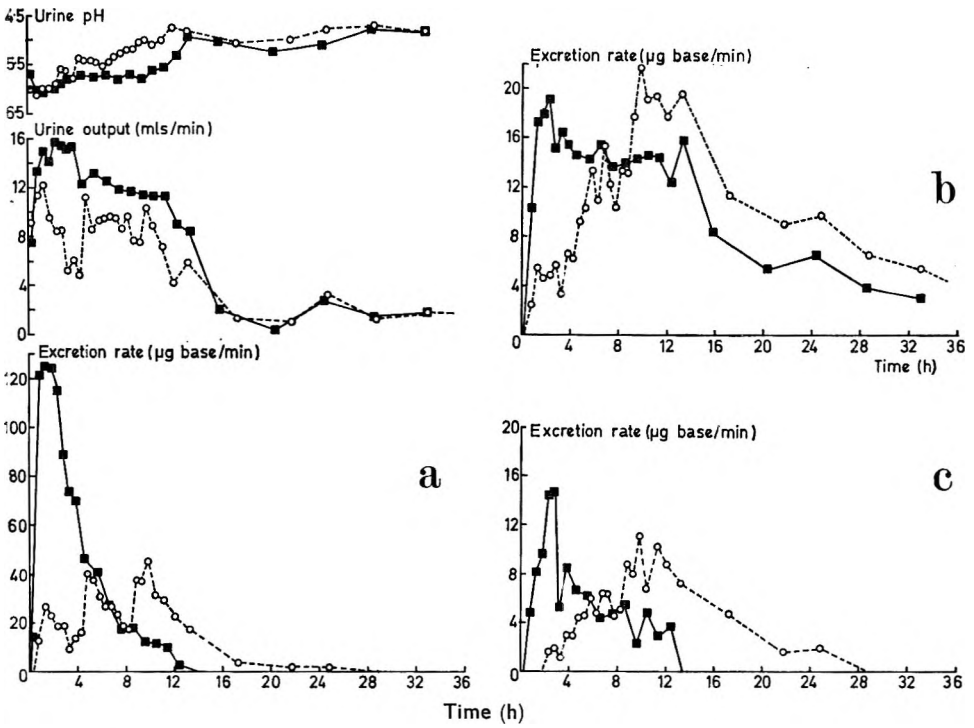



FIG. 2. Comparison of the urinary excretion of (a) unchanged drug, (b) compound II and (c) compound III after the oral administration of 3 × 50 mg of compound I HCl (○) at 4 h intervals and 150 mg of compound I in a single dose (■). Acidic urine and diuresis were maintained. Subject 2.

the parent compound I and is therefore not suitable for comparing the various dosage forms (Fig. 1A, B).

These results suggest that when the excretion of a drug is difficult to measure, it is possible to show the effectiveness of a sustained release formulation by minimizing

tubular reabsorption and by following the excretion of a metabolite which is formed rapidly and directly from the parent compound and comparing the metabolite's excretion profile after single and multiple doses with that after the sustained release product.

In the case of amphetamine, under similar conditions to the present experiment, it has been shown that the amount of amphetamine excreted in the urine is directly related to blood levels (Beckett, Salmon & Mitchard, 1969). If the levels of compounds I–III in blood are similarly related to those in urine, then higher blood levels of compound I is obtained from a single 150 mg dose or 3 × 50 mg doses than from the sustained release preparation. Thus the 150 mg sustained release preparation and the 3 × 50 mg doses at 4 h intervals will give similar blood levels of metabolites, and the former will give a steady level of unchanged drug for 14 to 16 h but eliminate the marked peaks and troughs in the blood levels of unchanged drug exhibited by the latter (Fig. 2b, c). This suggests that the lower constant and sustained blood levels of compound I obtained with the sustained release formulation could account for the observed lack of side effects with this preparation compared with the single 150 mg or 3 × 50 mg dose regimen.

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# The influence of stereochemistry on $pK_a$, rate of quaternization and partition coefficients of corynantheidine-type alkaloids

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In alkaloids of the corynantheidine group, the rate of quaternization at N-4 and the $pK_a$ values give a measure of the degree of steric hindrance at this site due to the axial hydrogen at C-3 and the ethyl group at C-20. These hinder the availability of the lone pair of electrons on N-4 to electrophiles. Partition coefficients indicate that lipid solubility is associated with planarity of the molecules; this explains why the more planar isomers (*allo* and *normal*) are more highly metabolized in microsomes than the less planar isomers (*pseudo* and *epiallo*).

Stereochemical features in corynantheidine-type alkaloids have been shown to influence the rate of metabolism in liver microsomes (Beckett & Morton, 1967); with methoxy substitution in the indole nucleus they have been used to explain the behaviour of these alkaloids on thin layers and in gas-liquid chromatography (Phillipson & Shellard, 1967; Beckett & Dwuma-Badu, 1968). The stereospecificity of biological receptors is well known (Beckett, 1959) for compounds such as analgesics, antibiotics and alkaloids.

The stereochemistry of some corynantheidine-type alkaloids has now been re-examined in the light of their $pK_a$ values, rates of quaternization, partition coefficients and metabolism by liver microsomes.

## EXPERIMENTAL

### *Materials*

Mitragynine and corynantheidine (Smith, Kline and French Laboratories, Philadelphia, U.S.A.); speciogynine and speciociliatine (Dr. J. D. Phillipson); mitraciliatine (Professor A. N. Tackie); hirsutine (Professor E. J. Shellard); dihydrocorynantheine (S. B. Penick and Co.); isocorynantheidine was prepared from corynantheidine (unpublished).

### *Apparatus*

A Cambridge Conductance bridge with a conventional conductivity cell having black platinized electrodes with large surface area (about 2 $cm^2$) was used for the rate studies. The cell was maintained at 25° ± 0·5°.

For the partition coefficients, a special glass stoppered test tube (50 ml capacity) was used. A thermostatically controlled water bath adjusted to 37° ± 0·5° fitted with a shaking device, which shook the tubes gently (60–80 strokes/min) along the XY plane was employed to attain equilibrium conditions. The pH values of the aqueous solutions were measured using Dynacap pH meter.

*Method*

(1) The methyl iodide for the rate experiments was distilled and treated with mercury to remove iodine present as impurity. Methanol, spectral grade (BDH), was distilled twice. The alkaloid (5 mg; 1·25 m mol) was added to methanol (10 ml) and the conductivity measured until it remained constant; methyl iodide (0·5 ml) (about five hundred fold excess) was then added rapidly from a microburette, a stop clock being started simultaneously. The conductivity was measured at one minute intervals for 15 min and then at 5 min intervals for a further 75 min. Finally a reading was made after three or four days to give an "infinity" reading.

(2) For the partition experiment, the Sorensen phosphate buffer was freshly prepared and its pH adjusted to 7·4 with either of the components using a pH meter; samples (0·25, 0·5, 0·75 and 1 mg) of the alkaloids were placed in a 10 ml volumetric flask and heptane (spectroscopic grade), previously saturated with phosphate buffer pH 7·4 for 24 h, was added. These solutions were shaken vigorously on a mechanical shaker until the alkaloids dissolved. The ultraviolet spectrum of each solution was determined using buffer saturated heptane in the reference cell and a calibration curve constructed using the ultraviolet peak for each alkaloid (280–292 nm). Owing to the insolubility of these alkaloids in the aqueous buffer phase (heptane saturated) very small quantities 0·05, 0·075 and 0·1 mg were weighed, each was made to volume in a volumetric flask and shaken for 15 h at 37° to dissolve and the ultraviolet calibration curve made similarly in the buffer phase pH 7·4 as above.

The partition coefficients of the alkaloids were determined by adding 10 ml of a solution of 1 mg of alkaloid in 30 ml of heptane to 10 ml of buffer pH 7·4. The mixture was shaken at 37° for 24 h. The phases were separated and the concentration of alkaloid in each was determined. The final pH of the aqueous phase was checked with a pH meter. The initial concentration of the alkaloid in heptane was checked as described above using the ultraviolet calibration curve. The determination was repeated on a second 10 ml portion of the solution of the alkaloid in heptane.

## RESULTS AND DISCUSSION

The $pK_a$ and the total percentage metabolism in rabbit liver microsomes recorded in Tables 1 and 2 were obtained from Beckett & Morton (1967). Fig. 1 shows a graph of conductance against time for the quaternization experiments on corynantheidine-type alkaloids. The conductivity of the solution, $G_t$, reflects the concentration of the

Table 1. $pK_a$ *and rate of quaternization of cornyantheidine-type alkaloids*

| | Alkaloid | Configuration[2] | R (in I) | Rate $K \times 10^{-4}$ | $pK_a$[1] |
|---|---|---|---|---|---|
| 1. | Speciogynine | *Normal* | OMe | 0·89 | 7·40 |
| 2. | Dihydrocorynantheine | *Normal* | H | 1·06 | 7·47 |
| 3. | Mitraciliatine | *Pseudo* | OMe | 3·40 | 7·95 |
| 4. | Hirsutine | *Pseudo* | H | 4·70 | 7·89 |
| 5. | Mitragynine | *Allo* | OMe | 0·14 | 7·06 |
| 6. | Corynantheidine | *Allo* | H | 0·16 | 7·15 |
| 7. | Speciociliatine | *Epiallo* | OMe | 0·82 | 7·44 |
| 8. | Isocorynantheidine | *Epiallo* | H | 0·65 | 7·45 |

[1] Beckett & Morton (1967).

[2] Tamelin, Aldrich & Katz (1956); Wenkert & Bringi (1959; Joshi, Raymond-Hamet & Taylor, (1963); Bartlett, Sklar & others (1962); Weisbach, & others (1965); Lee & others (1967); Trager & others (1967; 1968).

Table 2. *Partition coefficients in heptane/buffer* pH 7·4 *and percentage of total metabolism in rabbit liver microsomes of corynantheidine-type alkaloids*

| | Alkaloid | Configuration[2] | R (in I) | Partition $\frac{C_h}{C_b}$ | Coefficient $K_p$ | Relative % metabolism[1] by microsomes |
|---|---|---|---|---|---|---|
| 1. | Speciogynine | *Normal* | 9-OMe | 14·5 | 31·1 | 89 |
| 2. | Dihydrocorynantheine | *Normal* | H | 6·9 | 15·8 | 41 |
| 3. | Mitraciliatine | *Pseudo* | 9-OMe | 4·8 | 24·2 | 25 |
| 4. | Hirsutine | *Pseudo* | H | 2·7 | 12·4 | 21 |
| 5. | Mitragynine | *Allo* | 9-OMe | 68·0 | 103·7 | 69 |
| 6. | Corynantheidine | *Allo* | H | 23·1 | 34·7 | 33 |
| 7. | Speciociliatine | *Epiallo* | 9-OMe | 4·0 | 9·0 | 42 |
| 8. | Isocorynantheidine | *Epiallo* | H | 2·6 | 6·0 | 23 |

[1,2] See refs at foot of Table 1.

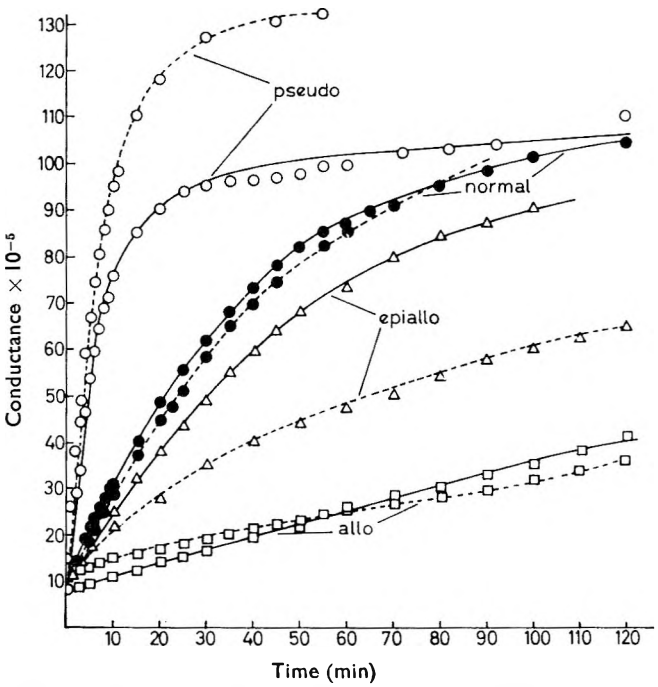



FIG. 1. The influence of stereochemistry on the rate of quaternization of corynantheidine-type alkaloids with methyl iodide. ○, *Pseudo.* ●, *Normal.* △, *Epiallo.* □, *Allo.* ————Methoxy alkaloids. - - - - - - Non-methoxy alkaloids.

methiodide at any time, t. Thus the value of $(G\infty - G_t)$, where $G\infty$ is the conductivity after complete quaternization, is a measure of the concentration of unquaternized alkaloid. The large excess of methyl iodide allows a first order treatment:

$$\ln (G_\infty - G_t) = \ln G\infty - kt \qquad (1)$$

where k is the pseudo first order rate constant. To permit comparison, the same excess of methyl iodide was used for each alkaloid, and the same quantity of alkaloid used in each case (Moss, 1962; Shamma & Moss, 1962). The *normal, allo* and *epiallo* alkaloids gave a linear plot of $\ln (G_\infty - G_t)$ versus t, but for the *pseudo* compounds the plot began to curve after some 7 min; the rate constants, k, are calculated therefore for the first 7 min. only.

For the calculation of partition coefficient values shown in Table 2, equation (2) was used:

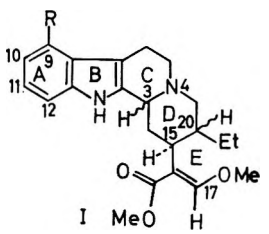
$$Kp = C_h/C_b \ (1 - \alpha) \qquad (2)$$

Where kp is the true partition coefficient, $C_h$ is the total concentration in the organic (heptane) phase, $C_b$ is the total concentration in the buffer phase and $\alpha$ is the degree of dissociation of the alkaloid. For bases, the correction factor $1/(1 - \alpha)$ is given by:

$$1/(1 - \alpha) = 1 + \text{antilog } (pK_a - pH)$$

where the pH value is that of the buffer phase after equilibrium.

The corynantheidine-type alkaloids have the basic structure (I) in which R = H or OMe. There are three asymmetric centres C-3, C-15 and C-20, the stereochemistry at C-15 and about the double bond is the same in all the isomers (Wenkert & Bringi,




R = H or O Me

1959; Weisbach, Kirkpatrick & others, 1956; Trager, Lee & Beckett, 1967) therefore four diastereoisomers are possible as follows:

| Configuration | C-3H | C-15H | C-20H |
|---|---|---|---|
| *normal* | α | α | β |
| *pseudo* | β | α | β |
| *allo* | α | α | α |
| *epiallo* | β | α | α |

Conformational analysis supported by physico-chemical data has shown that the *normal* and *allo* compounds exist to the extent of at least 95% in conformations AI and CI in which rings A, B, C and D are coplanar but with a C-20 ethyl group equatorial in the former compound and axial in the latter (Fig, 2). The *pseudo* isomer exists at least 95% in conformation BI in which ring D is at right angles to the coplanar rings A, B, and C with the C-20 ethyl group equatorial; on the other hand the *epiallo* isomer exists in an equilibrium between the non-planar conformation (DI), with a C-20 ethyl group axial, and the approximately planar DIII, with an equatorial C-20 group: conformer DI predominates (Trager, Lee & Beckett, 1967).

