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Science Papers

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J. Pharm. Pharmacol., 1965, **17**, *Suppl.*, 1S–6S Received February 3, 1965

Science Papers

Method of making spore papers of reproducible resistance

A. M. COOK AND M. R. W. BROWN*

A method of making papers impregnated with *Bacillus stearothermophilus* spores is described. Replicate experiments testing the resistance of these papers to steam at different temperatures have given reproducible results.

A PREVIOUS report (Cook & Brown, 1960) gave details of tests on a commercial brand of spore papers. The present work is mainly concerned with papers made in this laboratory.

The apparatus and method for testing heat resistance were those of Cook & Brown (1960). The method consists essentially of heating spore impregnated paper discs in a modified autoclave. Replicate discs recovered after suitable time intervals were then incubated in separate tubes of broth.

PREPARATION OF SPORE PAPERS

Spore suspensions of *Bacillus stearothermophilus* N.C.I.B. 8919 in water were prepared and viable counts made as described previously (Brown, 1962). Paper discs† were impregnated with a spore suspension in one of two ways.

Method A. A dropping pipette and needle (Cook & Yousef, 1953) was used to deliver a known volume of a standardised spore suspension separately to each disc. The discs were then dried at ambient room temperature before being stored in dark coloured screw-cap glass jars.

Method B. Sufficient discs were added to a standardised spore suspension present in a glass dish until only a small volume of suspension was left unabsorbed by the discs. Care was taken that each disc was thoroughly wet. The unabsorbed suspension was discarded and the discs then dried and put into jars as described above.

All spore papers were stored at ambient room temperature on the bench. Details of non-commercial spore papers are listed in Table 1.

Recovery conditions. Previous findings with commercial spore papers (Brown, 1962; Cook & Brown, 1960) have shown that the presence of bromocresol purple as an indicator in the broth recommended by the makers of these papers resulted in significantly reduced recovery after

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† Whatman Antibiotic Assay Discs, W. and R. Balston Ltd. Obtained from H. Reeve Angel and Co. Ltd., 9, Bridewell Place, London, E.C.4.

heat treatment. The medium used in this work contained dextrose 0·5%, tryptone (Oxoid) 1% in water and was used for both commercial and non-commercial papers. The spore papers were incubated for 3 days at 56° after heat treatment in the autoclave described by Brown (1962).

TABLE 1. DETAILS OF SPORE PAPERS

Paper code	Sporulation time of spores (days)	Method of preparation	Number of spores per paper
A	2	A	3×10^4
B	2	A	3×10^4
C	8	B	10^7
D	8	B	10^6

Presentation of results. When the percentage of tubes showing growth (positive) was plotted against time of exposure to steam at constant temperature the graph was typically of the form in Fig. 1. The "middle" straight line portion of the graph is important in the present work. In attempting to calculate the equation of this middle portion, it is necessary to exclude from the calculation points which occur on the distal portions of asymptotes of the curve. Consequently, where possible, the percentage of positive results was converted to probits and plotted against exposure time. This transformation enabled a better assessment of the linearity of the graph to be made.

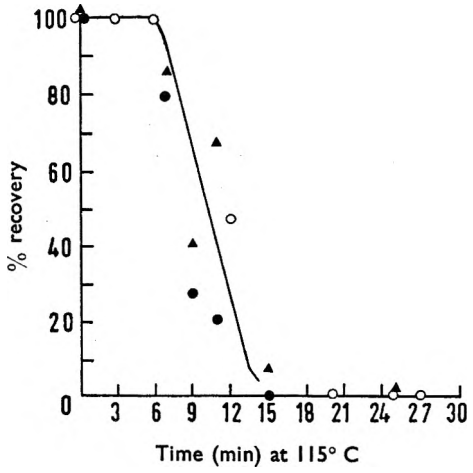

FIG. 1. Reproducibility of results of heat resistance tests on spore papers coded B. ○, Expt. 1. ▲, Expt. 2. ●, Expt. 3.

The equations calculated to fit these lines are not put forward as precise measurements of the rates of kill, but are useful for comparative purposes. This is because in most cases the number of degrees of freedom associated with these lines is small and the smallest interval possible on the % survivor or probit axis is large.

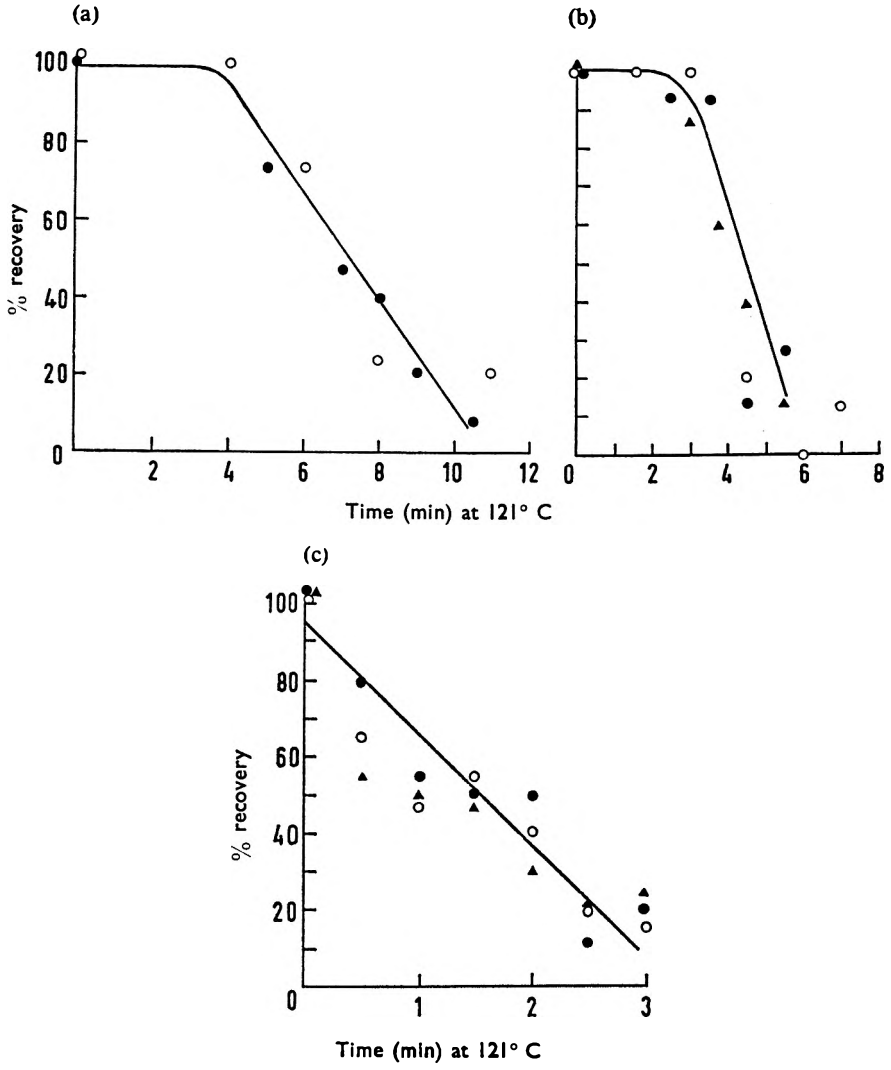

FIG. 2. Reproducibility of results of heat resistance tests on spore papers. (a) Coded C. (b) Coded D. (c) Commercial. ○, Expt. 1. ●, Expt. 2. ▲, Expt. 3.

Variability of results. Replicate experiments were made of the resistance to wet heat at 121° of papers coded C and D and Oxoid* spore papers (Ox. S) and at 115° of papers coded B. The results of these experiments are illustrated in Figs 1–3 and an analysis of variance of these results is recorded in Table 2. The results recorded in Fig. 2(b) show evidence of the reproducibility of the testing method but they are not suitable for probit transformation and are not recorded in Table 2.

*Oxide spore strips, Oxoid Division, Oxo Ltd., London.

TABLE 2. ANALYSIS OF VARIANCE OF RESULTS IN FIGURES 1 AND 2

Figure	Source of variance	Sum of squares	Degrees of freedom	Mean square	F	P	Equation of lines (Probit % +ive/Time)
1	Pooled residual (*a*)	1·8368	5				(1) $y=8·47-0·41x$
	Residual due to individual regressions (*b*)	1·1256	3	0·3752	1·0551	>0·2	
	(*c*)	0·7112	2	0·3556			(2) $y=7·89-0·28x$
2 (a)	Pooled residual (*a*)	2·1701	6				(1) $y=6·93-0·27x$
	Residual due to individual regressions (*b*)	0·4401	4	0·1100	7·8636	0·05–0·01	
	(*c*)	1·7300	2	0·8650			(2) $y=7·58-0·19x$
2 (c)	Pooled residual (*a*)	1·1420	16				(1) $y=5·7-0·28x$
	Residual due to individual regressions (*b*)	0·8807	12	0·0734	1·1240	>0·2	(2) $y=6·04-0·35x$
	(*c*)	0·2613	4	0·0653			(3) $y=5·37-0·20x$

Resistance at different temperatures. The resistance of A and B papers was tested at 110°, 115° and 121°. One batch of papers was tested at 110° and 121° and a second batch, made from the same spore suspension as the first batch, was tested at 115°. All the papers used in these experiments were stored for less than one week before being tested. The results are illustrated in Fig. 3.

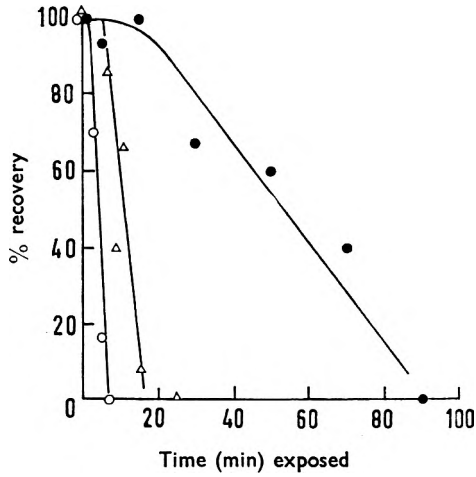

FIG. 3. Effect of steam at different temperatures on the recovery of spore papers. ○, 121° (A). △, 115° (B). ●, 110° (A).

The exposure time necessary to produce zero % positive results was estimated from the graph by eye for the three temperatures and the logarithm of this time was plotted against temperature (Fig. 4).

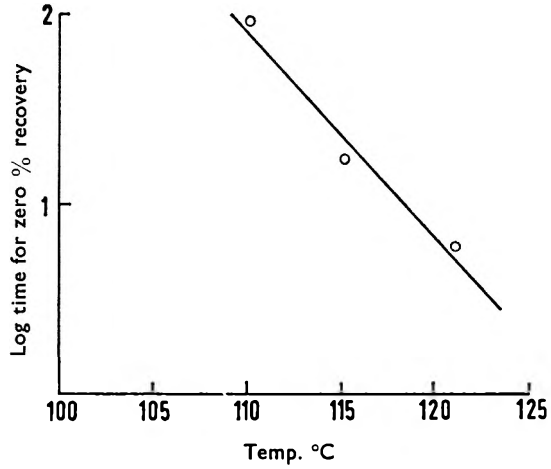

FIG. 4. Relationship between temperature and the log of exposure time for zero % positive results.

Discussion

The method of preparation of spore papers used in this work differs from that suggested by Kelsey (1961), who recommended that spore suspensions intended for impregnation of papers be heat activated immediately after harvesting, to kill vegetative bacteria. If the suspension had been stored, he recommended a second heat activation to kill extraneous vegetative organisms. He stated that "the pre-impregnation viable count of spores will be maximal" and also that this activation is important.

The suspensions used to impregnate papers in this work were not heat activated. Although Kelsey (1961) quoted Evans & Curran (1960) as showing that heat activated spores retain viability after prolonged storage at low temperatures (and at neutral or slightly alkaline pH), nevertheless these authors used buffered solutions. The suspensions used in this work and by Kelsey were made with distilled water.

Retention of heat resistance as well as viability is important with spore papers. In earlier papers, Curran & Evans (1945, 1947) have shown that there was a tendency for heat-activated spores in a nutritionally incomplete medium to lose viability, and that heat activated spores are more sensitive to heat than non-activated spores. If a count of heat-activated spores is required, this may be made with a sample of the suspension, leaving the bulk of the spores with their resistance unaltered.

Kelsey (1961), found that the cultural conditions he used did not affect survival from heated spore papers. However, he compared Oxoid dextrose tryptone broth (containing bromocresol purple) with nutrient broth. He varied the amount of broth by using "$\frac{1}{2}$ inch" and "3 inch" quantities. He did not test the effect of using dextrose tryptone broth without any dye—the medium we found to be most satisfactory in the present work.

Figs. 1–3 and Table 2 show that heat resistance experiments gave results which were satisfactorily reproducible.

It is necessary that the spores on the papers be sufficiently resistant to the lethal effects of heat to be of practical value. In hospital sterilisation practise, the main concern is the killing of pathogenic organisms such as tetanus and the gas-gangrene group. The M.R.C. report (1959) suggested that the minimum heat resistance for spore papers should be 5 min at 121° or 1 min at 130° wet heat. The review by Perkins (1954) of quoted thermal death times from the literature indicates that 5 min at 121° wet heat would be sufficient to kill pathogenic organisms.

Papers coded C which contained about 10^7 spores showed 100% positive results on subculture after exposure to steam at 121° for over 4 min (Fig. 2a). With these papers 90% and 10% positive recoveries occurred after 5 and 10 min respectively at 121°. Thus, if each of two C papers showed growth on subculture after exposure to steam at 121° then there would be a 100 to 1 chance ($P = 0{\cdot}99$) that they had been exposed for less than 10 min. Conversely if neither of the 2 papers showed growth then the probability that they had been exposed for at least 5 min would be $P = 0{\cdot}99$.

The maximum exposure times at 121° resulting in 100% recovery for other papers tested were as follows: Ox.S—3½ min, D (10^6 spores per paper)—3 min, A (3×10^4 spores per paper)—2 min.

Kelsey (1961) showed that bulk impregnation of spore papers did not significantly increase the variation compared to that obtained by individual impregnation. The results obtained here confirm this finding. With A and B (Figs 1 and 3), which were impregnated individually, results were similar to those obtained with spore papers prepared in bulk. The absence of an excessive number of "wild" negative or positive results with bulked papers is an indication that they are not more variable than the individually impregnated papers.

This work shows that papers impregnated with aqueous suspensions of *B. stearothermophilus* N.C.I.B. 8919, such that each paper contained about 10^7 spores, were of satisfactorily high and reproducible resistance.

The relatively low resistance shown by Ox.S papers (Fig. 2c) is probably correlated with the fact that this batch was stored in the laboratory for 4 months before this experiment.

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Effect of storage on the heat resistance of bacterial spore papers

A. M. COOK AND M. R. W. BROWN*

Papers impregnated with 10^4 *Bacillus stearothermophilus* spores in water and subsequently stored on the bench can lose heat resistance and viability. This loss of viability was accelerated with storage over silica gel. Papers containing more than about 10^6 spores retained significant heat resistance over long periods. The pH of the broth significantly affected recovery from heated spore papers.

THIS paper states results of tests on the viability and heat resistance after storage of papers impregnated with *Bacillus stearothermophilus* spores described previously (Cook & Brown, 1965).

Experimental

METHODS AND MATERIALS

Spore papers were prepared and tested as described by Cook & Brown (1965). In addition, papers were prepared using spores suspended in *Mist. Dessicans* (Fry & Greaves, 1951) which contained 7·5 g glucose, 1 g peptone, 0·5 g sodium chloride, 75 ml serum and water to 100 ml. Papers were either impregnated with spore suspension individually using a dropping pipette (Method A) or in bulk (Method B) (Cook & Brown, 1965). Spore papers were stored at room temperature in screw capped dark coloured jars either on the bench or over silica gel in a vacuum desiccator. Details of the spore papers are given in Table 1.

Effect of storage on viability. Unheated spore papers were incubated in broth after storage and the results for papers prepared as described

TABLE 1. DETAILS OF SPORE PAPERS

Code	Storage conditions	Sporulation time of spores (days)	Times washed	Suspending medium	Method of preparation	Number of spores per paper
E	Over silica gel	5	6	Water	A	10^4
	Bench					
G	Over silica gel	5	0	Broth culture	A	10^4
	Bench					
A	Bench	2	5	Water	A	3×10^4
D	Bench	8	5	Water	B	10^8
C	Over silica gel	8	5	Water	B	10^7
	Bench					
F	Over silica gel	8	5	*Mist. Dessicans*	B	10^7
	Bench					

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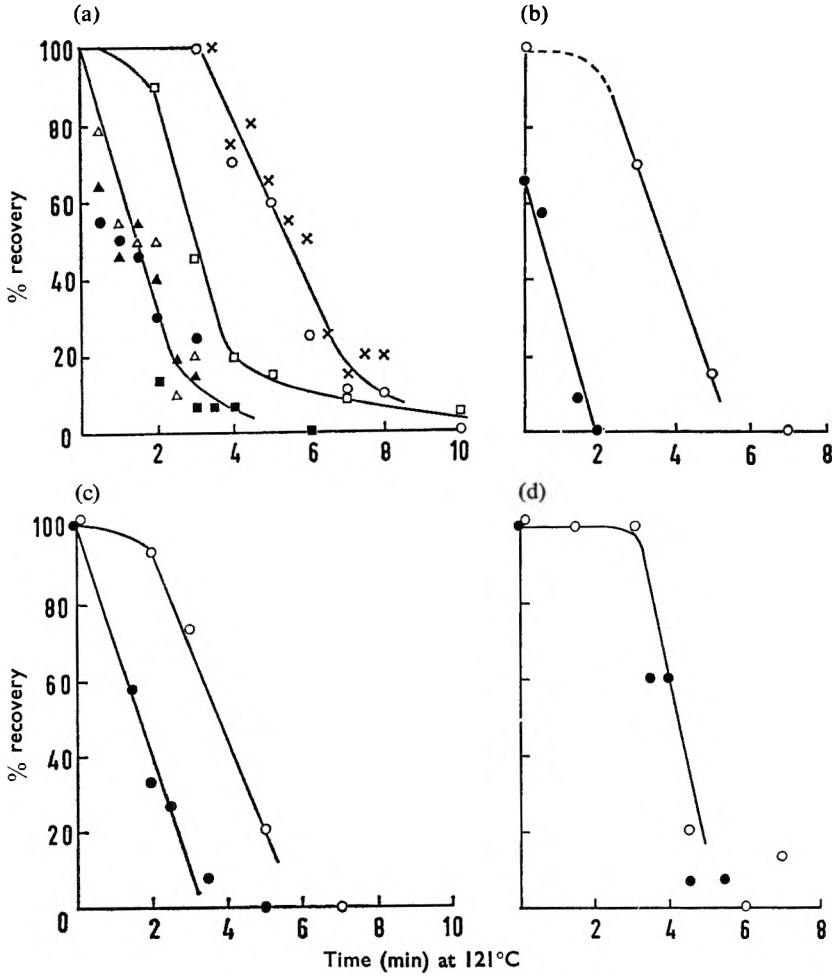

FIG. 1. Effect of bench storage upon heat resistance of spore papers. (a) Commercial papers. ○, ×, Zero storage time. □, Stored 5 weeks. ▲, △, ●, ■, Stored 16 weeks. (b) Papers coded A. ○, Zero time. ●, Stored 16 weeks. (c) Papers coded G. ○, Zero time. ●, Stored 16 weeks. (d) Papers coded D. ○, Zero time. ●, Stored 22 weeks.

above and for papers from a commercial source (Ox.S)* are recorded in Table 2.

Effect of storage on heat resistance. Spore papers prepared in different ways were tested for resistance to wet heat before and after storage. Results of such experiments for the papers coded Ox.S, A, G and D, stored on the bench are illustrated in Fig. 1. Results of experiments made to compare the effect of bench and desiccator storage conditions of C and F papers are given in Figs 2 and 3.

* Oxoid Spore Strips, Code number BR23, Oxoid Division, Oxo Ltd., London, E.C.4.

TABLE 2. EFFECT OF STORAGE UPON VIABILITY OF UNHEATED SPORE PAPERS

Paper	Weeks stored	Storage conditions	Number of papers tested	% showing viability
G	37	Bench	15	100
	20	Over silica gel	140	0
E	37	Bench	5	80
		Over silica gel	5	0
Ox.S	26	Bench	30	90

Effect of recovery medium pH. Unheated spore papers were incubated in broths of pH below 5·1 and above 6·3. It was observed that broths of pH 5·1 or less did not support the growth of the spores on the paper D but growth did occur at pH 6·3 or above. Spore papers coded D were also tested for resistance to wet heat and recovered in broths of differing pH (Fig. 4).

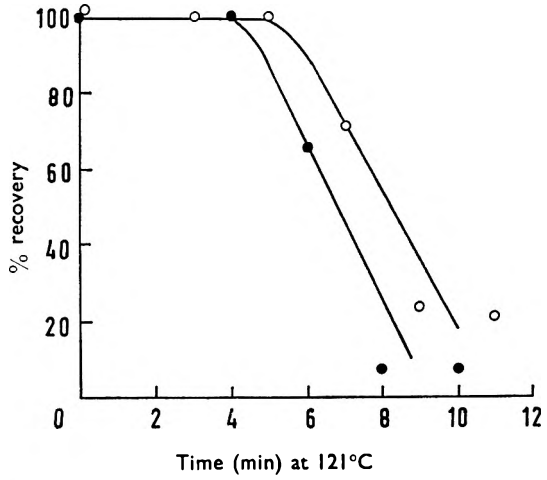

FIG. 2. Effect of storage over silica gel on heat resistance of papers coded C. ○, Zero time. ●, Stored 16 weeks.

Discussion

Spores can lose viability during storage on paper. Papers Ox.S and E (10^4 spores per paper) began to lose viability after 26 and 37 weeks bench storage respectively (Table 2). Papers coded G (10^4 spores per paper, unwashed) showed no loss in viability when stored on the bench for 37 weeks. Storage over silica gel resulted in complete loss of viability with E and G papers after 37 and 20 weeks respectively.

The papers with the smaller inocula (10^4 spores per paper) and Ox.S papers, lost heat resistance after less than 16 weeks bench storage (Fig. 1). Unlike G and Ox.S papers, papers coded A not only lost resistance

but also viability after 16 weeks bench storage (Fig. 1). G papers were inoculated with an unwashed suspension, and the spores in the finished papers may have been protected from harmful effects of storage by the presence of dried nutrients.

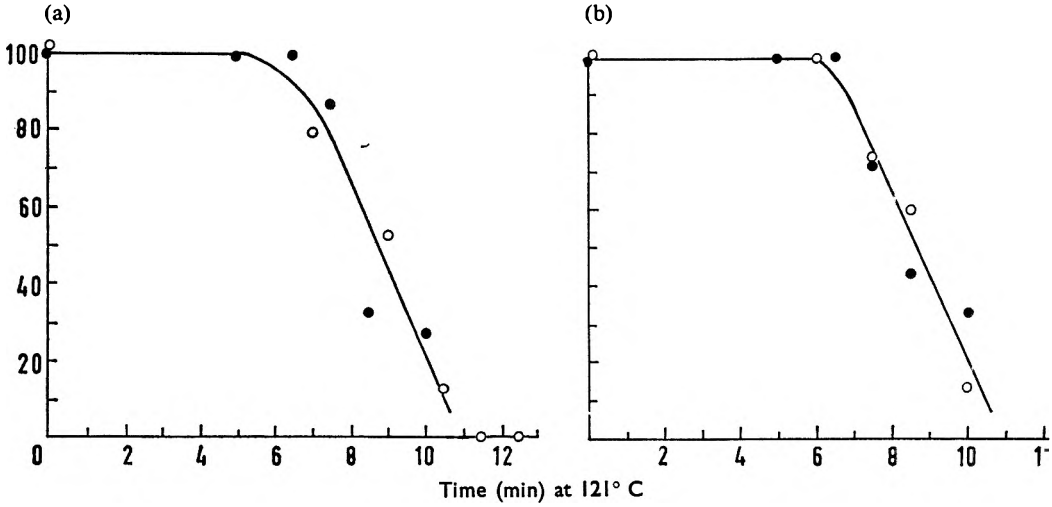

FIG. 3. Effect of storage on bench and over silica gel on heat resistance of papers coded F. Ordinate: % recovery. (a) Bench. ○, Zero time. ●, Stored 34 weeks. (b) Silica gel. ○, Zero time. ●, Stored 34 weeks.

With one exception, papers with more than about 10^6 spores showed no significant loss of heat resistance over the storage periods stated [Figs 1 (d), 2 and 3]. C coded papers appear to have lost some heat resistance after storage over silica gel for 16 weeks (Fig. 2). This finding accords with

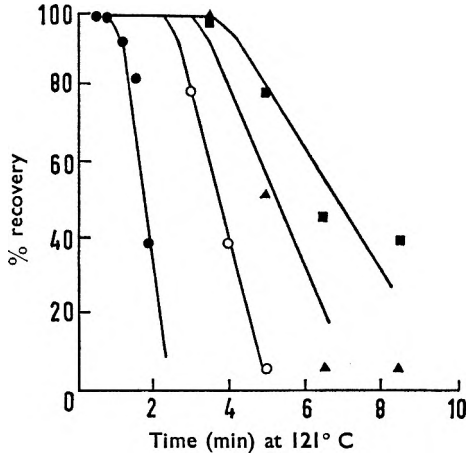

FIG. 4. Effect of pH of recovery broth on apparent heat resistance of papers coded D. ■, pH 7·4. ○, pH 6·8. ●, pH 6·3. ▲, pH 7·9.

loss of viability obtained after similar storage conditions for papers G and E (Table 2). No comparable experiment was made after 16 weeks bench storage with papers coded C, but no loss in resistance was observed after 5 weeks bench storage. F papers did not lose heat resistance when stored over silica gel or on the bench for 34 weeks (Fig. 3). The harmful effects of drying over silica gel were perhaps minimised in this instance by the presence of glucose and other ingredients in the *Mist. Dessicans* used to suspend the spores with which F papers were impregnated.

The pH of the broth used to recover heated spore papers is important; maximum recovery of heated spores occurred at about pH 7·4 (Fig. 4). These results agree with those for the recovery of heated spores in aqueous suspension (Brown, 1962) where increased pH of the medium, up to a maximum correlated with increased recovery.

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The viability of spores of some *Bacillus* species

G. RICHARDSON

The percentage of spores of *Bacillus subtilis* capable of giving rise to macro-colonies ("viability") has been determined. Under optimum conditions (heat-activation and inclusion of dextrose in the counting medium) about 30% of spores formed colonies. This figure appeared to increase to about 70% on prolonged cold storage (0–10°). The viability of spores decreased with increase in the manganese content of the sporulation medium. Spores of four other species of *Bacillus* examined were not heat-activatable and were unaffected by the dextrose content of the counting medium. They showed viabilities of 40–75%.

ATTENTION has recently been drawn by Cook & Brown (1964) to the fact that suspensions of spores prepared by conventional methods do not consist entirely of viable spore-forms. The percentage of viable spores depends on the method of preparation of the suspension and on the conditions under which viability is determined. Of particular importance in assessing viability is the role of any stimulus, especially heat-activation, which may be necessary to induce maximum germination. Two of the factors necessary for maximum viability of *B. subtilis* have been examined; these are the amount of heat-activation and the concentration of dextrose in the counting medium. The viability of some other *Bacillus* species has been determined under the same conditions.

Experimental

METHODS

The percentage viability of spore suspensions was calculated from the ratio of viable count (determined by a roll-tube method) to total count (determined using a counting chamber). Heat-activation factors were calculated from the ratio of viable count after heat treatment, to that before. Determination of viability was made on spore suspensions no more than 2 or 3 weeks old. (The viability of suspensions stored for longer periods in the refrigerator increased).

Test organisms and preparation of suspensions. Organisms were the following NCTC strains: *Bacillus subtilis* 8236, *B. subtilis* 3610, *B. pumilus* 8241, *B. cereus* 9946, *B. megaterium* 9848 and a laboratory isolate of *B. licheniformis*.

Spore suspensions were normally prepared by washing off the growth from 10 day cultures on nutrient agar slopes (Oxoid CM3) to which had been added 1 mg of $MnSO_4.5H_2O$ per litre. The initial concentration of manganese in the nutrient agar was <1 ppm (Oxoid Ltd., analysis of current material). For *B. megaterium* 200 mg per litre $MnSO_4$ was necessary to obtain a good spore yield.

Vegetative cell-free suspensions of *B. subtilis* and *B. licheniformis* spores were prepared by centrifuging and resuspending in water five times. For *B. cereus* spores, separation in a two-phase system of poly-

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ethylene glycol and phosphate buffer was used (Sachs & Alderton, 1961). *B. megaterium* and *B. pumilus* spore suspensions were subjected to ultrasonic irradiation (20 kc/sec: 30 min) using an M.S.E. ultrasonic disintegrator. After this treatment spores were readily separated from vegetative cell debris by centrifuging (cf. Mol, 1957; Grecz, Anellis & Schneider, 1962).

The purity of suspensions was examined by plating on nutrient agar, by staining with 1% methylene blue and also by the carbol fuchsin–methylene blue technique (Powell, 1950).

Effect of manganese. To investigate the effect of the manganese content of the medium on spore viability of *B. subtilis*, the concentration of manganese sulphate was varied between 1 mg and 1 g per litre.

