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SCIENCE PAPERS

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Science Papers

SHORT COMMUNICATION

The effect of ultrasonic energy on the aqueous extraction of senna pericarps

J. C. MORRISON AND R. WOODFORD

THE use of ultrasonic energy in the extraction of active constituents from plant material has been investigated by several workers (Adamski & Mizgalski, 1957; Kubiak, 1962, 1963a, b, 1964; De Maggio & Lott, 1964; Ovadia & Skauen, 1965) but little attempt has been made to investigate the various factors involved.

The effect of ultrasonic irradiation on the aqueous extraction of anthraquinone glycosides and free anthraquinone derivatives from senna pericarps was investigated since (i) both active and inactive constituents of this thin-walled tissue are extracted during maceration in water (ii) the active constituents are susceptible to hydrolysis and oxidation, and (iii) a method of assay for both free and combined compounds is available.

When an aqueous suspension of whole Alexandrian pericarps is insonated for up to 90 min there is an apparent increase in the amount of free anthraquinones and sennosides extractable by the method of Fairbairn & Michaels (1950). The increase over a similar sample not thus insonated is 200% of free anthraquinones and 20% of sennosides.

Over a 90 min period of ultrasonic irradiation the temperature of the irradiated suspension rose from 20° to approximately 50° and this rise in temperature accounts for about 70% of the above increase in extractable compounds. For the rest this is ascribed to a specific effect of ultrasonic energy.

Free radical formation is known to occur during insonation (Weissler, 1959) and in the present experiments hydrogen peroxide was formed in concentrations of $1{\cdot}10 \times 10^{-6}$ moles/litre (30 min) and $7{\cdot}00 \times 10^{-6}$ moles/litre (90 min) when purified water was insonated. The extraction rate of sennosides and free anthraquinones was not affected by adding up to fifty-fold these concentrations of peroxide. No change in pH was detected in the insonated extracts.

Examination of the pericarp extracts by paper and thin-layer chromatography showed no chemical degradation resulting from insonation. This was confirmed by ultrasonic irradiation of a prepared extract, when no change in the amount of free anthraquinones or sennosides was detected.

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EXPERIMENTAL

Whole Alexandrian senna pericarps (1 g) were suspended in purified water (100 ml) in a 150 ml glass beaker. The system was left at room temperature for 3 min then subjected to ultrasonic energy (MSE 60 W ultrasonic disintegrator, frequency 20 kc/sec, probe end diameter $\frac{3}{8}$ inch) for a further 90 min, the solvent volume being maintained constant throughout. The temperature rise was noted and the experiment repeated

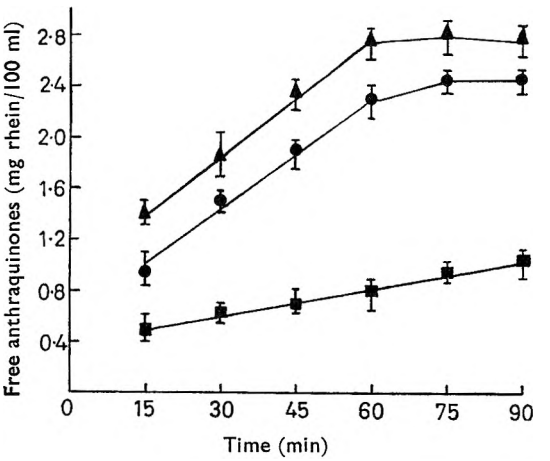

FIG. 1. Effect of ultrasonic energy and controlled heating on the extraction of free anthraquinones in aqueous extracts of senna pericarps. ■, Room temperature; ●, controlled heating; ▲, ultrasonic energy. Symbols indicate mean of 3 experiments and vertical lines indicate variation in 3 experiments.

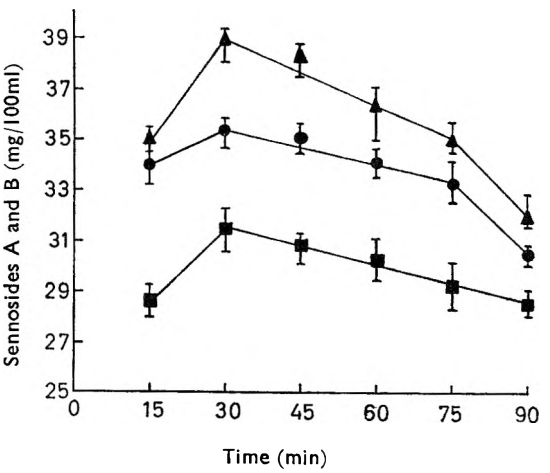

FIG. 2. Effect of ultrasonic energy and controlled heating on the extraction of sennosides A and B in aqueous extracts of senna pericarps. ■, Room temperature; ●, controlled heating; ▲, ultrasonic energy. Symbols indicate mean of 3 experiments and vertical lines indicate variation in 3 experiments.

omitting only the irradiation with ultrasonic energy. In each experiment 10 ml samples were withdrawn after 15, 30, 45, 60, 75 and 90 min and the content of free anthraquinones and sennosides A and B determined by the method of Fairbairn & Michaels (1950). The 10 ml volume removed at each time interval was replaced by 10 ml of purified water.

All experiments were made in triplicate and the results are shown in Figs 1 and 2.

The hydrogen peroxide content of purified water insonated as described above was determined by the method of Ovenston & Rees (1950).

Acknowledgement. The authors wish to thank Professor J. W. Fairbairn for samples of free anthraquinones.

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SHORT COMMUNICATION

Some effects of ampicillin on growing and glucose-starved cells of *Escherichia coli*

R. M. RYE AND DAVID WISEMAN

AMPICILLIN (α-aminobenzylpenicillin) is a semisynthetic penicillin with a relatively high activity against Gram-negative organisms. Some of its effects on *Escherichia coli* have been previously described (Boman & Eriksson, 1963). This communication reports the results of investigations into its effects on the growth, division and viability of cells of *E. coli*.

EXPERIMENTAL

Escherichia coli (NCTC 1013) was used. The growth medium, conditions of culture and methods for measuring radioactivity and absorbance have been described previously (Rye & Wiseman, 1966). Freshly prepared solutions of ampicillin in glucose-free medium were added to the cell suspensions to produce the required concentrations.

Viable counts were made by the pour-plate method using tryptone-soya broth in the dilutions and tryptone-soya agar as the plating medium. 1% "Wellcome" penicillinase was included in both the tryptone-soya broth and the agar. Colonies were counted after 24 hr incubation at 37°.

Total counts and size (volume) distributions were determined using a Coulter model B electronic particle counter fitted with a 30 μ orifice tube calibrated with polystyrene beads of 0·796 and 1·305 μ diameter. The electrolyte solution used consisted of 0·85% sodium chloride, 0·2% formaldehyde and 0·1% trihydroxymethylaminomethane and was adjusted to pH 7·5 by the addition of hydrochloric acid. This solution was freshly prepared and filtered through two membrane filters of 0·45 and 0·2 μ mean pore diameter. Samples of the bacterial suspensions were diluted with the electrolyte solution to produce a final cell concentration of about 6×10^5/ml for counting and size analysis. Total cell counts were obtained by counting all particles with the lower threshold set at the minimum possible value above the electronic noise and were corrected by subtracting the background counts obtained when using the electrolyte solution alone. Counts were also made with the lower threshold set at values corresponding to known particle volumes and from these counts the number of cells within each size range was calculated.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of different concentrations of ampicillin on the absorbance of logarithmically growing cells of *E. coli* and Table 1 shows the total count of these suspensions 25 and 120 min after the addition of ampicillin. Concentrations of ampicillin of 5 μg/ml or more caused lysis

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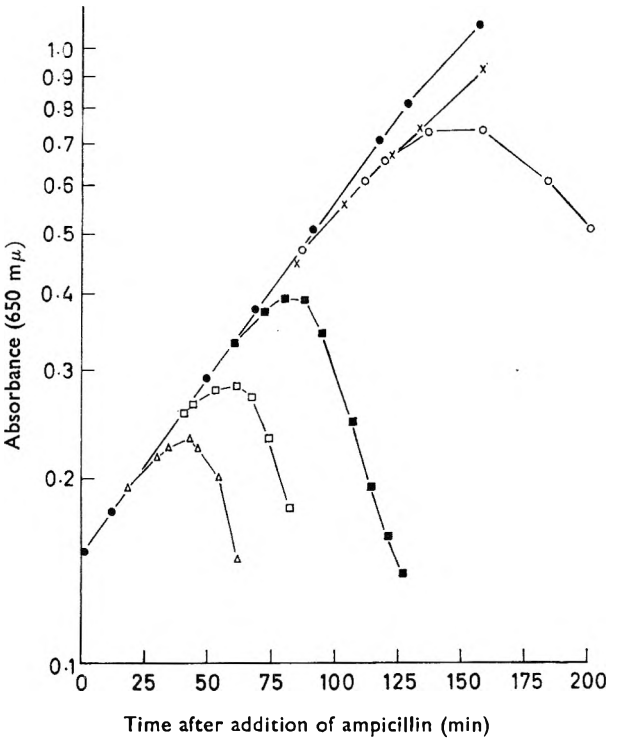

FIG. 1. The effect of ampicillin on the absorbance of logarithmically growing cells of *E. coli* at 37°. Ampicillin concentrations: ●, 0 μg/ml; ×, 2 μg/ml; ○, 5 μg/ml; ■, 10 μg/ml; □, 20 μg/ml; △, 50 μg/ml.

of the cells. This occurred after an interval of time which depended on the concentration of ampicillin and was indicated by a decrease in the absorbance and an increase in the noise level observed when using the Coulter Counter. The total cell counts in the suspensions containing ampicillin increased slowly during the first 15 min and then remained constant at the levels given in Table 1 for at least 2 hr or until lysis occurred.

TABLE 1. THE TOTAL CELL COUNT IN SUSPENSIONS OF *E. coli* AFTER 25 AND 120 MIN TREATMENT WITH AMPICILLIN

	Total cell count/ml × 10^{-8} at ampicillin concentration, μg/ml					
Time	0	2	5	10	20	50
0 min	1·42					
25 min	1·79	1·56	1·55	1·51	1·52	1·51
120 min	>7·5	1·56	1·57	—	—	—

Viable counts were made on suspensions containing 5 and 20 μg/ml of ampicillin. With the lower concentration, during the period of increase in absorbance before lysis, the viable count remained virtually constant; the onset of lysis was paralleled by a decrease in viability. With 20 μg/ml of ampicillin the viability decreased rapidly before lysis commenced.

The effect of ampicillin on non-growing bacterial suspensions was studied by harvesting logarithmically-growing cells by membrane filtration and suspending them at 37° in glucose-free medium containing [^{32}P] labelled phosphate (specific activity 50 μc/mM). Ampicillin (10 μg/ml) was added after maintaining these cells at 37° for 1 hr and glucose (1 mg/ml) after a further hr. The changes in absorbance, viability and total cell count of this suspension are shown in Fig. 2 and are compared with

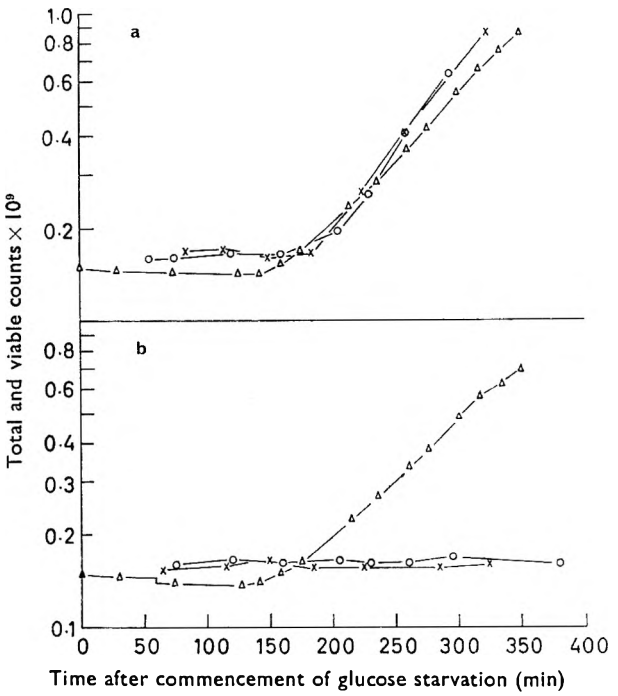

FIG. 2. Changes in the absorbance, viability and total cell counts of suspensions of *E. coli* in glucose-free medium at 37° before and after the addition of glucose, (a) in the absence and (b) in the presence of ampicillin. Ampicillin (10 μg/ml) was added after 60 min in (b) and glucose (1 mg/ml) after 120 min in both (*a*) and (b). △, absorbance; ×, viable count; ○, total cell count.

an identical suspension without added ampicillin. No change in the total or viable cell counts occurred in the presence of ampicillin either during glucose starvation or during the first 4 hr after the addition of glucose. The absorbance of this suspension however increased at a rate only slightly slower than that observed in the untreated suspension where cell numbers increased exponentially after the addition of glucose. The size (volume) distributions of both suspensions were determined during the recovery period and are shown in Table 2. The ampicillin-treated cells increased considerably in size: microscopical examination showed that this was due to an increase in cell length with many filaments greater than 25 μ in length being observed. The uptake of phosphorus and its incorporation into the "cold trichloroacetic acid insoluble" cell fractions

TABLE 2. THE PERCENTAGE OF CELLS OF *E. coli* FALLING WITHIN DIFFERENT SIZE RANGES AT VARIOUS TIMES DURING RECOVERY FROM GLUCOSE STARVATION (A) IN THE ABSENCE AND (B) IN THE PRESENCE OF AMPICILLIN

Time from commencement of glucose starvation	Size range (μ^3)						
	0–0·5	0·5–1·0	1·0–1·5	1·5–2·0	above 2·0		
(a) Untreated cells							
120 min	8·4	67·0	17·3	5·7	1·6		
160 min	6·2	64·8	22·6	4·6	1·9		
205 min	2·2	62·8	25·3	6·8	2·9		
230 min	2·3	66·9	23·5	5·1	2·3		
260 min	5·5	67·4	20·9	4·3	2·0		
	0–0·5	0·5–1·0	1·0–1·5	1·5–2·0	2·0–3·0	3·0–4·0	above 4·0
(b) Ampicillin treated cells							
120 min	6·2	68·5	20·3	3·8	1·2	—	—
160 min	4·9	64·6	23·2	5·6	2·0	—	—
205 min	1·5	49·9	32·0	11·1	5·5	—	—
230 min	0·5	31·0	40·2	13·8	14·6	—	—
260 min	0·6	15·7	34·4	21·7	15·5	11·1	—
300 min	0·1	9·2	15·4	20·4	27·3	13·1	14·6
	0–2·0	2·0–4·0	4·0–6·0	6·0–8·0	8·0–10·0	10·0–12·0	above 12·0
380 min	12·2	26·7	28·7	15·0	8·4	5·3	3·7

The above size distributions were obtained from the suspensions described in Fig. 2. Ampicillin 10 μg/ml) was added after 60 min in (b) and glucose (1 mg/ml) after 120 min in both (a) and (b).

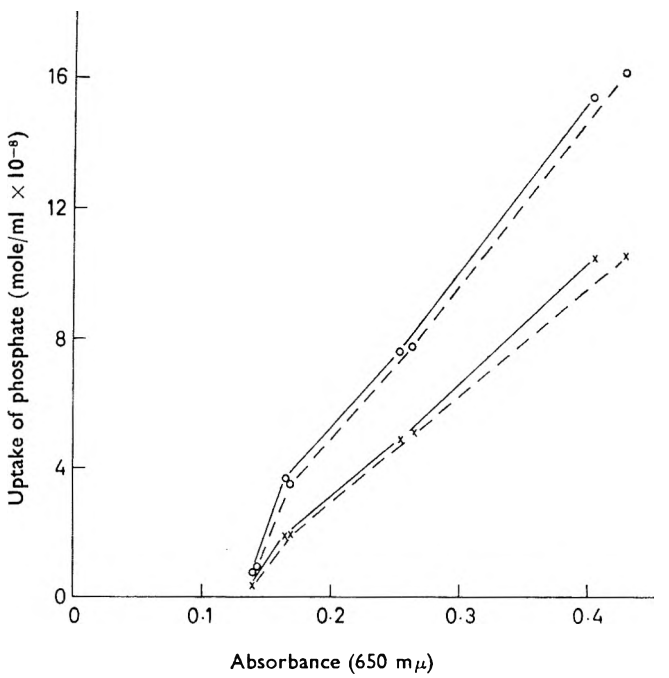

FIG. 3. The relationship between phosphate utilization and increase in absorbance in suspensions of *E. coli* recovering from glucose starvation at 37° (a) in the absence, and (*b*) in the presence of ampicillin (10 μg/ml). ○, phosphorus uptake; ×, phosphorus incorporation into the cold trichloroacetic acid insoluble cell fractions. — — — Untreated cells; ——— ampicillin-treated cells.

relative to the increase in absorbance was the same in both the untreated and ampicillin treated cells (Fig. 3). This suggests that ampicillin has little effect upon the synthesis of ribonucleic acid, deoxyribonucleic acid or phospholipids in which compounds most of the cellular phosphorus is found (Roberts, Abelson & others, 1957). No lysis of the cells was observed although the original concentration of ampicillin (10 μg/ml) was sufficient to lyse logarithmically growing cells within 90 min. Inactivation of some of the antibiotic possibly occurred during the 1 hr incubation at 37° with the glucose starved cells. Indeed, Boman & Eriksson (1963) showed a similar decrease in the activity of ampicillin during incubation with cells of *E. coli* in the lag phase of growth.

The development of elongated forms of Gram-negative organisms in cultures treated with penicillin has been described previously, and based on the theory of inhibition of mucopeptide synthesis (Rogers, 1962) a possible explanation of their formation has been suggested. However, after comparing the effect of eight semi-synthetic penicillins with that of benzylpenicillin on the growth and cell division of a species of *Erwinia*, Grula & Grula (1965) reported a partial independence of the growth inhibitory and cell division inhibitory properties of these substances. They suggested that the separation of these two effects cannot be explained on the hypothesis that inhibition of mucopeptide synthesis is the sole action of penicillin. Cole (1965), after reviewing some aspects of bacterial cell wall replication, suggested that the formation of peripheral wall is distinct from, and can proceed apart from, the process of division by crosswall formation even though the two processes may normally occur together.

Our results show that in glucose-starved suspensions, ampicillin prevents the resumption of cellular division which normally occurs after the addition of glucose even at a concentration in which no lysis or loss in viability occurs and when cellular growth (measured turbidimetrically) is almost unaffected. It is probable that a similar and immediate effect upon cellular division occurs with logarithmically growing cells, the slight increase in total count (approximately 9%) simply reflecting the percentage of cells in the culture in which cellular division had already commenced at the time of treatment. The Coulter Counter offers at present the most rapid and convenient method of measuring the early effects of antibiotics upon cellular division.

Acknowledgement. We are grateful to Mrs. S. Kaye for her technical assistance.

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Some factors affecting the sizing and counting of *Escherichia coli* with the Coulter Counter

R. M. RYE AND DAVID WISEMAN

The changes in the number and size of cells of *Escherichia coli* suspended in buffered sodium chloride solutions have been studied using the Coulter Counter. Cells in the exponential growth phase diluted with buffered normal saline continue to divide and also increase in size. The addition of 0·2% formaldehyde to the suspensions causes a slow decrease in cell number and size. Cells harvested by filtration before dilution are smaller than those obtained by dilution alone. Changes in sodium chloride concentration between 0·5 and 3·0% have little effect on the apparent cell size but at higher concentrations a decrease in size is observed.

SINCE Kubitschek (1958) showed that the Coulter electronic particle counter could be used to count and size bacteria, several reports of its use for these purposes have appeared. In all instances the cells were diluted and suspended in an electrolyte solution before size analysis or determination of total count. However, the methods of preparing these dilutions and the composition of the electrolyte solutions used have varied.

This paper describes the changes that occur in the number and size of cells of *Escherichia coli* suspended in electrolyte solutions of various compositions and discusses some of the requirements for the accurate sizing and counting of bacteria with the Coulter Counter.

Experimental

Escherichia coli (NCTC 1013) was used in this investigation.

The conditions of culture, growth medium and method of measuring absorbance have been described previously (Rye & Wiseman, 1966).

Electrolyte solutions consisted of sodium chloride (between 0·5 and 5%) in deionized water buffered with 0·1% trihydroxymethylaminomethane and hydrochloric acid. In some solutions up to 0·2% formaldehyde was included. The electrolyte solutions were filtered through two membrane filters of 0·45 and 0·20 μ mean pore diameter.

Preparation of dilutions. Samples were taken from cultures which had been growing exponentially for at least two generations and when the absorbance was between 0·100 and 0·150. In most experiments the samples were placed directly in the electrolyte solutions to produce a 1 in 200 dilution resulting in suspensions containing approximately 6×10^5 cells/ml. In some instances the growing cells were harvested by membrane filtration and washed with saline before dilution.

Total counts and size analyses were determined using a model B Coulter electronic particle counter fitted with a 30 μ orifice tube. The instrument was calibrated separately for each electrolyte solution with polystyrene beads of 0·796 and 1·305 μ mean diameter. The instrument settings used were those appropriate for the resistance of the electrolyte solutions and were selected so that the modal volume of the cells gave a reading of between 15 and 20 threshold units. Total counts were determined with

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the lower threshold set at the minimum value above the electronic noise and were corrected by subtracting the counts obtained when using the electrolyte solutions alone. The size (volume) distributions were obtained by making counts with a two threshold unit window and with lower threshold settings at two unit intervals. From these results the mode, median and mean volumes, and the coefficients of variation of the distributions were calculated.

Results

ADDITION OF FORMALDEHYDE AND HARVESTING OF CELLS

The changes in the number and size of cells occurring over a period of 3 hr in suspensions prepared by direct dilution of growing cultures with 0·85% sodium chloride electrolyte solution of pH 7·5, with and without 0·2% formaldehyde, are shown in Table 1. In the absence of formaldehyde

TABLE 1. CHANGES WITH TIME IN THE CELL SIZE AND TOTAL CELL COUNT OF SUSPENSIONS OF *E. coli* AT 20° SUSPENDED IN 0·85% SODIUM CHLORIDE BUFFERED AT pH 7·5 IN THE PRESENCE AND ABSENCE OF 0·2% FORMALDEHYDE.
Suspensions prepared by direct dilution of cultures with electrolyte solution.

	Time (min)	Cell size (μ^3)			Coefficient of variation	Change in total count %
		Mean	Median	Mode		
(a) Formaldehyde 0·2% ..	0	0·95	0·87	0·74	0·377	0
	100	0·89	0·83	0·68	0·382	−0·5
	170	0·89	0·81	0·68	0·382	−1·5
(b) No formaldehyde ..	30	1·13	1·01	0·90	0·373	+10·5
	140	1·38	1·24	1·08	0·361	+25·0
	200	1·40	1·29	1·10	0·359	+46·0

both the total count and cell size increased by over 40% during this period but in the presence of formaldehyde these changes were almost eliminated, there being approximately a 2% decrease in cell number and a 7% decrease in cell size.

The results of similar experiments using cells harvested by membrane filtration and washed free of growth medium before dilution are shown in

TABLE 2. CHANGES WITH TIME IN THE CELL SIZE AND TOTAL CELL COUNT OF SUSPENSIONS OF *E. coli* AT 20° SUSPENDED IN 0·85% SODIUM CHLORIDE BUFFERED AT pH 7·5 IN THE PRESENCE AND ABSENCE OF 0·2% FORMALDEHYDE.
Suspensions prepared from cells harvested by membrane filtration. Total counts were made on the suspensions immediately after preparation. The size of the cells before harvesting is also shown.

	Time (min)	Cell size (μ^3)			Coefficient of variation	Change in total count %
		Mean	Median	Mode		
(a) Formaldehyde 0·2% ..	80	0·85	0·78	0·68	0·369	−1·5
	150	0·82	0·75	0·65	0·373	−1·0
	270	0·82	0·74	0·63	0·379	−3·0
(b) No formaldehyde ..	20	0·84	0·77	0·67	0·370	+2·0
	60	0·83	0·76	0·65	0·367	+2·0
	130	0·82	0·75	0·65	0·377	+3·5
	240	0·80	0·74	0·63	0·381	+5·0
(c) Cells before harvesting	—	0·97	0·83	0·72	0·382	—

Table 2. This harvesting procedure resulted in a decrease in cell size of about 12%. In both the absence and presence of 0·2% formaldehyde, the cell size continued to decrease slowly with time and in the absence of formaldehyde this was accompanied by an increase in cell number.

CONCENTRATION OF FORMALDEHYDE

The effect of different formaldehyde concentrations on the changes in cell number occurring over a period of 2 hr in suspensions prepared by direct dilution is shown in Table 3. At both pH 7·5 and pH 9·0 concentrations of formaldehyde of 0·05% or less allowed an increase in cell

TABLE 3. THE TOTAL CELL COUNTS OF SUSPENSIONS OF *E. coli*, EXPRESSED AS A PERCENTAGE OF THE ORIGINAL COUNT, AFTER 2 HR AT 20° IN 0·85% SODIUM CHLORIDE CONTAINING DIFFERENT CONCENTRATIONS OF FORMALDEHYDE.

	Formaldehyde %							
	0	0·002	0·005	0·01	0·02	0·05	0·10	0·20
pH 7·5	142	133	107	105	102	101	99	97·5
pH 9·0	108	106	105	103	102	101	100	99

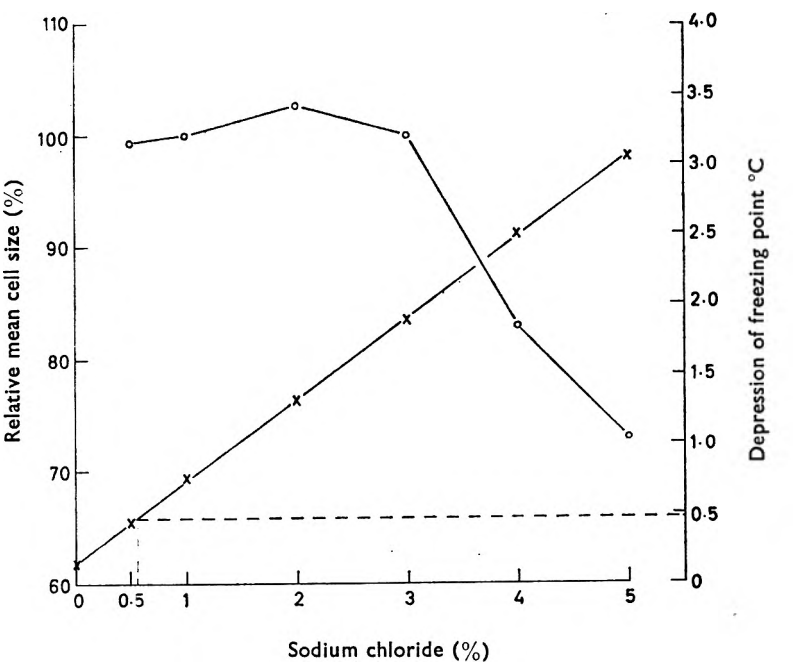

FIG. 1. The mean cell size of *Escherichia coli* after 30 min at 20° in different concentrations of saline containing 0·2% formaldehyde buffered at pH 7·5. The results are expressed as a percentage of the size in the 1% sodium chloride electrolyte solution. The freezing point depression of each solution is shown and that of the growth medium is indicated by a broken line. ○, cell size; ×, depression of freezing point.

numbers. The extent of this increase was greater at pH 7·5 than at pH 9·0 and in both cases it increased as the concentration of formaldehyde was decreased.

The percentage of cells having a volume greater than 1·0 μ^3 was determined in suspensions at pH 7·5. In concentrations of 0·05% formaldehyde or less this percentage increased over the 2 hr period thus indicating an increase in cell size. Little change in either cell number or size occurred in the presence of 0·1 or 0·2% formaldehyde.

CONCENTRATION OF SODIUM CHLORIDE

The effect upon the apparent cell size of varying the concentration of sodium chloride in the electrolyte solution is shown in Fig. 1. All solutions contained 0·2% formaldehyde and were buffered at pH 7·5. No satisfactory sizing of the cells was achieved with concentrations of sodium chloride of 0·4% or less and the lowest concentration selected for this experiment was 0·5%. Changes in the concentration of sodium chloride between 0·5 and 3·0% had little effect upon the measured cell size but increasing the concentration to 4·0 and 5·0% resulted in decreases in mean cell volume of 17 and 27% respectively. Fig. 1 also shows the freezing point depressions of those electrolyte solutions and that of the growth medium.

Discussion

Electrolyte solutions containing 0·85–0·9% sodium chloride have been used frequently for the counting and sizing of bacteria with the Coulter Counter (Lark & Lark, 1960; Toennies, Iszard & others, 1961; Parker, Barnes & Bradley, 1966). Hibbert & Tallentire (1966) however tested solutions of sodium chloride of different concentrations for instrument sensitivity and count stability and chose a 2% solution for the study of germinating bacterial spores. Mountney & O'Malley (1966) appear to have used peptone salt water for counting vegetative cells and in some reports the exact nature of the electrolyte solution is not stated (Allison, Hartman & others, 1962).

When growing cultures are diluted in saline solutions without the addition of a bactericide, sufficient culture medium may be added with the cells to allow growth to continue. In our experiments this growth continued for several hr and resulted not only in an expected increase in cell numbers but also in an unexpected increase in cell size. The use of such dilution techniques will thus give variable results depending upon the time interval between the preparation of the suspensions and the completion of the measurements. These variations will be independent of the final method of measurement and would affect results from microscopical analyses and from plate viable counts as well as from the Coulter Counter.

In an attempt to prevent these increases in cell size and number from occurring, the cells were harvested by membrane filtration and washed free from all traces of growth medium before resuspension in saline. This

procedure however resulted in a 12% decrease in the mean cell volume. It is not clear whether this was due to a decrease in the size of individual cells during harvesting, or to smaller cells being preferentially recovered from the filter during resuspension. Although freed from exogenous growth medium and diluted with saline, these cells continued to divide for some time. Presumably they were utilizing endogenous sources of energy and metabolites. Such multiplication resulted in an increase in cell number and a decrease in cell size.

Formaldehyde in different concentrations has sometimes been included in electrolyte solutions in order to fix the cells. Lark & Lark (1960) included 0·2% in their saline in some experiments and claimed that it preserved the samples and split a large proportion of double cells. Harvey, Marr & Painter (1967) used 0·04% formaldehyde and sized their suspensions between 1 and 3 hr after preparation. Garrett & Miller (1965) added a drop of formaldehyde to unstated volumes of dilutions and in some cases stored these suspensions in a freezer for up to 4 hr before counting. Toennies & others (1961) however stated that dilution with formaldehyde resulted in erratic counts and claimed that little change was observed over a period of 1 hr in suspensions in saline alone.

Our results show that the addition of sufficiently high concentrations of formaldehyde to the diluting solutions prevents the increase in cell size and number from occurring. Under the conditions of the experiments 0·1% formaldehyde was adequate but to allow either for variations in the volume of samples added or in cell sensitivity, a concentration of 0·2% is considered preferable. As a slight decrease in both cell size and number occurs at this concentration, measurements should be made promptly after preparing the dilutions.

If the volume of a bacterial cell alters with change in osmotic pressure; then electrolyte solutions exerting the same osmotic pressure as the growth medium should be chosen to obtain the correct size of growing cells. The freezing point of the medium used in this work was equivalent to that of the buffered electrolyte solution containing 0·6% sodium chloride and 0·2% formaldehyde but changes in cell volume were negligible for concentrations of sodium chloride between 0·5 and 3·0%. This suggests that these cells are normally in a state of internal pressure sufficient to maintain them at maximum size. In experiments with osmotically sensitive organisms it may be found necessary to adjust the electrolyte concentration to give an osmotic pressure equivalent to that of the growth medium which is used. Otherwise, a concentration of 1·0% sodium chloride appears to be suitable.

Buffered electrolyte solutions were used in this investigation to prevent fluctuations in pH which would cause changes in the surface charge of the cells and might result in some aggregation. The significance of the coefficient of variation of the size (volume) distribution of cells in logarithmically growing cultures has recently been discussed by Koch (1966). Any aggregation of bacteria or separation of cell doublets in the electrolyte solutions would not only result in an apparent change in cell size and

number, but would also alter this coefficient. In our experiments the coefficients of variation of the size distributions were not affected by the presence of formaldehyde or by storage of suspensions for up to 3 hr. It seems unlikely therefore that separation or aggregation of bacterial cells has contributed to the changes in cell size or number reported in this paper.

Acknowledgements. We thank Mr. K. Marshall for advice on the use of the Coulter Counter and Mrs. S. Kaye for technical assistance.

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The effect of antibacterial compounds on the electrical conductance of micellar sodium dodecyl sulphate

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The electrical conductance of aqueous 2% sodium dodecyl sulphate has been measured at 25° in the presence of benzyl alcohol, 2-phenylethanol, 3-phenylpropanol, *p*-hydroxybenzoic acid and its C_1-C_4 alkyl esters. Increases in conductance are observed and the effect becomes more pronounced with increasing hydrocarbon chain length of the additive. The effect is attributed to micellar penetration by the additives and is thus related to the distribution of the various compounds between aqueous and micellar phases. Micellar penetration causes separation of the closely packed dodecyl sulphate ions leading to a reduction in repulsion, to an increase in the proportion of free sodium counter-ions and hence to an increase in electrical conductance. The possible importance of these electrical changes is discussed for the antibacterial activity of the various compounds in the presence of ionic surfactants.

THE behaviour of antibacterial compounds in the presence of surface-active agents is of importance in the interaction between preservatives and emulsion ingredients (Wedderburn, 1964). The purpose of this paper is to provide information on the changes in electrical conductance which occur when antibacterial compounds are solubilized in sodium dodecyl sulphate solution. Recent work on the interaction between n-aliphatic alcohols (C_{2-7}) and micellar sodium dodecyl sulphate has indicated that, for any particular system, valuable information about the degree of counter-ion binding can be obtained by correlating electrical conductance and sodium ion activity measurements (Lawrence & Pearson, 1967). Preliminary results are now presented on the electrical conductance of a range of compounds of pharmaceutical interest.

Experimental

MATERIALS

Sodium dodecyl sulphate (B.D.H. specially pure grade) had a critical micelle concentration (CMC) of 0·0082M (0·24%) estimated from a plot of specific conductance against concentration. Recrystallization from water raised the CMC to 0·0086M, possibly due to removal of inorganic salts; soxhlet extraction (ether: 24 hr) had no further effect on this value. Distilled and de-ionized water equilibrated with atmospheric CO_2 was used throughout; it had a specific conductance of 0·89 μmhos. Antibacterial compounds used were benzyl alcohol, 2-phenylethanol and 3-phenylpropanol (B.D.H.); *p*-hydroxybenzoic acid (Koch Light "puriss" grade), methyl and n-propyl *p*-hydroxybenzoates (B.D.H.), ethyl and n-butyl *p*-hydroxybenzoates (Nipa).

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APPARATUS

A Mullard conductance bridge (type E 7566/3) was used operating at a frequency of 3 kc/sec in conjunction with Pye conductance cells (type 7407) with blackened platinum electrodes. Measurements were recorded when the additives had completely dissolved and after the solutions had equilibrated in a water bath at 25·0° ± 0·1°.

Results

The increase in conductance for ethanol was very small but for the other alcohols large increases were observed (Fig. 1); the initial slopes became steeper with increasing hydrocarbon chain length. Results were

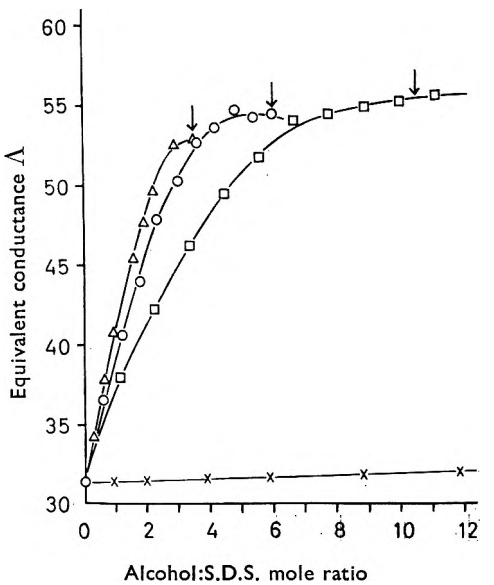

FIG. 1. Equivalent conductance Λ of 2% sodium dodecyl sulphate (S.D.S.) as a function of increasing alcohol concentration (mole ratio). □, Benzyl alcohol; ○, 2-phenylethanol; △, 3-phenylpropanol. Systems become turbid at the points marked by the arrows. Results for ethanol (×) taken from previous work (Lawrence & Pearson, 1967). All values recorded at 25°C ± 0·1°.

calculated after allowing for bulk dilution due to addition of the alcohols. There were no marked increases in viscosity and the systems remained isotropic until becoming turbid at the points denoted by the arrows. Benzyl alcohol produced the greatest overall increase in conductance ($\Lambda = 55{\cdot}2$) before phase separation occurred at an alcohol:sodium dodecyl sulphate mole ratio (R) of 10·5 (7·1% v/v). For 2-phenylethanol the corresponding values are $\Lambda = 54{\cdot}4$ at $R = 6{\cdot}0$ (4·8% v/v); for 3-phenylpropanol they are $\Lambda = 52{\cdot}8$ at $R = 3{\cdot}5$ (3·2% v/v). The phenyl-substituted alcohols depressed the CMC of the surfactant.

TABLE 1. EQUIVALENT CONDUCTANCE (Λ) OF 2% SODIUM DODECYL SULPHATE IN THE PRESENCE OF *p*-HYDROXYBENZOIC ACID AND ITS ESTERS

Compound	0·1% (w/v)		0·25% (w/v)		Saturation
	R*	Λ	R	Λ	Λ
p-Hydroxybenzoic acid	0·104	32·7	0·261	34·4	39·1
Methyl *p*-hydroxybenzoate	0·095	31·6	0·237	33·4	34·4
Ethyl *p*-hydroxybenzoate	0·087	31·6	0·217	33·4	34·8
n-Propyl *p*-hydroxybenzoate	0·079	31·6	0·199	33·4	35·5
n-Butyl *p*-hydroxybenzoate	0·074	31·6	0·185	33·0	36·9

* R is the additive: sodium dodecyl sulphate molecular ratio. All readings taken after approximately 40 hr.

Results for *p*-hydroxybenzoic acid and its esters are shown in Table 1. Since these compounds are much less soluble than the alcohols, the range of concentrations examined is much smaller. Thus the observed increases in conductance at saturation are not as great as those for the alcohols. (Fig. 1). However, the increases in Λ are about 7% for the esters at 0·25% (w/v) concentration (about 12% greater at saturation) and the results indicate that when compared on a molar basis, compounds of longer chain length have a greater effect. The parent acid seems anomolous in this respect but its enhanced values are probably due to dissociation.

Discussion

With increasing hydrocarbon chain length, polar additives show a greater tendency to penetrate the micelles, becoming solubilized with polar groups in the micellar surface (Lawrence, 1937). Introduction of such polar groups affects interaction between the surfactant ionic groups by a dielectric or screening effect and by separation of the charges. The former effect is difficult to estimate, the latter is probably of greater importance. It leads to a reduction in repulsion inversely proportional to the square of the separation distance; this accounts for the release of previously bound counter-ions, hence the increase in electrical conductance.

Previous work (Lawrence & Pearson, 1967) has shown that for 2% sodium dodecyl sulphate about 77% of micellar counter-ions are strongly bound and that this proportion decreases considerably as polar materials are solubilized. Initially, for 2% of the surfactant, $\Lambda = 31{\cdot}1$ but consideration of work by Flockhart & Ubbelohde (1953) and use of the Onsager equation shows that $\Lambda_{ideal} = 55$. It is therefore evident from Fig. 1 that the phenyl-substituted alcohols are producing almost complete micellar ionization. Because of their lower solubility, *p*-hydroxybenzoic acid and its esters produce smaller changes (Table 1). Ethanol, which shows no micellar penetration, produces an even smaller increase in Λ attributable largely to bulk dilution and dielectric effects.

Solubility considerations, particularly the distribution of additive between aqueous and hydrocarbon phases, are of importance in determining the antibacterial activity of compounds used as emulsion preservatives either alone or in the presence of surfactants (Alexander & Trim,

1946; Berry & Bean, 1948; Bean & Heman-Ackah, 1965). Shinoda (1954) has shown how the mathematical form of the partition depends on the hydrocarbon chain length of the penetrating molecules. It has been suggested (Lawrence & Pearson, 1967) that since alcohol molecules in the micellar phase are responsible for the increases in conductance, the slopes of the initial increases are related to the distribution coefficients. In this respect, the results presented for benzyl alcohol and 2-phenylethanol agree very closely with those previously observed for n-pentanol and n-hexanol respectively. This suggests that the hydrophobic behaviour of the substituted phenyl radical is equivalent to four normal aliphatic methylene groups. Effects similar to those described here have been observed with other anionic surfactants (Heckmann, 1954; Passinen & Ekwall, 1955) and cationic surfactants (Hyde & Lawrence, 1960).

The concentration of ions in the micelle surface has been estimated (Mukerjee, 1962) as equivalent to about a 3M solution of a 1 : 1 electrolyte (about 86% sodium dodecyl sulphate). In these terms the observed electrical changes may be considered as a "surface dilution" effect resulting from the introduction of various polar additives; subsequent micellar penetration then produces systems in which the ionic groups are less closely packed.

Acknowledgement. The author wishes to thank Miss F. M. Simm for technical assistance.

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Crystal growth in aqueous suspensions

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Factors controlling the sensitivity of crystalline suspensions to fluctuating temperatures have been investigated using a temperature-cycling apparatus. It is shown that surface-controlled crystal growth for a given temperature cycle increases with increase in particle solubility and with decrease in particle concentration. The effects of associated factors such as the slope of the solubility-temperature curve and sedimentation are discussed.

IT has been proposed (Gibbs, 1928) that a crystal grown under equilibrium conditions assumes a shape such that the expression

$$\frac{\Sigma (\gamma_j l_{ij} \operatorname{cosec} \omega_{ij} - \gamma_i l_{ij} \cot \omega_{ij})}{A_i} \quad \ldots \quad \ldots \quad (1)$$

has the same value for each face of the crystal. The expression (1) represents the summation around the i'th face of area A for all the faces j which are contiguous with it, l_{ij} being the length of each common edge and ω the angle between the i'th face and each neighbouring face (Fig. 1). For the generalized case of a parallelepiped of edge lengths a, b and c, with thermodynamic interfacial tensions γ_1, γ_2 and γ_3 respectively and

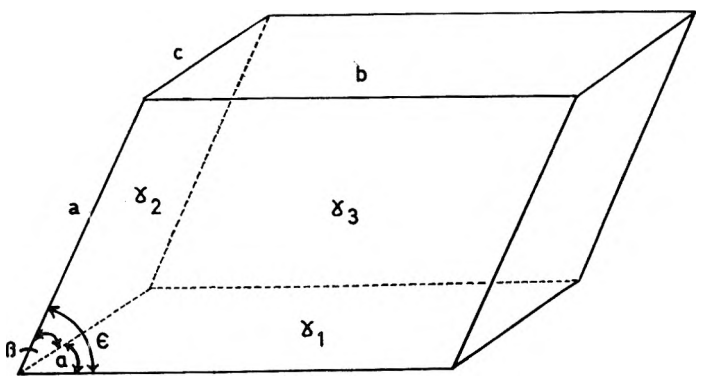


FIG. 1. Equilibrium crystal shape of parallelepiped. Crystallographic angles: α, β, ϵ. Edge lengths: a, b, c. Interfacial tensions: γ_1, γ_2, γ_3.

crystallographic angles α, β and ϵ (Fig. 1), it can be shown that the chemical potential μ_n of a crystal containing n moles is related to the bulk chemical potential μ_b by:

$$\mu_n - \mu_b = 2\left(\frac{M}{\rho\phi}\right)^{\frac{2}{3}}\left(\frac{2\gamma_1 \sin\alpha \cdot \gamma_2 \sin\beta \cdot \gamma_3 \sin\epsilon}{n}\right)^{\frac{1}{3}} \quad \ldots \quad (2)$$

where ρ is the density of the crystal, M is the molecular weight and ϕ is a constant characteristic of the crystallographic system.

From Imperial Chemical Industries Limited, Pharmaceuticals Division, Macclesfield, Cheshire.

It will be noted that, since the figures in parentheses represent positive quantities, μ_n is greater than μ_b; i.e. a small crystal has a higher solubility than the same material in bulk. Assuming a constant activity coefficient for the solute at the two different concentrations, the solubilities of the material in the small crystal L_n and in bulk L_b are related to n (and hence particle size) by:

$$\ln \frac{L_n}{L_b} = \frac{2}{RT} \left(\frac{M}{\rho\phi}\right)^{\frac{2}{3}} \left(\frac{2\gamma_1 \sin\alpha \cdot \gamma_2 \sin\beta \cdot \gamma_3 \sin\epsilon}{n}\right)^{\frac{1}{3}} \quad .. \quad (3)$$

R and T having the usual meanings.

Equation (3) represents the thermodynamic potential, in this case for the parallelepiped, for the well-known phenomenon of large particle growth at the expense of small ones. Experimental evidence for the increased solubility of small particles is given by Dundon (1923), Knapp (1922), Roller (1931) and others.

The substitution of typical values in equation (3) indicates that an increase in solubility of about 10% demands a reduction in particle diameter from a bulk crystal of 50–60 μ to about 1 or 2 μ. However, even a small solubility difference renders the system incompletely reversible and can result in greatly accelerated physical changes under fluctuating conditions. The marked increase in crystal growth rates with temperature cycling has been demonstrated by Carless & Foster (1966), using aqueous suspensions of sulphathiazole in the presence of various surface-active agents.

This report describes a machine for the investigation of temperature cycling phenomena of several samples simultaneously, together with preliminary results obtained by its use.

Experimental

DESIGN OF A TEST APPARATUS

As a means of carrying out temperature cycling under controlled conditions, a machine was designed for mechanically transferring samples between two thermostatted water baths. It was built from an industrial kit,* the quality of parts being such that continuous operation over several days was possible. In principle, the transfer is made by a rack and pinion mechanism. Two racks are used, one for each direction of transfer. A continuously rotating pinion is brought into mesh with one rack by a solenoid-actuated lever mechanism and subsequent movement of the rack causes the rotating samples to be transferred from one water-bath to the other. After a pre-selected time interval the solenoid is automatically de-energized and the pinion, under spring loading, engages the other rack. The second half of the cycle is then carried out, the test suspensions being transferred to the original bath. By means of a counter-balance arrangement, a large number of test suspensions can be loaded

* Fac. Construction System, Industrial Models Ltd, Ashton-under-Lyne.

on the immersion arm and cycled simultaneously. The apparatus also offers scope for testing the effect of a variety of containers on a given suspension.

TEST CONDITIONS

To examine the effect of bulk particle solubility on crystal growth, standard suspensions of the veterinary drug oxyclozanide (2,2′-dihydroxy-3,3′5,5′6-pentachlorobenzanilide) were prepared in an aqueous medium containing an anionic wetting agent and also in a water–acetone mixture. The suspensions were cycled between 20° and 50° (±0·5°) at 20 min intervals, a complete cycle lasting 40 min. The temperature of the suspensions became constant at each value within 2 min. Fig. 2 (A)–(C) show the increased growth of oxyclozanide under surface-controlled conditions. Virtually no growth was obtained when the solubility was below 2·5 mg/100 ml (Fig. 2B) but a significant increase in solubility produced the results shown in Fig. 2 (C).

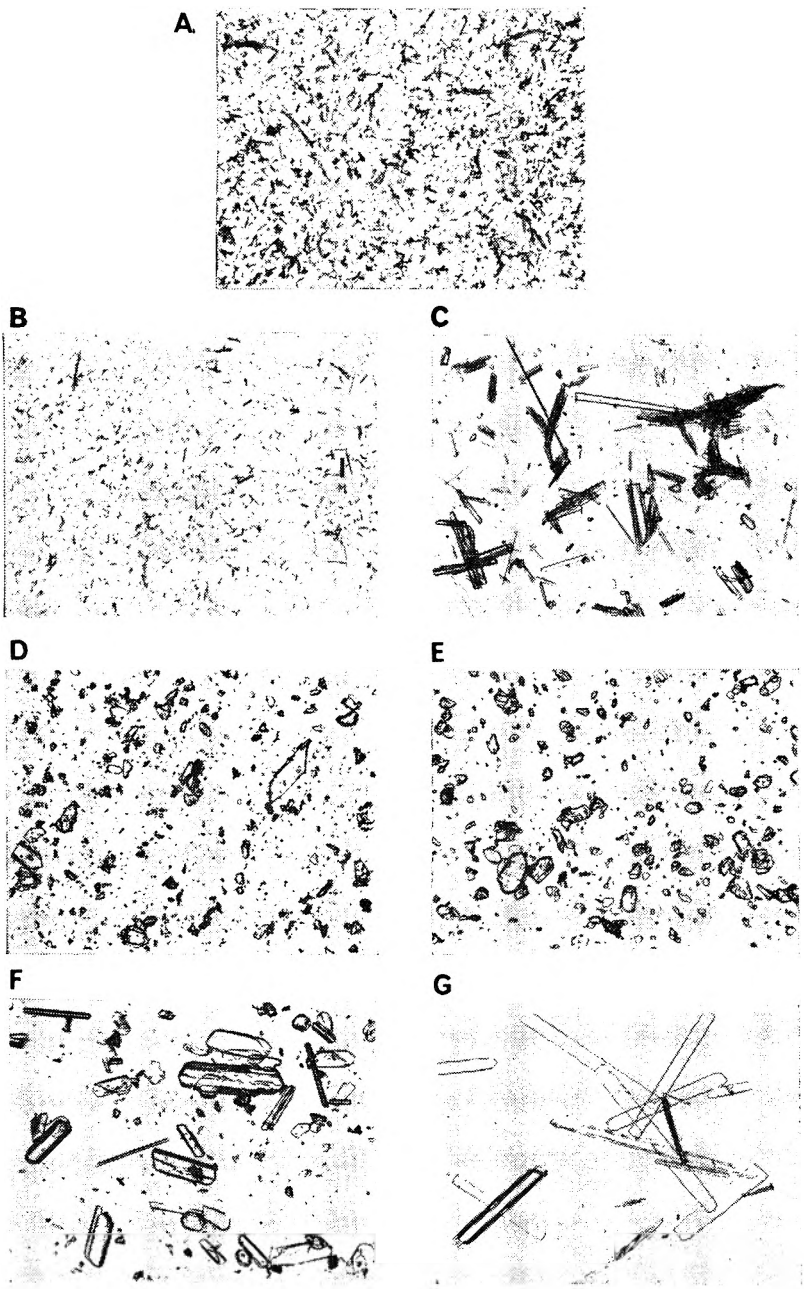
Sulphathiazole was used to observe the effect of particle concentration, this compound having a relatively high solubility in aqueous media. The cycling conditions were as for oxyclozanide. The 10% suspension was practically unaffected by temperature cyling, but subsequent ten-fold reductions in concentration gave much increased growth rates, as shown in Fig. 2 (E)–(G).

Discussion

During temperature cycling the solubility ratio L_n/L_b is virtually unchanged for the largest particles, but is much increased for the smallest crystals on the point of disappearing at the upper temperature limit. For a particle considered in isolation, the subsequent lowering of temperature would simply restore the size to the original value, the solubility being decreased in a reversible manner. In a suspension, however, some of the solute deposits on the larger particles of lower solubility, the net effect being to broaden the size distribution. This effect is demonstrated by the photomicrographs.

The following are controlling factors in the acceleration of crystal growth by temperature cycling, consideration being restricted to the solubility effects of size reduction.

1. The particle number concentration. In a dilute suspension a greater proportion of material must dissolve from a given particle to reach saturation at a higher temperature, and vice versa, thus increasing L_n/L_b. 2. The bulk particle solubility. A high solubility results in a large quantity of material transferred for a given L_n/L_b ratio. 3. The slope of the bulk solubility curve. The steeper the positive slope, the greater the reduction in particle size with a temperature increase. 4. The rate of stirring. 5. The temperature interval. 6. The frequency of fluctuation. 7. The crystal habit, the thermodynamic potential being a function of this. 8. The influence of surface-active agents.


A
B
C
D
E
F
G

The effects of all but the last two of these factors have been considered in the experimental work reported here.

The practical implications are:

1. Particle growth in suspensions is influenced by the addition of excipients which increase the particle solubility. The quantities of solubilizing and wetting agents used in a given formulation should therefore be kept to the minimum.

2. In a quiescent suspension, crystal growth is governed by the rate of diffusion of solute molecules. Consequently, since the mean diffusion path is greater in a more dilute suspension, the growth rate should be reduced under such conditions. Conversely, in a stirred suspension where crystal growth is surface controlled a low concentration under cycling conditions increases the growth rate.

3. Where sedimentation occurs, the local increase in particle concentration will increase particle growth due to the shortened inter-particle diffusion distances. A sedimenting formulation, therefore, even if easily re-dispersed, is prone to accelerate physical change within the system.

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FIG. 2. Photomicrographs showing crystal growth (magnification × 280). Suspensions contained in full 3 ml neutral glass ampoules. Temperature changed from 23° to 50° and vice versa at 20 min intervals (1 cycle = 40 min). A. Oxyclozanide in aqueous suspension containing 2% wetting agent* and 0·44% preservatives before temperature cycling. B. Suspension of oxyclozanide 0·1% w/v in same aqueous suspension after 100 temperature cycles. Solubility 0·0015% w/v at 23°; 0·0024% w/v at 50°. C. Suspension of oxyclozanide 0·5% w/v in 50:50 acetone–water suspension after 30 temperature cycles. Solubility 0·120% w/v at 23°; 1·33% w/v at 50°. D. Sulphathiazole in aqueous suspension containing 0·075% wetting agent† before temperature cycling. E. Sulphathiazole 10% in same suspension after 50 cycles. F. Sulphathiazole 1% in same suspension after 50 cycles. G. Sulphathiazole 0·1% in same suspension after 50 cycles.

* Perminal BXN (sodium alkylnaphthalene sulphonate)—I.C.I. Ltd.
† AP14 (an alkylphenol–ethylene oxide condensate)—I.C.I. Ltd.

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The effect of particle size on the strength of sodium chloride tablets

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A review of the literature concerned with the effect of particle size on the strength of sodium chloride tablets presents a confusing picture. In an attempt to rationalize opposing views, a wide range of narrowly classified powders was produced and compacted at five different pressure levels. The results show that there is no single simple relationship between crushing strength and particle size over the complete range of sizes studied (4–925 μ).

THE strength of compressed tablets depends on a number of factors amongst which the most important are the compacting pressure and particle size (Higuchi, Arnold & others, 1952). Several workers have examined the relations between particle size, compacting pressure and breaking strength, but often with conflicting results. Thus Huffine (1953) examined the relation between breaking strength and mesh size of sodium chloride, sucrose and boric acid. With sodium chloride, the effect on this relation of applying two compacting pressures, 926 and 397 kg cm^{-2}, was investigated. He found a maximum breaking strength at a particle size of 183 μ when compression was at the higher pressure; at the lower pressure the maximum was at 229 μ.

In explanation it was pointed out that for small particles there would be many more points of contact than with large particles. Whilst the material is deforming plastically, the total contact area for either coarse or fine particles would be constant for a given pressure, since the smaller number of contacts deform to a greater extent. However, if the material work-hardens, the area of contact would be less than that expected for the same pressure on a plastically deforming point of contact. Huffine suggested that sodium chloride *did* undergo work-hardening and that this would take place more rapidly where there were relatively few points of contact supporting a given pressure, i.e. at the larger particle size levels. Increasing the pressure would cause this effect to occur at a smaller particle size level. The work of Bowden & Tabor (1964) suggests that in compression up to 1,500 kg cm^{-2} sodium chloride will be deformed plastically with some degree of work-hardening. Their work also supports the findings of Huffine that the consolidation of the sodium chloride during tabletting will mainly be by plastic deformation and not by fracture.

In contrast, Henderson (1962) found that the hardness of sodium chloride tablets increased with increasing particle size in the region 180–1,435 μ (mean mesh size), using compacting pressures of 547 to 911 kg cm^{-2}.

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For sodium chloride at a mean pressure of about 780 kg cm^{-2}, work in this laboratory (Shotton & Ganderton, 1961) has shown that there is a logarithmic decrease of crushing strength with increasing particle size between 120 and 400 μ. This was explained on the basis of the Orewan (1949) concept of the strength of brittle polycrystalline materials.

Shotton & Ganderton also examined the type of failure that occurred with sodium chloride, with hexamine and with aspirin tablets. Where cross-grain failure was apparent, i.e. with sodium chloride and hexamine, the Orewan concept could be applied, but where round-grain failure occurred, as with aspirin, the crushing strength was almost independent of particle size. Further work by Shotton & Lewis (1964) showed a minimum crushing strength to exist when compressing particle size fractions of sodium chloride between 120–450 μ at compaction pressures of 1168 and 1485 kg cm^{-2}.

The effect of moisture on the strength of sodium chloride tablets made from 30–40 mesh fraction at mean pressures up to 2000 to 3000 kg cm^{-2} has subsequently been examined (Shotton & Rees, 1966).

It is evident from this review that the part played by particle size in the determination of the strength of sodium chloride is not fully understood. Without a clear understanding of this simple system it is difficult to envisage the problems posed by the tabletting of granules. The purpose of this communication is to examine the part played by particle size in determining the strength of sodium chloride tablets in the hope that such understanding may subsequently be related to the behaviour of granules under compression.

Experimental

A single batch of sodium chloride (British Drug Houses, B.P. quality) was sifted using an Endecott Model A Test Sieve Shaker to obtain fractions between 20 and 80 mesh sizes. Smaller sized fractions (100 to 350 mesh) were obtained by ball milling the original material and sifting the product on a Lavino Alpine Air-Jet Sieve. The sub-sieve material was produced by feeding the original material through a Gem fluid energy mill operating at 75 psig using a feed rate of about 5 g min^{-1}.

The particle size fractions were further subjected to a microscopic examination to determine the mean size and quality of the distribution. With the coarse fractions the sizes of some 250 particles were measured using a suitably calibrated eyepiece mounted vernier scale. The fine fractions were sized using British Standard method. The "freely poured" apparent density was determined, for each size fraction, by pouring the sample at 45° into a tared 25 ml measuring cylinder.

Unless otherwise stated, the samples were allowed to equilibrate with the atmosphere and the moisture content determined immediately before compression using a Cahn Gram Electrobalance.

An instrumented single punch tablet machine as described by Shotton & Ganderton (1960) was used in the present study. A tablet weight

equivalent to a 0·4 cm length at zero porosity was produced, by introducing the weighed powder into the 12 mm diameter die-cavity by hand. The motor, which in continuous running would produce 72 tablets per min, was used to operate the machine.

The dimensions of the ejected tablet were measured and the tablet immediately crushed using the strength test described by Shotton & Ganderton (1960). For observation of the fractured surfaces, a Watson 3D microscope was used.

Results

The properties of the sodium chloride powder fractions used are given in Table 1. The fractions showed a logarithmic normal distribution, although in the range 100 to 350 mesh, this distribution overestimates the size of the smallest particles (0·5% wt range). Additionally, a 50% mixture (C) of the dry sub-sieve fraction (A) was made with unclassified sodium chloride of mean size 345 μ (standard deviation 0·137).

TABLE 1. PROPERTIES OF SODIUM CHLORIDE FRACTIONS

Mesh size	Mean size by weight μ	Standard deviation	Moisture content %
20–30	925	0·049	0·000
30–40	665	0·068	0·000
40–60	430	0·080	0·000
60–80	285	0·065	0·000
100–120	200	0·063	0·012
120–200	177	0·081	0·012
200–240	120	0·058	0·010
300–350	93	0·061	0·057
−350	58	0·184	0·044
Sub-sieve (A) ..	4·2	0·169	0·000
Sub-sieve (B) ..	4·2	0·169	0·040

The relative densities of the materials from the poured density and during compression are given in Table 2. The values quoted are the mean of five determinations. Except for the subsieve fractions, a coherent compact was not formed at a pressure of 250 kg cm^{-2}. Tablets of the sub-sieve material exhibited capping on ejection from the die at the higher pressures.

TABLE 2. RELATIVE DENSITIES OF SODIUM CHLORIDE AT DIFFERENT COMPACTION PRESSURES

Mesh size or description	Mean compaction pressure kg cm^{-2}					
	0	250	637	997	1896	2345
20–30	0·563	—	0·814	0·874	0·954	0·970
30–40	0·571	—	0·809	0·874	0·947	0·961
40–60	0·547	—	0·804	0·870	0·946	0·965
60–80	0·524	—	0·802	0·868	0·959	0·963
100–120	0·504	—	0·798	0·862	0·936	0·964
120–200	0·497	—	0·796	0·865	0·937	0·963
200–240	0·484	—	0·792	0·853	0·934	0·956
300–350	0·461	—	0·790	0·847	0·913	0·958
−350	0·375	—	0·785	0·856	0·927	0·947
Sub-sieve (A) ..	0·312	0·677	0·780	0·841	0·917	—
Sub-sieve (B) ..	0·190	0·673	0·789	0·835	—	—
Mixture (C) ..	0·343	0·705	0·817	0·873	0·939	0·950

At zero pressure the 20–30 mesh fraction gave a lower relative density than the 30–40 mesh fraction. The reason for this relatively poor packing can be seen microscopically. Whereas all the other coarse fractions consist of discrete particles of the regular cubic form, the 20–30 fraction consisted of aggregates of two or three such crystals and were irregular in shape.

The results of the effect of particle size on crushing strength at the five different compacting pressures are shown in Fig. 1. The general shape of the relationship plotted on semi-logarithmic paper shows both a minimum and maximum within the particle size range studied.

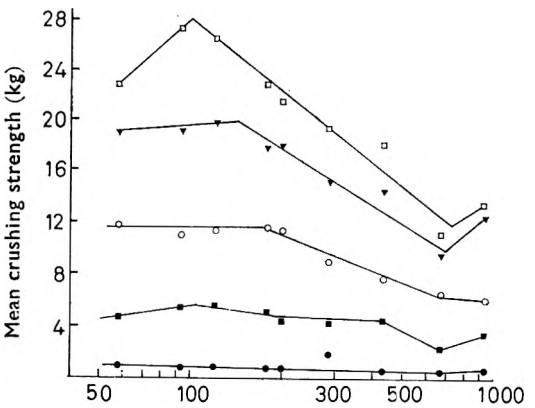

FIG. 1. The effect of particle size on the strength of sodium chloride tablets at different compaction pressures. □, 2345; ▼, 1896; ○, 997; ■, 637; ●, 250 kg cm^{-2}.

With the tablets made from sub-sieve sized material very much stronger tablets were produced at the lower compacting pressures, whilst "capping" was evident at the higher pressure levels (see Table 3).

TABLE 3. STRENGTHS OF COMPACTS OF SUB-SIEVE SIZED SODIUM CHLORIDE. CRUSHING STRENGTH IN KG.

Material	Mean compacting pressure kg cm^{-2}				
	250	637	997	1896	2345
Sub-sieve (A)	12·47	23·85	31·03	36·85	Capped
Sub-sieve (B)	9·99	23·07	31·12	Capped	Capped
Mixture (C)	1·53	9·37	16·49	30·09	12·25

The relationship between compacting pressure and crushing strength is linear for the particle size fractions studied except at higher pressures, where a deviation similar to that found by previous workers was evident (Fig. 2). In Fig. 2, because of the number and scatter of experimental results obtained for the sieved fractions, these results are enclosed by two broken lines. Deviations from this envelope of results are shown for the

very coarse particle sizes. The sub-sieve fractions (A) and (B) and the mixture (C) show linear relationships distinctly different from the sieved fractions.