*pKa and rate of quaternization*

Table 1 shows that there are no significant differences in the $pK_a$ values of the non-methoxylated and the methoxylated isomers of the same configuration. Although there are slight differences in the rates of quaternization (Table 1, cf cpd 1 with 2; 3 with 4; 5 with 6; 7 with 8); these are not as marked as the differences in the rate and $pK_a$ values due to changes in configuration.

The conductivity curves (Fig. 1) and the $pK_a$ values (Table 1) indicate that the *pseudo* and *normal* isomers, in that order, are the strongest bases and quaternize more quickly than the other compounds examined. The rate of quaternization is sensitive

to steric hindrance (Brown & Eldred, 1949) and two aspects deserve comment. In the preferred conformation of both the *pseudo* (BI) and *normal* (AI) isomers (Fig. 2) the bulky C-20 ethyl group is equatorial whereas in the other compounds, *allo* (CI) and *epiallo* (DI), it is axial and thus creates hindrance to attack by an electrophilic reagent at N-4. In addition, the *normal* series (AI) has the configuration at C-3 of a *trans*-quinolizidine and is more sterically hindered at N-4 than is the *pseudo* isomer (BI) which corresponds to a *cis*-quinolizidine.

The relationship between the *allo* (CI) and *epiallo* (DI) (see later) isomers are of the same kind as those between the *normal* (AI) and *pseudo* (BI) respectively (Fig. 2) and this provides an explanation of the more ready quaternization of the *epiallo* isomers compared to the *allo* isomers. Methoxy substitution in the *epiallo* series produces a basic strength and quaternization rate (Table 1 cf. cpd 7 with cpd 1) almost equivalent to that of *normal*, the compound with the C-20 equatorial ethyl group. This is probably due to the significant contribution of conformation DIII (*trans*-quinolizidine) in the *epiallo* configuration in which the C-20 ethyl group is now equatorial.

Thus the $pK_a$ and the rate of quaternization are in accord with the conformations suggested for the corynantheidine type alkaloids by Trager, Lee & Beckett (1967).

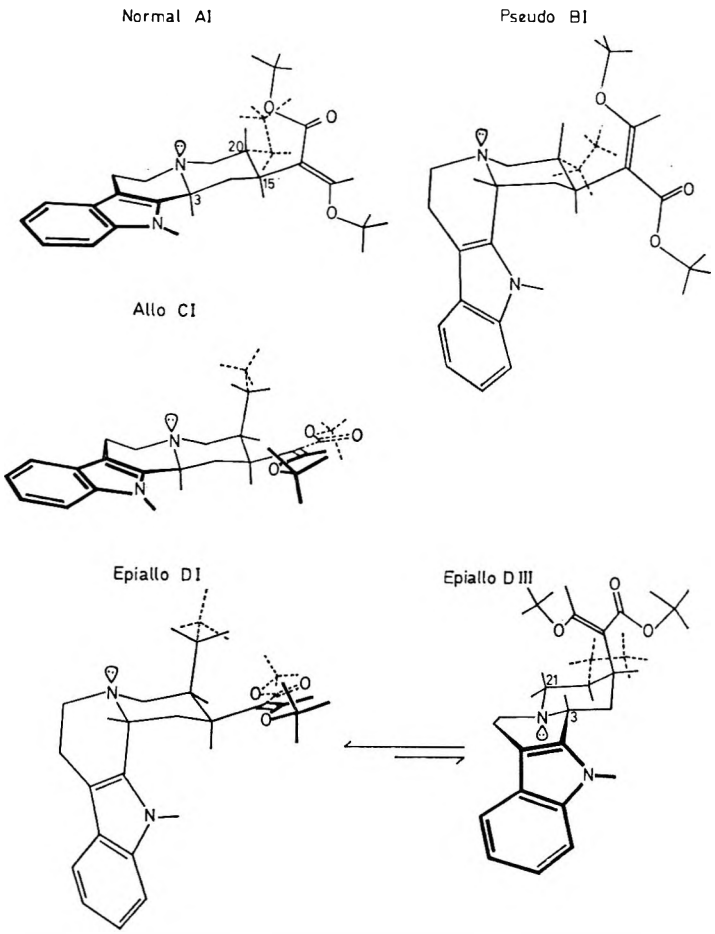



FIG. 2. The preferred conformations of Corynantheidine-type alkaloids (open E-ring); (Trager & others, 1967) AI, *normal*; BI, *pseudo*; CI, *allo*; DI and DIII *epiallo*.

*Partition coefficients and metabolism in liver microsomes*

It has been suggested by Mayer, Maickel & Brodie (1959) that the rates of entry of drugs into the cerebrospinal fluid (CSF) from plasma may be predicted from their partitition coefficients between heptane, benzene or chloroform and water at pH 7·4. These authors showed that drugs enter the CSF at rates which depend upon the lipid solubility of the unionized molecules and that rapid penetration is assured provided that there is a sufficient proportion of this form in the plasma. That lipid solubility is the physical property governing the passage of uncharged molecules across membrane barriers is supported by the work of Brodie, Kurz & Schanker (1960), Schanker (1959) and Kakemi, Arita & others (1967). It is also known that partition coefficients of molecules influence the rate of metabolism by liver microsomes (Ahmed, 1958; McMahon, 1961).

Partition coefficients for the corynantheidine-type alkaloids in the heptane–buffer pH 7·4 system and the metabolism by rabbit liver microsome of the isomers under identical conditions are shown in Table 2.

Each planar *normal* and *allo* isomer has a higher partition coefficient than its corresponding non-planar *pseudo* and *epiallo* isomer (Table 2 cf. cpd 1, 2, 5 and 6 with 3, 4, 7 and 8). Methoxy substitution increases the partition coefficients in all the isomers (Table 2 cf. cpd 1 with 2, 3 with 4, 5 with 6, 7 with 8). The change in the C-20 ethyl group from the *axial* to the equatorial position in the series *normal* and *allo* increases partition coefficient whereas the same change in the *pseudo* and *epiallo* compounds decreases it. This is probably because of the conformational equilibrium between DI and DIII in the *epiallo* compounds.

Each planar isomer is metabolized to a greater extent than its corresponding non-planar one whilst methoxy isomers are metabolized more than their non-methoxy analogues. This would be expected since the higher lipid solubility of the planar isomers favours metabolism. However, metabolism is also influenced by the affinity and activity of a molecule at a metabolic site as well as by its availability. Thus the change from a C-20 equatorial ethyl to a C-20 axial group in the planar compounds (Fig. 1, cf. AI with CI) reduces metabolism (Table 2, cf. 1, 2 with 5 and 6) despite the increase in partition coefficients; this is explicable in terms of the axial ethyl group constituting a steric hindrance to the reinforcement by the basic nitrogen lone pair to the binding of the flat indole nucleus to the metabolic site. The increase in retention times in gas-liquid chromatography, upon making this change, has been similarly explained (Beckett & Dwuma-Badu, 1968).

The change from the planar *normal* AI to the *pseudo* BI compounds, in which ring D is roughly at right angles to the rings A, B and C (Fig. 1), reduces not only the partition coefficients and the total metabolism but also produces a change in the route of metabolism (Beckett & Morton, 1967), thus emphasizing the importance of the site binding as well as the site availability of the molecules.

In contrast to the *pseudo* isomers (Table 2 cf. cpd 7 and 8 with 3 and 4), the *epiallo* isomers are not only metabolized by enol *O*-demethylation as are the planar *normal* and *allo* compounds (Table 2, cf. 1 and 5 with 7), but they are also metabolized to an extent greater than would be expected from their partition coefficients. This is in accord with the planar *epiallo* conformation DIII influencing both the route and amount of metabolism, a situation which exists only in this configuration since it has been established by Trager & others (1967) that the *normal*, *allo* and *pseudo* configurations exist to the extent of 95% in the conformations AI, CI and BI (Fig. 2) respectively.

The methoxy isomers have partition coefficients at least double those of their non-methoxy analogues in both planar (*normal* and *allo*) and non-planar *pseudo* isomers but with the metabolism results for the planar isomers, the methoxy group doubles the metabolism over their non-methoxy analogues while for the *pseudo* analogues only a small increase in the metabolism occurs. This is consistent with the change in metabolic route which results on the change from planar to non-planar isomers.

Only one of the isomers studied in Table 1 is active as an analgesic and antitussive agent; this is mitragynine (Table 1, *allo*) its activity could arise from its planarity which helps it to bind to a surface, from its $pK_a$ (7·06) which ensures sufficient concentration in the plasma and from its higher lipid solubility allowing easier penetration to the CNS. The planar non-methoxylated alkaloid of *allo* configuration (Table 2 compound 6) may be inactive not only because of its less favourable partition characteristics but because of its reduced electron availability in the indole nucleus.

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SHORT COMMUNICATION

# Some effects of bacterial pyrogens and activated charcoal on polarographic maxima

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The adsorption of pyrogens from injection solutions is well known (Lees & Levvy, 1940; Brindle & Rigby, 1946), and to monitor the removal of pyrogens (Micka & Kalvoda, 1956) a polarographic method has been proposed; this consists of observing the form of the polarographic reduction wave of cadmium. In the presence of pyrogen no maximum is seen at the crest of the wave whereas after treatment of the solution with activated charcoal an acute maximum (Fig. 1B) can be observed. It is claimed that this corresponds to adsorption of the pyrogen onto the charcoal, the minimum amount of charcoal required for full maximum formation being used as a measure of the amount of pyrogen present. Farkas & Bridicksa (1964) have employed this polarographic technique for the routine quality control of pyrogen-free water. The same authors have similarly used the polarographic oxygen maximum. The suppression of the oxygen maximum by some pyrogenic solutions has been reported previously by Suzuki (1955).

It is known (Heyrovsky & Vascautzanu, 1931) that because the half-wave potential of cadmium lies close to the electrocapillary zero potential of mercury, the cadmium wave does not normally exhibit a maximum. It therefore seemed desirable to investigate more closely the effect of charcoal on the cadmium wave and also to determine the sensitivity of polarographic maxima to pyrogens.

*Experimental*

A Tinsley type 14/3 polarograph was used with a dropping electrode system made entirely of glass. The drop-time was 2·5 s on closed circuit and zero applied potential. The cell anode was a mercury pool. One of the two electrolysis cells used permitted the introduction of solutions from a burette. Cell solutions were deoxygenated with solvent-saturated oxygen-free nitrogen, and polarograms recorded with minimal damping. The water used was from a still previously shown to produce apyrogenic water. Potassium chloride (0·1 M) was used as a supporting electrolyte. Analar chemicals were used where possible: the gelatin was of B.P. quality. Activated charcoal was Norit N.K. freed from water-soluble impurities. The pyrogens used were: the dried purified lipopolysaccharide from *Salmonella abortus-equi*, the O-somatic antigen from *Shigella dysenteriae* (the International Pyrogen Reference Preparation) and the purified "3922 lipopolysaccharide *Escherichia coli* 0111: B4", the latter supplied by Difco Laboratories Ltd.

Current-time recordings were obtained with a current-calibrated double-beam oscilloscope.

*Results and discussion*

We were able to record the acute cadmium maximum only when charcoal was present in the cell (Fig. 1B). Despite rigorous attempts at pyrogen exclusion we have

been unable to record this maximum in the absence of charcoal. In all cases when charcoal was not present in the cell during recording, the polarogram was of the form shown in Fig. 1A.

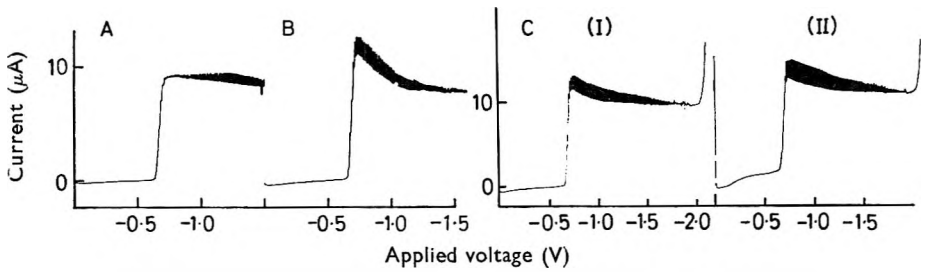


FIG. 1. Polarographic waves for (A) 1·0 mM cadmium chloride in 0·1M potassium chloride. (B) 1·0 mM cadmium chloride and 0·01% w/v activated charcoal in 0·1M potassium chloride. (C) 1·0 mM cadmium chloride and 0·01% w/v activated charcoal in 0·1M potassium chloride: (I) immediately after deoxygenation, (II) 18 h after deoxygenation without disturbing cell contents.

These results are at variance with those of Micka (1956), who stated that polarograms with maxima of identical height were recorded when the charcoal had been removed by sedimentation or by centrifugation. After centrifugation at 10,000 rev/min for 2 h we could not record the acute maximum from the supernatant liquid, whereas before centrifugation this had been possible, even with as little as 0·01% w/v charcoal present. After 18 h of undisturbed settling in the cell, the maximum was still in evidence (Fig. 1C II). Light scattering measurements showed that at this time there was still a detectable amount of charcoal in the capillary tip region (Fig. 2).

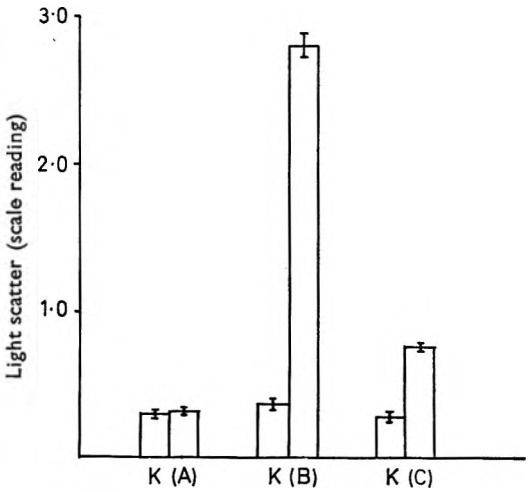


FIG. 2. Block diagrams to show the light scatter with standard errors produced by the cell contents: (A) before addition of charcoal, (B) immediately after deoxygenation of cell suspension containing charcoal, (C) 18 h after deoxygenation without disturbing cell contents. K is the cell solution (without added charcoal) as control.

The current-time curves obtained for charcoal suspensions both before and after sedimentation confirmed the presence of charcoal at the mercury drop surface (Fig. 3). All these observations led us to conclude that the role of charcoal in the recording

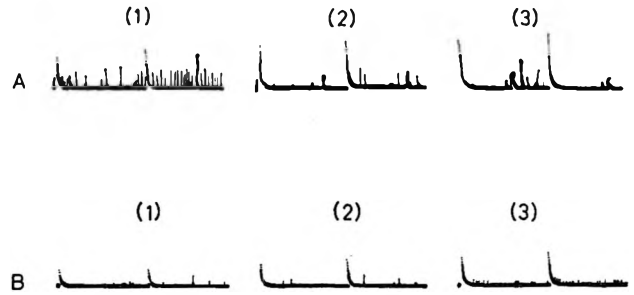


FIG. 3. Oscillographic current-time curves recorded from 0·1M potassium chloride containing 0·01% w/v activated charcoal: A, immediately after deoxygenation, B, 18 h after deoxygenation without disturbing cell contents. Potentials: A: (1) —609, (2) —699, (3) —781 mV. B: (1) —599, (2) —687, (3) —768 mV.

of the acute cadmium maximum is due to phenomena other than the adsorption of maximum-suppressing pyrogens—the explanation advanced by Micka & Kalvoda. We have previously reported the shifting of the electrocapillary zero potential of mercury by charcoal in suspension (Jones & Kaye, 1969) and feel that this provides an explanation of the appearance of the cadmium maximum, in accordance with Heyrovsky's rule (Heyrovsky & Vascautzanu, 1931; Heyrovsky, 1934).