Total counts. The counting method incorporated the recommendations of Cook & Lund (1962). Two 0·1 mm slides were used for each count which was made using normal microscopy of all spores in each of three fields of sixteen squares selected at random on each slide. Normal microscopy was preferred to phase-contrast microscopy since the object was to determine the total count of all spore forms.

The Index of Dispersion, χ^2, was calculated for the counts from 50 slides. Since $\sqrt{2\chi^2} - \sqrt{2n-1} < 1{\cdot}645$, χ^2 did not significantly exceed expectation (Fisher, 1948) and thus the counts did not differ significantly from a Poisson distribution.

Using 25 pairs of counts, the average coefficient of variation between duplicate slide counts was 5·9%; when duplicate counts showed a difference $> 10\%$ the count was repeated. The same two counting slides were used throughout the work and there was no indication of bias between the slides.

Additionally, ten duplicate counts were made on the same suspension on different days and the coefficient of variation for these was 8·3%. The major source of variation in total counts is likely to be the fit between cover-slip and slide. Results quoted show that the total count estimates are not subject to undue variation from this source and are reproducible.

Viable counts. Viable counts were determined by the roll-tube method (coefficient of variation 6%: Richardson, 1959). The effect of dextrose and soluble starch on viability was investigated by varying the concentrations of these substances in the nutrient agar counting medium.

Reproducibility of viability. Six suspensions of *B. subtilis* 8236 were prepared on different occasions and the viability of each crop determined immediately after preparation of the suspension. The six values had a mean value of $16{\cdot}2\% \pm 1{\cdot}7$ with a coefficient of variation of 10·5%. The variation in viability between spore crops prepared on different occasions is of the same order as the combined errors of total and viable count.

Heat-activation. The effect of different temperatures for varying periods of time was determined on the viable counts of suspensions of *B. subtilis* 8236 spores. Suspensions were sealed in 10×75 mm tubes and heated in a water-bath.

Attempted separation of heat-activatable fraction. An attempt was made to separate the heat-activatable fraction of the spore population so that its properties could be investigated. *B. subtilis* spores were allowed to germinate in a medium containing 1% peptone and 0·5% dextrose, and later in a medium identical to the counting medium except for the omission of agar, for periods from 5–16 hr. After incubation the cells were centrifuged and the ungerminated spores separated from the vegetative cells using the two-phase system. The heat-activation factor of the recovered spores was then compared with that of a normal population which had also been passed through the two-phase system.

Results and discussion

EFFECT OF MANGANESE IN THE SPORULATION MEDIUM

Manganese has been claimed to be an essential requirement for spore production in several species of *Bacillus* and work with these species has been summarised by Murrell (1961). The number of cells of *B. megaterium* giving rise to spores has been shown by Weinberg (1964) to be dependent on the manganese concentration in the medium. It therefore seemed possible that viability of spores might be affected by the manganese content of the sporulation medium. Fig. 1 shows that as the concentration of manganese sulphate in the medium increases, the

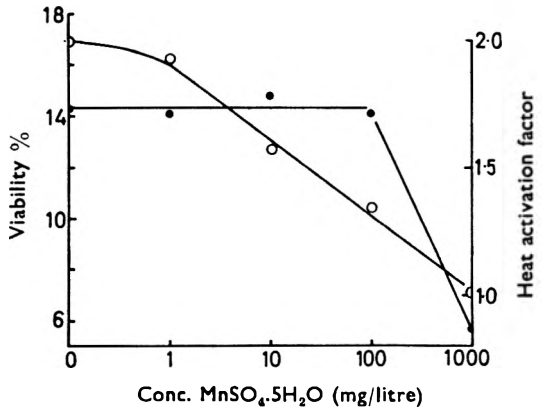

FIG. 1. The relationship of heat-activation and viability of *B. subtilis* spores to the manganese content of the sporulation medium. ○ = viability. ● = heat-activation.

percentage viability of *B. subtilis* falls significantly. At the highest manganese concentration growth was extremely variable. A suspension of spores was not obtained on every occasion from this medium, growth sometimes being restricted to the production of filamentous forms, with no tendency to sporulate. When spores were formed they were atypical. Growth and sporulation of *B. megaterium* and *B. licheniformis* were not inhibited by high concentrations of manganese.

These results suggest that it is not necessary to add manganese to the medium to induce the formation of viable spores of *B. subtilis*. But there is a marked variation of manganese tolerance with species and it seems that, even where manganese may be a requirement for sporulation, excessive manganese concentration may lead to a higher proportion of non-viable forms.

COMPOSITION OF COUNTING MEDIUM

The composition of the counting medium can markedly affect the viable count. The importance of dextrose and soluble starch in the medium, particularly for heated spores of *B. stearothermophilus*, has been demonstrated by Cook & Brown (1964). Fig. 2 shows that for both normal and pre-heated suspensions of *B. subtilis*, the count is much increased by the addition of dextrose. The highest increase in count is given by 0·5% dextrose. Higher concentrations do not result in further increases. Soluble starch in concentrations up to 1% did not affect the count and in media containing both dextrose and soluble starch the increase in count was no more than that attributable to dextrose alone. Thus for *B. subtilis* a medium containing 0·5% dextrose is recommended for viable counts.

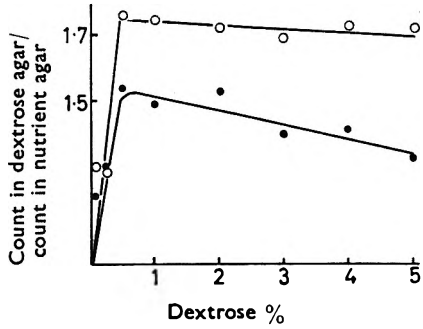

FIG. 2. The increase in viable count of *B. subtilis* spores caused by dextrose in the counting medium. ○ = heat-activated spores. ● = normal spores.

HEAT-ACTIVATION

Fig. 3 shows that as the temperature increased, activation occurred more rapidly and to a greater extent. Maximum activation occurred in 4–10 min at 85°. When suspensions were heated in capillary tubes instead of ignition tubes at this temperature, activation time was reduced to less than 30 sec. Since activation occurs at temperatures which are also rapidly sporicidal, the death of some viable spores may occur so that it is possible that the activation curve may represent less than the true number of potentially activatable spores.

Desrosier & Heiligman (1956) claimed that activation occurred with *B. thermoacidurans* and *B. globigii* only at temperatures which were subsequently lethal. This was not so in the present work with *B. subtilis* with which there was no decrease in count up to 5 hr at activating temperatures of 60° and 70°.

Powell & Hunter (1955) investigated the increase in the rate of germination brought about by heat-activation of *B. megaterium*. They plotted rate of germination against time of heating and claimed that the curve obtained was typical of the effect of temperature on a first order reaction. However it seems equally possible that the increase brought about by heat in numbers of spores forming colonies may be due to heterogeneous heat-activation characteristics existing among a spore population.

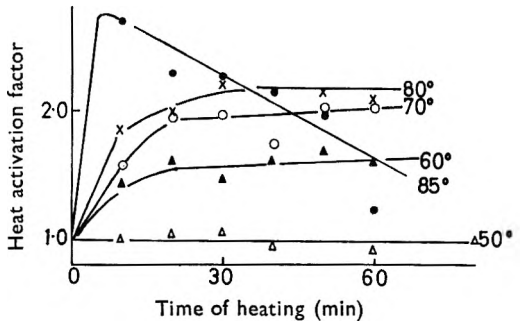

FIG. 3. Heat-activation curves for *B. subtilis* 8236.

Heat-activation and manganese content of sporulation medium. The spore suspensions prepared from media of varying manganese content were tested for heat-activation. The factors obtained remained constant except for spores from the medium of highest manganese content. Suspensions from the latter showed mortality rather than activation after heating at 85° for 10 min. This is compatible with the previous suggestion that spores produced on this medium are abnormal.

VIABILITY OF SPORES OF *Bacillus* SPECIES

The viability of several species was determined under conditions which were optimal for *B. subtilis* 8236. Viability was also determined after heating at 60° for 30 min and by counting on media with and without dextrose. The results are shown in Table 1. The heat-resistant *B. subtilis* even after activation had a lower viability than the other organisms. Under the optimum conditions *B. subtilis* showed a viability of about 30% (similar figures were obtained from both strains). Under the optimum conditions for *B. subtilis*, *B. licheniformis* showed a viability of 75%. The inclusion of dextrose in the counting medium did not result in an increase in count for organisms other than *B. subtilis*, although all are able to metabolise dextrose (Bergey, 1957). Dextrose may be a specific inducer of germination in some species (Powell, 1951).

Suspensions of *B. subtilis* which had been stored in the refrigerator for up to 8 months showed a marked increase in viable count although the total count remained constant. Results from the three suspensions available are presented in Table 2. It appears that concurrent with "storage

activation" there is a decrease in heat-activatability. The overall effect was that the viability rose from a comparatively low figure to a value at least equal to that for the other species. These results inevitably suggest that heat-activation is merely an acceleration of a process which occurs more slowly under normal environmental conditions.

TABLE 1. THE VARIATION IN % VIABILITY OF SPORES OF SOME *Bacillus* SPECIES WITH HEAT SHOCK AND DEXTROSE CONTENT OF MEDIUM

Organism	Conditions					
	No heat shock		60° for 30 min		85° for 10 min	
	NA	DA	NA	DA	NA	DA
B. subtilis 3610	10·5	13·1	11·2	15·8	17·2	28·5
B. subtilis 8236	12·1	16·2*			16·3	33·1*
B. cereus	40·0	41·7	29·6	32·3	19·2	22·5
B. pumilus	45·1	45·1	47·9	50·0	47·5	54·4
B. megaterium	56·2	56·9	47·8	50·7	36·9	39·1
B. licheniformis	57·9	55·0	75·5	62·3	43·5	22·7

NA = nutrient agar DA = dextrose agar
* Average value from six crops

None of the organisms showed a viability of 100% although most published work supports the findings that virtually all the spores in a suspension are able to initiate the germination process as indicated by the criteria of loss of refractility and change in staining reactions. The germination of *B. subtilis* 8236 was followed using as the criterion the change in staining reaction to carbol fuchsin and methylene blue (see Powell, 1950). It appeared that after 5–6 hr about 90% of the spores had

TABLE 2. THE INFLUENCE OF STORAGE-ACTIVATION AND HEAT-ACTIVATION ON THE VIABILITY OF SPORES OF *B. subtilis* 8236

Crop	Storage (months)	Viability of non-activated spores (%)	Storage-activation factor	Heat-activation factor of spores after storage	Viability due to combined effects of storage and heat-activation %
2	0	13·6	1·0	2·3	31·2
	8	—	3·3	1·2	53·8
3	0	18·4	1·0	1·3	23·9
	3	—	1·4	1·2	30·9
	6	—	2·3	1·0	43·3
4	0	17·5	1·0	2·5	43·7
	3	—	2·3	1·5	60·4
	6	—	3·1	1·3	70·5

$$\text{Heat-activation factor} = \frac{\text{viable count after heating—85°, 10 min}}{\text{viable count before heating}}$$

$$\text{Storage-activation factor} = \frac{\text{viable count after storage in refrigerator}}{\text{initial viable count}}$$

germinated (ratio of blue staining forms to total cells; about 800 cells being counted). Counts after this time were unreliable due to the proliferation of vegetative forms. After 8 hr only 2% of spores were present but this low figure may have been due to an increase in the total count.

The absolute accuracy of total cell counts using 0·02 mm slides has been questioned by Norris & Powell (1961). From their results it can

be calculated that the depth of liquid entrapped by such slides was on average about 30% in excess of the normal depth of the slide. However, using 0·1 mm slides, Cook & Lund (1962) have obtained counts which were about 70% of those using 0·02 mm slides, which suggests that the magnitude of absolute error with the deeper slides is much less, if indeed it does occur. It seems likely, therefore, that in the present work the estimates of viability using 0·1 mm slides are reliable and that the low viability reported is not due to errors of total count. The discrepancy between the figures for viability determined as described above and those for germination may be reasonably ascribed to the fact that not all germinated forms grow to give macro-colonies, an observation which has previously been made by Pulvertaft & Haynes (1951). They suggested that the freshly formed vegetative cells were liable to lyse due to their more exacting growth requirements.

ATTEMPTED SEPARATION OF HEAT ACTIVATABLE SPORES

It was expected that under the conditions of germination the heat-activatable fraction would not germinate and that these potentially viable spores could then be recovered. The recovered spores should then fail to germinate without heat treatment and should show a heat-activation factor of infinity.

No significant difference between the heat-activation factors for normal and recovered spores was found among seven separations. Results from two separations, for which viability of the recovered spores was also determined, are presented in Table 3.

TABLE 3. THE VIABILITY AND HEAT-ACTIVATION FACTORS OF NORMAL SPORES AND OF RESIDUAL SPORES RECOVERED AFTER A PERIOD OF GERMINATION

	Total count $\times 10^6$/ml	Viable count $\times 10^6$/ml		Viability of unheated spores (%)	Heat-activation factor
		Unheated	Heat-activated		
Normal spores	101·5	200	476	19·7	2·4
Recovered spores, 7 hr germination	33·9	20·8	45·0	6·1	2·2
Recovered spores, 8 hr germination	37·5	22·8	52·4	6·1	2·3

The sterile filtrate obtained after germination was added to a normal suspension before counting and also included in the counting medium, without having any effect on the count. Also, in a second experiment, after the initial germination, the cells were separated from the liquid and a fresh spore suspension added to the liquid. On further incubation the fresh spores germinated normally. Thus it appeared that germination in the original stage was not brought to a standstill by the products of germination.

It is difficult to explain satisfactorily the failure to separate the heat-activatable fraction by this method. It is hoped that it may prove possible to separate the heat-activatable spores by utilising properties which may correlate with heat-activation.

Acknowledgement. The technical assistance of Miss P. Collins is gratefully acknowledged.

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SHORT COMMUNICATION

The effect of yeast cells in the heating medium on the heat resistance of *Bacillus stearothermophilus* spores

A. M. COOK AND R. J. GILBERT

THE heat resistance of bacterial spores is greatly influenced by the nature of the medium in which the spores are heated. Organic matter present during heating can afford protection to spores and many workers have shown that carbohydrates, proteins and fats can provide this. Dead bacterial cells have protected living cells of the same species subjected to heat (Lange, 1922). No protective effect was found with *Clostridium botulinum* spores when vegetative cells or heat killed spores of the same species were added (Sugiyama, 1951), and similarly for *Bacillus coagulans* spores when vegetative cells were added (Frank & Campbell, 1957). However, the spores of *Clostridium sporogenes* were protected from heat by living cells or spores of micro-organisms of different species (Amaha & Sakaguchi, 1954); killed suspensions had no effect. The experiments reported are part of a series designed to investigate the nature and extent of this protective effect with reference to an acknowledged heat resistant spore. Yeast cells were used as a source of organic matter as they are of uniform size.

EXPERIMENTAL

Spores of *Bacillus stearothermophilus* NCIB 8919 were obtained using the method of Cook & Brown (1964), except that the sporulation conditions were seven days at 60°. Vegetative cells were separated from the spores by washing ten times using a refrigerated centrifuge. The heating and counting techniques used were those described by Cook & Brown (1964) but the recovery medium, antibiotic assay medium with 0·1% starch, was of pH 7·3.

A special moist yeast marketed as "Yeast for B.S.I. C/10 tests"* was used to prepare a 10% dry weight suspension of yeast in distilled water. The suspension was prepared, sterilised by heating at 115° for 20 min and assayed for moisture content using the methods given in B.S. 808: 1938.

RESULTS AND DISCUSSION

Typical time survivor curves for *B. stearothermophilus* spores heated in water and in a killed yeast suspension are given (Fig. 1). Both curves show a characteristic period of heat activation followed by an exponential death rate. The exponential part of the curve for spores heated in the presence of yeast is less steep than that of the control curve, and when both regression lines are extrapolated back to zero time they show nearly the

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* The Distillers Co. Ltd., Cheese Lane, Bristol.

same extrapolation number (Alper, Gillies & Elkind, 1960). This would indicate that the yeast cells afford some protection from heat to the *B. stearothermophilus* spores. If the difference between the slopes was due to initial differences in rate of heat transfer, then the exponential parts of each curve would be parallel. Thermocouple readings indicated that 115° was reached in less than 1 min for spores in water and in less than 2 min for spores heated in the yeast suspension.

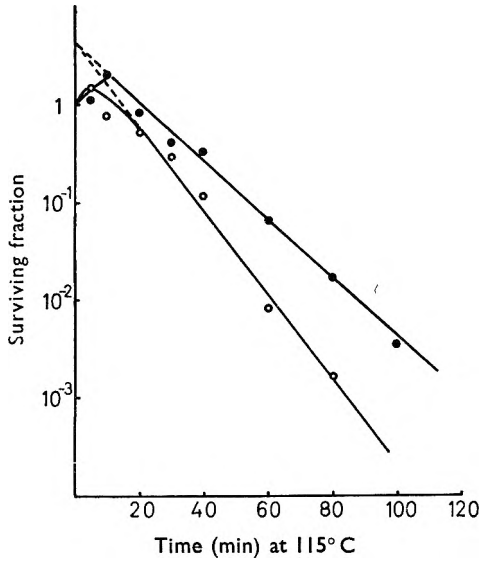

FIG. 1. Effect of heating at 115°C in different substrates upon the colony count of *B. stearothermophilus* spores. ○ Water ● 10% (dry weight) yeast suspension.

It was thought unlikely that yeast carried over from the heated suspension accounted for the increased recovery since the medium used already contained 0·3% yeast extract. This was confirmed by heating spores in water and subculturing into antibiotic assay medium containing additional heated yeast suspension; no significant increase in colony counts was observed. Experiments are continuing to examine this protective effect quantitatively and qualitatively.

Acknowledgement. One of us (R.J.G.) is a recipient of a Nuffield Foundation Food Science Scholarship.

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The influence of pH upon the antifungal activity of phenol and benzoic acid

B. E. WINSLEY* AND V. WALTERS†

The toxicity of phenol and benzoic acid to spores of *Aspergillus niger* is due to the unionised molecules.

WITH antimicrobial agents which are more active in the unionised form, e.g. organic acids (Cruess & Richert, 1929; Rahn & Conn, 1944), alteration in the pH of a reaction mixture will cause changes in the percentage ionisation and hence will influence the activity of these toxic agents. This effect is reported for the action of phenol and benzoic acid on *Aspergillus niger* spores.

Experimental

Buffered test solutions. (i) Phenol 2% in 0·05 M boric acid and 0·05 M potassium chloride solution adjusted to the required pH with 0·2 N sodium hydroxide. (ii) Benzoic acid 0·19% in 0·05 M potassium hydrogen phthalate solution adjusted to the required pH with 0·2 N sodium hydroxide or 0·2 N hydrochloric acid.

The pH values of the test solutions and of the reaction mixtures were determined with a "Cambridge" pH meter calibrated with standard buffers. Addition of the spore suspension to the test solutions caused a pH change of less than 0·1 unit and no further change occurred during the reaction period. The methods of preparation of spore suspensions and of evaluation of the fungicidal activity of the test solutions were as described by Winsley (1964) and are similar to those of Chauhan & Walters (1962). The reaction temperatures were 35 $\pm$ 0·1° with phenol and 25 $\pm$ 0·1° with benzoic acid.

Fungistatic activity. To appropriate quantities of concentrated buffered Oxoid Sabouraud liquid medium were added solutions of phenol 1% or benzoic acid 0·2%, water to 9 ml, and 1 ml of a suspension containing 5×10^7 *A. niger* spores. A series of concentrations was made for each pH and replicated ten times. The tubes were incubated at 36° for 5 days. Growth did not occur after this time in any tube previously negative. Controls without the test substances were made.

Results

Previous experiments showed that unmodified Sabouraud liquid medium of pH about 5·4, incubated at 36°, provided optimal recovery conditions for both damaged and undamaged spores. Approximately 99% of the latter were viable.

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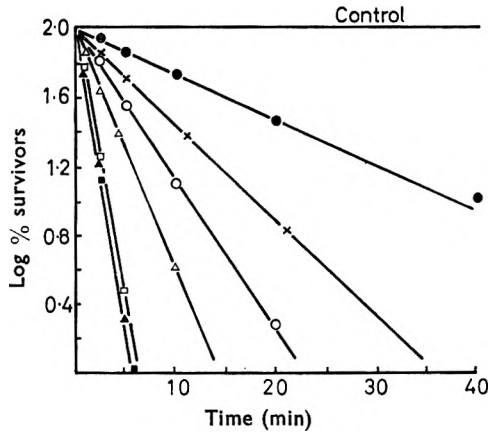

FIG. 1. Log survivor-time curves for the viability of *A. niger* spores in 1% phenol solutions at pH 5·3–9·5. Reaction temperature 35°. Control: buffer solution without phenol. □ = 5·3 (99·99). ▲ = 6·95 (99·5). ■ = 8·1 (98·7). △ = 9·1 (86·3). ○ = 9·2 (83·4). × = 9·3 (79·9). ● = 9·5 (71·5). Figures in brackets are percentages of phenol unionised.

Linear log survivor-time curves were obtained for the viability of *A. niger* spores in 1% phenol solutions buffered at pH 5·3 to 9·5 (Fig. 1). Fig. 2 shows the relationship between the pH and the percentage of undissociated phenol with time for 50 and 99% mortalities. Fig. 5 shows the same relationship for benzoic acid.

The calculated regressions of log percentage undissociated phenol and benzoic acid on log time to cause 50% and 99% mortalities are linear (Figs 3 and 6 respectively).

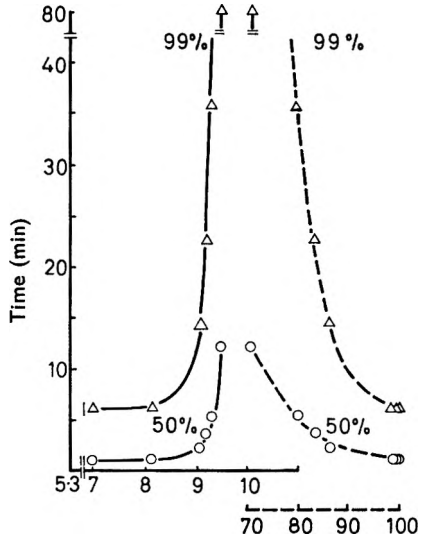

FIG. 2. Relationship between time for 50 and 99% mortality of *A. niger* spores in 1% phenol solution and pH (solid lines) and percentage of unionised phenol molecules (broken lines). Temperature 35°.

The times for 50% and 99% mortalities in a 1% solution of phenol containing only undissociated molecules were calculated to be 0·87 and 5·1 min, respectively. For benzoic acid the corresponding times are 15·9 and 32·8 min. With benzoic acid, as the reaction pH approached and exceeded the pK_a value (4·2), there was a decrease in mortality for a

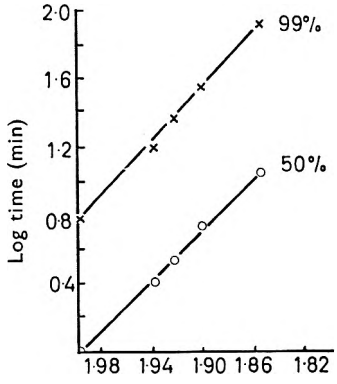

FIG. 3. Relationship between log percentage unionised phenol and log time for 50 and 99% mortality of *A. niger* spores in 1% phenol solution at 35°.

given contact time; curvilinear log survivor-time curves were obtained (Fig. 4). At least 98% of the spores were viable after exposure to buffer solutions alone (pH 2·2, 5·3 and 9·5) for 60 min (Figs 2 and 4).

The minimum inhibitory concentrations of phenol and benzoic acid at different pH values are summarised in Table 1.

TABLE 1. EFFECT OF pH ON THE FUNGISTATIC CONCENTRATION OF PHENOL AND BENZOIC ACID ON *A. niger* SPORES

pH (reaction mixture)	Percentage molecules unionised	Min. inhibitory conc. (%)	Conc. of unionised molecules in m.i.c. (%)
Phenol			
5·4	> 99·99	0·066	—
7·0	99·87	0·068	—
9·0	88·1	No growth in control	
Benzoic acid			
2·2	99·01	0·017	0·0168
4·0	61·32	0·032	0·0184
4·6	28·47	0·065	0·0185
4·9	16·63	0·11	0·0182
5·1	11·19	0·15	0·0168

Discussion

The shapes of the log survivor-time curves obtained respectively with solutions of phenol and benzoic acid of constant concentration but of different pH values (Figs 1 and 4) were identical to those obtained by

changing the concentration of these agents in unbuffered solutions in water (Winsley, 1964). Increasing the pH value of the reaction mixtures resulted in an increase in the amount of ionised phenol and benzoic acid present*; this was accompanied by a decrease in the death-rate. Apart from change of pH, all other reaction conditions were constant, therefore the change in death-rate with pH must have been due to change in the concentration of the effective form of the fungicide. The slopes of the regressions in Figs 3 and 6 may be considered as concentration coefficients for unionised molecules. To cause 50 and 99% mortalities these are 7·7 and 8·2 for phenol and 3·8 and 4·1 for benzoic acid.

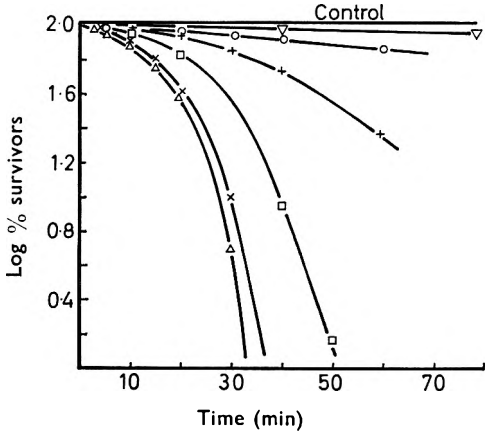

FIG. 4. Log survivor-time curves for the viability of *A. niger* spores in 0·18% benzoic acid solutions at pH 2·3–5·3. Reaction temperature 25°. Control: buffer solution without benzoic acid. △ = 2·3 (98·76). × = 2·9 (95·2). □ = 3·25 (89·9). + = 3·7 (76·0). ○ = 4·05 (58·5). ▽ = 5·3 (7·4). Figures in parentheses are percentages of unionised benzoic acid.

When the toxic agent is incorporated in the recovery medium, the pH of the medium affects not only the degree of ionisation of the agent, but also the growth of the fungus.

In general, the range of pH of the medium over which growth and germination of fungi occur is pH 3 to 8 with an optimum of 4·5 to 6·5 (Cochrane, 1958). The effect of ionisation on the fungistatic concentration of phenol could not be assessed since growth did not occur in the control at pH 9·0. With benzoic acid, whereas the concentration required to inhibit growth increased with pH, the corresponding concentration of unionised molecules remained almost constant from pH 2·2 to 5·1 (Table 1). Rahn & Conn (1944) likewise found that the inhibitory concentration of benzoic acid against *Saccharomyces ellipsoideus* increased with pH, varying from 0·042% at pH 3·5 to 1·27% at pH 5·8, whereas the corresponding percentages of unionised molecules were 0·035% and 0·028%. In contrast, Evans & Dunbar (1964) found that

* Ionisation $\% = \frac{100}{1 + \text{antilog } (pK_a - pH)}$ (Albert, 1965)

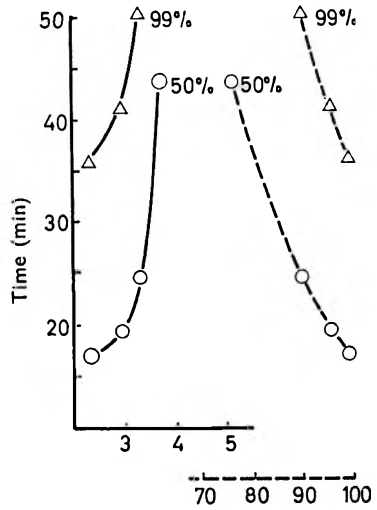

FIG. 5. Relationship between time for 50 and 99% mortality of *A. niger* spores in 0·18% benzoic acid solution and pH (solid lines) and percentage of unionised benzoic acid molecules (broken lines). Temperature 25°.

a decreasing amount of unionised molecules inhibited the growth of *A. niger* as the pH was raised above the pK_a, presumably because the benzoate anions possessed some of the activity of the intact molecules; 0·02% of undissociated acid was inhibitory at pH 4·0 but at pH 5·0, 0·006% was sufficient.

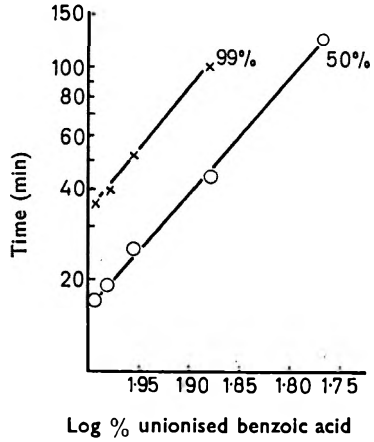

FIG. 6. Relationship between log percentage unionised benzoic acid and log time for 50 and 99% mortality of *A. niger* spores in 0·18% benzoic acid solution at 25°.

The results reported here indicate that unionised molecules are the predominantly active form; penetration to or beyond the cell membrane is more easily achieved by neutral molecules (Davson & Danielli, 1952; Albert, 1963, 1965).