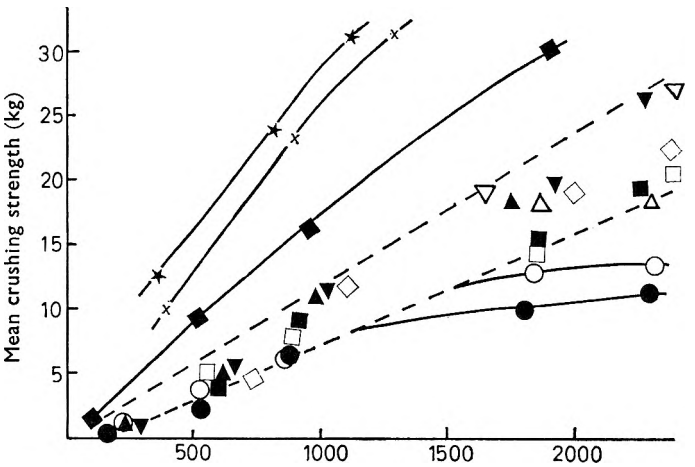

FIG. 2. The effect of compaction pressure on tablet strength using different particle size fractions. ★, Sub-sieve particles A. ×, Sub-sieve particles B. ◆, Mixture of sub-sieve and coarse particles. ◇, −350; ▽, 300–350; ▼, 200–240; △, 120–200; ▲, 100–120; ■, 60–80; □, 40–60; ●, 30–40; ○, 20–30 mesh fraction.

Discussion

Henderson (1962) and Shotton & Lewis (1964) reported an increase of tablet strength with increasing particle size in the region above about 200 μ. In the present work the increase in strength at 925 μ was probably due to fracture of the crystals. Unlike the smaller sized crystals, those of the 20–30 mesh fraction were composed of a number of smaller crystals aggregated together. Such aggregates would fracture comparatively easily when compressed and the resulting smaller primary crystals, together with the freshly cleaved surfaces (Bowden & Tabor, 1964), may be responsible for the observed increase in strength. Because of variations in the crystallization process, different batches of sodium chloride are likely to consist of differently sized primary crystals, together with their aggregates. The results of Henderson and of Shotton & Lewis may be due to the use of sodium chloride having a largest primary crystal size of about 200 μ.

Decreasing the particle size from 665 to about 100 μ has been shown to result in increasing tablet strength. This is in agreement with the results of Huffine (1953) and of Shotton & Ganderton (1961). The latter authors showed correlation with the Orewan concept of the strength of polycrystalline materials using the formula

$$S = KD^{-0.20}$$

where S is the crushing strength of the compact (kg), D is particle size (cm) and K is a constant. When plotted using double logarithmic co-ordinates, the present results give a relation in this region of particle size, for all pressures investigated, of the form

$$S = KD^{-0.33}$$

irrespective of the type of failure produced in crushing. The value of this index may vary between different batches of a single material since the number and size of crystal dislocations may vary between batches.

Smaller sized particles have only been examined by Huffine (1953), who demonstrated that maximum strength was produced by particles of 100–200 μ. The present work is consistent with Huffine's theory of the work-hardening of the contact areas between particles, where the maximum strength appears to shift to a larger particle size at lower pressures.

The effect of the moisture content of the different particle size fractions must be considered. Shotton & Rees (1966) observed an increase in strength of compacts of sodium chloride containing up to 0·55% moisture at pressures below about 1000 kg cm^{-2}, whereas at higher pressures, the strength was reduced compared with compacts of the anhydrous material. With increasing surface area, sodium chloride, in equilibrium with atmospheric conditions, contains increasing amounts of moisture up to about 0·05% (see Table 1). Although tablets made at a mean pressure of 1090 kg cm^{-2} from 350 mesh powder containing 0·04% moisture showed no significant change in strength on drying in an evacuated oven at 110° for 1 hr, or on drying the material before compaction, the presence of moisture would appear to be a contributing cause in the weakening of sodium chloride tablets made from fine powders, especially at high compaction pressures.

To examine the maximum strength in more detail the sub-sieve fractions were prepared. Tablets made from this material, however, showed a large increase in strength at low pressures, when compared with all other particle size fractions (see Table 3). This effect is probably due to a change in the surface properties and to the very large increase of surface area at this particle size. The increased cohesion of the material is evident from Fig. 2, which shows that significantly lower pressures are required to produce a given tablet hardness. The cohesion of the material, especially when dry, forming aggregates up to 5 mm diameter before compaction, was also observed. During compaction breakdown of these aggregates must occur to give similar relative densities to those of the more coarse fractions (Table 2). Such breakdown may also considerably influence the strength of the tablet which is similar to the increased strength of sodium chloride granulations observed by Shotton & Lewis (1964).

An equal mixture of the unclassified coarse fraction and the dry sub-sieve material, possessing much better packing characteristics, gave intermediate strength values.

Capping of sodium chloride tablets, which had not previously been reported, was experienced at high pressures for the sub-sieve material.

With hexamine, Shotton & Ganderton (1961) observed that the disruptive forces are more marked with finer particles, whilst Baba & Nagafuji (1965) report a similar experience using aspirin. It would appear that with fine particle sized material there will be very many contacts, the deformation of which will be mainly elastic. The tablet then exhibits considerable recovery on ejection from the die leading to failure by capping. In contrast, the extensive plastic deformation that would occur between relatively few contacts at the same pressure would lead to a much more stable tablet.

CONCLUSIONS

Tablet strength is not a simple function of particle size. For aggregates, the breakdown to the smaller primary crystals may result in a compact strength higher than that of compacts made from crystals of the same size as the aggregate. For simple crystals, the strength of the compact increases with a decrease in particle size in the region 600–100 μ, depending upon the applied pressure. Further reduction in size may lead to a slight reduction in crushing strength.

The use of very fine cohesive powders produces strong tablets at low pressures because of the nature of the surface and the breakdown of aggregates. However, at higher pressures, compacts of such fine powders exhibit capping.

Attempts to extrapolate tablet crushing strength data to particle sizes that have not been examined should be viewed with extreme caution.

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Buccal absorption of basic drugs and its application as an *in vivo* model of passive drug transfer through lipid membranes

A. H. BECKETT AND E. J. TRIGGS

A method of studying the buccal absorption of single and multicomponent mixtures of basic drugs at varying pH values is described. The mode of absorption is shown to be a partitioning into, or passage through, a lipid phase. The shape of the curves of drug absorption against pH permits classification of the drugs into four main classes. The shape also gives a better indication of the likely passage of drugs through biological lipid membranes than simple partition or rate of partition experiments between water and organic solvents. There is a direct relation between the drug absorption–pH curves and the renal tubular reabsorption of a number of drugs.

A REVIEW on the absorption of drugs through the oral mucosa (Gibaldi & Kanig, 1965) has described the sublingual administration of some cardiovascular drugs, steroids, barbiturates, insulin, heparin and enzymatic materials. The classic experiments of Overton (1902) have laid the foundation for the pH-partition hypothesis of drug absorption (Schanker, 1960), and although this theory falls short of explaining completely the innumerable complexities involved in the absorption of materials through mucous membranes, it does provide some basis for the understanding of drug absorption. The lipid solubility of a drug has been shown to be an important factor in the passage of drugs through the oral mucosa (Walton, 1935a,b). We have therefore investigated the method of buccal absorption of some basic drugs as an example of an *in vivo* model of passive drug transfer through a lipid membrane.

Experimental

Buffer solutions in the range pH 4·00 to 9·18 were prepared using potassium hydrogen phthalate (0·05M) for pH 4·00, sodium tetraborate (0·05M) for pH 9·18, and Sörenson's phosphate buffer to obtain intermediate pH values of 5·00–8·20 (Documenta Geigy, 1962). The pH values of the solutions were checked at room temperature (approximately 20°) with a pH meter.

Drug solutions. Solutions of the drugs listed in Table 1 were prepared by dissolving a suitable quantity of the salt of the drug in distilled water; each solution contained the equivalent of 2 mg base/ml.

BUCCAL ABSORPTION MEASUREMENTS

Men aged 20 to 30 were used.

General method. Drug solution (0·5 ml) (equivalent to 1 mg base) was pipetted into a 100 ml beaker and 24·5 ml of the appropriate buffer solution added; the pH of the resultant solution was checked. The

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solution was placed in the subject's mouth, and by movement of the cheeks and tongue circulated some 300–400 times round the mouth for 5 min, after which the solution was expelled. The subject quickly rinsed his mouth with distilled water (10 ml) for 10 sec, and expelled the rinsing. The expelled solutions were combined, adjusted to 200 ml, and an aliquot (5 ml) used for analysis.

TABLE 1. CHROMATOGRAPHIC CONDITIONS FOR SOME BASIC DRUGS

Drug	Retention time (min) t_R	Column	Oven temperature (°C)	Internal marker	Retention time (min) t_R
(+)-Amphetamine	11·4	"A"	155	*NN*-Dimethylaniline	9·2
(−)-Amphetamine	11·4	"	"	"	"
(+)-Methylamphetamine	10·8	"	"	"	"
(−)-Methylamphetamine	10·8	"	"	"	"
Ethylamphetamine	11·0	"	"	"	"
Norfenfluramine	10·0	"	"	"	"
(+)-Fenfluramine	8·8	"	"	"	"
(−)-Fenfluramine	8·8	"	"	"	"
Mephentermine	13·4	"	"	"	"
Furfurylmethylamphetamine	5·8	"B"	160	(+)-Benzphetamine	9·8
(+)-Benzphetamine	9·8	"	"	Furfurylmethylamphetamine	4·8
(−)-Benzphetamine	9·8	"	"	"	"
Chlorphentermine	4·7	"	135	(−)-Nicotine	4·0
(−)-Nicotine	4·0	"	"	Chlorphentermine	4·7
Lignocaine	6·2	"	190	Chlorpheniramine	8·0
Chlorpheniramine	8·0	"	"	Lignocaine	6·2
Pethidine	8·0	"	155	4-Benzylpyridine	6·0
Norephedrine	8·6	"	140	(2,6-Dimethylphenoxy)-ethylamine	3·2
Methylephedrine	4·7	"	"	"	"
Ethylephedrine	6·5	"	"	"	"
(±)-Pseudoephedrine	6·3	"	"	"	"
(+)-Norpseudoephedrine	8·4	"	"	"	"
Phenmetrazine	3·5	"	150	Furfurylmethylamphetamine	4·8
Phendimetrazine	2·0	"	"	"	"
(+)-Methadone	8·4	"	190	Tripelennamine	6·75
(−)-Methadone	8·4	"	"	"	"
Isomethadone	8·4	"	"	"	"
1-(2′Chlorobenzyl)-2-methyl-phenethylamine	9·8	"	180	1-(4′-Fluorobenzyl)-2-methyl-phenethylamine	4·5

Mouth contact time. The absorption of (+)-amphetamine at pH 8·22 and (+)-benzphetamine at pH 7·27 was determined after varying contact times.

Drug concentration. The absorption of (+)-methylamphetamine at pH 8·22 and chlorphentermine at pH 7·27 was determined within the range 1 to 7 mg of base per mouth wash.

Drug mixtures. Mixtures containing five to eight different drugs were investigated using the general procedure over the pH range 4–9·18. A quantity of the salt equivalent to 1 mg base of each drug was used, and the amount of each drug absorbed was determined. In experiments with different drug mixtures, the mixtures were prepared to contain one or two drugs in common.

Intra- and inter-subject variation. The absorption of a number of the drugs listed in Table 1 was determined on several days for some subjects, and the absorption of (+)-amphetamine, (−)-methylamphetamine and dimethylamphetamine, all at the pH values 9·18 and 8·12, was determined for seven different subjects on different occasions.

Analytical technique. The 5 ml aliquot of the diluted expelled solution was extracted using the method described by Beckett & Rowland (1965a) for amphetamine. Calibration curves for each drug were constructed by adding known amounts to water and saliva diluted to 200 ml, together with an internal marker. The bases were extracted (see Beckett and Rowland, 1965a) and submitted to gas chromatography. The ratio of peak height of the drug to peak height of the internal marker was plotted against drug concentration (μg/ml). Two sets of conditions were used for the analysis:

(i) Column "A," a 3 metre, $\frac{1}{8}$ inch o.d. stainless steel tube packed with Chromosorb G (acid-washed 80–100 mesh) coated with 5% w/w potassium hydroxide and 5% w/w carbowax 6000; injection block temperature, 250°, air pressure 28 lb/inch^2, hydrogen pressure, 20 lb/inch^2, and nitrogen pressure, 20 lb/inch^2.

(ii) Column "B," a 1 metre, $\frac{1}{8}$ inch o.d. stainless steel tube packed with Chromosorb G (acid-washed 80–100 mesh) coated with 5% w/w potassium hydroxide and 2% w/w carbowax 20 M; injection block temperature 250°, air pressure 25 lb/inch^2, hydrogen pressure, 15lb/ inch^2, and nitrogen pressure, 15 lb/inch^2.

Results and discussion

ANALYTICAL TECHNIQUE

Use of the gas-liquid chromatographic method made possible the rapid and specific analysis of drugs in the multicomponent drug mixtures used in these experiments. Calibration curves for each drug, separately or collectively, were linear over the range 0·1 to 8·0 μg base/ml of drug in water alone or diluted saliva solution, and the curves were identical for the two solutions. The chromatographic conditions for each drug are summarized in Table 1.

BUCCAL ABSORPTION MEASUREMENTS

The percentage buccal absorption of single drugs, e.g. (+)-amphetamine, (+)-benzphetamine, (+)-methylamphetamine and chlorphentermine over various time intervals and at various drug concentrations in one man is shown in Fig. 1A and B. Absorption increased rapidly with time of contact up to about 5 min; a contact time of 5 min was therefore chosen for subsequent experiments. A linear relation was observed between percentage absorption and initial drug concentration for *single* drugs up to concentrations of 3 mg base/25 ml of buccal contents; in the subsequent experiments, a concentration of 1 mg base/25 ml was chosen. With multicomponent mixtures in which *each* drug was present in a concentration of not more than 1 mg/25 ml, absorption was the same as when each drug was administered separately.

Duplicate absorption experiments on several of the drugs listed in Table 1 are given in Table 2 for one man on different days; intra-subject variation is surprisingly small. Experiments with (+)-amphetamine at

pH 8·22 on five different days gave a spread of results from 33·0 to 36·5%. Similar variation was observed using six volunteers and a variety of the drugs in Table 1.

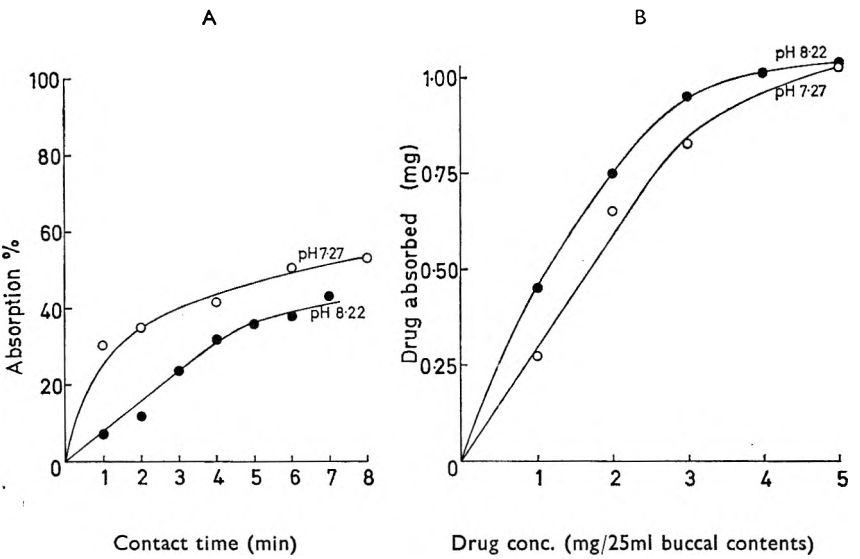

FIG. 1. A. The effect of solution contact time on the buccal absorption of (+)-amphetamine and (+)-benzphetamine. ○, (+)-benzphetamine; ●, (+)-amphetamine.

B. The effect of drug concentration on the buccal absorption of (+)-methylamphetamine and chlorphentermine. ●, (+)-methylamphetamine; ○, chlorphentermine

TABLE 2. INTRA-SUBJECT VARIATION OF BUCCAL ABSORPTION OF SEVERAL DRUGS

Drug	% Buccal absorption				
	Buffer pH 5·0	Buffer pH 6·0	Buffer pH 7·0	Buffer pH 7·5	Buffer pH 9·2
(+)-Amphetamine	0·0, 0·0	0·0, 0·0	13·5, 7·5	26·5, 24·0	70·5, 72·0
(+)-Methylamphetamine ..	0·0, 0·0	0·0, 0·0	7·5, 9·0	15·0, 12·0	69·0, 64·0
(+)-Benzphetamine	6·0, 7·5	12·0, 12·0	36·0, 32·5	51·0, 43·5	72·0, 77·5
(+)-Fenfluramine	10·5, 12·0	25·0, 23·5	43·5, 40·5	51·0, 51·0	85·0, 83·5

Inter-subject variation in these experiments with the volunteers and using the general method was also not great (Table 3); to date all seven subjects have produced a similar ranking order for each drug in these buccal absorption experiments at a particular pH when a mixture of drugs has been used. Thus, inter-subject variations will not interfere with the establishment of the order of lipid characteristics of series of drugs.

Fig. 2 shows that there is no stereochemical selectivity in the absorption process.

TABLE 3. INTER-SUBJECT VARIATION IN BUCCAL ABSORPTION OF DRUGS BY THE GENERAL METHOD

Subject	Amphetamine			Methylamphetamine			Dimethylamphetamine		
	Buffer pH 8·12		Buffer pH 9·18	Buffer pH 8·12		Buffer pH 9·18	Buffer pH 8·12		Buffer pH 9·12
E. J. T.	20·0	24·2	63·6	21·8	27·0	66·2	39·2	44·6	75·9
N. B.	21·6	30·6	69·2	20·6	29·4	70·7	40·1	44·0	79·4
A. C. M.	23·6	30·0	70·8	18·2	32·8	69·8	27·8	50·2	80·0
G. T. T.	30·0	33·2	64·0	25·8	34·6	62·4	41·2	53·4	76·4
C. W.	20·6	26·0	61·6	18·6	27·2	64·3	37·8	44·0	76·0
L. G. B.	22·6	36·0	58·6	17·0	29·6	62·6	38·0	35·0	73·6
J. F. T.	34·4	33·0	73·4	28·6	28·6	71·4	44·2	50·2	80·6
* J. H. M.	—	35·6	—	—	25·0	—	—	33·2	—
* A. S.	—	42·8	—	—	34·0	—	—	39·0	—

* The following instructions were given to two subjects who had *never* participated in these experiments before: "Take the solution into your mouth and circulate it by means of your tongue and cheeks about once/sec. Hold your head forward when you swallow. Keep the solution in your mouth for 5 min and then expel the solution into the beaker provided." The results are in close agreement with those obtained from the other subjects used.

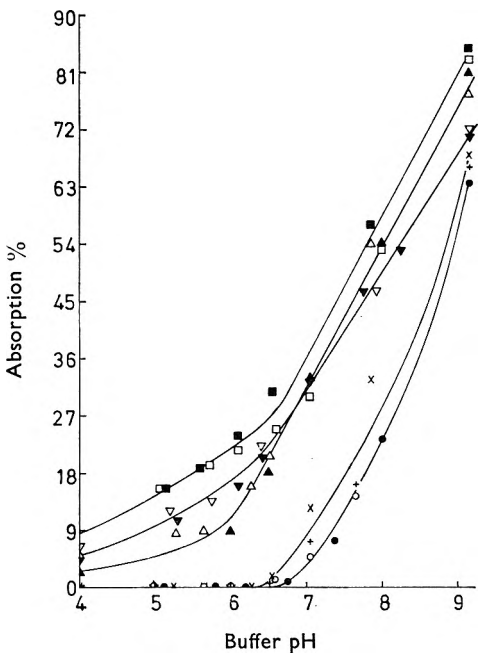

FIG. 2. The buccal absorption of pairs of drug enantiomorphs. ●, (+)-methylamphetamine; ○, (−)-methylamphetamine; ×, (−)-amphetamine; +, (+)-amphetamine; ▲, (−)-benzphetamine; △, (+)-benzphetamine; ▽, (−)-methadone; ▼, (+)-methadone; □, (−)-fenfluramine; ■, (+)-fenfluramine.

The absorption curves of a series of drug mixtures are shown in Figs 3–5; the percentage of the drug absorbed increases as the concentration of the unionized form of the drug increases. The shapes of the curves also vary with variation in pK_a values and the lipid solubilities of their unionized forms (see later).

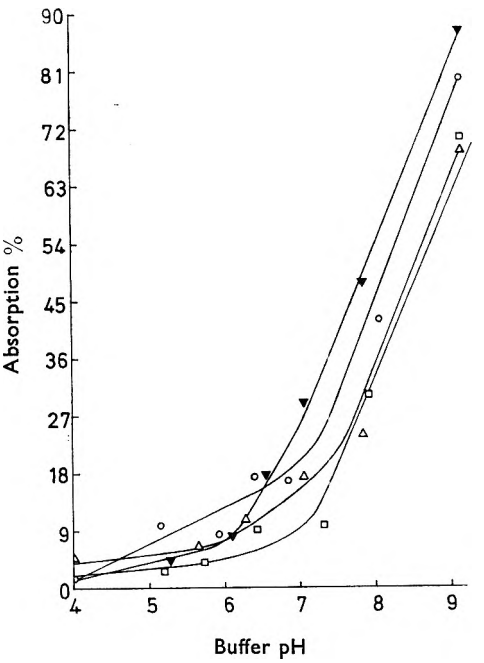

FIG. 3. The buccal absorption of some basic drugs listed in Table 1. △, dimethylamphetamine; ○, norfenfluramine; ▼, furfurylmethylamphetamine; □, ethylamphetamine.

From our observations it would seem that the buccal absorption of drugs may be considered to be a partitioning into, or passage through, a lipid phase because (i) stereo-selectivity was not evident; (ii) increasing the concentration of the unionized lipid-soluble form of the drug increases the percentage absorption in a regular manner; (iii) no difference in the percentage absorption of a drug at any given pH value was observed when the drug was given separately or in admixture with a number of other drugs.

Washing out the mouth with buffer of pH 4·0 after the absorption experiment causes *some* of the absorbed drug in the lipid to be partitioned back into this aqueous phase in the mouth. The possibility of the binding of drugs to plasma proteins and other tissues as the distribution approaches equilibrium will be discussed by Beckett, Boyes & Triggs (in preparation).

The shapes of the curves in Figs 2 to 5 permits classification of the basic drugs into four main classes; representative members of these classes with their absorption characteristics are recorded in Table 4.

The experiments we have described involve uptake of drug in a fixed time, i.e. 5 min. Drug binding is considered to play a role subsidiary to drug partitioning in view of the lack of competition of uptake between drugs when drug mixtures are used. The amount absorbed will be dependent on the pK_a of the base, on the *rate* of partition of the unionized

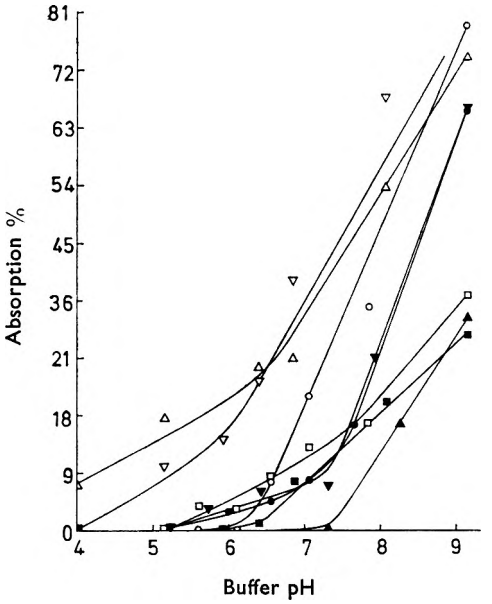

FIG. 4. The buccal absorption of some basic drugs listed in Table 1. △, isomethadone; ▽, 1-(2′-chlorobenzyl)-2-methylphenethylamine; ■, phenmetrazine; ▲, phendimetrazine; ●, pethidine; ▼, mephentermine; □, lignocaine; ○, chlorphentermine.

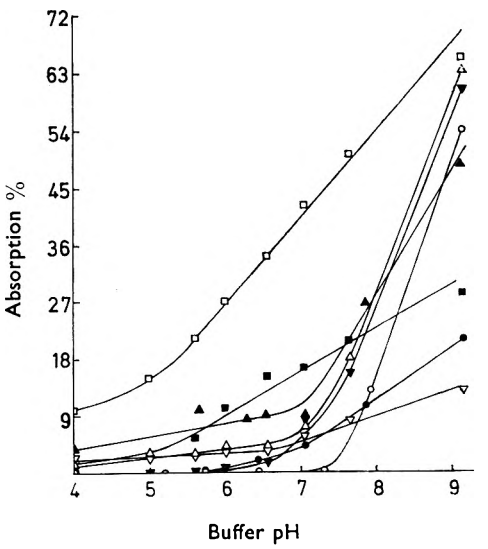

FIG. 5. The buccal absorption of some basic drugs listed in Table 1. □, chlorpheniramine; △, ephedrine; ▼, methylephedrine; ○, ethylephedrine; ▲, (±)-pseudoephedrine; ■, (−)-nicotine; ●, (+)-norpseudoephedrine; ▽, norephedrine.

TABLE 4. CLASSIFICATION OF DRUGS IN BUCCAL ABSORPTION

Class of drug	% Buccal absorption		
	Buffer pH 5·0	Buffer pH 6·0	Buffer pH 7·5
Class 1: (+)-amphetamine, (−)-amphetamine, (+)- and (−)-methylamphetamine, ephedrine, chlorphentermine, mephentermine, (±)-pseudoephedrine, and methylephedrine	0·0	0·0–3·0	10·0–23·0
	i.e. negligible uptake at pH 4 to 6·5 but a steep rise as pH becomes more alkaline		
Class 2: norephedrine, and (+)-norpseudoephedrine	0·0–3·0	1·0–5·0	6·0–9·0
	i.e. little uptake at pH 4 to 6·5 and only a slight rise as pH becomes more alkaline		
Class 3: chlorpheniramine, (+), and (−)-benzphetamine, (+)- and (−)-methadone, (+)- and (−)-fenfluramine, isomethadone, and 1-(2′-chlorobenzyl)-2-methylphenethylamine	7·0–15·0	21·0–27·0	40·0–54·0
	i.e. substantial uptake at pH 4 to 6·5 and a steep rise as pH becomes more alkaline		
Class 4: ethylephedrine, furfurylmethylamphetamine, phenmetrazine, phendimetrazine, lignocaine, (−)-nicotine, norfenfluramine, ethylamphetamine, pethidine and dimethylamphetamine	0·0–6·0	0·0–13·0	3·0–30·0
	i.e. characteristics *between* those of class 2 and 3		

form of the drug, on the lipid–water partition coefficient of the drug, and the pH of the solution. Thus, the *shape* of the absorption–pH curves for the various drugs is more likely to give a better indication of the likely passage of drugs through biological lipid membranes than partition coefficient experiments between water and organic solvents. For instance, it has been shown that, even in a simple series of drugs of the ephedrine class, the ranking of the compounds in terms of water–organic solvent partition coefficients varies with the organic solvent used (e.g. see Wilkinson, 1966a). Even in the rate of partition experiments the nature of the organic solvent can greatly influence the result, and non-physiological pH conditions may have to be used to get comparative values.

The excretion of a variety of basic drugs is dependent on the pH of the urine, and this is explicable in terms of increasing reabsorption of the unionized lipid-soluble form of the drug as the pH of the urine becomes less acid (Beckett & Rowland, 1965b,c; Beckett & Wilkinson, 1965a). Comparison of urinary excretion experiments with buccal absorption experiments is therefore of interest.

The urinary excretion of (+)- and (−)-amphetamine (Beckett & Rowland, 1965b), methylamphetamine (Beckett & Rowland, 1965c), ephedrine and methylephedrine (Beckett & Wilkinson, 1965a), all Class 1 drugs in buccal absorption, is maximal at a urinary pH less than about 5·0 (70% excreted unchanged); it is reduced at pH values greater than 7·5 (5–30% excreted unchanged). Smooth curves (see, for example, Wilkinson, 1966b) were obtained for these drugs and changes in the urinary flow rate did not affect the rate of their excretion at pH values of 5·0. On the other hand, the urinary excretion of norephedrine (Wilkinson, 1966b), a Class 2 drug in buccal experiments, was not greatly affected by changes in urinary

flow rate or by large changes in urinary pH, e.g. 94% excreted unchanged at pH 5·0 and 86·4% at pH 8·0. The urinary excretion rate of chlorpheniramine (Beckett & Wilkinson, 1965b) and methadone (J. F. Taylor, personal communication), Class 3 drugs in buccal experiments, was greater at pH 5·0 than at 8·5 but this difference was not so marked as with amphetamine; furthermore, at pH 5·0, small changes in pH caused fluctuations in the urinary excretion rate which were not observed with amphetamine with the pH about 5·5. Fluctuations in the rate of excretion of Class 3 drugs occurred unless the subject was water loaded throughout the trial to ensure a large and relatively constant urine flow rate.

Thus in examples which have been examined to date, there is a direct relation between the buccal drug absorption–pH curves, and the way in which renal tubular absorption of drugs changes with pH. Experiments such as we have described for the buccal absorption of drugs at different pH values give results of value when considering the potential of drugs to pass through lipid body membranes. Such experiments are likely to be of more value than simple partition coefficient experiments. Buccal absorption measurements have led to a rationalization of some apparent anomalies. For example, at a urinary pH of 5·0 dimethylamphetamine is largely excreted as unchanged drug and its metabolites methylamphetamine and amphetamine; the total drug recovered in the urine being 65% in 24 hr; under the same conditions benzphetamine is excreted only about 0·5% unchanged over 16 hr with less than 20% as methylamphetamine and amphetamine (Beckett, Tucker & Moffat, 1967). Significantly different routes or amounts of metabolism other than de-alkylation would not be expected in either drug. Examination of the differences in the buccal absorption curves for dimethylamphetamine and benzphetamine indicate that at pH 5·0 the biological partitioning characteristics of benzphetamine would result in its being completely reabsorbed in the kidney tubules and thus it would only be excreted in negligible quantities.

The proposed buccal absorption test may be used for rapid screening to determine the relative order of partitioning into biological lipids of a wide variety of drugs at widely different pH values. In addition, from the shape of the curves of absorption against pH, it is possible to predict the excretion profiles of these drugs in man under normal conditions and under conditions of controlled pH of urine provided hepatic recycling of the drugs does not occur. These predictions are especially valuable if a drug is not extensively metabolized in the body when the urinary pH is not controlled. If the calibration curves of a drug extracted from water and urine are different as is the case for fenfluramine and to a lesser extent norfenfluramine (Beckett & Brookes, 1967), then there may be less reabsorption in the kidney tubules than predicted from the buccal absorption test. To obtain the required information, only a fraction of a milligramme of a drug needs to be absorbed *into* the body and even this amount can be reduced by *immediately* rinsing out the mouth with buffer of pH 4·0 to extract some of the absorbed drug from the lipid of the mouth.

If, in the buccal absorption test, the shape of curve for the drug assigns it to Class 1, then it can be predicted that the rate of excretion of the drug will vary greatly with fluctuating urinary pH, i.e. normal conditions. It will be possible to obtain increased excretion and a smooth time excretion curve by making the urine acid (pH about 5); variation of pH between 4·8 and 5·1 will cause no fluctuations in the smooth curve. With a urinary pH of 7·5 and above, less than one-fifth of the amount of drug excreted in 16 hr at pH 5·0 will be excreted. At pH values more acidic than 6·0, normal changes in urinary flow rate will not cause fluctuations in the excretion-time curve. The biological half-life will be under 6 hr if the urine is kept about pH 5·0, and about 12 hr under normal conditions with urine fluctuating between pH 5·0 and 7·5 (mean value pH 6·2) throughout the trial.

If the buccal absorption test gives a curve which assigns the drug to Class 2, its rate of excretion will vary only slightly with the normal conditions of fluctuating urine pH values. Administration of ammonium chloride to make the urine acid will result in only a slight increase in the excretion of the drug, while administration of sodium bicarbonate to render the urine alkaline will produce only a slight reduction in the amount excreted in a given time. A substantial increase in urinary flow rate will cause increased drug excretion and the effect will be more pronounced under conditions of alkaline than acidic urine. The biological half-life will be less than 6 hr under conditions of acidic or normal urinary pH, and less than 8 hr even when the urine is pH 7·5 and above, provided that metabolism of the drug is proceeding slowly with at least 50% of the drug recovered unchanged where the urine is acidic.

If the buccal absorption test results in curves indicating that a drug belongs to Class 3, a fluctuating urinary pH will result in failure to obtain a smooth curve for an individual when excretion-time curves are plotted for the drug. Rendering the urine acid (pH 4·8 to 5·0) will increase the amount of drug excreted and reduce the fluctuations in the drug excretion-time curve but will not completely abolish them if small changes in pH (about $\pm 0{\cdot}2$ pH unit), are occurring. If urinary pH can be maintained at around 4·5, a further increase in the amount of excretion and reduction in fluctuations in the drug excretion-time curve will occur, but large changes in rate of urinary flow will still cause fluctuations in this curve. Maximum water loading to increase the urinary flow rate will give the maximum excretion and smoothest drug excretion-time curve for a drug of this type. Even under acidic conditions of pH, the biological half-life will be more than 16 hr if at least 30% is recovered unchanged. As the buccal absorption curve increases with change in pH from 5 to 7 for a series of drugs of this class, the amount of drug excreted unchanged in the urine decreases substantially, i.e. an absorption of 10–20% in this range in the buccal test and one of 5% would be expected to give recoveries of not more than 5% and not more than 30% respectively of unchanged drug provided their metabolic rate constants were similar.

For class 4 drugs, the prediction of the % excreted in the urine may be made from the relative shapes of the curves below and above pH 7·0. If the curve above pH 7·0 is steep relative to the portion below pH 7·0, urinary excretion will be pH dependent under normal pH conditions of urine; the level in the buccal absorption curve between pH 5·0 and 7·0 will indicate whether much kidney tubular reabsorption of the drug will occur under normal and acidic conditions of urine. If the buccal absorption curve has only a gentle upward slope between pH 5·0 and 8·5 and values above 5% occur between pH 5·0 and 7·0, then this drug will not show marked fluctuations in the drug excretion-time curve under uncontrolled urinary pH conditions and large differences between drug excreted under conditions of urinary acid and alkaline controlled pH will not occur.

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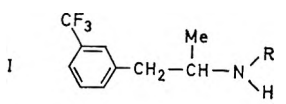
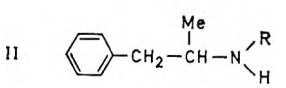
The absorption and urinary excretion in man of fenfluramine and its main metabolite

A. H. BECKETT AND L. G. BROOKES

The urinary excretion of fenfluramine and its main metabolite, norfenfluramine, was examined after oral administration of (±)- and (+)-fenfluramine hydrochloride and of (±)-norfenfluramine hydrochloride to man. The rate of excretion of both substances was shown to depend on urinary pH, fluctuations in excretion rate being associated with changes in urinary pH. The excretion of fenfluramine and norfenfluramine was measured after the administration of (±)- and (+)-fenfluramine hydrochloride to subjects whose urine had been made acidic or alkaline by their taking ammonium chloride or sodium bicarbonate respectively. Under these conditions fluctuation in rates of excretion were abolished (alkaline urine) or minimized (acidic urine), total recovery of both amines being greater when the subject's urine is acidic than when it is alkaline. *N*-De-ethylation of both (±)- and (+)-fenfluramine was considerable.

FENFLURAMINE [1-(3-trifluoromethylphenyl)-2-ethylaminopropane] (Ia) is a new, non-stimulant anorectic drug, superficially resembling amphetamine (IIa) and methylamphetamine (IIb). It is now known to be de-ethylated in man to norfenfluramine [1-(3-trifluoromethylphenyl)-2-aminopropane] (Ib).





Earlier work in man (Duhault & Fenard, 1965) suggested that after oral administration of fenfluramine hydrochloride 38% of the administered dose would be recovered from the urine over a five-day period, little metabolism to norfenfluramine was reported, but the analytical procedure was non-specific and would not have differentiated between fenfluramine and norfenfluramine. Using the more specific technique of gas chromatography for analysis, we have examined the effect of urine pH on the excretion of fenfluramine and norfenfluramine.

Experimental

ORAL ADMINISTRATION OF FENFLURAMINE AND NORFENFLURAMINE AND COLLECTION OF URINE

The general procedure adopted for diet and the collection of urine, was similar to that previously described by Beckett & Rowland (1965a). Urine was rendered alkaline and maintained thus by oral administration of

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sodium bicarbonate as previously described (Beckett & Rowland, 1965a). To produce and maintain an acidic urine, 1 g ammonium chloride (0·5 g enteric coated tablets) was administered five times daily on the two days preceding the first day of the trial: this dosage was continued throughout the trial. Urine was extracted by the method of Beckett & Rowland (1965a). Urine samples were analysed by gas-liquid and thin-layer chromatography. Preparative thin-layer chromatography was also used to obtain samples of fenfluramine and norfenfluramine for infrared analysis.

The subjects (males, age 25–45 years) were given an oral dose of 20 mg of either fenfluramine or norfenfluramine hydrochloride (equivalent to 17·27 and 16·96 mg of the base respectively) in 60–80 ml aqueous solutions. Urine, alkaline or acidic (for both drugs) or pH uncontrolled (fenfluramine), was collected hourly or 2-hrly for 48 hr.

The drugs, 20 mg in 5 ml aqueous solutions, were also administered intravenously to subjects with induced acidic urine. Urine was collected every 30 min for the first 4–5 hr, hourly for the next 10–12 hr, and 4-hrly on the second day of the trial with a final collection of the 40–48 hr sample.

The amount of norfenfluramine excreted was calculated as a percentage of the dose of fenfluramine hydrochloride administered.

GAS-LIQUID CHROMATOGRAPHY

Analysis by gas-liquid chromatography was as described for amphetamine and methylamphetamine (Beckett & Rowland, 1965a,b) using Column A below. Amphetamine sulphate (10 μg/ml in water) was used as an internal standard in place of *NN*-dimethylaniline, the peak for which interferes with those of fenfluramine and norfenfluramine. This aqueous solution was added to the urine at the start of the extraction procedure. Calibration curves were prepared using water, and eight samples from different men of urine which contained 0·1–10·0 μg/ml of both fenfluramine and norfenfluramine.

A Perkin Elmer F11 gas chromatograph with a flame ionization detector was used with the following columns:

Column A. Stainless steel tube, two metres in length $\frac{1}{8}$ inch o.d. packed with acid washed, DMCS treated Celite 545, coated with 5% potassium hydroxide and 10% Carbowax 6000. Column temperature 140°. Injection block temperature 250°. Nitrogen flow rate 27 ml/min at room temperature. Hydrogen pressure 15 lb/in^2. Air pressure 26 lb/in^2. Stream split ratio 1 : 5.

Column B. Stainless steel tube, one metre in length. $\frac{1}{8}$ inch o.d. packed with acid washed, DMCS treated Chromosorb G, coated with 5% potassium hydroxide and 2% Carbowax 20 M. Column temperature 177°. Injection block temperature 250°. Nitrogen flow rate 33 ml/min at room temperature. Hydrogen pressure 15 lb/in^2. Air pressure 20 lb/in^2. Stream split ratio 1 : 5.

Column C. Glass column, two metres in length, $\frac{1}{4}$ inch o.d. packed with acid washed, DMCS treated Chromosorb G coated with $2\frac{1}{2}$% SE 30.

Column temperature 160°. Injection block temperature 250°. Nitrogen flow rate 17 ml/min at room temperature. Hydrogen pressure 20 lb/in². Air pressure 28 lb/in². No stream splitter used.

In addition, some of the ether extracts of urine were treated as follows: (a) about 2 μl acetone was added to 5 μl of the concentrate, heated at about 60° for 1 hr then injected into column A (see Table 1), (b) two portions of the concentrate were treated separately with propionic and acetic anhydrides: each was injected into columns B and C. Fenfluramine and norfenfluramine were added to alkaline and to acidic urines (1 μg drug/ml), stored at 4°, and the urine solution assayed every third day for two weeks.

TABLE 1. RETENTION TIMES OF FENFLURAMINE AND NORFENFLURAMINE AND SOME DERIVATIVES ON COLUMNS

Column	Substance	Retention time (min)
A	Fenfluramine	4·8
	Norfenfluramine	5·8
	Norfenfluramine + acetone	5·0
B	Fenfluramine – acetyl derivative	1·7
	Norfenfluramine – acetyl derivative	3·0
	Fenfluramine – propionyl derivative	1·7
	Norfenfluramine – propionyl derivative	3·0
C	Fenfluramine – acetyl derivative	2·8
	Norfenfluramine – acetyl derivative	2·2
	Fenfluramine – propionyl derivative	3·5
	Norfenfluramine – propionyl derivative	2·5

THIN-LAYER CHROMATOGRAPHY

Glass plates, 20 × 20 cm, prepared with either silica gel G (Merck) or aluminium oxide G (Merck) 0·25 mm thick were developed at room temperature with the solvents (a) to (h) below. Fenfluramine was visualized by a Dragendorff's spray (Stahl, 1962), and norfenfluramine by diazotized *p*-nitroaniline (Wickström & Salvesen, 1952) oversprayed with Dragendorff's reagent. Alternatively both substances may be visualized by an ethanolic solution of Bromothymol Blue.

The following systems were used: (a) Chloroform–diethylamine (9 : 1). (b) Ethanol 96%–ammonia 25% (4 : 1). (c) Butanol–acetic acid–water (4 : 1 : 5). (d) Acetone–benzene–ethanol 96%–ammonia 880 (40 : 50 : 5 : 5) plus 2½ ml dilute sulphuric acid. (e) Methanol–chloroform (1 : 4). (f) Methanol–acetone–triethanolamine (50 : 50 : 1 : 5). (g) Methanol–acetone (1 : 1). (h) Chloroform–ethanol (8 : 2).

Results

STRUCTURE OF THE METABOLITE

Gas chromatographic analysis of ethereal extracts of urines from subjects taking fenfluramine hydrochloride gave two peaks. One was identical in retention time to that of authentic fenfluramine and the second to that of authentic norfenfluramine. That the two peaks were fenfluramine and norfenfluramine was further confirmed by preparing the propionyl and acetyl

derivatives of both bases, and also the acetone derivative of norfenfluramine, and comparing their retention times with those of corresponding derivatives of authentic samples of the bases. Identical retention times were obtained.

Thin layer-chromatography in systems (e), (f) and (g), gave an Rf value of the metabolite identical to that of norfenfluramine. Preparative thin-layer chromatography gave an oil whose infrared spectrum was identical to that of norfenfluramine.

Quantitative analysis of fenfluramine and norfenfluramine

From the gas chromatographic results, calibration curves were derived by plotting the ratios of the peak heights of either fenfluramine or norfenfluramine to amphetamine for known concentrations of the drugs in urine. The curves for fenfluramine and norfenfluramine were linear over the range 0·1–1·0 μg of drug/ml of urine. Interfering peaks were not found on analysing several samples of urine from each of many subjects to whom no drug had been administered. The calibration curves for fenfluramine and norfenfluramine obtained using water in place of urine were also linear. Whereas the extraction of both bases from water was absolute, from urine it was not so, and the ratios of the slopes of the calibration curves using urine and water were 1 : 1·23 (fenfluramine) and 1 : 1·08 (norfenfluramine). Extraction of both drugs was the same from the urine of several subjects. Constant levels of both drugs in acidic and alkaline urines indicated stability in these media for at least two weeks at 4°.

URINARY EXCRETIONS

After oral doses of fenfluramine. The excretion rates of fenfluramine and its metabolite norfenfluramine varied with urinary pH and were influenced by urine output (Fig. 1) (see Table 2). In subjects with an alkaline urine, the excretion of fenfluramine over 48 hr was 0·4–0·7% and 1·2–1·8% for norfenfluramine. In subjects with an acidic urine, excretion was much higher, and fluctuations in the excretion rate of fenfluramine and its metabolite observed during experiments where the pH of urine was not controlled were almost abolished. Typical excretion curves of fenfluramine and norfenfluramine are given in Fig. 2. The excretion rate of fenfluramine reached a maximum about 3–5 hr after administration of the drug and then fell exponentially. The excretion rate of norfenfluramine reached a maximum 7 hr after administration of fenfluramine.

After oral doses of norfenfluramine and with the urine acidic, excretion was similar to that of fenfluramine, the maximum excretion rate again occurring about 3–5 hr after drug administration.

After intravenous doses of fenfluramine. Fluctuations in the excretion rate of fenfluramine occurred during the first 5 hr, after which a logarithmic decline was observed. Table 3 gives the total urine recoveries of unchanged fenfluramine and its metabolite norfenfluramine over 48 hr after intravenous administration of fenfluramine hydrochloride to subjects with acidic urine.

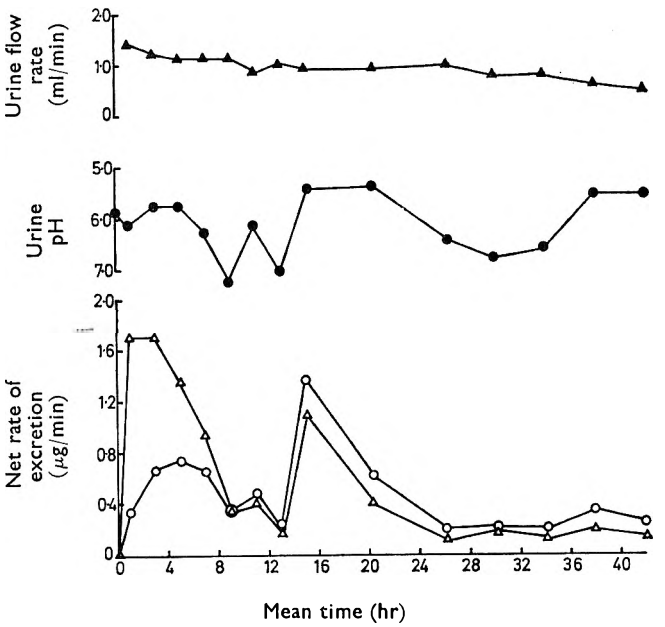

FIG. 1. Urinary excretion of fenfluramine and norfenfluramine over 48 hr from a subject (N.S.) with no urine pH control, who had taken an oral dose of 20 mg(+)-fenfluramine hydrochloride. △, Fenfluramine; ○, norfenfluramine; ●, urine pH; ▲, urine flow rate ml/min.

TABLE 2. URINARY EXCRETION OF FENFLURAMINE AND NORFENFLURAMINE OVER A PERIOD OF 48 HR IN SUBJECTS HAVING TAKEN A SINGLE ORAL DOSE OF 20 MG FENFLURAMINE HCl UNDER CONDITIONS OF ALKALINE AND UNCONTROLLED URINE pH

Subject	Fenfluramine hydrochloride	Uncontrolled pH			Alkaline urine		
		% dose excreted			% dose excreted		
		Fenfluramine	Norfenfluramine	Total	Fenfluramine	Norfenfluramine	Total
N.S.	(+)	7·8	8·4	16·2	0·73	1·62	2·35
C.A.	(+)	10·0	13·8	23·8	0·44	1·75	2·19
A.B.	(+)	2·7	3·1	5·8	—	—	—
L.B.	(±)	5·3	3·6	8·9	0·76	1·21	1·97

After intravenous doses of norfenfluramine. Fluctuations in the excretion rate of norfenfluramine were similar to those with fenfluramine, again these were followed by a logarithmic decline. Table 4 gives the urinary excretion data of norfenfluramine over 48 hr after intravenous injection of the hydrochloride to subjects with acidic urine.

Clinical Effects. No stimulant effects were observed after either (+)- or (±)-fenfluramine hydrochloride, but a slight nausea was experienced by two subjects taking norfenfluramine hydrochloride.

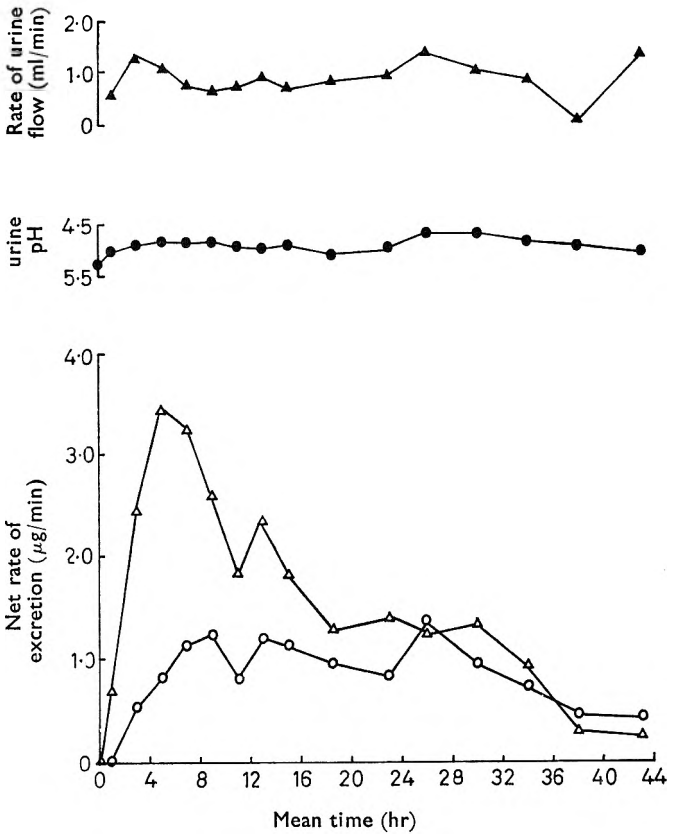

FIG. 2. Urinary excretion of fenfluramine and norfenfluramine over 48 hr from a subject (B.B.) under conditions of acidic urine, who had taken an oral dose of 20 mg (±)-fenfluramine hydrochloride. △, Fenfluramine; ○, norfenfluramine; ▲, urine flow rate ml/min; ●, urine pH.

TABLE 3. URINARY EXCRETION OF FENFLURAMINE AND NORFENFLURAMINE OVER 48 HR AFTER A SINGLE DOSE OF 20 MG FENFLURAMINE HYDROCHLORIDE TO SUBJECTS WITH ACIDIC URINE (pH ABOUT 5)

Subject	Fenflur-amine hydro-chloride	Oral administration % dose excreted			Intravenous injection % dose excreted		
		Fenflur-amine	Nor-fenflur-amine	Total	Fenflur-amine	Nor-fenflur-amine	Total
G.D.	(±)	29·1	22·3	51·4	33·0	17·5	50·5
C.B.	(±)	24·9	17·5	42·4	32·7	19·7	52·4
B.B.	(±)	23·4	13·1	36·5	24·5	11·4	35·9
L.B.	(±)	19·9	17·3	37·2	20·4	12·3	32·7
N.S.	(±)	17·9	15·5	33·4	—	—	—
A.B.	(−)	24·3	12·0	36·3	—	—	—
C.A.	(−)	23·1	22·2	45·3	—	—	—

TABLE 4. URINARY EXCRETION OF NORFENFLURAMINE OVER 48 HR AFTER A SINGLE DOSE OF 20 MG (±)-NORFENFLURAMINE HYDROCHLORIDE, GIVEN ORALLY OR INTRAVENOUSLY TO SUBJECTS WITH ACIDIC URINE (pH ABOUT 5)

Subject	% Dose excreted	
	Oral administration	Intravenous injection
B.B.	37·62	'43·4
G.D.	37·1	40·8
L.B.	24·33	31·1
G.K.	—	31·9

Discussion

De-alkylation similar to that yielding norfenfluramine has been shown for mephentermine (Walkenstein, Chumakow & Seifter, 1955) and methylamphetamine (Beckett & Rowland, 1965b).

Urinary recovery of fenfluramine and also norfenfluramine is similar after both oral and intravenous administration of the drugs. This indicates complete absorption of both drugs from the gastrointestinal tract. Slight differences in recoveries may be explained by difference in urinary pH (see later). The ratio of maximum to minimum excretion of fenfluramine between subjects with uncontrolled urine pH is 1:4·0, but between subjects with acidic urine is only 1:1·5. This demonstrates the constancy in urinary excretion of this drug, subject to subject, with acidic urine and, in contrast, the variation between subjects with no urinary pH control.

As with amphetamine, the observed fluctuations in the excretion rate of the compounds when the urinary pH was not controlled may be partly explained by the non-ionic diffusion of bases in the kidney (Milne, Scribner & Crawford, 1958; Weiner & Mudge, 1964) although the flow rate of urine appears to affect excretion of the compounds at any urine pH $> 5{\cdot}1$. After intravenous injection, the rapid fall in the rate of excretion of both fenfluramine and norfenfluramine, and the 3–4 hr fluctuation period suggests that the drug is undergoing distribution in the body tissues, with subsequent release into the blood stream as equilibrium is being reached in those tissues.

The relatively low recoveries (see Tables 2 and 3) of unchanged fenfluramine and norfenfluramine, even under conditions of acidic urine, might appear to suggest rapid metabolism of these compounds in man. Under similar conditions, the superficially related compounds amphetamine and methylamphetamine give urinary recoveries of approximately 70% (Beckett & Rowland, 1965a,b). For methylamphetamine and amphetamine the elimination half-lives are about 5·0 (Beckett & Rowland, 1965a,b) whereas for fenfluramine and norfenfluramine it is about 11 hr—if metabolism of these fluoro-compounds was more rapid than that of the amphetamines, then a much shorter half life for fenfluramine and norfenfluramine would be expected. The differences in urinary recoveries may be due to an inability of the body to excrete fenfluramine and norfenfluramine rapidly, either because of serum binding or of kidney tubular

reabsorption of the drugs occurring even at urine pH 5·0, with consequent retention in the body. Table 3 shows that recovery of norfenfluramine after oral or intravenous administration is about 35%. The recovery of norfenfluramine after administration of fenfluramine hydrochloride is about 20%, thus, the amount of norfenfluramine formed in the body after administration of fenfluramine hydrochloride is about 60%, indicating that de-ethylation of fenfluramine to norfenfluramine is the major metabolic route for this compound.

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SHORT COMMUNICATION

Blood concentrations of pethidine and pentazocine in mother and infant at time of birth

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DRUG-PLACENTAL transfer and problems related to its study have been extensively reviewed (Baker, 1960; Hagerman & Villee, 1960; Moya & Thorndike, 1962; Villee, 1965). There are a number of repetitive and conflicting clinical findings (Shnider & Moya, 1964), but a contrasting lack of controlled biokinetic studies, especially in man.

Owing to the use of non-specific assay methods the significance of the results to date is in doubt, since much previous work (Apgar, Burns & others, 1952) on the study of blood, plasma or urine levels of pethidine has been based on the methyl orange complexing method of Brodie, Udenfriend & others (1945) and Brodie, Udenfriend & Dill (1947). It is known that nicotine (pK_a 8·07) interferes with the methyl orange assay even when modifications such as washing with buffer solutions are used (Beckett, Rowland & Triggs, 1965). Such interference may reasonably be assumed for other bases of similar pK_a and lipid solubility.

Studies by Apgar & others (1952) on the ratios of maternal plasma drug levels to cord plasma drug levels have included results where local anaesthetics were administered. Beckett, Boyes & Parker (1965) have shown that lignocaine (pK_a 7·86), given epidurally during labour, traverses the placenta in significant concentrations. The similarity of the pK_a values of pethidine (8·72, Beckett, 1956), nicotine and some local anaesthetics means that unequivocal estimation of pethidine and pentazocine by the methyl orange technique would be almost impossible if these other bases were present.

The more specific method of gas-liquid chromatography allows accurate measurement of blood levels of pethidine and pentazocine, separately and combined, in the presence of other bases, and it has now been applied to a study of these drugs in man.

Measurements were made of the relative blood levels of pethidine and pentazocine in mother and infant at parturition. Since Low (1963) has shown that the blood of newborn infants is more acidic then that of the mother, it might be expected that the blood level of basic drugs in the infant would be higher than in the mother if placental transport occurred by rapid passive diffusion of the unionized drug. Results to date (Table 1) show that blood concentrations of pentazocine in the newborn are usually less than in the mother, whereas those of pethidine may be higher. Where each drug was administered singly to the mother during labour, assays

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revealed large intersubject variation in the ratios of drug concentrations in cord and maternal blood. To determine the blood concentrations of *both* drugs in the cord and mother under identical conditions, pentazocine and pethidine were administered *simultaneously* to the mother. In this type of experiment the ratio of concentration of drug in cord to the concentration of drug in maternal blood was higher for pethidine than for pentazocine (see Table 1).

TABLE 1. MATERNAL AND CORD BLOOD CONCENTRATIONS OF PETHIDINE AND/OR PENTAZOCINE AFTER ADMINISTRATION TO WOMEN DURING LABOUR

Drug	Patient	Dose(mg) and route	Time interval before birth hr	Time interval before birth min	µg analgesic base per ml blood Maternal	µg analgesic base per ml blood Cord	Ratio Cord level: Maternal level
Pentazocine	A	48 i.m.	1	50	0·25	0·11	0·4
"	B	" "	1	05	0·25	0·15	0·6
"	C	48 "	2	30	0·31	0·23	0·7
"	D	24 i.v. 48 i.m.	0 6	55 15	0·12	0·07	0·6
Pethidine	E	120 " " "	4 2	00 00	0·77	0·58	0·8
"	F	" "	1	50	0·56	0·37	0·7
"	G	" "	3	15	0·27	0·27	1·0
"	H	" "	1	50	0·27	0·32	1·2
"	J	" " " "	5 3	25 00	0·38	0·49	1·3
Pentazocine and pethidine	K	24 " 60 "	16 6	45 45	0·014 0·12	0·01 0·15	0·7 1·3
Pentazocine and pethidine	L	24 " 60 "	2	40	0·04 0·15	0·02 0·34	0·5 2·3
Pentazocine and pethidine	M	24 " 60 "	1	45	0·11 0·27	0·08 0·43	0·73 1·59
Pentazocine and pethidine	N	" "	2	00	0·10 0·28	0·07 0·33	0·70 1·18
Pentazocine and pethidine	O	" "	3	25	0·03 0·30	0·02 0·25	0·66 0·83
Pentazocine and pethidine	P	" "	5 1	47 & 57	0·06 0·30	0·04 0·36	0·67 1·20

These preliminary results may be explained if pethidine is transferred across the placenta more rapidly than pentazocine. Controlled studies on the excretion of pethidine and pentazocine in the urine of men indicate that pentazocine is metabolized to a greater degree than pethidine (see Table 2).

TABLE 2. THE RECOVERIES OF PETHIDINE AND PENTAZOCINE IN THE URINE FOR A 48 HR PERIOD FOLLOWING INTRAVENOUS ADMINISTRATION TO MAN

Subject	Drug	Dose	Urinary pH	Percentage unchanged drug recovered in the urine
P.A.	Pethidine	50 mg	5·03–5·29	35
J.F.T.	"	"	4·57–5·05	31
P.O'D.	Pentazocine	20 mg	4·67–4·87	11
J.F.T.	"	"	4·84–5·08	13

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her patients in the drug-transplacental studies; the Bayer Products Company, Surbiton, Surrey, for supplying pentazocine. One of us (J.F.T.) thanks the S.R.C. for a grant in support of this research.

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Neuromuscular blocking agents: structural modification of (+)-tubocurarine and (−)-curarine

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(+)-Tubocurarine chloride and *OO*-dimethyl-(+)-tubocurarine iodide have been *N*-demethylated to (+)-tubocurine and *OO*-dimethyl-(−)-tubocurine. These bases, (−)-curine and its *OO*-dimethyl ether, have been converted to a new series of quaternary derivatives analogous to (+)-tubocurarine chloride. The pharmacological action of these derivatives has been examined.

DESPITE widespread studies of bis- and poly-quaternary ammonium compounds of the tubocurarine type as potential neuromuscular blocking agents, the examination of substances derived directly from (+)-tubocurarine and its stereoisomers (Table 1) has been largely neglected with the exception of a few readily accessible derivatives. The isomeric (+)-chondocurarine (I, R,R^2 = Me; R^1,R^3 = H) which has the same absolute stereochemistry as (+)-tubocurarine (I, R,R^1 = Me; R^2,R^3 = H), but differs only in the positions of one methoxyl and one hydroxyl group, has been reported to be about three times more potent than (+)-tubocurarine in the rabbit head drop test [Wintersteiner (1959) quoting the unpublished work of Holaday and Varney]. Complete *O*-methylation of either (+)-chondocurarine or (+)-tubocurarine gives *OO*-dimethyl-(+)-tubocurarine (II) which is similarly reported by Wintersteiner (1959) and by Collier, Paris & Woolfe (1948) to have about nine times the potency of (+)-tubocurarine. The corresponding *OO*-diethyl, di-n-butyl and di-n-benzyl ethers, however, show diminishing potency with increasing size of alkylating substituent, though the diethyl ether still has approximately twice the potency of (+)-tubocurarine (Table 1).

The four tubocurarine stereoisomers (+)- and (−)-tubocurarine, and (+)- and (−)-curarine, show marked variation in potency on the rat diaphragm (King, 1946, 1947) and in the rabbit head drop test (Wintersteiner, 1959) (Table 1). Some rationalization of these observed potencies might be possible if their respective molecular conformations were considered from the standpoint of their component tetrahydroisoquinoline moieties. The restrictions placed on each of the heterocyclic rings by fusion with a benzenoid ring necessitate adoption of a half-chair or skew conformation in which four of the heterocyclic ring atoms are planar, and the remaining two (of which one is the quaternary nitrogen) are situated one above and one below the general plane of the rings.

Two conformations are possible, therefore, for a dimethotetrahydroisoquinolinium salt with the quaternary nitrogen centre either above or below the plane of the molecule as a whole. Of these, the former would seem to be preferred in the L-(+)-series (*S*) and the latter in the D-(−)-series (*R*) (Fig. 1). The linking of two such conformations in the four

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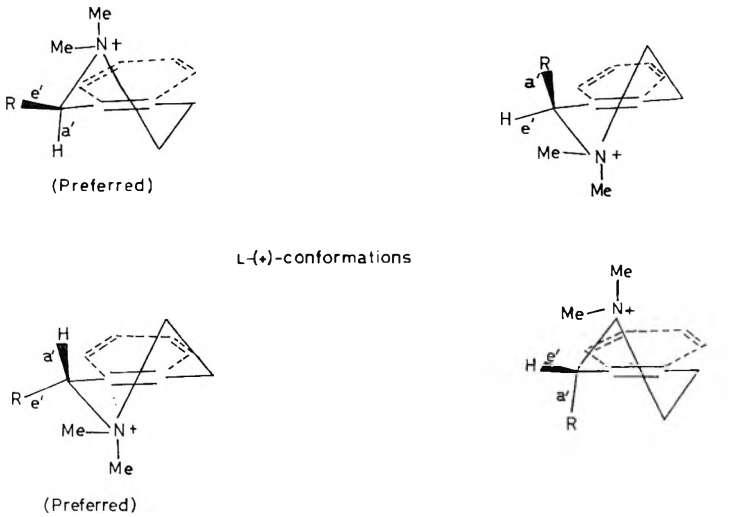


FIG. 1.

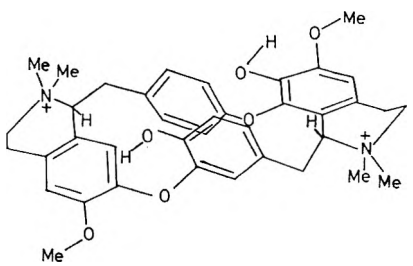


FIG. 2. (+)-Tubocurarine.

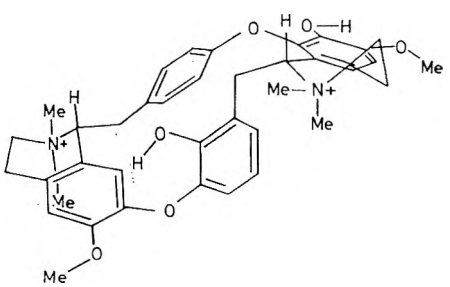


FIG. 3. (−)-Curarine.

tubocurarine isomers leads to the conformations shown in Figs 2 [(+)-tubocurarine] and 3 [(−)-curarine]. In the tubocurarines, the two quaternary centres project one above and one below the general plane of the molecule, whilst in the curarines both quaternary centres project to the same side.