Table 1. *Effect of some pyrogens and gelation on polarographic maxima.* Purified pyrogen solution (5·1 ml) was added to produce a cell concentration of 1 μg/ml.

| Maximum suppressor (Cell concentration) | Mean % suppression of the maximum produced by the polarographic electrolysis of: | | | | | |
|---|---|---|---|---|---|---|
| | $Cd^{++}$ | $Tl^{+}$ | Oxygen | $Co^{++}$ | $Ni^{++}$ | $Pb^{++}$ |
| Pyrogen from *Salmonella abortus-equi* (1 μg/ml) | —1·0 | 13·2 | 3·8 | 5·0 | 13·6 | —5·5 |
| Pyrogen from *Escherichia coli* (1 μg/ml) | 11·7 | 13·6 | 0 | 3·2 | 17·5 | 9·7 |
| Pyrogen from *Shigella dysenteriae* (1 μg/ml) | 6·3 | 10·7 | 4·5 | 17·5 | 10·5 | 26·5 |
| *Gelatin* (1 μg/ml) | 26·5 | 35·0 | 14·5 | 49·0 | 62·7 | 49·5 |
| Apyrogenic water (5·1 ml) | —6·7 | 5·0 | 1·6 | —1·2 | 3·0 | 4·0 |

(—ve sign indicates mean increase in maximum height.)

We have also examined the effects of known concentrations of three purified pyrogens on the acute cadmium maximum recorded in the *presence* of charcoal, and on other polarographic maxima recorded in the *absence* of charcoal. Table 1 shows that the cadmium and oxygen maxima are relatively insensitive to pyrogen concentrations of 1 μg/ml, a value which is very high in terms of the rabbit thermal response test, where for example 0·003 μg of the International Reference Preparation is known to elicit a febrile response (Humphrey & Bangham, 1959). For comparison, the results with gelatin, an effective maximum suppressor, are included. All the pyrogens tested are far less effective maximum suppressors than gelatin.

We conclude that the suppression of polarographic maxima does not afford a dependable method of pyrogen detection.

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# A model for the influence of emulsion formulation on the activity of phenolic preservatives

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Basic mathematical models are suggested for calculating the concentration of preservatives which is chemically available in the water of emulsions. It is influenced by the total concentration of preservative in the emulsion, the oil : water partition coefficient of the preservative, the oil : water ratio and the concentration of emulgent. The activity of the preservative is related to the concentration free in the water.

The ability of microorganisms to grow in emulsions is well established (Bennett, 1962; de Navarre, 1962; Wedderburn, 1964; Noble & Savin, 1966) but assessments of the ability of preservatives to prevent microbial invasion of emulsified products have depended largely on empirical tests involving inoculation of the finished product and examination during a prolonged period of storage (Wedderburn, 1964). More detailed and less empirical studies of the influence of emulsion components have used simplified models involving a minimum of components. A preservative added to an oil-water mixture partitions between the two phases (Bean, Richards & Thomas, 1962; Hibbott & Monks, 1961), its activity being controlled by the concentration in the aqueous phase which in turn is dependent upon the total concentration of preservative in the mixture, the oil : water partition coefficient of the preservative, the phase : volume ratio and the temperature (Bean, Richards & Thomas, 1962; Bean & Heman-Ackah, 1964; Heman-Ackah & Konning, 1967; Hibbott & Monks, 1961). A third component of even the simplest emulsion is the emulgent which usually is a micelle-forming surface-active agent. Anionic surface-active agents at concentrations below the critical concentration for micelle formation (CMC) increase the activity of phenolic preservatives but at concentrations above the CMC they depress the activity as a result of partitioning of the preservative between the micelles and the water (Alexander & Tomlinson, 1949; Bean & Berry, 1951; Evans & Dunbar, 1965). The CMC of non-ionic surface-active agents is much lower than that of anionics and in practice such solutions of non-ionics are rarely encountered at sub-CMC concentrations.

The precise nature of the interaction between non-ionics and preservatives is uncertain and may be either complex formation (Guttman & Higuchi, 1956; Mulley & Metcalf, 1956; Patel & Kostenbauder, 1958; Ansel, 1965) or solubilization of the preservative within the micelles of the non-ionic (Goodhart & Martin, 1962; Hurwitz, De Luca & Kostenbauder, 1963; Evans, 1964; Mulley, 1964). Irrespective of which theory may be correct, both have been successfully employed to verify the hypothesis that the antimicrobial activity of preservatives in aqueous solutions of non-ionics is, as in aqueous solutions of anionics, directly related to the concentration of free uncombined preservative (Pisano & Kostenbauder, 1959; Blaug & Ahsan, 1961; Mitchell, 1964).

Pisano & Kostenbauder (1959), advocates of the complexation theory, have shown that the concentration of free preservative in aqueous solutions of non-ionics is

$$C_W = \frac{C_A}{R} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where $C_W$ = concentration of preservative free in the water, $C_A$ = total concentration of preservative in the aqueous solution, R = ratio of total : free preservative.

The value of R increases as the concentration of non-ionic is increased (Pisano & Kostenbauder, 1959), and, for any specified value of $C_A$, the biological activity decreases (Mitchell, 1964; Malcolm, 1967).

Notwithstanding failures of conventional concentrations of preservatives to protect emulsions from microbial contamination (Noble & Savin, 1966), few systematic studies have investigated the activity of preservatives in complete emulsions. The present communication suggests two simple mathematical models to relate changes in the basic formula of an emulsion to the concentration of free preservative and relates this concentration to the activity.

## THEORETICAL

In a simple oil-water dispersion a preservative is partitioned between the oil and aqueous phases, according to the following mathematical model (Bean & Heman-Ackah, 1964),

$$C_W = \frac{C(\phi + 1)}{(K_W^o \phi + 1)} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where C = total concentration of preservative in the system, $C_W$ = concentration of preservative in the water, $\phi$ = oil : water ratio, $K_W^o$ = oil : water partition coefficient of the preservative.

In an emulsion, a small proportion of the total emulgent is at the oil-water interface, but most, particularly if it has negligible oil-solubility, is dispersed throughout the water as micelles where it behaves as an additional phase. Any preservative added to the emulsion is partitioned between the three phases according to the oil : water partition coefficient, micelle : water partitionc oefficient and the volumes of each of the phases.

Several mathematical models may be devised to illustrate how the concentration of free preservative in the water is influenced by the magnitude of the afore-mentioned physical parameters, but objections may be raised to some. For example, to a model embodying a term for the volume of micellar phase objection may be made that the volume of emulgent (e.g. Polysorbate 80) added to water to yield a specified volume of aqueous solution, may not represent the volume of the micellar phase since any hydration of the micelles would modify their volume.

Such objections may be avoided by using models which do not include a term denoting the *volume* of the micellar phase but which necessitate reference to experiments relating the value of R (equation 1) to the concentration of non-ionic in the aqueous phase. In this connection it must be recognized that an increase in the concentration of non-ionic increases the total concentration of preservative in the aqueous phase, and the oil : aqueous phase partition coefficient falls. The concentration in the aqueous phase at equilibrium can thus be described by a model similar to that of Bean & Heman-Ackah (1964).

$$C_A = \frac{C(\phi + 1)}{(K\phi + 1)} \quad .. \quad .. \quad .. \quad .. \quad (3)$$

where $C_A$ = concentration of preservative in the total aqueous phase, C = total concentration of preservative in the emulsion, $\phi$ = oil : water ratio, K = oil : total aqueous phase partition coefficient of the preservative.

The concentration of free preservative in the water may be calculated by invoking the model of Pisano & Kostenbauder (eqn 1).

$$C_W = \frac{C(\phi + 1)}{(K\phi + 1)} /R$$

or

$$C_W = \frac{C(\phi + 1)}{(K\phi R + R)} \quad .. \quad .. \quad .. \quad (4)$$

Although, with increasing non-ionic concentration the apparent oil : water partition coefficient (K) falls, the true oil:water partition coefficient ($K_W^o$) remains constant. Part of the additional preservative partitioned into the aqueous phase is complexed with the nonionic and $C_W = C_A/R$ (Pisano & Kostenbauder, 1959) and therefore

$$K_W^o = KR \text{ or } C_W = \frac{C(\phi + 1)}{(K_W^o\phi + R)} \quad .. \quad .. \quad .. \quad .. \quad (5)$$

Experimentally, this is probably a simpler expression to employ than (4).

Thus, the concentration of free preservative in the water of an emulsion ($C_W$) may be calculated from either (4) or (5) provided that the following parameters are known: (i) total concentration of preservative in the emulsion (C); (ii) oil : water ratio ($\phi$); (iii) the ratio of total : free preservative in the aqueous phase (R); and either (iv) the oil : total aqueous phase partition coefficient of the preservative for the concentration of non-ionic emulgent present (K), or (v) the true oil : water partition coefficient for the distribution of the preservation between the oil and the water ($K_W^o$).

## EXPERIMENTAL

### *Materials*

*Polysorbate* 80 conforming to the monograph of Polysorbate 80 in U.S.P. XVI and B.P.C. 1968. It is virtually insoluble in liquid paraffin. *Phenol* (A.R. quality). p-*Chloro*-m-*cresol* (Laboratory Reagent grade). *Liquid paraffin B.P.C.* 1963. S.G. 0·830–0·870. *Nutrient broth*, Oxoid granules (C.M.1) at a concentration of 1·3% w/v. *Indicator broth*, nutrient broth containing 1% w/v lactose and 0·0016% w/v bromocresol purple. *Nutrient agar*, nutrient broth solidified with 2% w/v Kobé agar ("Oxoid").

### *Organism*

*Escherichia coli* (NCTC 5933) cultivated and maintained as described by Bean & others (1962). The inoculum was a nephelometrically standardized suspension of the organism stored in sterile water at 4°.

### *Methods*

*Preservative-polysorbate* 80 *interaction.* Quantitative estimates of the interactions between phenol and polysorbate, and between chlorocresol and polysorbate were obtained using a dialysis technique similar to that used by Patel & Foss (1964). A

solution (20 ml) of preservative and polysorbate was pipetted into one compartment of a Perspex dialysis cell and water (20 ml) into the other compartment. The cell was stoppered and agitated in a water bath at 25 ± 0·1° for 5 days, after which samples from each compartment were assayed spectrophotometrically for the preservative, using the method of Johnson & Savidge (1958). This was repeated for a series of different concentrations of polysorbate.

At equilibrium the concentration of preservative in the cell compartment containing no surface-active agent was equal to the concentration of free preservative in the compartment containing surface-active agent. If the total amount of preservative in the cell is known, then the ratio of total to free preservative (R) may be calculated.

*Oil-water partition coefficients.* Oil-water-preservative mixtures each containing a known concentration of preservative were equilibrated at 25 ± 0·1°. After separation the aqueous phase was assayed spectrophotometrically for the preservative, using the method of Johnson & Savidge (1958) and the partition coefficients calculated from the assay results.

*Determination of extinction times.* Extinction times were determined by the method of Bean & Heman-Ackah (1964). Reaction mixtures of the required composition, containing 20 × $10^6$ viable *E. coli*/ml in the aqueous phase, were placed in glass-stoppered tubes and maintained at 25 ± 0·1°. At intervals corresponding to approximately 1/7th of the expected extinction time, 1 ml samples were transferred to 24 ml of nutrient broth and the mixture was shaken vigorously; 1 ml of the broth dilution was then transferred to 24 ml of indicator broth providing a ×625 dilution of the phenol in the original sample; the final dilution was incubated at 37° for 48 h. Bacterial growth produced a change in the colour of the indicator from purple to yellow thereby distinguishing turbidity due to growth, from turbidity due to oil droplets in the broth. Five replicate determinations were made for each extinction time.

## RESULTS

### *Preservative—polysorbate* 80 *interaction*

The ratio total: free preservative (R) was linearly related to the concentration of polysorbate for both phenol and chlorocresol (Fig. 1). The slopes of these regressions were 0·27 and 4·7 for phenol and chlorocresol respectively and the value of 0·27 for phenol compares with that of 0·26 found by Patel & Foss (1964).

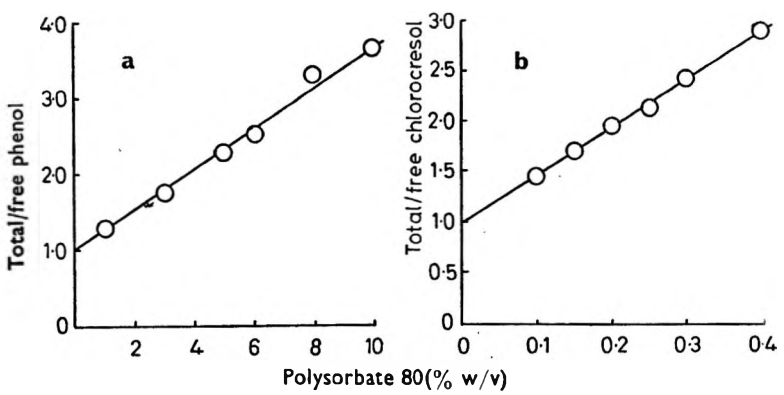


FIG. 1. The association of phenols and polysorbate 80. a. Phenol. b. Chlorocresol.

*The influence of polysorbate* 80 *on the apparent oil : water partition coefficients of phenol and chlorocresol*

The inclusion of polysorbate in a liquid paraffin-water dispersion reduces the proportion of phenol or chlorocresol remaining in the oil phase at equilibrium, and increases the proportion in the immiscible aqueous phase. That is, the oil : aqueous phase partition coefficient falls (Fig. 2) although the true oil : water partition coefficient

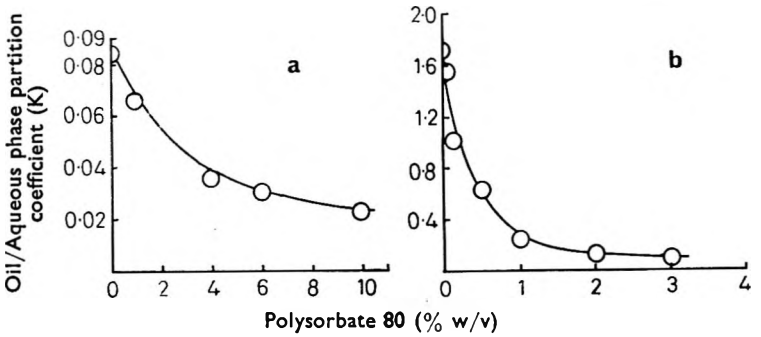


FIG. 2. Effect of polysorbate 80 on oil : aqueous phase partition coefficients of phenols between liquid paraffin and water. a. Phenol. b. Chlorocresol.

must remain unchanged on addition of polysorbate. If the oil : aqueous phase partition coefficient is known for any given polysorbate concentration, and the ratio of total : free phenols in the aqueous phase is known, the concentration of free preservative in the water may be calculated using equation (4).

*Effect of oil and polysorbate* 80 *concentration on the concentration of free preservative in the water*

For a fixed overall concentration of phenol and a fixed aqueous phase concentration of polysorbate, an increase in oil : water ratio produced an increase in the concentration of free phenol (Fig. 3), irrespective of the concentration of polysorbate.