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Effect of concentration and temperature on the inactivation of a bacteriophage by phenol

W. R. L. BROWN, A. M. COOK AND J. ODURO-YEBOAH

The concentration of phenol affects not only the rate of inactivation of phage but also the shape of the survivor-time curves. The shape of the curves obtained indicates that phenol may inactivate phage in two suggested stages involving different components of the phage protein. The concentration exponent for the inactivation depends on the level of inactivation chosen. The temperature coefficient is independent of the level of inactivation. The temperature of incubation of phenol treated phage does not obviously affect the recovery of the phage.

A NUMBER of published reports of studies of the inactivation of viruses have shown that the rate of inactivation may not follow the kinetics of a first order reaction. Gard (1960) and Hiatt (1964) have commented on the possible significance of the shapes of inactivation curves for viruses but little information is available on the effects of the concentration of inactivating agent and the temperature of the reaction on the kinetics of virus inactivation. The effects of these factors on the inactivation of a coliphage by phenol have now been investigated.

Experimental and results

The host organism (*Escherichia coli*) and the bacteriophage (Coliphage T6r) were cultivated by the methods described by Cook & Brown (1963). Inactivation of the bacteriophage was investigated by the procedure previously described (Brown, Cook & Oduro-Yeboah, 1964).

EFFECT OF CONCENTRATION OF PHENOL

Bacteriophage inocula for all the reaction mixtures were taken from the same phage stock (stored at 4°) which had an initial phage titre in the reaction mixtures of approximately $2{\cdot}5 \times 10^7$ phage particles per ml. The number of surviving infective phage particles was estimated by the surface drop method of counting. The course of the inactivation of the bacteriophage by various concentrations of phenol in aqueous solution at 25° (± 0·05°) are shown on a semilog plot in Fig. 1A. Fig. 1B shows the same results on a log-log plot. The relationship between log contact time and log phenol concentration for 90·0%, 99·0% and 99·9% inactivation (Fig. 2A) was linear ($r = 0{\cdot}9947$, 0·9769 and 0·9793 with degrees of freedom of 4, 4 and 3 respectively) and the calculated slopes (or concentration exponents) for the regressions were −8·628, −11·628 and −13·164 respectively.

INFLUENCE OF TEMPERATURE OF REACTION

Inactivation of the bacteriophage by 2·0% (w/v) aqueous phenol at various temperatures was investigated using a bacteriophage stock which

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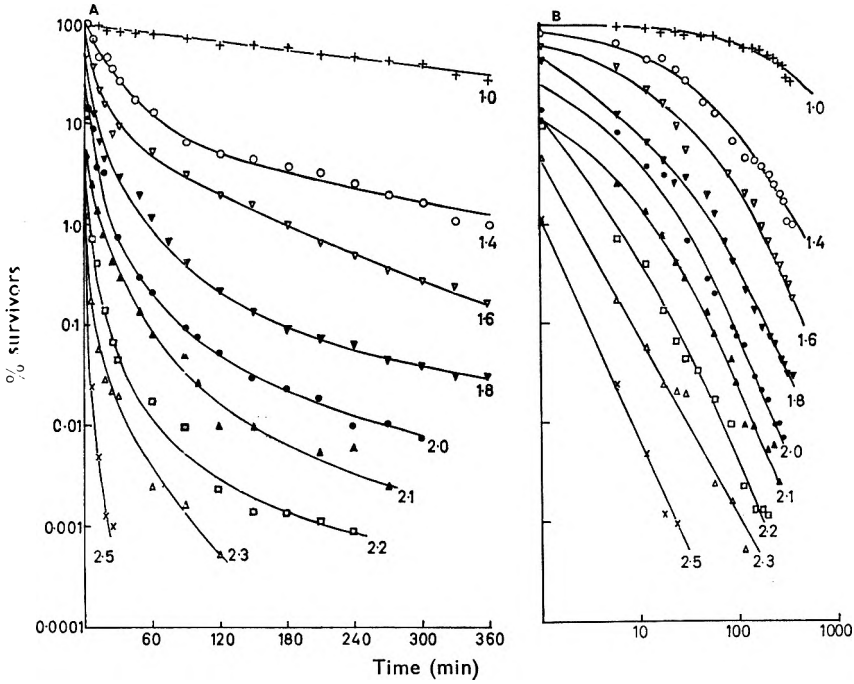

FIG. 1. Inactivation of coliphage T6r by phenol (25°). A, log% survivors plotted against time. B, log % survivors plotted against log time. Figures on curves are % concentrations of phenol.

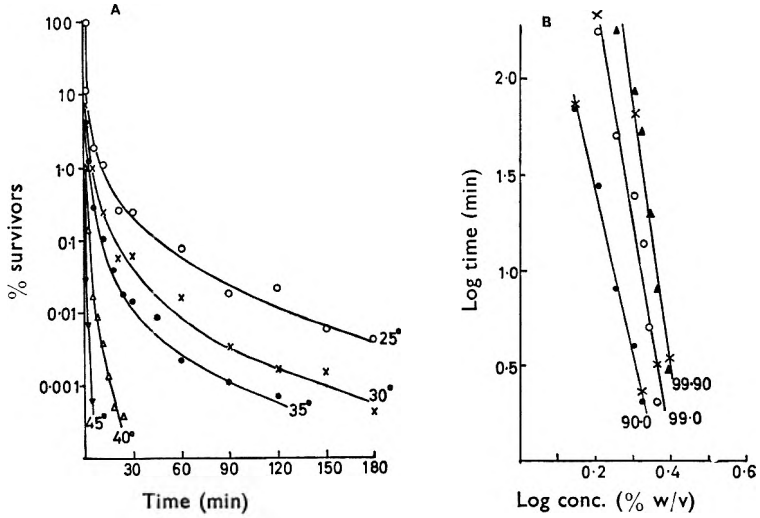

FIG. 2. A. Relationship between log contact time and log concentration of phenol for different levels of inactivation of coliphage T6r (25°). (×——×, calculated regression of log contact time upon log concentration of phenol). Figures on curves are % kill. B. Influence of temperature on inactivation of coliphage T6r by 2·0% phenol. Figures on curves are temperature °C.

had an initial titre in the reaction mixtures of approximately 6×10^7 phage particles per ml. Surviving phage particles were estimated by the soft agar layer method of counting. The reaction mixtures were maintained within $\pm$ 0·05° of the required temperature and the results are illustrated in Fig. 2B. Using the contact times required to effect 99, 99·9, 99·99 and 99·999% inactivation, the temperature coefficient (θ) for the inactivation of the phage was calculated from the formula $\theta^{T_1-T_2} = \frac{t_1}{t_2}$, where t_1 and t_2 are the times producing the required % inactivation at temperatures T_1 and T_2 respectively (Table 1).

TABLE 1. TEMPERATURE COEFFICIENTS (θ) FOR THE INACTIVATION OF COLIPHAGE T6r BY 2% W/V PHENOL CALCULATED FOR VARIOUS % INACTIVATION

Temperature range, °C	Temperature coefficient (θ) for % inactivation of phage			
	99	99·9	99·99	99·999
25–30	1·15	1·17	1·17	—
30–35	1·13	1·12	1·12	1·09
35–40	1·18	1·32	1·33	1·41
40–45	1·21	1·34	1·32	1·28

EFFECT OF THE TEMPERATURE OF INCUBATION DURING RECOVERY

Duplicate experiments were performed for the inactivation of the phage by 2·0% (w/v) phenol at 25° and 30°. For each reaction temperature one set of plates was incubated at 37° and the other set at 26°. The results are shown in Table 2.

TABLE 2. EFFECT OF THE TEMPERATURE OF INCUBATION ON THE RECOVERY OF COLIPHAGE T6r AFTER EXPOSURE TO 2% W/V PHENOL AT 25° AND 30°

Temperature of reaction mixture °C	Contact time (min)	% surviving phage at incubation temperature of °C	
		26	37
25	0	100·00	100·00
	30	0·139	0·080
	60	0·076	0·061
	120	0·025	0·018
	180	0·0010	0·0012
30	0	100·00	100·00
	12	0·256	0·202
	30	0·053	0·054
	60	0·016	0·023
	120	0·0026	0·0017

Discussion

The concentration of phenol to which the phage was exposed had a marked effect on the kinetics of the inactivation of the phage. The lowest concentration of phenol tested (1% w/v) showed a rate of inactivation which followed the kinetics of a first order chemical reaction. Increasing the concentration of phenol produced an increase in the initial rate of

inactivation; the rate became less as the time of contact increased. That fraction of the inoculum undergoing this rapid inactivation also increased as the phenol concentration rose until at 2·3 and 2·5% w/v the relationship between log % survivors and log contact time was linear. This relationship has previously been shown to persist, at least for 2·5% w/v phenol, until the inactivation of the phage is virtually complete (Brown & others, 1964).

Inactivation of poliovirus by formaldehyde shows a deviation from first order reaction kinetics similar to that shown here for concentrations of phenol of 1·4–2·2% w/v. Salk & Gori (1960) attributed the shape of the inactivation curve partly to the high resistance of a small fraction of the viruses present and partly to the aggregation of virus particles during the course of the reaction. They suggested that particles embedded in an aggregate were protected from inactivation. Gard (1957, 1960) suggested that the fall in the inactivation rate resulted from the formaldehyde causing hardening of the protein coat of some of the virus particles. Such hardening reduced the permeability of the protein and the rate at which formaldehyde can reach the vital deoxyribonucleic acid (DNA) core of the particle. Heicken & Spicher (1956) arrived at similar conclusions for the inactivation of the T-even coliphages with formaldehyde.

It is possible that the shape of the inactivation curves for coliphage T6r by phenol is due to effects similar to those suggested for formaldehyde. However, the effects of different concentrations of phenol on the shape of the curves suggest that the shape reflects the mode of action of the phenol on the phage and that at least two stages are involved. One stage occurs at low concentrations of phenol and gives a rate of inactivation which is slow but exponential with time. High concentrations of phenol would produce the other stage of inactivation at a rate which gives a linear relationship between log % survivors and log contact time. The transition in the shape of the inactivation curves with increasing phenol concentration from a linear semilog relationship to a linear log-log relationship then reflects the increasing predominance of the second stage as the phenol concentration increases. The possibility that there are two stages of inactivation by phenol is supported by the fact that phage protein consists of serologically distinguishable components, one associated with the protein coat surrounding the DNA in the "head" of the phage particle, another with the protein which constitutes the "tail" of the particle (Lanni & Lanni, 1953). Further, different proteins are precipitated by different concentrations of phenol (Cooper & Sanders, 1927, 1928). Denaturation of the phage tail protein will prevent the adsorption of the phage to the host cells (Hershey & Chase, 1952) and probably will affect the mechanism by which the phage DNA penetrates the host cell wall. The tail protein seems the more likely site of attack by low concentrations of phenol. Denaturation of the head protein may also inactivate the phage and earlier results (Cook & Brown, 1964) indicate that high concentrations of phenol also affect the association between the protein coat of the phage and its DNA.

The increase in the value of the concentration exponent for the

inactivation of the phage by phenol, when the exponent is calculated for increasing levels of inactivation, is confirmed by the previously reported values for the exponent of −14·659 calculated from mean single survival times and −15·140 from mean extinction times (Cook & Brown, 1963). This relationship is another manifestation of the effect of the concentration of phenol on the kinetics of the inactivation of the phage and it is interesting that Jordan & Jacobs (1944) found the same relationship for the inactivation of *E. coli* by phenol.

Increasing the temperature of the reaction between phage and phenol produced an increase in the rate of inactivation of the phage, but the changes in the shape of the inactivation curves corresponded to those produced by increasing the concentration of the phenol. No obvious alteration in the mechanism of inactivation therefore resulted from an increase in the temperature at which the reaction was carried out. The values of the temperature coefficient for the inactivation of the phage were independent of the level of inactivation chosen within any of the temperature ranges tested. A similar effect has been reported by Tilley (1942) and Jordan & Jacobs (1946) for the action of phenol on *E. coli*.

The temperature of incubation of phage culture plates had no effect on the number of phage particles recovering after exposure to phenol. It should be noted, however, that as the plaque counts were made using the soft agar layer method, the adsorption of the phage particles to their host took place at approximately the same temperature (46°) in all the experiments. The temperature at which adsorption takes place can affect the efficiency with which the phage infects its host (Garen & Puck, 1951) and it is at this stage that different temperatures may affect the recovery of phenol-damaged phage particles.

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Datura sanguinea R. and P., its seeds

T. E. WALLIS

The seeds of the Peruvian plant *Datura sanguinea* R. and P. contain hyoscine and are used in Peru as stramonium is used in Britain. Their structure is described and is compared with that of the seeds of stramonium and of *D. fastuosa* with a view to their identification either unground or in powder.

SOME years ago the seeds of *Datura sanguinea* R. and P. were examined by Drey & Foster (1953) for their content of solanaceous alkaloids and were found to contain about 0·172% total alkaloid which was almost entirely hyoscine. These seeds have been used in Peru since early times and J. Lindley in *The Vegetable Kingdom* (1847) when discussing the uses of seeds of *Datura spp.* especially *D. tatula* and *D. metel*, gives the following information "the seeds are the most powerful part of these plants, and are stated by some authors to have been used by the priests of the Delphic Temple to produce those frenzied ravings which were called prophesies. Such a practice certainly obtains, or obtained, in the Temple of the Sun in the city of Sagomozo, where the seeds of the Floripondio (*Datura sanguinea*) are used; the Peruvians also prepare from them an intoxicating beverage which stupifies if taken much diluted; but, when strong, brings on attacks of furious excitement."

MATERIAL

The seeds used for this investigation were part of the sample used by Drey & Foster for the determination of their alkaloidal content.

MACROSCOPICAL

The seeds are dark greyish brown, flattened wedge-shaped, either subreniform in outline with a slightly pointed end where the funicle was attached, Fig. 1 B, or the testa is extended towards the placenta giving a mutton-chop shape to the seed, Fig. 1 A; the two forms are present in about equal numbers. They are hard, about 10 mm long, 5 to 6 mm broad and 4 mm thick at the convex margin. The testa has a slightly granular surface with 4 or 5 wrinkled ridges at the thicker convex edge; the hilum is at the pointed end, see Fig. 1 A and B. The embryo, which is about 15 mm long and 1·5 mm in diameter, is embedded in a white oily endosperm and is narrowly cylindrical with the hypocotyl-radicle pointing towards the hilum and the two narrow cotyledons, placed face to face and curved until their tips nearly meet the radicle, see Fig. 1 E, F and G. The seeds are odourless and have a bitter taste; 100 seeds weigh about 4·0 g.

From the Museum of the Pharmaceutical Society of Great Britain, London, W.C.1.

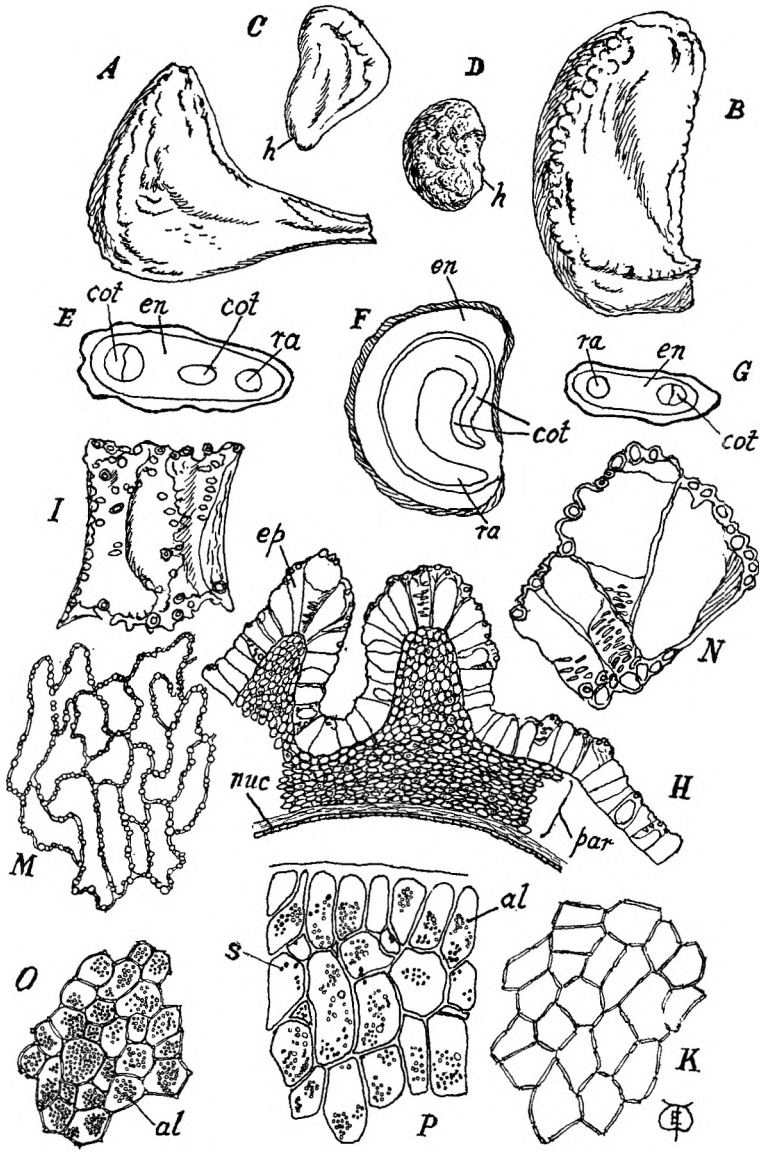

FIG. 1. Seeds of *D. sanguinea* R. and P., A, mutton-chop shape and B, reniform shape; C, seed of *D. fastuosa*; D, seed of *D. stramonium*. E to P, seed of *D. sanguinea*, E, longitudinal section; F, section parallel to the flat faces; G, transverse section; I, epidermal cell from the flat face in vertical section; N, epidermal cells from summit of a ridge in vertical section; H, transverse section of the testa; K, nucellus, surface view; M, epidermis in surface view from flat face; O, epidermis of the endosperm; P, transverse section of the endosperm; *al*, aleurone grain; *cot*, cotyledon; *en*, endosperm; *ep*, epidermis; *h*, hilum; *nuc*, nucellus; *par*, parenchyma; *ra*, radicle; *s*, starch. A to G, × 4; I and N, × 100; M and H, × 40; O, P and K, × 175.

MICROSCOPICAL

Testa. The seed has one seed-coat—the testa. The epidermal cells of the flat surfaces in surface view have irregularly wavy and pitted anticlinal walls with superior outgrowths or processes interlocking with those of neighbouring cells; there are similar, less thickened, processes from the base of the cells, Fig. 1 I. The cells vary in length from about 110 to **375** to 720 μ and about 40 to **60** to 100 μ wide, the width often varying much in the same cell; their height is about 125 to 150 μ; in vertical section they are sub-rectangular. On the ridges at the edges of the seed, where the testa is strongly folded over the ridges, the shape of the epidermal cells is modified; here in vertical section they are triangular in outline, Fig. 1 N, and of much greater height measuring about 250 to 275 μ; in surface view they are sub-rectangular about 85 to **250** to 325 μ long by 50 to 65 to 100 μ wide and the anticlinal walls have much less prominent processes. The thickness of the walls of all the epidermal cells is about 8 to 10 μ and the bases of all the epidermal cells are pitted with oval pits. Beneath the epidermis are about 12 to 15 layers of parenchyma consisting of thin-walled rounded cells, about 30 to **45** to 60 μ in diameter, having circular or oval pits in their walls; this is followed by a layer about 30 μ wide of collapsed cells and a well-marked epidermis of the nucellus consisting of rectangular tabular cells about 30 to **40** to 60 μ long and about 20 μ high in transverse section and in surface view sub-rectangular and about 38 to 45 μ in either direction, Fig. 1 K. All the cells of the testa and of the nucellus are lignified.

Endosperm. The endosperm is composed of polyhedral to rounded cells about 40 to **60** to 80 μ in diameter with a few small intercellular spaces. They contain fixed oil as an oil-plasma and numerous small aleurone grains measuring about 3 to 6 μ, most of them being small and amorphous and some of the larger ones enclosing a crystalloid and one or more minute globoids. A few small starch granules, mostly measuring about 4 μ, are present in many of the cells, being starch which has not been converted into oil (Jost & Gibson, 1907). The aleurone grains and the starch are best observed by mounting a defatted section in iodine water. In a section mounted and warmed in water, very numerous globules of oil separate from the plasma and are stained deep red by tincture of alkanet, Fig. 1 O and P.

Embryo. The embryo consists of cells rather smaller than those of the endosperm, about 20 to 40 μ in diameter; palisade and procambium are all evident in the embryo. The cell contents are similar to those of the endosperm.

COMPARISON WITH SEEDS OF OTHER SPECIES OF *Datura*

The unground seeds of species of *Datura* used medicinally are easily recognised as shown in Fig. 1 A, B, C and D, where seeds of three medicinal species are drawn to the same scale. Seeds of *D. stramonium* are reniform, nearly black and 100 seeds weigh about 0·85 g; seeds of *D. fastuosa* are ear-shaped, brownish yellow and have about the same weight as seeds of *D. stramonium.* As stated above, the seeds of *D. sanguinea*

are much larger, dark greyish brown, sub-reniform to mutton-chop shaped and 100 weigh about 4·0 g.

The powders of these seeds may be distinguished by the characters of the epidermis, see Fig. 2.

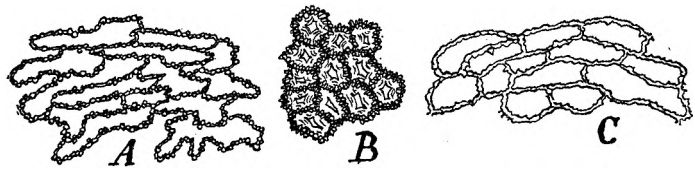

FIG. 2. A, epidermis of flat face of seed of *D. sanguinea*; B, epidermis of seed of *D. stramonium*; C, epidermis from a ridge of the seed of *D. sanguinea*; All × 40.

(1) *D. stramonium.* The epidermal cells are darkly pigmented, polygonal in surface view, measure about 95 to **125** to 160 μ and have numerous small superior outgrowths. These cells also have a band of very heavy thickening in the equatorial position giving a lumen of a somewhat hour-glass shape (Moll & Janssonius, 1923) which appears stellate in surface view.

(2) The epidermal cells of *D. fastuosa* closely resemble those of *D. stramonium*; they are less pigmented and are slightly larger, measuring up to 190 μ (Timmerman, 1927).

(3) The epidermal cells of *D. sanguinea* from the flat surfaces are narrow and much elongated, about 320 to 720 μ by 50 to 80 μ, with irregularly wavy walls; those from the ridges are sub-rectangular, about 80 to 300 μ by 50 to 100 μ. There is no equatorial band of heavy thickening.

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SHORT COMMUNICATION

The biosynthesis of tigloyl esters in *Datura*

W. C. EVANS AND J. G. WOOLLEY

TIGLIC acid esters have been isolated from various *Datura* species and the translocation of 3,6-ditigloyloxytropane, 3,6-ditigloyloxytropan-7-ol and meteloidine from the roots to the leaves of *D. innoxia*, and the subsequent metabolism of these alkaloids, has already been described (Evans & Griffin, 1964). The origin of the tiglic acid moiety, however, does not appear to have been investigated.

It has been suggested that the tiglic acid could arise from acetoacetic acid by *C*-methylation followed by reduction and dehydration (Leete, 1963). In animal tissue homogenates, however, α-methylbutyryl-CoA and tigloyl- (or angeloyl-) CoA are intermediates in the degradation of isoleucine to acetate and propionate (Coon & Abrahamsen, 1952; Coon, Abrahamsen & Greene, 1952; Robinson, Bachhawat & Coon, 1956). It has also been proposed that isoleucine may be the precursor of α-methylbutyric acid in the α-methylbutyryl ester alkaloids of the *Duboisia* species (Barger, Martin & Mitchell, 1938), whilst in *Ascaris lumbricoides*, it has been shown that α-methylbutyric acid may arise by condensation of acetate and propionate (Saz & Weil, 1960).

In this work we have administered sodium acetate-2-^{14}C (0·025 mc), sodium propionate-1-^{14}C (0·025 mc), isoleucine-1-^{14}C (0·02 mc) and isoleucine-U-^{14}C (0·025 mc) via the roots to groups of 8-months old *Datura meteloides*. Other batches of *D. meteloides* were placed in an atmosphere containing $^{14}CO_2$ (0·025 mc). After 48 hr the roots and aerial parts of the plants were separately dried and the root alkaloids, without dilution with inactive carrier, were isolated by chromatography. The picrates of the separated alkaloids were recrystallised until a constant m.p. and a constant value for the specific activities were obtained. With the exception of the isoleucine-U-^{14}C experiments (Table 1), no labelling of the alkaloids could be detected.

TABLE 1. INCORPORATION OF ISOLEUCINE-U-^{14}C INTO THE ROOT ALKALOIDS OF *D. meteloides*

Alkaloid	Specific activity of picrate (dpm/mM)	Total activity of isolated picrate (dpm)	% Total activity of alkaloid in tigloyl moiety	% Total administered activity incorporated
3,6-Ditigloyloxytropane	1·3 × 10^6	3·3 × 10^4	100	0·06
3,6-Ditigloyloxytropan-7-ol	4·4 × 10^5	1·6 × 10^4	100	0·03
Meteloidine	8·5 × 10^5	4·5 × 10^4	100	0·08
Hyoscine		0		
Hyoscyamine		0		

From the Department of Pharmacy, University of Nottingham.

The results demonstrate a fairly rapid, and appreciable, incorporation of isoleucine-U-^{14}C into the tigloyl moiety of all the relevant alkaloids. Isoleucine-1-^{14}C is not incorporated and this is in accordance with the degradation of this amino-acid in animal tissue. Provided all the administered compounds gain equal access to the site of synthesis, it would appear that isoleucine is a more immediate precursor of the tigloyl moiety than either acetate or propionate. No significant labelling of the tropane ring was obtained in any instance, but in experiments of longer duration the incorporation of acetate has been adequately demonstrated by a number of workers.

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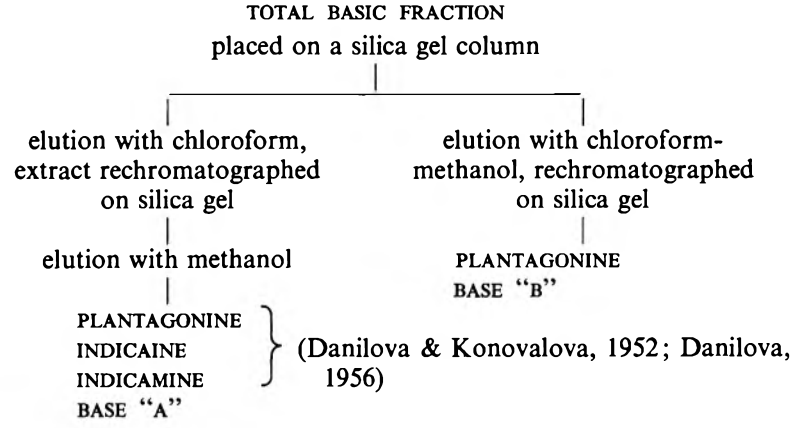
SHORT COMMUNICATION

Phytochemical studies of Egyptian *Plantago* species (alkaloids)

Z. F. AHMED, A. M. RIZK AND F. M. HAMMOUDA

THE alkaloidal content of eight species of *Plantago* common in Egypt has been examined. Authentication of the species has already been described (Ahmed, Batanouny & Hammouda, 1965). Extraction of the defatted seeds of *Plantago albicans* with 96% ethanol has yielded a basic fraction which was isolated either with mineral acid in the conventional manner or by precipitation with ammonium reineckate (Lee, 1960).

The behaviour of this extract on two-dimensional thin-layer chromatography is shown in Table 1. Column chromatography on silica-gel as outlined below has yielded five crystalline fractions.



Plantagonine had m.p. 220–222°, $[\alpha]_D^{20} + 29{\cdot}7°$ (*c*, 1·2, ethanol) (Danilova & Konovalova, 1952, cite m.p. 218–220°, $[\alpha]_D + 30{\cdot}8$).

The hydrochloride m.p. 227–229° (Danilova & Konovalova, 1952, cite m.p. 228–230°) gave no depression on admixture with an authentic specimen. The infrared spectra of the hydrochloride and of an authentic specimen were identical.

The picrate had m.p. 158–160° (Danilova & Konovalova, 1952, cite m.p. 159–160°).

Indicaine, an oil, gave a picrate m.p. 148–150° (Danilova & Konovalova, 1952, cite m.p. 149–150°). Oxidation of the oily base with silver oxide (Danilova, 1956) yielded plantagonine.

Indicamine picrate had m.p. 125–127° (Danilova & Konovalova, 1952, cite m.p. 124–127°).

From the Medicinal Plants and Crude Drugs Research Unit, National Research Centre, Dokki, Cairo, U.A.R.

TABLE 1. Rf* OF *Plantago albicans* BASIC FRACTION (TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY) ON KIESELGEL GF 254 (250 μ)

Spot No.	Base	Rf		Fluorescence colour (200–400 mμ)
		Solvent A	Solvent B	
1	Base "A"	0·03	0·05	Pale yellow
2	Plantagonine	0·06	0·07	" "
3	Base "B"	0·11	0·12	" "
4	Unidentified	0·28	0·07	Light bluish yellow
5	"	0·37	0·44	Blue
6	"	0·48	0·28	Greenish yellow
7	"	0·60	0·65	Light greenish yellow
8	Indicamine	0·72	0·45	Violet
9	Indicaine	0·80	0·54	"
10	Unidentified	0·84	0·92	Yellow
11	"	0·14	0·09	Faint violet
12	"	0·29	0·15	Yellowish green
13	"	0·37	0·54	Blue

* Mean values of five determinations.
Solvent A: Ether: methanol: diethylamine (85:10:5) (Sandberg & Michel, 1962)
" B: Chloroform: methanol: diethylamine (92:3:5). (" " ")
N.B.: Spots 5 and 13 may be interchangeable.