This apparent correlation of potency with conformation in the parent alkaloids, however, is confused by the anomaly that *OO*-dimethyl-(+)-tubocurarine is reported to be seven times more potent than *OO*-dimethyl-(−)-curarine in the rabbit (Table 1), though some uncertainty must be

TABLE 1. RELATIVE POTENCIES OF ETHERS DERIVED FROM (+)-TUBOCURARINE AND ITS STEREOISOMERS ON THE RABBIT HEAD DROP ASSAY[1]

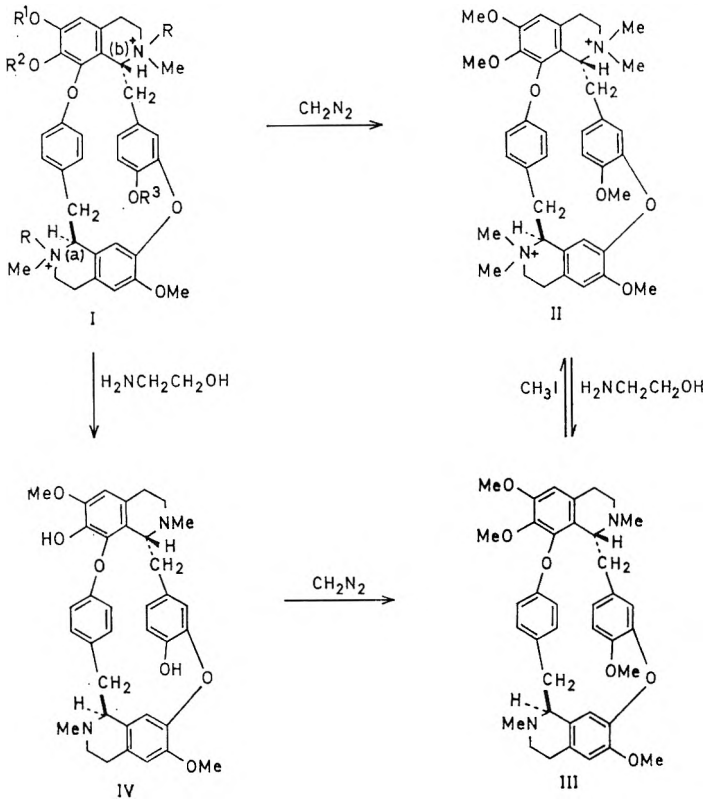
Compound	Structure				Absolute configuration[2]		Relative potencies
	R	R^1	R^2	R^3	Centre (*a*)	Centre (*b*)	
(+)-Tubocurarine	Me	Me	H	H	*S*	*R*	100
OO-Dimethyl-(+)-tubocurarine	Me	Me	Me	Me	*S*	*R*	870
OO-Diethyl-(+)-tubocurarine	Me	Me	Et	Et	*S*	*R*	190
OO-Di-n-butyl-(+)-tubocurarine	Me	Me	n-Bu	n-Bu	*S*	*R*	9
OO-Dibenzyl-(+)-tubocurarine	Me	Me	$PhCH_2$	$PhCH_2$	*S*	*R*	7
(−)-Tubocurarine	Me	Me	H	H	*R*	*S*	Slight[3]
(+)-Curarine	Me	Me	H	H	*S*	*S*	350 (63)[4]
OO-Dimethyl-(+)-curarine	Me	Me	Me	Me	*S*	*S*	– (133)[4]
(−)-Curarine	Me	Me	H	H	*R*	*R*	130
OO-Dimethyl-(−)-curarine	Me	Me	Me	Me	*R*	*R*	330
(+)-Chondocurarine	Me	H	Me	H	*S*	*R*	290
NN'-Diethyl-(+)-chondocurine	Et	H	Me	H	*S*	*R*	90
NN'-Dibenzyl-(+)-chondocurine	$PhCH_2$	H	Me	H	*S*	*R*	17

[1] Wintersteiner (1959).
[2] Cahn, Ingold & Prelog convention (1956).
[3] King (1947) 1/30–1/60 activity of (+)-tubocurarine on rat diaphragm.
[4] Marsh, Sleeth & Tucker (1948).

attached to these observations, since they are based on the unpublished work of Holaday and Varney, quoted by Wintersteiner (1959). Moreover, Collier & others (1948) have reported *OO*-dimethyl-(−)-curarine to be more potent than *OO*-dimethyl-(+)-tubocurarine in the mouse, though the relative potencies of the two compounds were reversed in the rat and no comparison appears to have been made in the rabbit.

The present work was therefore undertaken to confirm these results and to extend them by the examination of other factors, such as substitution at the quaternary centre, which may influence the shape of the molecule as a whole. Whilst both *NN'*-diethyl-(+)-chondocurine and *NN'*-dibenzyl-(+)-chondocurine are significantly less potent than chondocurine (Wintersteiner, 1959), the corresponding derivatives in the tubocurarine and curarine series have not been examined, nor has the effect of such substituents on the geometry of the molecule been considered. The present study therefore is concerned with the preparation and

pharmacological evaluation of analogous bis-quaternary salts of (+)-tubocurarine (−)-curarine and their respective *OO*-dimethyl ethers.



Chemistry

Preparation of the required tubocurarine derivatives depended on the availability of (+)-tubocurine (IV) and its *OO*-dimethyl ether (III), the tertiary bases corresponding to (+)-tubocurarine and *OO*-dimethyl-(+)-tubocurarine. Conditions were therefore required favouring *N*-demethylation of *OO*-dimethyl-(+)-tubocurarine (without racemization of the optical centres), rather than the alternative Hofmann elimination (King, 1939). *N*-Demethylation of *OO*-dimethyl-(+)-tubocurarine with lithium aluminium hydride (Kenner & Murray, 1950) was unsuccessful, in agreement with the results of Tomita & Ibuku (1962) for other bis-benzylisoquinolinium compounds.

Treatment of *OO*-dimethyl-(+)-tubocurarine chloride (II) with excess ethanolamine under reflux, however, yielded *OO*-dimethyl-(+)-tubocurine (III) in 58% yield (cf. Hünig & Baron, 1957). Similarly, *N*-demethylation of (+)-tubocurarine chloride proceeded in 48% yield. The identity of the products was confirmed by their ultraviolet absorption spectra, which

TABLE 2.

Compound	R in Structure (I) (R^1 = Me; R^2 = R^3 = H)	% Yield	M.p.	Reaction time (hr)	Formula	Found %		Required %	
						N	Halide	N	Halide
NN'-Diethyl-(+)-tubocurine iodide	C_2H_5	88	238–240°	24	$C_{40}H_{48}I_2N_2O_6.5H_2O$	2·85	25·2	2·8	25·4
NN'-Dipropyl-(+)-tubocurine bromide pentahydrate	C_3H_7–	50	232°	1·25	$C_{42}H_{52}Br_2N_2O_6.5H_2O$	3·0	17·8	3·0	17·2
NN'-Di-isopropyl-(+)-tubocurine iodide pentahydrate	iso-C_3H_7	70	238°	24	$C_{42}H_{52}I_2N_2O_6.5H_2O$	3·0	23·7	2·7	24·3
NN'-Dibutyl-(+)-tubocurine bromide pentahydrate	C_4H_9–	63	224°	24	$C_{44}H_{56}Br_2N_2O_6.5H_2O$	3·4	13·5	2·9	16·7
NN'-Dipentyl-(+)-tubocurine bromide pentahydrate*	C_5H_{11}–	77	229°	90	$C_{46}H_{60}Br_2N_2O_6.5H_2O$	3·2	16·4	2·8	16·4
NN'-Dihexyl-(+)-tubocurine bromide pentahydrate	C_6H_{13}–	69	200°	93	$C_{48}H_{64}Br_2N_2O_6.5H_2O$	2·6	16·4	2·8	15·8
NN'-Dioctyl-(+)-tubocurine bromide pentahydrate	C_8H_{17}–	65	193°	90	$C_{52}H_{72}Br_2N_2O_6.5H_2O$	3·4	13·6	2·6	14·9
NN'-Didecyl-(+)-tubocurine bromine pentahydrate	$C_{10}H_{21}$–	73	184°	140	$C_{56}H_{80}Br_2N_2O_6.5H_2O$	2·9	13·2	2·6	14·2
NN'-Diallyl-(+)-tubocurine bromide pentahydrate	$CH_2:CH.CH_2$–	72	218°	1·5	$C_{42}H_{48}Br_2N_2O_6.5H_2O$	3·0	18·6	3·0	17·2
NN'-(2-Ethoxyethyl)-(+)-tubocurine bromide pentahydrate	$C_2H_5OC_2H_4$–	95	217°	90	$C_{44}H_{56}Br_2N_2O_8.5H_2O$	3·1	14·9	2·8	16·0
NN'-(3-Phenylpropyl)-(+)-tubocurine bromide pentahydrate	$C_6H_5CH_2CH_2CH_2$–	82·3	214°	42	$C_{64}H_{60}Br_2N_2O_6.5H_2O$	3·2	15·6	2·9	16·2
NN'-(2-Phenylethyl)-(+)-tubocurine bromide pentahydrate	$C_6H_5CH_2CH_2CH_2$–	78	222°	60	$C_{62}H_{56}Br_2N_2O_6.5H_2O$	2·95	16·6	2·9	16·6

* Found: C, 54·7; H, 6·7. $C_{46}H_{60}Br_2N_2O_6.5H_2O$ requires C, 55·6; H, 7·7%.

TABLE 3.

Compound	R in Structure (I) ($R^1 = R^2 = R^3 = Me$)	% Yield	M.p.	Reaction time (hr)	Formula	Found %		Required %	
						N	Halide	N	Halide
NN'-Diethyl-*OO*-dimethyl-(+)-tubocurine iodide pentahydrate	C_2H_5-	81	210°	2	$C_{42}H_{52}I_2N_2O_6.5H_2O$	2·5	25·4	2·7	24·8
NN'-Dipropyl-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	C_3H_7-	60	202°	2·5	$C_{44}H_{56}Br_2N_2O_6.5H_2O$	3·1	18·4	2·9	19·4
NN'-Di-isopropyl-*OO*-dimethyl-(+)-tubocurine iodide pentahydrate	iso-C_3H_7-	69	218°	96	$C_{44}H_{56}I_2N_2O_6.5H_2O$	2·9	23·7	2·7	24·6
NN'-Dibutyl-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate*	C_4H_9	69	203°	48	$C_{46}H_{60}Br_2N_2O_6.5H_2O$	3·0	17·9	2·8	16·9
NN'-Dipentyl-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	C_5H_{11}	53	195°	24	$C_{48}H_{64}Br_2N_2O_6.5H_2O$	2·9	15·4	2·8	15·7
NN'-Dioctyl-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	C_8H_{17}	71	173°	90	$C_{54}H_{76}Br_2N_2O_6.5H_2O$	2·5	14·7	2·6	14·5
NN'-Didecyl-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	$C_{10}H_{21}-$	56	172°	48	$C_{58}H_{84}Br_2N_2O_6.5H_2O$	3·3	14·1	2·5	13·8
NN'-Dibenzyl-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	C_6H_5-	60	189°	48	$C_{50}H_{52}Br_2N_2O_6.5H_2O$	2·9	17·5	2·7	15·6
NN'-Diallyl-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	$CH_2 = CHCH_2-$	75	198°	1	$C_{44}H_{52}Br_2N_2O_6.5H_2O$	3·0	17·2	2·9	16·7
NN'-(2-Ethoxyethyl)-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	$C_2H_5OCH_2CH_2-$	100	211–213°	72	$C_{46}H_{60}Br_2N_2O_8.5H_2O$	2·6	15·6	2·7	16·0
NN'-(3-Phenylpropyl)-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	$C_6H_5CH_2CH_2CH_2-$	58·5	197°	42	$C_{56}H_{64}Br_2N_2O_6.5H_2O$	2·6	14·6	2·5	14·4
NN'-(2-Phenylethyl)-*OO*-dimethyl-(+)-tubocurine bromide pentahydrate	$C_6H_5CH_2CH_2-$	63	200°	10	$C_{54}H_{60}Br_2N_2O_6.5H_2O$	3·0	15·5	2·6	14·7

* Found: C, 55·4; H, 6·8. $C_{46}H_{70}Br_2N_2O_{11}$ requires C, 56·0; H, 7·1%.

showed maxima at 225 and 286 mμ in the same intensity ratio (*ca* 3:1) as the corresponding maxima in the spectrum of (+)-tubocurarine chloride. This is in contrast to the *cis*-methine resulting from the Hofmann elimination of laudanosine, which shows maxima at 215 and 294 mμ in the intensity ratio *ca* 2:1. *N*-Demethylation of laudanosine, isotetrandine, cycleanine and insularine methiodides by a similar method has been reported (Tomita & Takano, 1960).

The retention of optical configuration in the products was confirmed (*a*) by the conversion of (+)-tubocurine (IV) to *OO*-dimethyl-(+)-tubocurine (III) in a state of optical purity identical with that obtained by *N*-demethylation of *OO*-dimethyl-(+)-tubocurarine iodide (II), and (*b*) by methylation of *OO*-dimethyl-(+)-tubocurine (III) to *OO*-dimethyl-(+)-tubocurarine iodide (II).

Treatment of (+)-tubocurine, (−)-curine and their respective *OO*-dimethyl ethers with appropriate alkyl halides gave the quaternary compounds listed in Tables 2 to 5.

Cleavage of *OO*-dimethyl-(+)-tubocurine with sodium in liquid ammonia in benzene-toluene yielded D-(−)-laudanidine and L-(+)-*N*-methylcoclaurine. Isolation of these two fragments, and examination of their rotation in solvents of varying polarity provides further evidence for the absolute stereochemistry of (+)-*N*-methylcoclaurine as L(*S*). This was originally established by Tomita & Kunitomo (1962) and Ferrari & Deulofeu (1962). These observations also provide independent confirmation of the absolute stereochemistry of (+)-tubocurarine as indicated in (I).

EXPERIMENTAL

Throughout, Rf values refer to the system methanol : chloroform (1 : 10) on alumina (0·35 mμ).

OO-*Dimethyl-(+)-tubocurarine iodide.* (+)-Tubocurarine chloride (5·28 g) was heated (2 hr: 70°) with ethanolic potassium hydroxide (55 ml, 0·5N) and methyl iodide (7 ml). The solution was reduced to small bulk (*ca* 10 ml), cooled and the resultant solid (6·7 g) collected. This solid in water (200 ml) was re-precipitated with potassium iodide (0·9 g) yielding *OO*-dimethyl-(+)-tubocurarine iodide (6·27 g), m.p. 266°, $[\alpha]_D$ + 179° (*c*, 1·3 in MeOH). Dutcher (1946) gives m.p. 266°, $[\alpha]_D$ + 172° (*c*, 1·02 in MeOH).

OO-*Dimethyl-(+)-tubocurine* (from *OO*-dimethyl-(+)-tubocurarine iodide).

1. Lithium aluminium hydride (2·09 g) was refluxed in tetrahydrofuran (75 ml) (1 hr) then finely powdered *OO*-dimethyltubocurarine iodide (5·27 g) was added. The mixture was refluxed with continuous stirring until the evolution of methane ceased (*ca* 1 hr). Excess lithium aluminium hydride was decomposed at 0° with water (9 ml) and 20% sodium hydroxide (1·5 ml), and the solution filtered. The filtrate was acidified with hydrochloric acid, reduced to *ca* 15 ml, the base liberated by the addition of sodium hydroxide, and extracted with ether (3 × 50 ml). The combined

extracts (dried Na_2SO_4) yielded a syrup (98 mg) which was placed on alumina (12 g); benzene (75 ml), affording a yellow solid (80 mg), m.p. 90°.

2. *OO*-Dimethyltubocurarine iodide (1·18 g) in ethanolamine (60 ml) was refluxed (45 min), the solution cooled, and aqueous potassium hydroxide (1·14 g in 30 ml) added. The alkaline solution was extracted with light petroleum (b.p. 40–60°, 1·15 litre) in the usual way to yield OO-*dimethyl*-(+)-*tubocurine* (0·402 g, 58%), m.p. 98°, $[\alpha]_D$ + 168° (*c*, 1·25 in MeOH. Rf = 0·89, λ_{max} 225 mμ, 286 mμ, log ϵ 4·487 and 4·023 respectively. Found: C, 72·4; H, 6·5; N, 4·5. $C_{38}H_{42}O_6N_2 \cdot \frac{1}{2}H_2O$ requires C, 72·4; H, 6·8; N, 4·4%.

(+)-*Tubocurine*. Tubocurarine chloride (0·8 g) was refluxed (1 hr) in ethanolamine (10 ml): and solid carbon dioxide was added to the cooled solution. The resultant precipitate was extracted with ether, the ether solution dried in the usual way to yield a product, dried over phosphorus pentoxide at 62°/10 mm, (+)-*tubocurine* (0·51 g, 84%), m.p. 164°, $[\alpha]_D^{17}$ + 153° (*c*, 1 in CH_3OH), $[\alpha]_D^{17}$ + 144° (*c*, 0·5 in 0·1N HCl), $[\alpha]_D^{17}$ + 72° (*c*, 0·67 in pyridine). Rf 0·78. Found: N, 4·9. Equiv. (titration) 298. $C_{36}H_{38}N_2O_6$ requires N, 4·7%. Equiv. 297·5.

OO-*Dimethyl*-(+)-*tubocurine* (from (+)-tubocurine). An ethereal solution of diazomethane (from 3·5 g of nitrosomethylurea) was added to tubocurine (0·153 g) in ether (15 ml) and methanol (5 ml). After 3 days at room temperature a further quantity of ethereal diazomethane was added, and the solution again left for 3 days. Evaporation of solvent left a yellow powder which showed two components on thin-layer chromatography.

The mixture in light petroleum (b.p. 40–60°) was placed on alumina (12 g) and the same solvent used as eluant. Fractions of 10 ml were collected, of which 1–8 yielded *OO*-dimethyl-(+)-tubocurine (Rf 0·89). This was identical in m.p. and infrared spectrum and $[\alpha]_D$ with an authentic specimen. Fractions 9–12 showed two components. Rf = 0·89 and Rf = 0·78 ((+)-tubocurine).

Cleavage of OO-*dimethyl*-(+)-*tubocurine with sodium in liquid ammonia*. *OO*-Dimethyltubocurine (1·2 g) in toluene–benzene (1 : 1, 40 ml) was added dropwise to liquid ammonia (400 ml) containing a small piece of sodium. More sodium was added whenever the blue colour faded, until the reaction was complete as judged by the blue colour being stable for 30–40 min (sodium used = 1·2 g). After evaporation of the ammonia, water was added and the aqueous layer separated from the organic layer. The organic phase was washed with 2% sodium hydroxide (2 × 25 ml), dried (Na_2SO_4) and evaporated to yield a brown syrup (0·585 g). The combined alkaline solutions were carbonated with solid carbon dioxide, and the precipitate which formed extracted with ether (4 × 250 ml). The ethereal extracts yielded the phenolic fraction (0·444 g) as a yellow oil.

The pale brown phenolic syrup (250 mg) in a minimum volume of benzene was placed on alumina (12 g) and eluted with 1% methanol in benzene. Forty fractions (10 ml each) were collected, and examined by

TABLE 4.

Compound	R in Structure as (I) R^1 = Me; R^2 = R^3 = H)	% Yield	M.p.	Reaction time (hr)	Formula	Found %		Required %	
						N	Halide	N	Halide
NN'-Diethyl-(−)-curine iodide	C_2H_5–	80·6	238°	46	$C_{40}H_{48}I_2N_2O_8$	3·0	28·4	2·9	26·9
NN'-Dipropyi-(−)-curine bromide dihydrate	C_3H_7–	77	220°	10	$C_{42}H_{52}Br_2N_2O_6.2H_2O$	2·9	14·0	3·2	18·1
NN'-Di-isopropyl-(−)-curine bromide dihydrate	iso-C_3H_7–	65	240°	6	$C_{42}H_{52}I_2N_2O_6.2H_2O$	2·9	26·6	2·9	26·1
NN'-Dibutyl-(−)-curine bromide dihydrate	C_4H_9–	58	222–223°	48	$C_{44}H_{56}Br_2N_2O_6.2H_2O$	3·2	16·5	3·1	17·5
NN'-Dipentyl-(−)-curine bromide dihydrate	C_5H_{11}–	52	219°	65	$C_{46}H_{60}Br_2N_2O_6.2H_2O$	2·8	15·5	3·0	17·1
NN'-Dioctyl-(−)-curine bromide dihydrate	C_8H_{17}–	60	202°	67	$C_{52}H_{72}Br_2N_2O_6.2H_2O$	3·2	15·2	2·9	16·4
NN'-Didecyl-(−)-curine bromide dihydrate	$C_{10}H_{21}$–	62	177°	140	$C_{56}H_{80}Br_2N_2O_6.2H_2O$	2·9	14·3	2·6	14·9
NN'-(2-Phenylethyl)-(−)-curine bromide dihydrate	$C_6H_5CH_2CH_2$–	81	226°	95	$C_{52}H_{56}Br_2N_2O_6.2H_2O$	3·0	16·6	2·9	16·0
NN'-(3-Phenylpropyl)-(−)-curine bromide dihydrate	$C_6H_5CH_2CH_2CH_2$–	86·5	222°	90	$C_{54}H_{60}Br_2N_2O_6.2H_2O$	3·0	16·1	2·7	15·5
NN'-Diallyl-(−)-curine bromide dihydrate	CH_2 = $CH.CH_2$–	99	210°	90	$C_{42}H_{48}Br_2N_2O_6.2H_2O$	2·9	19·5	3·3	19·2
NN'-(2-Hydroxyethyl)-(−)-curine bromide dihydrate	$HO.CH_2CH_2$–	85	242–244°	60	$C_{40}H_{44}Br_2N_2O_6.2H_2O$	2·8	17·2	3·1	18·0
NN'-(2-Ethoxyethyl)-(−)-curine bromide dihydrate*	$C_2H_5OCH_2CH_2$–	76	225°	50	$C_{44}H_{56}Br_2N_2O_8.2H_2O$	3·3	16·4	3·0	17·1

* Found: C, 56·1; H, 6·3. $C_{44}H_{60}Br_2N_2O_{10}$ requires C, 56·1; H, 7·0%.

TABLE 5.

Compound	R in Structure as (I) ($R^1 = R^2 = R^3 =$ Me)	% Yield	M.p.	Reaction time (hr)	Formula	Found %		Required %	
						N	Halide	N	Halide
NN'-Diethyl-*OO*-dimethyl-(−)-curine iodide trihydrate	C_2H_5–	91	216°	91	$C_{42}H_{52}I_2N_2O_6.3H_2O$	3·3	27·7	2·8	25·7
NN'-Dipropyl-*OO*-dimethyl-(−)-curine bromide trihydrate	C_3H_7–	90	213°	92	$C_{44}H_{56}Br_2N_2O_6.3H_2O$	3·4	17·8	3·2	18·4
NN'-Di-isopropyl-*OO*-dimethyl-(−)-curine iodide trihydrate	iso-C_3H_7–	75	205°	92	$C_{44}H_{56}Br_2N_2O_6.3H_2O$	2·5	26·3	2·7	25·2
NN'-Dibutyl-*OO*-dimethyl-(−)-curine bromide trihydrate	C_4H_9–	90	214°	91	$C_{46}H_{60}Br_2N_2O_6.3H_2O$	3·1	16·8	2·9	16·8
NN'-(2-Phenylethyl)-*OO*-dimethyl-(−)-curine bromide trihydrate	$C_6H_5.CH_2CH_2$–	84	216°	92	$C_{54}H_{60}Br_2N_2O_6.3H_2O$	2·9	17·6	2·7	15·3
NN'-(3-Phenylpropyl)-*OO*-dimethyl-(−)-curine bromide trihydrate	$C_6H_5CH_2CH_2CH_2$–	76·6	201°	92	$C_{56}H_{64}Br_2N_2O_6.3H_2O$	2·6	16·2	2·6	15·0

* Found: C, 57·2; H, 6·8. $C_{44}H_{58}Br_2N_2O_9$ requires C, 57·1; H, 6·9%.

thin-layer chromatography. Fraction 1 contained only one substance Rf = 0·86; fractions 2–15 similarly contained a single component Rf = 0·68; fractions 16–40 gave two spots of Rf = 0·68 and 0·39. Elution was completed with methanol (50 ml), the eluate bulked with fractions 16–40, and evaporated to dryness. The residue was again chromatographed in an identical manner.

Elution with benzene (80 ml) again yielded one component (Rf = 0·85). Benzene in methanol (95:5) (250 ml) yielded laudanidine (10 mg), m.p. 184° (Rf = 0·69). $[\alpha]_D^{17}$ − 111° (*c*, 0·07 in benzene), $[\alpha]_D^{17}$ + 40° (*c*, 0·07 in MeOH), $[\alpha]_D^{17}$ = −98° (*c*, 0·07 in $CHCl_3$). Kidd & Walker (1954) report $[\alpha]_D^{16}$ − 99·2° (*c*, 0·11 in $CHCl_3$), m.p. 182–183°.

Elution with benzene–methanol (90:10) (80 ml) yielded *N*-methylcoclaurine (*ca* 8 mg), $[\alpha]_D^{17}$ + 66° (*c*, 0·07 in MeOH), $[\alpha]_D^{17}$ = +73° (*c*, 0·07 in $CHCl_3$), m.p. 175° Rf = 0·39. Kidd & Walker report $[\alpha]_D^{23}$ + 69·6° (*c*, 0·85 in $CHCl_3$), m.p. 176–177°.

Preparation of quaternary salts. Base (*ca* 200 mg), consisting of (+)-tubocurine, (−)-curine, *OO*-dimethyl-(+)-tubocurine, or *OO*-(−)-curine, was heated with the appropriate alkyl halide (5–7 ml) at 65° for 1–4 days, to yield the products described in Tables 2–5.

Note. The difficulty of obtaining good analytical figures for quaternary salts of curine, chondocurine and *OO*-dimethylcurine has been noted by Dutcher (1946), King (1939) and by Faltis & Newmann (1922). A full analysis for C, H, N and halogen was, therefore, completed on one compound only in each series. Compounds of this type crystallize almost invariably as hydrates; the assumed hydration was confirmed in one example in each series by drying to contant weight at 105° over phosphorus pentoxide in high vacuum.

Pharmacology

METHODS

The methods were as previously described (Edwards, Stenlake & others, 1961). All drugs and control solutions were injected in 0·9% sodium chloride solution.

All compounds were compared with (+)-tubocurarine chloride on the isolated rectus abdominis muscle preparation (Table 6) and on the gastrocnemius muscle-sciatic nerve preparation of the anaesthetized hen (Table 6). Selected compounds were studied on the anterior tibialis muscle preparation of the anaesthetized rabbit (Table 6).

The reversibility of selected compounds to neostigmine was examined in the hen and rabbit, and the duration of action of other representative compounds compared with that of (+)-tubocurarine on the rabbit anterior tibialis muscle (Fig. 4).

TABLE 6. INFLUENCE OF ONIUM SUBSTITUENTS UPON NEUROMUSCULAR BLOCKING POTENCY ON THE FROG ISOLATED RECTUS MUSCLE PREPARATION, HEN GASTROCNEMIUS MUSCLE-SCIATIC NERVE PREPARATION AND RABBIT ANTERIOR TIBIALIS MUSCLE (TUBOCURARINE = 100)*

Quaternary substituent (structure I, R = alkyl)	(+)-Tubocurine	Parent base *OO*-dimethyl-(+)-tubocurine	(−)-Curine	*OO*-Dimethyl-(−)-curine
Frog rectus muscle				
Methyl	100	58	32	97
Ethyl	158	43	68	424
Propyl	15	44	57	69
i-Propyl	54	77	9	82
Butyl	31	38	12	86
Pentyl	40	36	—	—
Hexyl	42	—	14	—
Octyl	72	25	32	—
Decyl	23	19	12	—
Hydroxyethyl	33	96	11	—
Ethoxyethyl	85	58	61	166
Allyl	34	29	40	—
Benzyl	—	20	19	—
Phenethyl	59	42	49	55
Phenpropyl	38	62	25	110
Hen gastrocnemius-sciatic nerve				
Methyl	100*	204	30	56
Ethyl	125	52	23	63
Propyl	12	15	9	27
i-Propyl	51	61	5	16
Butyl	42	16	10	29
Pentyl	20	40	—	—
Hexyl	22	—	3	—
Octyl	9	13	4	—
Decyl	7	4	—	—
Hydroxyethyl	25	45	9	—
Ethoxyethyl	46	32	5	21
Allyl	32	7	15	15
Benzyl	—	26	4	4
Phenethyl	19	7	3	3
Phenpropyl	16	26	9	9
Rabbit anterior tibialis muscle—				
Methyl	100*	933	117	143
Ethyl	163	261	32	105
Propyl	10	20	5	27
Hydroxyethyl	40	90	6	—
Ethoxyethyl	45	79	6	25

‡ Values expressed as $\frac{2}{3}$molar potencies relative to (+)-tubocurarine (100).

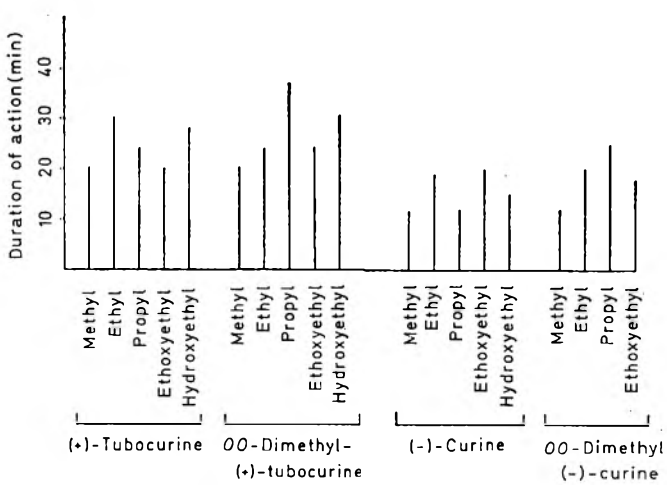
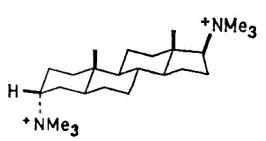


FIG. 4. Influence of onium substituents on duration of action on rabbit anterior tibialis muscle preparation (40–60% inhibition).

Results and discussion

The results recorded in Table 6 represent in each case the average of at least three experiments. They confirm the marked species differences observed by other workers, and apart from one or two striking exceptions, confirm the general conclusion that *OO*-dimethyl ethers in both stereochemical series are more potent than their parent quaternary alkaloids. Contrary to expectation, and in confirmation of the report of Wintersteiner (1959), (−)-curarine is only slightly more potent than (+)-tubocurarine in the rabbit (though significantly less potent in the frog and hen), whilst *OO*-dimethyl-(−)-curarine is significantly less potent than *OO*-dimethyl-(+)-tubocurarine in both the hen and the rabbit.

The folding of the molecule is significantly greater in the curarines than in the corresponding tubocurarines, because in the former both optical centres have the same absolute configuration. It would appear, therefore, that molecular folding is more significant than the actual disposition of the quaternary centres about the general plane of the molecule in determining the level of potency. This conclusion conforms with the observations that 3α,17β-bistrimethylammonium-5-α-androstane di-iodide (V) and 3β,17β-bistrimethylammonium-5-α-androstane di-iodide (VI) possess somewhat similar levels of neuromuscular blocking potency despite the difference in the relative dispositions of the onium groups in the two compounds (Dr. M. Martin-Smith, personal communication; Bamford, Biggs & others (1967)). Similarly, crystal structure analyses (Lonsdale, Milledge & Pant, 1965) have shown that the nitrogen atoms are disposed on opposite sides of the carbon chain in decamethonium and related quaternary salts in which the quaternary centres are separated by an even number of carbon atoms.



V

VI

Lonsdale & others (1965) suggest that the level of activity in this series is only indirectly related to the N . . . . N distance, but is perhaps more critically determined by the extreme (Me) . . . . (Me) distances as measured on each side of the chain, as well as the number of van der Waals' contacts with the receptor substance which are possible within these extremes. By analogy, therefore, it would appear that the observed potency differences between the tubocurarines and curarines may arise from a combination of the general molecular conformation and the number of contact points which the molecule, by virtue of both conformation and the number and nature of effective substituents, can make with the receptor substance. Thus there is considerable evidence for the involvement of methoxy and phenoxy substituents, since, for example, it has been shown that increasing substitution in the aromatic rings by methoxyl markedly

enhances potency in a series of decamethylenebis-tetrahydroquinolinium and -tetrahydroisoquinolinium salts (Collier & Taylor, 1949, 1950) and other analogous compounds (Stenlake, 1963). The influence of alkoxyl substitution, which appears to be linked with the intensification of the action of curare-like blocking drugs by diethyl ether, and the lower potency with increasing alkyl group size in a series of *OO*-dialkyl-(+)-tubocurarines (Holaday & Varney, 1959) accords with the view that ether oxygen functions represent important links for hydrogen-bond formation with the receptor molecule.

Fig. 2 (p. 54S) illustrates the general molecular conformation of (+)-tubo-curarine and its *OO*-dimethyl ether. Both molecules have only limited flexibility; the two tetrahydroisoquinolinium fragments are almost coplanar though slightly stepped with respect to each other, with the linking aromatic rings forced into roughly parallel planes almost at right angles to the tetrahydroisoquinolinium units. Such a conformation provides for a large measure of drug receptor interaction involving both quaternary centres (and/or their substituent alkyl group) and, in *OO*-dimethyl-(+)-tubocurarine, five of the six available methoxyl and aryloxy substituents.

Fig. 3 similarly illustrates the general molecular conformation of the curarines which, in contrast to that of the tubocurarines, is distinctly, though perhaps irregularly, step-shaped with each tetrahydroisoquinolinium unit representing a tread and the linking alkylaryloxy functions the risers. A molecule of such conformation would only be capable of a much more limited interaction with a receptor molecule of limited flexibility, which is known to show a high affinity for *OO*-dimethyl-(+)-tubocurarine. Such an interaction would involve only one of the two quaternary centres (and/or adjacent alkyl groups), and in *OO*-dimethyl-(−)-curarine only two of the six available methoxyl and phenoxy groups, in agreement with the significantly lower potency of this compound (Tables 1, 6, 7) compared with that of *OO*-dimethyl-(+)-tubocurarine.

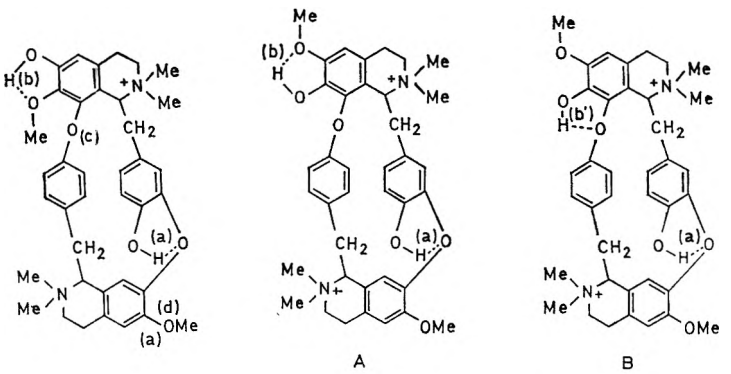
TABLE 7. RELATIONSHIP BETWEEN THE NUMBERS OF QUATERNARY AMMONIUM AND ALKOXYL CONTACT POINTS AND POTENCY OF TUBOCURARINE DERIVATIVES

Compound	Number of contact points			Potency		
	Quaternary ammonium	Non H-bonded alkoxyl	Total	Frog	Hen	Rabbit
(+)-Tubocurarine	2	1	3	100	100	100
(+)-Chondocurarine	2	2	4	—	—	290
OO-Dimethyl-(+)-tubocurarine	2	5	7	58	204	933
(−)-Curarine	1	1	2	32	30	117
OO-Dimethyl-(−)-curarine	1	2	3	97	56	143

The observed potencies of (+)-tubocurarine, (+)-chondocurarine and (−)-curarine may similarly be rationalized if potency is also considered to reflect the extent to which internal hydrogen-bonding (due to adjacent *ortho*-hydroxyl groups) limits the ability of phenoxy and methoxyl substituents to participate in drug-receptor interactions. Thus (+)-chondo-

curarine (VII) has two hydrogen-bonded oxygen functions [(a) and (b) in VII], so that only the two non-hydrogen-bonded aryloxy (c) and methoxyl (d) groups are available as effective contact points for receptor interactions, compared with five such groups in *OO*-dimethyl-(+)-tubocurarine.

(+)-Tubocurarine (VIII) and the curarine, similarly, have two hydrogen-bonded oxygen functions of which one is identical with that in (+)-chondocurarine. The second hydroxyl group (b), however, may hydrogen bond with either the adjacent methoxyl (VIIIA) or phenoxy group (VIIIB). This is evident from the pK_a of the two hydroxyl groups which were shown by Kalow (1954) to be 8·1 and 9·1 respectively; it is confirmed in the present work by the 100 mc/sec nuclear magnetic resonance spectrum of (+)-tubocurarine chloride, which shows two distinct proton signals (singlets) at 0·95 and 0·40 τ respectively, the signal at the lower field being assigned to the more heavily de-shielded proton. Accordingly, the ability of (+)-tubocurarine to hydrogen-bond to a receptor molecule by virtue of ether-oxygen lone pairs should be less than that of (+)-chondocurarine and corresponds to the observed potency difference. The significantly lower potency of (−)-curarine in the frog and hen compared with that of (+)-tubocurarine reflects the smaller number of contact points; the observed potency in the rabbit is, however, anomalous, in that it is greater than would be expected on the basis of our hypothesis.

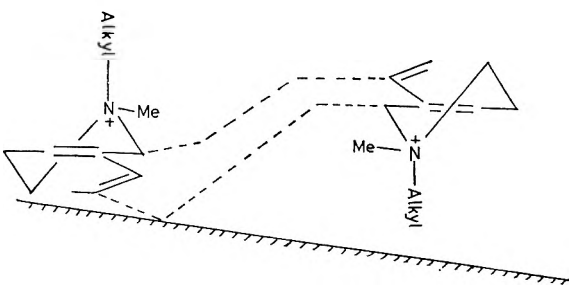


VII VIII

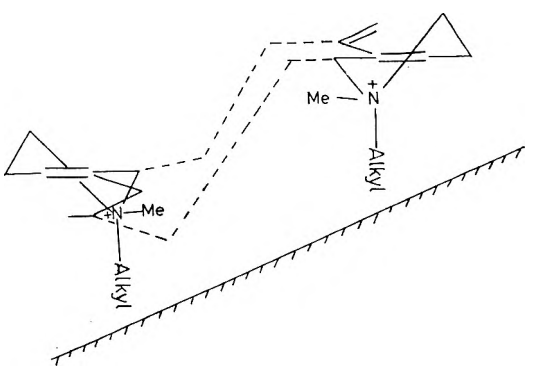
The relationship between the numbers of quaternary ammonium and alkoxyl contact points and observed potency is summarized in Table 7.

As shown in Table 6, replacement of one quaternary ammonium methyl substituent by an ethyl group increases the potency of (+)-tubocurarine in all three species examined, but other alkyl groups reduce potency significantly. This contrasts with the reduction in potency observed in *NN'*-diethylchondocurine and *NN'*-dibenzylchondocurine by Holiday & Varney (1959), and in *OO*-dimethyl-(+)-tubocurarine, (−)-curarine and *OO*-dimethyl-(−)-curarine when *N*-methyl is replaced by any other alkyl

group (including ethyl). These apparently conflicting observations are difficult to rationalize, but may be due to stereochemical factors, since the presence of two different alkyl substituents on each quaternary nitrogen gives rise to two new optical centres. The method of quaternization is such that the larger entering substituent is initially axial (McKenna & others, 1965a,b,c) and, whilst it is not impossible, both equilibration and major conformational changes appear unlikely. Preliminary observations, which still require to be confirmed, suggest that the compounds are stereochemically homogeneous, so that the preferred conformation would appear to be that with the larger alkyl substituents orientated axially. Such axially-orientated *N*-ethyl groups in (+)-tubocurarine could provide an additional contact point for van der Waals' adhesion in that tetrahydroisoquinolinium moiety which lacks a non-hydrogen bonded oxygen function. This would rationalize the observed increase in potency, and in (+)-chondocurarine provide a sufficiently bulky substituent to disrupt intermolecular hydrogen-bonding of the phenoxy (VIIc) and the methoxy (VIId) groups with the receptor. Large axial alkyl groups would, however, decrease the effectiveness of binding of the quaternary ammonium centre at the receptor as observed.



NN'-Dialkyl-(+)-tubocurine at assumed receptor.



NN'-Dialkyl-(−)-curine at assumed receptor.

FIG. 5.

Similarly, the enhanced potency of the *NN'*-diethyl compound in the *OO*-dimethyl-(+)-tubocurine series, compared with (+)-tubocurarine itself (Table 6) may be explained by the additional contact point. This, however, could only be achieved at the expense of full intermolecular *O*-hydrogen bonding with the receptor, and the compound is, therefore, less potent than *OO*-dimethyl-(+)-tubocurarine. The failure of the *NN'*-diethyl compounds to enhance potency in the (−)-curine and *OO*-dimethyl-(−)-curine series, finds a similar explanation in that any increase of van der Waals' bonding at the single effective quaternary centre would be outweighed by the consequent loss of intermolecular *O*-hydrogen bonding capacity, which resides entirely in the same tetrahydroisoquinolinium moiety. That the fall in potency with larger *N*-alkyl substituents is so much more profound in the curarine than the tubocurarine series is probably a reflection of the differences in the geometry of the tetrahydroisoquinolinium moieties in the two stereochemical series (Fig. 5). Thus in the *NN'*-dialkyl curines, both *N*-alkyl substituents project on the same side of the molecule, producing greater hindrance to receptor interaction than in the *NN'*-dialkyl tubocurines.

Compounds with *NN'*-dihydroxyethyl and *NN'*-diethoxyethyl substituents were only marginally more potent in the (+)-tubocurine and *OO*-dimethyl-(+)-tubocurine series than the corresponding *NN'*-dialkyl compounds, and no more effective in the (−)-curine and *OO*-dimethyl (−)-curine series. Further, the duration of action of the *NN'*-dihydroxyethyl and *NN'*-diethoxyethyl compounds was not significantly different from that of the parent *NN*-dialkyl compounds. In general, however, compounds in the (−)-curine and *OO*-dimethyl-(−)-curine series had a shorter duration of action than the corresponding compounds in the (+)-tubocurine series.

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Surface-active betaines: *N*-alkyl-*NN*-dimethylalanine hydrobromides and their critical micelle concentrations

A. H. BECKETT, G. KIRK AND A. S. VIRJI

Optically active and racemic forms of a series of *N*-Alkyl-*NN*-dimethylalanine hydrobromides (alkyl betaines) have been prepared. The use of optical rotatory dispersion and gas-liquid chromatography techniques to confirm their purity is described. Refractive index and surface tension methods have been used to determine the critical micelle concentrations of the alkyl betaines. An explanation of the differences in critical micelle concentrations between *N*-alkyl-*NN*-dimethylglycines and corresponding *N*-alkyl-*NN*-dimethylalanines is proposed.

OPTICALLY active and racemic forms of a series of *N*-alkyl-*NN*-dimethylalanine hydrobromides (alkyl betaines) were required for a study of their interactions in micelle form with optically active amino-acids and related compounds. We now report the preparation of these alkyl betaines.

Experimental

Materials. Optically active and racemic alanines were methylated as described by Bowman & Stroud (1950) to give DL-2-dimethylaminopropionic acid hydrochloride m.p. 148–149°; D-isomer m.p. 118–119° $[\alpha]_D^{18} - 12{\cdot}3°$ (*c*, 3·0 in H_2O); L-isomer m.p. 118–119° $[\alpha]_D^{18} + 12{\cdot}7$ (*c*. 3·0 in H_2O). Dodecyl, tetradecyl and hexadecyl bromides (Eastman Kodak) were examined by gas-liquid chromatography. A Perkin Elmer F.11 chromatograph was used with a 2 metre glass column $\frac{1}{4}$ inch o.d. packed with 2·5% S.E.30 on acid washed silanized (dimethyldichlorosilane) Chromosorb G (80–100 mesh). Operating conditions: hydrogen pressure, 20 lb/inch², air pressure, 25 lb/inch²; nitrogen pressure 20 lb/inch²; injection block temperature about 250°; oven temperature 160°. For each alkyl bromide the area of the main peak was expressed as a percentage of the total area of all peaks observed. Thus expressed the percentage purity of dodeceyl tetradecyl and hexadecyl bromides was 99·9, 98 and 100 respectively.

General method for the preparation of N-*alkyl*-NN-*dimethylalanine hydrobromides*

N-*Alkyl*-NN-*dimethylalanine hydrobromides.* A mixture of 2-dimethylaminopropionic acid hydrochloride (0·18 equiv.) alkyl bromide (0·45 equiv.) and Na_2CO_3 (0·45 equiv.), in methanol (4 ml/g bromide) was refluxed (70 hr) with stirring. The cooled reaction mixture was filtered, acidified with 47% w/v aqueous hydrobromic acid and evaporated to dryness under reduced pressure. The following process was repeated several times. The residue was treated with acetone (20 ml/g bromide), the undissolved sodium bromide filtered off and the filtrate evaporated to

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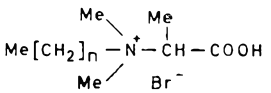
dryness. When all the sodium bromide had thus been removed the required product crystallized from the final filtrate. Five recrystallizations from acetone gave the pure product. Details of the compounds prepared are given in Table 1.

TABLE 1. *N*-ALKYL-*NN*-DIMETHYLALANINE HYDROBROMIDES



Alkyl chain	n	Isomer	Equiv. weight		m.p. °	Optical rotation $[\alpha]_D^{20}$	Micro-analytical data					
							Carbon		Hydrogen		Nitrogen	
			calc.	found			Calc.	Found	Calc.	Found	Calc.	Found
Dodecyl	11	(DL)	366·5	370	108–9	—	55·7	55·8	9·9	9·95	3·8	4·05
	11	(D)	366·5	368	100–1	(+)9·5°	55·7	56·1	9·9	9·9	3·8	4·3
	11	(L)	366·5	365	102–3	(−)9·5°	55·7	54·45	9·9	9·7	3·8	3·9
Tetra-decyl	13	(DL)	394	394	109–10	—	57·8	57·9	10·2	10	3·5	3·3
	13	(D)	394	398	107–8	(+)8·6°	57·8	58·1	10·2	9·8	3·5	3·7
	13	(L)	394	394·5	105–6	(−)8·9°	57·8	58·4	10·2	10·3	3·5	3·6
Hexa-decyl	15	(DL)	422·5	421·7	109–10	—	59·7	60·2	10·5	10·3	3·3	3·45
	15	(D)	422·5	425	109–10	(+)12·4°	59·7	60·1	10·5	10·5	3·3	3·0
	15	(L)	422·5	428	109–10	(−)12·5°	59·7	60·2	10·5	10·9	3·3	2·9

$[\alpha]_D^{22}$ values for dodecyl- and tetradecyl compounds obtained with 3% aqueous solutions; hexadecyl determined using 1% methanolic solutions.
Yields ranged from 5–15%.

The purity of the optically active forms of betaines and amino-acids was checked using an optical rotatory dispersion technique. Curves recorded in the range 19,000 to 48,000 cm^{-1} were of the "plain type" and for the D and L configuration of each compound they were mirror images. L-(−)-N-*Hexadecyl*-NN-*dimethylalanine.* L-(−)-*N*-Hexadecyl-*NN*-dimethylalanine hydrobromide (2·0 g) in distilled water was passed through a column of IRA-400 in the hydroxyl form. The eluate was evaporated, xylene added, and the residue dried by codistillation of the xylene water azeotrope. Crystallization from acetone gave L-(−)-N-*hexadecyl*-NN-*dimethylalanine* (1·0 g) m.p. 157–158°, $[\alpha]_D^{23}$–6·5 (*c*, 1·5 in acetone). Found: C, 73·6; H, 12·4; N, 3·9%; equiv. 350. $C_{21}H_{43}BrO_2$ requires: C, 73·8; H, 12·7; N, 4·1%, equiv. 341·3.

MEASUREMENT OF REFRACTIVE INDEX

A Hilger-Rayleigh interference refractometer (Model M 154) fitted with constant temperature water jacket was used with a tungsten lamp and a 10 cm cell.

Procedure. △ R, the difference in terms of instrument scale divisions between the refractive index of a betaine solution and that of distilled water as the reference liquid, was determined over a range of concentrations: cell and contents were allowed to equilibrate for 30 min before taking a reading. Duplicate determinations were made. A plot of △ R against molar concentration is shown in Fig. 2.

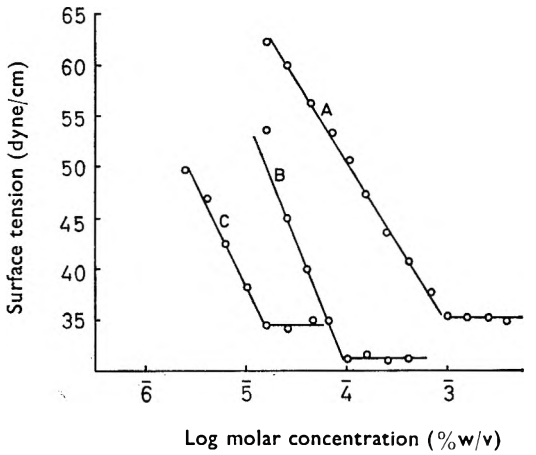


FIG. 1. Plot of surface tension against log molar concentration to determine the CMC of *N*-alkyl-*NN*-dimethylalanines. A, Dodecylbetaine; B, tetradecylbetaine; C, hexadecylbetaine.

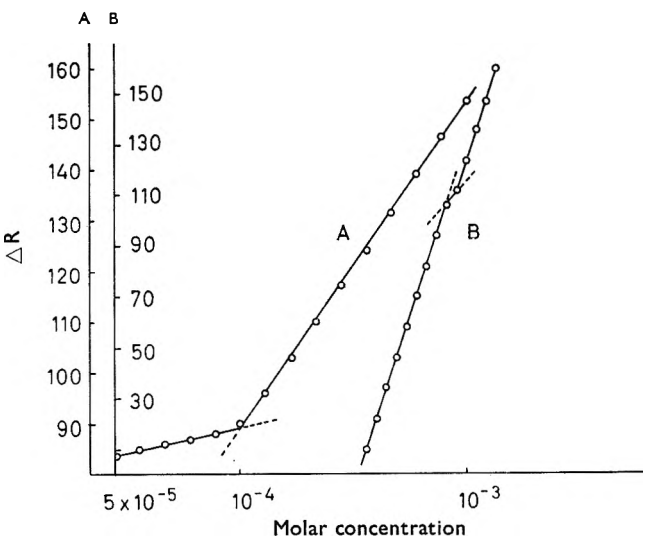


FIG. 2. Graphs of differential refractive index at 21° C plotted against molar concentration to determine the CMC of *N*-alkyl-*NN*-dimethylalanyl betaines. A, Tetradecyl betaine: ΔR plotted on ordinate A. B, Dodecylbetaine: ΔR plotted on ordinate B.

MEASUREMENT OF SURFACE TENSION

A Cambridge Du Nouy tensiometer was used with the ring detachment method, and the Harkins & Jordan (1930) corrections were applied to the

results. Measurements were made at 22° (±1°). A plot of surface tension against log molar concentration is shown in Fig. 1.

Results and discussion

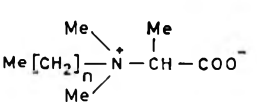
It has been shown (Beckett & Woodward, 1963) that the critical micelle concentrations for alkyl betaine hydrochlorides and for the free betaines are identical. Thus the salts may be regarded as completely dissociated in aqueous solution. Since L-(−)-*N*-hexadecyl-*NN*-dimethylalanine prepared as above was hygroscopic, the hydrobromides have been used in the present investigation; only the hexadecyl betaine was examined as the free base. It was not possible to determine the CMC of *N*-hexadecyl-*NN*-dimethylalanine hydrobromide by the refractive index method. As determined by the surface tension method the CMC of this compound is in the region of 10^{-5}M, and differences between the refractive index of such dilute solutions and distilled water are too small to be detected by the Hilger-Rayleigh differential refractometer.

The results of the surface tension method for each alkyl betaine gave a graph on which the CMC was interpreted from the intercept of two straight lines (see Fig. 1). Below this value, the surface tension decreased linearly with increase in log surfactant concentration, above it, the surface tension was almost constant.

The CMC results obtained for the *N*-alkyl-*NN*-dimethylalanine hydrobromides using both methods are in good agreement (Table 2).

TABLE 2. THE CMC VALUES FOR A SERIES OF *N*-ALKYL-*NN*-DIMETHYLGLYCINES AND THE CORRESPONDING *N*-ALKYL-*NN*-DIMETHYLALANINE HYDROBROMIDES

N-Alkyl-*NN*-dimethylalanine hydrobromide



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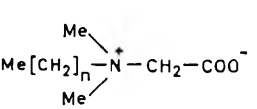
N-Alkyl-*NN*-dimethylglycine



II

Alkyl chain	n	CMC (moles/litre) Surface tension method	CMC (moles/litre) Refractive index method	Alkyl chain	n	CMC (moles/litre) Surface tension method	CMC (moles/litre) Refractive index method
Dodecyl ..	11	1×10^{-3}	$9{\cdot}6 \times 10^{-4}$	Dodecyl ..	11	$1{\cdot}8 \times 10^{-3}$	$2{\cdot}1 \times 10^{-3}$
Tetradecyl ..	13	$9{\cdot}5 \times 10^{-5}$	1×10^{-4}	Tetradecyl ..	13	$1{\cdot}8 \times 10^{-4}$	$1{\cdot}7 \times 10^{-4}$
Hexadecyl ..	15	2×10^{-5}	—	Hexadecyl ..	15	$2{\cdot}0 \times 10^{-5}$	$1{\cdot}6 \times 10^{-5}$

These results show that the CMC is dependent on the number of carbon atoms in the alkyl chain and decreases as the chain length increases. This would be expected since an increase in chain length will increase the hydrophobic character of the molecules and therefore the tendency to

form micelles at lower concentrations. No differences were observed between the CMC values of optical isomers and racemic compounds.

The CMC values obtained by Beckett & Woodward (1963) for a series of *N*-alkyl-*NN*-dimethylglycines are compared in Table 2 with those for the *N*-alkyl-*NN*-dimethylalanines.

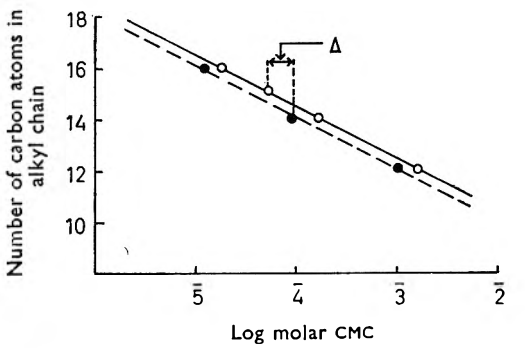


FIG. 3. Graphs of alkyl chain length against log molar CMC for the series of *N*-alkyl *NN*-dimethylglycines and *N*-alkyl *NN*-dimethylalanines. Δ represents the difference in CMC due to replacement of a hydrogen on the C atom between N^+ and COO^- groups by Me, and replacement of a hydrogen on the terminal Me group of the long chain alkyl group by Me ○—○, *N*-Alkyl-*NN*-dimethylglycines (Woodward, 1963); ●- - -●, *N*-alkyl-*NN*-dimethylalanines.

Alanyl betaines form micelles at lower concentrations than the corresponding glycyl betaines. An increase of one methylene group in the length of the long chain *N*-alkyl group of a glycyl betaine results in a decrease in CMC (Beckett & Woodward, 1963). It can therefore be assumed that the resulting decrease in CMC, when going from a glycyl to the corresponding alanyl betaine, is due to the methyl group on the asymmetric carbon atom being orientated in the same general direction as the long-chain alkyl group. The decrease in CMC observed between a glycyl and its corresponding alanyl betaine is approximately half that which occurs when the length of the *N*-alkyl chain of the glycyl betaine is increased by one methylene group (Fig. 3). The hydrogen atom on the asymmetric carbon atom of the alanyl betaine will then take up a position in the micelle-water interface.

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The ratio of orthogonal function coefficients as an index of purity

I. U. AGWU AND A. L. GLENN

Ratios of orthogonal function coefficients are superior to extinction ratios as purity indices. They reflect improved sampling of the absorption spectrum, can be made more robust to overall shifts of the spectrophotometer's wavelength scale and also exhibit simpler behaviour in the presence of impurities. An example is given of the use of such ratios to study the distribution of irrelevant absorption in pharmaceutical injections.

EXTINCTION ratios or simple functions thereof have long been recommended for the control of absorbing impurities in samples of absorbing compounds. Stearns' (1950) "Impurity Index" refers to a difference in ratios for sample and reference compound, respectively, whereas the "Spectrophotometric Purity Index" proposed by White (1962) consists of a ratio of ratios. To the extent that Beer's and Lambert's laws hold in given circumstances, such indices are independent of concentration and can therefore be measured on solutions of unknown concentration. On the other hand, for satisfactory results, a ratio requires greater attention to detail than does the measurement of a single peak extinction. Thus, if one or both extinctions should fall outside a suitable range (e.g. 0·4–2·0 when ample radiant power is available for measurement), Lambert's law deviations may well occur (Vandenbelt, Forsyth & Garrett, 1945). Cell blank errors also increase in importance as the lower extinction approaches zero. The ratio exhibits significant sensitivity to both wavelength and spectral slit width (Lothian, 1958; Rogers, 1965) if one extinction falls on a steep slope in the absorption curve, and although errors in setting the wavelength scale can be reduced by replication (Ismail & Glenn, 1964), it is more difficult to take account of the shifts in wavelength calibration which arise from thermal effects and mechanical wear. It follows that extinctions measured on steep slopes are a good deal more sensitive to minute differences between instruments than those measured at maxima or at minima. The ratios are correspondingly sensitive to instrument parameters.

Although an extinction ratio can be made to reveal absorbing impurities in specific regions of the spectrum by careful choice of wavelengths, it necessarily provides minimal information about band shape. For greater information, the band must be sampled at a larger number of wavelengths and in these circumstances, it is often more convenient to express the results in terms of orthogonal function coefficients than as a set of ratios of the kind included in the pharmacopoeial appendix on vitamin A.

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THE RATIO OF ORTHOGONAL FUNCTION COEFFICIENTS

Any experimental curve can be broken down (expanded) in the following way.

$$E(\lambda) = p_0P_0(\lambda) + p_1P_1(\lambda) + p_2P_2(\lambda) + \ldots + p_nP_n(\lambda) \quad .. \quad (1)$$

where $E(\lambda)$ denotes the extinction at wavelength, λ, the latter belonging to a set of $(n + 1)$ *equally spaced* values at which the orthogonal polynomials, $P_0(\lambda)$, $P_1(\lambda)$, $P_2(\lambda)$, etc. are each defined. The said polynomials represent a series of fundamental curve shapes (see Fig. 1) that may be scaled up or down by the appropriate coefficients (p_0, p_1, p_2, etc.) and then summed to reproduce the value of any $E(\lambda)$ within the set.

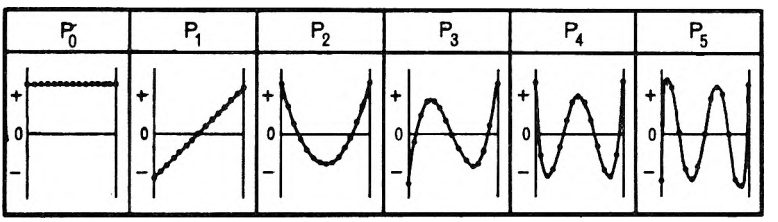

FIG. 1. Orthogonal polynomials. In each diagram, $P_j(\lambda)$ is plotted against a set of abscissa values, λ_s, $\lambda_s + k$, $\lambda_s + 2k$, ... $\lambda_s + 14k$, the same set being used in all six diagrams. Each set of points represents a fundamental curve shape, whose general characteristics are indicated by a line which joins them but otherwise has no significance. Thus, P_j does not exist at values of λ outside the given set.

In view of the orthogonality of the polynomials and the resultant mutual independence of the coefficients, calculation of the required coefficient entails no more than simple arithmetic (Glenn, 1967). Each coefficient moreover, is an *exact* linear function of the set of $E(\lambda)$ employed in its calculation. Hence, subject only to the usual Beer-Lambert limitations, a given coefficient is *exactly* proportional to concentration (Glenn, 1963).

RATIOS OF ORTHOGONAL FUNCTION COEFFICIENTS AS CRITERIA OF PURITY

Having measured an absorption band at $(n + 1)$ wavelengths (λ_s, $\lambda_s + k$, $\lambda_s + 2k$, ... $\lambda_s + nk$, where k denotes a constant wavelength interval), its shape over the range, λ_s to $(\lambda_s + nk)$, can be expressed in terms of n ratios of the form, p_j/p_0, where $j = 1, 2, \ldots . n$. As with extinction ratios, p_j/p_0 is independent of concentration subject only to Beer-Lambert limitations. On the other hand, p_j/p_0 is often superior to an extinction ratio in the matter of instrumental errors. If an extinction ratio is to provide information about band shape (as opposed to a relationship between adjacent bands), the need for one of the extinctions to fall on a slope endows the ratio with a corresponding sensitivity to wavelength scale errors. In contrast, p_j/p_0 can often be made substantially robust to such errors by suitable choice of wavelengths.

By way of example, the use of the particular ratio, p_2/p_0, as a quantitative expression of band distortion is illustrated in Fig. 2 where the band

is sampled at seven equally spaced wavelengths. A denotes the absorption curve of pure compound "A" and A + Z the curve produced by addition of a linear background, Z. Now, in view of orthogonality between P_2 and $(P_0 + P_1)$, coefficient, p_2, will be the same for the two curves, whereas p_0 will be greater for A + Z than for A. The numerical value of p_2/p_0 will therefore decrease in the presence of Z, thus indicating a decrease in purity. Moreover, unlike the extinction ratios, E_1/E_3, p_2/p_0 is independent of the slope of Z. For example, a decrease in the slope of Z produces a decrease in E_1/E_3 on the short wavelength side of the band (i = 0, 1, 2) and an increase on the long wavelength side (i = 4, 5, 6). In this respect, ratios of orthogonal function coefficients behave more simply than extinction ratios.

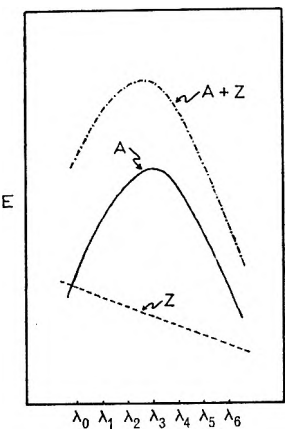

FIG. 2. Addition of a linear background absorption to the absorption curve of a pure compound.

In the general case, p_j/p_0 will not always decrease in the presence of irrelevant absorption. Sometimes it will increase and on rare occasions may even show no detectable change. p_2/p_0 and p_3/p_0 probably constitute the most useful ratios in practice, although higher ratios, such as p_5/p_0 may have value in detecting irrelevant absorption that possesses vibrational structure.

To sum up: relative to extinction ratios, ratios of orthogonal function coefficients, (i) reflect a better sampling of the band, (ii) are more robust to overall shifts in wavelength calibration and (iii) exhibit more simple behaviour in the presence of impurities.

PRACTICAL APPLICATION OF RATIOS OF ORTHOGONAL FUNCTION COEFFICIENTS

The ratio for a given sample is conveniently expressed as a percentage deviation (F) from the ratio for the corresponding reference substance. Thus,

$$F = 100 \left[\frac{(p_j/p_0)_{sample}}{(p_j/p_0)_{ref.}} - 1 \right] \quad .. \quad .. \quad .. \quad (2)$$

F can be made into a sensitive function of irrelevant absorption provided instrumental errors are minimized by differential spectrophotometry. The scheme for measuring F is then as follows.