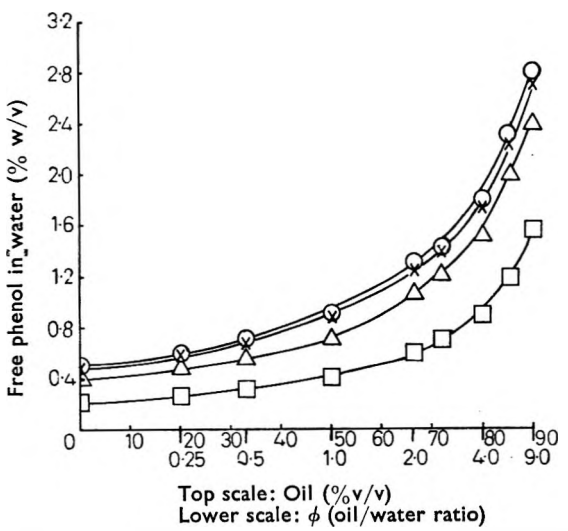


FIG. 3. Effect of proportion of oil in emulsions on concentration of free phenol in water when the total phenol concentration is 0·5% w/v. ○, Polysorbate concentration 0·0% w/v; ×, 0·04% w/v; △, 1·0% w/v and □, 5·5% w/v.

The curvilinear relations in Fig. 3 may be transformed to slightly sigmoidal near-linear relations with a positive slope by transforming the abscissa to $\sqrt{\text{oil : water ratio}}$.

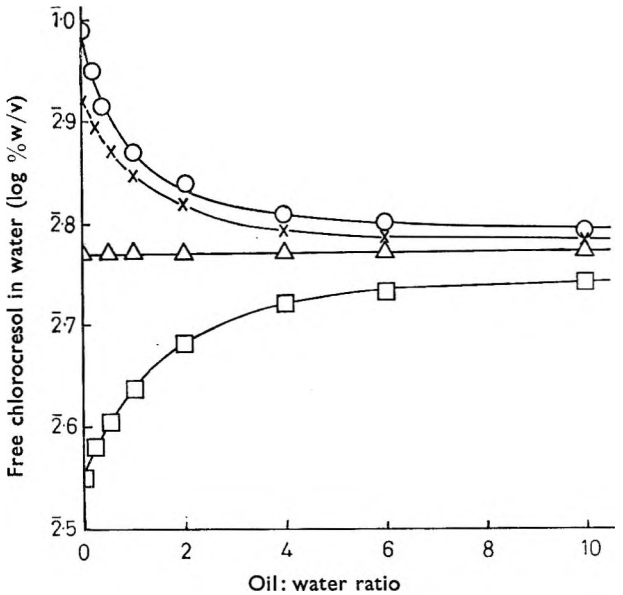


FIG. 4. Effect of oil : water ratio of emulsions on concentration of free chlorocresol in water when total chlorocresol concentration is 0·1% w/v. ○, Polysorbate concentration of 0·0% w/v; ×, 0·04% w/v; △, 0·15% w/v and □, 0·40% w/v.

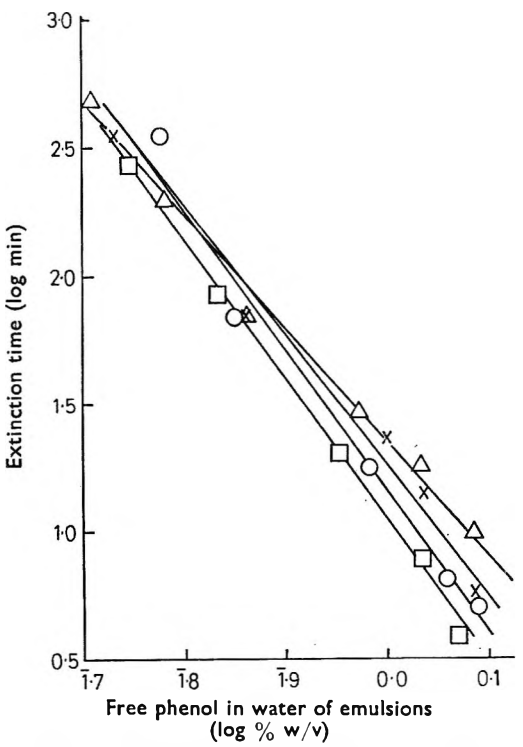


FIG. 5. Relationship between free phenol in emulsions and extinction time for *E. coli*. ○, Polysorbate concentration of 0·0% w/v; □, 0·04% w/v; ×, 1·0% w/v and △, 5·0% w/v.

With chlorocresol, the direction of change in concentration of the free preservative was influenced by both the oil:water ratio and the concentration of polysorbate (Fig. 4). At concentrations of polysorbate less than 0·15% w/v an increase in oil:water ratio produced a decrease in the concentration of free chlorocresol. When polysorbate was in excess of 0·15% w/v, an increase in the oil:water ratio produced an increase in the concentration of free chlorocresol, whilst when the polysorbate concentration was 0·15% w/v, the concentration of free chlorocresol was independent of the oil:water ratio.

*The activity of the preservatives in the emulsions*

*Phenol.* The bactericidal activity of phenol in emulsions containing 0–5·0% polysorbate is shown in Fig. 5 for oil:water ratios 0–9·0 (90% oil) where a linear relation is demonstrated between the free phenol in the emulsions and log extinction time.

*Chlorocresol.* For a fixed overall concentration of 0·1% w/v chlorocresol and an oil:water ratio of less than 2, the direction of change in activity with increasing oil:water ratio was dependent upon the concentration of polysorbate (Fig. 6). Increasing the oil:water ratio above 2, increased activity at all concentrations of polysorbate. For a fixed oil:water ratio, an increase in the concentration of polysorbate decreased the activity of the preservative in the emulsion.

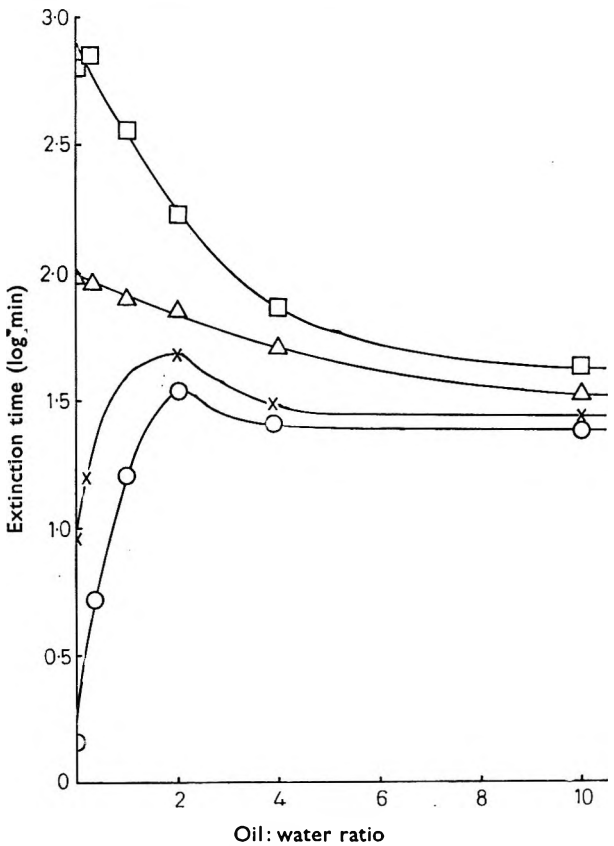


FIG. 6. Effect of oil : water ratio on extinction time for *E. coli* of emulsions containing 0·1% w/v total concentration of chlorocresol. ○, Polysorbate concentration of 0·0% w/v; ×, 0·04% w/v; △, 0·15% w/v and □. 0·40% w/v.

## DISCUSSION

As a preliminary to the evaluation of preservative activity in emulsions, simplified models comprising only two of the three essential components of an emulsion have been studied. Pisano & Kostenbauder (1959) examined systems containing water and emulgent and concluded that the activity of preservatives in such systems was related to the concentration of uncomplexed preservative . Similarly the activity of preservatives in oil-water mixtures is dependent primarily upon the concentration in the water, this being influenced by the oil : water partition coefficient of the preservative and the phase-volume ratio (Bean, Richards & Thomas, 1962; Bean & Heman-Ackah, 1964). In both cases mathematical models have proved invaluable in relating preservative activity to parameters of the preservatives and changes in the composition of the systems. Such models may be either, simple, such as those referred to, or complex like the integrated models of Garrett (1966) which incorporate terms to quantify all identifiable factors influencing preservative availability. Not all factors are identifiable and some are difficult to quantify. Experience has shown, however, that simplified models, though admittedly incomplete, are sufficiently accurate for formulation purposes. The more complex a formula the greater the number of factors influencing preservative availability and the greater the number of terms necessary in any operational model to relate changes in preservative availability to formulation changes. The two models suggested in this communication (eqns 4 and 5) are only slightly more complex than the earlier model (Bean & Heman-Ackah, 1964) for computing preservative availability in oil-water mixtures. Their validity has been confirmed by assay of the preservative in the aqueous phase of the emulsions.

For preservatives having an oil : aqueous phase partition coefficient of less than 1·0, an increase in the proportion of oil in the emulsion increases the concentration of free preservative in the water (Fig. 3), thus resembling the effect in simple oil-water mixtures. In fact, calculations show that an increase in the percentage of oil in a liquid paraffin emulsion simultaneously increases the concentration of phenol in the water, aqueous phase, oil-phase and micelles of the polysorbate emulgent.

The changes are more complex when chlorocresol is the preservative and are best understood by reference to Fig. 4. Increasing the proportion of oil reduces the free chlorocresol concentration in the water when the polysorbate concentration is below 0·15% and increases it when it is greater than 0·15%. This is because at these polysorbate concentrations the oil : total aqueous phase partition coefficient (K) is respectively above and below 1·0. When the emulsion contains 0·15% polysorbate the oil : aqueous phase partition coefficient is 1·0 and the concentration of free chlorocresol is independent of the oil : water ratio. The direction of concentration change is always the same for all phases (cf. Bean & others, 1962).

A plot of free phenol in the water of the emulsions against extinction time of *E. coli*, shows a linear relation (Fig. 5). This indicates that the activity of phenol in the emulsions is dependent upon the concentration free in the water, the latter being dependent in turn upon the total concentration of phenol in the emulsion, the oil : water ratio, and the concentration of non-ionic emulgent (eqns 4 and 5).

The relation between the activity of chlorocresol and the proportion of oil or emulgent in the emulsion is much more complex (Fig. 6) and reflects the complex changes in the concentration of free chlorocresol produced by altering the oil : water ratio or concentration of emulgent. To indicate the comparatively large changes in preservative activity which can result from comparatively small changes in the

composition of an emulsion, the chlorocresol results have not been transcribed to relate activity to free chlorocresol. But once again it can be shown that the activity is dependent upon the free chlorocresol in the water. It may be noted that for oil-water mixtures containing 25% v/v liquid parafin and 0·1% w/v total chlorocresol, the extinction time is about 5 min whereas the addition of 0·4% v/v polysorbate, a far lower concentration than is likely to be encountered in practical emulsions, reduces activity one hundred-fold—to about 550 min. On the other hand, if the proportion of liquid paraffin in an emulsion containing 0·4% polysorbate is increased from 25 to 80%, the activity is *increased* about 10-fold.

It is quite meaningless to add an arbitrary concentration of a preservative to an emulsion. The only rational and safe procedure is to decide upon the activity required of a preservative in an emulsion and then to calculate the total quantity required from a knowledge of the parameters discussed in this communication. The calculation may often reveal the unsuitability of the preservative selected.

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# Effect of organic matter and increased inoculum size on the minimum inhibitory concentration of crystal violet towards *Staphylococcus aureus* at various pH values

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The minimum inhibitory concentration towards *Staphylococcus aureus* of crystal violet at pH values 6, 7 and 8, increased when the inoculum size was increased or if egg albumen was added to the medium. When allowance was made for dye uptake, the minimum inhibitory concentration still exceeded that in the absence of organic matter. The increased minimum inhibitory concentration in the presence of muscle could in most cases be attributed to the removal of dye from solution.

It is well known that the presence of organic matter reduces the antibacterial activity of many substances, including that of basic dyes (Graham-Smith, 1919). The higher the concentration of peptone for example in the growth medium, the less effective are dyes against bacteria (Kligler, 1918).

It has also been shown by Churchman & Kahn (1921) that, although single cells of *Escherichia coli* do not grow on crystal violet agar, groups of 30 cells are able so to do —a phenomenon they called "communal activity" of bacteria.

Small pieces of animal tissue reduce the bactericidal action of serum-dye mixtures (Wels, 1922), while serum, yeast or large inocula diminish the effect of crystal violet, presumably due to combination with the dye (Stearn & Stearn, 1924).

I have carried out experiments to determine whether or not the increased minimum inhibitory concentration of crystal violet towards *Staph. aureus*, in the presence of organic matter, was due to absorption by the organic matter.

## EXPERIMENTAL

### *Materials*

Crystal violet B.P. recrystallized from ethanol [$E$ (1% 1 cm) = 2059 at 591 nm] was used to prepare a 0·01M aqueous solution.

Nutrient broth was prepared from granules (Oxoid CM1) and 1% potassium dihydrogen phosphate (Analar) added, and the pH adjusted to the required value (6, 7 or 8) using a freshly prepared solution of potassium hydroxide.

Cultures were prepared from freeze-dried *Staphylococcus aureus* (NCTC 7447).

Meat muscle was prepared by macerating granules of cooked meat medium (Oxoid CM 81) in water, replacing the water several times until the supernatant liquid was colourless, and drying the separated muscle particles at 60°. 10 mg and 40 mg quantities were autoclaved in test-tubes containing 2 ml water, which was then decanted and the muscle used.

Albumen was removed aseptically from eggs and mixed with sterile nutrient broth.

*Estimation of sorption of crystal violet*

*Bacteria.* Organisms were grown on nutrient agar, removed with quarter-strength Ringer solution, washed and centrifuged and a weighed amount of moist bacteria mixed with broth. A total cell count was performed, and a measured volume of suspension added to tubes containing varying amounts of crystal violet solution; broth (at pH 6) was added to produce 5 ml. The mixture was equilibrated at 37° (30 min), centrifuged (10 min at 2300 *g*) and the extinction of the supernatant liquid determined at 591 nm. A second series of tubes was prepared without organisms to act as dye controls, and the extinction determined. The sorption of dye by the organisms was thus calculated, and the experiments were repeated using different volumes of suspension, and at pH 7 and at pH 8.

*Muscle.* Amounts of broth (10 ml, pH 6) were transferred to test-tubes containing muscle (10 mg) and varying amounts of dye added. After 48 h incubation at 37° the extinction of the supernatant liquid was determined. Another series of tubes was prepared without muscle to act as dye controls, and the extinction again determined. The sorption of dye by the muscle was calculated, and the experiments were repeated with 40 mg muscle, and also at pH 7 and at pH 8.

*Albumen.* Dye binding was determined by the equilibrium dialysis method (Adams, 1968). Quantities of broth (8 ml; pH 6) were transferred to glass bottles, and bags made from Visking dialysis tubing placed therein. Volumes of 2% w/v albumen solution (0·7 ml) were placed in each bag, varying amounts of dye solution added, and the volume adjusted to 8 ml with broth. The bottles were placed in a reciprocating water bath (24 h at 37°), and the extinction of the liquid in the bottles (outside the bags) determined. Identical dialysis experiments were prepared without albumen to act as dye controls, and the extinction determined. The binding of dye to albumen was calculated, and the experiments were repeated with 2 ml albumen solution, and at pH 7 and at pH 8.

*Determination of minimum inhibitory concentration of crystal violet*

Different amounts of crystal violet solution were added to 10 ml volumes of broth at pH 6, and inoculated with one drop (about 1/35 ml containing 2–3 $\times$ $10^6$ organisms) of an overnight culture of *Staph. aureus.* The tubes were incubated (48 h at 37°) and examined for growth. The experiment was repeated with the addition of 10 mg and 40 mg muscle, 0·5 ml and 1·5 ml albumen solution (containing 10 and 30 mg dry weight albumen respectively), and with different inoculum sizes. The series of experiments was repeated at pH 7 and at pH 8.