Bases A and B have been obtained in crystalline form and characterised as follows.

Base A, m.p. 240°, λ_{max} (ethanol) 280 mμ. Found: C, 75·1; H, 8·9; N, 7·5. $C_{12}H_{17}ON$ requires C, 75·4; H, 8·9; N, 7·4%. Picrate m.p. 280–282° (decomp.). Found: C, 51·1; H, 5·6; N, 13·5. $C_{12}H_{17}NO.C_6H_3O_7N_3$ requires C, 50·9; H, 5·7; N, 13·2%.

TABLE 2. THE BASIC FRACTION OF THE SEEDS OF EGYPTIAN *Plantago* SPECIES (TWO-DIMENSIONAL CHROMATOGRAPHY ON KIESELGEL GF 254)

Species	Total alkaloids %	Alkaloid number												
		1	2	3	4	5	6	7	8	9	10	11	12	13
P. notata	0·02	+	+	+	+							+		
P. crypsoides	0·01	+	+	+	+	+	+							+
P. coronopus	0·02	+	+											
P. crassifolia	0·02	+	+		+	+					+			+
P. major	0·05	+	+	+	+	+				+	+		+	+
P. albicans	0·04	+	+	+	+	+	+	+	+	+	+	+	+	+
P. cylindrica	0·04	+	+	+	+	+								+
P. ovata	0·06	+	+	+	+	+			+		+			+

1: Base "A." 8: Indicamine.
2: Plantagonine. 9: Indicaine.
3: Base "B." 4, 5, 6, 7, 10, 11, 12 and 13: Unidentified.
Running solvents as in Table 1.

Base B, m.p. 176–178°, λ_{max} (ethanol) 239 mμ. Found: C, 69·9; H, 8·6; N, 10·3. $C_8H_{12}NO$ requires C, 69·6; H, 8·7; N, 10·1%. Picrate m.p. 268–269°. Found: C, 45·9; H, 4·2; N, 15·5. $C_8H_{12}ON$. $C_6H_3N_3O_7$ requires C, 45·8; H, 4·1; N, 15·3%.

The basic fraction of seven other species has been subjected to two-dimensional thin-layer chromatography with the results shown in Table 2.

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Carvone in the developing fruits of *Anethum graveolens* L. and *Carum carvi* L.

T. J. BETTS

The carvone content of the developing fruits of *Anethum graveolens* L. (dill) and *Carum carvi* L. (caraway) has been determined spectrophotometrically over three seasons. Three or four weeks after pollination, carvone in the fruits attains a level of 11–20 mg per 100 caraway fruits, or 4–9 mg per 100 dill fruits. Variations in the carvone content of the essential oils distilled from these fruits are probably due to variable quantities of limonene.

THE principal flavouring constituent of the umbelliferous fruits *Anethum graveolens* L. (dill) and *Carum carvi* L. (caraway), (+)-carvone, forms up to 63% of the essential oils of these two fruits (B.P.C.). The other main constituent, (+)-limonene, is probably the direct biogenetic precursor of (+)-carvone (Birch, 1963). The oils have similar physical constants and may be indistinguishable, although dill oil may contain small amounts of aromatic compounds such as dillapiole (Gupta, Chandra & Zaidi, 1955). The present communication examines the production of carvone in the fruits of these closely related species.

Experimental

Plant material. Caraway and dill plants, grown in the Myddelton House drug garden, Enfield, Middlesex, were identified by their botanical characters, the macroscopy of their fruits and the constituents of their essential oils. Compound umbels were marked when most of their flowers were open and insect pollination was taking place. Fruits were then sampled at various times after being marked, until they ripened and dropped. Samples were taken in this fashion during 1962, 1963 and 1964.

Distillate extracts. Each sample was of the same population of 100–200 accurately counted fresh entire cremocarps (fruits) taken from about five compound umbels on different plants. At the final, ripe stage, two mericarps were counted for each cremocarp. Carpophores and small amounts of pedicel were included with the cut off fruits, together, during the first week after pollination, with any petals remaining. Only healthy plants and full sized fruits were sampled.

The fruits were weighed and steam distilled the same day by co-distilling with about 50 ml water until 5 ml liquid remained in the still; the aqueous condensate was passed directly into a separating funnel containing 4 ml n-hexane. When the distillation was completed (40–60 min), the condenser was washed out into the separating funnel with 1 ml n-hexane and a little water. The funnel contents were shaken gently and

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allowed to separate and the separated hexane layer diluted to volume with hexane in a volumetric flask. In the first weeks of fruit development this volume was 5 ml; in later weeks, 10 or 20 ml, this being made up by re-extracting the aqueous condensate with more hexane.

In both 1962 and 1963, two identical samples were taken, the second being used to determine the loss of weight on heating at 105°. This value was subsequently corrected for the essential oil content of the sample to provide a value for the dry weight of each sample (fresh weight minus water only). The total oil content was taken as twice that of the carvone found.

Thin-layer chromatography of the above hexane extracts was carried out as previously described (Betts, 1964).

Carvone assay. A modification of the method of Stenlake & Williams (1957) was used. A suitable aliquot of the hexane extract of the distillate (0·5–2 ml) was diluted to 10 ml with n-hexane, absolute ethanol (5 ml) was added, and the solution was refluxed (4½ hr) with glacial acetic acid (0·5 ml) and Girard's Reagent T (200 mg). After cooling, N sodium hydroxide (7·5 ml) was added and the mixture was transferred to a separating funnel with water and ether. The organic phase was extracted three times with water, each extract being washed with ether. The combined aqueous extracts were diluted with water to a suitable volume (200–500 ml) and the absorbance determined at approximately 273 mμ. A blank assay was made with 10 ml n-hexane and used as the reference solution. The amount of carvone present in the sample was calculated as follows:

$$\text{Carvone, mg per 100 fruits} = \frac{d \times v}{1{\cdot}267 \times n}$$

Where d = absorbance reading at peak; v = volume (ml) of diluted aqueous extract prepared for reading d; n = number of fruits represented by v (i.e., by the hexane aliquot taken for assay).

The high extinction value of the water-soluble carvone complex [E(1%, 1 cm) 1267] means that the assay is sensitive enough to work with about fifteen developed fruits.

Results and discussion

Similar results were obtained for carvone assays on the developing fruits over three seasons, and illustrative figures are quoted in Table 1. At 1 week after pollination, caraway fruits contained little or no carvone, but by the third week 10 mg or more per 100 fruits was present. In subsequent weeks the carvone content varied between 12–20 mg per 100 fruits in 1962 and 11–14 mg in 1964. Dill fruits initially contained more carvone (1–2 mg per 100 fruits) than caraway fruits but about 3 weeks after pollination the carvone content was only 5–9 mg per 100 fruits in 1962 and 4–6 mg in 1964. These results support Luyendijk's observation (1957) that as the two fruits develop "the content of carbonyl compounds increases from traces until the specific level for the species is reached".

The distinction between carvone content expressed in terms of dry weight and as content of 100 fruits arises because the carvone content rises more rapidly than does the dry weight. Thin-layer chromatography confirmed that carvone was present at all stages of the development of both dill and caraway fruits. Dillapiole was not observed. From spot size and intensity, the terpene hydrocarbons (e.g. limonene) decrease in quantity as the fruits of caraway and dill develop and ripen.

TABLE 1. OBSERVATIONS ON DEVELOPING FRUITS 1963

Date marked i.e. in flower	Date collected	Days (and weeks) since marking	Fresh wt. 100 fruits[1] in mg	% fresh wt. not lost on drying	Calc.[2] dry wt. 100 fruits in mg	mg carvone	
						in 100 fruits[3]	per g dry wt.
			Caraway				
31 May	7 June	7 (*1*)	426	19	82	0·4	5
7 June	21 June	14 (*2*)	546	21	127	6	47
31 May	21 June	21 (*3*)	1,160	24	311	15	47
7 June	5 July	28 (*4*)	1,324	29	412	12	30
31 May	5 July	35 (*5*)	1,391	35	515	16	31
31 May	12 July	42 (*6*)	904	60	575	16	30
			Dill				
6 Aug.	15 Aug.	9 (*1*)	268	26	75	2	24
29 July	15 Aug.	17 (*2*)	701	23	174	6	33
6 Aug.	28 Aug.	22 (*3*)	838	29	257	7	28
29 July	28 Aug.	30 (*4*)	1,062	33	357	6	17
6 Aug.	11 Sept.	36 (*5*)	1,154	33	399	7	17
29 July	11 Sept.	42 (*6*)	513	58	313	8	26

[1] Fresh weights are averages of 2 or 3 lots.
[2] Dry weights obtained from percentage of fresh weight not lost on drying, corrected for loss of essential oil (taken to be twice the amount of carvone present).
[3] Average of 2–4 determinations.

Although the maximum yield of carvone can be obtained from a crop of fruits which has been allowed to develop for about 4 weeks, the essential oil from this will contain a higher proportion of limonene than that from more developed fruits and so will be sweeter and less intensely flavoured. Low carvone content of the essential oil is thus not necessarily the result of carvone deficiency in the fruit.

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SHORT COMMUNICATION

Some effects of fungal growth on the roots of *Rauwolfia oxyphylla* Stapf.

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DURING an investigation of the dried roots of *Rauwolfia oxyphylla* Stapf., a species indigenous to the swampy forests of Uganda, it was noticed that, with some samples, the xylem fibres isolated by Schulze maceration possessed abnormal characters, which appeared to be associated with certain micro-fungi. We have examined this association.

The external appearance of the roots was normal but the root-bark separated easily from the wood, often crumbling into flaky pieces. The internal surface of the bark was flaky and possessed white and black patches which corresponded with similar areas on the underlying wood surface. In ultraviolet light the inner surface of the bark, apart from white and black patches, appeared a deep orange instead of the normal dark brown. Smoothed transverse surfaces of roots frequently showed thin black lines running irregularly across them. From these roots, three cellulose-destroying micro-fungi, *Melanospora zamiae* Corda, *Chaetomium funicolum* Cooke and *C. globosum* Kunze were isolated by Dr. L. Jacobs and identified by the Commonwealth Mycological Institute. These species are normally found on plants rotting in damp conditions. They belong to that group of wood-attacking micro-fungi which cause "soft-rot" involving the production of pointed cavities within the middle layer of the secondary wall. Timber attack by these fungi has been known for many years and Schacht, in 1850, reported the characteristic pointed cavities. A detailed study has been made by Bailey & Vestal (1937); the term "soft-rot" was introduced by Savory (1954). Three other groups of fungi which attack wood are recognised by Mr. J. F. Levy (personal communication), but these are outside the scope of this report.

To study the effects of the infestation of *Rauwolfia oxyphylla* root by the individual fungi, the following procedure was adopted. Discs of normal *R. oxyphylla* root, about 3 mm in thickness, were sterilised in sealed polythene packets by irradiation (1·1 megarads over 2 hr). After being moistened with sterile water, the discs were aseptically transferred to sterilised petri dishes containing a layer of water. The roots were supported above the level of the water and their surfaces inoculated with the appropriate fungus which had been cultivated in potato-dextrin-agar or malt-agar media. The strains used were those isolated from the roots. The dishes were incubated at 26°, the humidity within the incubator being maintained at about 80% to prevent the specimens drying out. Samples of wood were examined at 7-day intervals for abnormal wood fibres.

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The results are recorded in Table 1. It is evident that the three fungi can each attack the wood fibres of *R. oxyphylla.* Cavities in the fibre walls, identical with those observed in the fibre walls of the original root samples, were produced by each of the individual fungi in the experimental material.

TABLE 1. PRODUCTION OF ABNORMAL FIBRES IN *Rauwolfia oxyphylla* ROOT-WOOD AS THE RESULT OF FUNGAL GROWTH

Incubation period (days)	Abnormal fibres produced after fungal growth			
	Melanospora zamiae	*Chaetomium funicolum*	*Chaetomium globosum*	Control (No inoculation with fungi)
7	None	None	None	None
14	None	None	None	None
21	About 5%	About 10%	About 50%	None
28	About 10%	75%	75%	None

It is possible to divide the digestion of the fibre wall into three stages (Fig. 1) as follows (Jefferies, 1965). An *early stage* in which numerous, narrow, elongated cavities with sharply pointed ends appear within the middle layer of the secondary cell wall. These are scattered along the length of the fibre, are relatively short, and do not give a spiral effect. They are all parallel to one another and in polarised light appear as dark lines in a "bright" fibre. A *middle stage* in which the cavities elongate so that some spiral effect is observed; some cavities may widen which makes the pointed ends even more prominent. The cavities remain parallel and in polarised light their dark spirals are very marked. A *late stage* in which the number of cavities is greatly increased so that many of them join to form wide areas of decomposed cell wall. The lines on the fibres are still approximately parallel and they are so frequent that it is difficult to distinguish normal cell wall areas from decomposed areas.

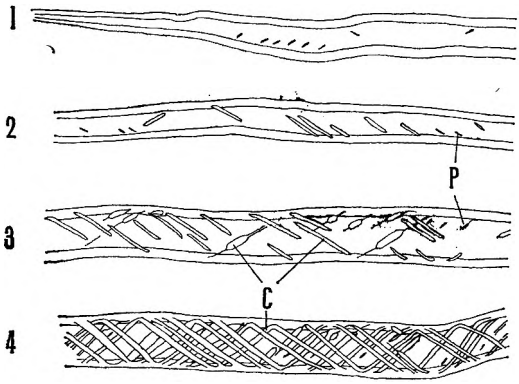

FIG. 1. Portions of wood fibres of *Rauwolfia oxyphylla* Stapf. All × 200. **1,** normal fibres; 2, 3, 4, fibres after four weeks incubation with *Chaetomium globosum* showing respectively, early, middle and late stages of fibre wall digestion. C, cavity; P, pit.

In polarised light these areas can be distinguished easily by the brightness of the unattacked cell walls. Eventually the unattacked residues of the cell walls are reduced to narrow spiral strips, still running parallel to one another; because the hardly visible, outer and inner layers of the cell walls remain to a large extent unattacked, they hold these few spirals of unattacked fibre wall together. Finally, even these areas are digested and the fibres in this condition do not survive Schulze maceration.

In transverse section the thin structure of the inner layer of the cell wall can be seen clearly either as an intact ring or broken (by the microtome) and lying within the lumen of the fibre, but in any event quite detached from the remainder of the cell wall. It does not appear bright in polarised light. The early stage of attack is difficult to see but can be detected by careful examination of suitable transverse sections. With the fungi studied, xylem parenchyma and xylem medullary ray cells do not normally show any effect of attack and vessels only rarely possess fine spiral cavities. Microchemical stains are available for the selective staining of fungal hyphae, making detection easier in cases of slight attack.

Acknowledgements. We thank Dr. L. Jacobs, of Bristol College of Science and Technology, for isolating the three fungi, and the Commonwealth Mycological Institute for confirming their identity.

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The effects of growth conditions on the yield and quality of essential oil of *Mentha piperita*

CHRISTOPHER O'CONNOR

The results of a factorial experiment designed to study the effects of nitrogen, phosphorus and potassium, alone and in combination, on the yield of green herb, and on the yield and composition of the essential oil of *Mentha piperita* are reported. The experiment was made on reclaimed peatbog in Ireland. The main conclusion is that there is a significant increase in the yield of essential oil when nitrogen is added in high concentration. This also results in a decrease in the menthol content of the oil. Superphosphate also reduces the percentage menthol content of the oil. Mixtures of all three fertilisers reduce the percentage oil yield.

PREVIOUS work has shown that virgin dried peat can produce satisfactory crops of *Mentha piperita* provided a minimum application of potash, phosphate and nitrogen is added, together with sufficient lime to give a pH between 5 and 6 (O'Connor, 1960).

The present work investigates the effect of nitrogen, phosphorus and potassium, alone and in combination, on (*a*) the yield of green or fresh herb and of essential oil, and (*b*) the composition of the oil of *Mentha piperita.*

Experimental

MATERIALS AND METHODS

Plants of *Mentha piperita* obtained from Kent in 1950 and propagated vegetatively were planted in rows 2 feet apart in the Spring of 1962. The plots were hand weeded during the growing period and the crop was harvested before flowering in September, 1962. The site of cultivation was dried peat bog as previously described (O'Connor, 1960).

The soil under the peat consisted of calcareous glacial drift having a pH of between 6·0 and 7·0 in the first 6 inches. The overlying peat had a pH of 5·2 to 5·7, its calcium content was between 2·5 and 3·5%, its phosphorus and potassium contents were 0·01 to 0·02% and 0·2 to 0·5% respectively, whilst its nitrogen content was approximately 1·5%. The levels of cobalt, copper and molybdenum in the peat were less than 0·01%.

SCHEME USED IN THE LAYOUT OF EXPERIMENTAL PLOTS

N = Nitrocal. P = Superphosphate. K = Potassium sulphate

Block I		Block III		Block III	
(1) NK	(2) NPK	(9) K	(10) Control	(17) NK	(18) P
(3) K	(4) Control	(11) P	(12) NPK	(19) N	(20) PK
(5) PK	(6) N	(13) N	(14) NP	(21) K	(22) Control
(7) P	(8) NP	(15) PK	(16) NK	(23) NP	(24) NPK

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Nitrogen, phosphate and potash were combined factorially and the experiment was laid down to a randomised block design of 3 replications. The layout is shown on the accompanying chart. The rates of addition for nitrogen, phosphate and potassium were 3, 6 and 2 cwt per acre respectively. Plot size was 15 sq yd and each plot received a basal application of 1 cwt of potassium sulphate and 3 cwt of superphosphate per acre to provide conditions for minimum growth of the plants. Nitrocal (N) which was used to supply nitrogen had the following analysis: 58% ammonium nitrate, 33·36% calcium carbonate, 3–6% magnesium carbonate, and the remainder silica. Phosphate and potassium were supplied as superphosphate (P) and potassium sulphate (K) respectively.

The crop from each of the experimental plots was harvested during one day using a motor mower. Fresh herb was weighed immediately and then was spread out in a thin layer under cover to wilt. When sufficiently dry and crisp, the herb from each plot was separately placed in polythene bags.

Distillation was carried out in a copper still using steam at 15 lb/in^2. The oil was separated from water, dried over sodium sulphate and weighed; it was not rectified. Samples of oil were placed in dry, brown glass bottles and stored in darkness. Each oil sample was examined by gas-liquid chromatography using an Aerograph Hi Fy 600 apparatus. Column: 20% hexose diacetate hexaisobutyrate (SAIB) on 60–80 mesh Embacel Kieselguhr. Length: 10 ft of $\frac{1}{8}$ inch diam. stainless steel tube. Oven temperature: 151–152°. Gas flow: 25 ml/min N_2 and H_2.

Identification of the peaks was effected by the use of authentic specimens of the known components. Peak areas, which are approximate, are given as height × width at half height; they were checked by counting squares.

A number of unidentified compounds were present in traces in the oil. Two compounds, eluted from the column after octan-3-ol but before menthofuran, were present in slightly larger quantities.

Results and discussion

YIELD OF HERB

Table 1 shows that nitrogen significantly increases the yield of fresh herb (P, 0·01) given by first year plants. Second or third year plantations produce much larger yields of fresh herb, but the yields represent a crop much in excess of that usually given by first-year crops on ordinary mineral soil.

Some interaction occurs between nitrocal and superphosphate (P, 0·05); thus in the absence of nitrocal, superphosphate increased the yield from 3·5 to 4·9 tons per acre, but with nitrocal present superphosphate had no effect. Similarly, potassium sulphate increased the yield from 3·4 to 5·1 tons per acre in the absence of nitrocal but there was no effect with nitrocal present (P, 0·001).

The replicates varied significantly from 4·4 to 6·1 tons per acre, which gives an indication of the variation encountered. This result suggests that with a high application of nitrogen, potassium and superphosphate

are unnecessary. In the absence of nitrogen, potash and superphosphate produce a beneficial effect.

It should be noted that only those plots treated with nitrogen alone, or in combination with phosphorus or potassium, contain nitrogen in excess of the 1·5% present in the natural peat.

TABLE 1. EFFECT OF NITROGEN PHOSPHATE AND POTASSIUM ON YIELD

	Herb tons/acre[1]		Oil lb/acre[2]		% limonene[3]		% menthol[4]		% menthone[5]	
	−P	+P	−P	+P	−P	+P	−P	+P	−P	+P
−N	3·5	4·9	2·6	4·5	1·1	1·5	61·9	60·6	17·0	16·8
+N	6·3	6·1	10·0	7·6	1·3	1·3	59·7	58·0	20·0	18·8
Significance of N main effect	***		**		*		*		*	
Significance of NP interaction	*		N.S.		N.S.		N.S.		N.S.	
	−K	+K	−K	+K	−K	+K	−K	+K	−K	+K
−N	3·4	5·1	2·6	5·5	1·1	1·5	63·2	59·3	15·9	17·9
+N	6·5	5·9	10·0	3·4	1·3	0·7	59·4	58·3	18·0	20·8
Significance of K main effect	N.S.		N.S.		N.S.		*		*	
Significance of NK interaction	**		**		*		N.S.		N.S.	
	−P	+P	−P	+P	−P	+P	−P	+P	−P	+P
−K	4·7	5·1	2·6	4·5	1·1	1·5	61·6	61·0	17·5	16·4
+K	5·1	5·9	5·5	6·8	1·5	1·7	60·0	57·9	19·5	19·2
Significance of P main effect	N.S.		N.S.		***		N.S.		N.S.	
Significance of PK interaction	N.S.		*		*		N.S.		N.S.	

[1]s.e. per plot = 0·9. [2]s.e. per plot = 1·8. [3]s.e. per plot = 0·2. [4]s.e. per plot = 2·3. [5]s.e. per plot = 2·5. N.S. = not significant. * 5%, ** 1%, *** 0·1% significance levels.

The above results are in good agreement with those of Baird (1957) for the growth of *Mentha piperita* on mineral soil, and also with those of Birkeli (1948). For *Mentha piperita* in water culture it has been shown that intermediate levels of nitrogen and potassium gave poorer growth than higher or lower concentrations of these nutrients (Crane & Steward, 1962). Maximum growth was obtained with a high level of calcium as would be expected since *Mentha piperita* behaves as a pronounced calcicole, occurring naturally on calcareous habitats.

YIELD OF OIL

Table 1 shows that the yield of oil in lb per acre was significantly increased when nitrogen was available (P, 0·01), while phosphate may have a slight beneficial effect (P, 0·1). This is in agreement with the work of Kotin (1950) and of Latypov (1960).

Significant interaction occurred between nitrogen and potassium; in the absence of nitrogen, potassium gave an increase of 2·9 lb of oil per acre, compared with a decrease of 6·6 lb per acre in the presence of nitrogen.

Interaction was also shown between phosphate and potassium in the presence of nitrogen. Absence of potassium in this case gave a particularly low yield of oil. This result conflicts with that obtained by Birkeli (1948) who found that both potassium and nitrogen are required in large quantities by *Mentha piperita* for the formation of the oil.

Oil yields per acre are exceptionally low compared with those usually obtained on ordinary mineral soil. The average percentage oil yield

ranged between 0·032 and 0·071%. For comparison, plants from the same stock were grown on ordinary soil in three different locations. The average percentage yield of oil was 0·18% which is almost four times that from plants grown on peat. This remarkably low yield may be due to the lack of some essential trace element(s). Preliminary survey of the overlying peat revealed a very low trace element content in the case of copper, cobalt and molybdenum and it is possible that other essential trace elements may be entirely absent or may be present in too low a concentration.

COMPONENTS OF THE ESSENTIAL OIL

The effects of the different fertiliser treatments on the amounts of the various components of the oil may be summarised as follows.

Nitrogen depresses the limonene and menthol contents while increasing the menthone and isomenthone components.

Phosphate increases α-pinene, β-pinene and especially limonene (P, 0·01).

Potassium increases the menthone content and reduces that of menthol.

Fertiliser interactions. There was no nitrogen–phosphate interaction on any of the components.

Nitrogen and potassium interactions affect the content of α-pinene, limonene and cineol. Potassium in the presence of nitrogen depressed the content of these constituents, although increasing it when nitrogen was not present.

Phosphate and potassium: for both α-pinene and limonene, potassium depressed the yield where phosphate was absent but increased the yield where phosphate was present.

Nitrogen, phosphate and potassium. A particularly low limonene content was obtained where phosphate was absent but nitrogen and potash were both present.

TABLE 2. TOTAL AVERAGE MENTHOL CONTENT (%) OF OIL SAMPLES OBTAINED UNDER THE DIFFERENT FERTILISER TREATMENTS

Plot	Menthol	Menthyl acetate	Neomenthyl	Total %
N	59	5	4	69
P	63	6	5	73
K	60	5	5	69
NP	59	6	5	70
NK	60	6	5	71
PK	59	6	5	69
NPK	57	6	5	67
Control	64	6	5	74

A notable feature of all the oil samples is the consistently high menthol content, irrespective of the fertiliser treatment (Table 2). Total menthol content varied between 67 and 74%. It was highest in the oil obtained from the control plots and lowest in that obtained from the crop receiving all three fertilisers at the high rate. This finding may be related to the prevailing cold and wet weather which was a feature of the summer of 1962, and in this connection Bankowski (1953) has reported that the oil

yield increases with a rise and decreases with a fall in temperature. Menthol content likewise rises with the temperature.

English Mitcham peppermint oil has a total menthol content between 42 and 64% and a menthone content between 29 and 42% (Parry & Ferguson, 1936). These figures for menthone are much higher than in our samples where the menthone content varied between 16% in the controls and 21% in plot NPK. The total menthol content of our samples is considerably higher than the maximum figure (64%) that they obtained.

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Comparative assays of some nitrofurans in urine

B. M. JONES, R. J. M. RATCLIFFE AND S. G. E. STEVENS

Polarographic and microbiological methods for the determination of nitrofurantoin and of *N*-(5-nitro-2-furfurylideneamino)-2-imidazoline-one (NF 246) in urine are described. In these the limit of detection is 1 μg/ml by the polarographic and 5 μg/ml by a cup plate microbiological technique. No preliminary separation of the drugs is required. The polarographic method is to be preferred, since it is a more sensitive assay and results can be obtained within an hour of receiving the sample.

THE use of nitrofurans in the treatment of urinary infections has found general clinical acceptance and the assay methods described below have been used to determine the amounts of nitrofurantoin and *N*-(5-nitro-2-furfurylideneamino)-2-imidazoline-one (NF 246) (O'Connor, Russell, Michaels, Newland & Carey, 1963) excreted in urine.

Experimental

MICROBIOLOGICAL

The cup plate assay as described for antibiotics in the B.P. (1963) was used. Nutrient agar, Oxoid CM 3, was poured in plates 12 × 12 inches and 36 cups 18 mm in diameter cut out and sealed. *Bacillus subtilis* NCTC 8236 was the test organism and spore suspensions were prepared as in B.P. (1963), page 1,105. Randomisation of experiments was achieved by means of a 6 × 6 Latin Square test design.

Nitrofurantoin assay. Dilute the urine with an equal volume of citrate phosphate buffer (pH 5·0–6·0) and pipette 1 ml into each cup.

NF 246 assay. This is in two parts: (i) to assess unchanged drug, the method is as for nitrofurantoin but using pH 6·8 buffer; (ii) for total nitrofurans as NF 246, heat urine at 70° for 60 min, cool and then treat as for the unheated sample.

Suitable standards of nitrofurantoin and NF 246 in dimethylformamide and citrate phosphate buffer were used in each test.

The plates were maintained at room temperature, protected from the light for 2 hr, then incubated at 30° for 18 hr.

Minimum inhibitory concentrations. Minimum inhibitory concentrations were measured by standard serial dilution techniques in liquid media using a 24 hr culture of *Escherichia coli* NCTC 8196 as the test organism. Nutrient broth, Oxoid CM 1, was used and the tubes incubated at 37° for 18 hr. Standards were prepared by adding the individual nitrofurans in dimethylformamide to normal male urine and proceeding as for test urines. All samples were pasteurised at 30° for 30 min.

We confirm the opinion of Carroll & Brennan (1954) that a medium of acid pH is preferable for the organisms used. The optimum pH

From Smith, Kline & French Laboratories Ltd., Welwyn Garden City, Hertfordshire.

range was 5·0–6·0 when testing nitrofurantoin, and 5·0–7·0 when examining NF 246. Under these pH conditions urea concentrations of up to 5% did not affect the results of the cup plate assays.

POLAROGRAPHIC

Supporting electrolyte. Dissolve Analar ammonium chloride (53·5 g) and Analar 0·88 ammonia solution (67·5 ml) in distilled water and dilute to volume (2 litres).

Standard solutions. Accurately weigh nitrofurantoin (50 mg) and dissolve in *NN*-dimethylformamide (25 ml); dilute to 100 ml with supporting electrolyte. Each ml contains 500 μg nitrofurantoin. Prepare standard solutions with supporting electrolyte to contain 25, 50, 100 and 200 μg nitrofurantoin per ml. In the same manner prepare standard dilutions in supporting electrolyte to contain 25, 50, 100 and 200 μg NF 246 per ml.