A solution of the reference substance is placed in the sample cell and measured with respect to solvent (in the reference cell). The resultant extinctions are then used to calculate p_0 and p_j for the reference substance. In the next stage, a solution of the sample under investigation is placed in the sample cell and measured against the solution of the reference substance (now in the reference cell). The set of extinction differences (ΔE) which result from this procedure are then used to calculate Δp_0 and Δp_j. We may then write:

$$p_{0(sample)} = p_{0(ref.)} + \Delta p_0 \quad .. \quad .. \quad .. \quad (3)$$

$$p_{j(sample)} = p_{j(ref.)} + \Delta p_j \quad .. \quad .. \quad .. \quad (4)$$

$$\text{so that } F = 100 \left[\frac{\left(\frac{p_{j(ref.)} + \Delta p_j}{p_{0(ref.)} + \Delta p_0} \right)}{(p_j/p_0)_{ref.}} - 1 \right] \quad .. \quad .. \quad .. \quad (5)$$

Although differential spectrophotometry (Crawford, 1959) has been mainly used to improve photometric accuracy, its value in the present context lies in a general cancellation of instrumental errors, particularly those due to uncertainties of wavelength and spectral slit width. In the course of determining an orthogonal function coefficient, most of the extinction measurements are made on the slopes of the absorption curve so that a major proportion of the variance of a coefficient must arise from wavelength setting errors. Moreover, for a given error in setting the wavelength scale, the resultant extinction error increases with the slope of the absorption curve at the wavelength of measurement (Ismail & Glenn, 1964). Therefore, any procedure that diminishes the slope will also reduce the residual variance of the orthogonal function coefficient. Hence, the main virtue of differential spectrophotometry in the present context, namely that when solutions of sample and reference substance are of equal concentration, negligible slopes are encountered on the ΔE curve.

There can be no such elimination of wavelength setting errors from the measurement of $p_{0(ref.)}$ and $p_{j(ref.)}$. Nevertheless, errors in these last quantities largely cancel out when F is small, as may be seen from equation (5).

Experimentally, there is no need for the critical adjustment of concentration, necessary in the compensation method (Jones, Clark & Harrow, 1951). Thus, F is independent of the concentrations of sample and reference substance so that avoidance of appreciable slopes in the ΔE curve is the sole reason for requiring these concentrations to be approximately equal.

Using a manual spectrophotometer, measurements at six wavelengths, for example, may take less time than is consumed by solution preparation and cell filling, whilst the calculations take only a few minutes on an electric desk calculator. Nevertheless, the principal objection to the manual method resides less in the labour than in the possibility of gross errors in setting the wavelength scale. This particular hazard increases with the number of wavelengths measured. Such problems may, however, disappear in view of recent trends towards automatic instruments capable of three decimal point precision and able to follow a specified programme of wavelength settings. Moreover, p_2/p_0 can also be obtained from an analogue computer, which gives a direct reading of p_2 or p_0 at the end of a spectral run on a recording spectrophotometer (Glenn, to be published).

TABLE 1. DIFFERENCES BETWEEN ESTIMATES OF THE RATIO OF ORTHOGONAL FUNCTION COEFFICIENTS

Injection	Wavelength (mμ)				(p_2/p_0)sample*		
	Mean	Initial	Final	Interval	by direct measurement	by differential measurement	% Difference
Aminophylline	272·5	256	289	3	− 8·5994	− 8·5031	−1·12
Nikethamide	264·5	248	281	3	− 8·4491	− 8·4380	−0·13
Mephenesin	269·5	253	286	3	−16·1538	−16·0979	−0·35
Emetine	284·0	273	295	2	− 8·2115	− 8·2389	+0·33
Tubocurarine	279·5	263	296	3	−13·0425	−13·1027	+0·46
Nalorphine	283·5	267	300	3	−13·3538	−13·3649	+0·08
Pethidine	260·0	249	271	2	− 6·9081	− 6·9549	+0·68
Gallamine	271·0	260	282	2	− 6·2628	− 6·2988	+0·57
Methylamphetamine	256·5	240	273	3	−14·2915	−14·0280	−1·84
Methadone	295·5	279	312	3	− 6·0403	− 6·0124	−0·46
Atropine sulphate	258·0	247	269	2	− 5·8124	− 5·7060	−1·83

* Unnormalized

Values of $(p_2/p_0)_{sample}$ for a number of pharmacopoeial injections are shown in Table 1. In the absence of prior experience in measuring ratios of orthogonal function coefficients, each determination of $(p_2/p_0)_{sample}$ by the differential method was accompanied by a direct determination of the same quantity. Differences between the resultant pairs of independent estimates corresponded with a coefficient of variation of 0·66 (0·39 after rejecting two outliers) for the determination of $(p_2/p_0)_{sample}$. These coefficients are somewhat larger than those (0·26 for $n = 5$ and 0·22 for $n = 23$) obtained from thirty direct estimates of the same ratio for phenol using mean extinctions based on duplicate settings of the wavelength scale (Wahbi & Glenn, to be published). On the other hand, failure to replicate wavelength settings in the present work was bound to produce a substantial increase in the variance of $(p_2/p_0)_{sample}$. Present measurements also involved an additional step, namely, the determination of ΔE, which did not arise in the phenol work. Nevertheless, since F is relatively insensitive to errors in $(p_2/p_0)_{ref.}$, a change of 0·3 in F is probably significant, particularly when F is small. This statement will not however apply if $|p_1|$ is too small for precise measurement.

CONVOLUTED ABSORPTION CURVES

Choice of interval and number of wavelengths is less critical in the present context than in quantitative elimination of irrelevant absorption and will be discussed elsewhere. Nevertheless, in most applications of orthogonal functions to spectrophotometric analysis, it is essential to minimize the effect of overall shifts in the spectrophotometer's wavelength scale. To this end, the coefficient relating to a specified interval

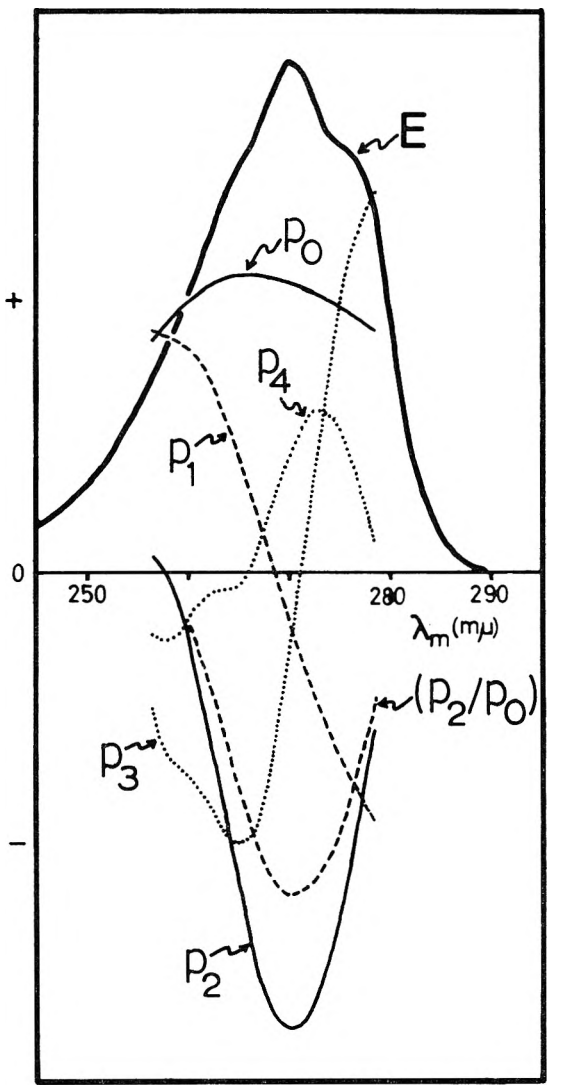

FIG. 3. Absorption curve of phenol and related convoluted curves. Convoluted curves (p_0, etc.) were obtained from E, the absorption curve of phenol in 0·1N H_2SO_4 aq., using 12 point orthogonal polynomials and 3 mμ intervals.

and number of wavelengths is plotted against λ_m, the mean of the set of wavelengths—a process that leads to a *convoluted absorption curve* (Blackman & Tukey, 1958). By way of example, several convoluted curves for phenol are given in Fig. 3 together with the parent absorption curve.

λ_m for the optimum range corresponds with a maximum or minimum in the convoluted absorption curve. The principles for choosing λ_m are in fact identical with those for choosing the wavelength of measurement in a normal "one substance" spectrophotometric analysis. Thus, for a given p_j, λ_m should correspond with that peak or minimum which is furthest removed from the abscissa scale. The same compromises in regard to wavelength sensitivity also arise, so that λ_m is better sited on a broad peak or minimum than on a narrow one. Sensitivity of the coefficient to overall shifts in the spectrophotometer's wavelength calibration is thereby minimized.

In the present context, careful choice of λ_m would be most important if $(p_2/p_0)_{sample}$ were measured directly and $(p_2/p_0)_{ref.}$ taken from published data, as might occur in routine analysis. Furthermore, λ_m should be chosen from a graph of p_2/p_0 against λ_m (which is not a convoluted curve in the strict sense). However, for a reasonably symmetrical band (Fig. 3), the optimum λ_m for p_2 is unlikely to differ greatly from that for p_2/p_0.

EXAMPLE OF THE USE OF RATIOS OF ORTHOGONAL FUNCTION COEFFICIENTS

This paper forms part of a programme of work on the use of a modification of Vierordt's method to correct for bactericide absorption in the spectrophotometric assay of pharmacopoeial injections. Earlier evaluations of the method were so conducted as to eliminate errors due to irrelevant absorption (Glenn, 1960; Ismail, 1964). Thus, irrelevant absorption errors due to batch differences were eliminated by using the *same* batches of active constituent and bactericide to establish the assay coefficients as were used to prepare the injection. Errors arising from general contamination of solutions were also minimized by suitable technique and under these conditions, the method gave excellent results.

TABLE 2. ASSAY OF ATROPINE SULPHATE INJECTIONS BY COMPENSATION AND MODIFIED VIERORDT METHODS

Sample	Compensation method		Modified Vierordt method					
			Using 257·5 mμ and 245 mμ			Using 257·5 mμ and 268 mμ		
	% w/v	% error	% w/v	% error	b/m	% w/v	% error	b/m
S_1	0·120	0	0·1224	+ 2·0	1·6	0·1124	− 6·3	1·9
S_2	0·125	+4	0·1292	+ 7·7	1·8	0·1276	+ 6·3	2·3
S_3	0·120	0	0·1270	+ 5·8	1·7	0·1150	− 4·2	2·0
S_4	0·125	+4	0·1734	+44·5	1·9	0·1556	+29·7	2·1
S_5	0·125	+4	0·1320	+10·0	1·8	0·1376	+14·7	2·8

% error relates to the labelled concentration (0·12%).
All five samples contained 0·5% w/v chlorbutol as bactericide. S_1 and S_4 were in rubber capped vials and the remainder in ampoules.

Similar conditions are unlikely to obtain in practice, the nearest approach being realized in the injection manufacturer's own control department where batch differences are easy to eliminate. In such a case, errors due to irrelevant absorption would arise from general contamination during manufacture. On the other hand, "outside analysts", unable to eliminate batch differences, are less favourably placed and it was therefore desirable to evaluate the method under these conditions. In this respect, atropine sulphate injection B.P. was an obvious choice, for in view of its very low peak E(1 cm), a spectrophotometric assay is likely to suffer from both kinds of irrelevant absorption—particularly that due to general contamination.

Results obtained for five samples of injection of atropine sulphate B.P. derived from commercial sources are given in Table 2. Concentrations evaluated by the modified Vierordt method showed a marked dependence upon wavelength, indicative of irrelevant absorption. Hence, despite its inability to discriminate between increments of less than 4% in atropine concentration, the compensation method gave the more reliable estimates. The same method also revealed substantial quantities of irrelevant absorption in all five injections, a typical result being shown in Fig. 4. In these circumstances, the modified Vierordt method was associated with much larger errors than hitherto (Ismail, 1964).

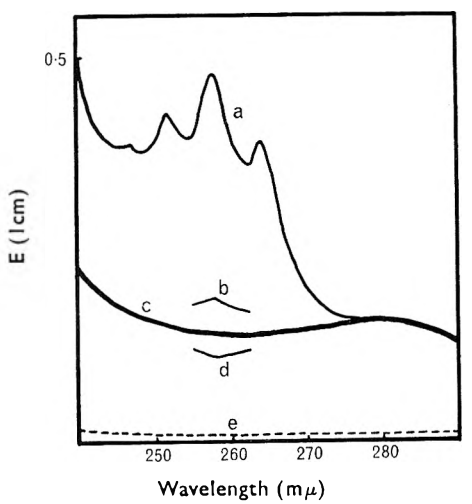

FIG. 4. Irrelevant absorption in atropine sulphate injection. Curve (a) is the absorption curve of diluted atropine sulphate injection (S_1). Curves (b), (c) and (d) are difference curves, using various concentrations of reference substance (R_1), (c) representing irrelevant absorption alone. Curve (e) is the absorption curve of chlorbutol at the labelled concentration.

The irrelevant absorption found in these injections probably originated from both sources referred to above. Large variations were observed between different batches of both atropine sulphate and chlorbutol (the

bactericide). On using the compensation method to compare six samples of atropine sulphate with a given sample (R_1), two samples showed negligible irrelevant absorption whereas the remainder betrayed four substantially different irrelevant absorption curves. As may be seen from Fig. 5, the irrelevant absorption in the worst sample (R_2) would create serious error in a one substance assay, whilst in the same circumstances, Vierordt's method would suffer even more.

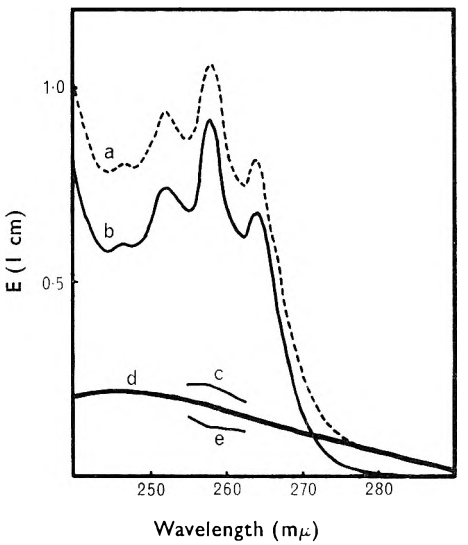

FIG. 5. Comparison of samples of atropine sulphate B.P. Curves (a) and (b) are absorption curves of 0·160% w/v solutions of samples R_2 and R_1, respectively. Curves (c), (d) and (e) are difference curves, using various concentrations of reference substance (R_1), (d) representing the irrelevant absorption in R_2.

The results in Table 2 may be typical of a class of injection for which peak E(1 cm) is too small and the active constituent unusually subject to batch differences. Moreover, in the likely event that, on average, general contamination makes a limited contribution to the peak E(1 cm), a simple relation between F and peak log E(1 cm) would not be unexpected. Thus, although batch differences may contribute a substantial fraction of the total irrelevant absorption in any injection, general contamination should only assume importance when peak log E(1 cm) is small. On this basis, circumstances that are favourable to the application of Vierordt's method may be distinguished from those which are not.

In order to obtain a preliminary assessment of the relationship between F and peak log E(1 cm), it was necessary to evaluate F for a set of injections, representative of a wide range of peak log E (1 cm) and obtained from commercial sources. In each case, the purest available specimen of active constituent was used as reference substance, the suitably diluted injection being treated as sample. None of the injections contained a

bactericide since this would have vitiated the measurement of F, values of which are given in Table 3 together with peak log E(1 cm). Furthermore, since errors in F were unlikely to exceed 0·3 in the present work, all but one of the results quoted in Table 3 relate to significant differences between sample and reference in the matter of irrelevant absorption.

TABLE 3. VALUES OF F AND PEAK LOG E(1 CM) FOR PHARMACOPOEIAL INJECTIONS

Injection	Concentration measured (%)	Peak log E(1 cm)	$(p/_2p_0)$ sample / (p_2/p_0) ref.	F
Aminophylline	0·002	4·0448	0·9804	− 1·96
Nikethamide	0·0025	3·8657	0·9680	− 3·20
Mephenesin	0·010	2·8949	0·9947	− 0·53
Emetine	0·005	2·8026	0·9859	− 1·41
Tubocurarine	0·008	2·0286	0·9937	− 0·63
Nalorphine	0·024	1·6119	0·9762	− 2·38
Pethidine	0·050	1·5623	0·9720	− 2·80
Gallamine	0·100	1·5519	0·9625	− 3·75
Methylamphetamine	0·100	1·2801	0·9676	− 3·24
Methadone	0·060	1·1951	0·9973	− 0·27
Atropine sulphate	0·060	−0·0841	0·8684	−13·16

In the regression of F upon peak log E(1 cm), the results in Table 3 gave a correlation coefficient (0·559) which almost reached significance (0·602) at $P = 0·05$. Successive omission of results then led to the following coefficients, each being followed by the revised value for significance at $P = 0·05$: atropine sulphate (0·04, 0·632), nikethamide (0·270, 0·666), aminophylline (0·445, 0·707).

It would be dangerous to generalize from so small a set of results, particularly in circumstances wherein a moderate correlation rests entirely upon one result (atropine sulphate). Nevertheless, the progressive improvement in correlation which arose from successive rejection of results for nikethamide and aminophylline may possibly reflect an underlying relationship between F and peak log E(1 cm).

The large value of F recorded for injection of atropine sulphate concords with high levels of irrelevant absorption found by the compensation method in other samples of the same injection. Nevertheless, only part of this irrelevant absorption may be attributed to general contamination in view of differences observed between batches of the active constituent. Hence, as a rough guide, general contamination should produce negligible error in the Vierordt assay, provided peak log E(1 cm) exceeds 1·5.

Experimental

All injections were obtained from well-known drug houses. Assay coefficients for the modified Vierordt method were established from samples of atropine sulphate (R_1) and chlorbutol free from irrelevant absorption. R_1 was also used as reference substance in the measurement of F for atropine sulphate injection. All other reference substances were of pharmacopoeial quality. Solutions were prepared in 0·1N aqueous sulphuric acid with suitable precautions to minimize contamination, which proved to be negligible in several blank experiments. Despite the

usual assumption that a recording spectrophotometer is essential to the compensation method, a Hilger Uvispek proved adequate for the present work.

CALCULATION OF F

Values of F were calculated in terms of 12 point orthogonal polynomials by the scheme detailed in Table 4 for nikethamide injection. The second and third columns contain values of E(1 cm) and ΔE(1 cm) for reference substance and injection (versus reference), respectively. Values of P_0 and P_2 in the next two columns were taken from tables (Fisher & Yates, 1957). To clarify the arithmetical procedure, full details are given in the last four columns.

TABLE 4. CALCULATION OF F FOR INJECTION OF NIKETHAMIDE

Measurements			Polynomials		Products			
mμ	E	ΔE	P_0	P_2	$E \times P_0$	$E \times P_2$	$\Delta E \times P_0$	$\Delta E \times P_2$
248	0·433	0·037	1	+55	0·433	+23·815	0·037	+2·035
251	0·476	0·039	1	+25	0·476	+11·900	0·039	+0·975
254	0·539	0·041	1	+ 1	0·539	+ 0·539	0·041	+0·041
257	0·604	0·044	1	−17	0·604	−10·268	0·044	−0·748
260	0·660	0·046	1	−29	0·660	−19·140	0·046	−1·334
263	0·691	0·046	1	−35	0·691	−24·185	0·046	−1·610
266	0·671	0·044	1	−35	0·671	−23·485	0·044	−1·540
269	0·614	0·042	1	−29	0·614	−17·806	0·042	−1·218
272	0·504	0·036	1	−17	0·504	− 8·568	0·036	−0·612
275	0·365	0·030	1	+ 1	0·365	+ 0·365	0·030	+0·030
278	0·245	0·023	1	+25	0·245	+ 6·125	0·023	+0·575
281	0·159	0·019	1	+55	0·159	+ 8·745	0·019	+1·045
Sums of products (unnormalized coefficients)					+5·961 p_0 (ref.)	−51·963 p_2 (ref.)	+0·447 Δp_0	−2·361 Δp_2

$$(p_2/p_0)_{ref.} = \frac{-51{\cdot}963}{+5{\cdot}961} = -8{\cdot}717$$

mean cell blank = − 0·0025
p_0 (cell blank) = − 0·0025 × 12 = − 0·030
Δp_0 (corrected for cell blank) = 0·447 − (− 0·030) = 0·477
p_0 (sample) = (+5·961) + (+0·477) = + 6·438 }
p_2 (sample) = (−51·963) + (−2·361) = − 54·324 }

$$\text{hence } (p_2/p_0) \text{ sample} = \frac{-54{\cdot}324}{+6{\cdot}438} = -8{\cdot}438$$

$$\text{Therefore } F = 100\left(\frac{-8{\cdot}438}{-8{\cdot}717} - 1\right) = -3{\cdot}20$$

Each product relates to the multiplication of two other numbers *in the same row*. For example, in the first row (248 mμ), the entry (+2·035) in the final column ($\Delta E \times P_2$) is the product of ΔE (+0·037) and P_2 (+55) in the same row. The sum of each column constitutes an unnormalized coefficient. Moreover, with a desk machine able to accumulate products, the calculation is much easier than would appear from Table 4. Thus, having entered the data with multiplication, it is only necessary to record the unnormalized coefficient at the end of the process. Calculations may be further simplified by the use of data sheets, so printed that after tabulating the observed extinctions in wavelength order, each extinction lies opposite the appropriate values of P_0 and P_j. Normalization is unnecessary in view of the cancellation of normalizing factors in the expression for F.

Ideally, the cell blank, evaluated at all 12 wavelengths, should be subtracted from both E and ΔE, before commencing the calculation. However, cell blanks are usually unimportant to $(p_2/p_0)_{ref.}$ in view of an effective cancellation of errors when F is small. Furthermore, since Δp_2 is usually robust to cell blank errors (which mainly contribute to p_0 and p_1), it only remains to correct Δp_0. For this purpose, the mean of the cell blanks, observed at initial, mean and final wavelengths, is multiplied by the number of wavelengths (12, in this case) and the product subtracted algebraically from Δp_0.

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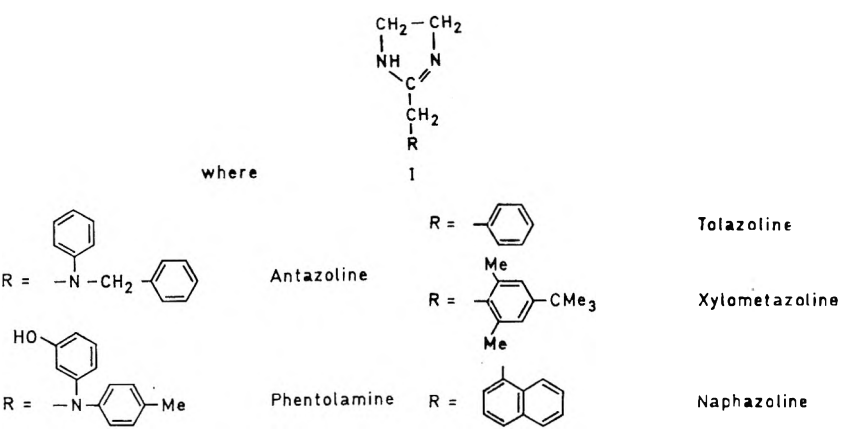
The gas chromatographic determination of imidazolines in pharmaceutical preparations

P. F. G. BOON AND W. SUDDS

The gas chromatographic determination of imidazolines in pharmaceutical preparations is described. Antazoline, naphazoline, tolazoline and xylometazoline are chromatographed at 197° on a column (4 ft) of potassium hydroxide (2%) and Carbowax 20M (1%) supported on Gas Chrom P; for certain formulations a shorter (1 ft) column has advantages. The method is shown by comparison with a colorimetric method based on reaction with nitroprusside, to be valid for obtaining stability data. The method is not applicable to the phenolic imidazoline phentolamine which is chromatographed after conversion to a trimethylsilyl derivative with bis-(trimethylsilyl)-acetamide on a column (3 inches) of silicone elastomer (10%) supported on Gas Chrom P.

SUBSTITUTED imidazolines of the general formula I find wide application in creams, tablets, nasal sprays and eyedrops. They may be formulated in admixture with each other, with other antihistamine compounds, corticosteroids, vasoconstrictors, antibiotics or sympathomimetics. The quality control of such complex formulations presents analytical difficulties, exacerbated when the preparations have undergone decomposition, for example as a result of accelerated storage treatment.

The most useful of the published procedures for the determination of imidazolines is the colorimetric method of Slack & Mader (1957) based on the reaction of imidazolines with alkaline nitroprusside. The reaction is apparently specific for the intact imidazoline ring (Stern, 1958), but it is not applicable to the phenolic derivative phentolamine and is often not directly applicable to complex formulations. Moreover, when applied to formulations containing mixtures of imidazolines, it can only give a total imidazoline figure.



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We have found the method of Schwartz, Kuramoto & Malspeis (1956), based on liquid-liquid chromatography, not to be generally applicable. Paper and thin-layer separations were explored but with their known quantitative limitations were not considered further.

Reports of the gas chromatographic detection of antazoline by MacDonald & Pflaum (1963, 1964) and Fontan, Smith & Kirk (1963) led to an extended examination of this technique.

In this laboratory we were particularly concerned to determine the minor components of the imidazoline mixtures: xylometazoline (0·05%)—antazoline (0·5%) and naphazoline (0·025%)—antazoline (0·5%). For this example and with many other routine assays where only one imidazoline is present, if instrumentation permits, a 1 ft column may be used with a solid injection system. This results in shorter retention times a higher throughput of samples and no solvent peak.

Experimental

Apparatus and conditions. A Pye Argon chromatograph was used throughout. Glass columns were packed with 4 ft or 1 ft of Carbowax 20M (1%) + potassium hydroxide (2%) on acid-and alkali-washed Gas Chrom P (100–140 mesh) rescreened* after preparation on a 150 mesh. Columns were normally operated at 197° with an argon flow rate of 40 ml/min.

PROCEDURE

Add a quantity of sample containing about 5 mg of imidazoline to 10 ml of chloroform in a separator, together with sufficient water to produce 20 ml of aqueous phase. Make alkaline with sodium hydroxide solution and extract immediately. When the phases separate, filter the lower chloroform layer through anhydrous sodium sulphate into a 20 ml volumetric flask. Extract the aqueous layer with a further 5 ml of chloroform, transfer the chloroform to the flask, dilute to 20 ml with chloroform and mix. Add 5 μl of this solution to a small square gauze and allow the solvent to evaporate. Chromatograph the sample on a column of suitable length. Calculate the area of the peak due to the imidazoline and by comparison with the area obtained by chromatographing a reference solution similarly prepared, the imidazoline content of the sample.

Results and discussion

On stationary phases such as silicone elastomer (SE30) (0·07%) and butanediol succinate (0·1%), the order of elution of the various substituted imidazolines is tolazoline, xylometazoline, naphazoline, antazoline in order of increasing molecular weight. Superior resolution with a minimum of tailing was achieved on a stationary phase of 1% Carbowax 20M on 2% potassium hydroxide (Fig. 1). Silanization of the prepared columns with bis(trimethylsilyl)acetamide was found advantageous.

* Rescreening led to an improvement in the performance (cf. Fontan & others, 1963).

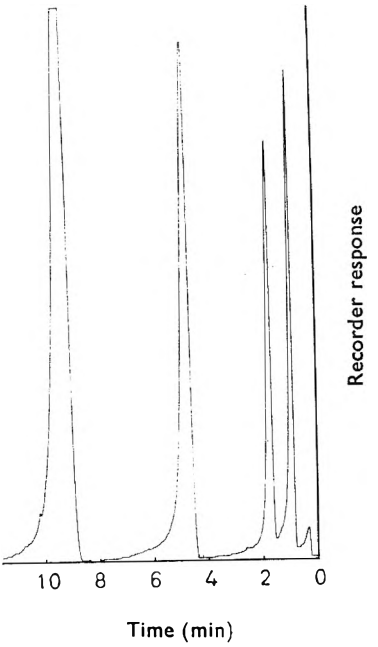


FIG. 1. Chromatography of tolazoline-xylometazoline-naphazoline-antazoline (elution order) on a column (4 ft) of Carbowax 20M (1%) and potassium hydroxide (2%) on Gas-Chrom P at 225° C.

The need for frequent recalibration of alkali-modified packing has been reported by Simonaitis & Guvernator (1967). This phenomenon, apparently due to adsorption onto active sites, was particularly serious in this work; repeated additions of standards to columns which had been out of use for a period or subject to mechanical shocks produced successively peaks of greater area.

The results obtained by applying the proposed method to various multicomponent formulations are shown in Table 1. Calibration curves

TABLE 1. RESULTS OBTAINED BY THE PROPOSED METHOD

Preparation	Prepared strength (% w/v)	Strength found (% w/v)
Nose drops containing antazoline sulphate and naphazoline nitrate	(i) antazoline sulphate 0·500 (ii) naphazoline nitrate 0·025	0·515 0·0245
Eye drops containing antazoline sulphate and xylometazoline hydrochloride	(i) antazoline sulphate 0·500 (ii) xylometazoline hydrochloride 0·050	0·480 0·0495
Nasal spray containing naphazoline nitrate, phenylephrine hydrochloride (0·25% w/v) and prednisolone (0·01% w/v)	0·0250	0·0258
*Solution containing naphazoline nitrate and ephedrine hydrochloride (1% w/v)	0·050	0·050

* This determination was carried out on a 4 ft column.

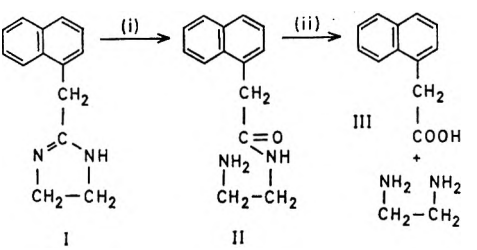
were rectilinear for all imidazolines over the range 0·94–1·56 μg. From routine use with duplicate runs of standard and sample the precision of the method is about $\pm 5\%$.

GAS CHROMATOGRAPHY OF PHENTOLAMINE

No response was obtained by adding phentolamine to any of the columns described. This is apparently due to the enhancement of the power to be adsorbed conferred by the phenol group since phentolamine is otherwise structurally similar to antazoline. Conversion of phentolamine to a trimethylsilyl ether was attempted with the conventional mixture of trimethylchlorosilane and hexamethyldisilazane but was unsuccessful. Klebe, Finkbeiner & White (1966) used bis-(trimethylsilyl) acetamide as a reagent for the silylation of hindered phenols. We found this reagent to react alone and rapidly with phentolamine base, the appearance of a less polar derivative being demonstrated by thin-layer chromatography. The trimethylsilyl derivative, however, could not be eluted from any of the standard 1 ft or 4 ft gas chromatography columns described above. It was successfully run at 210° on a short column (3 inches) containing a higher (10%) proportion silicone elastomer (SE30) to reduce adsorption. (Retention time was 3 min at 70 ml/min.)

APPLICATION TO DEGRADED FORMULATIONS

It has been suggested that the degradation of naphazoline (I) proceeds as follows (Schwartz & others, 1956):



and compounds II and III have been obtained from naphazoline by alkaline hydrolysis. It seems likely that decomposition of antazoline, tolazoline and xylometazoline will take place by an analogous route, while for phentolamine the phenol group is likely to be additionally involved. The four basic imidazolines were subjected to the alkaline hydrolysis conditions of Schwartz & others (1956) to produce II. Naphazoline and tolazoline each yielded a single basic product which did not give the nitroprusside reaction but which possessed ultraviolet absorption spectra similar to their parent compounds. With antazoline a mixture of two basic substances resulted; each showed a similar absorption spectrum to antazoline but neither gave the nitroprusside reaction.

These observations are consistent with reaction (i) occurring in each

case with perhaps a secondary degradation product for antazcline. Xylometazoline proved most resistant to hydrolysis, even the conditions used by Schwartz & others (1956) to produce III yielded unchanged starting material; this is to be ascribed to steric hindrance from the two *ortho* methyl groups.

Gas chromatography of the primary decomposition products of naphazoline and tolazoline yielded peaks which were well resolved from the parent compounds, having longer retention times consistent with their increased polarity. Chromatography of the hydrolysis mixture resulting from antazoline produced no detectable peaks. In addition to possessing very long retention times, these compounds may tail to such an extent as not to be detectable.

The nitroprusside and gas chromatographic methods have been applied to some experimental formulations subjected to accelerated storage tests. A comparison is made of the results in Table 2.

TABLE 2. COMPARISON OF COLORIMETRIC AND GAS CHROMATOGRAPHIC METHODS ON DEGRADED SAMPLES

Preparation	Treatment	Prepared strength (% w/v)	Strength found (% w/v)	
			Colour	GLC
Solution containing xylometazoline	3 years at 40°	0·100	0·085	0·084
Solution containing xylometazoline	3 years at 50°	0·100	0·059	0·060
Solution containing xylometazoline	3 years at 50°	0·100	0·048	0·049
Solution containing naphazoline	30 min at 100° at pH 8	0·100	0·082	0·084
Solution containing naphazoline	30 min at 100° at pH 8	0·0525	0·0254	0·0257

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A rapid thin-layer chromatographic method for the determination of noscapine (narcotine) and papaverine in opium and in pharmaceutical preparations

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When a simple ethanolic extract of opium or its preparations is run on Silica Gel G plates using ethyl acetate as running solvent, most of the impurities and all the alkaloids except noscapine and papaverine are retained on the starting line. Under the conditions described, quantitative recovery of the two alkaloids is possible in sufficiently pure form for spectrophotometric assay. The reproducibility of individual assays ($P = 0.95$) is about $\pm 2\%$ for noscapine and $\pm 4\%$ for papaverine.

A METHOD for the determination of noscapine and papaverine in poppy latex or opium has been described by El-Masry (1967) and is an adaptation of a similar paper chromatographic method for morphine, codeine and thebaine (Fairbairn & Wassel, 1963). Although satisfactory, the assay involved tedious area measurements and therefore we examined other methods. Mary & Brochmann-Hanssen (1963) describe a method based on elution followed by spectrophotometric measurement. However we found that this method leads to contamination of the eluted alkaloids with impurities which can cause errors of considerable magnitude in the assay. The method of Poethke & Kinze (1964) is based on elution and colorimetry but is not very sensitive. Both methods involve a preliminary separation of the alkaloids from the crude drug using column chromatography.

We then discovered a simple thin-layer system which could be used with untreated extracts of the crude drug. Most impurities and all the alkaloids except noscapine and papaverine are retained on the starting line. The latter are both well separated from each other and can be eluted quantitatively in pure form.

Experimental

Complete extraction of the two alkaloids from opium powder was effected by grinding with cold 80% ethanol; in the conditions described below neither alkaloid was detectable in the exhausted marc. Hot ethanol had no advantage over cold; the presence of acetic acid led to the extraction of fluorescent contaminants and to difficulties in the subsequent thin-layer chromatography. Grinding in the presence of acid-washed sand also led to the presence of contaminants. The elution of the alkaloids from the silica gel with a mixture of chloroform and ethanol (to which ammonia was added to liberate the free bases) was found most suitable as it also gave quantitative yields in small volume. Because of its volatility the eluate is evaporated to dryness and the residue dissolved in

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0·1N hydrochloric acid for spectrophotometry. Beer's law was obeyed for concentrations of 10–110 μg/ml for noscapine and 1–10 μg for papavarine.

METHOD OF ASSAY

Opium powder. Accurately weigh about 0·2 g of powdered opium and triturate thoroughly with 80% ethanol in small portions, filtering each portion into a 10 ml volumetric flask to volume. Transfer 0·2 ml of the extract in a fine streak about 10 cm long on the starting line of a 20 × 20 cm silica gel plate (250 μ thick).* Place marker spots of the extract on the starting line near both edges of the plate. After running the chromatogram† remove the plate from the tank and dry thoroughly in cold air. Separate the marker spots from the main bands by marking two vertical narrow channels. Protect the main bands and spray the two lateral series of marker spots with Dragendorff's reagent; the vertical channels prevent creep of spray reagent. Papaverine may be identified by the marker spots and as follows: Rf about 0·3, faint yellow fluorescence in ultraviolet light (366 mμ), noscapine has Rf about 0·5 and a distinct blue fluorescence. Another distinct greenish fluorescent band of Rf about 0·65 and a fainter one nearer the solvent front are also visible under ultraviolet light.

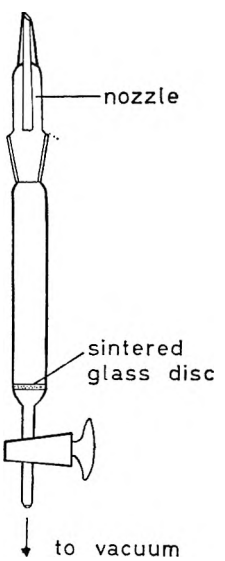

FIG. 1. Micro-vacuum cleaner (× ⅔).

* By Stahl's method (1956) using silica Gel G (Merck) tests showed that the organic solvent eluates of the alkaloids contained small amounts of contaminant, but only insignificant traces passed into the HCl solution. It is therefore unnecessary to use a silica gel eluate as a blank in the assay.

† Solvent: ethyl acetate. Conditions: room temperature (18–25°), saturation time 15 min, running time 30 min.

Remove each alkaloidal band separately by means of a suitable "micro-vacuum cleaner." This consists of a tube (about 12 × 1 cm) with a removable glass nozzle at the proximal end (modified from Truter, 1963; see Fig. 1).* The nozzle has a central channel about 3 mm diameter and its tip is ground flat at an oblique angle so that close contact can be made with the plate. At the distal end of the tube is a sintered glass disc, No. 2 porosity, and a tap connected to a vacuum. The nozzle is run over the marked area containing the alkaloid to transfer the adsorbent rapidly to the tube. When all the adsorbent has been thus transferred to the tube, invert it, tap down any powder in the nozzle, close the tap and release the vacuum. Wash the nozzle with about 2 ml of a mixture of chloroform–ethanol–ammonia (0·880) (90:10:1·5 v/v) and transfer the washings to the tube, gently shaking the adsorbent with the solvent. Allow to stand 15 min, percolate into a small glass evaporating basin and continue percolating with a further 5 × 2 ml of solvent. Evaporate the combined percolates to dryness by gentle warming.

Noscapine. Dissolve the appropriate dried percolate in 0·1N hydrochloric acid and make up to 5·0 ml with the acid. Determine the extinctions at 313 and 295 mμ against the acid. Calculate the quantity of noscapine from E (1%, 1 cm) (313 mμ) = 89·4 or from a calibration curve. The ratio of the extinction at 313 mμ to that at 295 mμ should be not less than 1·3.

Papaverine. Dissolve the appropriate dried percolate in 0·1N hydrochloric acid and make up to 10·0 ml with the acid. Determine the extinctions at 251 and 230 mμ against the acid and calculate the quantity of papaverine from E (1%, 1 cm) (251 mμ) = 1776. The ratio of the extinction at 251 mμ to that at 230 mμ should be not less than 2·0; between 251 mμ and 270 mμ it should be not less than 8·0.

Pharmaceutical preparations. For Injection of Papaveretum B.P.C. 60 μl were chromatographed directly for the assay: for Compound Spray of Adrenaline and Atropine B.P.C. 2 ml were diluted to 10 ml with ethanol and 40 μl chromatographed directly for the assay. For cough mixtures containing noscapine the latter was extracted with chloroform after rendering the mixture alkaline.

Results

The accuracy and reproducibility of the method was checked by making replicate assays on an artificially prepared extract containing known quantities of alkaloids and a sample of opium powder. The applicability to pharmaceutical preparations was checked using Injection of Papaverine B.P.C. and Compound Spray of Adrenaline B.P.C. Results are in Table 1. For Compound Spray of Adrenaline B.P.C. two assays gave 8·0 and 7·9 mg per ml of papaverine hydrochloride.

* The modified apparatus can be obtained from Oakes Eddon & Co. Ltd., Prescot Street, Liverpool, 7.

TABLE 1. RESULTS OF REPLICATE ASSAYS BASED ON THE RECOMMENDED METHOD

Sample	No. of assays	Noscapine	Papaverine
Artificial extract— Noscapine 54·8 mg/100 ml .. .. .. Papaverine 15·18 mg/100 ml	6	56·2 mg/100 ml (s.d. 0·21)	14·85 mg/100 ml (s.d. 0·13)
Powdered Opium	10	6·70% (s.d. 0·055)	1·80% (s.d. 0·036)
Injection of Papaveretum B.P.C.	2	4·44 mg/ml 4·46 mg/ml	0·71 mg/ml 0·72 mg/ml
Compound Spray of Adrenaline B.P.C. ..	2	— —	8·0 mg/ml 7·9 mg/ml

Discussion

The method is simple, accurate (98–103% of the alkaloids were recovered from the artificial extract), and reproducible. The coefficients of variation for the artificial extract values were 0·37 and 0·86% respectively for the noscapine and papaverine, and for opium 0·82 and 2·0% respectively. This degree of accuracy and reproducibility is achieved because the two alkaloids are well separated from each other and from contaminants. The method is also rapid, a complete assay of opium taking about 3 hr. The sensitivity is high, especially for papaverine. The extinction at the peak of 251 mμ, is about 8 times that at 279 mμ, recommended by Mary & Brochmann-Hanssen (1963) who, presumably, did not use the corresponding peak (239 mμ in methanol) because of heavy contamination at this wavelength.

Acknowledgements. We would like to thank Dr. W. E. Court for certain details of the modified micro-vacuum cleaner. This work forms part of a thesis to be presented by one of us (S.El-M.) for the degree of Ph.D. of the University of London.

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Chromatographic identification of cannabis

T. J. BETTS AND P. J. HOLLOWAY

Identification of cannabis can be achieved by using thin-layer chromatography together with gas chromatography. This is necessary because false positive reactions with some labiate herbs are given with one or other of the chromatographic methods alone. Tops and resin have been examined. The classic cannabis colour reactions have been used as sprays for thin-layer chromatography. For gas chromatography a 2% silicone elastomer on a silanized support was used, and the cannabinols run as their trimethylsilyl ethers.

CANNABIS is the general name for the reputedly narcotic products of *Cannabis sativa* L. A number of classic colour tests for cannabis exist which can give false positive reactions with other plant materials, especially labiate culinary herbs (UN Secretariat, 1960). Although the microscopy of cannabis is so distinctive, these colour tests are used possibly because they are felt to indicate more positively the presence of the true narcotic. The identification of cannabis by thin-layer chromatography should provide a more reliable means of chemical identification as the colour reactions can be used. We have correlated the results of thin-layer chromatography with results obtained using gas-liquid chromatography.

The active principles of cannabis are a mixture of cannabinols including cannabidiol, tetrahydrocannabinol isomers (THC) and cannabinol, the oxidation product of THC. Euphoric activity is attributed to the unstable, oily THC (Jacob & Todd, 1940). Cannabinols have been separated by partition chromatography on paper or thin-layers impregnated with dimethylformamide (de Ropp, 1960; Korte & Sieper, 1964) and separate in the sequence THC, cannabinol and cannabidiol (slowest), the Rf values depending greatly on the degree of impregnation of the paper or thin-layer. The spots are normally revealed by coupling with a diazo-compound, or by a modified Gibbs reaction (Krejčí, 1965). The phenolic cannabinols have been examined by gas chromatography (Farmilo & McConnell Davis, 1961; Kingston & Kirk, 1961; Lerner, 1963; Heaysman, Walker & Lewis, 1967) and also their trimethylsilyl ethers (Claussen, Borger & Korte, 1966; Heaysman & others, 1967) on low-loaded columns at temperatures between 170° and 250°. They always emerge in the sequence cannabidiol, THC, cannabinol (slowest).

Experimental

THIN-LAYER CHROMATOGRAPHY

Preparation of cannabis extracts. 0·1 g resin or 0·5 g tops (freed from seeds) is triturated with sand and light petroleum (b.p. 60–80°) in successive 10 and 2 × 5 ml portions. The combined, filtered light petroleum

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extracts are concentrated to a volume of about 1 ml before chromatography.

Plates. Approximately 250 μ layers of silica gel G for TLC are prepared on 20 cm square glass plates in the usual manner. Before use, each plate is impregnated in a chromatographic tank by allowing a mixture of dimethylformamide and carbon tetrachloride (equal parts by volume) to ascend the plate about 16 cm from the lower edge (cf. Korte & Sieper, 1964). Any plates on which the impregnating mixture does not rise evenly are rejected. The plate is then allowed to air-dry for exactly 15 min, towards the end of which time the cannabis extracts are applied; the plate is then put in the running solvent tank. This avoids excessive loss of impregnating agent, which causes decrease in the resolution of cannabinols.

Running solvent. Pure cyclohexane, with tank saturation ensured by lining the tank walls with absorbent paper. The cyclohexane is allowed to ascend 12 cm from the starting line (about 45 min at 22°).

Detection of cannabinols. After evaporation of the cyclohexane, the following colour reagents may be applied. Residual dimethylformamide does not prevent reaction and may even improve the response.

(*a*) Spray with 5% ethanolic potassium hydroxide and heat locally with a hair-dryer until cannabidiol spots appear [modified Beam test (1911) used by Farmilo, McConnell Davis & others, 1962]. Spray the alkali-treated plate with a fresh 0·5% ethanolic 2,6-dibromo-*p*-benzoquinone-4-chlorimine (Hopkin & Williams Ltd.) for a modified Gibbs reaction (1927).

(*b*) Spray with a freshly prepared 0·1% solution of tetrazotized di-*o*-anisidine (Fast Blue B, E. Gurr Ltd.) in chilled 3 N aqueous sodium hydroxide for a diazo-coupling reaction. This is the most sensitive test.

(*c*) Spray with 3% ethanolic vanillin containing 0·5% sulphuric acid by volume and heat in an oven at 110° for 5–10 min, or with 1% vanillin in 80% v/v sulphuric acid and heat with a hair-dryer [adapted from Ghamrawy test (1937) and Duquénois & Negm Moustapha test (1938)]. The colour response is modified by the amount of residual dimethylformamide present.

The identity of the three main thin-layer spots was confirmed from their colour reactions and relative positions on the plate as given in the literature. Korte & Sieper (1965) state that "cannabinolic components can be identified without using standard substances". Gas chromatographic peaks were also identified by comparison with their previously recorded positions and confirmed by removing the appropriate band from a partly sprayed thin-layer plate. The cannabinol was eluted with dry pyridine, its trimethylsilyl ether formed as described below, and examined on the gas chromatograph.

GAS-LIQUID CHROMATOGRAPHY

Preparation of cannabis extracts. The extract prepared for thin-layer chromatography is evaporated to dryness and the residue dissolved at room temperature (22°) in about 0·8 ml anhydrous pyridine in a 1 ml volumetric flask. Hexamethyldisilazane (0·1 ml) and one drop of trimethylchlorosilane is added and the contents made up to volume with dry pyridine and shaken for 1 min. After about 18 hr at room temperature, 1 μl aliquots are injected into the gas chromatograph. Retention times relative to n-eicosane were recorded, a trace of this C_{20} alkane being added to the pyridine mixture.

Gas-liquid chromatographic system. Pye 104 with a flame ionization detector. Glass columns (5 ft), internal diameter 4 mm, were packed with 2% Silicone elastomer SE-30 on silanized Chromosorb W (80–100 mesh). The method of Bohemen, Langer & others (1960) was used for support silanizing. Oven temperature 170°. Mobile phase nitrogen, inlet pressure 15 psi, flow rate at column exit 40 ml/min; the hydrogen supply to the detector being at the same flow rate.

Results

Table 1 gives the Rf values observed after thin-layer chromatography together with the appropriate colour responses. Table 2 gives the relative retention times found by gas chromatography and Fig. 1 shows a typical gas chromatogram. Table 3 gives the results obtained by the two chromatographic methods on various cannabis specimens.

No response was obtained by either chromatographic method from light coloured smoking tobacco or oven-dried banana peel. A positive thin-layer response (but none by gas chromatography) was obtained from thyme herb, whilst a misleading gas chromatographic peak (but no response by thin-layer) was obtained from sage and rosemary herbs. This last fact is of interest in view of the confusion by analysts between rosemary and cannabis (Farmilo & others, 1962).

TABLE 1. THIN-LAYER CHROMATOGRAPHIC RESPONSE OF CANNABINOLS AND THYME EXTRACT TO DETECTION SPRAYS

Spray reagent	THC (approx. Rf 0·65)	Cannabinol (approx. Rf 0·45)	Cannabidiol (approx. Rf 0·15)	Thyme ext. (approx. Rf 0·15)
(*a*) Alcoholic KOH and heat (Beam test)	—	—	violet (pale)	—
Followed by dibromoquinone chlorimide (Gibbs)	blue	pale blue	brown-violet	blue
(*b*) Alkaline fast blue B (diazo)	geranium lake	purple	vermilion	vermilion
(*c*) Vanillin and sulphuric acid (Ghamrawy–Duquénois)	pale green → blue-violet	violet-pink	dark green → blue-violet	bright red

TABLE 2. GAS CHROMATOGRAPHIC RELATIVE RETENTION TIMES ($t_r$rel) OF TRIMETHYLSILYL ETHERS OF CANNABINOLS AND SOME LABIATE HERB EXTRACTS

Cannabinol or etc. trimethylsilyl ether	$t_r$rel n-eicosane	$t_r$rel cannabidiol
Cannabidiol—isolated by thin-layer	2·88	—
—in resin extracts together with THC and cannabinol	3·05–3·11	—
—in tops extracts with cannabinol	2·81–3·12	—
—in tops extracts with THC	3·19	—
THC—isolated by thin-layer	4·15	1·44
—in resin extracts together with cannabidiol and cannabinol	4·34–4·52	1·43
—in tops extracts with cannabidiol	4·54	1·42
Cannabinol—in resin extracts together with cannabidiol and THC	5·97–6·21	1·97
—in tops extracts with cannabidiol	5·82–6·29	2·04
Sage or rosemary	6·08	—

TABLE 3. CANNABINOLS IDENTIFIED IN CANNABIS SPECIMENS EXAMINED BY THIN-LAYER AND GAS-LIQUID CHROMATOGRAPHY

Specimen type, description and origin*	Thin-layer			Gas-liquid		
	Canna-bidiol	Canna-binol	THC	Canna-bidiol	Canna-binol	THC
Resin A. Hard, flat, cloth-covered cake. Middle East	++	++	+?	++	+	+
Resin B. Brittle, thin discs about 5 cm diameter	++	++	++	++	++	++
Resin C. Very hard, flat cake, wax-covered	+?	+?	+?	—	—	—
Resin D. Soft cylinder found in tennis racquet handle	++	++	+?	++	+	+
Tops E. Recent police seizure	—	+	—	+?	++	−?
Tops F. Museum sample (1907) from Zanzibar	—	+	—	+	+	—
Tops G. Recent police seizure. English	—	+	—	—	+?	—
Tops H. Madras	—	+	—	+	+	—
Tops J. Transvaal	+	+	+?	+	++	—
Tops X. Museum sample. Very compressed masses	—	+	—	+	+	−?
Tops 6. Recent police seizure. Good green colour, not resinous	—	+?	+	+	—	++

++ indicates strong response, + indicates weak, but definite response, +? doubtful response, and — no response.

* Specimens A to J were kindly provided by the Metropolitan Police Laboratories, specimen X was from the School of Pharmacy Museum, and specimen "6" was sent to Professor J. W. Fairbairn in March 1967.

Discussion

Of the cannabis specimens examined, resin B should have been the most potent as it contained a high proportion of THC (see Fig. 1). Resins A and D were similar to each other, containing all three cannabinols, with much cannabidiol but little THC; resin C was almost devoid of cannabinols. Tops usually had only a large proportion of the oxidation product cannabinol (possibly a result of storage) with a little cannabidiol. The new, green specimen tops "6" was exceptional in containing THC with cannabidiol, and almost no cannabinol. Table 3 shows that trace amounts of cannabinols could sometimes be detected by one chromatographic method and not the other. Thus the use of the two methods in conjunction is particularly valuable in the identification of cannabis.

This is also important when examining labiate herbs, for thyme, sage and rosemary might be mistaken for cannabis using one method only. As tobacco gives no interfering response by either chromatographic method, it is possible to detect cannabis in admixture with it. Dried banana peel, which according to newspaper reports is used as a substitute for cannabis, gives no chromatographic response.

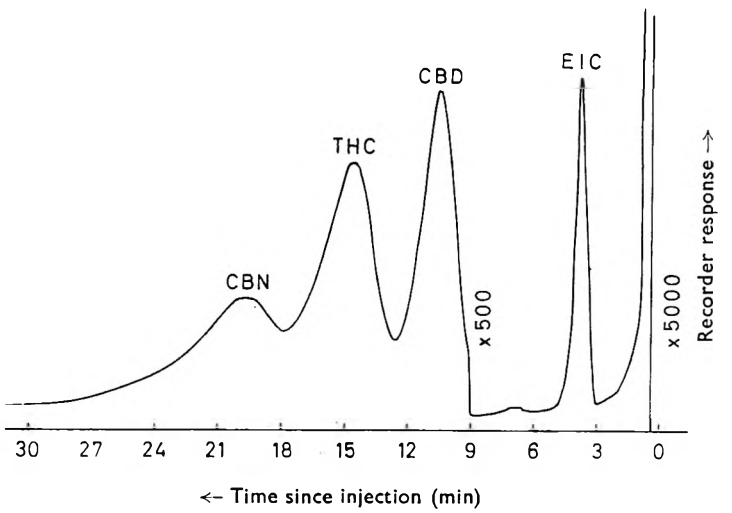

FIG. 1. Gas chromatogram of 1 μlitre of extract of resin B (0·1 g in 1 ml pyridine) trimethylsilyl cannabinol ethers and reference n-eicosane (EIC). Recorder attenuation × 5000 for first 9 min, then × 500. CBD = cannabidiol; CBN = cannabinol; THC = tetrahydrocannabinols.

The application of classic cannabis colour reactions to thin-layers should increase their value. The highly esteemed alkaline Beam test gives a specific response with cannabidiol, confirming the observation of Jacob & Todd (1940). Blackie (1941) found that a number of aromatic aldehydes gave colours in acid solution with cannabis extracts, and for a thin-layer spray, vanillin-sulphuric acid is an effective compromise between the Ghamrawy and Duquénois formulations. The constituent of thyme extract responding to the sprays and with an Rf value similar to cannabidiol is thymol, which is phenolic.

For gas chromatographic identification of cannabis, the routine formation of the trimethylsilyl ethers of the cannabinols is recommended, these being examined on a low-loaded column on a silanized support. This gives results free from the many interfering peaks observed by Kingston & Kirk (1961). The formation of the trimethylsilyl ethers is claimed to be rapid at room temperature (Wells & Makita, 1962) but an overnight period of reaction seems preferable for cannabinols, as was used by Claussen & others (1966). Cannabidiol reacts more slowly than the other cannabinols, and this period also allows the by-product precipitate of ammonium chloride to settle.

From examination of the internal standard, n-eicosane, it can be calculated that n-nonacosane (C_{29} alkane) which occurs naturally in cannabis (Farmilo & others, 1962) would not be resolved under the conditions used (Holloway, 1967). Variation in relative retention times observed here with the cannabinols (Table 2) may be due to their mutually affecting each other during passage through the column, or to varying quantities of cannabinols examined under non-linear partition conditions, or the use of an internal standard with a short retention time.

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SHORT COMMUNICATION

Colorimetric determination of vitamin D_2 with trifluoroacetic acid

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THE use of trifluoroacetic acid for the colorimetric determination of vitamin A has been reported by Neeld & Pearson (1963) and by Dugan, Frigerio & Siebert (1964), but its use for the determination of vitamin D does not seem to have been studied. Many other Lewis acids, such as antimony trichloride, have been used for both determinations (Carr & Price, 1926; Brockmann & Chen, 1936), and the reaction of vitamin D with antimony trichloride in the presence of acetyl chloride (Rogers, 1954, 1955; Stross & Brealey, 1955) is the basis of the colorimetric assays of Calciferol Solution and Calciferol Tablets in the British Pharmacopoeia 1963.

The antimony trichloride method suffers from a number of disadvantages (Stross & Brealey, 1955). The reagent is volatile, it becomes cloudy with even traces of moisture, and the spectrophotometer cuvette may become misty during the measurement. Because the colour appears within about $1\frac{1}{2}$ min and fades rapidly, skill is necessary to measure the maximum extinction. Values of E (1%, 1 cm) appear to vary from batch to batch of reagent and from day to day.

Trifluoroacetic acid gives an immediate blue colour with vitamin A, and the extinction at 616 mμ is directly proportional to the concentration of vitamin A over the range 0·6–3·0 μg/ml of the final solution (Neeld & Pearson, 1963). With a solution of vitamin D_2 (ergocalciferol) in chloroform, trifluoroacetic acid gives an immediate pink colour, changing after about 3–4 min to a yellow colour, which is suitable for quantitative spectrophotometric measurement. Repeated scanning of the visible spectrum shows that a maximum at about 496 mμ appears during the first 90 sec then rapidly disappears. A maximum at about 403 mμ appears during about 5 min and remains stable for at least 40 min.

The rate of formation of the absorption maximum at 403 mμ depends on the concentration of trifluoroacetic acid in the final solution, and also to some extent on the concentration of any ethanol or water that may be present in the chloroform used as solvent. The sensitivity varies with the concentration of trifluoroacetic acid; it appears to be but slightly dependent on the purity of the chloroform, and "reagent" grade containing 1 to 2% ethanol is satisfactory. The optimum concentration of trifluoroacetic acid, 20% v/v, which gives the highest sensitivity and the most

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convenient rate of development of colour, is independent of the concentration of ergocalciferol when this is in the range from 10 to 50 μg/ml.

The recommended concentration of ergocalciferol in the solution used for the colour development (see below) is the same whether trifluoroacetic acid or antimony trichloride is used. Two ml of ergocalciferol solution is required with trifluoroacetic acid; only 1 ml is required for the B.P. method but a greater proportion of antimony trichloride reagent is used.

The trifluoroacetic acid procedure is much more convenient. After the reagent has been mixed with the sample, the operator has 10 min before the measurement is made, and subsequent fading of the colour is very slow. Trifluoroacetic acid is more stable than the antimony trichloride reagent and it does not have to be specially prepared. Moderate amounts of water or ethanol are of little importance and do not produce a cloudiness. An extinction correction based on measurement at a second wavelength is unnecessary. No variation of E (1%, 1 cm) according to the batch of acid or from day to day has been found.

PROCEDURE

Take 2·0 ml of a chloroform solution containing from 20 to 100 μg of ergocalciferol and mix with 0·50 ml of trifluoroacetic acid (B.D.H. reagent grade) directly in a spectrophotometer cuvette of path length 1 cm. Set aside for 10 min and measure the extinction at 403 mμ. Calculate the concentration of ergocalciferol by comparison with the extinction of a similarly-treated standard solution.

The value of E (1%, 1 cm) for the colour produced at 403 mμ is about 200 and the molar extinction coefficient ϵ is about 7900.

The coefficient of variation based on 26 determinations of ergocalciferol in solutions containing 10 to 50 μg per ml is 1·73%. The corresponding coefficient of variation for 23 determinations by the antimony trichloride method is 1·55%, which statistically is not significantly different from 1·73%.

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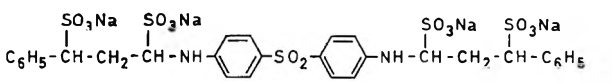
The chemical assay of solapsone

J. C. DEAVIN*, R. E. A. DREY AND G. E. FOSTER

The hydrolysis stage of the British Pharmacopoeial method of assay for solapsone has been reinvestigated and modifications are suggested whereby the formation of non-diazotizable polymeric material is avoided. Comparative assay results for a number of samples of solapsone and tablets of solapsone, when assayed by the official and modified methods, are given. Thin-layer chromatographic examination of solapsone has shown the presence of appreciable quantities of semi-solapsone [disodium salt of 4-(3-phenyl-1,3-disulphopropylamino)-4′-aminodiphenyl sulphone]; the significance of this observation is briefly discussed.

SOLAPSONE (sulphetrone; I) was first described by Buttle, Dewing & others (1938) and has since gained wide acceptance in the chemotherapy of leprosy. It is prepared by the interaction of dapsone with two or more equivalents of cinnamic aldehyde in the cold, followed by treatment of the resulting 4,4′-dicinnamylidene-aminodiphenyl sulphone with sodium bisulphite solution. The product contains not less than 5% of water.



I

The official method of assay (B.P. 1963, page 763), which is based on the work of Dewing & Foster (1948), involves hydrolysis with hydrochloric acid, followed by titrimetric determination of free sulphone with sodium nitrite.

Photocolorimetric procedures for determining solapsone by diazotization of the acid hydrolysate with sodium nitrite followed by coupling with *N*-(1-naphthyl)ethylenediamine or dimethyl-α-naphthylamine have been described by Brownlee, Green & Woodbine (1948), by Dewing & Foster (1948) and by Short (1951).

Although the composition of commercial solapsone is subject to variation, even uniform batches of the drug are liable to give results which are both variable and low when assayed by the official method. This is due to the formation of significant amounts of insoluble polymeric material in the hydrolysis stage. Such insoluble material is sodium-free and contains about 11% of sulphur and about 5% of non-diazotizable nitrogen, and it is therefore clear that the formation of this insoluble material is responsible, at least in part, for the low assay results obtained. A detailed examination of the hydrolysis stage of the official method of assay was consequently undertaken, with the object of minimising the

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formation of the insoluble compound and thus obtaining more accurate results. Commercial samples of solapsone have also been examined by thin-layer chromatography.

Experimental and results

ASSAY

Material used. Solapsone B.P. (Loss on drying, 8·66%.) All assay results are expressed with reference to the anhydrous material.

The effects of changes in the following variables on the assay results obtained by the official method were examined in turn: strength of acid; ratio of solapsone to acid, at various concentrations of acid; variations in the time of hydrolysis; and effect of carrying out the hydrolysis under reflux rather than in an open vessel, with intermittent replacement of water lost by evaporation.

Solapsone 0·5 g was boiled for 1 hr with hydrochloric acid (120 ml) of strengths from 0·1–4 N. The solapsone found varied from 80·1% with 0·1 N acid to 94·9% with 2 N acid falling to 67·3% with the 4 N acid.

The drug (0·5 g), hydrolysed for 1 hr with acid of strengths varying from 0·5 to 2·5 N used in volumes of 120, 250, 500 ml. Recoveries increased with increase of volume of acid and varied with acid strength; 91·5% was recovered with 120 ml of 0·5 N acid and 102·0% with 250 ml of 2 N acid.

The period of hydrolysis did not affect the recovery between 10 and 90 min (101·7%); it was 89·9% at zero time, when the end-point was indefinite, 103·2 and 105·2% at 5 min, and 100·2% at 120 min.

Formation of the insoluble material may be entirely prevented by increasing the volume of solution for a given weight of drug and by an increase in the acid content; no attempt was made to increase the volume of solvent above 500 ml however, due to loss of sensitivity in the detection of the end-point at this dilution. Boiling under reflux led to increased formation of insoluble matter, presumably due to reaction of condensed cinnamic aldehyde with diazotizable material.

The ease with which solapsone is hydrolysed in strong acid medium to dapsone was confirmed by following the reaction by thin-layer chromatography; after hydrolysis for 2 min, only trace amounts of solapsone and of semi-solapsone were detectable.

Proposed assay method. Dissolve solapsone (0·5 g), accurately weighed, in water (75 ml), add dilute hydrochloric acid (175 ml) giving a final normality of 2 N, and boil gently in a conical beaker for 30 min, replacing the water lost by evaporation. Cool to room-temperature, then stand for 15 min in melting ice and titrate with 0·1 M sodium nitrite, stirring magnetically. The end-point may be detected either with starch-iodide paper (Siggia, 1963) or by the dead-stop end-point method. Each ml of 0·1 M sodium nitrite is equivalent to 0·04464 g of $C_{30}H_{28}N_2Na_4O_{14}S_5$.

Make a blank determination and apply the necessary correction. (A blank determination should also be made when standardizing the nitrite solution.)

THE CHEMICAL ASSAY OF SOLAPSONE

Comparative assay results for six representative production samples of solapsone, and for two samples of solapsone tablets, when assayed by the official and by the modified methods, are given in Table 1.

TABLE 1. ASSAY OF SOLAPSONE AND SOLAPSONE TABLETS (0·5 g)

	Solapsone: $C_{30}H_{28}N_2Na_4O_{14}S_5$ content	
	B.P. method (%)	Proposed method (%)
Sample 1	97·1	101·0
" 2	96·5, 96·6	100·3, 100·2
" 3	94·4	100·5
" 4	96·7, 96·2	101·2, 101·3
" 5	98·1	101·4
" 6	97·7	102·7
	Tablets: $C_{30}H_{28}N_2Na_4O_{14}S_5$ content (expressed as % of stated amount)	
	B.P. method	Proposed method
Sample A	90·1, 88·9	94·4, 94·8
Sample B	87·1, 87·8	92·5, 93·3

THIN-LAYER CHROMATOGRAPHIC EXAMINATION OF SOLAPSONE

The following system proved suitable for the thin-layer chromatographic examination of solapsone.