## RESULTS

The concentrations of crystal violet preventing growth (48 h at 37°) are given in Table 1. Table 2 shows the effect of inoculum size on the minimum inhibitory concentration of crystal violet.

Fig. 1 shows the equilibrium concentrations of dye at pH 6, resulting from different initial concentrations, in the presence of muscle and albumen. The relation between reduction in concentration of dye and equilibrium concentration is shown in Fig. 2. The extent of reduction in concentration of dye by organic matter, under the conditions of the determination of the minimum inhibitory concentration, was calculated as follows. The initial concentration of dye in solution is known from the amount

Table 1. *Effect of organic matter on minimum inhibitory concentration of crystal violet towards* Staph. aureus (*incubated* 48 *h at* 37°).

| | With organic matter added to 10 ml broth | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Conc. of dye × $10^{-6}$M | | | | Conc. of dye × $10^{-6}$M | | | Without added organic matter |
| pH | Muscle (mg) | Initial | Uptake | Free dye | Albumen (mg) | Initial | Uptake | Free dye | |
| 6 | 10 | 0·65 | 0·30 | 0·35 | 10 | 0·59 | 0·08 | 0·51 | 0·40 |
| | 40 | 2·01 | 1·45 | 0·56 | 30 | 1·40 | 0·33 | 1·07 | |
| 7 | 10 | 0·40 | 0·29 | 0·11 | 10 | 0·41 | 0·02 | 0·39 | 0·25 |
| | 40 | 1·62 | 1·35 | 0·27 | 30 | 1·06 | 0·28 | 0·78 | |
| 8 | 10 | 0·056 | 0·035 | 0·021 | 10 | 0·141 | 0·015 | 0·126 | 0·035 |
| | 40 | 0·147 | 0·115 | 0·032 | 30 | 0·81 | 0·23 | 0·58 | |

Table 2. *Concentrations of crystal violet* (× $10^{-6}$M) *preventing growth of different inoculum sizes of* Staph. aureus (washed) *for* 48 *h at* 37°

| Crystal violet concentrations (× $10^{-6}$M) for inoculum sizes of: (× $10^{6}$/ml) | | | | |
|---|---|---|---|---|
| pH | 15 | 1·5 | 0·15 | 0·015 |
| 6 | 1·44 | 0·45 | 0·29 | 0·25 |
| 7 | 0·75 | 0·17 | 0·13 | 0·083 |
| 8 | 0·67 | 0·061 | 0·041 | 0·023 |

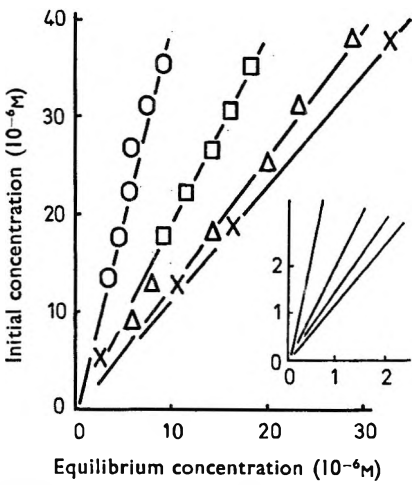


FIG. 1. Equilibrium concentrations of crystal violet produced by different initial concentrations in presence of muscle and albumen at pH 6. ○, 40 mg muscle; □, 10 mg muscle; △, 30 mg albumen; × 10 mg albumen. Inset: enlarged lower portion of graph.

added, and the corresponding equilibrium concentration is found from Fig. 1. The reduction in concentration of dye is obtained from Fig. 2, and this value is subtracted from the initial concentration to give the residual or free dye in solution (Table 1), e.g. at pH 6, in the presence of 40 mg muscle, the minimum inhibitory concentration was 2·01 × $10^{-6}$M. From Fig. 1 the equilibrium concentration is 0·47 × $10^{-6}$M, corresponding to a reduction in concentration of 1·45 × $10^{-6}$M (Fig. 2), leaving a concentration of free dye of 0·56 × $10^{-6}$M.

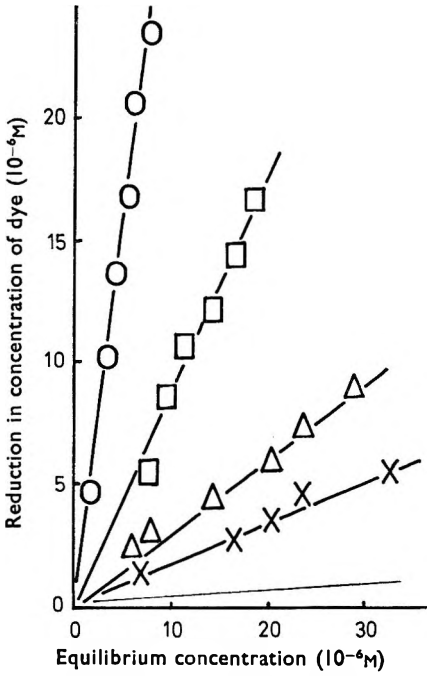


FIG. 2. Reduction in concentration of crystal violet produced by muscle, albumen and bacteria at different equilibrium concentrations at pH 6. ○, 40 mg muscle; □, 10 mg muscle; △, 30 mg albumen; × 10 mg albumen, plain line bacteria.

The graphs for dye sorption by muscle and by albumen at pH 7 and pH 8 were similar to those at pH 6, except that uptake by muscle was greatest at pH 7, slightly less at pH 8, and was least at pH 6; the amount of dye bound by albumen was greatest at pH 8 and least at pH 6.

Dye sorption by *Staph. aureus* itself was difficult to measure since the inoculum size used was so small as to be negligible. Sorption of crystal violet by mg amounts of bacteria is considerable (Adams, 1967), but in the present work it was necessary to extrapolate the results to the smaller inocula used. Fig. 2 includes an approximate indication of the dye uptake at pH 6 (which is similar to that at pH 7 and 8).

## DISCUSSION

The addition of 10 mg muscle to 10 ml broth increases the minimum inhibitory concentration *ca* 1·5 ×, and 40 mg 4–6·5 ×, and the effect is similar at the three pH values. Muscle removes crystal violet from aqueous solution slowly but continuously, probably both by absorption by muscle fibres and by reduction by unsaturated compounds such as linoleic acid, which explains its use in anaerobic media. Up to 75% of the dye is removed from solution within 48 h at 37°, e.g. when 10 mg is incubated in 10 ml broth at pH 7 containing $0{\cdot}40 \times 10^{-6}$M dye the concentration of free dye is reduced to $0{\cdot}11 \times 10^{-6}$M, which is below the minimum inhibitory concentration in the absence of muscle, viz. $0{\cdot}25 \times 10$—$^{6}$M. The concentration of free dye is not always below the minimum inhibitory concentration, but it is never much higher. Except at pH 6 with 40 mg muscle, the increased minimum inhibitory concentration in the presence of muscle can be attributed to the removal of dye from solution.

Under the conditions used, 10 mg albumen binds 5–13% of the dye present, and 30 mg binds 23–28%. Albumen reduces the effect of pH on the minimum inhibitory concentration, e.g. the ratio of the minimum inhibitory concentrations at pH 6 and pH 8 is 11 in the absence, but less than 2 in the presence, of 30 mg albumen. The concentration of free dye is usually much greater than that in the absence of albumen, so that in some way albumen must protect the organisms, in addition to combining with a portion of the dye. Indeed Sykes (1965) has already suggested that the reduction in efficiency of disinfectants in the presence of organic matter was "not so much through the inactivation of disinfectant as through protection of the bacteria". It is possible that the increased viscosity caused by the albumen assists growth of the organism, e.g. by facilitating build-up of $CO_2$ or by lowering the redox potential.

Increased inoculum size raises considerably the minimum inhibitory concentration of crystal violet. This applies especially at pH 8, the pH at which the organisms are most sensitive. Thus at pH 6 the minimum inhibitory concentration is raised about 6 × by a thousand-fold increase in inoculum size, while at pH 8 the figure is 30 ×. A large inoculum reduces the pH effect of crystal violet; thus the minimum inhibitory concentration at pH 6 is only double that at pH 8 using the largest inoculum, while 10 × the concentration of dye is required to inhibit growth at pH 6 compared with that at pH 8 using the smallest inoculum. This is similar to the effect of albumen discussed above. Churchman and Kahn (1921) observed that a large inoculum of *E. coli* could grow in the presence of crystal violet for some reason other than that of a lowered concentration due to uptake of dye by the bacteria. In the present work the uptake of dye was negligible, and it seems that growth in the presence of dye occurred by some "communal activity" of the organisms.

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SHORT COMMUNICATION

# Ulcerative colitis in the guinea-pig caused by seaweed extract

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Carrageenans are widely used as food ingredients as well as medicinally in various parts of the world (Bonfils, 1968). As yet there have been no reports of adverse effects following the use of these substances (Anderson, 1967).

Recently, we have found a high incidence of ulcerative colitis in several species of laboratory animals fed extracts of various red seaweeds (Marcus & Watt, 1969).

In this paper we present our findings on degraded and undegraded carrageenans derived from *Eucheuma spinosum* and fed to guinea-pigs over a relatively short time.

## *Method*

Adult male albino guinea-pigs, of 500 g average body weight, bred in our own laboratory, were housed in separate cages and fed a normal cube diet (S.G.1) supplemented daily with fresh cabbage.

One group of 10 animals received as drinking fluid a 1% aqueous solution of undegraded carrageenan[1] derived from *E. spinosum*. A second group of 10 animals received as drinking fluid a 5% aqueous solution of degraded carrageenan[2] also derived from *E. spinosum*. The degraded product, being less viscous, was used at a higher concentration than the undegraded product. Solutions of carrageenan were freshly prepared each day, stored at 4° and supplied in drinking bottles that were cleaned daily; no gross bacterial contamination was observed. Allowing for spillage from the drinking bottles, the daily intake per animal of undegraded carrageenan was not more than 1·5 g/kg and of degraded carrageenan not more than 2 g/kg body weight. A third control group of 10 guinea-pigs received water *ad lib* but without added carrageenan.

At frequent intervals throughout the experiment, the stools were examined for occult blood using the Hematest method. The animals were killed, using ether anaesthesia, between 20 and 30 days after the start of the experiment. The gastrointestinal tract was entirely removed. The large bowel was emptied of faeces, distended with 10% formol saline, and after fixation, carefully examined. Histological sections were prepared from various parts of the colon and stained with haematoxylin and eosin, and also with toluidine blue.

## *Results*

In the first group which received undegraded carrageenan, four guinea-pigs were killed after 20 days and two of these showed multiple ulcerative lesions in the caecum. The remaining six animals were killed after 30 days; traces of occult blood in the faeces were present from day 23. These animals had ulcerative lesions in the caecum

[1] Undergraded carrageenan was obtained from J. W. Cumming & Son Ltd, Salford.

[2] Degraded carrageenan was kindly supplied by Laboratories Glaxo-Evans, Paris.

and two had lesions extending into the colon for a short distance. The overall incidence of ulceration in this group was 80%.

In the second group, which received degraded carrageenan, most of the animals showed looseness of the stools by the end of 10 days; from the 20th to the 30th days, all had occult blood in the faeces. In this group the incidence of ulcerative colitis was 100%. In five animals killed between the 20th and 25th days, the lesions were mainly in the caecum; in the remainder, killed between the 26th and 30th days, ulceration had extended into the lower colon and rectum.

In both experimental groups the lesions were multiple, numbering sometimes in the hundreds. Macroscopically, they consisted of pin-head sized ulcers, rounded or slightly irregular in outline. Some had coalesced to form larger lesions, often linear in shape. Histologically, the ulcerations involved mainly the mucosa and showed features of both acute and subacute inflammatory infiltration, as well as crypt abscesses (Fig. 1). Macrophages containing metachromatic material were frequently seen in toluidine blue stained sections.

In the control group, the animals remained healthy throughout the 30 days. There was no occult blood in the stools and no pathology in the caecum, colon or rectum.

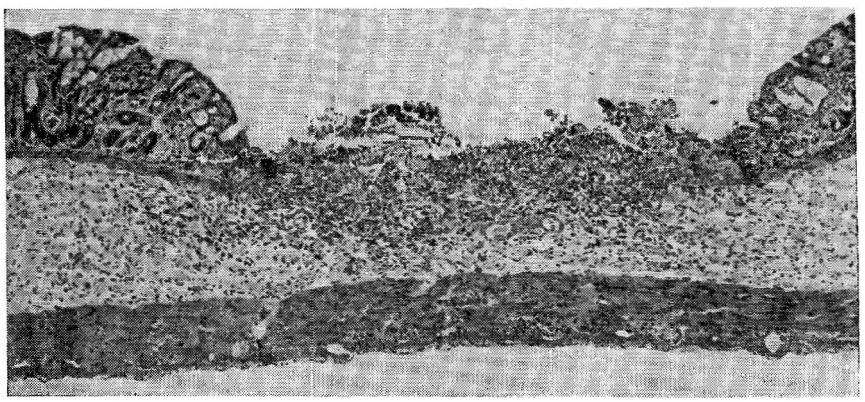

FIG. 1. Subacute ulcer in colon of a guinea-pig fed carrageenan: heavy cellular infiltrate in ulcer base and in submucosa. H. & E. × 56.

*Discussion*

Our results have revealed a hitherto undescribed effect of carrageenan, namely ulceration of the caecum and colon in the guinea-pig. The lesions produced are not readily visible unless the bowel is emptied of faeces and examined using transmitted light. This is perhaps why such lesions have not previously been observed.

The more severe ulceration in the animals on degraded carrageenan is more likely to be due to the greater total amount of carrageenan consumed by these animals than to possible contaminants arising as a result of the degradation process.

The ulcerative lesions in the guinea-pig are not identical in distribution with those typically seen in ulcerative colitis in man. Nevertheless, their histological appearance and their confinement largely to the mucosa are features common to both. The significance of our results in relation to human ulcerative colitis is at present only speculative and must await more comprehensive investigation.

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# The haemolytic activity of sols containing progesterone solubilized by phosphatidylcholine and lysophosphatidylcholine

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Lysis of rat erythrocytes by aqueous dispersions containing lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and progesterone has been studied. The haemolytic activity of LPC was reduced by saturating it with progesterone; mixed LPC–PC micelles owed their lytic activity to the LPC alone. Cholesterol solubilized together with progesterone by LPC greatly reduced the lytic activity of the LPC, and when solubilized with progesterone by a 1:1 mixture of LPC and PC, prevented haemolysis. Triolein also inhibited the haemolytic properties of the sols.

The dispersion of steroids in aqueous solutions offers the possibility of producing alternative dosage forms for these compounds. In this work the co-dispersion of progesterone with phospholipids has been explored. Saunders, Perrin & Gammack (1962) reported that large concentrations of cholesterol can be co-dispersed with egg phosphatidylcholine (PC) to give clear and stable aqueous sols. Hoyes & Saunders (1966) and Kellaway & Saunders (1967) have also reported on the co-dispersion of other steroids with PC. In general, cholesterol-like steroids with only one polar group in the molecule are readily dispersed, whereas the hormones and other steroids with polar groups at opposite ends of the molecule are much less co-dispersed. Kellaway & Saunders have shown that the bipolar steroids can be dispersed up to a concentration greater than 2% in water by using mixed sols of PC and lysophosphatidylcholine (LPC). Use of LPC as a dispersing agent introduces undesirable lytic effects and the present work describes studies on the lysis of rat erythrocytes by these sols and of methods for eliminating this effect.

This work is a preliminary investigation on the haemolytic activity of LPC when co-dispersed with other lipids; no attempt has been made to produce a formulated product which would have entailed a more detailed study of haemolysis using human erythrocytes at 37° and rates of release of the steroid from the micelles.