Determination of nitrofurantoin. Dilute 1 ml of urine to 25 ml with supporting electrolyte. Pipette 5 ml of this dilution into a polarographic cell containing a mercury pool anode, and maintained at 25 $\pm$ 0·5°. De-aerate the system with nitrogen (5 min) and measure the peak current of the derivative circuit at −0·32 V.* This step represents the reduction of the nitro-group (Stradins, Hillers & Jur'ev, 1959).

To 1 ml of urine add 1 ml of standard nitrofurantoin solution (25 μg/ml) and dilute with supporting electrolyte to 25 ml. Measure the peak current as above. Repeat using the standards containing 50, 100 and 200 μg/ml. Micrograms of nitrofurantoin per ml urine is given by the following formula.

$$\frac{i_d \text{ (sample)} \times \mu\text{g nitrofurantoin per ml standard solution}}{i_d \text{ (sample + standard)} - i_d \text{ (sample)}}$$

where i_d = peak current of the solution.

The determination of NF 246 is made in a similar manner.

Over the voltage range −0·1 V to −0·6 V, freshly voided urine contains no substances which interfere with this assay. For any given concentration of the nitrofuran, the diffusion current varies in urine samples from different subjects and even with the same subjects it varies with time. This fact was not appreciated by Marciszewski (1960) who also used a polarographic assay. The standard addition technique overcomes this difficulty since each sample is related to four standards.

VALIDITY OF METHODS

The validity of these methods was established by adding amounts of 25–150 μg/ml of either nitrofurantoin or NF 246 to Ringer solution, to sterilised urine and to freshly voided urine from healthy male subjects and assaying.

By the cup plate method the average drug recovery was 96·6% (s.d. 9·1).

By the polarographic assay the recovery of either drug was 102·2% (s.d. 7·5).

* A southern instrument K 1000 was used.

NITROFURANTOIN AND NF 246 IN URINE

Healthy male subjects were given doses of the drugs in accordance with the regimens outlined below. Urine was assayed by both methods. Results are shown in Figs 1 and 2 and Table 1.

TABLE 1. COMPARISON OF RESULTS OBTAINED BY POLAROGRAPHIC, CUP-PLATE AND MINIMUM INHIBITION CONCENTRATION METHODS ON A MAN TAKING NF 246

Time (hr)	µg/ml NF 246 excreted		
	Cup-plate method*	Minimum inhibition concentration	Polarographic method
08.00	100	96	112
10.00	46	48	55
12.00	92	96	87
14.00	92	96	88
16.00	244	192	270
18.00	184	192	182
20.00	348	192	338
22.00	296	192	325
32.00	164	96	181
34.00	92	48	117
36.00	50	48	63
38.00	92	48	88
40.00	276	192	244
42.00	400	384	381
44.00	138	192	138
46.00	82	96	77
48.00	146	192	138
50.00	nil	nil	1

* Heated sample.

In all cases the bladder was emptied before the start of the experiment.

Nitrofurantoin (100 mg) was administered at 07.00, 12.00, 17.00 and 22.00 hr. Urine was collected by draining the bladder at 2 hourly intervals from 07.00 to 21.00 hr; a further sample was obtained at 07.00 hr, 24 hr after administration.

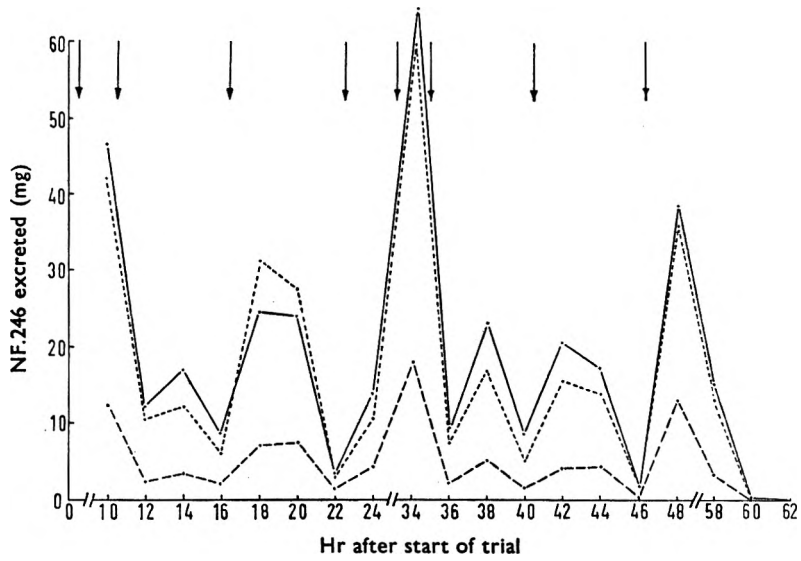

FIG. 1. Drug excretion pattern of NF 246 in urine. ——— Results of polarographic assay. - - - - - Results of heated microbiological assay. – – – – – Results of cold microbiological assays. At arrows 200 mg NF 246.

NF 246 (200 mg) was administered at 08.00, 14.00, 20.00, 22.30 hr and repeated over a second day. Urine was collected by draining the bladder at 2 hrly intervals between 08.00 and 22.00 hr.

33–49% of the nitrofurantoin administered was recovered from the urine by both methods, and 21–23% of NF 246 was recovered by a polarographic method and by a microbiological test applied to heated specimens.

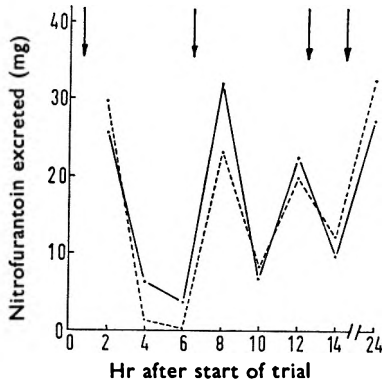

FIG. 2. Drug excretion pattern of nitrofurantoin in urine. ——— Results of polarographic assay. - - - - - Results of microbiological assay. At arrows 100 mg nitrofurantoin.

Urine from subjects receiving NF 246 showed that the polarographic and tube dilution methods gave higher results than were obtained using a simple cup plate assay. It should be noted that the tube dilution method was preceded by a sample pasteurisation, whereas for the simple cup plate assay unheated urine was used.

Pretreatment of the urine at 70° for 1 hr resulted in an increase in the *apparent* amount of drug present, and the results by cup plate assay were then in fair agreement with those obtained by the polarographic method. Urine containing nitrofurantoin does not require preliminary heating.

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The analysis of methisazone

J. C. DEAVIN AND D. H. MITCHELL

The physical and chemical properties of methisazone (1-methylindoline-2,3-dione 3-thiosemicarbazone) are described. Iodimetric titration is suggested as being the most convenient method of assay. Each molecule of methisazone reacts with eight atoms of iodine and the mechanism of this reaction is discussed. Methisazone was examined for impurities by thin-layer chromatography and details are given of the technique employed. Reference is made to the change in light absorption of methisazone solutions when exposed to light.

DURING the past decade an extensive programme of work devoted to the chemotherapy of pox virus infections has been carried out (Bauer, Dumbell, Fox-Hulme & Sadler, 1962). In particular, the discovery that some derivatives of isatin β-thiosemicarbazone possess chemotherapeutic activity against variola major infections in mice led eventually to the introduction of methisazone (1-methylindoline-2,3-dione 3-thiosemicarbazone; *N*-methylisatin β-thiosemicarbazone) for the treatment of smallpox contacts as an alternative to vaccination. Methisazone has now become of sufficient interest to create a demand for information about its physical and chemical properties and, as little published data is available, the present communication describes analytical methods used successfully in our laboratories.

IDENTIFICATION AND PURITY

Description. When freshly prepared, methisazone consists of fluffy orange-yellow micro-needles, m.p. about 250° with decomposition. For pharmaceutical purposes it is used as an ultra-fine powder, the particles of which have a characteristic property of slowly developing outgrowths in the form of "whiskers". This is of considerable importance, as the drug must be freshly ground before being incorporated in a pharmaceutical preparation and the presence of such outgrowths is a useful guide to the suitability of the powder for pharmaceutical purposes.

Gordon (1964) has given an interesting account of the formation of "whiskers" particularly in metals. When ductile metals are subjected to stress the growth of "whiskers" is sometimes provoked. Prolonged grinding is required to reduce methisazone to an ultra-fine powder and it is suggested that the particles, so produced, may possess internal strain thus leading to the phenomenon of outgrowths.

Solubility. Methisazone is practically insoluble in water; it dissolves in about 2,000 parts of methanol, in 800 parts of chloroform and 250 parts of acetone.

THIN-LAYER CHROMATOGRAPHIC EXAMINATION

Thin-layer chromatography of methisazone was carried out by established procedures using readily available apparatus. The more important details are as follows.

From the Wellcome Chemical Works, Dartford.

Materials. Glass plates, 20 × 20 cm, coated with a 250-μ layer of Kieselgel G (Merck & Co.) These were dried for 30 min at 110° and stored over silica gel.

Running solvent. Chloroform: glacial acetic acid (95: 5). Spots corresponding to methisazone and likely impurities are coloured and easily located but the colour may be enhanced by exposure to ammonia vapour.

It is routine to test methisazone for likely impurities and these consist of *N*-methylisatin, isatin and isatin β-thiosemicarbazone. Thin-layer chromatography enables these impurities to be readily detected. Normally no more than a trace, if any, of *N*-methylisatin has been detected. Approximate Rf values and other data are:

	Rf Value	Colour of spot
Methisazone	0·59	Yellow
N-Methylisatin	0·62	Orange
Isatin β-thiosemicarbazone ..	0·47	Yellow
Isatin	0·37	Orange

Not less than 0·1 μg of each compound can be detected.

When examined by thin-layer chromatography, solutions prepared in the dark yielded only single spots, whilst those exposed to daylight gave two distinct spots. When each spot was extracted separately from the chromatogram and its solution again exposed to light, each yielded two identical spots when re-examined. It is therefore suggested that methisazone in solution undergoes reversible isomerisation on exposure to light with the formation of *syn* and *anti* isomers. Prolonged exposure causes irreversible decomposition.

ULTRAVIOLET LIGHT ABSORPTION

Solutions of methisazone exhibit characteristic light absorption. In methanol solution, prepared in the dark and examined immediately, methisazone shows at the following wavelengths: maxima 241 mμ, E(1%, 1cm) 515; 274 mμ, E(1%, 1 cm) 565; 365 mμ, E(1%, 1 cm) 954; [372·5 mμ, E(1%, 1 cm) 975 in chloroform]. There are minima at 224 mμ, 263 mμ, and 294 mμ.

The absorption spectrum of methisazone is modified by exposure of the substance to light, this change being reversible provided the solution is not subjected to prolonged irradiation; the significant factors are the time and intensity of irradiation and the solvent. After standing for 1 hour in bright daylight a methanol solution of methisazone shows maxima at the following wavelengths: 241 mμ, E(1%, 1 cm) 624; 273 mμ, E(1%, 1 cm) 471; and 357 mμ, E(1%, 1 cm) 790 [360 mμ E(1%, 1 cm) 785 in chloroform].

INFRARED ABSORPTION SPECTRUM

The infrared absorption spectrum of methisazone (potassium chloride disc) is shown in Fig. 1.

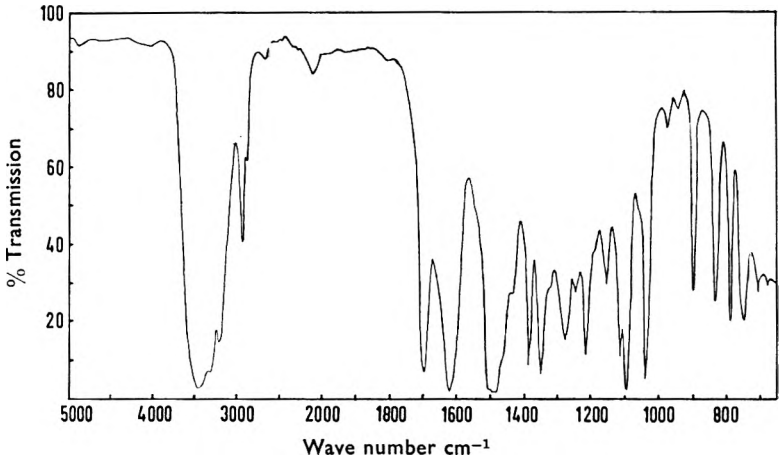


FIG. 1. Infrared spectrum (KCl disc) of methisazone.

ASSAY

Elemental analysis, light absorption, non-aqueous titration and iodimetric titration have all been used in our laboratories for quantitative analysis of methisazone. The most suitable of these, the iodimetric method, may be carried out as follows: transfer 100 mg ± 5 mg accurately weighed to an iodine flask and dissolve in 10% sodium hydroxide solution (12 ml); cool. Add 0·1N iodine solution (50 ml), close the flask, place 0·1% potassium iodide solution (5 ml) in the neck of the flask and set aside for 30 min. Add dilute hydrochloric acid (15 ml) and titrate the excess of iodine with 0·1N sodium thiosulphate solution using starch mucilage as indicator. Carry out a blank determination on the reagents.

Each ml of 0·1N iodine solution is equivalent to 2·928 mg of $C_{10}H_{10}ON_4S$.

When assayed by the iodimetric procedure, production batches of methisazone have seldom given figures corresponding to a content of $C_{10}H_{10}ON_4S$, less than 97·5 %. This figure is calculated with reference to the material dried at 110°.

Discussion

The assay of methisazone is based on the reaction of 1 molecule of the drug with eight atoms of iodine. Oxidation potentials are influenced by hydrogen ion concentration and the experimental conditions described in the assay must be closely followed. Thus exactly 12 ml of 10% sodium hydroxide solution should be used to dissolve 100 mg ± 5 mg of methisazone since excess of sodium hydroxide results in high assay figures. If warming is necessary to dissolve the methisazone the solution should be cooled to 25° before adding the 0·1N iodine solution.

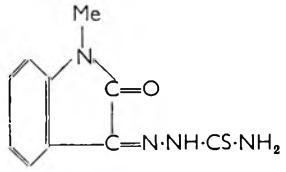
On acidifying the reaction mixture a yellowish brown precipitate is formed, and as this adsorbs some iodine, the mixture must be shaken vigorously at the end of the titration with 0·1N sodium thiosulphate

solution. At the end of the iodine reaction, sulphate is present in the mixture, and estimation of this by barium sulphate precipitation shows that one molecule of methisazone yields one sulphate ion. Eight iodide ions are formed at the same time and all the iodine used in the reaction is thus converted to iodide.

The precipitate formed on acidifying the mixture before the final titration has not been obtained in a highly purified state. Elemental analysis gave the following figures for the dried material: C, 58·8; H, 4·1; N, 28·7. $C_{10}H_8N_4O$ requires C, 60·0; H, 4·0; N, 28·0%.

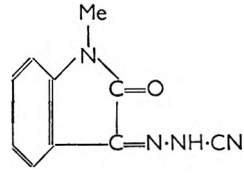
It is significant that the substance contains neither sulphur nor iodine. Its infrared absorption spectrum is similar to that of methisazone except for the presence of additional bands at 2240 cm^{-1} and 2150 cm^{-1} characteristic of nitrile and isonitrile groups respectively.

It is possible to represent the reaction during assay as follows:



$$+ 4I_2 + 4H_2O \rightarrow 8HI + H_2SO_4 + C_{10}H_8N_4O \qquad \text{(I)}$$

A possible structural formula for (I) is



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A limit test for *p*-chloroacetanilide and other impurities in paracetamol and phenacetin using thin-layer chromatography

R. A. SAVIDGE AND J. S WRAGG

A thin-layer chromatography procedure is described suitable as a limit test for *p*-chloroacetanilide in paracetamol and phenacetin. The sample is chromatographed on silica gel together with a standard using the solvent mixture cyclohexane: acetone: diisobutylketone: methanol: water (100:80:30:5:1) and detection is by irradiation with ultraviolet light 253·7 mμ followed by examination in light of wavelength 365 mμ. The procedure may be used to limit the *p*-chloroacetanilide content of tablets containing paracetamol or phenacetin. Other possible impurities in paracetamol and phenacetin are also detected and may be limited by similar procedures.

THE undesirable side-effects of phenacetin may be due to phenacetin itself or to impurities such as *p*-chloroacetanilide. Harvald, Valdorf-Hansen & Nielsen (1960) have suggested that renal damage arises from the methaemoglobin-forming activity of *p*-chloroacetanilide rather than from any direct effect of phenacetin or related drugs.

Methods for the determination of *p*-chloroacetanilide in phenacetin include Raney nickel reduction (Hald, 1951), a polarographic procedure (Jones & Page, 1964), paper chromatography (Ritter, Mutter & Hoffstetter 1961) and a spectrophotometric procedure (Crummett, Simek & Stenger, 1964). The content of *p*-chloroacetanilide in phenacetin is limited as follows: $\nless$ 0·11% (British Pharmacopoeia: Raney nickel reduction); $\nless$ 0·3% (Pharmacopoeia Nordica: Raney nickel reduction); $\nless$ 0·03% (United States Pharmacopeia: paper chromatography). The content of *p*-chloroacetanilide in paracetamol is limited to $\nless$ 0·11% (British Pharmacopoeia: Raney nickel reduction).

In our hands the paper chromatograms obtained by the U.S.P. method proved difficult to interpret because of the diffuse nature of the *p*-chloroacetanilide spots. The thin-layer chromatography procedure described below is more rapid, more sensitive and more easily interpreted.

Experimental

The following procedures are designed to limit the *p*-chloroacetanilide content of paracetamol or phenacetin or tablets containing these substances to not more than 0·03% of the drug.

Apparatus and reagents. Microcap pipettes, 2 and 5 μl capacity. Sources of ultraviolet light of 253·7 mμ and 365 mμ.* Kieselgel G (Merck): 0·25 mm layer spread on 20 × 20-cm glass plates using standard techniques; activated at 110° for 1 hr; stored over anhydrous silica gel.

From the Analytical Development Group, Standards Department, Boots Pure Drug Company Ltd., Station Street, Nottingham.

* A 15 W germicidal lamp (Mazda) was used for the former and a 125 W mercury vapour lamp for the latter.

Solvent mixture: analytical grades of cyclohexane, acetone, diisobutylketone, methanol and water (100:80:30:5:1).

Paracetamol and phenacetin free from p-chloroacetanilide may be obtained by repeated recrystallisation of the compounds. Alternatively, they may be conveniently obtained from manufacturers who do not use chlorinated intermediates in their preparation. Freedom from *p*-chloroacetanilide may be established either by the spectrophotometric method (Crummett & others, 1964; limit of detection, 10 ppm) or by the thin-layer procedure using a 900 μg loading on a 0·5 mm layer of Kieselgel G, running the chromatogram 7·5 cm with diisobutylketone as solvent and irradiating for 15 min at 253·7 mμ (limit of detection: 30 ppm).

I. PROCEDURE FOR PARACETAMOL AND PHENACETIN

Standard Solution A: Dissolve *p*-chloroacetanilide-free phenacetin (300 mg) in dichloromethane (8 ml), add 1 ml of 0·009% w/v solution of *p*-chloroacetanilide in dichloromethane and dilute to 10 ml with dichloromethane.

Standard Solution B: Dissolve *p*-chloroacetanilide-free paracetamol (1·50 g) in methanol (8 ml), add 1 ml of 0·045% w/v solution of *p*-chloroacetanilide in methanol and dilute to 10 ml with methanol.

Sample Solution A: Dissolve phenacetin sample (300 mg) in dichloromethane (8 ml) and dilute to 10 ml with dichloromethane.

Sample Solution B: Dissolve paracetamol sample (1·50 g) in methanol (8 ml) and dilute to 10 ml with methanol.

Method. According to whether phenacetin or paracetamol is being examined, apply 2 × 5 μl portions of Sample Solution A or 1 × 2 μl of Sample Solution B as a single spot about 2·5 cm from one edge of the chromatoplate. On the same spotting line apply, as appropriate, 2 × 5 μl portions of Standard Solution A or 1 × 2 μl of Standard Solution B as a single spot. The spots should be not less than 1·5 cm apart. Place the plate in the tank and allow the solvent to rise 15 cm past the spotting line. Dry the plate in a stream of cold air (10 min), hold it within 2–3 cm of the source of ultraviolet light (253·7 mμ) for 10 min and then examine under ultraviolet light (365 mμ). (Optimum irradiating conditions must be determined for the particular source used.) The intensity of fluorescence of the spot due to *p*-chloroacetanilide in the standard is compared with that of the spot with the same running distance in the sample.

Up to 6 samples of phenacetin or paracetamol can be examined on one plate, loading sample and standard solutions alternately. The total working time for the examination of 6 samples is about 90 min.

None of the undernoted substances interfere with the separation of *p*-chloroacetanilide from phenacetin or paracetamol or with its subsequent detection: maize starch, lactose, sucrose, acacia, alginic acid, gelatin, stearic acid, magnesium stearate, calcium stearate, talc, polyvinylpyrrolidone, sodium benzoate, acetylsalicylic acid, caffeine and codeine phosphate.

II. PROCEDURE FOR TABLETS OF PHENACETIN AND TABLETS OF PARACETAMOL

Use procedure I, replacing Sample Solutions A and B by the following:

Sample Solution C: To powdered tablet equivalent to 300 mg phenacetin in a 10 ml centrifuge tube, add sufficient dichloromethane to give a volume of 10 ml. Shake for 20 min, adjust to 10 ml with dichloromethane if necessary and centrifuge (5 min) at 1,000 rpm. Apply 2 × 5 μl portions of the supernatant liquid to the chromatoplate.

Sample Solution D: Repeat the extraction for Sample Solution C using powdered tablet equivalent to 1·50 g paracetamol and replacing dichloromethane by methanol. Apply a 2 μl portion of the supernatant liquid to the chromatoplate.

III. PROCEDURE FOR COMPOUND TABLETS OF CODEINE

Use procedure I, replacing Sample Solution A by Sample Solution C and Standard Solution A by Standard Solution D: Dissolve *p*-chloroacetanilide-free phenacetin (300 mg) and acetylsalicylic acid (300 mg) in dichloromethane (8 ml), add 1 ml 0·009% w/v solution of *p*-chloroacetanilide in dichloromethane and dilute to 10 ml with dichloromethane.

IV. PROCEDURE FOR COMPOUND TABLETS OF ACETYLSALICYLIC ACID

Use procedure I replacing Sample Solution A by Sample Solution C and Standard Solution A by Standard Solution E: Dissolve *p*-chloroacetanilide-free phenacetin (300 mg), acetylsalicylic acid (500 mg) and caffeine (100 mg) in dichloromethane (8 ml), add 1 ml 0·009% w/v solution of *p*-chloroacetanilide in dichloromethane and dilute to 10 ml with dichloromethane.

Standard solutions must contain the same amount of phenacetin and paracetamol as the sample solutions or false evaluations will result. Since acetylsalicylic acid and caffeine are soluble in dichloromethane and affect spot sizes and running distances, it is considered necessary to include them in the standards used for examining the compound tablets.

Under the conditions I to IV above, *p*-chloroacetanilide gives a bluish-white fluorescent spot which can be detected down to a level of 0·01%. There is a reasonably good gradation in intensity for standards containing 0·01, 0·02, 0·03, 0·04 and 0·05% of *p*-chloroacetanilide in paracetamol or phenacetin and an unknown in this range can be placed to the nearest 0·01%.

Results and discussion

Under the condition of Procedure I, *p*-chloroacetanilide runs approximately 8 cm and is separated from phenacetin and paracetamol and other impurities. The approximate running distances of some relevant compounds, relative to *p*-chloroacetanilide, are given in Table 1.

Samples of pharmaceutical-quality paracetamol and phenacetin and tablets containing these materials were examined by the recommended procedures and the results are given in Tables 2 and 3. The samples of paracetamol and paracetamol tablets contained no detectable *p*-chloroacetanilide, nor did three of the phenacetin samples, two of which were

probably manufactured by routes that do not involve chlorinated intermediates. With one exception, the tablets containing phenacetin did not contain more than 0·06% of *p*-chloroacetanilide. The exception contained 0·7%, a result which was confirmed by the spectrophotometric method (Crummett & others, 1964) and the Raney nickel reduction procedure (Hald, 1951).

TABLE 1. SEPARATION OF PHENACETIN, PARACETAMOL AND ASSOCIATED IMPURITIES UNDER CONDITIONS GIVEN IN PROCEDURE I

Compound	Running distance relative to *p*-chloroacetanilide	Approx. limit of detection as percentage in phenacetin or paracetamol
p-Nitrophenetole	1·50	†
Diacetyl-4-phenetidine	1·20	0·02
p-Nitrophenol	1·09	†
p-Phenetidine	1·07	0·04
p-Chloroacetanilide	1·00	0·01
Acetanilide	0·89	0·03
O-Acetylparacetamol	0·77	0·02
p-Aminophenol	0·70	0·01
Phenacetin	0·84 (0·73–0·95*)	
Paracetamol	0·54 (0·41–0·66*)	0·02

* Measured from rear to front of spots at 300 µg loading.
† These compounds do not give the bluish-white fluorescence which is given by the other compounds.

To check the recovery of *p*-chloroacetanilide, laboratory batches of tablets of phenacetin B.P.C. and compound tablets of acetylsalicylic acid B.P.C. were prepared from phenacetin containing 0·05% *p*-chloroacetanilide and were examined as described above. There was no apparent loss of *p*-chloroacetanilide.

TABLE 2. SAMPLES OF PARACETAMOL AND PHENACETIN

Samples	*p*-Chloroacetanilide %	
	Thin-layer procedure	Spectrophotometric procedure
Phenacetin 1	< 0·01	< 0·01
,, 2	0·15	0·15
,, 3	0·05	0·05
,, 4	< 0·01	< 0·01
,, 5	0·02	0·02
,, 6	0·10	0·12
,, 7	< 0·01	< 0·01
,, 8	0·08	0·10
Paracetamol 6 samples	each < 0·01	—

A number of other possible impurities in phenacetin and paracetamol are separated and detected by procedure I. An estimate of the approximate amounts of each present may be obtained by methods analogous to those described for *p*-chloroacetanilide. One such impurity found in several batches of paracetamol had the same running distances as *O*-acetylparacetamol and comparison with suitable standards gave the approximate contents of this impurity, samples of paracetamol Nos 1–6 giving < 0·02, 0·03, 0·02, 0·04, < 0·02, 0·09 % respectively.

Although the thin-layer procedure we have described is capable of giving an approximate estimate of the amount of *p*-chloroacetanilide in phenacetin and paracetamol, it is better suited for use as a limit test.

TABLE 3. TABLETS CONTAINING PARACETAMOL AND PHENACETIN

Samples	*p*-Chloroacetanilide as percentage of the phenacetin
Tablets phenacetin B.P.C. 1	0·04
" " " 2	0·05
" " " 3	0·01
" " " 4	0·03
" " " 5	< 0·01
" " " 6*	0·05
Tablets paracetamol B.P. 8 samples	each < 0·01
Compound tablets acetylsalicylic acid B.P.C. 1	0·02
" " " " " 2	0·7
" " " " " 3	0·06
" " " " " 4	< 0·01
" " " " " 5*	0·05
Compound tablets codeine B.P. 1	0·03
" " " " 2	0·01
" " " " 3	0·03
Soluble compound tablets codeine B.P. 1	0·03
" " " " " 2	0·05
" " " " " 3	< 0·01

* Laboratory-prepared tablets using phenacetin sample 3 (Table 2).

Compared with other procedures it is rapid, especially when more than one sample is to be examined, and it may readily be adapted to the determination of *p*-chloroacetanilide in tablets containing phenacetin or paracetamol. It has the advantage over non-chromatographic procedures that it will also indicate the presence of other impurities and thus give additional information about the purity of the phenacetin or paracetamol.

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SHORT COMMUNICATION

The configuration of dextromoramide

A. F. CASY

THE (+)-enantiomorph of 2,2-diphenyl-3-methyl-4-morpholinobutyryl-pyrrolidine [dextromoramide, (+)-I] is a potent analgesic in mice [the (−)-isomer is almost inactive]§ and knowledge of its configuration is of importance in relation to the stereospecificity of the analgesic receptor site (Beckett & Casy, 1965). This paper describes the correlation of dextromoramide (I) with (−)-isomethadone (VII), of known configuration (Beckett, Kirk & Thomas, 1962).

Treatment of levomoramide [(−)-I] with sodamide removed the amide function and gave the (−)-hydrocarbon II; by analogy therefore dextromoramide [(+)-I] must be related to the enantiomorphic (+)-hydrocarbon II. This was shown to have the same configuration as the (+)-nitrile III and the (−)-ketone IV by chemical methods of unambiguous stereochemistry (see Fig. 1). These three compounds II, III and IV of related configuration were correlated with the dimethylamino-analogues (+)-V, (+)-VI and (−)-VII [(−)-isomethadone], also of identical configuration, by a method based upon comparisons of molecular rotational changes in solvents of increasing polarity (cf. Beckett & Casy, 1957), and also by optical rotatory dispersion studies.