Adsorbent. Silica Gel GF_{254} (Merck). *Mobile phase.* Amyl alcohol–ethyl acetate–water–methanol (6 : 5 : 5 : 4). *Detection.* Either by viewing in ultraviolet light (254 mμ), or by spraying with 0·1 N hydrochloric acid, heating at 105° for 5 min, cooling, exposing to "nitrous fumes"* for 10 min, removing excess fumes with the aid of a current of air and finally spraying with a freshly-prepared 0·5% solution of *N*-(1-naphthyl)-ethylenediamine hydrochloride in 90% ethanol.

Solapsone and semi-solapsone were applied in 1% aqueous solution; dapsone was dissolved in ethanol.

Representative Rf values and colours of spots are given in Table 2.

TABLE 2. THIN-LAYER CHROMATOGRAPHY DATA FOR SOLAPSONE AND RELATED COMPOUNDS

	Solapsone	Semi-solapsone	Dapsone
Representative Rf value	0·25	0·47	0·78
Colour of spot:			
Ultraviolet light	fluorescent blue	mauve	purple
After spraying	reddish-purple	reddish-purple	reddish-purple
Least amount detectable:			
Viewing in ultraviolet light	0·5 μg	0·5 μg	0·1 μg
After spraying	0·2 μg	0·2 μg	0·05 μg

A semi-quantitative assessment was made of the impurities contained in five samples of solapsone B.P.; the results are presented in Table 3. The optimum loading was found to be 50 μg.

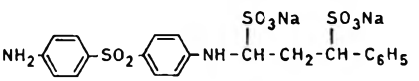
* 50% sulphuric acid is added gradually to an aqueous solution containing 10% sodium nitrite and 3% of potassium iodide.

TABLE 3. IMPURITIES CONTAINED IN SOLAPSONE B.P.

Sample	Semi-solapsone (%)	Dapsone (%)	Impurity Rf 0·66 (%)
1	15	0·5	nil
2	20	1	0·5
3	20	2	0·5
4	7	1	nil
5	20	1·5	nil

Discussion

Table 1 shows that the proposed method gives appreciably higher assay results than those obtained by the official method. The fact that results exceed 100% is presumably due to the presence of semi-solapsone (II) and of small quantities of dapsone (Table 3), which would be expected to lead to high assay results.



II

Whilst the method of assay fails to differentiate between solapsone and its most likely contaminants semi-solapsone and dapsone, the presence of these two compounds may readily be detected by thin-layer chromatography. Discrepancies between the content of solapsone and of semi-solapsone, as given in Table 3, and the assay figures given in Table 1, may be ascribed to small quantities of sodium bisulphite which it is not economic to remove from the commercial material.

In view of the lability of the disodium phenyl-disulphopropyl group it is not unexpected that relatively large amounts of the half-sulphone were found in commercial samples of solapsone. The presence of semi-solapsone in commercial material has also been reported by Bushby & Woiwod (1955). At the same time, it has been shown that solapsone itself is largely inactive, and owes its action to *in vivo* breakdown to the active semi-solapsone (Cochrane, 1952).

Semi-solapsone is not unique in displaying greater biological activity than the corresponding di-substituted sulphone: the mono-substituted analogues of sodium glucosulphone and of sodium sulphoxone are also more potent than the corresponding di-substituted derivatives (Smith, Jackson & Bauer, 1949). The toxicity of semi-solapsone is similar to that of solapsone.

Attempts to assay solapsone by titration in non-aqueous medium using various titrants (0·1 M sodium methoxide, tetra-n-butylammonium hydroxide, etc.) were unsuccessful.

Acknowledgements. The authors are grateful to Mr. M. Saleem and to Mr. D. Wheeler for assistance with the experimental work, and to Dr. C. G. Raison and Dr. S. R. M. Bushby for providing a sample of semi-solapsone.

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Structure and rheology of sodium dodecyl sulphate–cetyl alcohol–water: continuous shear experiments*

B. W. BARRY† AND E. SHOTTON

The nature of systems formed when sodium dodecyl sulphate, cetyl alcohol and water are heated, mixed and cooled has been investigated. Systems R_1 to R_{10} (where the subscript refers to the molar ratio alcohol to sulphate) consisted of frozen liquid crystal and solid alcohol dispersed in aqueous solution. The systems were metastable and with time their consistency decreased and crystals were deposited. In continuous shear at 25°, all systems underwent irreversible shear breakdown. Each flow curve showed a hysteresis loop and systems R_4 to R_9 had a spur on the upcurve. The loop area, shear stress, shear strain and shear rate at the spur increased with alcohol content. Between 25° and 62·5° system R_8 showed maximum apparent relative viscosity at 42·5°.

IN a review, Pickthall (1950–51) discussed emulsifying agents based on a mixture of cetyl alcohol with a surface-active agent such as sodium cetyl sulphate or phosphate. He considered that, when this type of emulsifier was heated with water, the water extracted the alkyl sulphate or phosphate, which formed a complex with the alcohol at the interface. The excess alcohol forms an oil phase and is emulsified in the water. It has also been shown (Munzel & Ammann, 1954) that if the concentration of emulsifying wax is sufficiently high, then addition of water produces ointment-like products in which the lipophilic constituents are stored within the micelles of the hydrophilic emulsifying agent. In the present work we have investigated the effect of the alcohol–sulphate ratio on the structure and rheology of the systems formed by dispersing the mixed emulsifiers in water.

Experimental and results

MATERIALS

Water. Mains water, distilled from an all glass still.

Sodium dodecyl sulphate (Marchon Products Ltd., Whitehaven) with the following manufacturer's analysis: sodium dodecyl sulphate 98·09%, free lauryl alcohol 0·66%, water content 0·72%, sodium sulphate 0·13% and sodium chloride 0·40%. Approximately 1 g of this material was refluxed (4 hr) with 30 ml water and 10 ml hydrochloric acid. The mixture was cooled and the alcohols extracted with ether. The alcohols were chromatographed in n-heptane solution using a Perkin-Elmer Fractometer Model 451 with a hot wire detector at oven temperature 184°. Hydrogen was used as the carrier gas at a flow rate of 100 ml/min and the column

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(length 105 cm) was 2% Versamid 900 in 60–80 mesh acid washed silanized Chromasorb W, obtained from F. & M., London. Results showed that the alkyl sulphate was composed of 98·6% C_{12}, 1·0% C_{10} and 0·4% C_{14} homologues.

Cetyl alcohol. This was obtained from Sipon Products Ltd., London, and was stated to be 99·9% pure and to contain no homologues or isomers. One peak only was obtained on gas chromatography as above at oven temperature 206°. The melting point was 49·5° (literature values = 49–50°).

PREPARATION OF SYSTEMS

The systems were prepared according to the general formula: sodium dodecyl sulphate 4 g, cetyl alcohol varied, water 400 g.

The proportion of alcohol was varied to give a molar ratio, alcohol to sulphate, varying by unit steps from system R_1 to system R_{10}, where the subscript indicates the molar ratio concerned. The ratio of sulphate to water was maintained constant throughout.

Mixing of the ingredients was carried out with a Silverson Multi-Purpose High-Speed Laboratory Mixer fitted with an axial flow head and emulsor mesh to ensure that there was no aeration; this was essential since not all the systems released entrapped air on standing. The rotor shaft of the standard model was enclosed in a stainless steel sleeve attached to the top of the stationary part of the working head so that the rotor shaft was not in contact with the mix. The motor of the mixer was connected to a variable output transformer so that low speeds were available, and mixing was in a 600 ml stainless steel beaker placed in a container through which cooling water could be passed.

The sodium dodecyl sulphate was added to 400 g water at about 85° and mixed at a low speed, for 1–2 min. This procedure allowed any trapped air to rise to the surface. The alcohol was melted and when both liquids were at approximately 65° it was poured in a thin stream into the sulphate solution. The rate of stirring was adjusted to ensure good mixing without entrapping air. The beaker and contents were cooled rapidly and as the temperature dropped, a marked increase in consistency occurred with all systems other than those of low alcohol content. When the consistency was such that mixing ceased, the material was packed in one ounce wide mouthed jars and stored at 25°. Each system was checked microscopically and discarded if air bubbles were present.

APPEARANCE OF SYSTEMS

Systems R_1 to R_3 were mobile, turbid, off-white liquids which increased in consistency from R_1 to R_3. Viscoelastic properties became noticeable from R_4 to R_{10}. System R_5 was off-white, shiny and translucent and showed noticeable elastic recoil and yet flowed slowly under its own weight. There was a steady increase in consistency up to R_{10} which was a white, glossy, smooth soft solid.

The systems were examined individually at a magnification of × 200 using a polarizing microscope and a Kofler Micro Hot Stage. Photomicrographs of system R_8 were taken between crossed polars, using a Practika camera and 35 mm Ilford F.P.3 film (Fig. 1).

At room temperature all systems showed a similar appearance to Fig. 1. In ordinary light, scattered small globules were visible, often embedded in larger, roughly circular masses which were only faintly seen. Between crossed polars, the large circular masses were strongly anisotropic, generally with a somewhat deformed black cross—that is, they showed the optical properties of a uniaxial crystal.

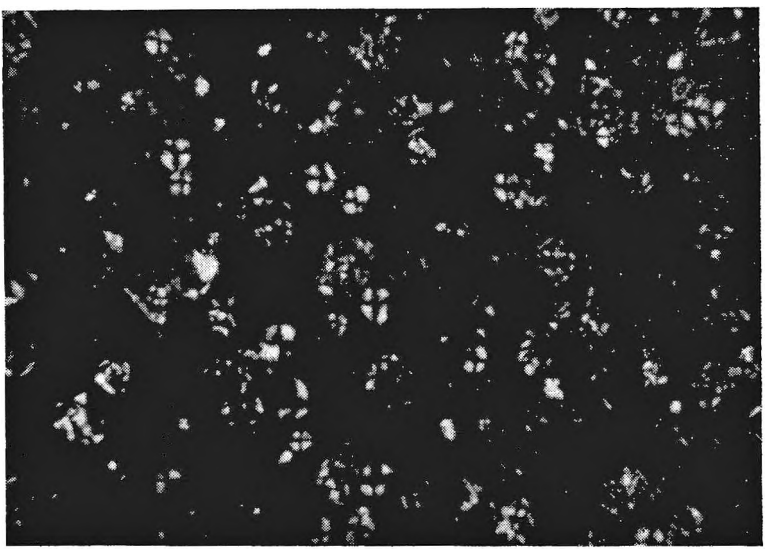

FIG. 1. Photomicrograph of system R_8: crossed polars.

Anisotropic structures were of two kinds: (*a*) cetyl alcohol, which can crystallize from a melt in the form of a uniaxial crystal; (*b*) frozen liquid crystals (often containing unreacted alcohol at their centres), formed from the interaction of sodium dodecyl sulphate, cetyl alcohol and water.

They were differentiated by their reaction on warming. Some of the anisotropic material melted and formed isotropic globules at about 50°: these appear to be mainly cetyl alcohol. Other structures maintained their anisotropic appearance to well above the melting point of cetyl alcohol, often to 65°: these are probably frozen liquid crystals. As the ratio of alcohol to sulphate increased from system R_1 to system R_{10}, the proportion of cetyl alcohol particles increased.

STABILITY OF SYSTEMS

With time, all systems underwent a change in appearance, becoming more mobile and forming silvery crystalline deposits. In systems of low alcohol content (particularly R_1) this deposit was very noticeable as it

formed a characteristic "pearly" layer adhering to the inner surface of the container, and excess was deposited at the bottom of the jar; it was thus denser than pure cetyl alcohol. Systems R_4 to R_{10} progressively lost their rigidity and became sufficiently liquid to flow under their own weight. Such changes could be hastened by subjecting the samples to the fluctuations in laboratory temperature. One jar containing system R_1 showed no evidence of this characteristic crystallization. The contents were divided into two parts, one portion being seeded from a container exhibiting crystallization, the other serving as a control. After four days storage in the open laboratory, more crystals were present in the seeded portion than in the control.

Microscopic examination of these samples showed features in common, regardless of the original composition of the system. There were present many thin, flat, polyhedral crystals, generally hexagonal; also a number of acicular crystals. A sample of R_1 containing these crystals was warmed on the micro hot stage. At 28° the flat crystals melted first, followed by the acicular crystals, although no temperature increase was detected. If the preparation was warmed until the flat crystals just started to melt and the heat was switched off, then on cooling, acicular crystals grew out of the sides of the polyhedral crystals, indicating a change in crystal habit. When systems were stored at a constant temperature immediately upon preparation, no crystallization occurred in less than fourteen days. Before rheological measurements were made, the systems were examined microscopically to confirm the absence of these crystals.

Under the conditions obtaining in the present work, hydrolysis was negligible.

RHEOLOGICAL ANALYSIS

This was made using a Ferranti-Shirley Cone and Plate Viscometer (McKennell, 1954, 1956, 1960; Van Wazer, Lyons & others, 1963a). Preliminary experiments showed that, at a given rate of shear, the shear stress fell steadily with the time of shearing; in general, an equilibrium shear stress at a set shear rate was not obtained even after 30 min. Microscopic examination of samples which had been sheared for long periods showed that most of the complex structures originally present had been broken down. Thus even if flow curves of equilibrium shear stress versus shear rate were constructed by rotating the cone for longer times, the curves derived would bear little relationship to the rheological properties of the original systems. The Ferranti-Shirley equipment was used therefore in conjunction with an automatic flow curve recorder unit designed to provide a standardized shearing procedure for materials which exhibit shear and time dependent flow behaviour. The control unit was designed to give uniform acceleration of the cone from zero to a preset maximum rev/min and then to decelerate uniformly: the recorder unit was set to give a maximum shear rate of 1632 sec^{-1} and a sweep time of 600 sec. The resulting flow curves were displayed on an X–Y Autoplotter.*

* Model XY–IP, Scientific Furnishings Ltd.

The performance of the complete instrument has been described elsewhere (Barry, 1967).

Fig. 2 shows a typical plot of the average of three runs for each system at 25° ± 0·1°. In general, repeat runs were within 5% as measured on the torque axis. System R_1 gave deflections too small to be measured with reasonable accuracy and results are not included in this paper.

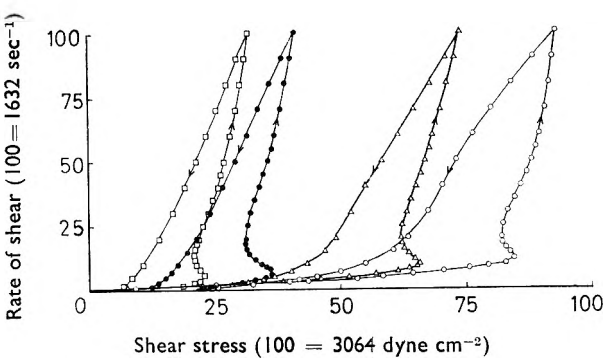

FIG. 2. Flow curves for systems R_5 —□—, R_6 —●—, R_8 —△— and R_9 —○—. Ferranti-Shirley viscometer used in automatic mode at 25°.

All experimental plots showed a common hysteresis effect in that the "down" curve lay to the left of the "up" curve. The area enclosed by the loop increased with increase in molar ratio from R_2 to R_{10}. From R_4 onwards there was a "spur" on the upcurve which virtually disappeared with R_{10} as there was no temporary fall in the shear stress. There was no evidence of a yield value; even when the downcurve intersected the stress axis at a positive value (shear rate zero) the stress did not represent a true yield value since it decayed to zero over a short period of time.

The total shear applied to the material up to the spur point was calculated from the angular equation of motion

$$\theta = \omega_0 t + \tfrac{1}{2}\alpha t^2$$

where θ is the angular displacement of the cone in radians; ω_0 is the initial angular velocity in radians sec^{-1} (zero in this case); α is the angular acceleration of the cone, assumed constant (radians sec^{-2}); t is the time elapsed from the start of rotation to the spur point (sec). The shear is then given by θ/ψ where ψ is the cone angle in radians.

The areas enclosed by the loops and the values of shear stress, shear rate and shear strain at the spur point for the different systems are given in Table 1.

A series of experiments was made at 25° ± 0·1° to determine if the systems were thixotropic, i.e. if a structure was reformed in the material after shearing had stopped. A flow curve was obtained as above and repeated on the same sample, varying the times for which the sample was allowed to rest: a typical result was that obtained for system R_5

TABLE 1. AREAS OF HYSTERESIS LOOPS AND VALUES OF SHEAR STRESS, SHEAR RATE AND SHEAR STRAIN AT SPUR POINT

System number	* Area of hysteresis loop (cm^2)	Shear stress at spur point (dyne cm^{-2})	Shear rate at spur point (sec^{-1})	Shear strain at spur point
2	2·6	—	—	—
3	5·6	—	—	—
4	21·4	361·5	97·9	1760
5	44·6	704·5	97·9	1760
6	55·3	1122	114·2	2400
7	57·7	1446	146·9	3970
8	66·6	2015	163·2	4900
9	101·9	2589	195·8	7060
10	167·8	—	—	—

* 1 cm^2 = 6·40 × 10^3 dyne cm^{-2} sec^{-1}.

(Fig. 3). A total of nine flow curves on the same sample were obtained, the characteristic spur occurring only on the first run. The loops of subsequent runs were displaced towards the shear rate axis, the displacement increasing in the order in which the runs were made. There was an initial large decrease in loop area from the first to the second run; subsequent decreases were of a much smaller magnitude.

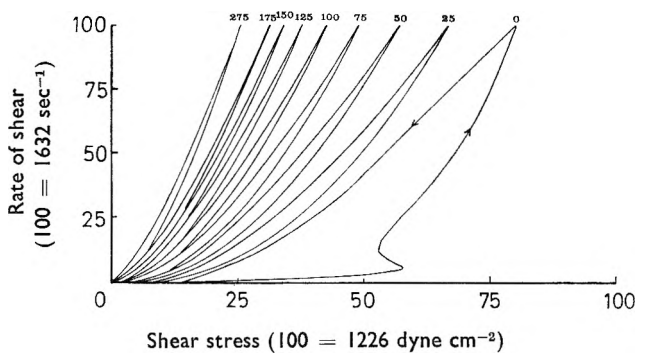

FIG. 3. Flow curves for system R_5. Ferranti-Shirley viscometer used in automatic mode at 25°—nine curves derived on the same sample. (Numerals on curves refer to time, in min, from onset of experiment.)

It was concluded that for all systems investigated the structure broken down by shear in the viscometer was rebuilt to a negligible extent on standing, thus as the structural breakdown was irreversible, the systems did not exhibit true thixotropy.

The effect of varying the temperature on the rheological properties of the systems was investigated. System R_8 was selected because at all temperatures in the range 25–60° an adequate torque was produced without exceeding the torque scale capacity. For temperatures greater than 60°, all systems gave very low readings. At temperatures greater than 25°, samples were stored overnight in a water bath at the required temperature. The shear stress at the maximum and half maximum rate of shear was derived on both the upcurve and the downcurve. The

approximation was made that the system under the conditions of testing behaved as a simple liquid, i.e. the elastic component was zero. An *apparent relative viscosity* was defined as:

$$\text{Apparent relative viscosity} = \frac{\text{Apparent viscosity of the system at T°}}{\text{Viscosity of water at T°}}$$

$$= \frac{\text{Shear stress in system at T°}}{\text{Shear stress in water at T°}}$$

both stresses being obtained at the same rate of shear. The shear stress in water at T° was obtained from tables (Lange, 1961) by multiplying the value for the viscosity of water at each temperature by the appropriate rate of shear. This eliminates the effect on the consistency of the system of a fall in the viscosity of the water in the system due solely to temperature rise.

Results in Fig. 4 show a maximum apparent relative viscosity at about 42·5°, at which temperature the characteristic sharp spur on the upcurve also disappeared.

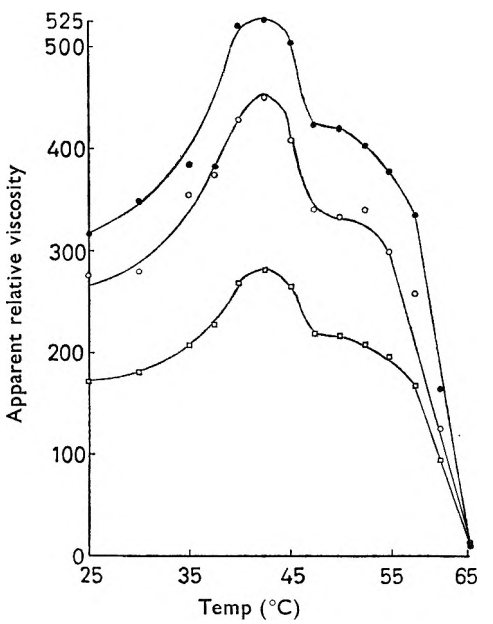

FIG. 4. Apparent relative viscosity versus temperature for system R_8: —●— at 816 sec^{-1} for upcurve; —○— at 816 sec^{-1} for downcurve; —□—at 1632 sec^{-1}.

Discussion

THE NATURE OF THE EXPERIMENTAL SYSTEMS

The types of molecular interaction, together with their resultant phase at equilibrium, which can occur when a surfactant, water and an amphiphile are mixed together has been examined by Lawrence (1961a,b) and Barry

(1967). For such interaction to take place the temperature of the amphiphile, and thus the system in general, must be at or above a certain critical temperature which, for the aqueous sodium dodecyl sulphate solution at a concentration of 1% w/w, is approximately 46°. To prepare the systems, the cetyl alcohol at approximately 65° is poured into the sodium dodecyl sulphate solution, maintained at the same temperature, and the mixture agitated. The molten alcohol disperses into droplets and the sulphate and water penetrate these to produce a highly viscous liquid crystal phase. This phase will tend to be drawn out along the complex flow lines in the mix, and some of it may finally dissolve to form an isotropic mixed micellar solution. Thus, individual globules of molten cetyl alcohol stream through the hot mix and produce elongated threads of liquid crystal, whilst they themselves diminish in bulk as they form this smectic phase. The fluidity of the liquid crystal phase increases as more water enters the structure, surface tension forces act, and spherulites are formed. When the temperature is rapidly lowered by forced cooling to below 46°, the alcohol solidifies and further interaction with sodium dodecyl sulphate solution is prevented. We made no attempt to ensure that phase equilibration occurred before cooling was initiated since particularly with amphiphiles of long chain length, equilibration may require protracted treatment (Dervichian, 1957, 1960; Ekwall, Danielsson & Mandell, 1960a,b). The liquid crystal phase is frozen and a metastable system is formed (a pseudomorphic state; Gray, 1962) in which the intimate mixture of alcohol, sulphate and water present in the smectic phase is maintained, and thus the system still shows optical anistropy at room temperature (Fig. 1). For systems of low alcohol content (R_1 to R_3) the result is a mobile suspension containing solid alcohol and frozen spherulites. The latter are somewhat deformed due to flow forces and partial crystallization, and often contain unreacted alcohol at their centres. Any frozen smectic threads have little effect on the consistency of the system. As the percentage of alcohol in the system rises (from R_4 to R_{10}) two effects become important:

(*a*) The effective disperse phase volume of the system rises. In a simple dispersion of a long chain alcohol in water, the disperse phase is the alcohol, but in the present systems this phase is increased in volume due to the incorporation of a small amount of alkyl sulphate and a relatively large amount of water. Even if the water is not bound, a honeycomb structure will result which will entrap continuous phase and the effective disperse phase volume will be increased.

(*b*) It is postulated that the threads of frozen liquid crystal are now abundant enough to form a submicroscopic network which entraps both solid alcohol and frozen spherulites. The result will be a typical gel-like structure exhibiting solid properties.

Microscopic evidence for this type of structure is shown in Fig. 1: the frozen threads are too fine to be seen in ordinary light and too thin to show up between crossed polars. Indications of a network structure, however, were obtained by introducing air bubbles into the mobile systems R_3 and R_4 and examining them microscopically. The bubbles were allowed

to traverse the field of view when a striated effect was seen in the flow lines surrounding them: this is strongly suggestive of a fibrillar structure.

THE STABILITY OF SYSTEMS

The systems were metastable and formed in time a pearly-crystalline deposit. The melting point of the crystals, determined in aqueous suspension, was 28°, some 21° below that of pure cetyl alcohol. The crystals fell to the bottom of the container whereas pure cetyl alcohol floated. It is probable that the crystals are solid adducts of sodium dodecyl sulphate with cetyl alcohol. This view appears reasonable when the work of Lawrence (1958), Epstein, Wilson & others (1954, 1956) and Kung & Goddard (1963, 1964, 1965) is considered. These authors have shown that it is relatively easy to form crystalline adducts between long chain alkyl sulphates and their alcohols, particularly when their chain lengths are the same. The most easily formed adducts are those in which the sulphate to alcohol ratio is 2:1, and this could explain why systems of low alcohol content, especially R_1, most readily crystallized. These crystals have not been investigated further.

RHEOLOGICAL PROPERTIES UNDER CONTINUOUS SHEAR

The area of the hysteresis loop (Fig. 2) is a measure of the amount of breakdown which has taken place, as well as being a function of the total solid content of the system. Unlike a thixotropic material, there is no evidence of any significant rebuilding of the structure on standing (Fig. 3), and the systems undergo irreversible shear breakdown or irreversible work softening (Scott Blair, 1949; Van Wazer & others, 1963b). This is in accordance with the view that the systems are in a metastable state and the fragile structure which lends strength to the material is readily broken down although a heating, mixing and cooling cycle will rebuild it.

Systems R_4 to R_9 show a pronounced spur on the upcurve at low rates of shear, and system R_{10} shows a pronounced bulge. Such spurs and bulges, when they occur in the flow curves of thixotropic materials, are thought to be due to a three dimensional gel-like structure (Martin, Banker & Chun, 1964). Similar spurs for various systems were obtained by Axon (1954), McKennell (1954), Ober, Vincent, & others (1958), and Boylan (1966). DeButts, Hudy & Elliott (1957) examined sodium carboxymethylcellulose solutions (1–3% w/w) and with some samples, depending on the method of preparation, the flow curve obtained was in the form of a hysteresis loop. Where, in addition, a spur on the upcurve occurred, they referred to the system as a gel. It was considered that the spur was a measure of the gel strength of the system and that the structure had to be broken down before flow could occur. The suggestion was made that in the solution there were regions of crystalline, poorly soluble material where a small percentage of the sodium carboxymethylcellulose was not molecularly dispersed, but was held together by crystalline remnants from the original cellulose. Such material functioned as a gel

centre, which entrapped a relatively large amount of soluble material in a three dimensional network held together by electrostatic and van der Waals' forces. This network it was suggested, gave the gel appreciable mechanical strength and caused the spur on the upcurve of the rheogram.

The spur in the flow curves of the present systems may be explained similarly. Here the "gel centres" are frozen spherulites and the network structure is made up of intermeshed frozen smectic phase in the form of threads. When the systems are at rest, these give considerable mechanical strength and provide most of the solid properties. They must be broken before a significant amount of deformation can take place and thus, when the systems are sheared, a spur is formed on the upcurve. Unlike the soluble sodium carboxymethylcellulose molecules these threads are unstable, and when fractured by shear they do not reform; hence the spur on the flow curve is not reproducible after one cycle (Fig. 3). After the network structure is disrupted by shear, what may be termed secondary breakdown will occur by fracture of the remaining frozen smectic phase, and this releases entrapped solution. Evidence for this was obtained microscopically when, after shearing, it was seen that most of the frozen spherulites had been broken down. It is this secondary effect which is primarily responsible for the hysteresis loops on the flow curves of subsequent runs on the same sample. There may also be a small amount of true thixotropic build up, due to normal solid particle-particle interaction in the system, but this does not have a significant effect on the shape of the flow curve. The shear stress value at the apex of the spur may be used as a measure of the strength of the system; this value increases with the alcohol content of the system (Table 1). Similarly, the amount of shear which systems will undergo and the shear rate they will support before significant breakdown occurs also increases with alcohol content (Table 1).

It had been noticed, when preparing systems, that during the cooling stage samples often exhibited a maximum consistency between 40° and 50°, before they reached the final more or less gelled state. Rheograms were developed at different temperatures for system R_8 and the apparent relative viscosities determined at three rates of shear (Fig. 4). This experiment measured primarily the ability of the system to resist structure breakdown in continuous shear, and the relationship between this ability and temperature. It was shown that system R_8 exhibited a maximum apparent relative viscosity at 42·5°. This temperature may represent the transition temperature from frozen smectic phase to liquid smectic phase, which may be a little lower than the penetration temperature of 1% w/w sodium dodecyl sulphate solution into pure cetyl alcohol (46°). After the maximum point, the liquid crystal phase will tend to round up into globules and the disturbance to flow will fall with a decrease in the apparent relative viscosity. Between 47·5° and 57·5° the liquid crystal phase is fairly stable to shear but above 57·5° the apparent relative viscosity drops markedly as the smectic phase dissolves to isotropic solution.

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Rheology of sodium dodecyl sulphate–cetyl alcohol–water: small strain experiments*

B. W. BARRY† AND E. SHOTTON

The rheology of systems formed when sodium dodecyl sulphate, cetyl alcohol and water are heated, mixed and cooled has been investigated under conditions of small strain. Systems R_5 and R_{10} (the numerals referring to the molar ratio of alcohol to sulphate) in sinusoidal oscillation between 0·01 cycles/sec and 5 cycles/sec at 25° exhibited phase angles of approximately 10° and they thus behaved essentially as solids. System R_8 in creep (25°) suggested a mechanical model of three Voigt units in series with a residual spring and a residual dashpot.

THE nature of the systems formed when sodium dodecyl sulphate, cetyl alcohol and water are heated, mixed and cooled has been considered previously (Barry & Shotton, 1967). The systems had the general formula sodium dodecyl sulphate (4 g), water (400 g), and cetyl alcohol which was varied to give a molar ratio of alcohol to sulphate of one for system R_1, increasing by unit steps to system R_{10}, where the molar ratio was ten. It was shown that the systems were in metastable equilibrium and when examined by continuous shear methods they suffered irreversible shear breakdown. These methods monitor the complex phenomenon of breakdown as a whole although classical parameters such as viscosity and elasticity are not measured as such. An alternative treatment is to examine a system in its ground state, i.e. when the method of testing does not significantly alter the structure, and where applicable to interpret the results of such tests on the basis of simple linear viscoelastic theory (see e.g. Turner Alfrey & Gurnee, 1956; Ferry, 1958, 1961; Leaderman, 1958). With the present systems this implied straining the materials to an extent such that the labile structure was not broken, and the samples did not change their physical properties during the test.

Experimental and results

Materials. As described by Barry & Shotton, (1967).

OSCILLATORY EXPERIMENTS

The systems were subjected to forced sinusoidal oscillations between a cone (2° 1′) and plate arrangement of a Weissenberg Rheogoniometer (Van Wazer, Lyons & others, 1963a). The instrument used was a model R_{14} rheogoniometer extensively modified to bring it up to R_{16} standards (Barry, 1967). In addition, the constant temperature chamber enclosing the cone and plate was converted to a humidifying chamber to minimize drying out of the samples, and the temperature was measured with three

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thermistors attached to the plate. Variation between the three thermistors was not more than 0·1° and the temperature of testing was 25 ± 0·2°.

The maximum shear strain below which the systems gave a linear response in oscillation was found by oscillating a sample at a minimum amplitude at the maximum frequency of oscillation used in the present work (5 cycles/sec). The input strain wave and the output stress wave were recorded with an ultraviolet recorder. The amplitude of oscillation was increased by small increments until the shape of the output sine wave deviated from that of the input; the shear strain corresponding to this amplitude was taken as the maximum for linearity.

The limit of linearity may be expressed as a percentage shear strain defined as $\frac{\text{Amplitude of oscillation}}{\text{Cone angle}} \times 100$. For systems R_4 to R_{10} the maximum percentage shear strain for linearity was in the region of 7 to 22%. In the systems of lower alcohol content the consistency was too low to be measured.

The variation of the phase angle with frequency was determined for each system. The cone was oscillated at an amplitude lower than the limit for linearity, starting at 0·01 cycles/sec and increasing in logarithmic steps to 5 cycles/sec. The phase angle between the input strain wave and the output stress wave was calculated at each frequency from the linear displacement of the stress wave from the strain wave. The phase angles for systems R_4 to R_{10} were in the region of 10°. In Fig. 1, tan phase angle is plotted against frequency, using logarithmic scales, for systems R_5 and R_{10}. Due to the low consistencies of systems R_1 to R_3, only an estimate of their phase angles could be obtained by testing at maximum amplitude and the highest frequency of oscillation available. The phase angles were in excess of 70°.

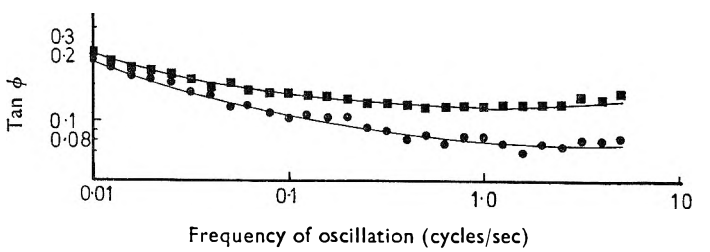

FIG. 1. Variation of tan phase angle (ø) with frequency of oscillation—system R_5 and R_{10}. —■— System R_5; —●— system R_{10}.

CREEP EXPERIMENTS

One of the simplest methods for studying viscoelastic behaviour is the creep test, in which a stress is suddenly imposed on a sample at zero time and then maintained constant. The time-dependent strain response to this steady stress is called the creep curve (Turner Alfrey & Gurnee, 1956). As the systems to be examined varied from a liquid to a soft gel, a

concentric cylinder geometry was chosen, and the apparatus was constructed to use the facilities of the rheogoniometer. Details of this apparatus will be published.

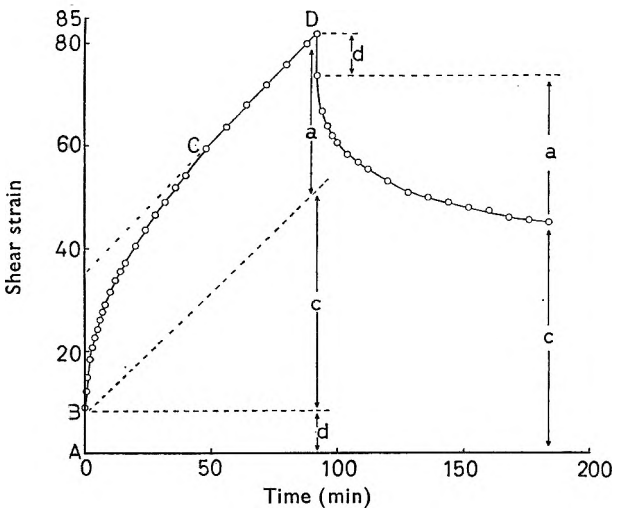

FIG. 2. Shear strain against time. System R_8. For each unit on the shear strain axis, $\gamma = 17{\cdot}98 \times 10^{-4}$.

The systems which were investigated in creep were those with pronounced solid properties as in systems R_4 to R_{10}. The creep curves obtained had the general shape shown in Fig. 2, which is the experimental curve obtained for system R_8. The model proposed to represent the behaviour is a Maxwell unit in series with a number of Voigt units (see e.g. Van Wazer, Lyons & others, 1963b). A-B represents an instantaneous elastic component and is associated with an uncoupled (residual) Hookean spring. C-D is the region of viscous flow and is associated with the uncoupled (residual) Newtonian dashpot. B-C is the region where the flow is viscoelastic and the model representation consists of a number of Voigt units (a spring in parallel with a dashpot) connected in series. In theory, the number of these Voigt units may be infinite; in the present work three only were resolved but these, together with the residual dashpot and spring, were sufficient to characterize the systems. Since the systems became non-linear in oscillation above shear strains varying from 7 to 22%, the shear stress was adjusted so that the maximum percentage shear strain never exceeded 4·5%. All measurements were taken at 25° ± 0·2°.

Loading the apparatus tended to disturb the metastable structure of the systems and reproducibility of results was not good. System R_8 is selected as an example of a viscoelastic system and the results of a typical run are given (Fig. 2) to illustrate the order of magnitude of the parameters viscosity, rigidity and retardation time.

Analysis of the creep curve (Fig. 2). The following definitions apply: γ = shear strain; γ_1, γ_2, γ_3 = shear strain due to Voigt units 1, 2 and 3

respectively; γ_T = total shear strain due to all Voigt units; these quantities are all functions of time: γ_∞ = shear strain at full extention; γ_{∞_1}, γ_{∞_2}, γ_{∞_3} = shear strain due to Voigt units 1, 2 and 3 respectively at full extension; γ_{∞_T} = total shear strain due to all Voigt units when fully extended; $\dot{\gamma}_m$ = arithmetic mean shear rate; σ_m = arithmetic mean shear stress = 79·81 dyne cm^{-2}; τ = retardation time = η/G; τ_1, τ_2, τ_3 = retardation time of Voigt units 1, 2 and 3 respectively; η = coefficient of viscosity; G = modulus of rigidity = σ_m/γ_∞; t = time.

Instrument constants. For 0·001 inch movement of the strain transducer, $\gamma = 17{\cdot}98 \times 10^{-4}$; for 0·001 inch sec^{-1} movement of the strain transducer, $\dot{\gamma}_m = 19{\cdot}34 \times 10^{-4}$ sec^{-1}. Maximum movement in transducer = 0·025 inch = 100 units deflection of the Kent Recorder.

From Fig. 2:

Instantaneous elastic deformation (AB) = 8 units = 0·002 inch

$$\therefore \text{Modulus of rigidity of residual spring } G = \frac{\sigma_m}{\gamma} = \frac{79{\cdot}81}{2 \times 17{\cdot}98 \times 10^{-4}}$$

$$= 2{\cdot}22 \times 10^4 \text{ dyne cm}^{-2}.$$

Slope of linear portion of experimental curve = 46·5 units in 92 min

$$= \frac{46{\cdot}5 \times 0{\cdot}25}{92 \times 60} = 2{\cdot}106 \times 10^{-3} \text{ thousandths of an inch sec}^{-1}$$

$$\therefore \dot{\gamma}_m = 2{\cdot}106 \times 10^{-3} \times 19{\cdot}34 \times 10^{-4} = 40{\cdot}73 \times 10^{-7} \text{ sec}^{-1}$$

$\therefore$ Coefficient of viscosity of residual dashpot,

$$\eta = \frac{\sigma_m}{\dot{\gamma}_m} = \frac{79{\cdot}81}{40{\cdot}73 \times 10^{-7}} = 1{\cdot}96 \times 10^7 \text{ poise}$$

For the viscoelastic region of the curve (BC) $\gamma_{\infty_T} = 27{\cdot}3$ units. The equation for a single Voigt unit in creep is

$$\gamma = \gamma_\infty(1 - e^{-t/\tau})$$

$$\text{or } \ln_e\left(\frac{\gamma_\infty - \gamma}{\gamma_\infty}\right) = -\frac{t}{\tau}$$

Thus in the experimental work $\log_{10}\left(\frac{\gamma_{\infty_T} - \gamma_T}{\gamma_{\infty_T}}\right)$ is plotted against time as in Fig. 3, and the slope of the linear portion of the plot equals $-\frac{1}{2{\cdot}303\tau_1}$.

The retardation time $\tau_1 = 17$ min. The initial deviation from the straight line indicates the presence of further Voigt units of shorter retardation times and includes the strains due to their extension. In the linear portion these additional units are fully extended. Extrapolation of the linear portion of the curve to the log axis gives the value of log Z_0' at zero time where Z_0' is the value of $\left(\frac{\gamma_{\infty_T} - \gamma_T}{\gamma_{\infty_T}}\right)$ representing the fraction of γ_{∞_T} due to γ_{∞_1}.

Thus $\gamma_{\infty 1} = Z_0' \gamma_{\infty T} = 0{\cdot}5781 \times 27{\cdot}3 = 15{\cdot}8$ units deflection, and $\gamma'_{\infty 2} = \gamma_{\infty T} - \gamma_{\infty 1} = 27{\cdot}3 - 15{\cdot}8 = 11{\cdot}5$ units deflection to a first approximation.

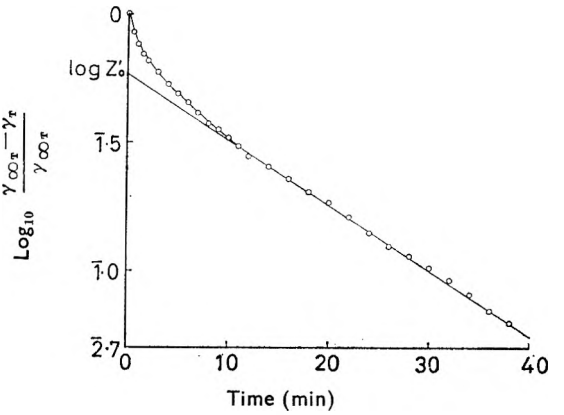

FIG. 3. $\text{Log}_{10} \dfrac{\gamma_{\infty T} - \gamma_t}{\gamma_{\infty T}}$ against time

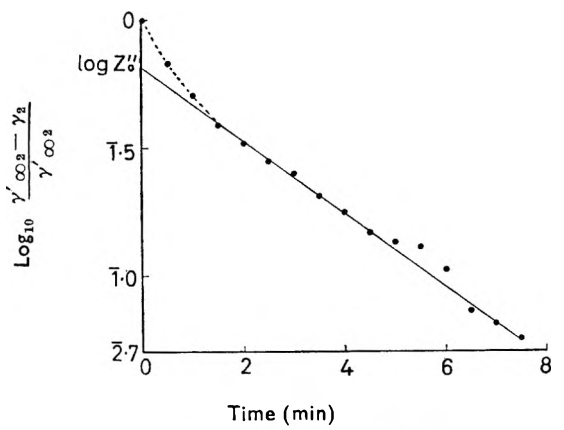

FIG. 4. $\text{Log}_{10} \dfrac{\gamma'_{\infty 2} - \gamma_2}{\gamma'_{\infty 2}}$ against time.

The true value of $\gamma_{\infty 2}$ for the second Voigt unit may then be derived from $\gamma'_{\infty 2}$ by using the non-linear results in Fig. 3 over the first 8 min of shear. Thus, $\log_{10}\left(\dfrac{\gamma'_{\infty 2} - \gamma_2}{\gamma'_{\infty 2}}\right)$ is plotted against time in Fig. 4. γ_2 is obtained by difference, $\gamma_2 = \gamma_T - \gamma_1$ at each time interval and γ_1 is derived from

$$\gamma_1 = \gamma_{\infty 1}\left(1 - e^{-t/\tau_1}\right) = 15{\cdot}8\left(1 - e^{-t/17}\right)$$

From Fig. 4 the slope of the linear portion gives $\tau_2 = 3{\cdot}05$ min, the

intercept on the log axis gives a value for Z_0'' and the true values for $\gamma_{\infty 2}$ can be derived:

$$\gamma_{\infty 2} = Z_0''\gamma'_{\infty 2} = 0{\cdot}6457 \times 11{\cdot}5 = 7{\cdot}43 \text{ units deflection}$$

The difference ($11{\cdot}5 - 7{\cdot}43 = 4{\cdot}07$) represents the extension of further Voigt units over the first min of shear and is represented by $\gamma'_{\infty 3}$. Insufficient experimental data are available at these short times to determine more than one further Voigt unit. The approximation may then be made that there exists only one further Voigt unit and $\gamma'_{\infty 3} = \gamma_{\infty 3}$. After 1 min, values for γ_1 and γ_2 may be calculated from the Voigt equations thus

$$\gamma_1 = 15{\cdot}8\left(1 - e^{-1/17}\right) = 0{\cdot}902 \text{ units}$$

$$\gamma_2 = 7{\cdot}43\left(1 - e^{-1/3{\cdot}05}\right) = 2{\cdot}08 \text{ units}$$

thus $\gamma_3 = \gamma_T - (\gamma_1 + \gamma_2) = 6{\cdot}6 - (2{\cdot}08 + 0{\cdot}902) = 3{\cdot}62$ units where $\gamma_T = 6{\cdot}6$ units and is the experimental shear strain after 1 min.

τ_3 may now be calculated from $\gamma_3 = \gamma_{\infty 3}\left(1 - e^{-t/\tau}\right)$

$$3{\cdot}62 = 4{\cdot}07\left(1 - e^{-1/\tau_3}\right)$$

$$\tau_3 = 0{\cdot}454 \text{ min}$$

Inokuchi (1955) has used a similar method to the above for the analysis of the creep curve of a surface film and Shama & Sherman (1966) and Sherman (1966) have applied Inokuchi's method to ice cream, using parallel plate and concentric cylinder viscometers.

TABLE 1. VISCOELASTIC PARAMETERS OF SYSTEM R_8 IN CREEP (25°)
$\sigma_m = 79{\cdot}81$ dynes cm^{-2}

	Values of viscoelastic parameters			
	τ (sec)	γ_∞	$G = \frac{\sigma_m}{\gamma_\infty}$ (dyne cm^{-2})	$\eta = \tau \times G$ (poise)
Voigt unit 1 ..	$1{\cdot}02 \times 10^2$	$71{\cdot}02 \times 10^{-4}$	$1{\cdot}12 \times 10^6$	$1{\cdot}14 \times 10^7$
Voigt unit 2 ..	$1{\cdot}83 \times 10^2$	$33{\cdot}40 \times 10^{-4}$	$2{\cdot}39 \times 10^6$	$4{\cdot}37 \times 10^8$
Voigt unit 3 ..	$2{\cdot}72 \times 10$	$18{\cdot}30 \times 10^{-4}$	$4{\cdot}36 \times 10^6$	$1{\cdot}46 \times 10^8$

Residual elastic component, $G = 2{\cdot}22 \times 10^6$ dyne cm^{-2}.
Residual viscous component, $\eta = 1{\cdot}96 \times 10^7$ poise.

The results for the analysis of system R_8 are given in Table 1. The general equation for the retarded viscoelastic region (B-C Fig. 2) of the experimental curve is thus:

$$\gamma_T = \sum_{n=1}^{3} \gamma_{\infty n}\left(1 - e^{-t/\tau_n}\right)$$

To check the accuracy of this equation the retarded viscoelastic region of the curve was regenerated using the above expression (inserting the

derived values of $\gamma_{\infty n}$ and τ_n) and the result compared with the experimental curve (Fig. 5). Agreement is good.

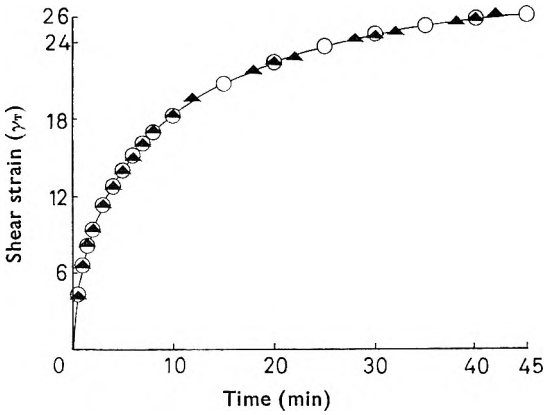

FIG. 5. Shear strain γ_t (theoretical) and shear strain γ_T (experimental) against time. —○—, Theoretical points; —▲—, experimental points. For each unit on the shear strain axis, $\gamma_T = 17{\cdot}98 \times 10^{-4}$.

Discussion

To provide information about a viscoelastic material at very short times of testing, dynamic methods such as sinusoidal testing may be used, where the stress (or strain) is varied periodically at a frequency of f cycles/sec or ω ($=2\pi f$) radians/sec. If the viscoelastic behaviour is linear, i.e. when the amplitude is very small, the amplitude of strain will be proportional to the amplitude of stress and the strain will also alternate sinusoidally but will be out of phase with the stress. The tangent of this phase angle (tan ϕ) is known as the *loss tangent* and is a measure of the ratio of energy lost to energy stored in a cyclic deformation. ϕ is zero for a Hookean solid and 90° for a Newtonian liquid.

Tan ϕ has been plotted against the frequency of oscillation for systems R_5 and R_{10} over a frequency range of two and a half decades (Fig. 1). The most obvious features are the low values of tan ϕ (corresponding to ϕ varying approximately from 12·5° to 4°) and the flatness of the curve. The former feature indicates that the systems, under the conditions of test, are exhibiting mainly elastic properties. The latter feature indicates that viscous properties will not become evident unless the frequency of testing is further reduced; the way in which the curve rises slightly as the frequency drops towards the minimum is in agreement with this view. It can also be assumed that if models are derived for these systems on the basis of combinations of viscous and elastic elements, then the values of η will be high with respect to G, that is, the retardation times will be long.

To investigate the behaviour of the systems at longer times the following considerations apply. A periodic experiment at frequency ω radians/sec is qualitatively equivalent to a transient experiment at times $t = \frac{1}{\omega}$.

The time scale of the proposed experiment should thus be greater than $\frac{1}{2\pi f} = 16$ sec, and a creep experiment was therefore selected. In a creep experiment, as in oscillatory work, the response of the system to the applied stress must be linear and the information obtained in oscillatory testing was used to set a limit to the total allowable strain—at all times the shear strain was less than 4·5%. Thus it may be concluded that negligible structural breakdown occurs. The symmetry of the experimental curves (see e.g. Fig. 2) confirms this; values for a, c and d for the creep curves were within 2% full scale deflection of the values for a, c and d for the recovery curves.

The model postulated to represent the behaviour of system R_8 in creep is shown in Fig. 6. That this model does in fact give a good representation of the system may be seen from Fig. 5, where the experimental strain and the theoretical strain (derived from the model equation) are plotted against time for the retarded viscoelastic region of the creep curve.

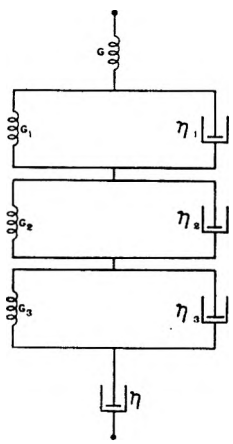

FIG. 6. Modified generalized Voigt model for system R_8. G, modulus of rigidity; η, coefficient of viscosity.

The instantaneous elastic deformation of the system in the creep experiment is associated with the residual spring which has a modulus of rigidity G of the order of 2×10^4 dyne cm^{-2}. This weak spring represents bonds being stretched elastically and represents extension of the network structure in the system. In any non-destructive test or manipulation, where a stress is applied to system R_8 for a very short period of time, the elastic strain will be provided by this element and this strain is recovered instantaneously when the stress is removed.

Where the stress is applied for a longer period of time, the Voigt units will be extended. In these the strains do not appear instantaneously (or rather with acoustic speed) but are delayed by the viscosity of the appropriate dashpot. Similarly, on removing the stress, the strain in each unit decays exponentially with time. These units represent that part of the

structure in which secondary bonds are breaking and reforming during the test. The retardation time τ is the time during which bonds break and reform, and as all bonds do not break and reform at the same rate, a spectrum of retardation times exists. For system R_8, only three Voigt units were required to simulate the viscoelastic region of the curve, although in theory an infinite number could be postulated. The minimum retardation time for a Voigt unit is of the order of 27 sec and this explains why oscillatory testing gave little indication of any liquid properties. The frequency of testing was at all times too high to allow significant extension of the Voigt units and thus little energy was dissipated in the oscillatory cycle.

When the stress has been applied for sufficient time to ensure that all the Voigt units are essentially fully extended (for absolute maximum extension, infinite time would be required, as strain development is exponential with time), further deformation is in the nature of viscous flow. This strain is non-recoverable and is represented by the residual dashpot. In this region the experimental system is flowing as a solid dispersion in a liquid medium and the viscosity is high, of the order of 2×10^7 poise. This residual viscosity prevents the system having a yield value as strictly defined; any stress, however small, will cause an observable flow providing the period of observation is long enough, and this flow will continue indefinitely as long as the stress is applied.

The development of strain as a function of time for the above model may thus be represented by

$$\gamma(t) = \frac{\sigma_m}{G} + \sigma_m \sum_{n=1}^{3} \frac{1}{G_n}\left(1 - e^{-t/\tau_n}\right) + \frac{\sigma_m t}{\eta}$$

where G is the rigidity of the residual spring and η the viscosity of the residual dashpot.

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The influence of soap concentration on the creaming of an emulsion system

E. SHOTTON AND S. S. DAVIS*

The creaming of potassium laurate–liquid paraffin emulsions has been examined and a measurement of the creaming rate of aggregated systems obtained using a conductivity method. The results have been analysed by theories of hindered setting. An estimate of aggregate size was obtained by means of a modified Stokes's equation involving the mean aggregate density and the viscosity of the emulsion at zero shear rate. A maximum aggregate size at 5·0% soap concentration is in agreement with rheological analysis.

STOKES'S law, which is valid for the velocity of a rigid sphere moving under gravity in an infinite amount of fluid, cannot be applied directly to emulsions. Garrett (1962) found that dilute emulsions (14%) followed the Stokes relation and other workers have reported modified forms for more concentrated systems, to take into account the interactions of the numerous globules upon each other. Aggregation of an emulsion will result in an increased particle diameter and an increased rate of creaming. Higuchi (1958) considered that concentrated systems could be represented as the continuous phase flowing through an immovable bed of the disperse phase. The Kozeny (1927) expression, modified by Carman (1937) could then be applied.

The conventional method of measuring creaming rate has been visual observation (Cockbain, 1952) but recently more sophisticated techniques have been reported including radioisotopes (Appino, Christian & Banker, 1962), capacitance changes (Kaye & Seager, 1965), and fluorimetry (Mima & Kitamori, 1966). The acceleration of creaming rates by centrifugation has been widely used for studies on emulsion stability (Vold & Groot, 1962; Rehfeld, 1962).

Experimental

Materials. Liquid paraffin (B.P.); lauric acid (99%) which was checked by gas chromatography; potassium hydroxide (A.R.); distilled water from an all glass still.

Particle size analysis. A Coulter Counter, model A Industrial was used to size the emulsion systems employing a 30 μ aperture tube calibrated with polystyrene lattices.

Viscometry. A Couette viscometer was used which was basically that described by Perrin & Saunders (1966): it was calibrated against pure distilled water and light liquid paraffin of known viscosity.

Estimation of creaming. A conductivity method was adopted and two creaming cells constructed. In both, creaming was followed by recording

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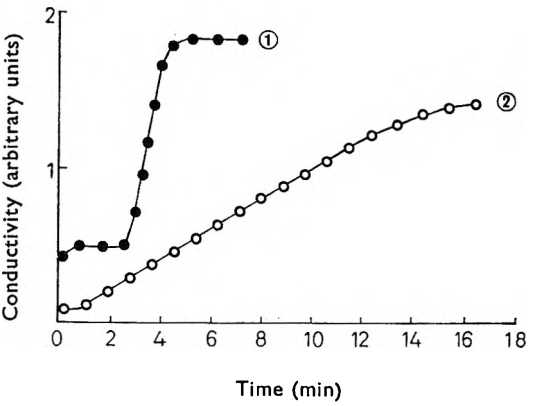

FIG. 1. The change in conductivity with time of creaming for two types of creaming cell. (1) 1 cm electrodes; (2) 12 cm electrodes.

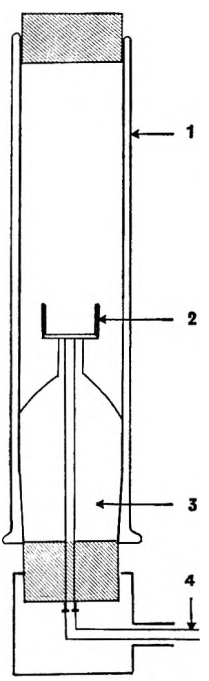

FIG. 2. The creaming cell. 1. Glass tube 12 cm long, 1·0 cm radius. 2. Platinized platinum electrodes, 1 cm square. 3. Philips conductivity cell. 4. Connexion to universal bridge.

the change in conductivity with time using a Wayne Kerr universal bridge with Autobalance Adaptor. Output facilities were available on a digital voltmeter with a print-out device. The two cells had electrodes of different lengths, 12 cm and 1 cm respectively. The cell with the longer electrodes gave a result similar to that obtained by visual methods (Fig. 1, curve 2). The change in conductivity with time was not linear over the length of the electrode thus correlation between conductivity and creaming rate was possible only when the cell had been calibrated previously for each system. The curve obtained with 1 cm electrodes (Fig. 1, curve 1) shows the conductivity remaining almost constant until the cream interface reaches the *bottom* of the electrodes. Then for emulsions of volume fraction less than about 0·35 the conductivity changes linearly with time until the interface passes the *top* of the electrodes whereupon it is once more constant. The creaming rate can be calculated directly from Fig. 1, curve 1. The construction of the 1 cm electrode cell is shown in Fig. 2.

The creaming rate of an emulsion measured in both cells gave similar values of 0·653 cm min^{-1} and 0·666 cm min^{-1}.

TABLE 1. FORMULATION DETAILS AND RESULTS FROM PARTICLE SIZE AND RHEOLOGICAL ANALYSIS

Potassium laurate conc. % w/w	Liquid paraffin volume fraction ϕ	Relative limiting viscosity	Particle size data	
			Mean volume diameter (μ)	Standard deviation σ
10·0	0·05	1·40	2·77	2·80
	0·11	1·92	2·56	2·57
	0·16	2·25	3·10	2·87
	0·22	3·14	2·20	2·28
	0·33	5·11	3·15	2·47
	0·43	7·86	2·09	2·88
5·0	0·05	1·41	2·95	2·55
	0·11	1·86	3·62	2·54
	0·16	2·75	2·88	2·58
	0·22	3·31	3·06	2·31
	0·33	5·89	3·35	2·11
	0·43	10·90	2·81	2·35
2·5	0·05	1·45	3·00	2·00
	0·11	1·80	3·98	2·45
	0·16	2·20	3·04	2·52
	0·22	2·75	4·07	2·31
	0·33	5·54	3·52	2·37
	0·43	8·15	3·41	1·99
1·0	0·05	1·25	3·50	2·52
	0·11	1·42	3·98	2·45
	0·16	1·72	3·34	2·49
	0·22	1·98	3·94	2·19
	0·33	2·75	3·97	2·24
	0·43	6·06	4·26	2·27
0·75	0·05	1·30	4·10	2·34
	0·11	1·45	4·10	2·34
	0·16	1·65	3·44	2·25
	0·22	2·02	4·17	2·23
	0·33	2·97	3·29	2·21
	0·43	5·32	3·92	2·16
0·5	0·11	1·49	5·12	2·18
	0·22	1·98	5·08	2·22
	0·33	3·03	4·45	2·10
	0·43	5·17	5·31	2·18

Formulation details are given in Table 1. Emulsions were prepared from weighed quantities of liquid paraffin and potassium laurate solution by initial dispersion with a dispersator and then by passage through a hand homogenizer.

After 4 days storage to allow for interfacial equilibrium and the escape of entrapped air, the emulsions were examined microscopically and a portion sized using the Coulter Counter.

A flow curve was obtained with the Couette viscometer over the shear rate range of 0–120 sec^{-1}.

Creaming was studied by three different methods: (i) a creaming rate determination using the creaming cell; (ii) the measurement of the ratio of cream volume to the total volume of emulsion after a creaming time of 100 min (Cockbain, 1952); (iii) the final volume of cream after a storage period of three months.

Results and discussion

Microscopic examination showed that aggregation of the emulsions occurred when the soap concentration in the aqueous phase reached 1·0% w/w. The aggregates at 1·0% soap were smaller than those at higher soap concentrations where differentiation between aggregate size was not possible. The aggregate size was influenced by dilution and the manner in which the cover slip was placed on the slide. Small aggregates increased in size with time and Hiestand (1964) has described similar effects for flocculated suspensions. Droplets in the unaggregated emulsions were characterized by the Coulter Counter but aggregated emulsions were broken down on dilution and only the individual particles could be sized (Table 1).

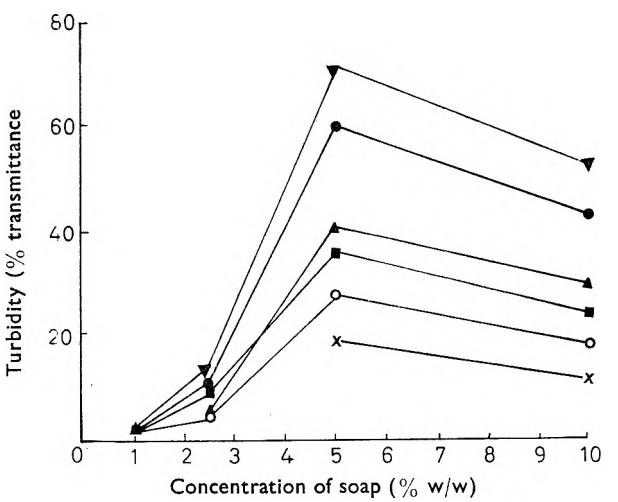

FIG. 3. The change in serum turbidity of potassium laurate–liquid paraffin emulsions after creaming, with soap concentration (compared with water in a Spekker absorptiometer). Volume fractions: ×, 0·05; ○, 0·11; ■, 0·16; ▲, 0·22; ●, 0·33; ▼, 0·43.

The flow properties of the emulsions were Newtonian until the soap concentration in the aqueous phase reached 1·2% when pseudoplastic flow was exhibited. The limiting viscosity of these systems was calculated by measuring the gradient of the straight line produced when the pseudoplastic flow curve became linear at high shear rates. A maximum relative limiting viscosity was obtained at 5·0% soap concentration (Table 1).

The aggregated emulsions creamed rapidly, leaving behind a lower layer whose turbidity was dependent on the emulsifier concentration (Fig. 3). Cockbain's method of creaming demonstrated the rapid build up of the cream layer for the aggregated emulsions (Fig. 4) whilst the unaggregated systems creamed negligibly.

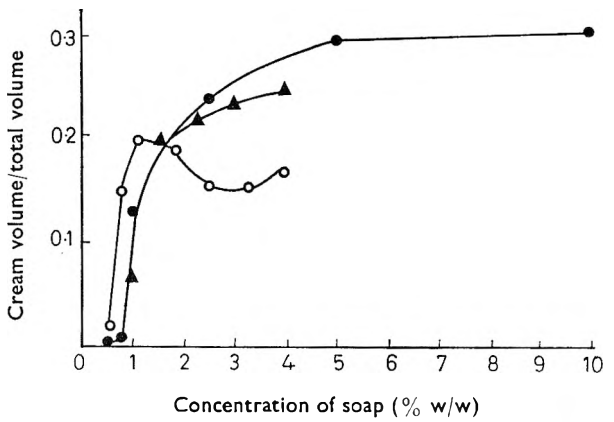

FIG. 4. Variation in cream volume with soap concentration after 100 min creaming (after Cockbain, 1952). $\phi = 0{\cdot}11$. ●, Present work; ▲, laurate/paraffin, and ○, laurate/benzene (Cockbain, 1952).