*The aqueous dispersion of progesterone*

Although virtually insoluble in water at 20°, progesterone may be dispersed directly by ultrasonic irradiation to yield a colloidal or semicolloidal solution (Misek & Skauen, 1958a, b); the addition of surface-active materials facilitates the disruption of the progesterone aggregates (Misek & Skauen, 1958a) by displacing adsorbed air from around the solid surface thus allowing the wetting of the solid and reduction of the energy necessary for the disruption of the aggregates. When the concentration of the surfactant exceeds its critical micelle concentration, solubilization of progesterone occurs. Several workers (Ekwall & Sjöblom, 1950; Diczfalusy, Ekwall &

Sjöblom, 1952), have reported the preparation of colloidally dispersed progesterone in an aqueous medium using high concentrations of surface active materials. Concentrations of progesterone from 0·5–5·0 mg/ml were solubilized in aqueous solutions of sodium lauryl sulphate (10%) and polysorbate 20 (20%) (Diczfalusy & others, 1952). Biological activity was retained by these preparations.

*The haemolytic activity of progesterone*

Amongst the neutral steroids, progesterone is particularly haemolytic to human erythrocytes (Tateno & Kilbourne, 1954; Palmer, 1964). In concentrations of $7{\cdot}5 \times 10^{-4}$ to $1{\cdot}0 \times 10^{-3}$M it is among the most haemolytic of all steroids for rabbit erythrocytes (Weissmann & Keiser, 1965), although at lower concentrations its haemolytic activity is greatly reduced.

Table 1. *Results of haemolyses by phospholipid sols*

| Sol (ratios by weight) | Concentration of phospholipid % w/w | Time for 50% haemolysis (s) |
|---|---|---|
| PC | 0·12 | No haemolysis in 15 min |
| LPC | $5{\cdot}0 \times 10^{-4}$ | 66 |
| | $6{\cdot}25 \times 10^{-4}$ | 34 |
| | $7{\cdot}5 \times 10^{-4}$ | 26 |
| | $8{\cdot}75 \times 10^{-4}$ | 20 |
| | $12{\cdot}5 \times 10^{-4}$ | 15 |
| LPC/PC 1 : 1 | $12{\cdot}5 \times 10^{-4}$ | 61 |
| | $18{\cdot}75 \times 10^{-4}$ | 34 |
| | $25{\cdot}0 \times 10^{-4}$ | 30 |
| | $31{\cdot}25 \times 10^{-4}$ | 27 |
| PC saturated with progesterone | 0·12 | No haemolysis in 15 min |
| LPC saturated with progesterone | $5{\cdot}0 \times 10^{-4}$ | 46·5 |
| | $6{\cdot}25 \times 10^{-4}$ | 37·0 |
| | $7{\cdot}5 \times 10^{-4}$ | 28·5 |
| | $10{\cdot}0 \times 10^{-4}$ | 25·5 |
| LPC/PL 1 : 1 saturated with gesterone | $12{\cdot}5 \times 10^{-4}$ | 76 |
| | $18{\cdot}75 \times 10^{-4}$ | 53 |
| | $25{\cdot}0 \times 10^{-4}$ | 45 |
| | $37{\cdot}5 \times 10^{-4}$ | 37 |
| LPC/PC 1 : 1 saturated with cholesterol | 0·12 | No haemolysis in 15 min |
| LPC saturated with progesterone and cholesterol | $37{\cdot}5 \times 10^{-4}$ | 65 |
| | $50{\cdot}0 \times 10^{-4}$ | 39·5 |
| | $75{\cdot}0 \times 10^{-4}$ | 31·5 |
| | $100{\cdot}0 \times 10^{-4}$ | 26·5 |
| LPC/PC 1 : 1 saturated with progesterone and cholesterol | 0·12 | No haemolysis in 15 min |
| PC/triolein 1 : 1 | 0·12 | No haemolysis in 15 min |
| LPC/PC/triolein | $19 \times 10^{-4}$ | 200 |
| | $38 \times 10^{-4}$ | 82 |
| | $57 \times 10^{-4}$ | 65 |
| | $76 \times 10^{-4}$ | 55 |
| | $133 \times 10^{-4}$ | 50 |

*The haemolytic activity of phospholipids*

Investigating the haemolytic activity of LPC (containing some "lysocephalin") Gorter & Hermans (1943) showed that haemolysis was rapid over the first few minutes and that it then ceased. They found that a given amount of lysophosphatide is capable of haemolysing a fixed number of erythrocytes owing to adsorption of the LPC onto the cell membrane and it was estimated that haemolysis resulted when the equivalent of a unimolecular layer of LPC had become adsorbed. The rate of haemolysis is accelerated as the volume of the cell increases (Wilbur & Collier, 1943) and the quantity of LPC required to lyse a given number of cells varies with the concentration of the cell suspension (Collier, 1951–52; Nygaard, Dianzani & Bahr, 1954). The lysing action is temperature dependent, more 'lysin' being adsorbed on the cells at lower temperatures. This would suggest that an equilibrium exists between the LPC in solution and that absorbed by the cell. Collier (1951–52) on the basis of results obtained with rabbit erythrocytes has suggested that the LPC reacts to form a complex with the free cholesterol within the cell membrane. Sublytic concentrations of LPC produce alterations in the erythrocyte membrane: "sphering" occurs, hypotonic fragility decreases and the rate of thiourea penetration is greatly increased while calcium ions cause much shrinkage. The partial all-or-none characteristics of LPC haemolysis have been demonstrated by Feeney, MacDonnell & Fraenkel-Conrat, 1954) using rat and rabbit erythroctyes.

The action of PC in haemolysis is less well defined. Domonkos (1962) found that haemolysis by LPC was inhibited by phosphatides and Gjone (1961) observed this to be true for PC extracted from normal human serum. Using synthetic phospholipids and bovine erythroctyes, Reman & Van Deenen (1967) showed that certain diacyl phosphoglycerides possessed haemolytic activity, the didecanoyl PC being much more active than its lyso derivative.

## EXPERIMENTAL

*Preparation of phosphatidylcholine* (PC). The starting material was a Merck A.G. commercial sample of egg phospholipids. Alumina (Savory & Moore) was packed in chloroform and the crude phospholipid fraction (20 mg/g of alumina) dissolved in the minimum quantity of chloroform, was applied to the top of the column. Elution was accomplished by a chloroform–methanol mixture of 8:1 and 3:1 v/v, the latter extracting the PC fraction. All fractions extracted giving a positive ninhydrin reaction were rejected, the negative fractions extracted by the 3:1 solvent mixtures were bulked and evaporated below 40°. The dried residue of crude PC was further purified by dissolving it in the minimum quantity of chloroform and applying it to a column of silicic acid (Malinckrodt) mixed with celite (Koch-Light 545) 2:1 (Hanahan, Dittmer & Warashina, 1957) by weight and packed in chloroform. Elution was by chloroform–methanol 4:1 and 3:2 v/v, collecting 100 ml fractions. Fractions containing only PC were identified by thin-layer chromatography bulked and evaporated at 35°. The PC residue was recrystallized several times from warm methyl ethyl ketone and acetone and the final product stored under dry acetone at 5°. Yield 30–40%. Immediately before use, the PC was checked for purity by thin-layer chromatography and recrystallized if necessary, until a single spot resulted. Analytical data: found N 1·78, P 3·79%. N/P 1·04. Iodine value 71.

*Preparation of lysophosphatidylcholine* (LPC). This was prepared essentially by the method of Hanahan, Rodbell & Turner (1954). PC (10 g) in ether (750 ml) was

shaken with 5 ml of an aqueous solution (pH 7·4) containing Russell Viper Venom (10 mg), 1% NaCl and 0·005M $CaCl_2$. The solution was allowed to stand for several hours, the ether was evaporated and the residue taken up in the minimum quantity of chloroform: 2 ml aliquots were pipetted into centrifuge tubes and ether (23 ml) added. Refrigerated centrifugation for 10 min brought the light precipitate to the base of the tubes, enabling separation by decantation. This procedure was repeated several times to remove any unreacted PC and venom. The final product was obtained by recrystallizing three to four times from hot ethanol (Saunders, 1957) and was stored under dry acetone at 5°. Analytical data: found: N 2·7, P 5·9%. N/P 0·99.

*Progesterone and cholesterol.* Pure samples were obtained from BDH and Fluka respectively.

*Triolein.* This was purified by silica gel column chromatography using a mixed ether–light petroleum (b.p. 40–80°) solvent system and was a gift from M. C. R. Johnson.

*Rat erythrocytes.* Rat blood was heparinized, centrifuged and the erythrocytes washed five times with 0·9% NaCl to give a 50% haematocrit.

*Preparation of the sols.* The dispersion of progesterone and the separation of undissolved solids was according to Hoyes & Saunders (1966).

*Examination of haemolysis.* Saline (20 ml, 0·9%) was placed in the cell of a Unicam SP1400 Prism Absorptiometer, previously calibrated with reference to a neodymium filter ($\lambda$ = 537 nm). 0·1 ml of the erythrocyte suspension was added to the cell and the transmission adjusted to read 10 at 625 nm. The phosphatide was added, the cell contents rapidly mixed and lysis (measured as an increase in transmission) recorded as a function of time.

*Spectrophotometric determination of progesterone concentration.* Progesterone was assayed by measuring the absorbance at 240 nm. Dilution of the sols by ethanol to give a measurable absorbance in 1 cm cells also dissolved the phospholipid micelles, thereby eliminating errors due to light scattering.

*Haemolysis curves and treatment of results.* From the experimental plot of transmission scale reading against time (Fig. 1), which gave haemolysis curves for lysophosphatidyl choline (concentration w/v), the time to produce 50% haemolysis was determined and these values are plotted against the concentration of lysin in Fig. 2.

## DISCUSSION

The haemolytic activity of LPC towards rat erythrocytes is reduced by saturating it with progesterone. Assuming that the LPC monomers are a more active haemolytic species than the micelles, then the addition of progesterone will result in a shift to the right of the equilibrium,

$$n \text{ (monomers)} \rightleftharpoons \text{micelle},$$

thus reducing the overall haemolytic activity of the sol. Surface tension studies by Robinson & Saunders (1958) indicated a critical micelle concentration of 4 to 18 mg litre$^{-1}$ for LPC in water at 25°. The mixed phospholipid micelle owes its haemolytic activity to the LPC alone, the PC slightly reduces the haemolysis by LPC. The sol with progesterone solubilized by the mixed phospholipids was less lytic than either the sol containing progesterone solubilized by LPC alone, or the mixed phospholipid sol in the absence of co-dispersed progesterone (Table 1).

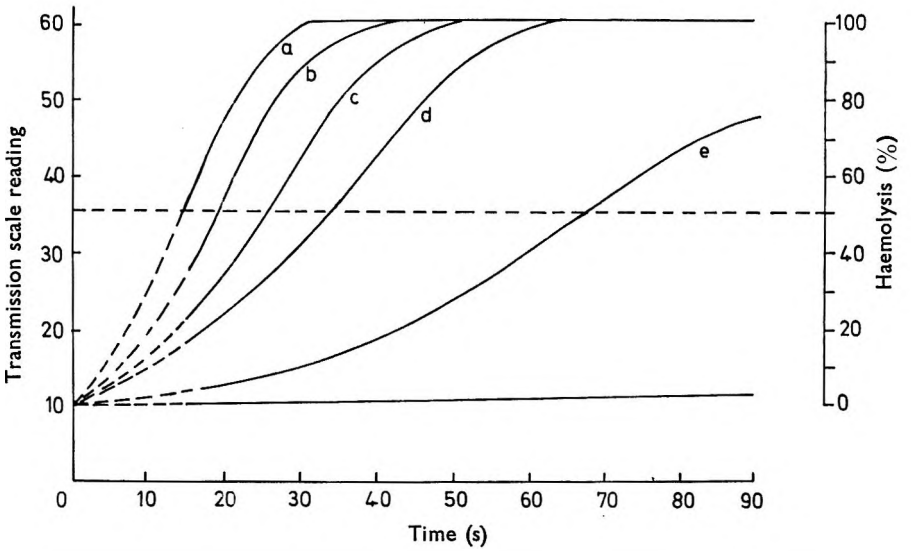


FIG. 1. Haemolysis curves for lysophosphatidylcholine concentrations in percentage w/w. a, $12{\cdot}5 \times 10^{-4}$. b, $8{\cdot}75 \times 10^{-4}$. c, $7{\cdot}5 \times 10^{-4}$. d, $6{\cdot}25 \times 10^{-4}$. e, $5{\cdot}0 \times 10^{-4}$. f, $3{\cdot}5 \times 10^{-4}$.

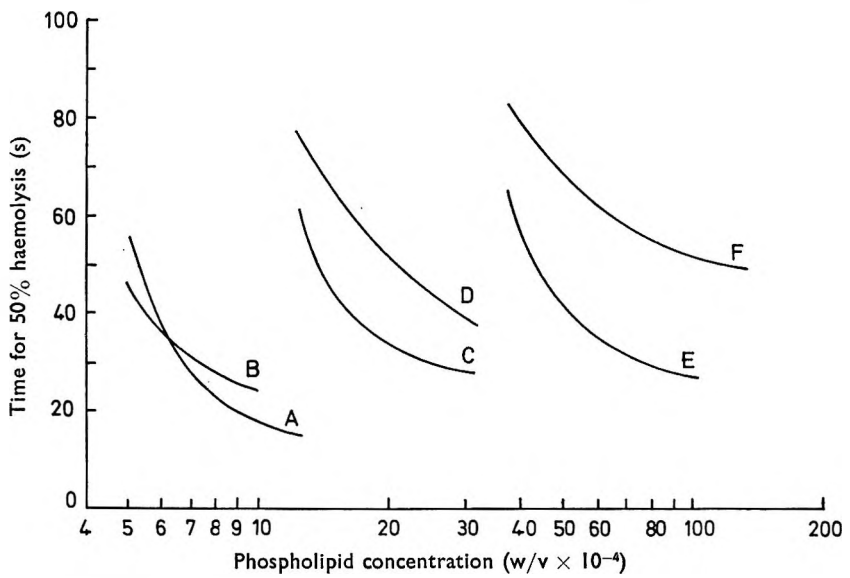


FIG. 2. Haemolysis–concentration graphs. A = LPC. B = LPC/progesterone sol. C = LPC/PC sol. D = LPC/PC/progesterone sol. E = LPC/progesterone/cholesterol sol. F = LPC/PC/triolein sol.

Cholesterol co-dispersed with progesterone by LPC, greatly inhibited the lytic activity of the LPC, and with the mixed phospholipid sol was sufficient to prevent haemolysis of the rat erythrocytes. The ability of cholesterol to inhibit the haemolysing activity of LPC was first reported by Minz (1908), who believed the effect to be due to molecular fixation of cholesterol by LPC. A complex is formed between cholesterol and LPC having no haemolytic activity (Delezenne & Fourneau, 1914), but no compound formation takes place.

The LPC/PC/triolein system (equal parts of each by weight) showed a marked reduction in haemolytic activity over the mixed phospholipid system, thus indicating a steric inhibiting capacity of the triolein within the micelle, or a marked reduction in the number of LPC monomers present in the sols.

Kinetic equations were applied to the haemolysis of red blood cells by LPC, but neither first order, two stage first order nor second order kinetics gave satisfactory solutions to the problem.