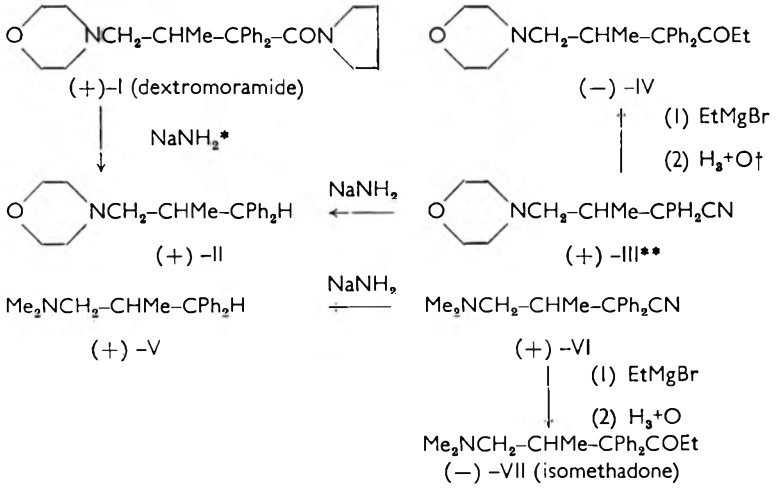

FIG. 1. Reactions employed in configurational studies.

* Relationship established using (−)-I(levomoramide). ** Resolved by means of camphor-10-sulphonic acid. † Refluxed 7 hr with conc. HCl

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§ ED50 mg/kg mice (hotplate test): (±) 1·25, (+) 0·64, (−) >150 (Janssen & Jageneau, 1957).

Molecular rotational values for the morpholino-compounds (II–IV) and for the dimethylamino-derivatives (V–VII) are recorded in Table 1. Within each trio of compounds, displacement of the sign of [M] (caused by increases in solvent polarity) is not uniform in direction, nor are regular variations in [M] values observed. It is important to note, however, that the [M] values of *corresponding members* of the two groups are displaced in the *same* direction: [M] values of the nitriles III and VI, for example, are both displaced towards increasing dextrorotation as solvent polarity increases. Corresponding members also show the same form of variation in the value of [M]: thus for both the ketones IV and VII, [M] values in benzene are contrary to the overall laevorotatory trend. These results indicate the configurational identity of the pairs II–V, III–VI and IV–VII.

Evidence for the configurational identity of the nitriles III and VI and the ketones IV and VII is also provided by optical rotatory dispersion data*. The former pair exhibit positive plain curves (rotations recorded to

TABLE 1. MOLECULAR ROTATIONS $[M]_D$ AT 21°±2° (*c* 1−1·5%)* OF 3-DIMETHYLAMINO AND 3-MORPHOLINO-1,1-DIPHENYL-2-METHYLPROPANE DERIVATIVES (A-CH_2·CHMe·CPh_2-B)

Compound	A	B	Solvent				Direction of sign displacement as solvent polarity increases
			C_6H_{12}	C_6H_6	EtOH	H_2O**	
II		H	+62	+59	+117	+126	+
V	Me_2N-	H	+70	+90	+134	+156	+
III		CN	+131	+108	+151	+196	+
VI	Me_2N-	CN	+151	+149	+192	+236	+
IV		COEt	−160	−61	−112	−279	−
VII	Me_2N-	COEt	−95	zero	−69	−232	−

Notes. * Variations due to concentration and temperature are much less than those due to change of solvent
** Hydrochloride

275 mμ) and the latter, negative Cotton effects ($[\alpha]_{316}$−3730°, trough; $[\alpha]_{277}$+5060°, peak; the curve then flattens and rises again near 256 mμ). In both cases the curves are virtually superimposable. Hence, *like* configurations are assigned to the (+)-hydrocarbon V, related to (−)-isomethadone (VII), and to (+)-II, related to dextromoramide: it follows that dextromoramide has the same configuration as *S*-(−)-isomethadone. Since it has already been shown that dextropropoxyphene (at its 3-C centre) and (−)-phenampromid have likewise the same configuration as *S*(−)-isomethadone (Casy & Myers, 1964, and references cited), configurational identity among the more pharmacologically active enantiomorphs of

* Recorded on a Polarmatic 62 photoelectric spectropolarimeter, in water (conc. 1 mg/ml).

analgesics containing the structural feature $>NCH_2CHMe$, is therefore firmly established.

Acknowledgments are made to Dr. Paul Janssen and to Glaxo Laboratories for supplying (−)-I and (±)-III respectively.

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SHORT COMMUNICATION

Dihydrostreptomycin derivatives: glycosides of dihydrostreptobiosamine

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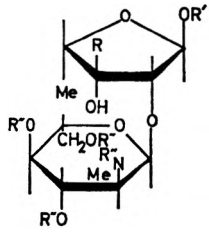
THE *in vitro* activity against *Mycobacterium tuberculosis* of streptomycin derivatives, in particular of certain glycosides of streptidine, has been investigated by Comrie, Mital & Stenlake (1960). In this communication we describe some glycosides of dihydrostreptobiosamine (I). This has allowed us to evaluate transglycosidation as a method of synthesising streptomycin-like compounds with the same stereochemistry as the natural antibiotic.

The anomeric mixture of methyl dihydrostreptobiosaminide hydrochlorides (II) was prepared by methanolysis (Fried & Wintersteiner, 1947), and separated into α- and β-methyl penta-acetyldihydrostreptobiosaminide (III), $[\alpha]_D^{20}$ −117° and −34° respectively, by differential solubility of the acetates in ether (Brink, Kuehl, Flynn & Folkers, 1946). This original anomeric assignment has been confirmed by nmr and optical rotation evidence from streptomycin and various of its derivatives, in particular from the α- and β-methyl *N*-acetyldihydrostreptobiosaminides (McGilveray & Rinehart, 1965).



I	R = CH_2OH, R′ = R″ = H.
II	R = CH_2OH, R′ = CH_3, R″ = H.
III	R = CH_2OAc, R′ = CH_3, R″ = Ac.
IV	R = CH_2OH, R′ = CH_2Ph, R″ = H.
V	R = CH_2OAc, R′ = CH_2Ph, R″ = Ac.
VI	R = CH_2OH, R′ = CH_2CH_2Br, R″ = H.
VII	R = CH_2OH, R′ = Ph, R″ = H.

Benzyl (IV) and 2-bromoethyl (VI) dihydrostreptobiosaminide hydrochlorides have been prepared by treatment of the anomeric mixture of methyl dihydrostreptobiosaminide hydrochlorides with the appropriate alcohol in the presence of hydrogen chloride for 48 hr at 50°. The product in each case was a hygroscopic solid.

The phenyl glycoside (VII) was prepared by fusion of methyl dihydrostreptobiosaminide with phenol in the presence of hydrogen chloride.

The benzyl derivative was also obtained as outlined in Scheme I. Carefully dried dihydrostreptomycin sulphate was treated (96 hr) with benzyl alcohol containing 2N hydrogen chloride. Streptidine was filtered off and the solution concentrated *in vacuo*: the addition of

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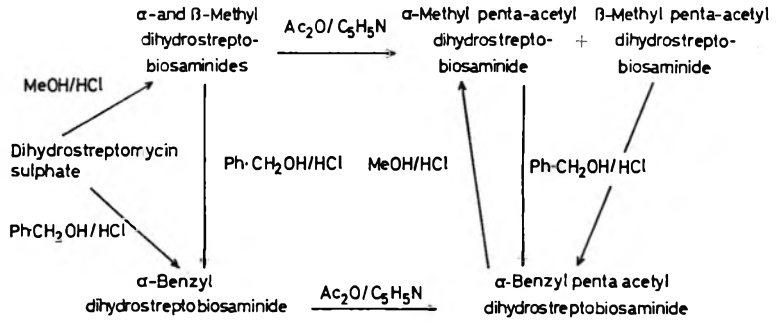
anhydrous ether yielded a hygroscopic precipitate of benzyl dihydrostreptobiosaminide hydrochloride.

The stereochemical homogeneity of the benzyl glycoside was indicated by thin-layer chromatography on silica gel using the organic phase of n-butanol-ethanol-water, 4 : 1 : 5, as solvent with periodate-permanganate (Lemieux & Bauer, 1954) as detecting agent (single spot with R glucosamine value, 3·0). This was confirmed by acetylation of the base (obtained

Scheme I



from the hydrochloride by means of anionic exchange resin) with acetic anhydride in pyridine to a single anomer of benzyl penta-acetyldihydrostreptobiosaminide (V), m.p. 141–142°, $[\alpha]_D^{20}$–138 (*c*,1 in $CHCl_3$). Found: C, 57·2; H, 6·6; N, 2·2. $C_{30}H_{41}NO_{14}$ requires C, 56·7; H, 6·5; N, 2·2%. This product showed solubility properties identical with those of α-methyl penta-acetyldihydrostreptobiosaminide, and, in contrast to the corresponding mixed α- and β-methyl penta-acetates, could not be fractionated by differential solubility in ether.

TABLE 1. DIHYDROSTREPTOBIOSAMINIDES

Glycoside	m.p.	$[\alpha]_D^{20}$	Found			Required as			
			C	H	N	C	H	N	
Benzyl HCl ..	210°	−110°	49·2	6·5	3·0	51·5	6·7	3·0	$C_{20}H_{32}ClNO_9$
2-Bromoethyl HCl	125–130°	−100°	35·3	6·0	3·1	37·3	5·8	2·9	$C_{16}H_{30}BrClNO_9$
Phenyl HCl ..	115°	−131°	51·1	6·7	2·9	50·05	6·5	3·1	$C_{19}H_{30}ClNO_9$
α and β-Methyl (*ca* 90% α)† ..	—	−135°*	—	—	—	—	—	—	

* Fried & Wintersteiner (1947).
† Brink & others (1946).

Assignment of the configuration of benzyl dihydrostreptobiosaminide as α-L is supported by rotational evidence (Table 1). This should be considered together with the specific rotations of α- and β-methyl penta-acetyldihydrostreptobiosaminides $[\alpha]_D^{20}$–117° and −34° respectively (Brink & others, 1946) and of the derived α- and β-methyl *N*-acetyldihydrostreptobiosaminides ($[\alpha]_D^{25}$–160° and −32° respectively) (McGilveray & Rinehart, 1965). This assignment as the α-anomer was confirmed as follows. α-Methyl penta-acetyldihydrostreptobiosaminide was treated (24 hr) with benzyl alcohol-hydrogen chloride. Subsequent acetylation with acetic anhydride in pyridine followed by chromatography

on alumina gave the same benzyl penta-acetyldihydrostreptobiosaminide (crystals from ethanol), m.p. 141–142° (undepressed on admixture with authentic material), $[\alpha]_D^{20}$ –138°($CHCl_3$). In the same way methanolysis of benzyl penta-acetyldihydrostreptobiosaminide gave α-methyl penta-acetyldihydrostreptobiosaminide as the sole product, in further support of the α- assignment to the benzyl glycoside.

Although the course of the transglycosidation reaction has not been investigated, a carbonium ion intermediate would be anticipated by analogy with the Fischer glycoside synthesis (Shafizadeh, 1958; Bishop & Cooper, 1962, 1963; Capon, Loveday & Overend, 1962). Thermodynamic considerations should therefore control the stereochemistry of the reaction products. This is consistent with the experimental evidence that the bulkier benzyl group allows formation of only one anomer, namely α-L-benzyl dihydrostreptobiosaminide. Some indirect evidence that this form is sterically favoured comes from the experiments of Bishop & Cooper (1963) on the formation of D-lyxofuranosides in which only the α-D-methyl lyxofuranoside was obtained. Indeed, contrary to previous reports, the streptose-streptidine link in streptomycin itself has now been assigned the α-L configuration (McGilveray & Rinehart, 1965). More directly, we find that when β-methyl penta-acetyldihydrostreptobiosaminide is treated with benzyl alcohol-hydrogen chloride it is the α-benzyl anomer which is produced. This we regard as significant evidence for the greater stability of the α- over the β-anomer.

MICROBIOLOGICAL RESULTS

Phenyl dihydrostreptobiosaminide (VII) and 2-bromoethyl dihydrostreptobiosaminide (VI), tested as their hydrochlorides at a concentration of 250 μg/ml in nutrient broth at 37°, were inactive against *Staphylococcus aureus* 898, *Bacillus subtilis* 814E, *Escherichia coli* 741; and *Pseudomonas pyocyanea* 150E. The same compounds were also inactive against *Mycobacterium tuberculosis* 666 at the same concentration in Dubos medium after two weeks at 37°.

Acknowledgements. We thank Glaxo Laboratories Ltd., for supplies of streptomycin and dihydrostreptomycin and for the microbiological results. We are indebted to the D.S.I.R. for a Research Studentship (G.K.J.F.) and to NATO for a Post-doctoral Fellowship (I.J.McG.).

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Some studies of friction and lubrication using an instrumented tablet machine†

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Samples of crystalline aspirin, hexamine, and sodium chloride, and a granulation of hexamine were compressed on an instrumented tablet machine. Further samples of these materials, together with samples of sucrose and sucrose granulation, were lubricated with 2% magnesium stearate −100 mesh powder and compacted under similar conditions. The effect of magnesium stearate on die wall friction depends on the ease of deformation of the base particles. For the materials used there is a linear relationship of the form $F_d = k_d(P_m.A)$ and a linear relationship exists between F_e and F_d up to the point where F_e becomes constant. Granulations required larger ejection forces at a given pressure than the corresponding crystalline material, particularly when the materials were lubricated; constant values of F_e were associated with compaction pressures at which the density of the ejected tablets became maximal. Values of F_e for tablets of unlubricated sodium chloride and hexamine granulation depend on particle size; the size of other materials used caused no such effect. Lubrication eliminated this size effect.

WHEN materials are compacted in dies to form tablets by movement of the upper punch only, frictional effects at the die wall cause the force transmitted to the stationary lower punch to be less than the force applied at the upper punch. If both punches move during the consolidation stage, as in rotary tablet machines, the system is equivalent to two single-ended pressings.

Instrumented eccentric tablet machines have been used to investigate force transmission during tabletting (Higuchi, Nelson & Busse, 1954; Markowski, 1958; Hasegawa, 1959; Shotton & Ganderton, 1960; Riad & Zobel, 1962; Fuhrer, 1962) and a rotary machine instrumented with resistance strain gauges on the punches has been reported (Shotton, Deer & Ganderton, 1963). However, most of the recorded work is concerned with the behaviour of pharmaceutical granulations (Nelson, Naqvi, Busse & Higuchi, 1954; Nelson, Busse & Higuchi, 1955; Nelson, 1955; Strickland, Nelson, Busse & Higuchi, 1956; Markowski, 1958; Strickland, Higuchi & Busse, 1960). With the advent of forced-feed devices on tablet machines and the possibility in certain cases of eliminating the granulation process, more information is needed about the behaviour of simple crystalline and powder systems.

Experimental

30–40 mesh samples of aspirin, hexamine crystals, and hexamine granulation, and 40–60 mesh sodium chloride crystals were compacted at mean compaction pressures ranging from 400–2,200 kg/cm². An

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instrumented single punch eccentric tablet machine, incorporating a ½ inch plane faced punch and die set, was used (Shotton & Ganderton, 1960). Further samples of these materials lubricated with 2% magnesium stearate (−100 mesh powder) were compacted over this same pressure range. These experiments were repeated with similarly lubricated samples of 30–40 mesh sucrose and sucrose granulation.

The granulations were prepared from −100 mesh powder using only distilled water as an adhesive agent (Shotton & Lewis, 1964). It was not possible to tablet sucrose in any form in the absence of a lubricant.

Samples of sodium chloride, hexamine, hexamine granulation, and aspirin, varying in size from 20–30 mesh to 80–100 mesh were compressed both unlubricated and when lubricated with 2% magnesium stearate −100 mesh powder. Lubricated samples of sucrose and sucrose granulation were also compressed. For any one material the mean compaction pressure was constant, but the pressure level chosen varied with the nature of the base material.

Sample weights tabletted were calculated to give a tablet 0·4 cm thick at zero porosity.

Results and discussion

All results represent the mean of five tablets. From the experimental measurements values were calculated for mean compaction pressure, P_m (the average of the top and bottom punch pressures), ejection force, F_e, force lost to die wall, F_d (difference between upper punch force and lower punch force), and the ratio of lower punch force to upper punch force, R. The relative density, ρ_R, of the ejected tablets was calculated from their weight and dimensions.

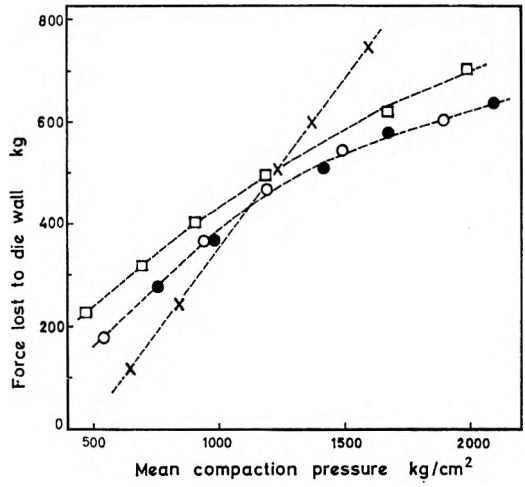

FIG. 1. Relationship between mean compaction pressure and force lost to die walls: unlubricated materials. × Sodium chloride. □ Aspirin. ● Hexamine. ○ Hexamine granulation.

EFFECT OF MEAN COMPACTION PRESSURE

Force lost to die wall. A linear relationship was found between P_m and F_d for unlubricated sodium chloride, but results for aspirin, hexamine, and hexamine granulation are best represented by curves (Fig. 1). The results for hexamine granulation follow closely those for hexamine crystals.

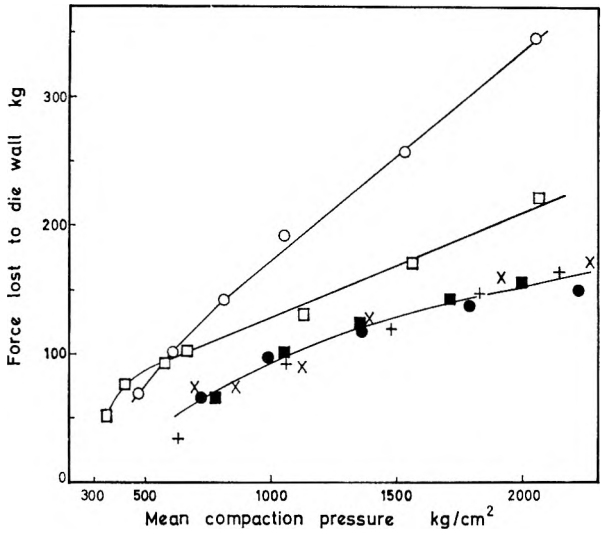

FIG. 2. Relationship between mean compaction pressure and force lost to die walls: materials lubricated with 2% magnesium stearate. × Sodium chloride. □ Aspirin. ● Hexamine. ○ Hexamine granulation. ■ Sucrose. + Sucrose granulation.

The presence of lubricant causes a marked reduction in values of F_d at any one pressure level (Fig. 2); also for any given increment of P_m, the increase in F_d is very much less for the lubricated materials. Results for lubricated samples of sucrose, sucrose granulation, hexamine, and sodium chloride can be represented by a single line, whilst lubricated aspirin and hexamine granulation show quite a different relationship. These latter materials seem to maintain a greater influence on die wall friction during consolidation.

Train (1956) quotes the results of Nelson & others (1954) in support of his own work which indicated an empirical relationship for ejection force,

$$F_e = c_1 (P_a.A)^n \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where P_a = applied pressure; A = apparent area of die wall/compact contact; n = a constant, a property of the material; c_1 = a constant, a property of the surface condition of the walls. He suggested a possible linear relationship between the ejection force and the maximum friction at the die wall during compaction.

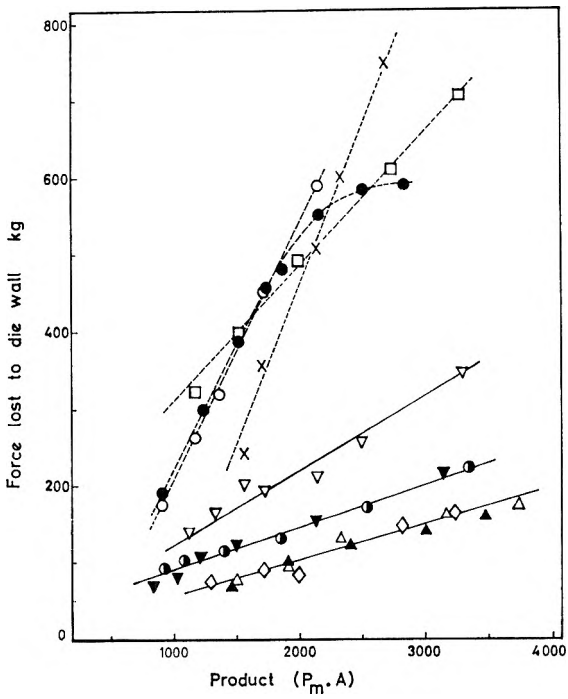

FIG. 3. Relationship between force lost to die wall and the product (P_m.A). × Sodium chloride, unlubricated. △ Sodium chloride, lubricated. ● Hexamine, unlubricated. ▼ Hexamine, lubricated. ○ Hexamine granulation, unlubricated. ▽ Hexamine granulation, lubricated. □ Aspirin, unlubricated. ◑ Aspirin, lubricated. ◇ Sucrose granulation, lubricated. ▲ Sucrose, lubricated.

If such a relationship exists

$$F_d = c_2 (P_a A)^n \quad .. \quad .. \quad .. \quad .. \quad (2)$$

Using P_m instead of P_a and calculating values of A ($= \pi DI$) from the length (l) of the ejected tablet it is found (Fig. 3) that there is a simple relationship between F_d and the product (P_m.A), and that n has a value of unity, i.e.

$$F_d = k_d (P_m.A) \quad .. \quad .. \quad .. \quad .. \quad (3)$$

This relationship is demonstrated by all materials, with the exception only of unlubricated hexamine, over the complete range of compaction pressures studied. Values of F_d for unlubricated hexamine tend to a maximum value at 600 kg, but there is a linear relationship below this value.

The slope of the line, k_d, for unlubricated materials varies with the type of material compacted.

e.g.	Slope, k_d
Sodium chloride	2·44
Aspirin	1·06
Hexamine crystal and granulation	2·00

When lubricated with magnesium stearate the base materials fall into 3 groups. Group 1: sucrose, sucrose granulation and sodium chloride. Group 2: hexamine and aspirin. Group 3: hexamine granulation.

The slopes of the lines for group 1 and group 2 materials are almost equal, i.e. 0·30 and 0·32 respectively. If the term k_d is a function of the surface condition of the die (cf. Train's constant c_1) it is apparent that the magnesium stearate determines the surface condition to the same extent for all the lubricated materials except hexamine granulation.

Force necessary for ejection, F_e. For tablets of unlubricated hexamine, hexamine granulation, and aspirin, a linear relationship exists between F_e and values of P_m up to 1,200 kg/cm² (Fig. 4). At greater values of P_m for these materials F_e becomes constant. Sodium chloride shows no maximum value of F_e over the range of pressures studied.

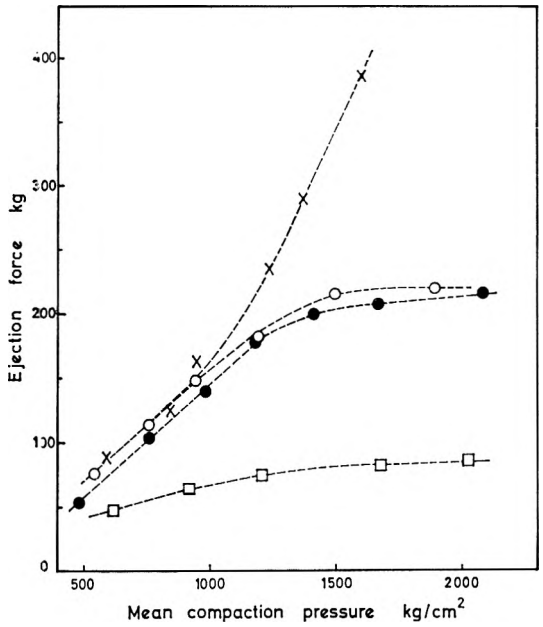

FIG. 4. Effect of mean compaction pressure on ejection force: unlubricated materials × Sodium chloride. ○ Hexamine granulation. ● Hexamine. □ Aspirin.

The magnesium stearate caused a marked reduction in F_e for all materials (Fig. 5). Values of ejection force for tablets of lubricated hexamine and hexamine granulation also became constant at pressures greater than 1,200 kg/cm², but values of F_e for lubricated aspirin tablets reached a constant value at a lower compaction pressure than previously. Ejection forces for lubricated sodium chloride tablets approach a limiting value at the maximum compaction pressure used, and these results are followed very closely by those for lubricated sucrose. There was no evidence of a maximum F_e value when lubricated sucrose granulation was compressed.

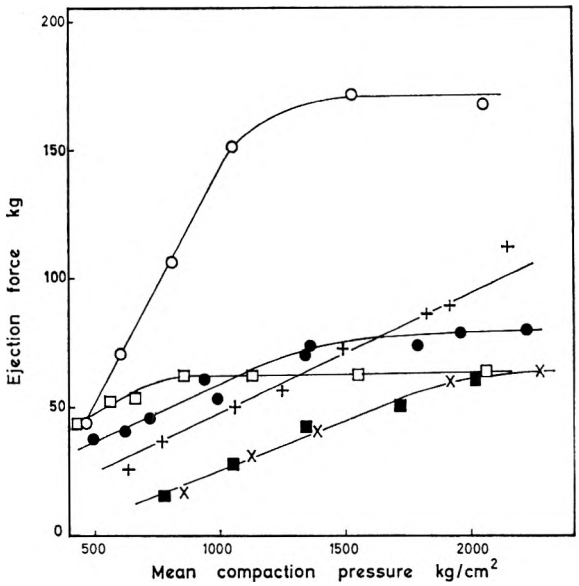

FIG. 5. Effect of mean compaction pressure on ejection force: lubricated materials. × Sodium chloride. ○ Hexamine granulation. ● Hexamine. □ Aspirin. ■ Sucrose. + Sucrose granulation.

Tablets made from the lubricated granulations required greater ejection forces at any one compaction pressure than the respective crystalline materials. Sucrose and sucrose granulation exhibit such large frictional forces that it was not possible to tablet the unlubricated materials, even at low pressures, because the machine jammed at the ejection stage. Both crystalline sucrose and sodium chloride require large ejection forces when unlubricated, yet when lubricated produced the smallest ejection forces over the whole range of compaction pressures.

A linear relationship between F_e and P_m was also found for tablets of lubricated materials until the limiting value of F_e was attained (Fig. 5). With the exception of hexamine granulation the linear portions of the graphs for lubricated materials are almost parallel to each other. In this case it is suggested that in the presence of a lubricant the "frictional condition" of the die surface is the same regardless of the base material, and that in this situation the observed differences in magnitude of F_e, at any one pressure, are dependent on the individual base materials and the residual radial stresses present in the tablet when the compacting force is removed before ejection of the tablet from the die.

The value of P_m at which ejection force for tablets of unlubricated materials approaches a constant value, corresponds to the pressure at which the relative density of the tablet becomes constant at its maximum value (Figs 6–8). The apparent peak value of ρ_R for hexamine is due to the fact that when tablets of this material are compacted at pressures greater than 1,250 kg/cm² they exhibit a marked lamination, which

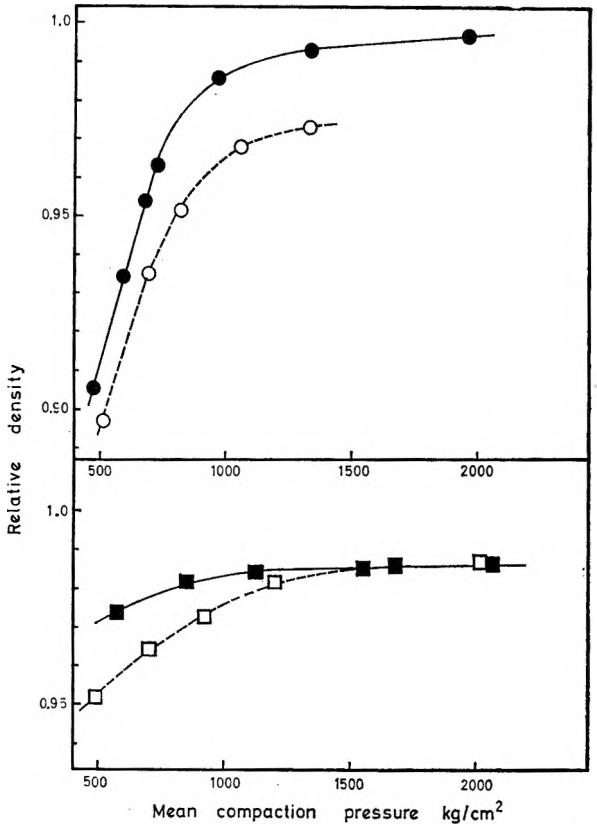

FIG. 6. Effect of mean compaction pressure on density of compact. ● Hexamine granulation, lubricated. ○ Hexamine granulation, unlubricated. ■ Aspirin, lubricated. □ Aspirin, unlubricated.

produces an apparent increase in the measured thickness of the tablet, and consequently a lower value for density.

Similarly, tablets of unlubricated sodium chloride show no sign of attaining a maximum value of F_e because the maximum value of ρ_R is 0·94 (Fig. 7); lubrication facilitates consolidation to a relative density of 0·99, and the ejection force approaches a maximum value. It is notable that at the maximum pressures used (Fig. 8), tablets of lubricated sucrose and sucrose granulation have low ρ_R values compared to the other materials. They also show no terminal value of F_e.

Up to the point where ejection force becomes constant, a linear relationship exists between F_e and F_d (Fig. 9). Such a relationship was reported for sulphathiazole granulation by Nelson & others (1954).