The cream volume (v_1) measured after three months was greater than the volume of oil (v_2) originally used for the emulsion. The ratio $v_1/v_2 = h$ which was calculated may be considered as a self-crowding factor. An emulsion of uniform spheres with a volume fraction of 0·7402 would occupy the whole volume and h would have a value of 1·35. In a polydisperse emulsion where droplets can be deformed, the maximum packing density exceeds 74·02% and h will have values below 1·35. For the laurate emulsions h falls as the soap concentration is increased from 0·5 to 0·75% (Table 2) and this is due to an increase in polydispersity as shown by the increase in the standard deviation of the particle size distribution (Table 1). At 1·0% soap, aggregation occurs and the value of h rises due to the entrapped continuous phase within the aggregate. A knowledge of the amount of entrapped liquid enables calculation of the aggregate density (Table 2) from:

$$\rho_1 = \frac{\left\{\begin{matrix}\text{Volume of}\\ \text{oil used}\\ \text{for emulsion}\end{matrix}\right\} \times \text{density} + \left\{\begin{matrix}\text{Volume of}\\ \text{entrapped}\\ \text{continuous}\\ \text{phase}\end{matrix}\right\} \times \text{density}}{\begin{matrix}\text{Volume of}\\ \text{oil used}\end{matrix} + \begin{matrix}\text{Volume of entrapped continuous}\\ \text{phase}\end{matrix}}$$

TABLE 2. THE CREAMED EMULSION

Soap conc. % w/w	ϕ_2 Cream volume fraction	ϕ_1 Oil used for original emulsion	$h = \phi_2/\phi_1$	Fraction of entrapped continuous phase	Aggregate density g cm^{-3}
0·5	0·145	0·112	1·29	0·033	
0·75	0·120	0·112	1·07	0·008	
1·0	0·146	0·112	1·31	0·034	0·902
2·5	0·166	0·112	1·48	0·054	0·915
5·0	0·184	0·113	1·67	0·071	0·924
10·0	0·215	0·113	1·90	0·102	0·937
0·5	0·256	0·221	1·20	0·044	
0·75	0·241	0·221	1·09	0·020	
1·0	0·265	0·221	1·20	0·044	0·891
2·5	0·280	0·222	1·26	0·058	0·896
5·0	0·310	0·222	1·40	0·088	0·908
10·0	0·336	0·223	1·51	0·114	0·917
0·5	0·425	0·327	1·29	0·094	
0·75	0·380	0·327	1·16	0·053	
1·0	0·410	0·328	1·26	0·082	0·897
2·5	0·440	0·328	1·34	0·112	0·903
5·0	0·500	0·328	1·52	0·172	0·915
10·0	0·561	0·330	1·69	0·230	0·925
0·5	0·570	0·431	1·32	0·139	
0·75	0·475	0·431	1·10	0·044	
1·0	0·520	0·431	1·21	0·089	0·892
2·5	0·620	0·431	1·44	0·189	0·908
5·0	0·680	0·432	1·57	0·248	0·919
10·0	0·750	0·433	1·73	0·317	0·926

Mean aggregate density for the 4 values of ϕ

Laurate conc. %	1·0	2·5	5·0	10·0
Mean aggregate density g cm^{-3}	0·895	0·902	0·917	0·926

The change in creaming rate with volume fraction at different soap concentrations is shown in Fig. 5. All the curves show a maximum in the region of a volume fraction of 0·10. At infinite dilution ($\phi \rightarrow$ zero) the aggregates will have been broken down and the creaming rate will be that calculated for the individual particles, in the region of 10^{-3} cm min^{-1}.

The curves may be considered in two parts: (a) up to about 10% oil content the aggregate size increases as the volume fraction, and thus the creaming rate, increases; (b) further increases in the volume fraction give rise to mutual interference between the aggregates, and hindered settling results. This is characterized by the formation of a sharp interface between the cream and the separating continuous phase (Orr & Dalla-Valle, 1959). The effect is to modify the rate of creaming by mutual interference and results in all particles moving at about the same rate regardless of size.

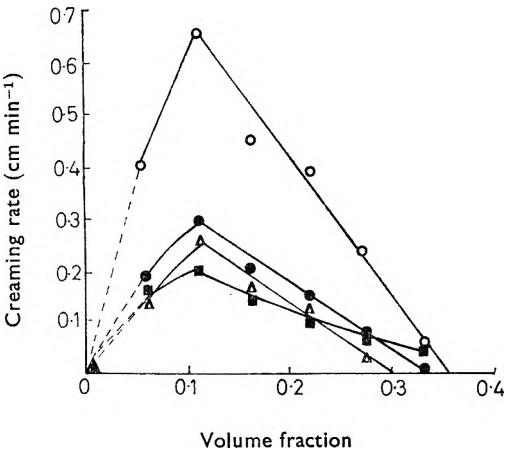

FIG. 5. Change in creaming rate with volume fraction for liquid paraffin–potassium laurate emulsions at different soap concentrations. Concentration of soap % w/w: △, 10·0; ●, 5·0; ○, 2·5; ■, 1·0. ▲, Creaming rate for individual particles.

Steinour (1944) and Michaels & Bolger (1962) have considered hindered settling in detail for both flocculated and non-flocculated suspensions. They modified Stokes's equation to the general form,

$$U = V_s f(\epsilon) \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where U is the rate of fall (or rise) of the interface, V_s the settling velocity of a single particle of average radius and $f(\epsilon)$ is some function of the porosity of the suspension. Higuchi (1958) on the other hand regarded the material as acting as a permeable plug and applied the Kozeny-Carman equation.

The creaming rates at different volume fractions found in this work, were examined by both methods. In neither case did a linear relation result between creaming rate and $f(\epsilon)$. Extrapolation to infinite dilution to obtain the creaming rate of the aggregate in the absence of hindered settling was not possible.

To determine the effect of emulsifier concentration on creaming rate, a volume fraction of 0·22 was chosen (Fig. 6). In Stokes's equation the viscosity factor is that of the continuous phase but the presence of particles can be considered to increase the effective local viscosity of the fluid due to their interaction. Hawksley (1951) has emphasized that the viscosity will be that at the low shear rate of creaming and not necessarily that obtained from measurements in a viscometer. This will be especially true in the case of the pseudoplastic aggregated emulsions in Table 1 where the viscosity decreases with increase in shear rate.

The shear rate ($\dot{\gamma}_c$) for a sphere of radius r, creaming velocity U in a cylinder radius R is given by equation (2) (Williams & Fulmer, 1938)

$$\dot{\gamma}_c = U/(R - r) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

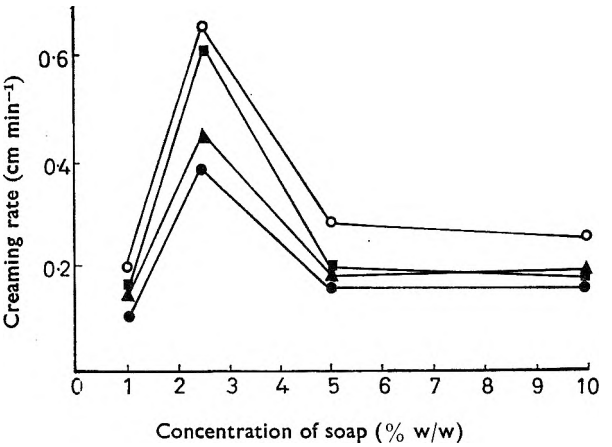

FIG. 6. Change in creaming rate with soap concentration for liquid paraffin–potassium laurate emulsions of different volume fraction. Volume fraction: ■, 0·05; ○, 0·11; ▲, 0·16; ●, 0·22.

It may be assumed that the shape of an aggregate on creaming will be approximately spherical (Michaels & Bolger, 1962). The aggregate size will be negligible in relation to the radius of the tube (1 cm) so that

$$\dot{\gamma}_c = U \text{ sec}^{-1} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

For the laurate emulsions the shear rate will be in the region of 0·005 sec^{-1}. Since the shear rate of creaming is very small the viscosity at zero shear rate was used in the Stokes's equation and this was calculated using the equation of Cross (1965)

$$\eta = \eta_\infty + \frac{(\eta_0 - \eta_\infty)}{(1 + \alpha\dot{\gamma}^{2/3})} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where η_0 is the limiting viscosity at zero shear rate, η_∞ the limiting viscosity at infinite shear rate, η the viscosity of the system at each shear rate $\dot{\gamma}$ from the flow curve, and α is a constant associated with the rupture of linkages. Rewriting (4)

$$\eta(1 + \alpha\dot{\gamma}^{2/3}) = \eta_\infty\alpha\dot{\gamma}^{2/3} + \eta_0 \quad .. \quad .. \quad .. \quad .. \quad (5)$$

$$= \eta_0(1 + \frac{\eta_\infty}{\eta_0}\alpha\dot{\gamma}^{2/3}) \quad .. \quad .. \quad .. \quad .. \quad .. \quad (6)$$

for low shear rates $\frac{\eta_\infty}{\eta_0}\alpha\dot{\gamma}^{2/3} \ll 1$

$$\therefore 1 + \alpha\dot{\gamma}^{2/3} = \eta_0/\eta \quad .. \quad .. \quad .. \quad .. \quad (7)$$

$$\text{or } \frac{1}{\eta_0} + \frac{\alpha\dot{\gamma}^{2/3}}{\eta_0} = 1/\eta \quad .. \quad .. \quad .. \quad .. \quad (8)$$

Thus a plot of $1/\eta$ against $\dot{\gamma}^{2/3}$ will be linear with an intercept of $1/\eta_0$.

TABLE 3. CALCULATION OF AGGREGATE SIZE

Soap conc. % w/w	Viscosity at zero shear rate η_0 (centipoises)	Emulsion density ρ_2	Aggregate density ρ_1	Aggregate size = r (μ) from equation (9)
1·0	1·98	0·974	0·895	12·5
2·5	5·82	0·975	0·902	46·5
5·0	28·3	0·976	0·917	74·5
10·0	24·2	0·980	0·926	72·5

The aggregate size was then calculated by substituting the values of the viscosity at zero shear rate (η_0), together with the aggregate density (ρ_1) and emulsion density (ρ_2) into the Stokes's equation (Table 3).

$$U = 2gr^2(\rho_1 - \rho_2)/9\eta_0 \quad .. \quad .. \quad .. \quad .. \quad .. \quad (9)$$

where U is the creaming rate, g the acceleration due to gravity, r the aggregate radius.

Fig. 7 is a plot of these results together with those obtained from the Coulter Counter for the unaggregated systems. A maximum aggregate size at 5% laurate is indicated.

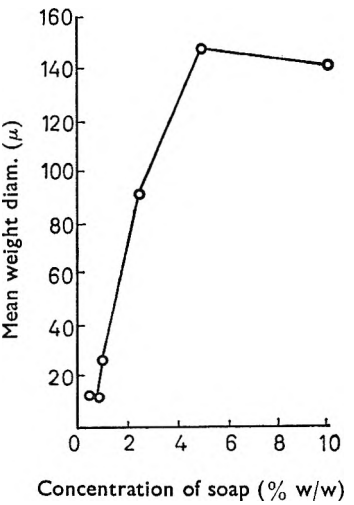

FIG. 7. Variation in aggregate size with soap concentration for liquid paraffin–potassium laurate emulsion (volume fraction = 0·22).

CONCLUSIONS

The measurement of the creaming rate of aggregated emulsions has enabled calculation of aggregate size where microscopic and Coulter Counter methods have proved unsatisfactory. For the system potassium laurate–liquid paraffin a maximum aggregate size was obtained at 5·0% potassium laurate concentration. This result correlates with a minimum serum turbidity after creaming, and a maximum relative viscosity found

in rheological analysis. Aggregation commences at 1·0% potassium laurate content and increases to a maximum at 5·0%.

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The stabilization of oil-in-water emulsions by the non-ionic surfactant cetomacrogol 1000

P. H. ELWORTHY AND A. T. FLORENCE

The stabilization of anisole and of chlorobenzene in water emulsions by the non-ionic surfactant cetomacrogol 1000 has been examined by following particle size changes with time. Rates of coalescence were calculated and found to be about 10^{-7} sec^{-1}. The effect of cetomacrogol concentration and phase volume, ϕ, on the stability were investigated. Increasing the surfactant concentration caused a slight increase in the rate of coalescence and increase in phase volume a slight decrease. Electrophoretic data indicated that the concentration effect was due to a reduction in zeta potential. Interfacial tension measurements showed that the film at the oil-water interface was more expanded than at the air–water interface; at 1% cetomacrogol concentrations the interfacial tension in both systems was about 5 dynes cm^{-1}. Spontaneous emulsification occurred above the critical micelle concentration in both systems. Emulsions are stable, no oil separation occurring even after nine months, although the mean volume particle size showed continual increase. The influence of hydrational, entropic and electrical stabilizing effects is discussed.

THE mechanism of stabilization of oil-in-water emulsions by non-ionic surfactants of the polyoxyethylene ether class is not clearly understood. The van der Waals' forces of attraction between two emulsion particles of radius 1–10 μ will be large; repulsive forces will, at first examination appear to be largely entropic in nature, as there exists uncertainty about whether particles stabilized by non-ionic surfactants are charged (Ginn, Anderson & Harris, 1964). To assess the contributions to stability from electrical, hydrational and entropic sources, we have investigated the ageing and other characteristics of emulsions stabilized by a series of synthetic polyoxyethylene cetyl ethers with varying polyoxyethylene glycol chain length. In this paper, we discuss the behaviour of the commercial non-ionic surfactant cetomacrogol 1000, $C_{16}H_{33}[OCH_2CH_2]_{22-24}OH$.

Experimental

MATERIALS

The oils used were anisole (α) and chlorobenzene (β) which were redistilled BDH materials. Decane (γ) was also used and was redistilled before use. The oils had the following refractive indices at 20° [literature values (Timmermans, 1950) in brackets]: anisole, 1·5169 (1·5170); chlorobenzene, 1·5241 (1·5246); decane, 1·4117 (1·4120). Cetomacrogol 1000 (Macarthy's Ltd.) was dissolved in water or methanol and the solution passed at least three times down a column of Biodeminerolit mixed bed resin; the solvent was removed at low temperature. Water was deionized and, for interfacial tension measurements, redistilled from potassium permanganate solution. Assay of the cetomacrogol by the method of Siggia, Starke & others (1958) showed the mean polyoxyethylene chain length to be 25 units.

PREPARATION OF EMULSIONS

The emulsions of anisole and chlorobenzene were prepared by an electrical dispersion method after the procedure of Nawab & Mason

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(1958). The apparatus consisted of a burette, with a reservoir bulb to provide a constant flow of oil, to which was attached, through a Luer-Lok joint, a metal needle. The joint was connected to a variable (0–5·5 KV) DC high voltage source. The needle passed through an earthed brass ring (diameter 1·5 cm). On applying voltages of about 4 KV, the anisole and chlorobenzene dispersed into fine sprays which were collected by rapid stirring in an earthed solution of the surfactant. Non-polar oils such as decane could not be dispersed electrically at up to 5·5 KV with our present apparatus and emulsions of decane for electrophoresis were prepared by ultrasonic dispersion in an M.S.E. ultrasonic disintegrator with a titanium probe. Dispersions of pure oil and water were also prepared ultrasonically for electrophoretic mobility measurements.

PARTICLE SIZE ANALYSIS

The particle size of emulsions was measured at intervals using a microscope with projection head and oil immersion lens. With the present system and microscope, 0·5 μ diameter particles could be observed, although this was the lower limit. A minimum number of 500 particles of each sample was counted but usually 1,000 particles were sized. The emulsions were stored at 20 $\pm$ 2°.

INTERFACIAL TENSION MEASUREMENTS

Interfacial tension measurements were made on a pendant drop apparatus based on the design of Andreas, Hauser & Tucker (1938) by photographing the drop on 35 mm film. The cell containing the drop was placed in a thermostat bath regulated to 20° $\pm$ 0·01°. The negatives were measured on a Cambridge Measuring Machine. Photographs were taken at rapid intervals up to a drop age of 60 sec and at longer intervals up to 24 hr; some confirmatory results were obtained at 48 hr. Equilibrium was generally reached after 5 hr.

The shape factors of Andreas & others (1938), Fordham (1948) and Stauffer (1965) were used. The latest computations of Stauffer (1965) were essential, as drops of water in anisole were almost spherical and factors for such drops are not contained in earlier tables.

ELECTROPHORESIS

Electrophoretic mobility measurements were made on a Zeta-Meter apparatus (Zeta-Meter Inc.) using the platinum anode and a glass Teflon cell calibrated with KCl. The emulsions, generally 0·1 ml, were diluted into water or into surfactant solutions (35 ml) and at least 10 determinations of mobility were made in each direction of travel. A mean time was taken and zeta potential calculated from the equation

$$\zeta = \frac{4\pi\eta\nu}{X\epsilon}$$

where the symbols have the usual meaning, the mobility (u) being equal to ν/X.

DISTRIBUTION DATA

Weighed portions of oil and surfactant solution were equilibrated for up to 10 weeks in a thermostat bath at 20° ± 0·01° or at 30° ± 0·01°. Weighed portions of each layer were assayed for cetomacrogol by drying to constant weight in a vacuum oven. In some cases high speed centrifugation was necessary to clarify the layers. The apparent partition coefficient K_W^O was calculated as K_W^O = conc. of surfactant in oil/conc. of surfactant in water.

STORAGE AND STERILITY

The emulsions were stored at room temperature in glass containers. They did not support bacterial growth and remained uncontaminated during storage. We thank Mr. M. S. Parker of this Department, for the bacteriological report.

Results and discussion

Although the electrical dispersion method has been stated to give monodisperse emulsions under certain conditions (Nawab & Mason, 1958), we were unable to obtain emulsions with a size distribution limited enough to be termed "monosized". The factors affecting the preparation of emulsions by this method are being examined. A typical particle size distribution for an emulsion prepared by electrical dispersion is shown in Fig. 1. The mean number diameter is in the region of 1 μ.

Similar distributions were obtained at intervals up to 10 months. The general shape of the distribution curve remains the same over this period,

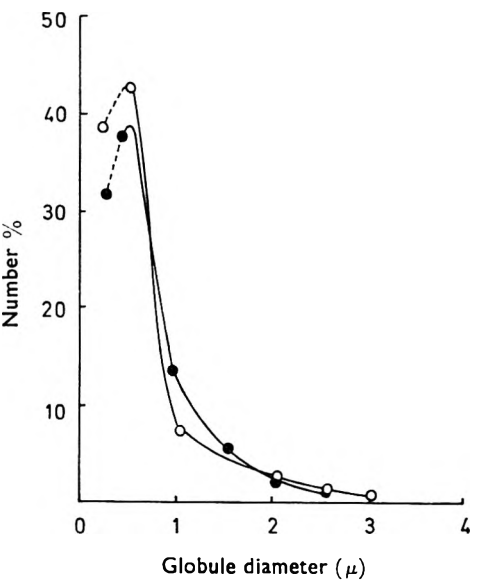

FIG. 1. Typical particle size distributions obtained with the electrical dispersion apparatus. ○, 5% cetomacrogol, ϕ = 0·20; ●, 1% cetomacrogol, ϕ = 0·56; oil phase anisole in both cases.

suggesting that particles in the size range of 0·5 μ–1 μ are stabilized by cetomacrogol. This is in contrast to the behaviour of emulsions stabilized by shorter chain surfactants where the peak of the distribution shifts rapidly to higher particle sizes (Elworthy & Florence, to be published). There is some growth of particle size however, as the mean volume diameter (d_{v_m}) increases with time (Table 2). The value d_{v_m}, $\left(\frac{\Sigma n_i d_i^3}{\Sigma n_i}\right)^{1/3}$ is very sensitive to the appearance of globules of large diameter (d_i) and for this reason the interpretation of the microscope sizing is difficult if d_{v_m} is relied upon alone. For this and other reasons $\Sigma n_i d_i^3$, $\Sigma n_i d_i^2$ and Σn_i have been determined and the results corrected to the same volume of oil (the same Σnd^3) at time $t = 0$. In this way the change of n with time can be followed, where n is the number of globules in the system.

Particle sizing data on emulsions can be treated in a number of ways. Lawrence & Mills (1954) plotted the mean volume ($\bar{v}$) of the emulsion globules against time and obtained a straight line relation with dilute emulsions of volume fraction ($\phi = 0{\cdot}01$), the ratio of the gradient of this line to $B\phi$ being $p = A\exp(-E/RT)$ where p is a fraction of the collisions effective in bringing about coalescence. Jellinek & Anson (1950) found

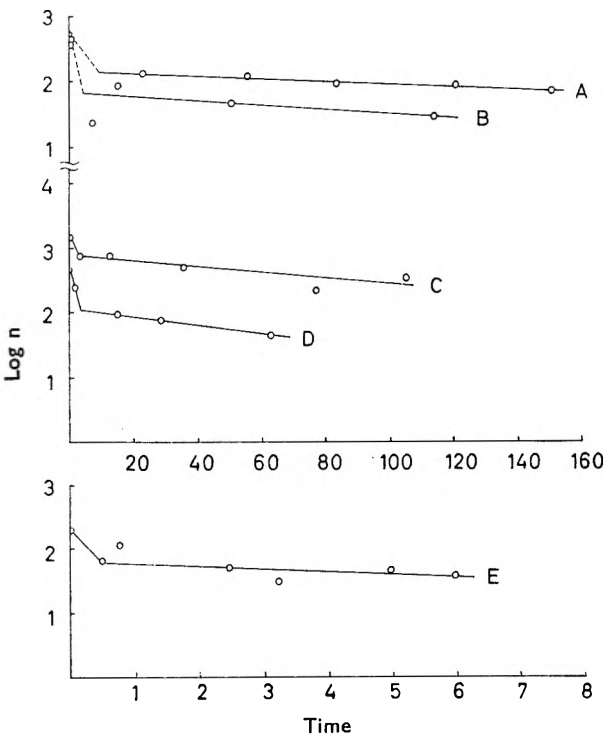

FIG. 2. Variation of log n (number of globules) with time in days for Anisole A, 0·1/0·17; B, 5·0/0·20; C, 1·0/0·56 and D, 1·0/0·05. Time in hr for E, 0·001/0·20 (see Table 1 for code).

TABLE 1. RATES OF COALESCENCE (SEC^{-1}) OF ANISOLE AND CHLOROBENZENE EMULSIONS STABILIZED BY CETOMACROGOL

Emulsion*	Rate 1(k_1 sec^{-1}	Rate 2 (k_2) sec^{-1}
Anisole 0·001/020	$26{\cdot}1 \times 10^{-4}$	$6{\cdot}94 \times 10^{-5}$
0·1/0·17	$8{\cdot}4 \times 10^{-7}$	$0{\cdot}29 \times 10^{-7}$
1·0/0·05	$13{\cdot}3 \times 10^{-7}$	$0{\cdot}69 \times 10^{-7}$
1·0/0·10	—	$0{\cdot}64 \times 10^{-7}$
1·0/0·31	—	$0{\cdot}64 \times 10^{-7}$
1·0/0·56	$13{\cdot}0 \times 10^{-7}$	$0{\cdot}52 \times 10^{-7}$
5·0/0·20	$20{\cdot}8 \times 10^{-7}$	$0{\cdot}87 \times 10^{-7}$
Chlorobenzene 0·1/0·16	$13{\cdot}3 \times 10^{-7}$	$0{\cdot}44 \times 10^{-7}$
1·0/0·05	$11{\cdot}6 \times 10^{-7}$	$0{\cdot}78 \times 10^{-7}$
1·0/0·10	$30{\cdot}0 \times 10^{-7}$	$0{\cdot}67 \times 10^{-7}$
1·0/0·19	$33{\cdot}6 \times 10^{-7}$	$0{\cdot}65 \times 10^{-7}$
1·0/0·50	$35{\cdot}0 \times 10^{-7}$	$0{\cdot}58 \times 10^{-7}$
5·0/0·22	$31{\cdot}8 \times 10^{-7}$	$0{\cdot}87 \times 10^{-7}$
10·0/0·20	$28{\cdot}7 \times 10^{-7}$	$1{\cdot}13 \times 10^{-7}$

* Code used for identification of emulsions 0·1/0·17 represents an emulsion stabilized with 0·1% cetomacrogol solution with an oil phase volume of 0·17.

a linear variation of specific interfacial area with time. Van den Tempel (1957) obtained in some cases, after an initial rapid fall, a linear variation of the logarithm of the number of particles with time. The slope of this line gave a constant k, the rate of coalescence. Our data have been treated in this way and some results are shown in Fig. 2 and Table 1. The calculation of rates of coalescence from the raw data obtained from microscopic sizing serves to quantify the changes which otherwise could only be followed visually. Table 2 shows changes of mean volume diameters of the particles of a number of representative emulsions in this series.

TABLE 2. CHANGE IN PARTICLE MEAN VOLUME DIAMETERS (d_{v_m}) OF FIVE REPRESENTATIVE EMULSIONS OF THE SERIES*

Age (days)	Chlorobenzene 1·0/0·05 $d_{v_m}(\mu)$	Chlorobenzene 1·0/0·10 $d_{v_m}(\mu)$	Chlorobenzene 5·0/0·2 $d_{v_m}(\mu)$
0	1·1	1·4	1·0
10	2·1	2·2	3·0
20	2·3	2·3	3·2
40	2·5	2·4	3·7
60	2·8	2·5	4·1
80	3·0	2·7	4·5
100	3·2	3·0	4·9
120	3·4	3·1	5·3
140	3·8	3·2	5·8
160	4·0	3·3	7·2
Age	**Anisole 1·0/0·31 $d_{v_m}(\mu)$**	**Anisole 1·0/0·55 $d_{v_m}(\mu)$**	
0	1·0	1·0	
10	2·0	3·0	
20	2·8	3·6	
30	3·4	3·9	
40	3·6	4·2	
60	3·8	4·3	
80	4·1	4·4	
100	4·5	4·4	
120	6·0	4·6	

* Values at even days interpolated from actual data for comparison.

More recently Hill & Knight (1965) have developed a kinetic theory of droplet coalescence applicable to slowly coagulating emulsions. An equation:

$$\frac{1}{\Sigma} = \frac{11BkT}{4a} t + \text{constant}$$

was obtained, B being a constant of proportionality, k the Boltzmann constant, a the available volume and Σ the surface area in cm^2, T the absolute temperature and t the age of the system. A plot of $\frac{1}{\Sigma}$ against time should be linear. Hill & Knight (1965) found that the mean volume of their particles was proportional to Σ^{-3} and, as a test of the applicability of Smoluchowski's (1917) theory which takes the form $\Sigma^{-3} = Bt + \text{constant}$ one could compare the variation of Σ^{-1} and Σ^{-3} with time. Selected results are shown applying this test (Figs 3 and 4).

In the Smoluchowski (1917) treatment for coagulation of dispersions every collision is regarded as effective in bringing about coalescence. In slow coagulation such as occurs in cetomacrogol stabilized emulsions, only a fraction of the collisions between the particles will result in coalescence. Hill & Knight (1965) approached the problem of coagulation in such emulsions by considering the possibility of coalescence of two droplets as a function of their radii and relative velocity, assuming that the droplets have a Maxwell distribution of velocities, and averaging the effects for all sizes of droplets. The theory is applicable to systems stabilized primarily by steric and hydrational mechanisms; the possibility of electrostatic forces is ignored. It would be expected, then, that our results would fit the theoretical prediction of a linear increase in Σ^{-1} with time if electrical forces are minimal in these systems.

Fig. 2 shows that there are two rates of coalescence, termed k_1 and k_2 (Table 1). The second rate of coalescence is a slow rate—for stable emulsions in the region of 10^{-6} to 10^{-7} sec^{-1}—and follows an initial fast rate which applies up to about four days and which is probably the result of re-equilibration at the interface. It was found that k_2 fell with increasing phase volume, a phenomenon also observed by van den Tempel (1957). It also increases slightly with increasing surfactant concentration (Table 1). After an initial rise the mean *number* diameter tends to level off at longer time intervals. The mean *volume* diameter shows a similar trend. The rapid initial size changes can be seen from plots of mean volume diameter and mean number diameter with time.

The effect of phase volume on the rates of coalescence is difficult to explain. In concentrated emulsions the droplets of disperse phase have a higher co-ordination number than those in dilute emulsions and the coalescence of one droplet with another will not affect the co-ordination number to the same extent as in a dilute emulsion. In a concentrated emulsion the droplets will not attain the same kinetic energy because of hindrance to movement. Hence it might be concluded that these globules will show less tendency to coalesce.

The effect of oil type and concentration of surfactant on the mobility of the oils in the emulsions was examined. Decane dispersions prepared ultrasonically with cetomacrogol as emulsifier were compared with emulsions of chlorobenzene and anisole. Pure samples of these compounds

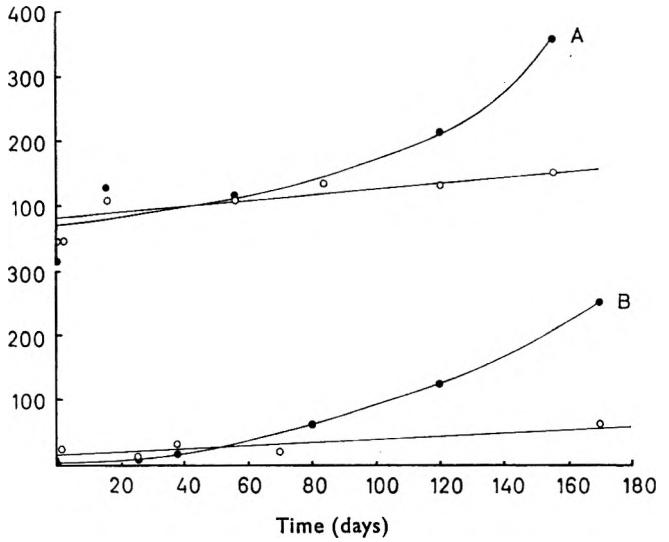

FIG. 3. Plots of ○ Σ^{-1} and ● Σ^{-3} with age of two emulsions of anisole A, 0·1/0·17 and B, 1·0/0·10 following the treatment of Hill & Knight (1965). In A, $\Sigma^{-1} = 10^3/\pi$ (interfacial area in cm²) and Σ^{-3} is adjusted to bring the plot onto the same ordinate scale. In B, $\Sigma^{-1} = 10^5/\pi$ (interfacial area in cm²) and Σ^{-3} is also adjusted to fit scale.

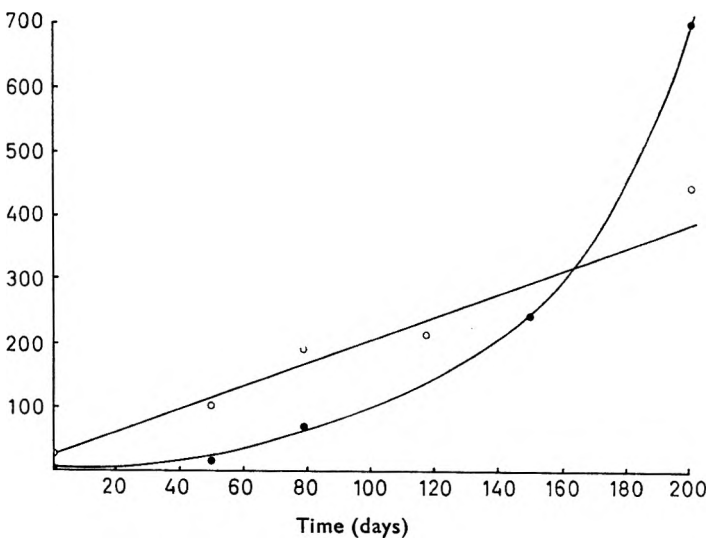

FIG. 4. Plot of ○ $\Sigma^{-1} = (10^4/\pi$ (interfacial area in cm²) and ● Σ^{-3} (adjusted as above) for the anisole emulsion 5·0/0·20, showing linearity of Σ^{-1} with time, within experimental error and curvature of Σ^{-3}.

TABLE 3. ELECTROPHORETIC MOBILITIES AND ZETA POTENTIALS OF EMULSION GLOBULES AT 20° ± 1°

	Concentration of cetomacrogol (%)	Mobility[a]	Zeta potential (ζ)[b]
Anisole	0	−5·3	−83·4
0·1/0·17	0·1	−2·0	−31·5
1·0/0·10	1·0	−1·1	−17·3
1·0/0·18	1·0	−1·4	−22·0
5·0/0·20	5·0	−0·4	−6·3
10/0/0·2*	10·0	0	0
Chlorobenzene	0	−3·08	−48·5
0·1/0·20	0·1	−1·7	−26·8
1·0/0·1	1·0	−1·3	−20·5
5·0/0·22	5·0	−0·35	−5·5
10·0/0·2	10·0	0	0
Decane	0	−3·21	−50·5
0·1/0·2	0·1	−0·71	−11·2
1·0/0·2	1·0	−0·2	−3·1
5·0/0·1	5·0	−0·2	−3·1
10·0/0·2*	10·0	0	0

(a) μ sec^{-1} V cm^{-1}

(b) mV calculated from $\zeta = \frac{4\pi\eta u}{\varepsilon}$ where u = v/X

* Extrapolated values.

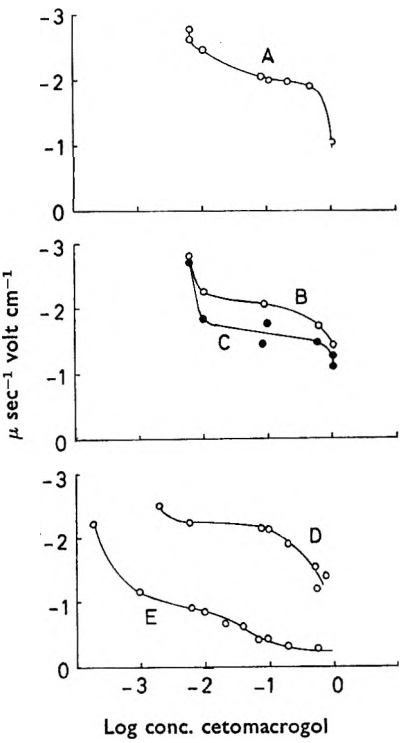

FIG. 5. Variation of electrophoretic mobility with concentration of cetomacrogol in electrophoresis cell. A, anisole 0·1/0·17; B, anisole 1·0/0·18; C, anisole 1·0/0·10; D, chlorobenzene 1·0/0·10; E, decane 1·0/0·10.

were dispersed ultrasonically in deionized water, the preparations being stable for a sufficiently long time for mobility measurements to be undertaken. Decane has a mobility of $-3{\cdot}2$ μsec^{-1} V cm^{-1}, which may be compared with the value of $-3{\cdot}1$ $\mu sec^{-1}V$ cm^{-1} given by Anderson (1959). Chlorobenzene had a mobility of $-3{\cdot}08$ and anisole $-5{\cdot}3$ $\mu sec^{-1}V$ cm^{-1}. Davies & Rideal (1961) have suggested that the high negative mobility of hydrocarbons in water is due to the selective desorption of hydrogen ions from their surfaces.

The effect of surfactant on the mobilities is shown in Table 3 and in Fig. 5. In 10% cetomacrogol the zeta potential of the oil globules is zero. The zeta potential of an oil-in-water emulsion stabilized by an octyl phenyl polyoxyethylene ether has been reported to be $-3{\cdot}2$ mV by Mochlova & Nikitina (1966); others (Mathai & Ottewill, 1962; Ottewill, 1967) have observed a reduction in mobility of charged particles in the presence of non-ionic surfactants. Ottewill (1967) states that "the interpretation of the results would appear to be that in the region of low surface coverage the non-ionic surfactant molecules are lying in a looped or extended form on the surface and only small decreases in mobility occur". Above the critical micelle concentration (CMC) the decrease in mobility is due to the shift of the plane of shear further into the solution. The very sharp fall in mobility which can be seen in the concentrated surfactant solutions is possibly due to an accentuation of this effect through multilayer formation. In chlorobenzene emulsions prepared with 5% and 10% cetomacrogol, extensive clumping of the particles occur, suggesting some sort of interaction between the particles, perhaps through bridging between the multilayers.

The zeta potentials of the decane emulsions are much lower than those of the polar oils in spite of the fact that the mobilities of the pure oils, chlorobenzene and decane are similar. This might suggest a different mode of packing or orientation of the cetomacrogol molecules, at the two interfaces.

A plot of the second rate of coalescence, k_2, against ζ^2 is linear whereas the plot of k_2 against ζ is not (see Fig. 6). This suggests that electrical factors are important in the stabilization of emulsions by non-ionic surfactants, the secondary stability of the emulsions being affected since even when the zeta potential is zero the emulsions are reasonably stable. At concentrations of surfactant near the CMC, however, the rate of coalescence k_2 increases to $6{\cdot}9 \times 10^{-5}$ sec^{-1} and the emulsions are very unstable, showing separation of oil after a few min. Increasing the surfactant concentration decreases the zeta potential of the particles and therefore increases the tendency to clumping and the rate of coalescence. The effect is not a drastic one, suggesting that the zeta potential is not a primary factor in the stabilization of these emulsions. This will be discussed by Elworthy & Florence (to be published). Fig. 7 shows the plot of log k_2 against ζ^2, employing points obtained from both anisole and chlorobenzene emulsions, and from this we obtain a linear relationship:

$$\log k_2 = C + M\zeta^2$$

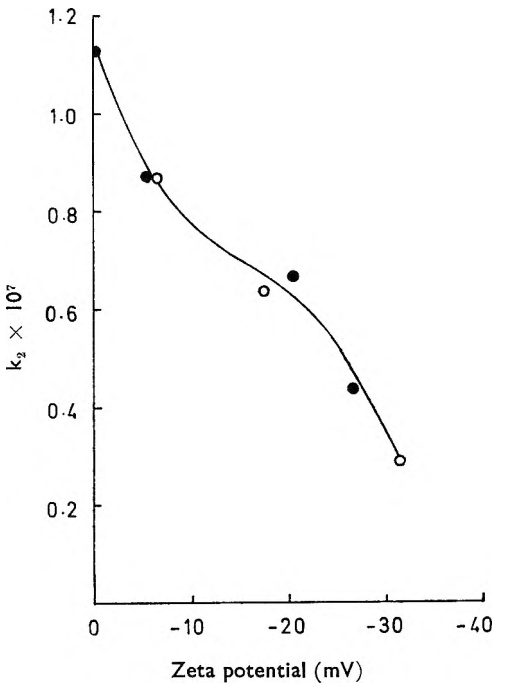

FIG. 6. Change of second rate constant k_2 (sec^{-1}) with zeta potential (mV) of globules in a series of: ○, anisole emulsions; ●, chlorobenzene emulsions stabilized with cetomacrogol.

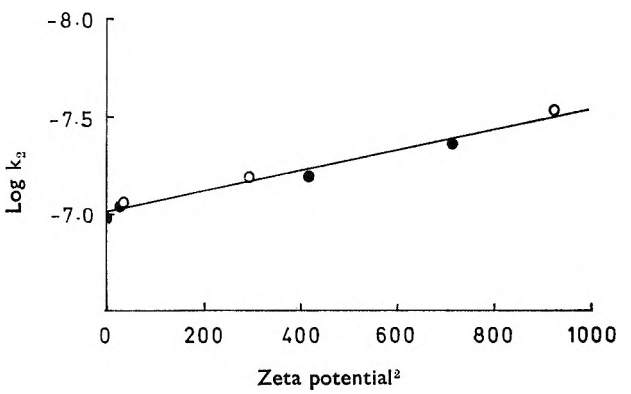

FIG. 7. Linear plot of log k_2 (cf. Fig. 6) against the square of zeta potential (mV^2).

Davies and Rideal found that the rate of coalescence can take the form

$$\text{rate} = A_1 \exp\left(\frac{-B\psi^2}{RT}\right)$$

where ψ is the surface potential in millivolts.

If ψ is replaced by ζ a value of B of $-0{\cdot}69$ is obtained. Using $\zeta = 0{\cdot}55\ \psi$, as suggested by Davies & Rideal (1961), B $=-0{\cdot}23$, which compares well with the value of $-0{\cdot}24$ obtained by these authors. The relation between measured zeta potential and the surface potential has occasioned much discussion. In the plots of zeta potential against the logarithm of cetomacrogol concentration there is a small plateau region above the CMC followed by a rapid fall at higher concentrations. To explain the similar behaviour of particles stabilized by ionic surfactants, it has been suggested that direct absorption of highly charged micelles or hemi-micelles occurs (Fuerstenau, 1956; Ottewill & Watanabe, 1960).

The variation of equilibrium interfacial tension with cetomacrogol concentration is shown in Fig. 8. The areas per molecule at the anisole–water and chlorobenzene–water interface are 154 Å^2 and 180 Å^2 respectively, which can be compared with the smaller value of 120 Å^2 at the air-water interface (Elworthy, 1960). The interfacial tension at the CMC

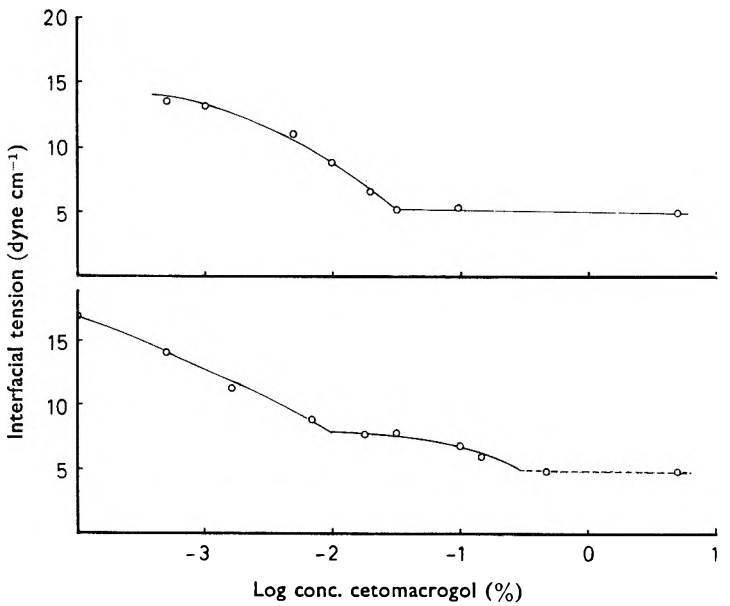

FIG. 8. Equilibrium interfacial tension against log conc. cetomacrogol for anisole systems (upper plot) and chlorobenzene systems (lower plot). In the lower plot the dotted line represents the region of heavy spontaneous emulsion formation, illustrated in Fig. 10.

is 7·0 dynes cm^{-1} for the chlorobenzene system and 5·0 dynes cm^{-1} for the anisole system.

During these experiments the formation of emulsions at the interface between the oil and aqueous surfactant phase was evident at concentrations above the CMC. The process is shown in Fig. 10. In no case in which this phenomenon was observed did the interfacial tension reach zero, a condition previously thought to be necessary for bringing about spontaneous emulsion formation. No such process, however, occurred

at the non-polar decane surfactant interface even in the presence of 10% cetomacrogol. The necessity of a polar constituent in this process has already been commented on by Lawrence (1957).

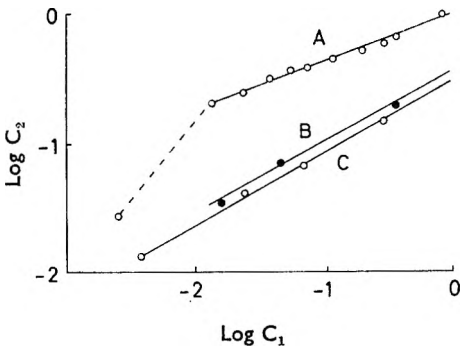

FIG. 9. Plot of log C_2 (concentration of cetomacrogol in oil phase) against log C_1 (concentration of cetomacrogol in aqueous phase) for A, chlorobenzene–water–cetomacrogol (emulsified system); B, the same but equilibrated two phase system; C, anisole–water–cetomacrogol, two phase system. Concentrations are equilibrium concentrations in g/10 ml^{-1}.

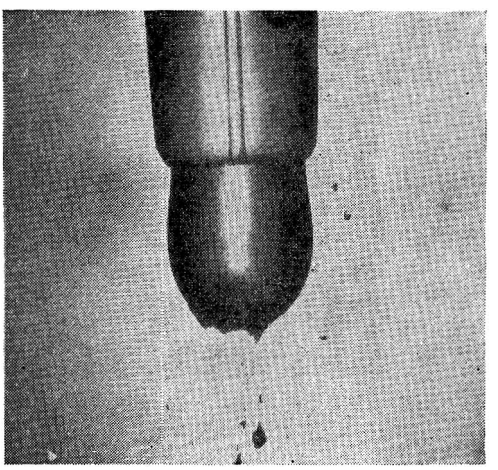

FIG. 10. Spontaneous emulsion formation at the interface of a drop of chlorobenzene suspended in an aqueous (5·0%) cetomacrogol solution.

Spontaneous emulsification at planar interfaces probably occurs due to diffusion of the cetomacrogol into the oil phase along with its hydrating water, thus forming regions of zero interfacial tension: such a concept would be supported by the distribution data. The systems anisole–aqueous cetomacrogol and chlorobenzene–cetomacrogol take approximately four weeks to reach equilibrium at 20°. Distribution data were obtained for a range of concentrations of surfactant above the CMC and

the apparent distribution coefficient, K_w^o calculated. K_w^o decreases with increasing concentration of surfactant above the CMC. Table 4 gives some values for the anisole, chlorobenzene and decane systems. The distribution of the surfactant to the oil follows the trend chlorobenzene > anisole > decane.

TABLE 4. APPARENT DISTRIBUTION COEFFICIENTS (K_w^o) OF CETOMACROGOL 1000 AT 20°: VARIATION WITH CETOMACROGOL CONCENTRATION

	Original aqueous concentration (%)	Final aqueous concentration (%)	K_w^o
Anisole*	0·491	0·1255	5·4
	0·507	0·1665	4·1
	0·979	0·484	2·1
	4·897	3·835	0·55
	9·794	8·561	0·29
Chlorobenzene*	0·0630	0·0047	11·34
	0·0813	0·0038	20·46
	0·200	0·0239	8·82
	0·408	0·0723	9·33
	1·006	0·293	4·87
	4·794	3·164	1·03
	9·794	8·004	0·45
Decane	0·103*	0·080	0·279
	5·00†	4·885	0·071
	10·00†	9·770	0·071
	30°		
Chlorobenzene*	4·794	2·679	1·58
	9·794	6·936	0·795

* 5 ml oil, 10 ml aqueous phase.
† 2 ml oil, 6 ml aqueous phase.

C_2 is the concentration of the surfactant in the oil and C_1 the concentration of the surfactant in the water; plots of log C_2 against log C_1 are linear for the systems studied. Similar relations have been found for the distribution of the non-ionic surfactants between water and iso-octane (Greenwald, Kice & others, 1961), whilst Lawrence & Stenson (1957) obtained similar relations for the distribution of cetyltrimethylammonium bromide between butanol and heptanol and water. The following general relation can be written

$$\log C_2 = S. \log C_1 + \text{constant}$$

Representative plots are given in Fig. 9, where S is the gradient of the plot. So for the system water–iso-octane and the surfactant octyl phenyl heptaoxyethylene glycol ether, S is 0·379 (Greenwald & others, 1961), below the CMC, the slope is 1·1. For cetomacrogol–anisole–water S is 0·36 at 20°; for chlorobenzene–water S is 0·54 at 20° and 0·43 at 30°. In the latter system at 20° the value obtained from a series of emulsions in which the oil had settled leaving a clear aqueous layer for analysis, S was 0·38. It is seen from Fig. 9 that below the CMC, S is also 1·1. From the law of mass action treatment, we may write

$$C_1 = x_1 + n_1 m_1$$

and

$$C_2 = x_2 + n_2 m_2$$

where n_1 is the number of monomers in the micelle and the micellar concentration is m_1 in the aqueous phase, and n_2 and m_2 are the equivalent

values in the oil phase and x_1 and x_2 are the concentration of monomers in both phases respectively. Thus

$$K_1 = \frac{m_1}{x_1^{n_1}} \text{ and } K_3 = \frac{x_1}{x_2}$$

where K_1 is the equilibrium constant for micelle formation in the aqueous phase and K_3 is the apparent distribution constant as defined. Treating the distribution of surfactant between the oil and water, and assuming that micelle formation takes place in both phases, we obtain a relation:

$$\log C_2 = \frac{1 + n_2}{1 + n_1} \log C_1 + \text{constant}$$

$$\text{i.e. } S = \frac{1 + n_2}{1 + n_1}$$

Below the CMC we would expect that $n_2 = n_1 = 1$ and the slope of the plot would be unity: this is indeed as found. For chlorobenzene above the CMC, knowing that $n_1 = 80$ (Elworthy, 1960), we obtain $n_2 = 30$. If this formulation is correct this would give a means of determining the micelle size in non-aqueous solvents.

The depletion of surfactant from the aqueous phase of an emulsion might be expected to affect the stability of the emulsions. This indeed may be so at low surfactant concentrations where K_w^o is high and where drastic reduction in the aqueous concentration may occur. This equilibration, however, may occur with little change in interfacial tension. Below the CMC, Greenwald & others found the distribution coefficient to be constant. During the interfacial tension experiment with chlorobenzene, there was a drop of about 0·1 ml in a cell containing 15 ml of surfactant solution; even with a distribution coefficient of 10 and an original surfactant concentration of 0·01% in the aqueous phase, the distribution coefficient will be satisfied with transfer to 1×10^{-4} g of surfactant which would not affect the concentration to any sensible degree. The distribution results support the theory that it is the transfer of surfactant from water to the oil which results in spontaneous emulsification. The distribution data for the decane systems show that the K_w^o is very low and these do not exhibit the phenomenon on spontaneous emulsification. However, in all cases K_w^o falls with increasing concentration and the spontaneous emulsion forms only at higher surfactant concentrations. It seems likely that a packed interface is essential for the process. Whether such effects are important at the curved 1 μ globule surface is not known and it is difficult to conjecture the effect of spontaneous emulsification on emulsion stability. The low interfacial tension values obtained with cetomacrogol at the anisole and chlorobenzene interfaces show that it is an emulsifier with which emulsions can be readily prepared with the minimal expenditure of energy. It is evident that the packing of the surfactant is closer at the anisole interface. This is reflected in the electrophoretic behaviour as can be seen in plots of the decrease of zeta potential against concentration of surfactant (decrease in zeta potential

$=\zeta_{H_2O}-\zeta_{surfactant}$). The decrease in zeta potential is much greater in the anisole system although at a particular surfactant concentration the mobility of the chlorobenzene emulsions are actually lower. These effects do not appear to reflect any differences in the stability of anisole and chlorobenzene emulsions.

CONCLUSION

Cetomacrogol can be used to produce emulsions of anisole and chlorobenzene which are stable for periods extending up to about 9 months with little or no separation of oil occurring in this time. At low concentrations of cetomacrogol, electrical effects appear to contribute to the stabilization of the emulsion. Increasing the surfactant concentration decreases the zeta potential and increases the tendency to coalescence. The main stabilizing influences, it is concluded, must be either steric or hydrational factors. These are being investigated further by observing the stabilization of the same oils with a series of synthetic surfactants with varying polyoxyethylene chain lengths.

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The rate of evaporation of liquids from beds of powder

K. RIDGWAY AND J. A. B. CALLOW

The drying rates of beds of magnesium carbonate powder wetted with various liquids have been measured. The powder had a median particle size of 20 μ and was formed into cylindrical beds with a circular upper surface area of 25 cm^2. The bed was held in a lightweight container machined from a nylon block, suspended in a 10 ft/sec air stream. The weight loss of the bed was recorded continuously. Bed depths of 1, 2, 3 and 4 cm were used, and drying rates measured for water, ethanol, isopropanol and chloroform. The magnesium carbonate was granulated using a 10% aqueous solution of polyvinyl pyrrolidone and the drying rate of the wet granules similarly measured. There is a linear relationship between log (drying rate) and liquid content during the falling rate period. Analysis of the constant and final drying rates indicates that vapour diffusion through the dry portion of the bed was the limiting process throughout the drying operation.

THE preparation of pharmaceutical tablets in large quantities by using a rotary tabletting machine depends on obtaining the material to be compressed in a free-flowing form which will run easily from the feed hopper into the die. The usual method of preparing a suitable feedstock is to wet-granulate the powder and to dry the granules. Since a tablet may contain only a few tens of the granules, movement of soluble constituents during the drying process is important. The present work was initiated with a view to studying solute migration during granule drying. However, the mechanics of the drying process itself proved to be of interest, and we report here the results of the drying of beds of an inert, insoluble powder wetted with pure liquids, the complication of a migrating solute being avoided.

Lewis (1921) first postulated that the drying of a solid involves a balance between the evaporation of moisture from the solid surface and the rate of moisture movement from within the solid to the drying surface. The Lewis model was extended theoretically by Sherwood (1931) and by Newman (1931) who solved diffusion-type equations for various geometries and boundary conditions believed to be applicable to the drying process. Sherwood discussed the concepts of a constant rate and a falling rate period, suggesting that the latter was divided into two parts; one of these was non-saturated surface drying, with a decrease of wetted area; the other was a period of control by internal moisture movement. It was pointed out by Sherwood & Comings (1933) however, that the actual mechanism of moisture movement through the solid was unknown, and that Newman's model did not fit experimental curves for the drying of sand and clay.

It was later shown (Ceaglske & Hougen, 1937) by examining moisture distributions, that in many materials liquid moved to the surface by capillary action rather than by diffusion. Newitt and his co-workers (Oliver & Newitt, 1949; Pearse, Oliver & Newitt, 1949) based their theory upon the restriction of liquid movement by frictional forces calculated

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from the Kozeny equation for single phase flow in particle beds. Bell & Nissan (1959) and Nissan, Kaye & Bell (1959) suggested that rate control was related to the ratio between the heat and mass transfer resistances of the porous medium, and they successfully predicted the value of the pseudo wet-bulb temperature of the evaporative surface, which is higher than the theoretical wet-bulb temperature. The concept of a receding evaporative plane within the bed, first put forward by Gilliland (1938) was developed further by Nissan, George & Bell (1960). Adams (1962) showed experimentally that the transition from constant to falling rate conditions does not represent a fundamental change in the nature of the drying process, but only the cessation of transport of liquid to the surface by capillary action. During the falling rate period liquid must be transported by *in situ* vaporization and subsequent diffusion.

The theoretical approach used by Morgan & Yerazunis (1967), following the boundary layer analysis of Luikov (1963), requires only that the location of the evaporative interface be determined experimentally. The present work, by utilizing an automatic recording balance for the first time in drying measurements, has shown that there is a linear relationship between the logarithm of the drying rate and the liquid content of the bed over the whole of the falling rate period. Together with the concept of the recession of an evaporative front into the bed, it has been shown that the initial and final drying rates are consistent with vapour diffusion being the controlling factor.

Experimental

The overall dimensions and layout of the drying tunnel are indicated in Fig. 1. The heating, calming and test sections were made of asbestos board, fastened to a welded mild steel framework. A Perspex window was incorporated in the test section to allow observation of the bed during the drying operation. An air flow-rate of 10 ft/sec could be obtained from the 6 inch diameter axial flow fan, and the air temperature was controllable at any value up to about 70° by altering the voltage applied to the nichrome heating coils. The straightening vanes, the calming

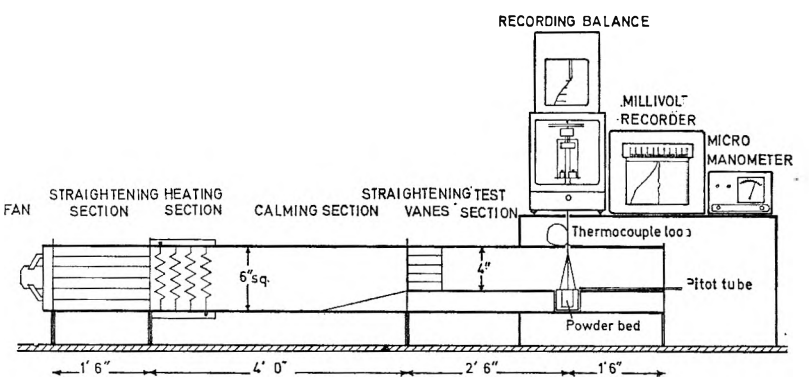

FIG. 1. Drying tunnel.

section and the inclined base immediately upstream of the powder container were adjusted to give as uniform a velocity profile as possible in the test section, as determined by a pitot survey.

The powder container was made of nylon. The bed area was 25 cm^2, and the depth of powder could be altered by inserting packing plates of 1, 2 or 3 cm thickness, as required. The sides and base were hollow to minimize weight and heat conduction. It was hung by thin wires from the pan of a Stanton automatic recording balance. Three thermocouples were placed in the bed, and six at various positions in the tunnel. All were connected to a Kent recorder. Air speed was recorded by means of a Hilger micromanometer connected to a pitot tube.

Heavy magnesium carbonate B.P., $3MgCO_3$, $Mg(OH)_2.4H_2O$ was used as the powder for this work. The particle size was measured with a Coulter Counter, and the results confirmed by microscopy. The mean particle size by weight was 20 μ, and the range was 95% by weight $>10\,\mu$, 5% by weight $>32\,\mu$. The liquids used were purified water, ethanol, isopropanol and chloroform, all of B.P. quality, being selected as liquids frequently used in wet granulation. Powder beds were initially prepared by making a 50% w/v suspension of powder in the appropriate liquid, and packing this into the bed container. Beds of granules were made by pouring the granules into the container, with light tapping.

For the granulation of the powder, polyvinyl pyrrolidone was chosen as binding agent. Heavy magnesium carbonate (250 g) was placed in a coating pan, which was rotated at 30 rev/min: a 10% aqueous solution of the binding agent was sprayed on to the powder, until the required wetness was obtained. This was found to require 100 ml of the solution. The damp mass was passed through a 22 mesh sieve, and the moist granules thus obtained placed in the bed container.

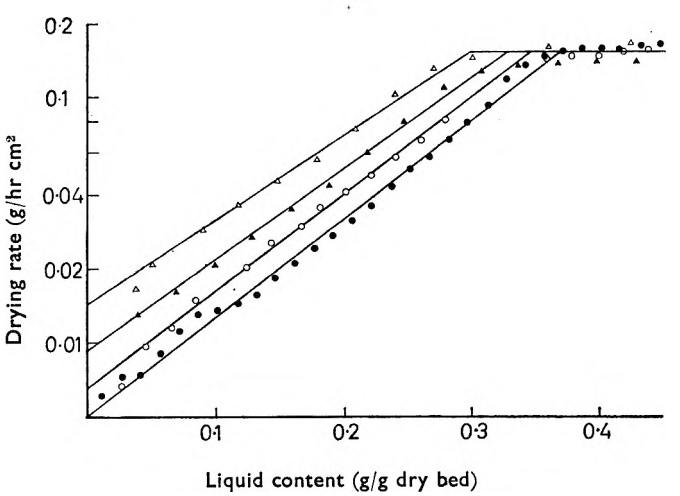

FIG. 2. Evaporation of water from beds of $MgCO_3$, △, 1 cm; ▲, 2 cm; ○, 3 cm; ●, 4 cm bed depth.

Results and discussion

The results for the four bed depths and the four liquids are shown in Figs 2–5, where the drying rate is plotted as a function of liquid content. Plotting the logarithm of the drying rate against liquid content gives a linear plot once the constant drying rate period has elapsed. This has not, to our knowledge, been noticed previously.

These graphs show (a) the rate of evaporation in the constant rate period, (b) the final drying rate, obtained by extrapolating the straight line plot of the falling-rate period to zero moisture content (or to the equil-

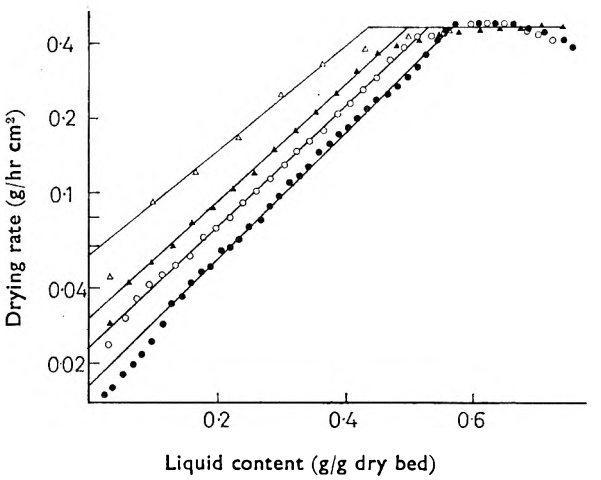

FIG. 3. Evaporation of ethanol from beds of $MgCO_3$. See Fig. 2 for key.

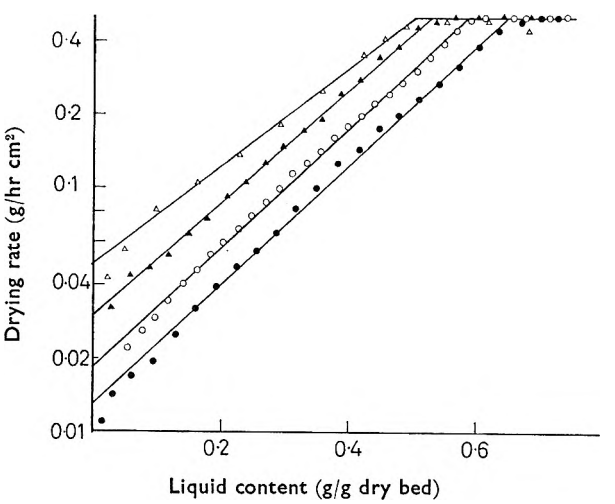

FIG. 4. Evaporation of isopropanol from beds of $MgCO_3$. See Fig. 2 for key.

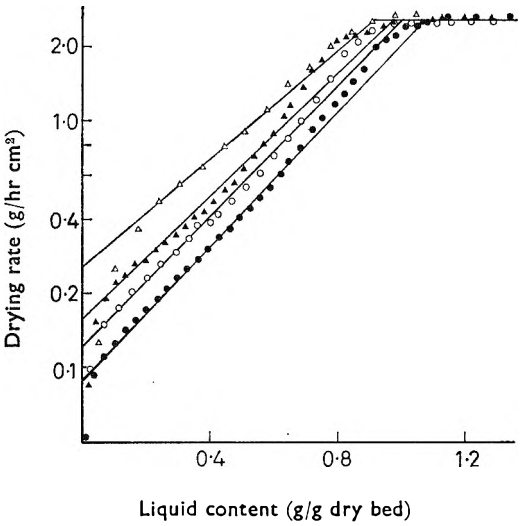

FIG. 5. Evaporation of chloroform from beds of $MgCO_3$. See Fig. 2 for key.

brium moisture content in the case of water because of the fact that anhydrous air was not used), and (c) the critical liquid content at which the change-over from constant to falling-rate occurs. Further comments on these are taken in the above order.

(a) CONSTANT RATE PERIOD

As expected the constant rate is independent of bed depth since it is governed by the stationary air film above the bed. An estimate of the boundary layer thickness could in principle be made on either a heat or a mass transfer basis. Table 1 gives the required information for heat transfer.

TABLE 1. DRYING RATE DATA RELEVANT TO HEAT TRANSFER FROM THE BED

Liquid	Constant drying rate g/cm²/hr	Latent heat of evaporation cal/g	Heat transfer cal/cm²/hr
Water	0·16	560	89·5
Ethanol	0·48	230	110
Isopropanol	0·50	185	92·5
Chloroform	2·62	64	168

The expected heat transfer coefficient for the air stream (Perry, 1964) is 1·4 cal/cm²/hr/°C. The tunnel air temperature was always 52°, and the measured surface temperature of all the beds during the constant rate period was 29°. Thus $\Delta t = 52 - 29 = 23°$ and for a bed area of 25 cm², the heat transferred is $25 \times 23 \times 1{\cdot}4 = 800$ cal/hr.

For the first three liquids, the required rate of heat transfer to produce the observed rate of evaporation is approximately $100 \times 25 = 2500$

cal/hr and for chloroform it is even greater. It is thus apparent that despite the precautions taken, heat is transferred from the air stream to the powder over the entire upper surface of the cylindrical container. This is confirmed by the equality

$$\frac{\text{actual heat transferred}}{\text{calculated for bed surface}} = \frac{2500}{800} = \frac{\text{area of entire container top}}{\text{area occupied by bed surface}}$$

Heat transfer data are thus of limited use for determining boundary layer thickness.