*Acknowledgement*

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SHORT COMMUNICATION

# The metabolism of thioamides by the supernatant fraction of rat liver homogenate

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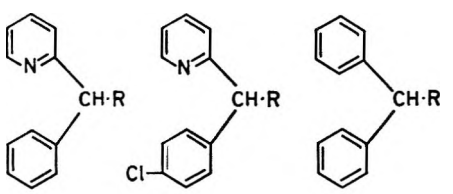
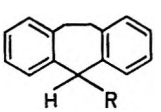
α-Phenyl-α-(2-pyridyl) thioacetamide (SC 15396; antigastrin) is metabolized by the supernatant fraction of rat liver homogenate to give two metabolites. The major metabolite has been identified as α-phenyl-α-(2-pyridyl)acetonitrile. Three other thioamides, analogues of SC 15396 also give the corresponding nitriles in these conditions.

α-Phenyl-α-(2-pyridyl)thioacetamide (SC 15396, antigastrin, G. D. Searle & Co.) (Ia) has been shown to inhibit gastric secretion when administered by the intravenous or oral routes (Gillespie, McCusker & others, 1968; Connell, Sircus & others, 1967). Since little is known about the metabolic fate of thioamides we have examined the effect of incubating SC 15396 and some closely-related thioamides with the supernatant fraction of rat liver homogenate, as a preliminary to examining the fate of the drug *in vivo*.

## EXPERIMENTAL




I II III




IV

a R= −CS·NH$_2$
b R= −CN

*Materials and methods*

The thioamides examined, α-phenyl-α-(2-pyridyl)thioacetamide (Ia), α-(4-chlorophenyl)-α-(2-pyridyl)thioacetamide (IIa), diphenylthioacetamide (IIIa) and 10,11-dihydro-5*H*-dibenzo-(*a*,*d*)-cycloheptene-5-thioamide (IVa) were obtained by courtesy of G. D. Searle & Co. α-Phenyl-α-(2-pyridyl)acetonitrile (Ib) and diphenylacetonitrile (IIIb) were obtained from Ralph N. Emanuel Ltd.

The two other nitriles (IIb) and (IVb) were most conveniently prepared by addition of sodium plumbite solution (10% w/v) to alcoholic solutions of the corresponding thioamides. Lead sulphide was precipitated and the nitriles extracted with ether.

Crystallization from ether/light-petroleum gave the pure nitriles (IIb) m.p. 63–65° (Brocades-Stheeman, 1962) and (IVb) m.p. 153° (Sperber, Papa & others, 1951).

### In vitro *metabolism*

Male Wistar rats, weighing about 300 g were used in all experiments. They were killed, the livers quickly removed, blotted, weighed and homogenized with 2 volumes of isotonic potassium chloride solution at 0–5° in an Ultra-Turax homogenizer (3 × 12 s). The homogenates were centrifuged 30 min at 10,000 *g* to remove cell debris and the supernatant fraction decanted.

To the supernatant (2·0 ml), in a 25 ml incubation flask there was added NADP (1mg), glucose-6-phosphate (5 mg), magnesium chloride (20 μmol), nicotinamide (60 μmol) and SC 15396 (1a) (10 μmol) as a fine suspension in Sørensen phosphate buffer (pH 7·4) to make a total volume of 7 ml. Control experiments were conducted from which the substrate was omitted.

To eliminate any possibility of purely chemical degradation of the drugs, some control experiments used liver supernatant, denatured by being heated at 65–75° for 45 min followed by further homogenization for 1 min.

The flasks were incubated 1·5 h at 37° in a shaking incubator, cooled and extracted three times with peroxide-free ether (3 × 8·0 ml). The ether extracts were evaporated to dryness at 37° and the residue dissolved in benzene (0·2 ml). The benzene extracts were chromatographed on 20 × 5 cm silica gel plates (Merck G, 250μm) by ascending chromatography with the three solvent systems ether, ether–formic acid (15 : 1 v/v) and methylene chloride. Extracts from blank control flasks and from flasks containing denatured supernatant fraction, co-factors and substrate were chromatographed at the same time. Spots due to metabolites and, where appropriate, the original materials were located by their fluorescence under ultraviolet light and by exposing the plates to iodine vapour. Results are shown in Table 1.

The large scale separation of the metabolites of SC 15396 (Ia) was conducted with the supernatant fraction from six livers (102 g) and the appropriate quantities of co-factors and substrate. The extracts were chromatographed on large (50 × 20 cm) thin-layer plates using ether as solvent.

The metabolite was eluted from the appropriate portion of the plate with methylene chloride, the solution evaporated *in vacuo*, and the residue dissolved in spectroscopic chloroform (0·2 ml.).

The infrared spectrum of this solution was determined on a Unicam SP 100 spectrophotometer in a 0·5 mm cell and compared with that of an authentic sample of (Ib) (200 mg) chromatographed and extracted in an identical manner.

## RESULTS

The chromatographic separation of the metabolites is shown in Table 1. Spots due to substrate and to materials extracted from the supernatant fraction can be identified by comparison of the control extracts with those containing substrate and active supernatant fraction. Apart from a small amount of material on the base-line in most cases, the main components extracted after incubation are the thioamides and the derived nitriles.

For SC 15396 (Ia) the infrared spectrum of the metabolite was identical with that of an authentic sample of the nitrile (Ib). No spot corresponding to this nitrile was present either in blank experiments or in those using denatured liver fractions.

Table 1. *Rf values of the thioamides, their metabolites and corresponding nitriles*

| Experiment | Solvent systems I | II | III |
|---|---|---|---|
| Metabolism of Ia | 0·40†, 0·56, 0·9 | 0·34, 0·74 | — |
| Control* | 0·38 | 0·34 | — |
| Ia | 0·40 | 0·28 | — |
| Ib | 0·55 | 0·74 | — |
| Metabolism of IIa | 0·39, 0·59† | 0·40, 0·78 | — |
| Control* | 0·39 | 0·42 | — |
| IIa | 0·38 | 0·36 | — |
| IIb | 0·57 | 0·79 | — |
| Metabolism of IIIa | 0·60, 0·72 | — | 0·28, 0·61† |
| Control* | 0·57 | — | — |
| IIIa | 0·62 | — | 0·26 |
| IIIb | 0·74 | — | 0·60 |
| Metabolism of IVa | 0·60, 0·75† | — | 0·32, 0·68 |
| Control* | 0·59 | — | — |
| IVa | 0·62 | — | 0·31 |
| IVb | 0·77 | — | 0·67 |

All results represent values from 6 repeat experiments.
* Denatured liver fraction, co-factors and drugs.
† A very small additional spot at Rf $< 0{\cdot}05$.
Solvent systems: I peroxide-free ether; II ether–formic acid 15:1 v/v; III methylene chloride.

## DISCUSSION

There are few previous reports of the metabolic fate of thioamides. Ethionamide (2-ethylisonicotinic acid thioamide) and its sulphoxide are excreted by man as 2-ethylisonicotinamide and 2-ethylisonicotinic acid (Bieder & Mazeau, 1964; Bieder, Brunel & Mazeau, 1966). The same metabolites are reported by Johnston, Kane & Kibby (1967) after administration of ethionamide to rats and mice.

2,6-Dichlorothiobenzamide (Prefix) however, in rats and dogs, is metabolized to the extent of about 40%, to 2,6-dichloro-3-hydroxybenzonitrile (Griffiths, Moss & others 1966), and the pattern of metabolic products is the same from the thioamide or the nitrile whichever is administered.

All four compounds examined in the present investigation are metabolized in the same way *in vitro*; simple hydrolysis of the thioamide or replacement of the sulphur by oxygen can represent only a minor pathway in these conditions. These metabolites can be detected as base-line spots on TLC plates but only traces of base-line materials were produced from extracts of the thioamide metabolic products.

These observations are interesting because of the unexpected course of the metabolic process. The only previous report of this nature, that of Griffiths & others (1966), concerned 2,6-dichlorothiobenzamide, a compound for which the more obvious hydrolytic pathway, would be hindered by the presence of substituents *ortho* to the thioamide group. It is conceivable therefore that in this case, oxidative removal of $H_2S$ is favoured. In the series of compounds we have used however, this explanation is untenable since all the thioamides may readily be hydrolysed.

It seems likely therefore that the formation of nitrile is an oxidative process, mediated by the oxidising enzyme system in the microsomal fraction of rat liver homogenate and further work is in hand to verify this. Preliminary work reported here suggests this may be true since inactivation of the microsomal enzyme system led to recovery of the thioamides unchanged.

The other significant feature is the possibility that a chemically reactive nitrile can be produced, *in vivo*, by the operation of a detoxication process.

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# The preparation and pharmacological properties of ψ-corbasil

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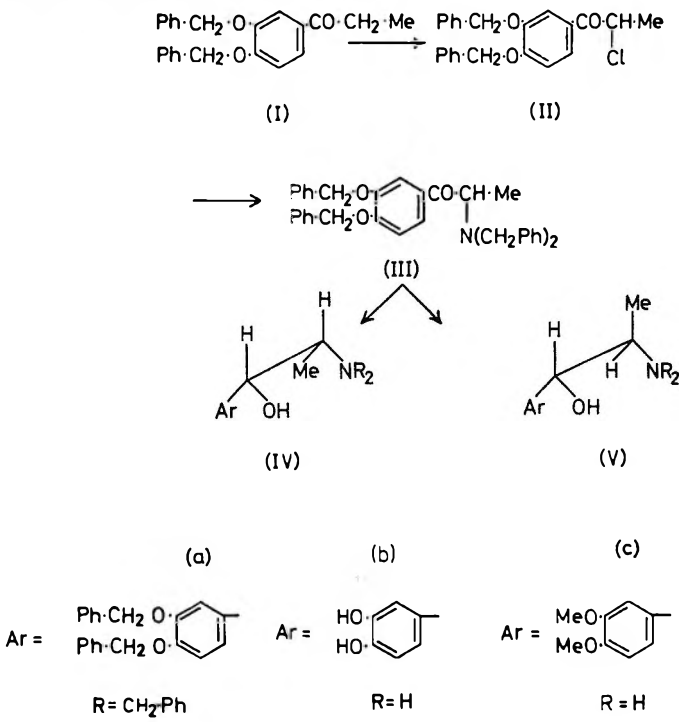
The synthesis is described of *threo*-α-methyl noradrenaline (ψ-corbasil). The assignments of the *erythro* and *threo* configurations to corbasil and ψ-corbasil respectively are supported by the evidence of the nmr spectra. Some preliminary results are reported for the relative pharmacological activity of the two isomers at α- and β-adrenergic receptors.

The sympathomimetic amine, 2(3',4'-dihydroxyphenyl)-2-hydroxyisopropylamine (α-methylnoradrenaline; corbasil) has been described (Bruckner & Fodor, 1943) and its pharmacological properties examined (Luduena, Hoppe & others, 1958). Since corbasil has two asymmetric centres it can exist as two pairs of racemic mixtures. The (±)-*erythro* pair, corbasil, has been resolved and each epimer examined pharmacologially (Luduena, Euler & others, 1957; Luduena & others, 1958).

The corresponding *threo* pair, ψ-corbasil, has not been prepared although Waldeck (1968) reported that treatment of the *erythro* compound, corbasil, with hot acid gave the *threo* isomer, characterized by its non-identity with corbasil and by its fluorescence spectrum which differed from that of corbasil.



In our hands this method gave a dark-coloured material of variable composition consisting mainly of oxidation products of corbasil.

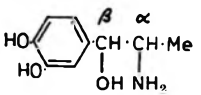
We now report the synthesis of authentic $\psi$-corbasil and its configurational assignment to the *threo* series.

The dibenzyl ether (I) was converted to the chloroketone (II) with sulphuryl chloride, a procedure that avoided the disproportionation which occurs on direct treatment of I with halogens. Treatment of II with dibenzylamine in acetone gave the tertiary amine (III) which served as starting material for preparation of the *threo* and *erythro* alcohols (IV) and (V).

Catalytic reduction of III gave the *erythro* compound (IVb) by simultaneous reduction of the carbonyl group and hydrogenolysis of all four benzyl groups to yield (±)-corbasil.

Reduction of the carbonyl group of the aminoketone (III) without affecting the benzyl groups, was achieved with lithum aluminium hydride in ether when (±)-*threo*-2-(3′,4′-dibenzyloxyphenyl)-2-hydroxy-*NN*-dibenzyl isopropylamine (Va) was obtained in high yield. Catalytic debenzylation gave Vb as the free base forming a stable hemihydrate whose composition was verified by analysis and by the broad background absorptions at 3500–2400 and 1800–1000 $cm^{-1}$, in the infrared spectrum. These bands are characteristic of compounds containing traces of water.

Table 1. *Nmr spectra of corbasil and $\psi$-corbasil*, 10% *w/v in trifluoroacetic acid*



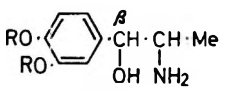

| Isomer | Group | τ (ppm) | Integral | Multiplicity | J (Hz) |
|---|---|---|---|---|---|
| Corbasil | Aromatic H | 3·05 | 3 | Multiplet | – |
| | α-H | 6·08 | 1 | Multiplet | – |
| | α-Me | 8·44 | 3 | Doublet | 6 |
| | β-H | 4·92 | 1 | Doublet | 5 |
| | OH, $NH_3$ | 3·15 | 6 | Singlet | – |
| $\psi$-Corbasil | Aromatic H | 3·02 | 3 | Multiplet | – |
| | α-H | 6·02 | 1 | Multiplet | – |
| | α-Me | 8·58 | 3 | Doublet | 7 |
| | β-H | 5·45 | 1 | Doublet | 7·5 |
| | OH, $NH_3$ | 2·54 | 6 | Singlet | – |

The configurational assignments for corbasil and $\psi$-corbasil are supported by the nmr spectral data shown in Table 1. Due to the insolubility of the compounds in the usual solvents the spectra were determined in trifluoroacetic acid. The dimethyl ethers of corbasil (IVc) and of $\psi$-corbasil (Vc) were therefore prepared. Details of the spectra of these in deuterochloroform and deuterium oxide are given in Table 2.

Comparison of the spectra of the *erythro* and *threo* pairs of the corbasils (IVb and Vb) and of their methyl ethers (IVc and Vc) shows that their only point of difference lies in the chemical shifts of the β-CH doublets, that for the *erythro* isomer lying downfield from the corresponding signal for the *threo* isomers. The coupling constant of the *erythro* isomer is also smaller than that of the *threo* isomer (Table 2). Similar findings have been reported for the ephedrine isomers (Hyne, 1961; Lyle & Keefer, 1966), as well as for the isomers of 2-(2-dimethylaminomethylphenyl)-1,2-diphenylethanol (Randall, Vaulx & others 1965). Hyne (1961) reported

the β-proton doublet of ephedrine (*erythro*) as 55 Hz downfield of the corresponding signal in ψ-ephedrine (*threo*) but apart from this the spectra were almost identical. For the pair corbasil/ψ-corbasil, this change in chemical shift is 32 Hz in the same direction (solvent-trifluoroacetic acid) (Table 2). For the free bases and hydrochlorides of the methyl ethers, the shifts, again in the same direction, are 16 Hz and 17 Hz in deuterochloroform and deuterium oxide respectively (Table 2).

Table 2. *Comparison of chemical shifts and coupling constants of β-proton for* threo *and* erythro *isomers*




| R | Solvent | τ(*threo*) | τ(*erythro*) | $\tau_T - \tau_E$ | $J_T$ | $J_E$ |
|---|---|---|---|---|---|---|
| H | T.F.A. | 5·45 | 4·92 | 0·53 | 7·5 | 5 |
| Me | $CDCl_3$ | 5·90 | 5·64 | 0·26 | 7 | 6 |
| Me | $D_2O$ | 5·30 | 5·02 | 0·28 | 10 | 4·5 |

The coupling constant for the α–β protons is also different in the *erythro* and *threo* isomers, the value for the *erythro* compound being smaller than that of the *threo* isomer in every case (Table 2). Similar relations have been reported by several authors for *threo/erythro* pairs of a variety of compounds (Hyne, 1961; Randall, 1965, Kingsbury & Best, 1967). For the *erythro* isomers of corbasil (IVb), the dimethylether (IVc) and its hydrochloride, $J_{\alpha\beta}$ = 7–10 Hz whereas for the corresponding *threo* isomers Vb, Vc and Vc hydrochloride $J_{\alpha\beta}$ = 4·6—6 Hz (Table 2).