Punch force ratio, R. Three entirely different effects were observed when R was plotted as a function of P_m for the compression of unlubricated materials (Fig. 10). When magnesium stearate was added as lubricant to sodium chloride, sucrose, sucrose granulation, and hexamine,

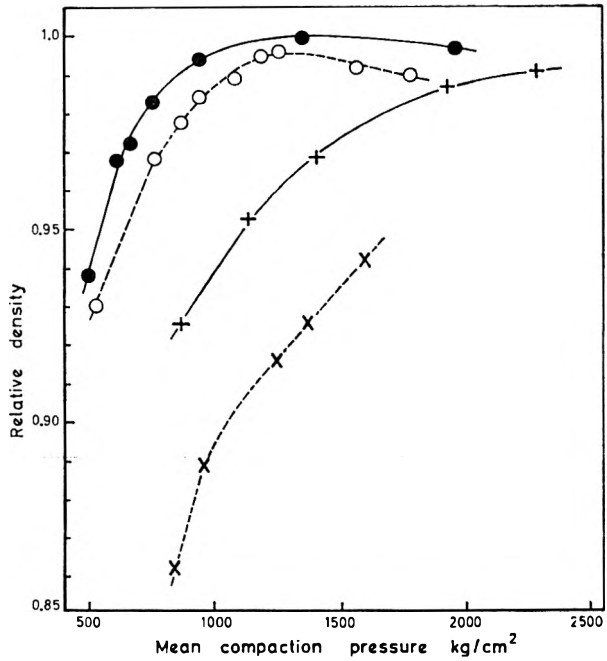

FIG. 7. Effect of mean compaction pressure on density of compact. × Sodium chloride, unlubricated. + Sodium chloride, lubricated. ○ Hexamine, unlubricated. ● Hexamine, lubricated.

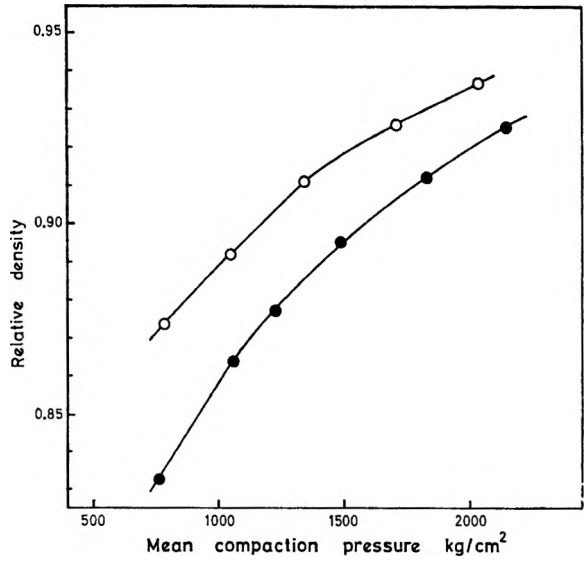

FIG. 8. Effect of mean compaction pressure on density of compact. ○ Sucrose, lubricated. ● Sucrose granulation, lubricated.

the proportion of the applied force transmitted to the lower punch was independent of the magnitude of P_m and the results are best represented by a single horizontal line, $R = 0{\cdot}94$. As shown in Fig. 2 lubricated aspirin and hexamine granulation behave in a distinctive manner.

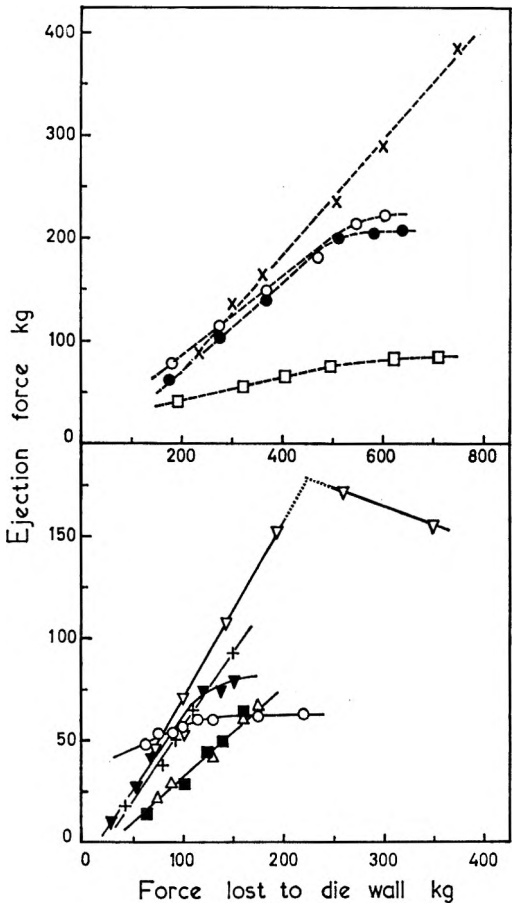

FIG. 9. Relationship between ejection force and the force lost to die wall. Unlubricated materials: × Sodium chloride. ○ Hexamine granulation. ● Hexamine. □ Aspirin. Lubricated materials: ▽ Hexamine granulation. ▼ Hexamine. + Sucrose granulation. ○ Aspirin. △ Sodium chloride. ■ Sucrose.

INFLUENCE OF BASE PARTICLE SIZE

For graphical representation of results the particle size is assumed to be equivalent to the mean of the sieve apertures by which the range is classified (Shotton & Lewis, 1964).

With the exception only of unlubricated sodium chloride and hexamine granulation, the force necessary to eject tablets compressed at a constant pressure is independent of the size of material being compacted (Fig. 11).

The increase in F_e with reduction in particle size of sodium chloride is probably due to the increased area presented to the die wall when the

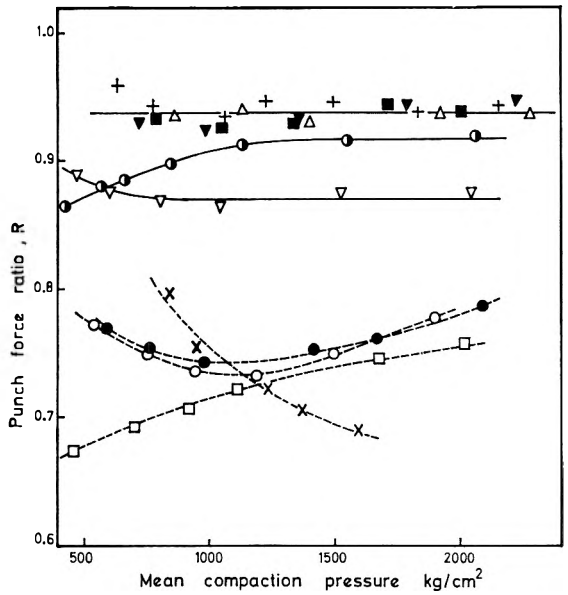

FIG. 10. Effect of mean compaction pressure on punch force ratio. Unlubricated materials: × Sodium chloride. ● Hexamine. ○ Hexamine granulation. □ Aspirin. Lubricated materials: △ Sodium chloride. ▼ Hexamine. ▽ Hexamine granulation. ◑ Aspirin. + Sucrose granulation. ■ Sucrose.

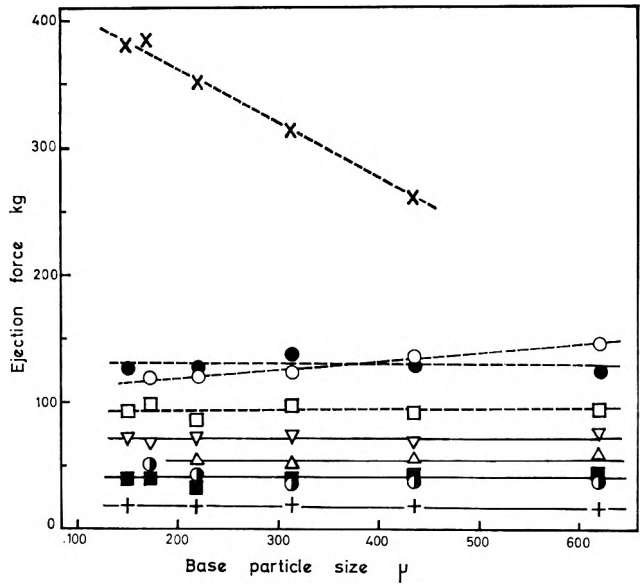

FIG. 11. Effect of base particle size on ejection force. Mean compaction pressures (kg/cm²) as indicated. Unlubricated materials: × Sodium chloride (1485). ○ Hexamine granulation (744). ● Hexamine (802). □ Aspirin (1925). Lubricated materials: △ Sodium chloride (1405). ▽ Hexamine granulation (691). + Sucrose (889). ■ Sucrose granulation (885). ◑ Aspirin (2083). Hexamine (753) results are coincident with those for sodium chloride, lubricated.

smaller material is tabletted. Aspirin, hexamine, and hexamine granulation readily deform so that the area of contact will be approximately constant irrespective of particle size, whereas sodium chloride is harder and more resistant to deformation so that the area of material in contact with the die will depend more on the particle size. The size effect is eliminated by magnesium stearate which subjugates the frictional characteristics of the sodium chloride crystal.

For tablets of unlubricated hexamine granulation F_e values decrease when granule size is reduced. A formed granule undergoes appreciable fragmentation during the compression cycle (Higuchi, Rao, Busse & Swintosky, 1953; Elowe, Higuchi & Busse, 1954; Higuchi, Elowe & Busse, 1954), and the degree of fragmentation will be greatest where the granule is large. It is proposed that the fresh surface produced by compaction of 20–30 mesh granules adheres more strongly to the die wall, and higher ejection force results. Again the effect is eliminated by lubrication.

With the exception of unlubricated sodium chloride, values of R and F_d at a constant compaction pressure were independent of particle size of the base material, whether lubricated or unlubricated. F_d values increased with a decrease in particle size of sodium chloride and there was a corresponding decrease in values of R as shown in Table 1.

TABLE 1. THE INFLUENCE OF PARTICLE SIZE ON FRICTIONAL LOSSES WHEN SODIUM CHLORIDE IS TABLETTED

Mean compaction pressure: 1485 kg/cm²

Particle size μ	Force lost to die wall F_d, kg	Punch Force ratio R
435	550	0·75
315	690	0·69
220	704	0·68
170	712	0·68

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A comparison of tablet lubricant efficiencies for a sucrose granulation using an instrumented tablet machine†

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Thirteen materials have been assessed as lubricants when added to a sucrose granulation and compressed on an instrumented tablet machine. Values of ejection force, force lost to die wall, and punch force ratio for each lubricated sample were compared, and the order of efficiency as lubricants was compared with the shear of lubricant powder compacts. The most efficient lubricants are those materials of lowest melting point, and not those with the smallest shear strength value as measured in a punch penetration test.

IN the production of tablets, substances are usually added to the materials to be compressed to improve the flow of granules and to reduce the friction between tablet material and the die wall. Those materials facilitating flow have been termed "glidants", and those reducing friction, "lubricants" (Munzel & Kagi, 1954).

Evaluations of various materials as lubricants have been made by Wolff, DeKay, & Jenkins (1947), Nelson, Naqvi, Busse & Higuchi (1954), Patel & Guth (1955), Markowski (1958), Strickland, Higuchi & Busse (1960) and Maly (1961). More importance is attached to the quantitative results of Nelson and Strickland and their colleagues and Markowski, which were obtained using conventional tabletting machines instrumented with strain gauge equipment. Although there is general agreement between workers that the best tablet lubricants are found in that class of materials normally called "boundary lubricants" in the general field of lubrication, e.g. salts of stearic acid, there are discrepancies between the various orders of merit. Only Markowski (1958) attempts an explanation of this variation and points out that his rating of the stearates seems to be related to the valency of the metal involved; he suggests that the "size of the molecule" may influence lubricant efficiency. Thus of all materials examined by this worker sodium stearate reduced the ejection force to the greatest extent and also had the smallest molecular size.

The magnitude of the die wall friction will depend on the shear strength of the friction junction and the area of contact (Bowden & Tabor, 1954). Good lubricants would be expected to have low shear strength values and the shear strength of a number of materials has been measured by Lewis & Train (1965).

The present work is an attempt to evaluate the lubricating efficiency of these same materials using a tablet machine instrumented with strain

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gauges as described by Shotton & Ganderton (1960). The relative efficiency of a lubricant is then compared with its shear strength value.

Experimental

MATERIALS AND APPARATUS

A granulation of sucrose was prepared from material ball-milled until all passed a 100 mesh sieve. The powder was moistened with distilled water and pressed through a 30 mesh sieve. The granules were dried at room temperature (2 hr) then at 60° (2 hr). They were sieved on an Inclyno machine for 15 min and the 30–40 mesh fraction used throughout this work.

To samples of 30–40 mesh granulation, 2% of the following materials was added as −100-mesh powder: stearic acid, palmitic acid, lithium stearate, sodium stearate, potassium stearate, magnesium stearate, zinc stearate, zinc oleate, calcium stearate, "synthetic wax,"* boric acid, and talc.

Hard paraffin was applied to the granulation as a solution in light petroleum (b.p. 60°–80°). The granules were coated with this in a coating pan, using a jet of hot air to remove the solvent; they were then dried (2 hr) at 60°/20 mm.

METHODS

Preliminary experiments indicated that the sucrose granulation possessed desirable pressing characteristics and high frictional resistance, making it suitable as a base material. When lubricated with −100-mesh magnesium stearate powder, this material gave a linear relationship between ejection force and mean compaction pressure over the range 500—2000 kg/cm². The proportion of the applied pressure transmitted to the lower punch over this range was constant. Values of the punch force ratio, R, force lost to die wall, F_d, and ejection force, F_e, were unaffected by variations in base granule size, and of all the lubricants examined 2% −100-mesh magnesium stearate affected these values to the maximum extent.

Samples weighing 700 mg were filled into the die with the aid of a funnel, levelled, and were compacted at a mean compaction pressure of 1033 kg/cm². The die was conditioned by compressing five tablets from the material in question; these were rejected and a further five tablets prepared. Measurements were made of upper punch force, lower punch force, ejection force, weight of tablet, and length of tablet.

Between the evaluation of each lubricated sample the die and punches were removed from the machine, cleaned with metal polish, polished, and then degreased with a mixture of equal parts of carbon tetrachloride and acetone before conditioning with the next sample.

* Synthetic wax flake WC 5956: Wilkins, Campbell and Co.

Results and discussion

Nelson & others (1954), Strickland, Nelson, Busse & Higuchi (1956), Strickland & others (1960) and Markowski (1958) favoured the ratio (R) of force transmitted to the lower punch to that applied by the upper punch as a means of comparing lubricant efficiencies. The better the lubricant, the greater the proportion of the applied force transmitted to the lower punch: values of R thus tend to unity.

The use of this parameter was criticised by Maly (1961) as being insufficiently sensitive to distinguish between good lubricants, and a lubricating factor based on ejection forces was proposed.

$$\text{Lubricating factor } M = \left(1 - \frac{T_2}{T_1}\right) \times 100$$

where

T_2 = ejection force for a given pressure for lubricated sample

T_1 = ejection force at same pressure for unlubricated sample of base material

For a given base material this factor (M) offers no advantage over the use of the ejection force F_e. Comparisons based on ejection force were also made by Patel & Guth (1955), Smilek, Cosgrove & Guth (1955), Appino, Banker & De Kay (1959) and Munden, De Kay & Banker (1960), but the method of instrumentation was not as sensitive as the strain gauge equipment used by other workers.

Present results for ejection force, F_e, punch force ratio, R, and force lost to die wall, F_d, are presented in Table 1.

TABLE 1. EVALUATION OF TABLET LUBRICANTS

Material	Ejection force, Fe, kg	Punch force ratio, R	Force lost to die wall, Fd, kg	Melting point °C.	Shear strength,* kg/cm²
Hard paraffin	15	0·94	80	50	—
Stearic acid	22	0·94	78	54	13·7
Palmitic acid	24	0·95	68	57	12·3
Synthetic wax	26	0·84	224	105	50·5
Sodium stearate	38	0·93	93	240–243	23·9
Zinc oleate	40	0·93	93	170–174	—
Lithium stearate	41	0·95	63	215–218	6·0
Potassium stearate	43	0·94	89	252–255	21·3
Zinc stearate	45	0·94	82	120	9·3
Calcium stearate	48	0·93	103	140	15·0
Magnesium stearate	50	0·93	104	186	20·0
Boric acid	346	0·63	584	—	73·0
Talc	353	0·59	664	—	—
Unlubricated	371	0·55	682	—	—

* Lewis & Train (1965). Spread of results ± 7%. Values measured under zero load conditions.
Base material: 30–40 mesh sucrose granulation
Lubricant concentration: 2%
Mean compaction pressure: 1033 kg/cm²
Sample weight: 0·7g

Most values of R fall in the close range 0·93–0·95 making the use of this factor unsatisfactory as a means of differentiating between the lubricating efficiencies of most of the substances tested. This parameter is insufficiently sensitive to distinguish between the soap lubricants but could serve

as a first classification to separate "efficient" lubricants, such as magnesium stearate, from "inefficient" ones such as talc.

Ejection forces allow of slightly sharper differentiation. The most efficient lubricants in this respect are the fatty acids and waxes, those materials with the lowest melting point. Salts of stearic acid melt with decomposition at a much higher temperature than the acid itself (see Table).

Bowden & Tabor (1954) have shown that considerable temperatures are generated even in the lightest frictional contacts, and that in addition, where boundary layer lubrication conditions exist, the efficiency of soap lubricants and fatty acids drops appreciably when the surface temperature reaches the value of their melting-points.

The addition of fatty acids to metals with which they react, results in the formation of soap films possessing strong lateral cohesion and a melting-point much higher than that of the acid. It is clear that the application of a metallic soap need not necessarily be as effective a lubricant as the soap formed *in situ*. The present results suggest that 2% lubricant is sufficient to make boundary layer conditions unimportant, so that the greatest efficiency of lubrication is not dependent on the high melting-point of a lubricant. A low melting-point probably facilitates the formation of wax or fatty acid films on the die wall.

Table 1 shows that the ratings of lubrication efficiency, as assessed by comparison of ejection forces, are not explained by the values of shear strength measured by the punch penetration method (Lewis & Train, 1965). Lithium and zinc stearates, which have the lowest shear strengths, gave ejection forces nearly twice those for hard paraffin, stearic and palmitic acids. The absence of such correlation may well be due to the properties of the sucrose granulation substrate. Jones (1960) reports for metal powders that the best lubricant and optimum concentration depends on the powder being pressed, and Daoust (1960) presents evidence that the efficiency of lubricants used with pharmaceutical granulations may vary with the type of granulating agent used. Such interdependence between substrate and lubricant may well account for different workers producing different orders of efficiency.

On the other hand the shear strength of compacted lubricants under zero load conditions may not be the important value for comparison purposes. When the compressive force has been removed and before ejection from the die, the tablet exerts a radial force on the walls of the die. The lubricant film will therefore be subjected to a constraining load and it is possible that the shear strength of the lubricant under an applied compressive load is the value that is needed.

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Adjuvant effect of aluminium monostearate paraffin gels on antitoxin response

C. L. J. COLES, KATHLEEN R. HEATH, MARJORIE L. HILTON, K. A. LEES, P. W. MUGGLETON AND C. A. WALTON

Clostridium welchii Type D toxoid has been dispersed in aluminium monostearate-hydrocarbon gels. When these are injected subcutaneously in laboratory animals the antitoxin response is influenced by the method of preparation of the gels. The level and duration of the antitoxin titre in the blood is related directly to the viscosity of the vaccine preparations. Six months after injection, the residual antitoxin titre produced by the most successful treatments was still as good as the peak titre obtained from simple aluminium hydroxide adsorbed vaccine.

THE blood level of antitoxin produced by a single dose of toxoid is frequently inadequate to develop satisfactory immunity so that a second or "booster" dose is required. When aluminium hydroxide gel is used as an adjuvant for toxoid vaccines, it significantly increases the response to the antigen, but it may also produce reactions at the site of injection. Moreover, the blood antitoxin levels fall fairly rapidly, even after a "booster" dose.

Improvement of antitoxin levels by dispersing antigen in pharmaceutical vehicles immiscible with serum has been demonstrated on several occasions, and the mechanisms of action of such systems has been reviewed (McKinney & Davenport, 1961; Davenport, 1961). Oil : aluminium monostearate gels have recently been shown to enhance antitoxin blood levels (Woodhour, Metzgar, Stim, Tytell & Hilleman, 1964; Stokes, Weibel, Drake, Woodhour & Hilleman, 1964).

It is well known that the use of such gels lowers the rate of release of procaine penicillin or the active principle in pollen extract from the site of injection (Bristol, 1952, 1954).

Our attention was drawn to the enhanced blood antitoxin levels obtained from injections of *Clostridium welchii* Type D toxoid when presented in liquid paraffin containing aluminium stearate (Dr. C. Moller, personal communication). The present communication describes an examination of liquid paraffin aluminium stearate suspensions and gels in this connection.

Experimental

MATERIALS

The paraffin oil used was a mixture of 75 parts (by volume) liquid paraffin B.P. and 25 parts light liquid paraffin B.P. 1958. It was sterilised by heating to 150° for 1 hr. Aluminium monostearate* had the following characteristics: Al_2O_2 (ash) 16·6%; free fatty acid (as stearic acid) 6·7%; water soluble salts 0·5%. It was sterilised by exposure

From Glaxo Laboratories Ltd., Greenford, Middlesex.

* Mallinkrodt special M grade.

to formalin vapour (24 hr), excess formalin vapour was then removed under reduced pressure.

The antigen used was a freeze-dried *Clostridium welchii* Type D purified formol toxoid comminuted by successive passage through 60 mesh and 100 mesh sieves. It had a potency of approximately 40 Lf units/mg.

PREPARATION OF PARAFFIN GELS

Two general methods were used.

Method A. Aluminium monostearate was dispersed in sufficient paraffin oil to constitute 20% by volume of the final vaccine. The suspension was then maintained at the required temperature for a fixed period (72° or 100° for 10 min, or 150° for 1 hr). The products were cooled to room temperature without agitation. The antigen was dispersed in the remainder of the cool sterilised oil, and the paraffin gel was then dispersed in this suspension; the mixture was finally passed once through an Ormerod homogeniser Type QR at 1,000 p.s.i. At 72° only partial gelation occurs; the product corresponds to that described by Moller.

Method B. Aluminium monostearate was dispersed in sufficient paraffin oil to produce 80% of the final vaccine volume. This suspension was heated to either 100° or 150° and stirred for 1 hr at this temperature; it was then cooled with continuous agitation. The antigen was dispersed in the remainder of the cool sterilised oil and blended with the paraffin gel; the vaccine was passed once through an Ormerod homogeniser, as above.

Suspensions of aluminium stearate (2% w/v) in paraffin oil decreased in viscosity as they were heated slowly to 80°, owing to a reduction in the viscosity of the oil. Microscopical examination of the suspensions indicated that the soap particles began to swell at 80 to 89°. At 95° complete solution of the soap yielded a visco-elastic gel. A further rise in temperature, to 135°, was accompanied by a sharp drop in the viscosity; this may be attributed to dissociation of the soap molecules by thermal motion.

Heating the gels to 100° followed by rapid cooling without agitation yielded a thick visco-elastic gel (Method A), whereas cooling associated with vigorous agitation yielded a thinner though still viscous gel (Method B). Rapid cooling of the gel from 150° (Method A) produced a solid grease which if dispersed in paraffin oil, yielded a product of viscosity similar to that cooled from 100°. If this was again heated to 150° (Method B) and stirred continuously while cooling, the least viscous gel of all was produced; it was much thinner than that produced at 100°.

An attempt was made to measure the viscosity of the gel systems with a rotary viscometer but no single parameter was found to express adequately the different characters of the gels. For this reason only visual observations of viscosity have been reported.

Antitoxin was induced in the serum of rabbits by subcutaneous injection of 2 ml (equivalent to 130 Lf units of toxoid) of each of these vaccines. Four weeks later a similar booster dose was given. The antitoxin levels

obtained are shown in Fig. 1. The titres presented as control were obtained by adsorbing antigen, from the same batch, on aluminium hydroxide gel.* Antigens before and after freeze drying were used for this purpose.

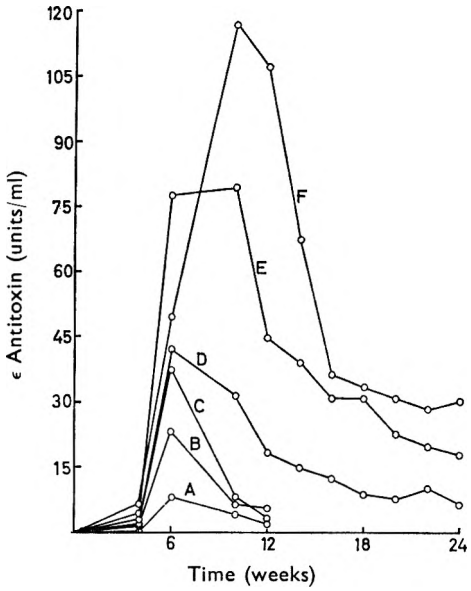

FIG. 1. Epsilon antitoxin titres produced in rabbits by a 2 ml subcutaneous injection of *Clostridium welchii* type D vaccine, followed by a booster dose at four weeks, A. Ungelled aluminium monostearate suspension. B. Reconstituted freeze-dried toxoid, aluminium hydroxide adsorbed. C. Aluminium hydroxide adsorbed toxoid. D. Aluminium monostearate gel prepared at 72°C. E. Aluminium monostearate gel prepared at 150°C. F. Aluminium monostearate gel prepared at 100°C.

A second series of vaccines was prepared both by methods A and B at the temperatures that had given most enhancement in the first experiments. The amount of aluminium stearate varied, 0·5, 1·0 and 2·0% being used. Antitoxin levels in guinea-pigs (after single 1 ml subcutaneous injection of vaccine) are shown in Table 1.

The addition of traces of many compounds, particularly those with pronounced co-ordinating properties, reduces the viscosity of gels (Gray & Alexander, 1949; Alexander & Gray, 1950). Non-ionic surface-active agents have this effect. A series of gels with a range of aluminium monostearate concentrations and incorporating 0·5% polysorbate 60† were prepared by Method B at 150°.

Guinea-pig and rabbit sera were titrated for their content of *Clostridium welchii* ε antitoxin. Approximately twofold dilutions of sera were

* Alhydrogel—Danish Sulphuric Acid and Superphosphate Works Ltd., Denmark.

† Tween 60—Honeywill-Atlas Ltd.

titrated in mice against standard *Clostridium welchii* ϵ toxin by the method of Batty & Glenny (1947).

TABLE 1. ANTITOXIN LEVELS (GUINEA-PIGS) FROM DIFFERENT ALUMINIUM STEARATE CONCENTRATIONS AND GELATION TEMPERATURES

Method of preparation	Temperature of gelation, °C	Aluminium monostearate %	Antitoxin units/ml				
			2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
A	100	0·5	0·06	0·28	0·4	0·4	0·14
		1·0	0·14	2·8	2·8	1·4	1·4
		2·0	0·14	5·6	4	5·6	5·6
A	150	0·5	0·14	0·8	1·4	1·4	0·56
		1·0	0·28	1·4	1·4	1·4	1·4
		2·0	2	14	14	14	4
B	100	0·5	0·56	0·8	2	2	2·8
		1·0	0·56	5·6	5·6	5·6	5·6
		2·0	1·4	28	28	14	4
B	150	0·5	0·28	0·04	0·28	0·14	0·08
		1·0	0·4	4	4	5·6	2·8
		2·0	0·28	2·8	2·8	5·6	4
Aluminium hydroxide adsorbate suspension			0·2	2·8	1·4	1·4	1·4

Results

The results in Fig. 1 show that a base prepared by the gelation of paraffins with aluminium monostearate increases and prolongs the titre of antitoxin in the blood. As the content of aluminium monostearate rises from 0·5 to 2%, the antitoxin level in guinea-pig serum rises irrespective of the method of preparation or the temperature of gelation (Table 1). Six months after injection the residual titre with the most successful vaccines was still as high as the peak titre obtained with a simple aluminium hydroxide adsorbed vaccine. The viscosity of the vaccines showed a positive correlation with the degree of enhancement and the prolongation of the antitoxin levels.

Products obtained by Method A confirm the effect of temperature on gelation although the results for vaccines prepared by Method B do not. The conclusion drawn from Fig. 1 is confirmed, namely that antitoxin titres and the viscosity of the vaccine are related.

TABLE 2. ANTITOXIN LEVELS IN GUINEA-PIGS FROM GELS WITH AND WITHOUT POLYSORBATE (P) 60

Aluminium monostearate %	Percentage P 60	Antitoxin units/ml				
		2 weeks	4 weeks	6 weeks	8 weeks	12 weeks
0·5	—	0·2	0·4	2·8	2·8	2
0·5	0·5	0·06	0·4	0·56	0·56	0·56
1·0	—	0·04	2·8	2·8	5·6	5·6
1·0	0·5	0·2	1·4	1·4	0·56	0·56
2·0	—	0·28	4	5·6	5·6	8
2·0	0·5	0·14	4	2·8	2·8	2·8

Table 2 confirms that antitoxin level is enhanced as the concentration of aluminium stearate increases. The addition of polysorbate 60 depresses antitoxin levels which moreover seem less prolonged. These effects are also related to the lower viscosity of these bases.

Acetoglycerides also reduce the viscosity of the gels, but they could not be evaluated in animals because gross reactions were produced by them at the site of injection.