TABLE 2. DRYING RATE DATA RELEVANT TO MASS TRANSFER FROM THE BED

Liquid	Drying rate g/cm²/hr	Conc. of sat. vap. at drying surface, g/cm³ × 10⁴ at 30°	Diffusivity at 40° cm²/sec	Boundary layer thickness cm
Water	0·16	0·30	0·295	0·148
Ethanol	0·48	1·89	0·144	0·204
Isopropanol	0·50	1·97	0·108	0·153
Chloroform	2·62	1·46	0·126	0·253

The mass transfer data are given in Table 2. The value of the boundary layer thickness for chloroform is rather larger than the values for the other liquids for the following reason. Fig. 6 shows that there is a first falling

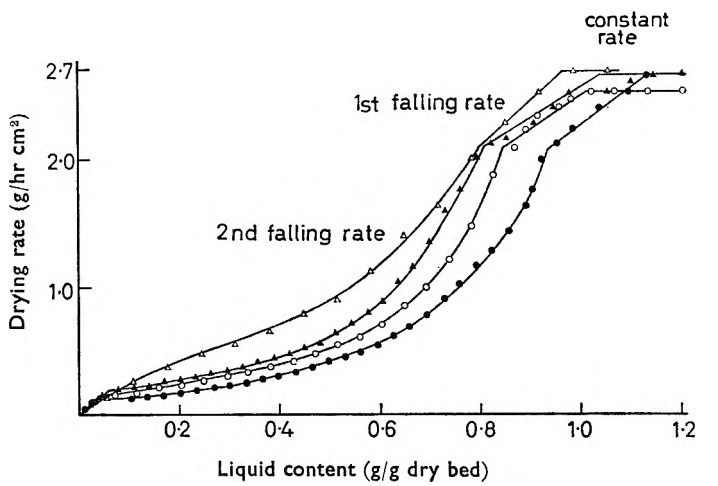

FIG. 6. Evaporation of chloroform from beds of $MgCO_3$. See Fig. 2 for key.

rate period as described by Oliver & Newitt (1949), where the drying rate is directly proportional to liquid content. Chloroform is the only liquid which shows a separate linear first falling rate period and this is produced by the surface area available for evaporation decreasing from the total surface area of the bed to the free space at the surface (Oliver & Newitt, 1949). The first falling rate period is independent of bed depth, and ends at 2·1 g/cm²/hr in all cases. Thus it must be a surface phenomenon.

Assuming that evaporation at this point is from the free space in the surface, i.e. that

$$\text{area of evaporation} = (\text{actual surface area}) \times \text{voidage},$$

Fig. 6 indicates that evaporation is from 76% of the surface during the constant rate period. Adjustment of the calculation of boundary layer thickness (Table 2) for this factor changes the value to 0·193 cm which is in much better agreement with the other figures.

Results for the four bed depths for moist granules are shown in Fig. 7 in which a linear relation is again obtained between log (drying rate) and liquid content. However, there is no constant drying rate period as the initial liquid content of the bed is too low.

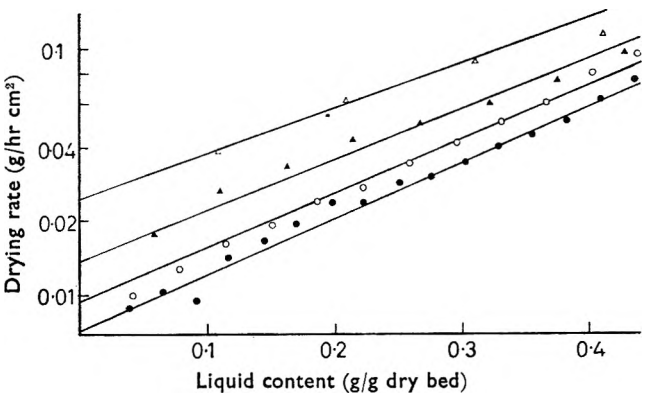

FIG. 7. Evapcration of water from beds of $MgCO_3$ granules. See Fig. 2 for key.

(b) FINAL DRYING RATE

The final drying rates obtained by extrapolation to zero liquid content are given in Table 3.

TABLE 3. FINAL DRYING RATES, G/CM²/HR

Liquid	Bed depth, cm			
	1	2	3	4
Water	0·0143	0·0093	0·0066	0·0050
Ethanol	0·056	0·031	0·0233	0·0163
Isopropanol	0·049	0·030	0·0184	0·0130
Chloroform	0·257	0·158	0·122	0·089

There are two possible mechanisms which may operate in the bed:

(1) The Newitt mechanism, in which liquid is gradually depleted from the pores of the bed, successively smaller pores losing their liquid as drying proceeds, as described by Oliver & Newitt (1949) and Pearse & others (1949).

(2) The Nissan mechanism, where an evaporative front recedes into the bed, leaving dry material above it through which vapour must pass by diffusion in order to reach the upper surface, as described by Nissan & others (1960).

Our results indicate that (2) is the operative mechanism in the system we have examined. Thus we have calculated the final drying rate for the case where the last trace of liquid evaporates at the base of the bed, and diffuses through the full bed depth: the diffusivity of the vapour in the bed interstices was corrected for the bed voidage, ϵ. In Table 4 which gives the relevant data, the temperature at the base of each bed is the value measured by means of a thermocouple. These temperatures are higher than the corresponding wet-bulb temperatures for the free liquid surface, which is additional evidence in favour of mechanism (2). The concentration of the vapour is calculated from the vapour pressure of the liquid.

TABLE 4. CALCULATION OF FINAL DRYING RATES BASED ON THE ASSUMPTION THAT VAPOUR DIFFUSION IS THE LIMITING FACTOR

Liquid	Temperature at base of bed °C	Concentration of saturated vapour at this temperature g/cm³ × 10⁴	Measured bed voidage	Diffusivity at mean temperature cm²/sec	Calculated drying rate g/cm² hr			
					1 cm	2 cm	3 cm	4 cm
Water ..	41·5	0·463	0·58	0·273	0·022	0·012	0·0083	0·0064
Ethanol ..	38	2·890	0·63	0·144	0·079	0·043	0·030	0·023
Isopropanol ..	38	3·090	0·63	0·108	0·063	0·035	0·024	0·018
Chloroform ..	34	17·50	0·61	0·126	0·40	0·22	0·15	0·115

The bed voidage was determinable as a by-product of the drying; since the beds did not shrink or crack, the change in weight during the experiment was due to removal of liquid from the voids, and this was measured. The diffusivity is taken at the mean of the bed base and air stream temperatures; the diffusion length is bed depth + boundary layer thickness, and the area for diffusion is bed area × voidage.

The calculated drying rates follow the same pattern as the experimental drying rates, but are approximately 40% larger. This is because the effect of voidage in powder beds is greater than a simple proportionality would indicate, due to the smallness of the passages and to their tortuosity, both of which tend to make the effective diffusivity smaller than the literature values, determined in a large air space.

The final drying rates calculated by means of the Bruggeman (1935) equation ($D_{eff} = D\epsilon^{3/2}$) are shown in Table 5.

TABLE 5. CALCULATED FINAL DRYING RATES ASSUMING A PERMEABILITY OF (VOIDAGE)$^{3/2}$, G/CM^2/HR

Liquid	Bed depth cm			
	1	2	3	4
Water	0·016	0·0094	0·0065	0·0048
Ethanol	0 057	0·033	0·024	0·018
Isopropanol ..	0 049	0·030	0·019	0·0145
Chloroform ..	0·30	0·19	0·114	0·091

Comparison of Table 5 with Table 3 shows that good agreement between extrapolated and the calculated final drying rates is obtained for most of the runs.

The mean voidage of the beds was increased from 0·59 to 0·77 by granulation of the magnesium carbonate. The calculated final drying rates at the increased voidage are again in good agreement with observed values (Fig. 7) where the lines for each bed depth have been drawn to intercept the axis at the calculated final drying rates.

(c) CRITICAL LIQUID CONTENT

The critical liquid content can be obtained from Figs 2–5 with some precision, due to the plot of log (drying rate) versus liquid content giving straight lines during both the constant and falling rate periods. The values are given in Table 6.

TABLE 6. CRITICAL LIQUID CONTENTS, G/G DRY BED

Liquid	Bed depth cm			
	1	2	3	4
Water	0·298	0·308	0·355	0·38
Ethanol	0·435	0·50	0·535	0·57
Isopropanol ..	0·49	0·524	0·576	0·64
Chloroform ..	0·90	0·98	1·033	1·10

Let a be the thickness of the boundary layer above the bed, which is of depth L and cross-sectional area A, and let the concentration of the saturated vapour be P and its diffusivity in air be D. If the bed has permability λ, so that the effective diffusivity of the bed is λD, then the drying rate by vapour diffusion will be equal to the concentration gradient multiplied by the diffusivity. During the falling rate period the concentration gradient operates across the boundary layer plus the depth of dry bed above the plane of evaporation which is, say, at a depth x below the bed surface.

For the boundary layer, diffusivity $= D$

For the dry bed, effective diffusivity $= \lambda D$

$$\therefore \text{Drying rate} = \frac{P}{\left[\frac{a}{D} + \frac{x}{\lambda D}\right]} = \frac{\lambda DP}{a\lambda + x} \quad .. \quad .. \quad .. \quad (1)$$

During the constant rate period, $x = 0$

$$\therefore \text{constant drying rate} = \frac{DP}{a} \text{ g/cm}^2\text{/hr} \quad .. \quad .. \quad .. \quad (2)$$

If evaporation is from the base of the bed, $x = L$

$$\therefore \text{final drying rate} = \frac{\lambda DP}{a\lambda + L} \text{ g/cm}^2\text{/hr} \quad .. \quad .. \quad .. \quad (3)$$

These are the formulae used for the derivation of the boundary layer thickness in Table 2 and the final drying rates in Table 5. The permeability is a function of the bed voidage, channel tortuosity, particle shape

and probably other parameters in addition. We find good agreement with experimental measurements using $\lambda = \epsilon^{3/2}$ which may be compared with the Kozeny permeability $\lambda = \dfrac{\epsilon^3}{(1-\epsilon)^2}$. The two equations are substantially identical for values of ϵ close to 0·5.

The linear relationship between log (drying rate) and moisture content, shown in Figs 2–5, can be expressed as

$$\frac{\log_{10}\left(\frac{dW}{d\theta}\right) - \log_{10}\left(\frac{dW}{d\theta}\right)_f}{\log_{10}\left(\frac{dW}{d\theta}\right)_c - \log_{10}\left(\frac{dW}{d\theta}\right)_f} = \frac{W}{W_c},$$

where $\left(\frac{dW}{d\theta}\right)$ is the drying rate, $\left(\frac{dW}{d\theta}\right)_c$ is the constant drying rate, and $\left(\frac{dW}{d\theta}\right)_f$ is the final drying rate. W is the liquid content and W_c is the critical liquid content.

By rearranging and using natural logarithms,

$$ln\frac{\frac{dW}{d\theta}}{\left(\frac{dW}{d\theta}\right)_f} = \frac{W}{W_c}\, ln\frac{\left(\frac{dW}{d\theta}\right)_c}{\left(\frac{dW}{d\theta}\right)_f} \quad .. \quad .. \quad .. \quad .. \quad (4)$$

$$\text{i.e. } \frac{dW}{d\theta} = \left(\frac{dW}{d\theta}\right)_f e^{\frac{W}{W_c}\, ln\left[\frac{\left(\frac{dW}{d\theta}\right)_c}{\left(\frac{dW}{d\theta}\right)_f}\right]} \quad .. \quad .. \quad (5)$$

Substituting (1), (2) and (3) into (4) gives

$$ln\left[\frac{\frac{\lambda DP}{a\lambda + x}}{\frac{\lambda DP}{a\lambda + L}}\right] = \frac{W}{W_c}\, ln\left[\frac{\frac{DP}{a}}{\frac{\lambda DP}{a\lambda + L}}\right]$$

$$\text{or } ln\left[\frac{a\lambda + L}{a\lambda + x}\right] = \frac{W}{W_c}\, ln\left[\frac{a\lambda + L}{a\lambda}\right]$$

$$\therefore \frac{-dW}{dx} = \frac{W_c}{(a\lambda + x)\, ln\left(\frac{a\lambda + L}{a\lambda}\right)} \quad .. \quad .. \quad .. \quad (6)$$

where dW is the weight lost when the drying surface recedes by a depth dx to $x + dx$.

When evaporation is taking place at a distance of dx from the base of the bed, i.e. when the last increment of liquid is evaporating, the concentration of liquid at the base of the bed will be $\frac{dw}{A\,dx}$. Let this final concentration be C. Then, substituting into equation (6)

$$C = \frac{W_c}{A(a\lambda + L)\,ln\left(\frac{a\lambda + L}{a\lambda}\right)} \quad .. \quad .. \quad .. \quad .. \quad (7)$$

Values of C for each run have been calculated from the critical liquid contents given in Table 6 and are shown in Table 7.

TABLE 7. VALUES OF C OBTAINED FOR EACH RUN, G/CM³

Liquid	Bed depth cm			
	1	2	3	4
Water	0·071	0·068	0·072	0·069
Ethanol	0·097	0·102	0·102	0·102
Isopropanol ..	0·112	0·115	0·108	0·109
Chloroform ..	0·199	0·208	0·187	0·185

These results indicate that C is independent of bed depth; this is to be expected as the liquid concentration C is in equilibrium with the saturated vapour concentration P. However, the exact relationship between C and P will depend on bed properties, and as all bed properties have been held constant in this work, values of C cannot be predicted for other drying situations.

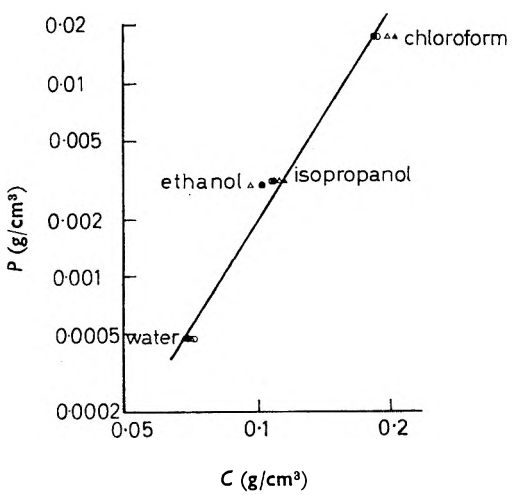

FIG. 8. Correlation of values of C for each liquid at each bed depth. See Fig. 2 for key.

Fig. 8 shows C plotted against P on a logarithmic scale and indicates that the relation between these two quantities is

$$C = 0{\cdot}55\ P^{0.27}$$

By integration it is possible to obtain a relation between time and moisture content during the falling rate period.

Combining equation (7) with (2), (3) and (4) gives

$$ln\frac{dW}{d\theta} = \frac{W}{CA(a\lambda + L)} + ln\left(\frac{\lambda DP}{a\lambda + L}\right)$$

$$\text{i.e. } \frac{dW}{d\theta} = \frac{\lambda DP}{a\lambda + L}\ e^{\frac{W}{CA(a\lambda + L)}}$$

Separating the variables

$$-\int_{W_c}^{W} e^{\frac{-W}{CA(a\lambda + L)}}\, dW = \left(\frac{\lambda DP}{a\lambda + L}\right)\int_{o}^{\theta} d\theta$$

remembering that dW is negative.

$$\text{Thus } CA(a\lambda + L)\left[e^{-\frac{W}{CA(a\lambda + L)}} - e^{-\frac{Wc}{CA(a\lambda + L)}}\right] = \frac{\lambda DP\theta}{a\lambda + L},$$

and since $W_c = CA(a\lambda + L)\ ln\left(\frac{a\lambda + L}{a\lambda}\right)$, we have

$$\theta = \frac{CA(a\lambda + L)^2\, e^{-\frac{W}{CA(a\lambda + L)}} - CAa\lambda(a\lambda + L)}{\lambda DP}$$

Putting $W = 0$ in this equation gives a value for θ_T, the total drying time:

$$\theta_T = \frac{CAL(a\lambda + L)}{\lambda DP}$$

Since $\left(\frac{DP\lambda}{a\lambda + L}\right)$ is the final drying rate and AL equals the bed volume V, we may write

$$\theta_T = \frac{CV}{\text{Final drying rate}}$$

If the initial liquid content is W_1, the overall drying time starting from any liquid content will be

$$\vartheta_{\text{overall}} = \frac{W_1 - W_c}{\text{Constant drying rate}} + \frac{CV}{\text{Final drying rate}}$$

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Assessment of homogeneity of powder mixtures

J. A. HERSEY

Currently used mixing indices for correlating the homogeneity of powder mixtures have been examined and applied to different proportions of potassium dichromate dispersed in two different particle size grades of exsiccated sodium sulphate. To overcome discrepancies encountered using existing terms for the description of homogeneity of powder systems, a new term, the "mixing margin", and a new mixing index are proposed. The "mixing margin" is the difference between the theoretical standard deviation of sample concentration allowed by the mixture specification. assuming 95% confidence limits and a normal distribution, and the theoretical standard deviation of sample concentrations of the fully randomized mix at the given sample size. The "mixing margin" can therefore be used to estimate the probability of obtaining a desired degree of homogeneity in practice. The mixing index is the ratio of the standard deviation of sample concentration estimated from a finite group of samples and the theoretical standard deviation of sample concentration allowed by the mixture specification. This index, unlike those based on the randomized mixture, gives a rapid indication of attainment of the desired degree of homogeneity.

POWDER mixing is a frequent precursor to many processes in industry. In the British pharmaceutical industry, it has been estimated (Ashton & Valentin, 1966) that some 10,000 tons of powder are mixed annually at a cost, based on mixing plant alone, of £50,000. Recent developments in this field have been reviewed by Wiedenbaum (1958) and Valentin (1965).

Problems of powder mixing not infrequently derive from an inability to describe the homogeneity of the powder mixture also referred to as the "degree of mixedness". Without a proper measure of homogeneity of the powder mixture, the correlation of mixer performance or the assessment of design features of industrial mixers becomes intuitive.

Proposals for a "mixing index", as a value to describe the "degree of mixedness", have been made. These stem from the work of Lacey (1943), which provided the necessary stimulus for the study of mixing theory. Of the indices proposed, those based on the standard deviation of a set of normally distributed values of the proportion of one ingredient in a binary mixture are the most favoured. Lacey's early work established the limiting values for the standard deviation of two unmixed powders and two randomly mixed powders.

If σ_0 is the standard deviation of an unmixed system, then

$$\sigma_0 = \sqrt{xy}$$

where x and y are the proportions of minor and major constituents in the binary mixture.

If σ_R is the standard deviation of the completely randomized system, then

$$\sigma_R = \sqrt{\frac{xy}{N}}$$

where N is the number of particles in the sample taken.

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This latter equation is simple to use for uniformly sized particles, and the treatment of Stange (1954), as extended by Poole, Taylor & Wall (1964) enables it to be used for all particle size distributions.

Indices of mixing are usually dimensionless ratios of the standard deviation, σ, calculated from a number of samples withdrawn from the mixture, and the theoretically derived standard deviation for the random mixture. The simple ratio σ_R/σ was proposed by Lacey (1943), but its reciprocal σ/σ_R has found more frequent use (Poole & others, 1964).

Use has sometimes been made of the standard deviation of the unmixed system σ_0, and Lacey (1954) proposed the following index based on variance:

$$M = \frac{\sigma_0^2 - \sigma^2}{\sigma_0^2 - \sigma_R^2}$$

As mixing proceeds, this index rapidly tends to unity making differentiation between mixtures difficult and to overcome this problem, Ashton & Valentin (1966) suggested an index of the form

$$M^2 = \frac{\log \sigma_0^2 - \log \sigma^2}{\log \sigma_0^2 - \log \sigma_R^2}$$

Use has been made of both Poole's simple ratio and the more complex ratio of Ashton & Valentin to describe the experimental results in this paper.

Experimental

The properties of powders that might be expected to influence their mixing characteristics include density, particle size and distribution, particle shape and surface characteristics. To eliminate density effects, potassium dichromate (sp.gr. 2·68) and exsiccated sodium sulphate (sp.gr. 2·70) were chosen (Mallick, 1964).

Particle size was controlled by sieving on an Alpine Air-Jet Sieve. For the potassium dichromate, that fraction passing a 152 μ mesh sieve was

TABLE 1. PARTICLE SIZE ANALYSIS AND CALCULATION OF NUMBER OF PARTICLES IN UNIT WEIGHT OF POTASSIUM DICHROMATE FRACTION

Particle size μ	No. of particles	Weight fraction undersize	Range fraction (f)	Particle weight μg	Mean weight in range (w) μg	fw
170	93	1		6·942		
			0·3468		4·692	1·627
120	342	0·6532		2·442		
			0·4484		1·655	0·742
85	377	0·2048		0·868		
			0·1757		0·587	0·103
60	162	0·0291		0·305		
			0·0266		0·207	0·005
42·5	41	0·0025		0·108		
			0·0024		0·073	—
30	7	0·0001		0·038		
			0·0001		0·026	—
21	5	0		0·013		
			0		0·009	—
15	3	0		0·005		

$\Sigma fw = 2{\cdot}477$ μg.
$\therefore$ No. of particles/g $= 4{\cdot}04 \times 10^6$.

used and this was further subjected to microscopical size analysis to ascertain the size range. Results of this analysis were used to calculate the number of particles in unit weight by Poole's (1964) method (see Table 1).

Narrow sieve fractions (32–64 and 65–104 μ) of exsiccated sodium sulphate were used. Microscopically, these appeared as loose aggregates of very much smaller particles. Since it was difficult to estimate how much dispersion would take place in preparing a sample for microscopic analysis and in view of the narrowness of the sieve fractions, the arithmetic mean sizes, i.e. 48 and 84 μ respectively, were used to calculate the numbers of particles in unit weight ($6{\cdot}4 \times 10^6$ and $1{\cdot}2 \times 10^6$ particles/g, respectively).

The mixing operation used was a modification of that of Mallick (1964). 500 g of the powders were mixed in a Kenwood planetary mixer fitted with a K-beater operating at 60 rev/min. Because of regions of dead space, the contents were emptied at 5 min intervals and replaced in the bowl. At selected time intervals, the contents were carefully transferred to a cubical container sealed with a grid in which 100 sampling points were available. An open-ended glass tube sampling thief was inserted through randomly selected sample points and between 10 and 20 samples taken of each sample weight. The sample weight (50–500 mg) was obtained by variation in diameter of the thief.

The samples were weighed and dissolved in 0·1N sulphuric acid. Serial dilutions were made to estimate the potassium dichromate content, at a measured extinction between 0·2 and 0·6 at 275 mμ using 1 cm cells and 0·7 mm slit width, with a Hilger Uvispek. The validity of this assay in the presence of relatively large quantities of sodium sulphate had previously been shown by Mallick (1964). Pure potassium dichromate sampled through the same processes as the mixture samples, gave an assay reproducibility of $\pm 1{\cdot}5\%$ for ten determinations at 95% confidence limits.

Results

Throughout this work, the results obtained by analysis of samples have been assumed to be normal about the mean. A χ^2 test has been suggested by Wiedenbaum (1958) to test this hypothesis but the difficulty of describing other distributions precludes them from being used for the analysis of the systems investigated.

The rate of mixing of 10% potassium dichromate in the 48 μ fraction of exsiccated sodium sulphate is shown in Fig. 1 where the mixing index used is that of Poole & others (1964). The graph shows the beginning of the expected exponential relationship observed in rate of mixing studies. Although mixing was still continuing after a 40 min time interval, the degree of homogeneity attained was judged sufficient to enable examination of other parameters.

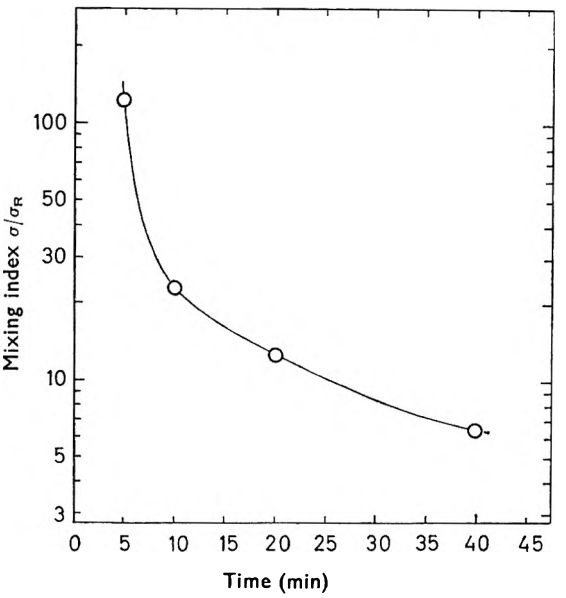

FIG. 1. Rate of mixing of 10% potassium dichromate in exsiccated sodium sulphate (32 to 64 μ).

One of these was sample size. Train (1960) has considered the relation between sample size, particle size and the required standards for a mixture and he warned of the danger of taking a sample size larger than the quantity which was to be used by a subsequent consumer, i.e. the "scale of scrutiny". Poole & others (1964) have investigated this relation and showed that the coefficient of variation obtained from samples decreased with increase in particle number according to the equation,

$$\log C = m \log N + \text{constant}$$

where C is the coefficient of variation between the samples and m has the value of $-0{\cdot}5$ for a *completely* randomized mixture and between $-0{\cdot}3$ and $-0{\cdot}5$ for *practical* mixes. This relation has since been treated theoretically by Bourne (1965). However, in common with Ashton & Valentin (1966), I found the present results could not be treated with confidence using this method. With the narrow range of sample weights taken (50–500 mg) any reduction in variance could be masked by estimating the variance from only a few samples (10 in this case) where the actual variance lies between 69 and 183% of the measured value (Davies, 1957).

The two parameters considered in Fig. 2 are (i) the effect of the concentration of the minor ingredient and (ii) the effect of changing the particle size of the major ingredient on the mixing index σ/σ_R, after a 40 min mixing operation. This index reduces to unity when a completely randomized state has been attained.

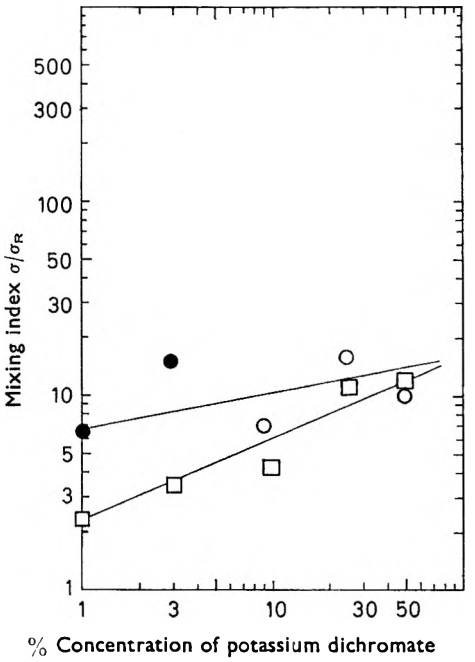

FIG. 2. Effect of concentration and particle size on the ratio of measured standard deviation to standard deviation of the random mix (sample size 100 to 500 mg). ●, Exsiccated sodium sulphate, mean size 48 μ. Results of Mallick (1964). ○, Exsiccated sodium sulphate, mean size 48 μ. □, Exsiccated sodium sulphate, mean size 84 μ.

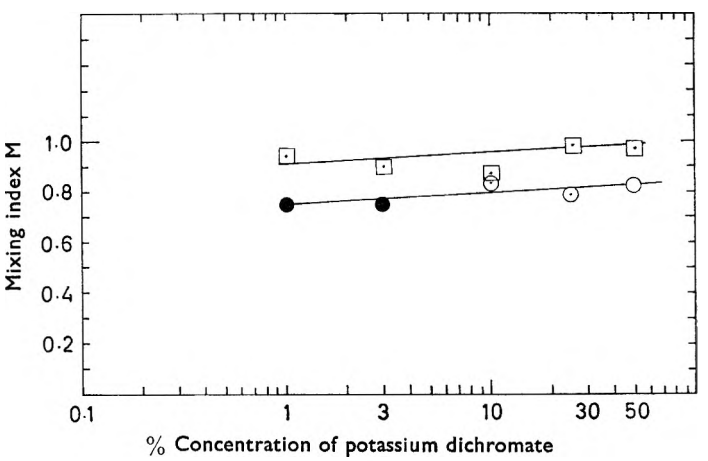

FIG. 3. Effect of concentration and particle size on the mixing index M (sample size 100 to 500 mg). $M^2 = \frac{\log \sigma_o^2 - \log \sigma^2}{\log \sigma_o^2 - \log \sigma_R^2}$

M = 1 is the condition for a randomized mixture. ●, Exsiccated sodium sulphate, mean size 48 μ. Results of Mallick (1964). ○, Exsiccated sodium sulphate, mean size 48 μ. □, Exsiccated sodium sulphate, mean size 84 μ.

The degree of scatter shown in the results must be expected from the number of samples examined but in common with the results of Poole & others (1964) and of Ashton & Valentin (1966), two tendencies are apparent. The smaller the concentration of the minor ingredient and the more coarse the particle size, the more closely the mixture approaches the randomized state.

The second mixing index $\frac{\log \sigma_0^2 - \log \sigma^2}{\log \sigma_0^2 - \log \sigma_R^2}$ is used in Fig. 3 to describe the same mixtures as in Fig. 2. This index varies between zero for the unmixed system and unity for the randomized mixture. Examination of this index confirms that the coarser the particles used, the more closely does the system approach the randomized state. However, in contrast to σ/σ_R, the index appears to suggest that the higher the proportions of the minor ingredient (up to equal proportions) the more closely the system tends to the randomized state.

Discussion

The two indices used to describe the results of this work appear to agree that coarse particles are more likely to result in a randomized mixture than fine particles. By incorporating a term for the unmixed condition, the index suggested by Ashton & Valentin largely eliminates the effect of proportion of the two ingredients. Nevertheless the two indices appear to show opposing tendencies in relation to the effect of concentration in the results shown.

To distribute a small quantity of a potent material equally throughout a large mass of diluent, recommended dispensary practice is to mix equal proportions, subsequently adding the diluent in equal quantities to the mass already mixed. Clearly, this traditional pharmacy practice favours mixing in equal proportions.

On the question of particle size, before mixing, all crystals are finely ground in a pestle and mortar at the pharmacy bench. Yet the mixing indices proposed both suggest that the coarse powder will give a more randomized mixture.

TABLE 2. THE EFFECT OF CHANGE IN MIXTURE PROPORTIONS AND PARTICLE SIZE ON THE PROPERTIES OF RANDOM MIXTURES OF EXSICCATED SODIUM SULPHATE AND POTASSIUM DICHROMATE

Size of exsiccated sodium sulphate μ	Proportion of minor ingredients	Standard deviation σ_R $\times 10^{-4}$	Coefficient of variation % $\times 10^{-4}$	Range* $\pm 1{\cdot}96\sigma_R$ $\times 10^{-4}$	Range as % about mean
84	0·01	1·30	130	± 2·5	±2·50
84	0·03	2·88	92	± 5·6	±1·87
84	0·10	3·77	37·7	± 7·4	±0·74
84	0·25	5·79	23·2	±11·3	±0·45
84	0·50	9·14	22·8	±17·9	±0·34
48	0·01	0·57	57	±1·12	±1·12
48	0·03	1·03	34·3	±2·02	±0·67
48	0·10	2·30	23·0	±4·51	±0·45
48	0·25	2·60	6·5	±5·10	±0·20
48	0·50	4·21	8·4	±8·25	±0·17

* 95% confidence limits for normal distribution.

These considerations must lead to an examination of the properties of a random mixture (see Table 2). The figures were computed using a nominal sample size of 500 mg and the proportions and properties of the powders used in the practical work.

Table 2 shows that, for a given mixture of constant particle size, the limits about the mean are very much higher for the 1% mixture and decrease up to the 50% value. Similarly, for the coarser mixture, the limits are considerably higher for a given proportion of minor ingredient.

If these limits are controlled, say to within $\pm 1\%$ or 5%, assuming 95% confidence limits for a normal distribution, it is possible to calculate the theoretical standard deviation, σ_q, in these circumstances (see Table 3), where $1{\cdot}96\sigma_q = \pm 1\%$ or 5% of the mean.

TABLE 3. CALCULATION OF THEORETICAL STANDARD DEVIATION FOR 1% AND 5% LIMITS OF MEAN CONTENT

Proportion of minor ingredient	Range $\pm 1\%$ $\times 10^{-4}$	σ_q* 1% level $\times 10^{-4}$	Range $\pm 5\%$ $\times 10^{-4}$	σ_q* 5% level $\times 10^{-4}$
0·01	1	0·51	5	2·55
0·03	3	1·50	15	7·65
0·10	10	5·10	50	25·5
0·25	25	12·76	125	63·8
0·50	50	25·51	250	127·6

* Confidence limits of 95% for normal distribution.

The value of σ_q at either level gives the standard deviation of a mixture complying with the required limits about the mean. Thus the value of $(\sigma_q - \sigma_R)$ will give a measure of the "mixing margin", i.e. the difference between the required standard deviation and that theoretically attainable. If this value is negative it follows that mixing to this level cannot be achieved.

TABLE 4. VALUES OF "MIXING MARGIN" FOR MIXTURES OF POTASSIUM DICHROMATE AND SODIUM SULPHATE (50 MG NOMINAL SAMPLE WT.) $\pm 1\%$ AND $\pm 5\%$ ABOUT MEAN

Size of exsiccated sodium sulphate μ	Proportion of minor ingredient	$(\sigma_q - \sigma_R) \times 10^{-4}$ $\pm 1\%$ level	$(\sigma_q - \sigma_R) \times 10^{-4}$ $\pm 5\%$ level
84	0·01	−0·79	+1·25
84	0·03	−1·38	+4·77
84	0·10	+1·33	+21·73
84	0·25	+6·97	+58·04
84	0·50	+16·37	+118·46
48	0·01	0	+1·98
48	0·03	+4·70	+6·62
48	0·10	+2·80	+23·20
48	0·25	+10·16	+61·2
48	0·50	+21·30	+123·4

Table 4 shows the values calculated for the "mixing margin" for the systems under examination. On theoretical grounds, it is impossible to mix 1% and 3% potassium dichromate with the coarser exsiccated sodium sulphate to a range about the mean of $\pm 1\%$ at the particle sizes and sample size used. With all the other mixes, it *is* theoretically possible to reach the required limits. However, since the random condition may not be attained

using practical mixing apparatus, it is desirable to have as high a value as possible for the "mixing margin". The latitude of mixing as illustrated by the "mixing margin" increases with an increase in concentration of the minor ingredient and with reduction in particle size.

This treatment also enables the definition of a new mixing index of the form σ/σ_q, the limits of which will be from σ_0/σ_q through unity, when the desired specification has been attained, to σ_u/σ_q. The practical results

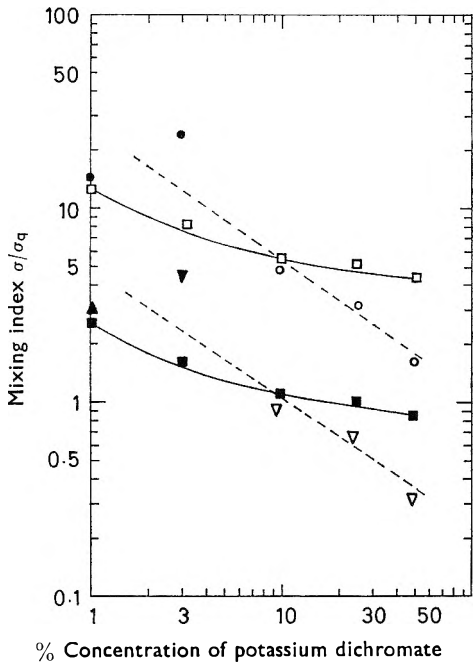

FIG. 4. Effect of concentration and particle size on the ratio of measured standard deviation to the standard deviation complying with specified limits. $\sigma/\sigma_q = 1$ condition for the mixture attaining specified limits. Conditions for $\pm 1\%$ about mean: ●, Exsiccated sodium sulphate 48 μ, Mallick (1964). ○, Exsiccated sodium sulphate 48 μ. □, Exsiccated sodium sulphate 84 μ. Conditions for $\pm 5\%$ about mean: ▼, Exsiccated sodium sulphate 48 μ, Mallick (1964). ▽, Exsiccated sodium sulphate 48 μ. ■, Exsiccated sodium sulphate 84 μ.

treated in this way are shown in Fig. 4. With the coarse sodium sulphate, only mixtures containing 25% and 50% have attained the desired mixing condition of $\pm 5\%$ of the mean after the 40 min mixing. With the finer grade of sodium sulphate, the 10% mixture of potassium dichromate has also attained this desired standard.

It is also possible to suggest other treatments of the practical data along the lines shown in this work. For example a higher degree of confidence might be chosen for the range about the mean. In this context a value of 3σ has been suggested by Train (1960). Alternatively, the effect of concentration can be overcome, by using a constant coefficient of variation, in place of a constant percentage range about the mean.

This latter treatment might find considerable usefulness in the evaluation of mixer performance for mixtures of differing proportions.

The mixing indices in common use are based on a comparison of the measured standard deviation with the theoretical standard deviation for the randomized mixture. In general they are not suitable for correlating the degree of homogeneity of mixtures containing different proportions of ingredients nor of mixtures containing different particle sized constituents.

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The influence of pH on the bactericidal activity of the isomeric monofluorophenols

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The bactericidal activities of the isomeric monofluorophenols have been determined by the Rideal–Walker test and by viable counting. Both methods show the *m*-compound to be the most active. Solutions in phosphate buffer are more toxic than would be expected if their activity were due solely to the unionized fraction. The increased effect is due, at least in part, to the buffer.

HALOGEN substituents enhance the bactericidal activity of phenol. In general the effect increases with the increase in atomic weight of the halogen (Suter, 1941; Burton, Clarke & Gray, 1964; Sykes, 1965). The fluorophenols have received the least attention of the halogenated phenols. We report some effects of pH on the antibacterial activity of the monofluorophenols.

Experimental

MATERIALS

Bactericides. Phenol, A.R. quality. *o*-, *m*- and *p*-Fluorophenols purified by preparative gas chromatography. An Aerograph Model 700 Autoprep was used with a 7 ft × 3/8 inch diameter aluminium column packed with 25% Apiezon L on Embacel. A column temperature of 145°, and carrier gas (hydrogen) at approximately 150 ml/min were used. *o*- and *m*-Fluorophenols are liquids and were injected onto the column as such; the solid *p*-isomer was injected as a concentrated solution in acetone.

Buffer solutions were prepared with potassium acid phosphate and sodium hydroxide A.R. (Britton, 1955).

Organisms. Salmonella typhi (NCTC786) maintained as described in British Standard 541. *Escherichia coli* (NCTC5933) grown and counted on nutrient agar (pH 7·2) consisting of "Oxoid" peptone 1% and sodium chloride 0·5%, gelled with "Davis" agar 1·5%.

METHODS

Phenol solutions. (i) Non-buffered: stock solutions were diluted as required for the Rideal–Walker coefficient and viable counts respectively. (ii) Buffered: 10 ml of a suitable concentration of fluorophenol in 0·2 M KH_2PO_4 and sufficient 0·2 M NaOH to produce the required pH, were made up to 50 ml with water.

E. coli suspensions were prepared daily from the washed 24 hr surface growth of *E. coli*. The suspensions were diluted with water

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to give about 4×10^9 viable cells per ml (% viability of the initial suspensions was 30–50%).

Bactericidal evaluations. These were by: (i) the Rideal–Walker coefficient (B.S. 541) and (ii) by viable counts. The suspension (0·25 ml) was added by a standard dropping pipette (Cook & Yousef, 1953) to 5 ml of phenol solution at 25° ± 0·1°; samples were counted by the method of Miles & Misra (1938).

All solutions were made with sterile distilled water. The pH was measured with a standardized Pye "Dynacap" pH meter. Addition of the organisms to the buffered solutions produced a pH change of less than 0·05 unit.

Results and discussion

The coefficients of variation for the Rideal–Walker coefficients of the *o*-, *m*- and *p*-fluorophenols (Table 1) were 2·6, 2·4 and 3·5% respectively; the greatest activity was shown by the *m*-compound. Stuart, Ortenzio & Friedl (1958) considered a value of 4·5% to be normal for replicate determinations within the same laboratory. The most active isomer of the chlorophenols was also the *m*-isomer according to the phenol coefficient results of Cooper & Woodhouse (1923), Kuroda (1926), Klarmann, Shternov & von Wowern (1929) and Wolf & Westveer (1952). Burton, Clarke & Gray (1964), using *Pseudomonas aeruginosa*, found the *p*-isomers of all the halogeno-phenols to have the greatest activity.

TABLE 1. THE BACTERICIDAL ACTIVITY OF *o*-, *m*- AND *p*-FLUOROPHENOLS

Compound	Rideal–Walker coefficient	Concentration Exponent for *E. coli*	Calculated % conc. for 99·9% mortality of *E. coli* in 50 min (A)	Ratio: Conc. phenol (A) / Conc. fluorophenol (A)
Phenol	—	6·20	0·83	—
o-Fluorophenol ..	1·31	7·76	0·60	1·38
m-Fluorophenol ..	1·89	8·19	0·47	1·77
p-Fluorophenol ..	1·37	7·76	0·60	1·38

The log survivor-time curves (not shown) were linear for concentrations of 0·6% and above of *o*- and *p*-fluorophenols and 0·45% or more of the *m*-isomer. They became curvilinear with decrease in concentration. A goodness of fit test on 100 χ^2 values calculated from data for all points of the survivor curves, gave a value of $\chi^2 = 11{\cdot}3$ (10 d.f.), $P = 0{\cdot}3$–$0{\cdot}5$. The results therefore formed a Poisson distribution and the counting method was satisfactory (Berry & Michaels, 1947).

The curves relating log time for 99·9% mortality (taken by interpolation from the linear parts of the log survivor-time curves) to log concentration are linear (Fig. 1). From the data the concentrations producing 99·9% mortality in 50 min were calculated and are compared in Table 1. The tabulated ratios, like the Rideal–Walker coefficients,

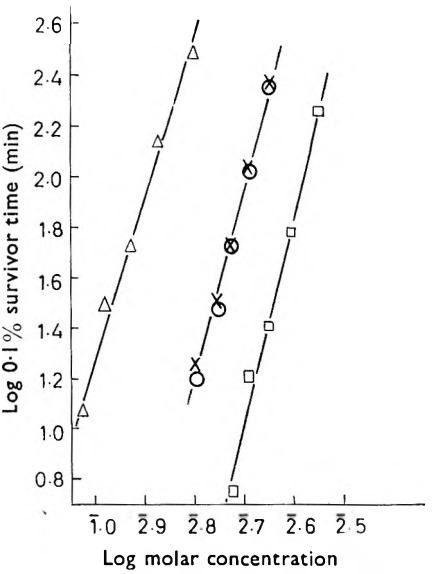

FIG. 1. Concentration exponent plots for phenol and monofluorophenols against *E. coli*. △ = Phenol. Fluorophenol: *o*- = ○; *m*- = □; *p*- = ×.

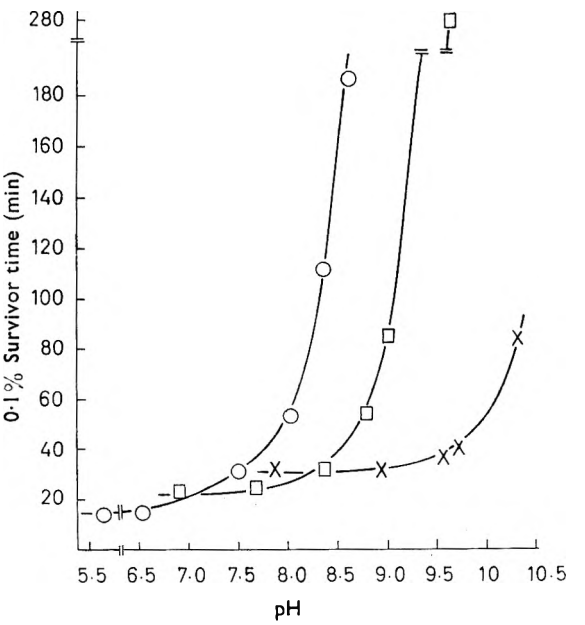

FIG. 2. Relationship between pH and the 0·1% survivor time for *E. coli* in solutions of monofluorophenols. Fluorophenol: 0·7% *o*- = ○; 0·5% *m*- = □; 0·65% *p*- = ×. Buffer = 0·04M KH_2PO_4/NaOH.

show the *m*-isomer to be the most active. The calculated slopes of the regressions in Fig. 1 give the values in Table 1 for the concentration exponents.

The effect of pH upon the disinfectant activity of phenols is related to the degree of molecular dissociation (Hoffman, Schweitzer & Dalby, 1941; Ordal, 1941; Albert, 1965). The relative activity of such compounds may therefore be obtained by comparison of solutions having the same degree of dissociation and not the same pH. The pK_a values of phenol, *o*-, *m*- and *p*-fluorophenol are 10·00, 8·70, 9·21 and 9·91 respectively (Biggs & Robinson, 1961). To compare solutions containing 100–30% unionized molecules, a pH of 6–10·5 is therefore necessary. Such solutions were prepared with the KH_2PO_4/NaOH buffer of Britton & Wellford (Britton, 1955), thus eliminating any variation in bactericidal activity which might have resulted from the use of more than one buffer.

0·1% survivor times, taken from the log survivor-time curves for constant concentrations of the fluorophenols at varying pH, were plotted against the pH of the solutions (Fig. 2). The curves show a marked decrease in activity with rise in pH, similar to the results of Winsley & Walters (1965).

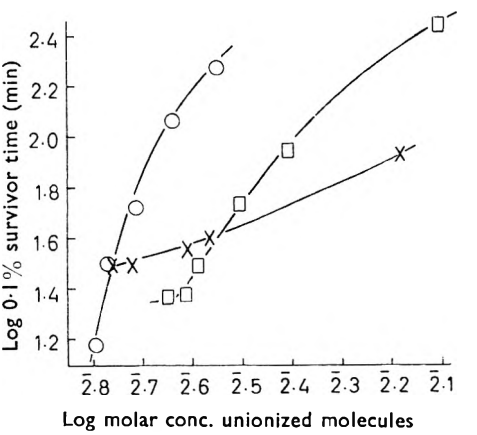

FIG. 3. Relationship between log 0·1% survivor time for *E. coli* in buffered solutions of monofluorophenols and the log molar concentration of unionized molecules. Symbols and buffer as in Fig. 2.

If the activities of the fluorophenols reside solely in their undissociated fraction, the regressions of log 0·1% survivor times on log concentration of unionized molecules should coincide with those for the corresponding concentrations in unbuffered aqueous solutions, where the ionization is less than 0·1%. A comparison of Fig. 3 with Fig. 1 shows that this does not happen. The deviation is greatest with the *p*-compound. A decrease in the % of unionized *p*-fluorophenol molecules from 99·9 to 65% produced no significant change in the 0·1% survivor time (Fig. 4): an analysis of variance and an F test on these results indicate that at

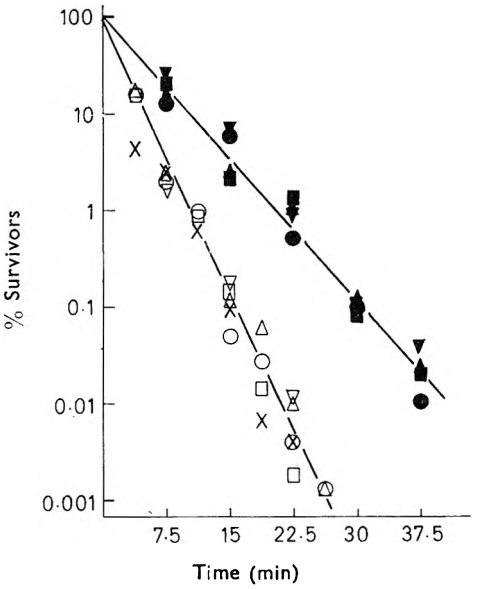

FIG. 4. Log survivor-time curves for *E. coli* exposed to 0·7% *p*-fluorophenol (pH 7·72–9·64) and 0·8% phenol (pH 8·04–9·58). *p*-Fluorophenol: × = pH 7·72 (99·9); □ = 8·88 (91·5); ○ = 9·12 (86·0); △ = 9·54 (70·1); ▽ = 9·64 (65·0). Phenol: ■ = pH 8·04 (98·9); ● = 8·69 (95·3); ▲ = 9·26 (84·6); ▼ = 9·58 (72·4). Figs. in brackets are percentages of unionized phenols.

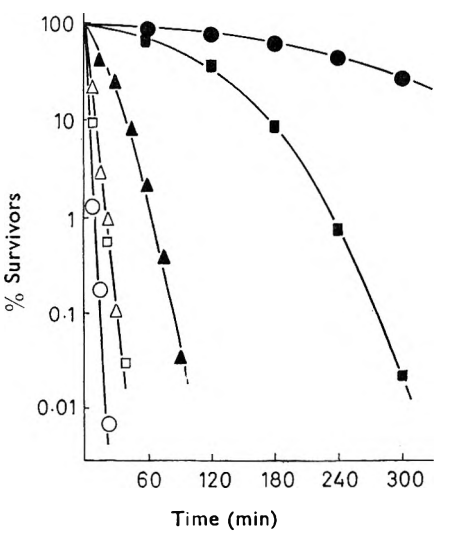

FIG. 5. Log survivor-time curves for *E. coli* exposed to monofluorophenols with buffer to produce about 70% ionization, and without buffer. Fluorophenols in water: 0·7% *o*- = ○; 0·5% *m*- = □; 0·65% *p*- = △. In 0·04M buffer: 0·7% *o*-, pH 9·14 (73·4) = ●; 0·5% *m*-, 9·62 (71·5) = ■; 0·65% *p*-, 10·30 (71·5) = ▲. Figs in brackets are percentages of ionized phenols.

$P = 0{\cdot}05$, the regressions for the five different pH values can be represented by a common line. The ratios of the 0·1% survivor times for the same overall concentrations of fluorophenols in aqueous solutions to those in buffer at about 70% ionization (Fig. 5) are 2·8, 10·7 and $\geqslant$20, for the *p*-, *m*- and *o*-isomers respectively.

The results suggest that the ionized molecules have bactericidal activity, particularly those of *p*-fluorophenol. Phenol itself gave results similar to the latter compound (Fig. 4). To determine whether the buffer had any effect, *E. coli* was exposed to 0·04 M buffer at pH 5·5 and 9·8; no change in viability occurred over a period of 6 hr. However, the activity of the compounds in 0·01 M buffer was much less than that in 0·04 M buffer (Table 2). It may be concluded that the higher bactericidal activity of these ionized solutions is due at least in part to an effect of the buffer.

TABLE 2. THE EFFECT OF CONCENTRATION OF KH_2PO_4/NaOH BUFFER ON THE BACTERICIDAL ACTIVITY OF PHENOL AND MONOFLUOROPHENOLS AGAINST *E. coli*

Compound	% conc. in 0·01 or 0·04M buffer	pH	% Ionization	Equivalent % conc. unionized phenol in water (A)	Ratio: 0·1% survivor time in (A) / 0·1% survivor time in 0·04M buffer	Ratio: 0·1% survivor time in 0·01M buffer / 0·1% survivor time in 0·04M buffer
Phenol	0·8	9·65	31·4	0·55	12·2	2·6
o-Fluorophenol	0·7	8·30	28·5	0·50	1·4	2·0
m-Fluorophenol	0·5	8·60	19·7	0·40	6·4	2·3
p-Fluorophenol	0·7	9·50	28·0	0·50	9·0	3·0

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The persistence of *Pseudomonas aeruginosa* in aqueous solutions of phenols

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Several strains of *Pseudomonas aeruginosa* were killed by a concentration of phenol lower than was needed to kill *Escherichia coli*, but *Ps. aeruginosa* persisted even in saturated solutions of substituted phenols of lower water solubility and in which *E. coli* succumbed. The persistence of *Ps. aeruginosa* in the latter solutions is due in part to a period of cell multiplication following a period of mortality; it is also due to the substituted phenols of low water solubility not being "available" to *Ps. aeruginosa* in sufficiently high concentration to kill this organism.

THE organism *Pseudomonas aeruginosa*, a common contaminant of pharmaceuticals, is capable of metabolizing a wide variety of substrates, including non-ionic surfactants frequently incorporated in pharmaceuticals (Barr & Tice, 1957). It has the ability to resist chemical agents used as disinfectants and preservatives (Lowbury & Collins, 1955; Mackenzie, 1961; Brown, Foster & others, 1964; Hugo & Foster, 1964; Sykes, 1965) and can utilize a variety of phenols as sole sources of carbon (Davey & Turner, 1961; Beveridge & Hugo, 1964; Ribbons, 1966).

We now report observations on the response of *Ps. aeruginosa* to several phenols and relate them to some biophysical properties of the bactericidal systems.

Experimental

MATERIALS AND METHODS

Phenolic bactericides used were phenol (A.R.), *m*-cresol (b.p. 202°), 3,5-xylenol (m.p. 64°), chloroxylenol (m.p. 115·5°) and benzylchlorophenol, i.e. 5-chloro-2-hydroxydiphenylmethane (m.p. 48·5°). With the exception of phenol all were redistilled or recrystallized.

Nutrient media. "Oxoid" (CM.1) nutrient broth granules at a concentration of 1·3% w/v in water, solidified when necessary by incorporating 1·5% w/v New Zealand agar and sterilized at 121° for 20 min.

Organisms. Escherichia coli (NCTC 5933) and *Pseudomonas aeruginosa* (NCTC 7244, 6750, 8203 and a laboratory strain). The organisms were maintained in the freeze-dried state. At monthly intervals a freeze-dried culture was suspended in nutrient broth and incubated at 37°. Subcultures were made daily for 4 days and the growth from the last day used for preparing the stock culture employed for the experiments. The broth culture was centrifuged, the deposited cells washed three times with water and resuspended in water to produce a cell density of approximately $4{\cdot}5 \times 10^{10}$ viable cells per ml, the resulting suspension being stored at 4° until required (Bean & Heman-Ackah, 1964).

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Estimation of bactericidal activity. The reaction mixtures were prepared by adding 5 drops (0·08 ml) of the stored bacterial suspension from a standard dropping pipette to 20 ml of an aqueous solution of the phenol under test, equilibrated at 25°. The reaction mixtures thus contained about $1{\cdot}8 \times 10^8$ viable cells/ml.

Extinction time determinations were made by removing samples at intervals with a loop from thoroughly shaken reaction mixtures, transferring the samples to 10 ml of nutrient broth and incubating at 37° for 48 hr. All experiments were triplicated.

For viable counts, the samples removed from the reaction mixtures were appropriately diluted with quarter-strength Ringer solution; drops of the dilution were delivered from a standard dropping pipette onto the surface of overdried nutrient agar plates (Miles & Misra, 1938) which were incubated at 37° for 48 hr. All experiments were quadruplicated.

Results

The extinction times (Table 1) indicated that all strains of *Ps. aeruginosa* examined were more susceptible to phenol than was *E. coli* (NCTC 5933). This result does not accord with the many statements in the literature about the resistance of *Ps. aeruginosa* to phenols. It does accord, however, with the results of Hess & Speiser (1959) who found *Ps. aeruginosa* less resistant than *E. coli*, *Staphylococcus aureus* or *Streptococcus faecalis* to several phenols, aromatic alcohols, organomercurials and quaternaries. After examining a number of typical freshly isolated strains, Hess and Speiser were convinced that a general high resistance of *Ps. aeruginosa* to disinfectants does not exist.

TABLE 1. EXTINCTION TIMES OF *E. coli* AND *Ps. aeruginosa* IN AQUEOUS PHENOL SOLUTIONS

Organism	Extinction time (min)	
	Phenol 10 mg/ml	Phenol 5·5 mg/ml
E. coli NCTC 5933	35	360
Ps. aeruginosa NCTC 7244	3	—
,, ,, 8203	2	60
,, ,, 6750	—	25
,, laboratory strain	—	60

Since the extinction time evaluations failed to detect a single strain of *Ps. aeruginosa* more resistant than *E. coli* (NCTC 5933), a strain commonly used for testing disinfectants, recourse was made to viable counting methods.

The phenol-resistance of the four strains of *Ps. aeruginosa* was roughly equivalent, so one strain (NCTC 7244) was selected as species representative and was used for a series of viable counts in which a range of concentrations of each of the five phenols was evaluated. Plots of survivors-on-time and log time-on-log concentration were made using a 95% mortality level (LT95) (Withell, 1942; Jordan & Jacobs, 1943;

Bean & Das, 1966). The concentration of each phenol to produce 95% mortality in 10 min (the equitoxic concentration) was read from the log LT 95-on-log concentration regression and is recorded in Table 2 together with the corresponding concentrations for *E. coli* (calculated from Das, 1960).

TABLE 2. CONCENTRATION OF PHENOLS (MG/ML) PRODUCING 95% MORTALITY OF *E. coli* AND *Ps. aeruginosa* IN 10 MIN

Compound	Solubility mg/ml	Concentration producing 95% mortality in 10 min	
		Ps. aeruginosa	*E. coli**
Phenol	85	6·0	8·5
m-Cresol	25	2·8	4·2
Xylenol	4·3	1·1	—
Chloroxylenol	0·37	0·3	0·12
Benzylchlorophenol	0·16	Greater than saturation concentration	0·04

* From Das, 1960.

The equitoxic concentrations for phenol and *m*-cresol were *lower* for *Ps. aeruginosa* than for *E. coli* whereas they were *higher* for chloroxylenol and benzylchlorophenol. Even a saturated solution of benzylchlorophenol did not produce 95% mortality of *Ps. aeruginosa* in 10 min. For saturated

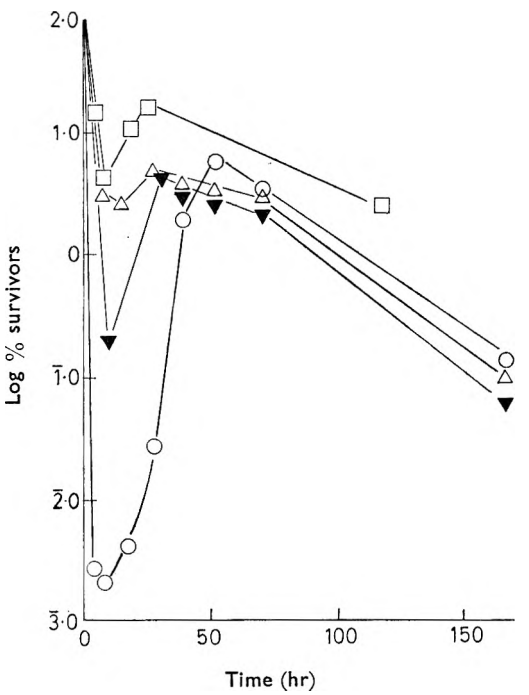

FIG. 1. Viability of *Ps. aeruginosa* in solutions of benzylchlorophenol 0·15 mg/ml (□), or chloroxylenol 0·37 (○), 0·23 (△) and 0·30 (▼) mg/ml.

chloroxylenol solution (0·37 mg/ml) survivors-on-time regression for *Ps. aeruginosa* (Fig. 1) showed an initial lag period, during which there was little mortality, followed by a period of marked bactericidal activity during which the mortality exceeded 99·99% in 10 min. This was succeeded by a period of multiplication (about 40 hr) when a 3000-fold increase in the viable organisms occurred; the count rose to about 4% of the original number, then gradually decreased again over a prolonged time period.

Lower rates of kill were produced by lower concentrations of chloroxylenol and also by a saturated solution of benzylchlorophenol, which required 7 hr to produce 95% mortality (Fig. 1). These lower rates of kill were followed by periods of multiplication: the greater the number of cells killed by the bactericide the greater the number of cells produced during the multiplication phase.

In all instances the peak of the multiplication phase was reached after the reaction had been in progress between 25 and 52 hr and the greater the mortality in the bactericidal phase, the later the peak of multiplication was reached. The ultimate decline in the number of viable cells was slow, extending over days or weeks (cf. Bean & Walters, 1955). The rate of decline was independent of the previous history of the population, suggesting the operation of a common (unidentified) control mechanism in all suspensions.

Discussion

Rapid death followed by almost equally rapid multiplication has been reported previously, both in aqueous solutions of bactericides (Bean & Walters, 1955) and in logarithmically growing cultures to which antibacterials have been added (Brown & Garrett, 1964; Brown & Richards, 1964). Bean & Walters reported a reduction of viable *E. coli* in aqueous solutions of benzylchlorophenol (0·05 mg/ml) from $1{\cdot}2 \times 10^9$ per ml to about $2{\cdot}0 \times 10^4$ per ml (>99·99% mortality) followed by a 500-fold multiplication in the following 250 hr. In the experiments now reported, viable *Ps. aeruginosa* were reduced from $1{\cdot}8 \times 10^8$/ml to $3{\cdot}8 \times 10^3$/ml (>99·99% mortality) and increased 3000-fold during 50 hr. Thus it appears that *Ps. aeruginosa* survivors of a bactericidal reaction can multiply to a greater extent and at a faster rate than those of *E. coli*.

Multiplication in a system initially bactericidal has been observed only in the presence of bactericides of high intrinsic activity and which are therefore employed in low concentration (Bean, 1967). We have now found the extent of the multiplication to be related to the activity of the solution.

When bacteria are added to a phenolic solution they absorb a proportion of the bactericide thereby reducing its concentration in the supernatant. Only a comparatively small proportion of the more water-soluble phenols is absorbed whilst a much larger proportion of the less water-soluble and more active phenols is absorbed (Bean & Das, 1966; Bean, 1967; Judis, 1966). Thus during the bactericidal reaction, solutions of the

most active phenols lose a bigger proportion of their initial activity than do solutions of the less active phenols. The marked loss of activity from solutions of the most active phenols may explain the *persistence* of *Ps. aeruginosa* in these solutions without the acceptance of any concept of *resistance* to the more water-soluble phenols—a resistance which certainly does not exist.

Multiplication of *E. coli* in solutions of phenols has been attributed to the ability of this organism to utilize exudate from dead and dying cells (Bean & Walters, 1961), the extent of multiplication being related to the concentration of exudate. The amount of exudate absorbing at 260 mμ produced by *Ps. aeruginosa* is greater than that produced by *E. coli* and this contributes to the greater rate of multiplication with this organism, the multiplication being aided by the marked reduction in concentration of bactericide in the supernatant.

It is obvious that *Ps. aeruginosa* has a tendency to be susceptible to some phenols and not to others (Table 1). If it is accepted that phenols are non-specific bactericides (Allawala & Riegelman, 1954) and produce death by the disruption of the cytoplasmic membrane, an explanation of the susceptibility of *Ps. aeruginosa* to different phenols is provided in physico-chemical terms by the classical work of Ferguson (1939) without invoking a concept of cellular resistance. He showed that the biological activity of structurally non-specific substances is related linearly to the thermodynamic activity (represented by solubility) of the toxic substances. On this basis, Fig. 2 compares data from the present paper with those of Allawalla & Riegelman (1954) for *Staph. aureus* and of Das (1960) for *E. coli.*

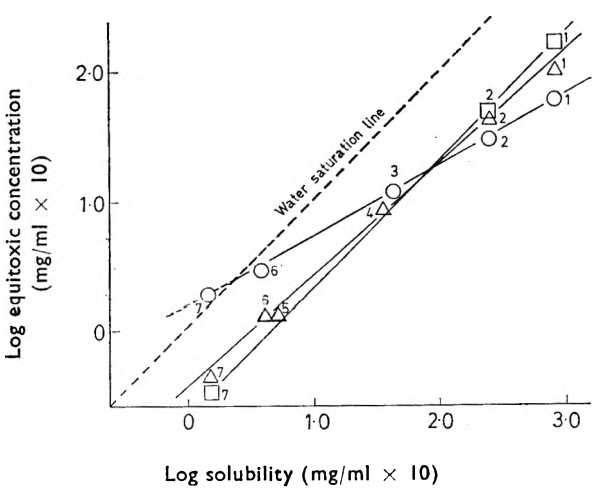

FIG. 2. Relationship between solubility and equitoxic concentration. □, *Staph. aureus* (from Allawala & Riegelman, 1954); △, *E. coli* (from Das, 1960); ○, *Ps. aeruginosa.* Phenols examined: phenol (1), *m*-cresol (2), xylenol (3), chlorocresol (4), hexylresorcinol (5), chloroxylenol (6), benzylchlorophenol (7).

For all three organisms there is a linear relation between thermodynamic activity (represented by solubility) and the equitoxic concentrations of the phenols. The slopes of the regressions are different for each organism and depict the reversal of activity against *E. coli* and *Ps. aeruginosa* for the phenols of high and low water solubility. The regression line for *Ps. aeruginosa* is cut off by the saturation line and extrapolation of the former indicates that the equitoxic concentration of benzylchlorophenol would be in excess of its water-solubility (Table 2) and that of chloroxylenol very close to saturation.

Since the bactericidal-activity of phenols is related to the cell water partition coefficient of the bactericides (Ferguson, 1939; Bean & Das, 1966), and since a large portion of the initial amount of the less water-soluble and more active phenols is absorbed by bacteria, the data presented here suggest that the persistence of *Ps. aeruginosa* in solutions of the less water-soluble phenols is explained by their low water solubility precluding the attainment of a bactericidal concentration within the cell.