These values serve to distinguish the *erythro* and *threo* compounds from each other.

## PHARMACOLOGICAL ACTIVITY

Muscholl & Lindmar (1967) reported that corbasil and ψ-corbasil cannot be distinguished from each other on the basis of their uptake, storage or release at adrenergic sites, whilst Waldeck (1967) observed the *threo* isomer to have slightly lower affinity for storage sites than the *erythro* isomer. Thus the relative potency of the two isomers at adrenergic receptors should be a measure of their direct effect at the receptor and should not arise from differences in their ability to release endogenous catecholamines.

(±)-Corbasil and (±)-ψ-corbasil were compared with noradrenaline for α-stimulant potency on the vasopressor response in the spinal rat and as β-stimulants in guinea-pig isolated atria. Results are shown in Fig. 1, a–d.

Fig. 1a shows the dose-response curves for (±)-corbasil and (−)-noradrenaline as α-stimulants. The maximum response to corbasil (at about 40 μg) is less than that due to noradrenaline. The corresponding results for ψ-corbasil are shown in Fig. 1b. The maximum response to ψ-corbasil (at about 200 μg) is lower than that due to corbasil and much lower than that due to noradrenaline. The results indicate that both corbasil and ψ-corbasil have a lower affinity for α-receptors than has noradrenaline.

Fig. 1c, shows the comparison of (±)-corbasil with (−)-noradrenaline at β-receptors. The β-stimulation of corbasil is positive but is much less than that due to noradrenaline whilst the maximum response to corbasil (at 25 μg/ml) is attained at a lower dose than that to noradrenaline; this suggests that corbasil may have a

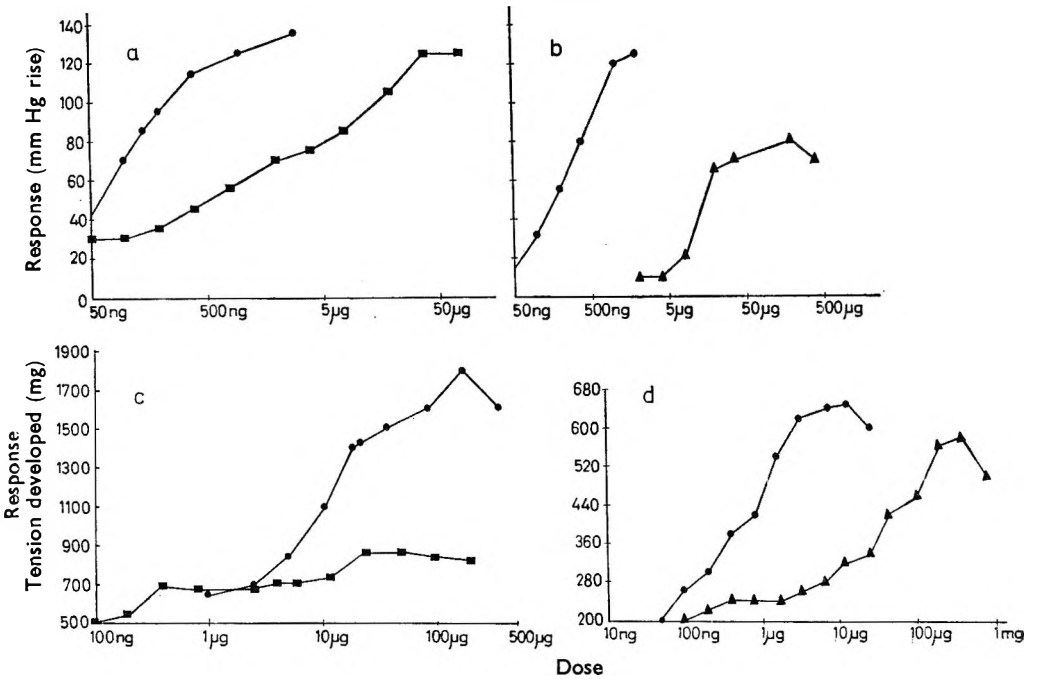



FIG. 1. Dose response curves for corbasil (■), noradrenaline (●) and ψ-corbasil (▲) on the spinal rat (α-stimulation; a, b) and guinea-pig isolated atria (β-stimulation; c, d).

greater affinity for β-receptors than has noradrenaline even though it has a lower activity. Since the dose - response curves are not parallel it is not possible to calculate a meaningful ratio for the potency.

The results for β-stimulation by ψ-corbasil are shown in Fig. 1d, the maximum response occurs at about 400 μg/ml. ψ-Corbasil therefore appears to have less affinity than noradrenaline for β-receptors although its activity at the receptor is comparable with that of noradrenaline.

Since corbasil and ψ-corbasil displace endogenous catecholamines to the same extent, the difference in the α- and β-stimulant activity of these two drugs must therefore be due to the differences in their activity at the receptor sites. The relatively high β-stimulation of ψ-corbasil compared with corbasil might possibly contribute to the smaller vasopressor effect of ψ-corbasil (Fig. 1a and b) for the result of β-stimulation is vasodilatation which would oppose the vasoconstriction due to α-stimulation.

Further work is in progress to prepare the two optical isomers of ψ-corbasil and to study their pharmacological action in the presence of α- and β-blocking agents.

## EXPERIMENTAL

Infra-red spectra were recorded as Nujol mulls on a Unicam SP200 spectrophotometer.

Nmr spectra were determined on a Perkin–Elmer R 10 instrument, operating at 60 mHz.

Melting points are uncorrected.

3,4-*Dibenzyloxy-α-chloropropiophenone* (*II*). A solution of sulphuryl chloride (10 ml) in methylene chloride (20 ml) was added dropwise to a stirred solution of 3,4-dibenzyloxypropiophenone (Bockmöhl, 1934) (40 g) in methylene chloride (100 ml). The mixture was stirred at room temperature until no hydrogen chloride or sulphuryl chloride could be detected by starch–KI paper. Evaporation under reduced pressure at room temperature gave a pale yellow oil which crystallized from cyclohexane - light petroleum (b.p. 40–60°) to give 3,4-*dibenzyloxy-α-chloropropiophenone* as colourless crystals (31 g, 75%) m.p. 65·5–67°. Found: C, 72·8; H, 5·2; Cl, 9·4%. $C_{23}H_{21}ClO_3$ requires: C, 72·5; H, 5·6; Cl, 9·3%. Nmr spectral details are in Table 3.

(±)-3,4-*Dibenzyloxydibenzylaminopropiophenone* (*III*). Dibenzylamine (25 ml) was added to 3,4-dibenzyloxy-α-chloropropiophenone (25 g) and potassium iodide (0·5 g) in acetone (150 ml). After 3½ days at 10°, ether was added to complete the precipitation of dibenzylamine hydrochloride, and this, together with the potassium iodide, was filtered off: solvent was evaporated under reduced pressure. The residual brown oil was examined by thin-layer chromatography on silica gel, using chloroform as solvent and shown to consist of dibenzylamine (Rf 0·2) and a second component of Rf 0·9. The residual oil in benzene (80 ml) was passed through a column of alumina (125 g type H) in benzene. Elution with benzene (600 ml) was monitored by thin-layer chromatography and gave one component only. This was recrystallized three times from methanol to give (±)-3,4-dibenzyloxydibenzylaminopropiophenone as colourless crystals (23 g, 65%), m.p. 78–80° (Bochmöhl (1934) gives m.p. 84–86°). Found: C, 81·4; H, 6·5; N, 3·2%. Calc. for $C_{37}H_{35}NO_3$: C, 82·0; H, 6·5; N, 2·6%. Nmr spectral details are in Table 3.

(±)-threo-2-(3′,4′-*Dibenzyloxyphenyl*)-2-*hydroxy*-N-*dibenzylisopropylamine* (*Va*). A solution of (±)-3,4-dibenzyloxydibenzylaminopropiophenone (1 g) in dry ether (40 ml) was added dropwise to a stirred suspension of lithium aluminium hydride (100 mg) in dry ether (10 ml). The reaction mixture was then refluxed gently (1 h). After cooling, excess reagent was decomposed cautiously with water (2 ml). The mixture was allowed to stand for a short time and then filtered through kieselguhr. The ethereal layer of the filtrate was dried ($Na_2SO_4$) and the solvent evaporated. The yellow residual oil crystallized from methanol to give (±)-threo-2-(3′,4′-*dibenzyloxyphenyl*)-2-*hydroxy*-N-*dibenzylisopropylamine* off-white crystals (0·82 g, 81%), m.p. 96–97°. Found: C, 81·3; H, 7·1; N, 2·6%. Calc. for $C_{37}H_{37}NO_3$: C, 81·7; H, 6·9; N, 2·6%. Nmr spectral details are in Table 3.

(±)-threo-2-(3′,4′-*Dihydroxyphenyl*)-2-*hydroxyisopropylamine* (*Vb*) (*ψ-corbasil*). Concentrated hydrochloric acid (1·9 ml) was added to a suspension of (±)-*threo*-2-(3′,4′-dibenzyloxyphenyl)-2-hydroxy-*N*-dibenzylisopropylamine (10 g) in 96% ethanol (250 ml). The mixture was warmed gently to yield a slightly acid solution and when cool this was hydrogenated over 10% palladium-charcoal (2 g) at room temperature until uptake ceased. The catalyst was filtered off and the solvent evaporated under reduced pressure. The oily residue in water (5 ml) was basified with conc. ammonia. The mixture, which darkened rapidly, was scratched and the solid which slowly precipitated filtered off and washed with a little water. Recrystallization from water gave (±)-threo-2-(3′,4′-*dihydroxyphenol*)-2-*hydroxyisopropylamine* as a light buff powder (1·37 g, 40%), m.p. 112–114° (decomp.). Found: C, 55·2; H, 7·2; N, 7·1%. Calc. for $C_9H_{13}NO_3 \cdot \frac{1}{2}H_2O$: C, 56·2; H, 7·3; N, 7·3%. The nmr spectral details are shown in Table 1.

(±)-erythro-2-(3′,4′-*Dimethoxyphenyl*)-2-*hydroxyisopropylamine* (*IVc*). Dry hydrogen chloride was passed through a solution of (±)-*erythro*-2-(3′,4′-dihydroxyphenyl)-2-hydroxyisopropylamine in dry methanol (10 ml) until solution was complete. Nitrogen was passed to remove excess hydrogen chloride, and the solution was kept under nitrogen, to prevent atmospheric oxidation. A chilled solution of diazomethane (1·03 g, 10·5 mol) in ether was added quickly portionwise to the chilled solution of amine hydrochloride and the reaction mixture, securely stoppered to prevent access of atmospheric moisture, was allowed to stand (16 h) at 0° then 24 h at 20°. Evaporation of solvent gave a brown crystalline residue which was refluxed in benzene and filtered. Evaporation gave a sticky yellow solid (0·48 g), m.p. 117–124°, whose methanolic solution gave no green colour with $FeCl_3$.

Table 3. *Nmr spectra of compounds II, III, IVc, Va and Vc*

| | Group | τ | Integral | Multiplicity | J value (Hz) |
|---|---|---|---|---|---|
| Compound II (10% w/v in $CDCl_3$) | Aromatic hydrogen | 2·70 | 13 | Multiplet | — |
| | Benzyl $CH_2$ | 4·85 | 4 | Doublet | — |
| | Side-chain CH | 4·90 | 1 | Quartet | 6 |
| | Side-chain $CH_3$ | 8·35 | 3 | Doublet | 6 |
| Compound III (6% w/v in $CDCl_3$) | Phenyl aromatic hydrogen | 2·68 | 23 | Multiplet | — |
| | Benzyl aromatic hydrogen | 2·84 | | Singlet | — |
| | Benzyloxy $CH_2$ | 4·90 | 4 | Doublet | 9 |
| | Side-chain CH | 5·74 | 1 | Quartet | 6 |
| | *N*-Benzyl $CH_2$ | 6·40 | 4 | Singlet | — |
| | Side-chain $CH_3$ | 8·70 | 3 | Doublet | 6 |
| Compound IVc hydrochloride (12·5% w/v in $D_2O$) | Aromatic hydrogen | 2·95 | 3 | Multiplet | — |
| | Side-chain βCH | 5·02 | 1 | Doublet | 4½ |
| | Aromatic methoxy | 6·12 | 6 | Doublet | — |
| | Side-chain αCH | 6·28 | 1 | Octet | αH–βH 4·5<br>αH–$CH_3$ 7·5 |
| | Side-chain α$CH_3$ | 8·74 | 5 | Singlet | — |
| | OH, $NH_3^+$ | 5·25 | 5 | Singlet | — |
| Compound IVc free base (7% w/v in $CDCl_3$) | Aromatic hydrogen | 3·20 | 3 | Multiplet | — |
| | Side-chain βCH | 5·64 | 1 | Doublet | 6 |
| | Aromatic methoxy | 6·16 | 6 | Singlet | — |
| | Side-chain αCH | 6·94 | 1 | Octet | αH–βH 6<br>αH-$CH_3$ 6 |
| | Side-chain α-$CH_3$ | 9·04 | 3 | Doublet | 6 |
| | OH, $NH_2$ | 7·96 | 3 | Singlet | — |
| Compound Va (8% w/v in $CDCl_3$) | Benzyl aromatic hydrogen | 2·72 | 20 | Multiplet | — |
| | Phenyl aromatic hydrogen | 3·35 | 3 | Multiplet | — |
| | Benzyloxy $CH_2$ | 5·00 | 4 | Doublet | 7 |
| | Side-chain βCH | 5·87 | 1 | Doublet | 10 |
| | *N*-benzyl $CH_2$ | 6·40 | 4 | Quartet | 14 |
| | Side-chain α CH | 7·32 | 1 | Octet | αH-βH 10<br>αH-$CH_3$ 7 |
| | Side-chain α $CH_3$ | 9·24 | 3 | Doublet | 7 |
| Compound Vc (7·5% w/v in $CDCl_3$) | Aromatic hydrogen | 3·24 | 3 | Multiplet | — |
| | Side-chain βCH | 5·90 | 1 | Doublet | 7 |
| | Aromatic methoxy | 6·18 | 6 | Singlet | — |
| | Side-chain αCH | 7·05 | 1 | Octet | αH-βH 7<br>αH-$CH_3$ 6 |
| | Side-chain α$CH_3$ | 9·05 | 3 | Doublet | 6 |
| | OH, $NH_2$ | 7·78 | 3 | Singlet | — |

Re-crystallization from benzene-light petroleum (b.p. 40–60°) gave (±)-erythro-2-(3′,4′-*dimethoxyphenyl*)-2-*hydroxyisopropylamine*, as a yellow powder (0·28 g, 49%), m.p. 136–8°. Fodor, Bruckner & others (1949) give m.p. 139° for material prepared by hydrogenation of 3,4-dimethoxy-2′-aminopropiophenone. Nmr spectral details are shown in Table 3.

(±)-threo-2-(3′,4′-*Dimethoxyphenyl*)-2-*hydroxyisopropylamine* (Vc), prepared similarly, had m.p. 128–130°. Pfeiffer, Breitbach & Scholl (1940) give m.p. 128–129°. Nmr spectral details are shown in Table 3.

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