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Admixture of oils as a method of controlling the activity of phenol in oil-water dispersions during storage at different temperatures

S. M. HEMAN-ACKAH AND G. H. KONNING*

A mixture of liquid paraffin 79% v/v and arachis oil 21% v/v has been used to form the oil phase of an oil–water dispersion in which the oil–water partition coefficient of phenol is unity over a range of temperature 5–45°. The concentration of phenol maintained in the aqueous phase, and hence the concentration per unit area of the oil–water interface of the dispersion, does not vary with change in the oil–water ratio or temperature, over the range examined. Consequently bactericidal parameters of phenol in the dispersion, such as concentration exponent (n) and temperature coefficient (θ), likewise remain unchanged.

FAILURES in preservation have been encountered in cosmetic and pharmaceutical creams stored under tropical conditions and it has been claimed that these are caused by chance contamination by different organisms "insensitive" to the particular preservatives used (de Navarre, 1962). It has been concluded however from studies on the influence of temperature on bactericidal activity in oil–water dispersions (Bean & Heman-Ackah, 1965), that variations in the temperature coefficient (θ) of preservatives in oil–water dispersions are a more likely cause of such failures. A rise in temperature may cause the preservative to be partitioned more in favour of the oil phase, where it is no longer available to exert its action, and may also reduce the effect of the interface on preservative activity, because with rise in temperature molecules migrate from the interface. Consequently, the product may be attacked by contaminants more readily in tropical than in temperate climates.

Of the total amount of preservative, that proportion remaining in the aqueous phase of a dispersion is determined by the magnitude of the oil–water partition coefficient (K_w^o) which may be temperature dependent. However, the ultimate concentration of the preservative in the aqueous phase, and the total concentration at the oil–water interface, is controlled by the oil–water ratio (Bean, Heman-Ackah & Thomas, 1965b). The combined effect of K_w^o, oil–water ratio and temperature on the activity of a given overall concentration of preservative in a dispersion, led to a hypothesis (Heman-Ackah, 1965) that an antibacterial agent with a value of K_w^o of less than, but near to, unity over a wide range of temprature, would probably be the ideal for preserving oil–water formulated products.

We report a method of shifting the K_w^o of phenol in oil–water systems to unity at various temperatures and an examination of the effect of this on bactericidal activity.

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Experimental

MATERIALS

Oils. (*a*) Liquid paraffin, B.P.C. 1963 (s.g. 0·830–0·970). (*b*) Arachis oil (locally produced by Nzima Oil Mills Ltd. and complying with the requirements of the B.P. 1963, wt/ml = 0·9132).

Phenol. A.R. (B.D.H.) was dried under vacuum for 24 hr before use.

Nutrient media. Nutrient broth, indicator broth, nutrient agar were as described by Bean & Heman-Ackah (1963, 1964).

Organism. Escherichia coli (NCTC 5933) was maintained and cultivated as described by Bean, Richards & Thomas (1962).

DETERMINATION OF K_w^o OF PHENOL BETWEEN A MIXTURE OF OILS AND WATER AT DIFFERENT TEMPERATURES

For an oil–water system in which a preservative has $K_w^o = 1$, three methods were considered: (i) the use of a preservative equally soluble in both the oil and water phases of the system; (ii) alteration of the distribution of a preservative between the phases by additives such as inorganic salts, glycols, etc., to bring the K_w^o to unity; (iii) the admixture, in suitable proportions, of an oil in which a preservative is slightly soluble with another oil in which the preservative is very soluble, to form an oily phase in which the preservative is ultimately soluble to the same extent as in an aqueous phase.

The last method was adopted because it was easy and practicable. Further, an interference with antibacterial action due to specific effects of additives such as salts and glycols in the system (Heman-Ackah, 1965) would not arise. At 25°, the K_w^o for phenol in liquid paraffin–water is 0·069 (Bean & Heman-Ackah, 1964) and in arachis oil–water, 5·5 (Bean & others, 1962). A suitable mixture of the two oils to form the oil phase of a dispersion in which the K_w^o for phenol would be unity was produced thus: Liquid paraffin and arachis oil were allowed to attain 25° in a thermostated bath. Mixtures of the oils consisting of 10, 20, 40, 60 and 80% v/v arachis oil in liquid paraffin respectively were prepared. The K_w^o of phenol for each mixed oil–water system was determined at 5, 15, 25, 35 and 45°, a spectrophotometric method being used to assay the concentration of phenol in the aqueous phase (Bean & Heman-Ackah, 1964).

The interfacial surface tension between oil and water containing appropriate concentrations of phenol was measured with a Du Noüy tensiometer.

For the evaluation of bactericidal activity, an extinction time method was employed (Bean & Heman-Ackah, 1963, 1964).

Results and discussion

Table 1 shows the K_w^o for various phenol–oil–water systems over a range of temperature 5–45°. The magnitude and direction of change of K_w^o with rise in temperature appears to be controlled by the percentage of arachis oil (f) in the oil phase of the oil–water system. The value

TABLE 1. PARTITION COEFFICIENT (K_w^o) OF PHENOL IN OIL–WATER SYSTEMS AT VARIOUS TEMPERATURES

Proportion of arachis oil (f) in mixture of liquid paraffin and arachis oil	K_w^o				
	5°	15°	25°	35°	45°
0	0·057	0·065	0·067	0·123	0·132
10	0·360	0·378	0·430	0·463	0·449
20	0·895	0·968	0·990	0·806	0·813
40	2·045	2·053	1·919	1·717	1·668
60	3·285	3·458	3·309	2·885	2·839
80	4·710	4·603	4·000	3·959	3·707
100	5·782	5·422	5·696	5·098	5·075

of f at which $K_w^o = 1$ was calculated from regressions of K_w^o on f for each of the temperatures 5, 15, 25, 35 and 45° (Table 2). A mean value of f = 21·1% v/v (coefficient of variation 3·71%) was obtained. This was verified experimentally and employed as the composition of the oil phase of the dispersions in which phenol has $K_w^o = 1$ at 5–45°.

TABLE 2. THE PERCENTAGE ARACHIS OIL IN LIQUID PARAFFIN TO GIVE AN OIL–WATER PARTITION COEFFICIENT OF 1·0 FOR PHENOL

Temperature (°C)	(f)
5	22·0
15	21·5
25	20·0
35	21·52
45	20·72
Mean	= 21·1
Standard deviation	= 0·78
Coefficient of variation	= 3·71%

ACTIVITY OF PHENOL IN OIL–WATER DISPERSIONS ($K_w^o = 1$) AT 25°

The oil phase of the dispersion consisted of a mixture of 21 parts arachis oil and 79 parts liquid paraffin. The activity of the dispersions with oil–water ratios 0·2, 1·0 and 10·0 : 1 and aqueous phase phenol concentrations between 0·5 and 1·1% against *E. coli* was determined. The results are shown in Fig. 1.

Now Bean & Heman-Ackah (1964) showed that

$$C = C_w \left(\frac{K_w^o \phi + 1}{\phi + 1} \right)$$

where C = the overall phenol concentration, C_w = concentration of phenol in the aqueous phase, ϕ = oil–water ratio. Because $K_w^o = 1$, $C = C_w$. Hence the overall concentration of the phenol in the dispersions remains the same as that in an aqueous reference solution. Even so, the dispersions are more active than the aqueous reference solution. For instance, when the aqueous phase phenol concentration is 0·5% dispersions of oil–water ratios 0·2, 1·0 and 10·0 : 1 are respectively 1·5, 1·6 and 7·1 times more active than the 0·5% aqueous phenol solution. The enhanced effect on the activity of the dispersions must be due to a relative increase in the total phenol concentration at the oil–water interface as a result of the increase in surface area.

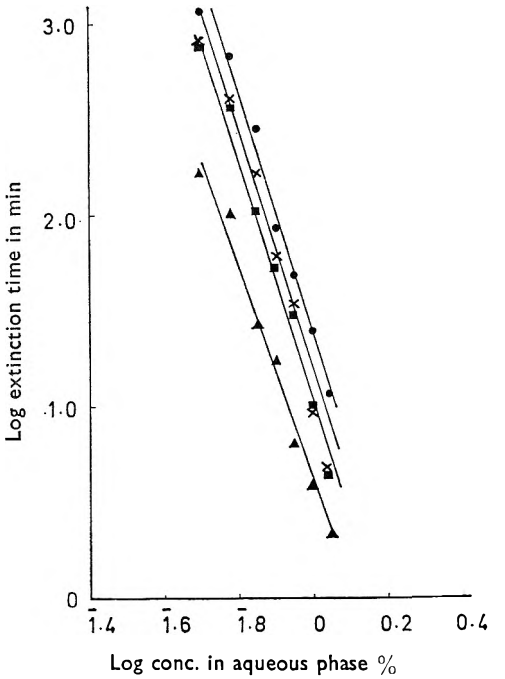

FIG. 1. Activity against *E. coli* of phenol in oil-water dispersions ($K_w^o = 1$) at 25°. ●, Aqueous reference. Oil-water ratios: ■, 0·2; ×, 1·0; ▲, 10·0.

For any given oil–water ratio, an increase in the phenol concentration in the aqueous phase increases bactericidal activity. The regressions of log extinction time on log aqueous phase phenol concentration for different oil–water ratios are linear (P = 0·01–0·001) and parallel over the range of aqueous phase phenol concentrations 0·5 to 1·1%. This contrasts with the observations of Bean & others (1962) who found that the regressions relating log t (extinction time) to log C_w (concentration in aqueous phase) in liquid paraffin–water dispersions $K_w^o < 1$ converged at an aqueous phase phenol concentration of 0·9–1·0%. The observed differences in the activity of phenol in the two types of dispersions, however, appear to follow closely the pattern of physical adsorption of the phenol at the oil–water interfaces as shown by interfacial tension (γ) measurements (Fig. 2). In a phenol–oil–water system ($K_w^o = 1$) there is a slow rate of decrease of γ with increase in aqueous phase phenol concentration over the range 0·5 to 1·1%. On the other hand, a phenol–liquid paraffin–water system ($K_w^o < 1$) shows a rapid decrease of γ with increase in aqueous phase phenol concentration up to 0·9–1·0%, and in excess of this concentration γ becomes constant and independent of the aqueous phase phenol concentration, i.e. the interface becomes saturated with phenol in accordance with the Gibb's equation.

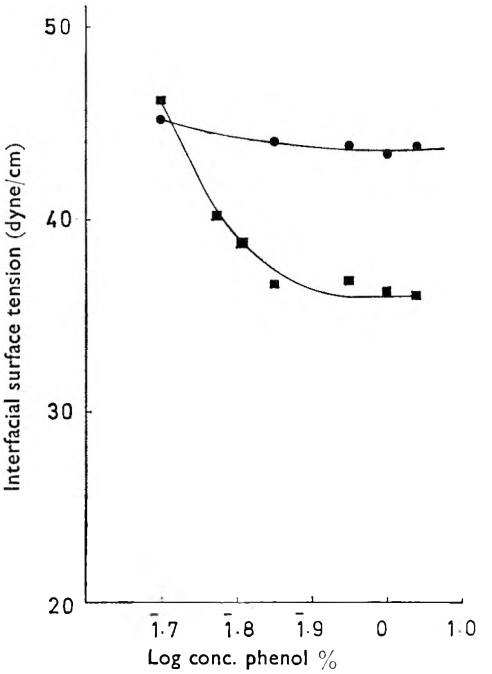

FIG. 2. Relationship between interfacial surface tension and aqueous phase phenol concentration at 25°. ●, Oil-water system ($K_w^o = 1$); ■, liquid paraffin - water system ($K_w^o = < 1$).

Further, γ is certainly higher for the phenol–oil–water system than for phenol–liquid paraffin–water system over the range of aqueous phase phenol concentrations used (0·5–1·1%). The extent of adsorption of phenol at the oil–water interface and its effect on activity of the dispersion must therefore be less in the former than the latter system and this was found to be the case.

THE CONCENTRATION EXPONENT (n) OF PHENOL IN OIL–WATER DISPERSIONS ($K_w^o = 1$) AT 25°

The parameter "n" is characteristic of the bactericide–bacterium reaction (Wills, 1955) and it relates only to the system in which the reaction takes place. This is because the value depends on the availability of the bactericide to microbial cells in the environment.

TABLE 3. THE CONCENTRATION EXPONENT OF PHENOL IN OIL–WATER DISPERSIONS ($K_w^o = 1$) AT 25°

Oil-water dispersion	Concentration exponent (n)
0 (aqueous reference)	6·03
0·2	6·58
1·0	6·52
10·0	5·76

The slope of the regression of log extinction time on log aqueous phase phenol concentration (Fig. 1) is the concentration exponent (n) of phenol in the dispersion. Table 3 shows that "n" for the dispersions is not significantly different from that of an aqueous reference solution. This indicates that the activity of the dispersions is determined largely by the concentration of phenol available in the aqueous phase. It also signifies that there is little interfacial effect to modify the fundamental biological action.

INFLUENCE OF TEMPERATURE ON THE ACTIVITY OF PHENOL AGAINST *E. coli* IN OIL–WATER DISPERSIONS ($K_w^o = 1$)

Extinction times of *E. coli* were determined in 0·5% aqueous solutions of phenol at 5, 15, 25 and 35° and also at each temperature in oil–water dispersions of an overall phenol concentration of 0·5% and oil–water ratios 0·2, 1·0, and 10·0:1. The results are shown in Fig. 3.

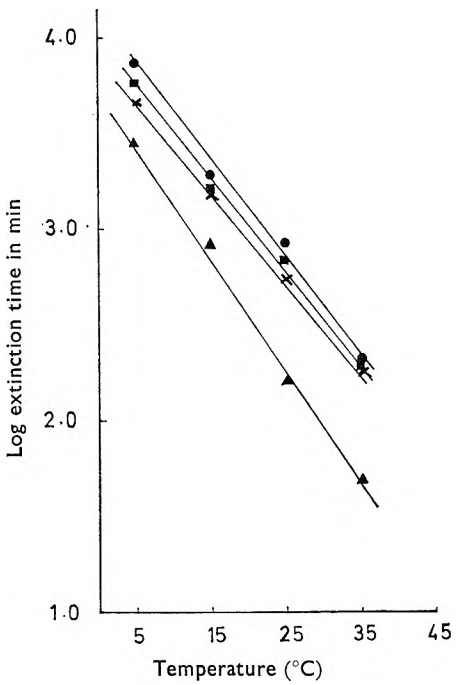

FIG. 3. Effect of temperature on the bactericidal activity of 0·5% overall phenol concentration against *E. coli*. ●, Aqueous reference. Oil - water dispersions ($K_w^o = 1$): ×, 1·0, ■, 0·2; ▲, 10·0.

Since $K_w^o = 1$, over the range of temperatures 5–35°, the aqueous phase phenol concentration of the dispersions is maintained the same independent of changes in oil–water ratio or temperature. Therefore, except for effects due to the oil–water interface, the influence of temperature changes on the activity of the dispersions should be the same as on an aqueous reference solution.

TABLE 4. THE TEMPERATURE COEFFICIENT (θ) OF PHENOL IN OILS-WATER DISPERSIONS ($K_w^c = 1$) AT 5–35°

Oil–water ratio	Temperature coefficient (θ)
0 (aqueous reference)	1·12
0·2	1·11
1·0	1·11
10·0	1·15
—	Mean = 1·12

The regressions of log extinction time on temperature for the aqueous reference solution and for the dispersions (Fig. 3) are linear ($P = 0{\cdot}01$–$0{\cdot}001$). Table 4 shows that the temperature coefficient (θ) of phenol in the dispersions, as derived from the regression coefficient of log extinction time on temperature, does not change significantly with the oil–water ratio and is of the same order as in aqueous solution. So, as the temperature is raised, the activity of phenol in the dispersions is increased to the same extent as in aqueous solution. The dispersions are, however, more active than an aqueous reference solution at each temperature. The relative increase in activity is dependent on the oil–water ratio. It is, however, practically of the same order for each of the temperatures 5, 15, 25 and 35° in a dispersion of specified oil–water ratio (Table 5). The inference is that the enhanced effect of the oil–water interface on activity is relatively uninfluenced by temperature changes.

TABLE 5. RELATIVE ACTIVITY OF DISPERSIONS HAVING AN OVERALL CONCENTRATION OF PHENOL OF 0·5% COMPARED WITH A 0·5% AQUEOUS SOLUTION OF PHENOL

Temperature (°C)	$\frac{\text{Extinction time in aqueous reference}}{\text{Extinction time in dispersions}}$ Oil : water ratio		
	0·2	1·0	10·0
5	1·3	1·6	2·5
15	1·2	1·3	2·3
25	1·2	1·6	5·0
35	1·0	1·0	4·3
Mean change in activity at 5–35°	1·2	1·3	3·7

CONCLUSIONS

Thus the fundamental bactericidal activity of phenol is not altered in oil–water dispersions when K_w^o is unity. Consequently, parameters of phenol such as concentration exponent (n) and temperature coefficient (θ) remain the same in the dispersions as in aqueous solutions. The enhanced effect of the oil–water interface on activity is small and is not temperature dependent. Changes in parameters of phenol in oil–water dispersions as previously reported by Bean & others (1962, 1965a, 1965b), Bean & Heman-Ackah (1963, 1964, 1965) may therefore be apparent and not real. Such changes occur because the K_w^o of phenol in the dispersions is remote from unity and is temperature dependent. Under these conditions the availability of the phenol in the dispersions is controlled by oil–water

ratio and temperature. Also, the effect of the interface on activity may be so marked as to suggest a modification of the fundamental biological action in the dispersions.

To conclude, it is possible to approach the formulation of oil–water dispersions in such a manner as to maintain the preservative equally distributed between oil and water phases over a wide range of temperature. If such a formulation has physical properties which minimize the tendency for adsorption of preservative at the oil–water interface, then the efficiency of the preservative in dispersions under different storage conditions may be predicted from its performance in aqueous solutions during laboratory screening tests.

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The bactericidal activity of phenol in a solid-liquid dispersion

H. S. BEAN AND G. DEMPSEY

The bactericidal activity of phenol in aqueous suspensions of carbon is dependent on the residual aqueous phenol concentration after the adsorption equilibrium between phenol and carbon has been established allowance having been made for the effect on activity of a leached extract from the carbon. In contrast to oil-water systems, the interface between the two phases has no direct effect on the bactericidal reaction.

THE activity of preservatives in liquid-liquid systems has been investigated by several workers and among the factors known to control the activity are the liquid-liquid partition coefficient, concentration of preservative in the aqueous phase and the phase volume ratio (Atkins, 1950; Bennett, 1962; Bean, Richards & Thomas, 1962). There is little documented information on the activity of preservatives in solid-liquid systems. However, Chick & Martin (1908) observed that the activity of tar acid dispersions was reduced in the presence of particulate organic matter, while Batuyios & Brecht (1957) noted a marked depression in the activity of some quaternary ammonium compounds in aqueous suspensions of talc and kaolin.

It is also known that interfaces are often the site of biological activity and that the activity at an interface may be different from that in bulk solution. Phenol tends to be adsorbed at air-water interfaces (Goard & Rideal, 1925) and exhibits greater disinfectant activity in small droplets than in bulk solution since organisms can more readily reach the interface in the droplets (Valentine, 1957). The bactericidal activity of oil-water dispersions containing phenols or organomercurials is greater than can be accounted for by the bactericide in the aqueous phase and is dependent on the phase volume ratio. The enhanced activity has been ascribed to the adsorption of both bacteria and bactericidal molecules at the oil-water interface (Bean & others, 1962; Bean & Heman-Ackah, 1964).

The present communication seeks to evaluate the influence on activity of suspended solid particles and of the solid-liquid interface in a suspension. To produce the maximum degree of adsorption (and possibly the greatest influence on activity), an adsorbent of high specific surface was sought. Activated carbon was chosen since it satisfied the adsorptive requirements without necessitating very concentrated suspensions.

Experimental

MATERIALS AND METHODS

Activated carbon. Ultrasorb ZX (British Carbo Norit Union Ltd.) was washed several times with distilled water and dried at 105° for two days. The specific surface (1091 m^2/g) was determined by nitrogen adsorption using a Perkin-Elmer Sorptometer. *Phenol* (A.R. quality). *Nutrient broth.*

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Oxoid granules (CM1) at a concentration of 1·3% w/v. *Indicator broth.* Nutrient broth containing 1% w/v lactose and 0·0016% w/v bromocresol purple as indicator. *Nutrient agar.* Nutrient broth solidified with 2%w/v Kobe agar (Oxoid).

Organism. Escherichia coli (NCTC 5933, IMViC^{++--} 44$^{\circ+}$) cultivated and maintained as described by Bean & others (1962). A viable count was made on each new stock suspension using the surface viable method of Miles & Misra (1938); from this suspension suitable dilutions were made for use in extinction time determinations.

Adsorption isotherm of phenol on Ultrasorb ZX at 25°. 10 ml of aqueous phenol solution of appropriate concentration was added to each of a duplicated series of weighed quantities of carbon in 25 ml ground-glass stoppered conical flasks. The flasks were placed in an oscillating water-bath at 25° for 24 hr. The suspensions were then centrifuged, aliquots removed from the supernatant and analysed by measuring the extinction at 270 mμ.

Reaction mixture. The bactericidal activity was determined for phenol in dispersions of carbon in water. The concentration range of phenol employed was from 0·6 to 1·5% w/v using 25 to 400 mg of carbon per 20 ml (i.e. 0·125 to 2·0% w/v carbon).

The adsorbent was equilibrated overnight at 25° with the solution of bactericide; the adsorption equilibrium established, the inoculum of *E. coli* was then added to give a final viable population of 20×10^6 organism/ml.

Determination of extinction times. Extinction time determinations (five replicate experiments) were made at 25° in glass-stoppered tubes. The reaction mixtures were shaken and sampled at intervals of approximately one seventh of the expected extinction times (Cook & Wills, 1954). Samples of 1 ml were transferred to 24 ml of nutrient broth thereby reducing the phenol concentration to a maximum of 0·06% w/v, and the mixture was shaken vigorously; 1 ml was then transferred from the broth dilution to 24 ml of indicator nutrient broth and incubated at 37° for two days. The second dilution further reduced the phenol concentration to a maximum of 0·0024% w/v (well below the minimum inhibitory concentration). The indicator broth, which changed colour from purple to yellow on growth of *E. coli*, facilitated detection of bacterial growth and ensured that turbidity arising from suspended particles was not interpreted as bacterial growth.

Results

The adsorption isotherm for phenol on Ultrasorb ZX at 25° is shown in Fig. 1 and the relationship between overall concentration of phenol and final equilibrium concentration, after adsorption by varying amounts of carbon, is shown in Fig. 2. The effect of dilution showed that the adsorption was reversible and therefore physical in nature.

The application to the adsorption data of the BET equation (Brunauer, Emmett & Teller, 1938) for determining the pattern of adsorption,

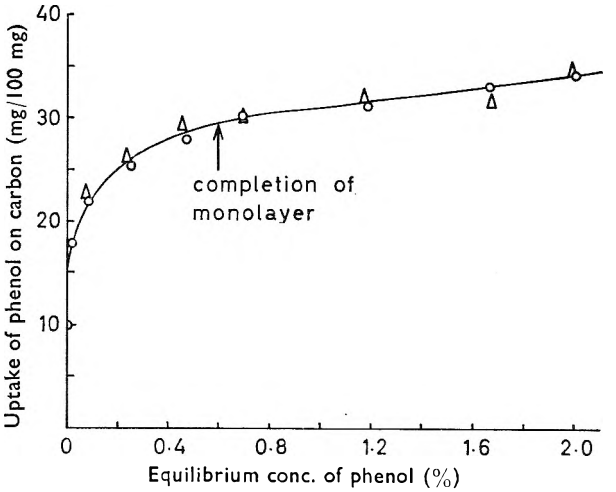

FIG. 1. Adsorption isotherm for phenol on Ultrasorb ZX at 25°.

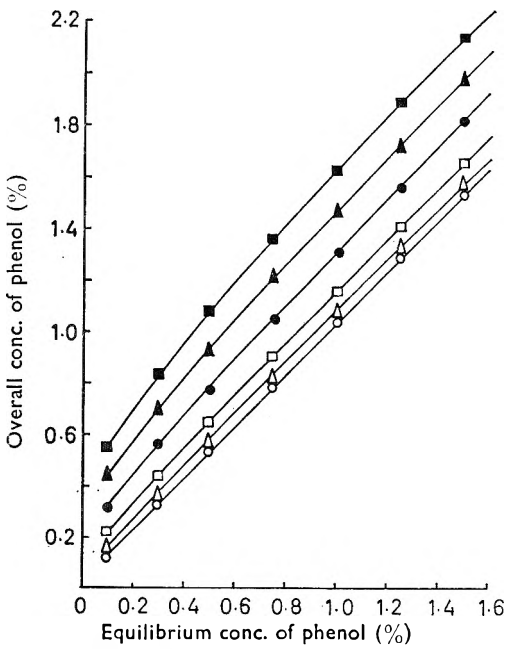

FIG. 2. Relationship between equilibrium concentration and overall concentration of phenol for its adsorption on Ultrasorb ZX at 25°. Carbon concentrations %: ○, 0·125; △, 0·25; □, 0·50; ●, 1·0; ▲, 1·50; ■, 2·0.

suggested a monolayer capacity of 0·295 g phenol/g carbon. Thus a monolayer would be complete at an equilibrium concentration of 0·6% w/v phenol (Fig. 1) which would necessitate an initial concentration of 0·63% w/v for a carbon concentration of 0·125% w/v and of 1·19% w/v for a carbon concentration of 2·0% w/v (Fig. 2).

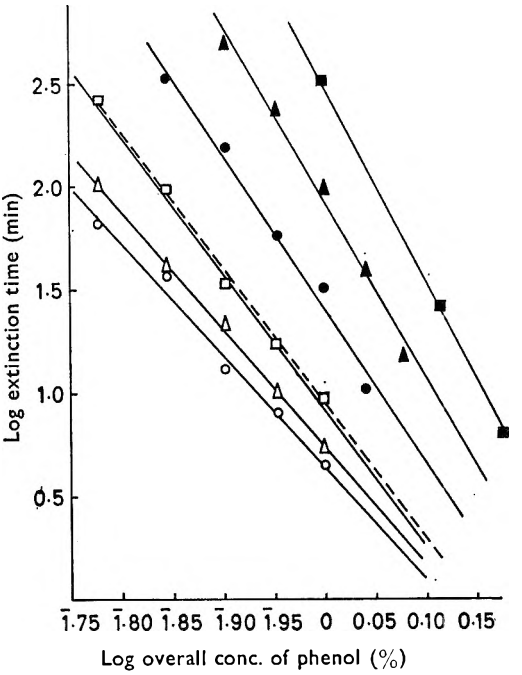

FIG. 3. Extinction times of *E. coli* in aqueous solutions of phenol containing suspended carbon. Carbon concentrations as in Fig. 2. - - -, aqueous phenol solutions not brought into contact with carbon.

The bactericidal activities of the carbon-phenol systems are represented in Fig. 3. For any overall phenol concentration the biological activity was related to the amount of carbon present. Suspensions containing less than 0·5% w/v carbon were more active than the corresponding aqueous solution and those containing more than 0·5% w/v carbon less active; the decrease in activity was approximately thirty-fold at an overall phenol concentration of 1·0% w/v and a carbon concentration of 2% w/v. Supernatant liquors removed from contact with carbon by centrifugation possessed a much higher activity than would have been expected for the concentration of phenol left in solution (Table 1). The activities of the

TABLE 1. THE OBSERVED AND EXPECTED ACTIVITIES OF SUPERNATANT PHENOL SOLUTIONS

Adsorbent adsorbate system	Supernatant conc. (%)	Extinction time (min)	
		Observed	Expected*
50 mg/0·95%	0·862	8	22
100 mg/0·90%	0·746	20	59
300 mg/1·14%	0·700	25	89
50 mg/0·72%	0·640	40	168
100 mg/0·77%	0·616	47	207
300 mg/1·02%	0·590	70	280

* Expected extinction times represent the activities of aqueous phenol solutions equivalent in concentration to those in the supernatant.

supernatants were in fact the same as those of the suspensions from which they had been removed.

Logarithmic plots of equilibrium concentration against extinction times produced a common regression line irrespective of the amount of carbon present (Fig. 4). Coincident with this line was the regression for supernatant phenol concentrations on extinction time.

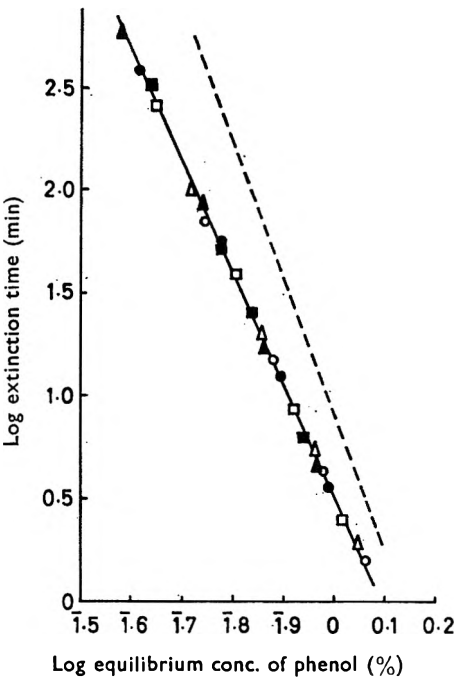

FIG. 4. Extinction times of *E. coli* with suspensions of carbon in aqueous phenol solutions. Carbon concentrations as in Figs 2 and 3.

Discussion

After phenol solutions had been in contact with Ultrasorb ZX, the supernatant liquors exhibited enhanced antibacterial activity, presumably due to the ability of phenol to leach from the carbon materials which are not extracted by water. The extent of the increased activity was independent of the amount of carbon, within the limits of the experiment. Thus the true aqueous references were represented by the activities of these supernatants removed from contact with the carbon and not by phenol solutions which had not been in contact with carbon. The close agreement between the true aqueous reference line and the common equilibrium concentration-activity regression of Fig. 4 indicated that the activities of the suspensions were totally determined by the equilibrium concentrations of phenol remaining after adsorption and that the carbon-water interface *per se* in no way influenced the activity of the system.

The overall effect of adding carbon to a phenol solution was to decrease bactericidal activity due to adsorption of the solute from the aqueous phase: it was thus similar to the effect of adding animal charcoal to tar-acid dispersions (Chick, 1908) or adsorbents such as talc and kaolin to solutions of quaternary ammonium compounds (Batuyios & Brecht, 1957).

If the solid-liquid interface had been playing a direct part in the bactericidal reaction and produced enhanced activity due to the close proximity of the bacterial cells to the adsorbed layer of phenol, then for any specified equilibrium concentration (i.e. fixed degree of saturation of the solid surface), the activity would have been expected to increase with the surface area of the carbon and therefore with the weight of carbon added to a phenol solution.

The suspension system chosen represented an extreme case since the very high specific surface, the major part of which was internal surface and therefore not "available" to the bacteria, would not normally be found in suspensions of medicinal importance requiring preservation. For example, the specific surface for a specimen sample of hydrocortisone acetate has been given as 4·4 m^2/g (Chapman & Neustadter, 1965) and the surface area of the crystalline insulin zinc suspension of the B.P. is probably of the order of 0·4 m^2/g assuming cubic crystals with an edge of 10 μ. In both these cases the degree of adsorption of phenol onto aqueous suspensions would be negligible, assuming a similar pattern of adsorption to that on carbon. In consequence, the activity of the suspension systems would be very similar to that of an aqueous phenol solution of the same total concentration as the corresponding suspension.

It is concluded that solid particles suspended in a solution of bactericide have a much smaller influence on the bactericidal activity of the system than do suspended oil droplets, provided the solid itself does not slowly pass into solution and thereby endow the supernatant with enhanced activity.

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The effect of pH on the bactericidal activity of crystal violet and its sorption by various bacterial species

E. ADAMS

The sorption (uptake) of crystal violet from aqueous buffer solutions of pH 5·5–8·5 by *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Bacillus subtilis* increased as the amount of dye present was increased. The absorption isotherms were of the L type. The bactericidal action of crystal violet against *E. coli* and *Str. faecalis* increased with rise in pH from 7·0–8·5, but with *Staph. aureus* and *B. subtilis* change in pH over this range had no effect on the activity of the dye. At a given initial concentration of dye the effect of pH over the range 5·5–8·5 on sorption of dye was negligible for all species. At a given equilibrium concentration, *Staph. aureus* showed an increase in sorption with rise in pH; the other organisms showed a fall.

IN 1924 Stearn & Stearn showed that *E. coli* sorbed small amounts of gentian violet (an impure form of crystal violet) at pH 2. As the pH was raised the sorption increased, but at pH 7 the authors described it as only "moderate." *Staph. aureus*, however, was found to sorb moderate amounts at pH 2, and sorption increased appreciably as the pH was raised to 7·7.

I have examined the effect of pH on the bactericidal activity of crystal violet and its sorption by *E. coli*, *Staph. aureus*, *Str. faecalis* and *B. subtilis*.

Experimental

MATERIALS

Crystal Violet B.P. was re-crystallized from ethanol (E 1%, 1 cm, at 591 mμ = 2059). The purity was verified by thin-layer chromatography using several solvent systems.

Buffer solutions to cover the pH range 5·5–8·5 were prepared from 1% Analar potassium dihydrogen phosphate and potassium hydroxide. Nutrient broth was prepared from granules (Oxoid CM1) and adjusted to the same pH range as the buffer solutions.

Test organisms. Cultures were prepared from freeze-dried specimens of *Escherichia coli* 1 (NCTC 8196), *Staphylococcus aureus* (NCTC 7447), *Streptococcus faecalis* (NCTC 370), and *Bacillus subtilis* (NCTC 3610).

ESTIMATION OF SORPTION OF CRYSTAL VIOLET BY BACTERIA

Preliminary experiments showed a negligible difference between sorption of the dye by resting cells and sorption by the same amount of freeze-dried bacteria resuspended in buffer solution over the pH range 5·5–8·5. The latter were therefore used for convenience. Organisms were grown on nutrient agar, washed in quarter-strength Ringer solution, then with sterile water, and finally freeze-dried.

2 ml of a solution of crystal violet (1–40 $\times$ 10^{-5}M) in buffer (pH 5·5–8·5) was added to a weighed amount of the organism, mixed and equilibrated

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at 37°. Preliminary experiments showed that 5 min allowed complete sorption to occur; 15 min was allowed in subsequent experiments. The mixture was centrifuged at 2300 *g* (10 min) and the extinction of the supernatant liquid determined at 591 mμ. The supernatant was shaken with portions of isobutanol to extract the dye and the extinction of the supernatant at 591 mμ re-determined. Negligible extinction indicated that no material absorbing at 591 mμ was released from the bacteria by treatment with the dye. This was repeated using different concentrations of dye. The absence of material absorbing at 591 mμ was confirmed after a suspension of each bacterial species (untreated by dye) was subjected to disintegration by ultrasonic vibration for 30 min from an MSE Ultrasonic Power Unit (60 W, 20 Kcycles/sec at maximum power).

EVALUATION OF BACTERICIDAL ACTIVITY

A suspension of the organism was prepared in quarter-strength Ringer from a 24 hr growth on an agar slope (about 14 hr for *B. subtilis*.) This suspension was then filtered through sterile Whatman No. 1 filter paper to remove clumps of bacteria and small pieces of agar. The total count was adjusted to about $6{\cdot}0 \times 10^7$ organisms/ml, and measured amounts (0·1 ml) were transferred by calibrated needles to tubes of broth (previously warmed to 37°) of known pH value and known concentration (2–20 $\times 10^{-6}$M) of crystal violet. Samples were then removed at intervals from 15 min to 8 hr to measured volumes of quarter-strength Ringer solution at 37°, cooled to 20°, and viable counts made by the drop-plate method; ten drops were applied to each overdried plate. The survivors were counted and the mortalities % converted to probits and plotted against log time of exposure to dye.

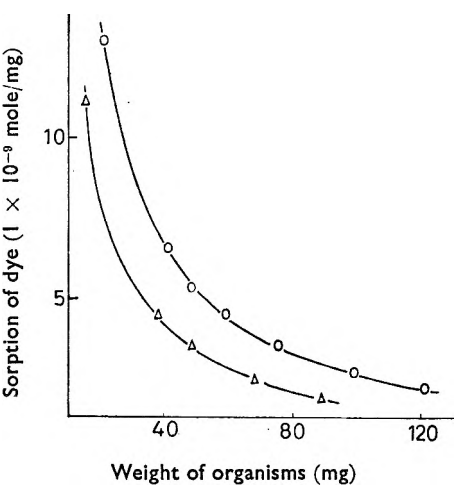

FIG. 1. Effect of weight of *B. subtilis*, and concentration of dye, on sorption of crystal violet. ○, 150×10^{-6}M dye; △, 100×10^{-6}M dye.

Results

EFFECT OF WEIGHT OF ORGANISMS ON DYE SORPTION

Different weights of bacteria were treated with a constant amount of dye and the sorption of dye measured. As would be expected when different amounts of bacteria and dye were used a series of parallel curves was obtained (Fig. 1). A high proportion of dye was sorbed by each organism from all concentrations of dye used, viz., *Staph. aureus* 97–99%, *E. coli* 92–96%, *Str. faecalis* 90–95%, and *B. subtilis* 83–90%. A fourfold change in the volume of the dye solution caused little change in the amount of dye sorbed.

EFFECT OF pH ON DYE SORPTION

The sorption of dye by the bacteria can be represented by absorption isotherms of the L type (Giles, MacEwan & others, 1960). At *equilibrium* concentration of crystal violet for any system for *Staph. aureus* there was an increase in sorption with rise in pH of the buffer solution, while the other species showed a fall in sorption. Fig. 2 shows the graph for *Staph. aureus* and *E. coli*. For a given *initial* concentration of dye, change of pH caused no change in dye sorption.

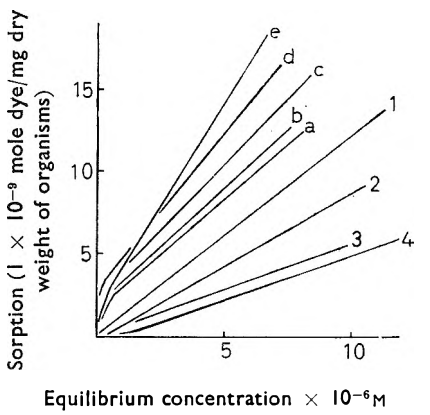

FIG. 2. Effect of pH value on sorption of dye at various equilibrium concentrations of crystal violet. *Staph. aureus*: a, pH 5·5; b, pH 6·0; c, pH 6·9; d, pH 7·5; e, pH 8·0. *E. coli*: 1, pH 6·0; 2, pH 7·5; 3, pH 8·0; 4, pH 8·5.

VIABLE COUNTS

The technique of viable counts was examined by counting the colonies from a diluted suspension of each organism in quarter-strength Ringer solution in ten drop areas on a nutrient agar plate. Tests were carried out on untreated organisms and on the survivors after exposure to dye. Statistical analysis showed satisfactory reproducibility with each species.

COMPARATIVE BACTERICIDAL ACTIVITY OF CRYSTAL VIOLET AT DIFFERENT pH VALUES

Probit mortality-log time graphs (not shown) were rectilinear or slightly sigmoid, and a comparison of the bactericidal activity of the dye at

different pH values was made by plotting the values of log time required to produce 50, 90, and 99% mortalities against pH values. There was a fall in log time with rise in pH from 7·0–8·5 for *E. coli* and *Str. faecalis*, indicating an increase in bactericidal activity from pH 7·0–8·5 (Figs 3 and 4). For *Str. faecalis* there was also a dip in the curve at pH 6·3

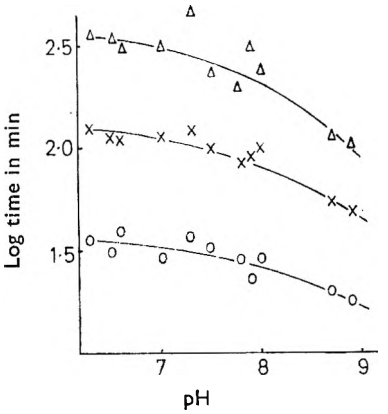

FIG. 3. Effect of pH on values of log time required to produce given % mortalities of *E. coli*. △, 99%; ×, 90%; ○, 50%.

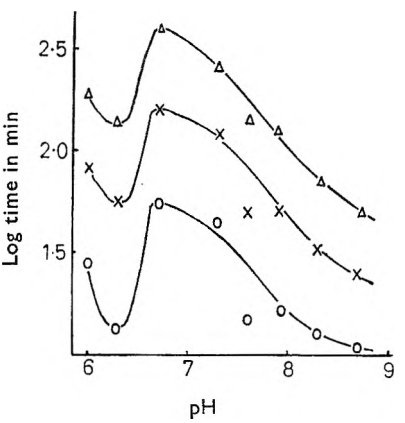

FIG. 4. Effect of pH on values of log time required to produce given % mortalities of *Str. faecalis*. △, 99%; ×, 90 %; ○, 50%.

indicating an increase in bactericidal activity which fell away on either side of this value. The effect of pH on the bactericidal activity of crystal violet against *Staph. aureus* and *B. subtilis* was not significant over the pH range 5·5–8·5.

Discussion

Normal (or Langmuir) absorption isotherms were obtained for crystal violet with each species at all pH values examined. These L-type isotherms are usually shown by molecules which are adsorbed flat on the surface (Giles & others, 1960); crystal violet is a near-planar molecule.

It has previously been stated that dye sorption is proportional to bacterial mass (Stearn, 1933): it has also been stated that when the ratio of dye concentration to bacterial mass is constant, the uptake of dye is constant (Gale & Mitchell, 1947). Results (Fig. 1) show that the latter, but not the former, is true. The upper curve (150×10^{-6} M) shows sorption to be 1·5 times that of the lower curve (100×10^{-6} M), i.e. when more dye is present, more is sorbed. This suggests that the dye is partitioned between the external aqueous phase and the bacteria.

Bartholomew & Finkelstein (1954) attempted to distinguish between dye retention and total dye sorption. In the present work efforts to remove the dye by replacing the dye solution by fresh buffer of the same pH were only partly successful; 1–4% was removed from *E. coli* and *Staph. aureus*, up to 14% from *Str. faecalis*, and up to 20% from *B. subtilis*. If buffer of a different pH were used, more dye might be desorbed; this occurred with *B. subtilis* when the eluting buffer was at a higher pH than that used initially.

Borzani & Vairo (1959) have claimed that the sorption of crystal violet by Gram-positive organisms, but not by Gram-negative organisms, followed Langmuir's adsorption law. *Staph. aureus*, alone of the organisms I examined, obeyed this law (Fig. 5), but the values of $Y = PC\ C_f/C_i - C_f$ (where C_i = initial dye concentration, C_f = dye concentration at equilibrium point, C = cell concentration, P = % dead cells) were approximately constant for the other species. Barbaro & Kennedy (1954) stated that Gram-negative bacteria sorbed only traces of crystal violet but Gram-positive organisms an appreciable amount. Each species used by me showed considerable sorption. *E. coli* (Gram-negative)

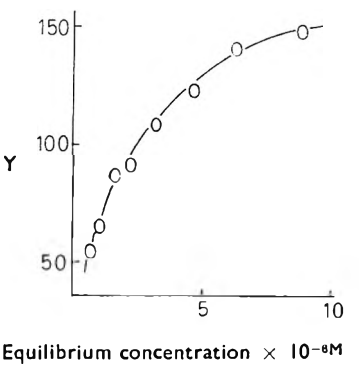

FIG. 5. Uptake of crystal violet by *Staph. aureus* showing compliance with Langmuir's absorption law. $Y = PC\ C_f/C_i - C_f$, where C_i = initial dye concentration, C_f = equilibrium dye concentration, P = % dead cells, C = wt of organisms in mg.

sorbed less dye than the other three species (Gram-positive) at a given *initial* dye concentration (Fig. 6), although Bartholomew & Finkelstein (1954) found no correlation between crystal violet sorption and the Gram characteristic of the organism.

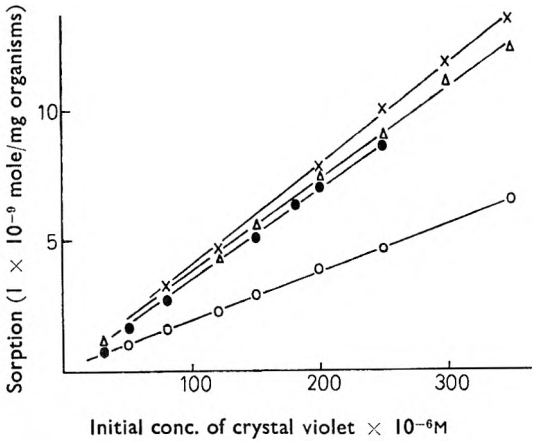

FIG. 6. Sorption of crystal violet 1×10^{-9} mole/mg organism against initia concentration of dye $\times 10^{-6}$M at pH 9·2. ×, *Staph. aureus*; △, *Str. faecalis*; ●, *B. subtilis*; ○, *E. coli*.

Kennedy & Woodhour (1956) found that *Staph. aureus* sorbed more crystal violet than did *E. coli* at pH 7·5, but less at pH 5·2. I found that, over the pH range used, *Staph. aureus* sorbed more dye than *E. coli* for a given *equilibrium* concentration (Fig. 2), and also for a given *initial* concentration of dye (Fig. 6).

Change in pH over the range 5·5–8·5 had only a small effect on the absorption isotherms of the species used, an observation which may be due to the presence of potassium ions in the buffer used. Indeed, Finkelstein & Bartholomew (1954) found that an increase in hydrogen ion concentration or the presence of metallic ions reduced the dye uptake, and suggested that salt cations had more effect on dye sorption than small pH changes.

Surprisingly, no correlation was evident between the effect of pH on bactericidal activity of crystal violet, and the effect of pH on sorption of the dye. The increased antibacterial effect of crystal violet as the pH value rose [bactericidal for *E. coli* and *Str. faecalis* shown here, and bacteriostatic for all four species (Adams, 1967)], is evidently not due to increased sorption, as sorption is not affected by pH.

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The recovery of phenylmercuric nitrate-treated bacteria using sodium thioglycollate

J. P. RICHARDS AND A. E. E. EL KHOULY*

The effect of sodium thioglycollate on the recovery of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* has been examined when the sodium thioglycollate was included in the fluid used to dilute the treated bacterial suspension or when it was included in the nutrient agar recovery medium. The effect of thioglycollate on untreated organisms of the same three species was also studied; *Ps. aeruginosa* was found to be the most sensitive. For bacteria treated with the mercurial, the optimal thioglycollate concentration for recovery depended upon the species of organism, on the time of treatment with the bactericide and also on the manner in which thioglycollate was used. When it was used in the diluting fluid thioglycollate was more effective as a recovery agent than when it was incorporated in the nutrient agar. In the diluent, the optimal concentration of thioglycollate for the recovery of phenylmercuric nitrate-treated *E. coli* was approximately 0·4%, for *Ps. aeruginosa* it was 0·2% and for *Staph. aureus* 2–3%. The possible mechanisms involved in the recovery processes are discussed.

MERCURIAL compounds, particularly organo-mercurials, are widely used as preservatives and bactericides, not only because they are effective in low concentration but because of their low toxicity at this level. In general, mercurial antibacterial agents are more active against Gram-positive organisms, thus the lethal dilution of phenylmercuric nitrate for *Escherichia coli* is 1 in 48,000 but 1 in 200,000 for *Staphylococcus aureus* (Birkhaug, 1933).

It is known that sulphydryl compounds inactivate the antibacterial action of mercury and sodium thioglycollate is the most widely used compound, being a normal constituent of anaerobic media (Brewer, 1940). Inhibition of growth and subsequent death of the organisms due to mercury compounds is attributed to their –SH binding properties (Fildes, 1940). In support of this hypothesis it has been reported that certain enzymes such as oxidases and dehydrogenases with free –SH groups may be inactivated with mercurial compounds and later reactivated with sulphydryl compounds (Cook, Perisutti & Walsh, 1946; Davie, Koningsberger & Lipmann, 1956; DeLuca, Wirtz & McElroy, 1964).

Cook & Steel (1960) found that sodium thioglycollate was the most efficient of several mercurial antagonists, glutathione was the least efficient and cysteine occupied an intermediate position: this was held to be due to differences in lipid solubility. Glutathione is insoluble in lipids and therefore reacts only with the free mercury present in solution or that adsorbed onto the bacterial cell. Sodium thioglycollate on the other hand is soluble both in the aqueous phase and to some extent in lipids; it is thus able to penetrate the bacterial cell and antagonize mercury within the cell cytoplasm. Further, Cook & Steel (1959c) have shown that the theoretical amount of sulphydryl compound will inactivate mercuric chloride when

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added before the bacteria are introduced to the system, whereas a greater amount of antagonist is required if the bacteria have previously been in contact with the bactericide.

The present work was undertaken to establish the most effective concentration of thioglycollate to bring about the recovery of three species of bacteria treated with phenylmercuric nitrate.

Experimental

Phenylmercuric nitrate (Laboratory Reagent quality, BDH). 0·0025 and 0·00125% solutions were prepared aseptically and stored at ambient temperature until required for use.

Sodium thioglycollate was prepared aseptically as follows. Thioglycollic acid (Laboratory Reagent quality, BDH) was neutralized with N sodium hydroxide solution and diluted so that the final solution contained 10% w/v sodium thioglycollate; it was stored at 4° and used within one month of preparation (cf. Cook & Steel, 1959a). When required for use the solution was warmed to 25° and diluted aseptically to the desired concentration.

Media. Nutrient broth was prepared from Oxoid CM 15 granules (16 g/litre in distilled water) and solidified when necessary with 15 g/litre New Zealand agar (Davis Gelatin Ltd., Warwick). Nutrient agar containing sodium thioglycollate was prepared aseptically from double strength nutrient agar held at 48°. Approximately 20 ml quantities of both types of solid media were used for the overdried plates on which bacteria were counted.

Organisms. Escherichia coli type 1 (IMViC++−−, 44° +) strain II of Harris, Richards & Whitefield (1961); *Pseudomonas aeruginosa* (NCTC 7244) and *Staphylococcus aureus* (NCTC 4163).

METHODS

Suspensions. A 24 hr broth culture was used to inoculate a large nutrient agar slope and the 24 hr growth from this was harvested with sterile distilled water. The organisms were washed three times and the resultant aqueous suspension was standardized nephelometrically and diluted to give a concentration of the order of 10^{10} viable cells/ml. Suspensions were stored at 4° and used within 24 hr of preparation.

Treatment of bacteria. To 9 ml of phenylmercuric nitrate solution at 25°, 1 ml of the bacterial suspension, also at 25°, was added and samples were removed and diluted as desired. Depending upon the period of treatment with the bactericide and thus the mortality level, as many as three 1 in 100 dilutions were required to give reasonable numbers for counting. When more than one dilution was required, either sodium thioglycollate was included in the first diluent only, subsequent dilutions being made in water, or all diluents used contained thioglycollate.

When the sodium thioglycollate was included in the nutrient agar, dilutions were made in distilled water.

Viable counts. Plate counts were made by the surface viable method using ten replicate drops of about 0·017 ml and counting after incubation

for 24 or 48 hr, depending upon the conditions of treatment with the bactericide.

Results

The results are presented in Figs 1–4. Each point plotted is based on the mean of two trials. Some of the results for untreated cells given in Fig. 1 are repeated in Figs 2A, 3A and 4A for comparative purposes.

THE EFFECT OF SODIUM THIOGLYCOLLATE ON UNTREATED BACTERIA

Fig. 1 shows that when thioglycollate was included in the first diluent only, neither *E. coli* nor *Staph. aureus* were markedly affected although

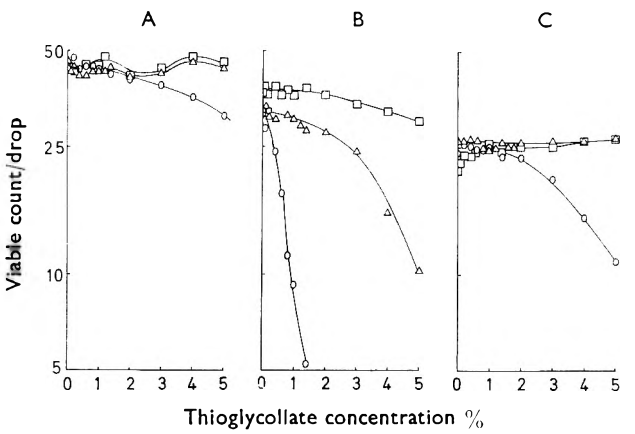

FIG. 1. The effect of sodium thioglycollate on the viable count of untreated bacteria. A. *E. coli*. B. *Ps. aeruginosa*. C. *Staph. aureus*. △, First dilution in thioglycollate solution; ○, all dilutions in thioglycollate solution; □, thioglycollate in nutrient agar.

high concentrations were lethal to *Ps. aeruginosa*. This effect was greater if thioglycollate was included in *all* the diluting fluids, then *Ps. aeruginosa* was particularly sensitive. Incorporation of thioglycollate in the nutrient agar in concentrations of up to 5% had little effect on any of the test organisms. These observations agree with those of Cook & Steel (1959b). When thioglycollate was included in the nutrient agar alone, increased viable counts of *Staph. aureus* were observed (t 5·27; d.f. 38; $P < 0{\cdot}001$ at 1% thioglycollate). With higher concentrations the rise in viable counts was gradual, the count on agar containing 5% thioglycollate being 126% of that on media with none added (t 6·57; d.f. 38; $P < 0{\cdot}001$). On the other hand, if thioglycollate was added to *all* solutions used as diluents all concentrations above 2% were detrimental to growth.

THE EFFECT OF SODIUM THIOGLYCOLLATE ON BACTERIA TREATED WITH PHENYLMERCURIC NITRATE

Escherichia coli. Fig. 2A illustrates the effect of sodium thioglycollate on *E. coli* treated with 0·00225% phenylmercuric nitrate for 35 min,

24 and 36 hr respectively. The optimal concentration was approximately 0·4% irrespective of the time of treatment; the greater the mortality the greater was the adverse effect of non-optimal concentrations.

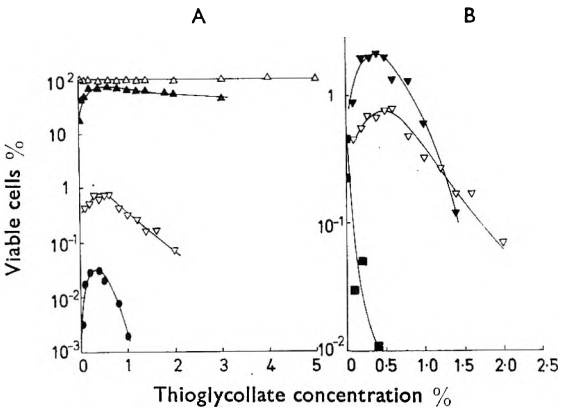

FIG. 2. The effect of sodium thioglycollate on the recovery of *E. coli* treated with 0·00225% phenylmercuric nitrate. △, Untreated cells, first dilution in thioglycollate solution; ▲, cells treated for 35 min, first dilution in thioglycollate solution; ▽, cells treated for 24 hr, first dilution in thioglycollate solution; ●, cells treated for 36 hr, first dilution in thioglycollate solution; ▼, cells treated for 24 hr, all dilutions in thioglycollate solution; ■, cells treated for 14 hr, thioglycollate in nutrient agar only.

When thioglycollate was incorporated in the diluent, the number of cells recovered was more than when it was present in the nutrient agar (Fig. 2B). By using thioglycollate at its optimal concentration in the diluent, viable cells could be recovered after 24 hr treatment with phenylmercuric nitrate, the level of mortality being about 98–99%. But, when it was incorporated in the nutrient agar at its optimal concentration, viable cells could only be recovered after 14 hr treatment, the level of mortality being about 99·5%.

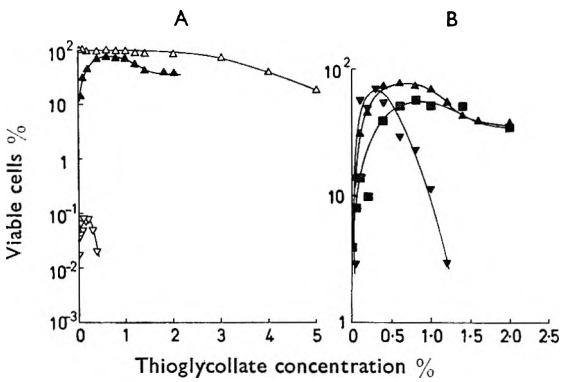

FIG. 3. The effect of sodium thioglycollate on the recovery of *Ps. aeruginosa* treated with 0·001125% phenylmercuric nitrate; △, Untreated cells, first dilution in thioglycollate solution; ▲, cells treated for 30 min, first dilution in thioglycollate solution; ▽, cells treated for 7 hr, first dilution in thioglycollate solution; ▼, cells treated for 30 min, all dilutions in thioglycollate solution; ■, cells treated for 30 min, thioglycollate in nutrient agar only.

Pseudomonas aeruginosa. This organism was found to be more sensitive to phenylmercuric nitrate than either *E. coli* or *Staph. aureus.* Thus, treatment for 7 hr with 0·001125% phenylmercuric nitrate resulted in a mortality level of about 99·96%, based on the optimal thioglycollate concentration. This optimal thioglycollate concentration varied according to the time of treatment with phenylmercuric nitrate (Fig. 3A) and the method of thioglycollate application (Fig. 3B). The optimal concentration fell from 0·4–0·8% to 0·2–0·3% for cells treated for 30 min when thioglycollate was present in all diluents. Fig. 3B also shows that, when used in all diluents, the higher concentrations of thioglycollate were more detrimental to the damaged cells.

Staphylococcus aureus. After 40 min treatment with phenylmercuric nitrate and using concentrations of sodium thioglycollate over the optimal range 1–4% in either diluent or agar, no death was detected (Figs 4A and B). However, when thioglycollate was included in all diluents at concentrations in excess of 1% it was toxic to cells which had been treated for 40 min (Fig. 4B). Fig. 4C shows that with cells treated with the mercurial for 24 hr, sodium thioglycollate in all diluents at concentrations higher than 2% were toxic, and that thioglycollate included in the nutrient agar was less effective for recovery. Unlike the other organisms, *Staph. aureus* required higher concentrations of thioglycollate as the time of treatment with the bactericide increased, for example, for cells treated for 48 hr, the optimal thioglycollate concentration was 3–4% (Fig. 4A).

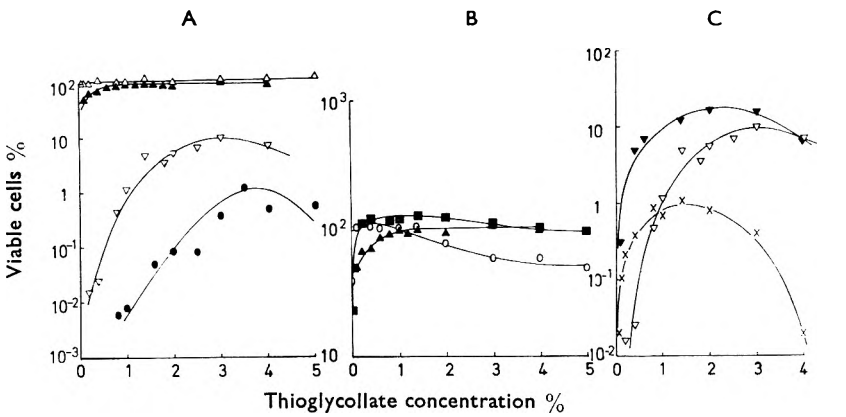

FIG. 4. The effect of sodium thioglycollate on the recovery of *Staph. aureus* treated with 0·0025% phenylmercuric nitrate. △, Untreated cells, first dilution in thioglycollate solution; ▲, cells treated for 40 min, first dilution in thioglycollate solution ▽, cells treated for 24 hr, first dilution in thioglycollate solution; ●, cells treated for 48 hr, first dilution in thioglycollate solution; ○, cells treated for 40 min, all dilutions in thioglycollate solution; ■, cells treated for 40 min, thioglycollate in nutrient agar only; ▼, cells treated for 24 hr, all dilutions in thioglycollate solution; X, cells treated for 17 hr, thioglycollate in nutrient agar only.

Discussion

Even after fairly long periods of treatment, thioglycollate reduces the bactericidal effect of phenylmercuric nitrate, enabling viable organisms to

be recovered. In theory, for each atom of mercury two –SH groups should be present to bring about inactivation. During the present investigation, however, it was found that using an –SH : Hg ratio of 2 : 1 the apparent mortality level of a suspension of phenylmercuric nitrate-treated *Ps. aeruginosa* was greater than 80%. But, when the –SH : Hg ratio was increased to 100,000 : 1 the apparent mortality level fell to 20%. Also, with treated *Staph. aureus* the count was increased from about 75% of the original using no thioglycollate to a count greater than the original using 1% thioglycollate.

The optimal thioglycollate concentration range for maximum recovery is relatively narrow and varies according to the species and the technique adopted for the application of the thioglycollate. The experiments in which undamaged cells were used revealed that thioglycollate was toxic particularly at high concentrations. Thus there is a balance between the inherent toxicity of thioglycollate and its ability to antagonize phenylmercuric nitrate.

In general, the number of cells which could be recovered increased as the thioglycollate concentration increased. This is in agreement with the observation that mercury compounds form complexes with metabolites containing sulphydryl groups (Barron & Kalnitsky, 1947). The equilibrium between the components of the complexes will be altered on the addition of –SH compounds as these will also complex with the mercury. Klarmann (1950) suggested that if the dissociation constant of an inhibitor-antidote complex was low compared with the dissociation constant of an enzyme-inhibitor complex, reactivation of the enzyme could be expected. It is suggested that the very large concentration of the antidote (thioglycollate) found to be necessary was required to alter the equilibrium between the mercury and the ligand. The mercury may also have reacted with other functional groups (Thomas & Cook, 1947) and thioglycollate would be involved in the restoration of these in addition to sulphydryl groups.

Other factors are known to affect the recovery and revival of phenylmercuric nitrate-treated bacteria. Harris & others (1961) cited instances where the recovery conditions affected the response, depending upon the previous treatment of the organisms. The more severe the treatment the greater was the response. Similar effects were observed in the present work. For example, with *E. coli* (Fig. 2A), the differences in the viable counts using diluent containing no thioglycollate and diluent containing 0·4% thioglycollate were much greater for the 36 hr treated cells than for the 24 hr treated cells. Prolongation of treatment, however, had no effect on the optimal concentration of thioglycollate required for recovery. But, for all species examined, at low mortality levels the concentration of thioglycollate required for optimal recovery of phenylmercuric nitrate-treated cells was much less critical than at high mortality levels.

The optimal concentration of the thioglycollate is affected by its method of application. Of the three different techniques used, that in which thioglycollate was present in all diluting fluids was the most effective while

that in which thioglycollate was included in the nutrient agar alone was the least effective. Cook & Steel (1960) reported similar findings using mercuric chloride-treated *E. coli* and they attributed their results to oxidation of the thioglycollate during the drying of the agar plates. However, in addition to this possibility, diffusion of thioglycollate from the semi-solid environment of the nutrient agar to the bacterial cell would be hindered, the rate being considerably less than would be the rate of diffusion from an aqueous solution. Friedman & Kramer (1930) found that diffusion of solutes was much slower in a gelatin gel than in water.

The results suggest that penetration of the thioglycollate into the damaged cells is required for their subsequent recovery. The molar ratio of –SH : Hg for optimal recovery for all species was about 10^5 : 1. The somewhat slow bactericidal effect produced by the phenylmercuric nitrate and the revival of the cells by thioglycollate is consistent with the view that the reaction is intracellular. Hess & Speiser (1959) consider that the effect of a mercury compound adsorbed onto the cytoplasmic membrane is a bacteriostatic effect becoming bactericidal only when the compound penetrates into the cell cytoplasm.

It was observed that the viable count of untreated *Staph. aureus* when diluted in thioglycollate solutions or allowed to grow on thioglycollate agar media was higher than the original count. This effect was also seen with cells treated with phenylmercuric nitrate for 40 min (Fig. 4C), where the viable count of treated cells counted on media containing 1% thioglycollate was higher than the viable count of untreated cells counted on media without thioglycollate (t 6·36; d.f. 48; $P < 0{\cdot}001$). Thus the thioglycollate must be exerting an effect additional to reversal of the action of mercury which may be on the oxidation-reduction potential of the environment.

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