Discussion

Hydrocarbon-aluminium stearate gels comprise a two-phase structure. One phase consists of a network of more or less solvated materials, whose interstices are filled with its saturated solution (Rideal, 1950). It is possible that particles of antigen are situated within the network of solvated material so that their diffusion from the vaccine is hindered. This hypothesis received some support from an experiment in which the freeze-dried toxoid was milled in oily suspension, to provide particulate toxoid: by microscopical appearance this exhibited a range of particle sizes. The samples of milled toxoid in paraffin-aluminium stearate gel however show negative correlation of particle size and antitoxin response.

The way in which oil adjuvant vaccines enhance and prolong antibody production is not clearly established. In simplified general terms two mechanisms have been suggested (Davenport, 1961): (*a*) local irritation at the site of injection attracts certain cells to the site so that an "antibody producing organelle" is formed and (*b*) that slow continuous release of antigen from the site of inoculation promotes more efficient utilisation of the antigen for its specific purpose. The local reactions from all the vaccine preparations (except those prepared with acetoglycerides) were macroscopically indistinguishable from each other and from those produced by the simple suspension of aluminium monostearate in paraffin oil.

These results suggest that, because the viscosities of the products described correlate directly with antitoxin response, the rate of release of the antigen from the injection site plays an important part in determining the adjuvant effect. In the absence of histological data the significance of other mechanisms which may operate at a cellular level cannot be assessed.

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A new method for the determination of emulsion stability by dielectric constant measurement: a preliminary report

R. C. KAYE AND H. SEAGER

The creaming of emulsions of liquid paraffin in water stabilised by cetomacrogol 1000 has been studied in a specially designed capacitance cell. Before visual separation of the emulsion is detectable, changes in emulsion composition in the upper part of the cell result in a measurable decrease of dielectric constant. Differences in the creaming rates of a number of emulsions are easily detected and the rate of change of dielectric constant with time is dependent on globule size.

EMULSIONS are thermodynamically unstable systems which reach stability only when demulsification has occurred. The fundamental measurement of the instability therefore is the rate at which the globules of the emulsion coalesce. Creaming, or phase separation without coalescence, is quite a different phenomenon from demulsification but one that is often used as an indication of instability because the resulting closer association of the globules greatly facilitates their coalescence.

Creaming is difficult to detect visually in the early stages (except in very unstable emulsions) and there is no simple and rapid method for its quantitative determination. Andreasen's method (Andreasen, 1928) is feasible but tedious and methods which depend on changes in optical density, for example photoelectric sedimentometry, are not applicable because pharmaceutical emulsions are too opaque.

Since water has a high, and oil a low, dielectric constant the ascent of oil globules into the upper layers of the emulsion, and their replacement in the lower layers by the aqueous phase, should cause dielectric changes in these two regions of the emulsion. Such changes have been investigated using a specially designed capacitance cell.

Experimental

APPARATUS FOR THE MEASUREMENT OF DIELECTRIC CONSTANT

The dielectric constants of the emulsions were measured by the heterodyne beat method. In agreement with theoretical considerations [see equation (1) below], composition changes are more readily detected in the upper regions of the emulsion and hence a capacitance cell of the type shown in Fig. 1 was used. The cell responds to changes in dielectric because its capacitance is related to the dielectric constant by the following equation (Blaedel & Petitjean, 1956):

$$C = \frac{[(2\pi f)^2 C_0\epsilon(C_0\epsilon + C_g) + K^2]C_g}{(2\pi f)^2(C_0\epsilon + C_g)^2 + K^2} \quad .. \quad .. \quad .. \quad (1)$$

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where C = capacitance of cell when filled with emulsion
C_o = capacitance due to air in the empty cell
C_g = capacitance due to the glass walls of the cell
ϵ = dielectric constant of emulsion
K = conductance of emulsion
f = frequency of electrical field.

The equation shows that the cell capacitance is sensitive to dielectric changes only in emulsions of low conductivity. In highly conducting emulsions such as those prepared with ionic surfactants, the terms $(2\pi f)^2 C_o\epsilon(C_o\epsilon + C_g)$ and $(2\pi f)^2(C_o\epsilon + C_g)^2$ may be neglected in comparison with K^2 when the equation reduces to

$$C = C_g \quad .. \quad .. \quad .. \quad .. \quad (2)$$

The cell capacitance is now independent of the dielectric constant of the emulsion and is determined only by the capacitance due to its glass walls. Thus the measurement of emulsion creaming by the dielectric method is limited to those emulsions of low conductivity prepared with non-ionic surfactants.

The cell (Fig. 1) consists of a glass vessel (1) having two concentric walls (2) and (3) defining an annular space (4) between them. The vessel is filled with emulsion through an opening (5) provided near one end of the vessel. The capacitor plates consist of two conducting layers (6) and (7) formed by the deposition of silver on the surface of the glass. An electrical connection to the inner plate is made by the threaded terminal

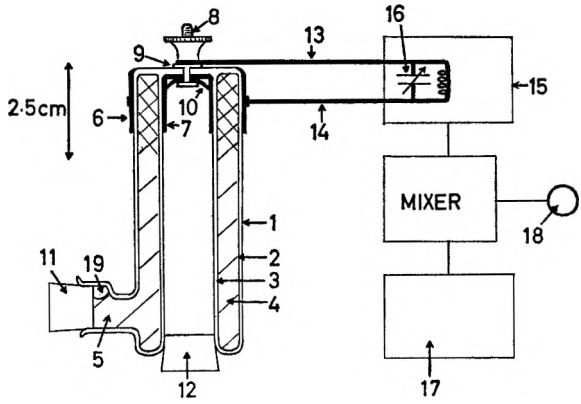

FIG. 1. Capacitance cell and block diagram of heterodyne beat circuit. For explanation see text.

(8) inserted through the end of the glass wall of the vessel. The terminal is secured by means of a nut (9), fibre washers being inserted between the metal and glass surfaces to prevent cracking of the glass. The terminal can be made more secure by using a suitable bonding compound. Electrical connection from the terminal to the inner silver coating is provided by means of a thin springy copper washer (10) inserted under

the head of the terminal. Electrical contact with the outer capacitor plates is made by wrapping three turns of wire around the silver coating and soldering the wire to it.

Opening (5) is closed with a rubber stopper (11). The central space within the inner wall is also closed with a rubber stopper (12) to prevent the entry of dust and fumes which might impair the electrical contact to the inner conducting plate.

The two electrical leads (13) and (14) of the cell are taken to a variable frequency valve oscillator (15). The cell acts as a capacitance in the frequency-determining circuit of the oscillator (15). This circuit is tuned by means of a precision variable condenser (16). The output from the oscillator circuit is mixed with the output from a fixed frequency oscillator (17) (crystal controlled at 1·79 M cycles/sec) and the resultant beat frequency is monitored aurally on a loud speaker and visually on an indicator tube of the "magic eye" type (18).

In operation, the cell is filled with the emulsion under test and inverted. Initially the variable condenser in parallel with the cell is adjusted so that the frequency of the variable frequency oscillator is equal to that of the crystal reference oscillator. This is indicated by the absence of a note from the loudspeaker and of a sign on the indicator tube. As the emulsion gradually separates and oil globules float to the top, the electrical capacitance of the cell decreases causing a frequency shift in the output of the variable frequency oscillator and an audible note of increasing frequency from the loudspeaker. The amount by which the variable condenser must be adjusted to return the frequency to that of the reference crystal oscillator is a direct measure of the capacitance change brought about by creaming.

METHOD OF MEASURING CREAMING RATES

Emulsions containing 50% w/w of liquid paraffin B.P. in a 3% w/w aqueous solution of cetomacrogol 1000 B.P.C. were prepared in an Ato-mix M.S.E. Emulsifier. Details of the emulsions used are given in Table 1.

TABLE 1. DETAILS OF EMULSIONS USED

Emulsion	1	2	3	4	5	6
Homogenisation time min.	10	3	2	1·5	1	0·5
Average of log of globule diameter	0·46±0·01	0·49±0·01	0·50±0·01	0·56±0·01	0·60±0·01	0·65±0·01
Standard deviation of log of globule diameter	0·19	0·19	0·21	0·22	0·27	0·29
Number of oil globules measured for statistical analysis	501	582	509	509	546	476
Observed amounts of creaming (i.e. depth of translucent liquid at bottom of cell)	None after 7·24 hr	1·5 mm After 8·06 hr	2 mm After 8·24 hr	—	3·5 mm After 6·12 hr (Creaming line was diffuse)	4 mm After 2·21 hr (Creaming line was very diffuse)

50 ml of the emulsion was withdrawn from the emulsifier, gentle agitation of the latter being maintained to ensure uniform distribution of the globules. Air was removed from the sample by gentle rotation for 10 min in a flask under vacuum. Gentle shaking was continued as the emulsion was then warmed to 25°.

The cell, maintained at 25° in a small Perspex air chamber, was disconnected from the oscillator and filled with part of the emulsion. The rubber stopper was inserted and the cell was inverted so that the remaining air bubble was trapped in the small space provided (19) (Fig. 1). The gap between the capacitor plates was thus entirely filled with emulsion free from air bubbles.

A clock was immediately started, the cell was quickly reconnected to the oscillator and the thermostat box was replaced. The frequency of the variable oscillator was returned at suitable time intervals to that of the reference oscillator. The readings of the variable condenser were converted to dielectric constant units, the value at zero time being estimated by extrapolation.

MEASUREMENT OF GLOBULE SIZE

The remainder of the emulsion sample was used for the determination of globule size. One drop was diluted with a few drops of water and a little placed on a haemocytometer slide. Photomicrographs of randomly chosen fields were taken during the determination of creaming rate. The magnification of all finished photographs was the same and the diameters of all globules in each photomicrograph were measured to $\pm 0{\cdot}1\,\mu$. A total of about 500 globules was recorded for each emulsion.

Histograms in which the frequency of globule size was plotted against the logarithm of the globule diameters, showed the globule size distributions to be logarithmically normal. The means and standard deviations of the logarithms of the globule diameters were therefore used to characterise the emulsions.

Results and discussion

Many of the factors affecting the dielectric constant of emulsions have been discussed previously (e.g. Piekara, 1929, 1932; Heymann, 1934; Kruyt, 1952; Smyth, 1955; Hanai, Koizumi & Gotoh, 1962). It is apparent that the dielectric constants of liquid paraffin in water emulsions prepared with non-ionic surfactants, and measured in the way described, depend chiefly upon the relative concentrations of liquid paraffin and water between the capacitor plates. An increase in the liquid paraffin concentration results in a decrease of dielectric constant. In the present work therefore, the decrease in the dielectric constant of emulsions with time (see Fig. 2) is due to an increase in the concentration of liquid paraffin between the capacitor plates.

Comparison of Table 1 with Fig. 2 shows that whereas the dielectric change is quite marked, visual inspection of the emulsion in the lower part of the cell is a relatively insensitive method of detecting phase

separation. It has been found that stable emulsions in which no visible creaming can be detected for several days will provide a readily measurable change of dielectric constant after only a few minutes or at most a few hours. Detection of phase separation by the dielectric method is therefore rapid and precise.

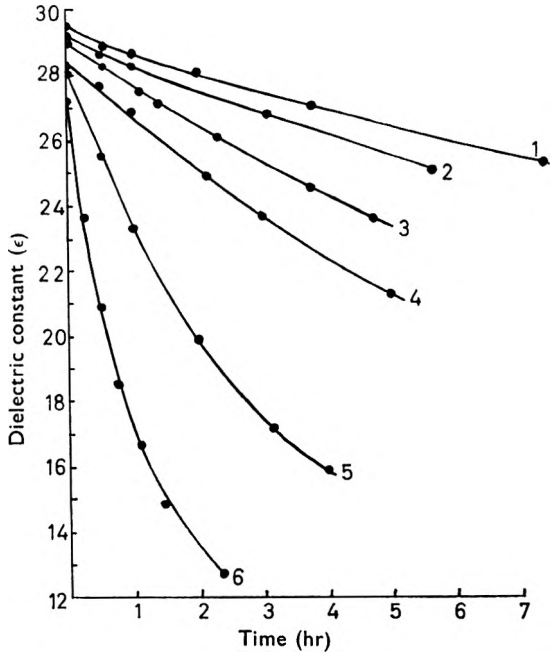

FIG. 2. Relation between dielectric constant and time for emulsions.

An important factor governing the rate of emulsion creaming is the globule size. Comparison of the dielectric constant changes for emulsions 1 and 5 in Fig. 2 and the photomicrographs of these same emulsions (Fig. 3) illustrates the greater creaming rate of the coarser emulsion. A quantitative relation between globule size and creaming rate of the systems studied is given in Fig. 4. The logarithm of the rate of change of dielectric constant $d\epsilon/dt$, 30 min after the commencement of the experiment, has been plotted against the mean of the logarithms of the diameters and also against the standard deviations of the logarithms of the diameters. This shows that the rate of change of dielectric constant with time is markedly dependent on globule size, small changes of which lead to large changes in the gradient of the dielectric curve. Dielectric measurements therefore may provide a simple method for determining differences in the globule size of two emulsions.

The initial readings of dielectric constant for emulsions of a given concentration vary with globule size. This effect is much greater than can be explained by the $\pm 0{\cdot}1\%$ experimental error in dielectric constant measurement and the phenomenon is not yet fully understood.

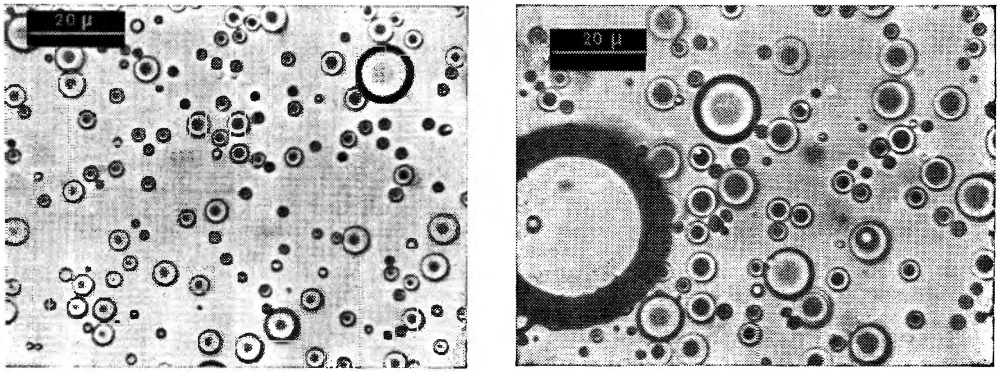

FIG. 3. Photomicrographs of emulsions 1 and 5.

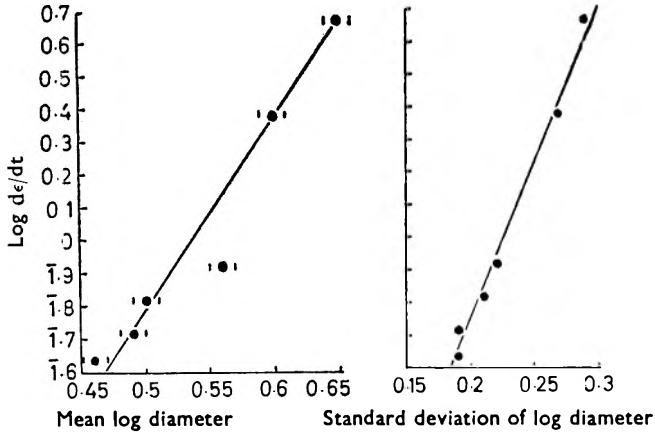

FIG. 4. Relation between log rate of change of dielectric constant dε/dt and mean of log diameter and standard deviation of log diameter.

The apparatus described is the subject of the United Kingdom Patents Application No. 4885/64.

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Interaction of aluminium with bacteria isolated from wounds

T. J. BRADLEY, F. FISH AND M. S. PARKER

The effect of aluminium as metal foil and as a weak solution of potassium aluminium sulphate on the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus vulgaris*, has been examined over a range of pH values. Aluminium foil, and an aluminium metal film deposited by evaporation on a bonded cellulose fabric, failed to inhibit the growth of the organisms on agar plates. In contact with saline metal foil yielded trace amounts of aluminium in solution but no detectable amounts of aluminium were found in human serum which had been in prolonged contact with aluminium metal. *Staph. aureus* and *Ps. aeruginosa* were adsorbed from aqueous suspension by aluminium surfaces. Below pH 5 the growth of these organisms was inhibited by low concentrations of aluminium in aqueous solution. Preliminary measurements of the uptake of aluminium by these sensitive organisms have been made.

ALUMINIUM foil has been used surgically as a wound covering since the time of Lister. Brown, Farmer & Franks (1948) described the use of aluminium foil in the management of burns and, more recently, other applications of aluminium foil have been reported such as the treatment of venous ulcer of the leg (Haeger, 1963). An aluminised cellulose fabric dressing has also been described (Meyer, 1960).

Aluminium is reputed to have antibacterial properties at very low concentrations (Berger & Einstmann, 1959) and the present work was undertaken to provide more information on the interaction of aluminium and its ions with bacterial cells.

Methods and results

MATERIALS

The aluminium foil and chemicals were analytical grade unless otherwise stated. The aluminium-coated cellulose bonded-fibre fabric was supplied by Messrs. Wallace, Cameron & Co. Ltd., Glasgow, S.5. 2,3,5-*Triphenyltetrazolium chloride* (B.P.) in aqueous solution (10%) was sterilised by filtration. *De-ionised water* had a resistance of 4 mega ohms. *Nutrient agar.* Oxoid granules (C.M.3). *Peptone-free nutrient agar.* Oxoid beef extract (1%), Oxoid yeast extract (2%), dextrose (1%), sodium chloride (0·5%), Oxoid Ion agar No. 2 (1%) in de-ionised water. *Agarose.* Prepared by the method of Hjertèn (1962).

Organisms. *Staphylococcus aureus* (893), *Pseudomonas aeruginosa* (899), *Escherichia coli* (891), and *Proteus vulgaris* (894) were isolated from infected wounds. Cultures were freeze-dried and catalogued (bracketted numbers) in the Department of Applied Microbiology, University of Strathclyde. Bacterial suspensions in de-ionised water were prepared by washing 24 hr cultures from nutrient agar slopes which had been

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incubated at 31°. Suspensions were centrifuged and resuspended alternately to wash cells free from nutrient material. The final suspensions contained about 120 × 10^6 viable cells per ml.

EFFECT OF ALUMINIUM ON THE GROWTH OF ORGANISMS

By gel diffusion. Pieces (4 cm^2) of aluminium foil and aluminium-coated cellulose fabric were sterilised by heating in an autoclave (120°/20 min). They were placed in petri dishes and covered with a thin layer of peptone-free nutrient agar containing triphenyltetrazolium chloride solution (0·1 ml %) and seeded with the organism under test. After storage at 4° for 24 hr the plates were incubated (31°/7 days), then examined.

Similar experiments were made replacing the 1% Oxoid Ion agar in the peptone-free nutrient agar with 0·3% agarose, to enhance ion mobility.

No zones of inhibition were observed around the pieces of aluminium foil or aluminised fabric in any of the tests performed.

In aqueous suspension. Sterilised pieces (4 cm^2) of aluminium foil and aluminium-coated cellulose fabric were placed in normal saline (100 ml) containing triphenyltetrazolium chloride solution (0·1 ml). Each flask was separately inoculated with 0·1 ml cell suspension of the organism under examination and maintained at 31° for 72 hr. Subcultures were taken at intervals to assess viability.

Suspensions of the organisms were exposed, under the same conditions, to the cellulose fabric without the aluminium coating.

In contact with aluminium, *E. coli* and *P. vulgaris* gave positive subcultures throughout the experimental period of 72 hr. *Staph. aureus* and *Ps. aeruginosa* were negative on subculture after contact periods of 18 and 24 hr, respectively, and in both cases a red film was observed on the aluminium. This feature, *viz*, the red film of formazan, the reduced form of triphenyltetrazolium (Barnes, 1956), indicated that the organisms had been adsorbed on the metal surface from aqueous suspension. The adsorption was confirmed by microscopic examination.

DETERMINATION OF ALUMINIUM LEACHED OUT OF METALLIC ALUMINIUM BY SERUM AND SALINE

Using plastic containers, pieces (4 cm^2) of aluminium foil and aluminium-coated cellulose fabric were placed in separate 10 ml volumes of fresh human serum or in 0·9% sodium chloride solution in de-ionised water. Solutions were maintained at 31° for 40 days. The aluminium squares were removed and the fluid assayed for aluminium content by the method of Jones & Thurman (1958). A wet ash extraction procedure was used for the human serum (Sandel, 1950). Some samples of ash from the experiments using human serum were extracted with hydrochloric acid/water (1 : 1), the extracts mixed and evaporated to dryness on a rotary film evaporator and the residue of chlorides examined for aluminium by mass spectrometry.

No aluminium was detected in any of the experiments using human serum or when the aluminium-coated fabric had been immersed in saline.

After 40 days at 31° the sodium chloride solution in which the foil had been immersed contained 0·08–0·9 μg/ml aluminium, mean value 0·33 μg/ml (s.d. = 0·29).

EFFECT OF A WEAK SOLUTION OF POTASSIUM ALUMINIUM SULPHATE ON THE GROWTH OF ORGANISMS OVER A pH GRADIENT

Plates (16 cm × 24 cm) of peptone-free nutrient agar containing the following systems in wedge-shaped portions, one superimposed on the other, were prepared as described by Sacks (1956), 150 ml volumes composing each wedge to eliminate any irregularities in the surfaces and to give sufficient material for pH measurement after dissecting out. Sodium hydroxide solution (20 g/litre; 15 ml) was included in the lower wedge which was poured with the plate suitably tilted; the plate was levelled after 1 hr and the upper wedge containing potassium aluminium sulphate solution (176 g/litre, pH 3·2; 15 ml) poured and allowed to set. To provide other pH gradient plates without the aluminium salt, for comparison, similar plates were prepared incorporating sodium hydrogen sulphate solution (103 g/litre, pH 1·1; 15 ml) in the upper wedge. The prepared plates were dried at 31° for 1 hr and the standard aqueous suspensions of wound organisms containing 0·1% of the triphenyltetrazolium chloride solution streaked on each plate in the direction of the pH gradient. The plates were incubated at 31° for 72 hr and the pH at the limits of growth measured, first by laying narrow-range indicator papers on the agar surface adjacent to the upper and lower limits of growth and subsequently, by dissecting out a narrow strip of agar at the boundaries of growth, melting, cooling to 45° and inserting a glass electrode assembly. The pH meter was standardised to buffer solutions at 31°.

The upper limit of growth was in the region of pH 10 in all cases. On the sodium hydrogen sulphate pH gradient plates, the lower limits of growth occurred at pH values 4·0 for *E. coli* and 4·5 for *P. vulgaris*, *Ps. aeruginosa* and *Staph. aureus*. In the presence of the aluminium salt the lower limits of growth had the following pH values: *E. coli* 5·2, *P. vulgaris* 5·7, *Ps. aeruginosa* 5·5 and *Staph. aureus* 6·5. The calculated concentration of aluminium in the areas of the lower growth boundaries was about 8 μg/ml.

EVALUATION OF THE BACTERICIDAL EFFECT OF AN ALUMINIUM SOLUTION ON *Staph. aureus* AND *Ps. aeruginosa* AND ESTIMATION OF THE ALUMINIUM BOUND

Volumes (50 ml) of potassium aluminium sulphate solution (0·3518 g/litre in de-ionised water, adjusted to pH 4·5 (using 0·1N ammonium hydroxide solution), were mixed with 50 ml volumes of suspensions of the organisms containing about 12×10^6 viable cells per ml and maintained at 18°. At intervals, samples (1·0 ml) were suitably diluted and 0·5 ml used for surface counting on dried agar plates. Simultaneously, 10 ml samples were withdrawn and centrifuged (10 min/2° at 3,200 revs/min); 5 ml of the supernatant liquid was assayed for aluminium content by the method

of Jones & Thurman (1958). A similar suspension of the organisms in a solution containing sodium hydrogen sulphate (0·1024 g/litre) was sampled to compare the viable count at the same pH level in the absence of aluminium.

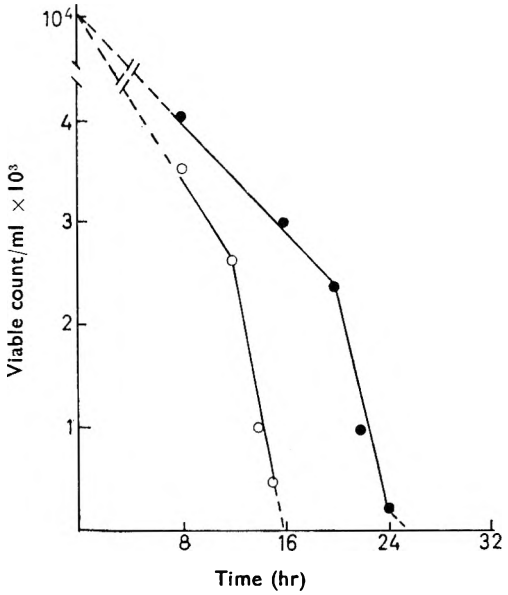

FIG. 1. The fall in viable count of suspensions of *Staph. aureus* and *Ps. aeruginosa* in a solution of potassium aluminium sulphate containing 10·1 μg aluminium/ml; at 18° and pH 4·5. ○ = *Staph. aureus.* ● = *Ps. aeruginosa.*

There was no significant fall in the viable count of the aluminium-free suspensions. The viable count of the suspensions which did contain aluminium salt, fell as indicated in Fig. 1 in which each point represents the mean of 10 counts. The count of *Staph. aureus* fell to zero in 16 hr and that of *Ps. aeruginosa* within 30 hr.

Staph. aureus bound about 60% of the aluminium ions available at the level of 10·1 μg/ml and about 75% of this total binding occurred within 2 min of contact: uptake by *Ps. aeruginosa* reached about 50% of available aluminium and about 90% of this was bound in the first 2 min (Fig. 2).

Discussion

The absence of zones of inhibition in the gel diffusion studies and the absence of detectable amounts of aluminium in human serum which had been in contact with the metal for 40 days, indicate that ions do not pass into solution under these conditions. Whereas aluminium ions have been detected in solution when aluminium foil remained in contact with saline for 40 days, no ions were detected when the foil was replaced by aluminised fabric. The layer of oxide present on aluminium exposed to

the air was probably much thicker on the metal film deposited by evaporation and this could account for the failure to remove ions from the metallised fabric.

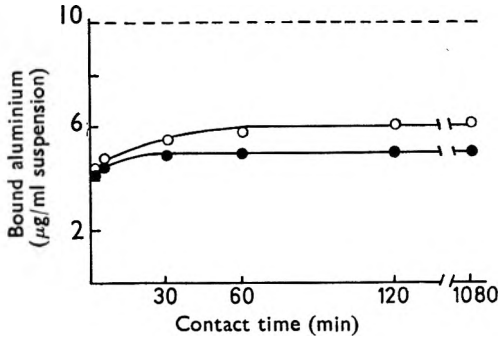

FIG. 2. The rate of binding of aluminium by *Staph. aureus* and *Ps. aeruginosa* from aqueous solution, pH 4·5 at 18°. ○ = *Staph. aureus*. ● = *Ps. aeruginosa*. – – – – Initial concentration of aluminium 10·1 μg/ml.

The oxide layer is responsible for adsorbing certain organisms from aqueous suspension as indicated by the appearance of a red film on the metal when the sensitive growth indicator triphenyltetrazolium chloride was used. It is significant that in the case of organisms which were adsorbed, *Staph. aureus* and *Ps. aeruginosa*, no growth was obtained on subcultures after 18 and 24 hr, respectively. With organisms which were not adsorbed, *E. coli* and *P. vulgaris*, growth was obtained from subcultures after 72 hr.

To investigate the inhibitory effect a soluble aluminium salt was used. Blank & Dawes (1960) have shown that aluminium acetate and basic aluminium chloride, in solution at pH 4·5 and 4·0 respectively, inhibit the growth of micrococci on thin sheets of human callus. Their work indicates that the inhibition is due not to low pH values alone, but to aluminium ions both free and complexed with cutaneous proteins. In the present work the inhibitory effect of aluminium ions in solution was clearly demonstrated on pH gradient plates where all the organisms studied were inhibited below pH 5·2; in this region the concentration of aluminium ions was about 8 μg/ml.

When suspensions of *Staph. aureus* and *Ps. aeruginosa* were exposed to a concentration of 10 μg/ml of aluminium at pH 4·5, the organisms were killed within 16 hr and 30 hr respectively. The cells of both organisms bound aluminium rapidly from solution, the uptake curves being similar to those obtained with *Staph. aureus* for the binding of iron (Beckett, Vahora & Robinson, 1958) and iodine (Hugo & Newton, 1964). The lethal effect demonstrated is probably due to an interaction between aluminium ions and bacterial proteins facilitated by the surface characteristics of these sensitive organisms.

Acknowledgements. The authors thank Dr. L. G. Bruce, Victoria Infirmary, Glasgow for supplying the test organisms, Dr. G. C. Wood,

of this Department for a supply of agarose and Dr. E. Clayton, University of Glasgow for mass spectrophotometric determinations.

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Identification and determination of ephedrine and its congeners in urine by gas chromatography

A. H. BECKETT AND G. R. WILKINSON

A sensitive and specific assay for ephedrine-type compounds in urine is described. The procedure involves the removal of neutral and acidic compounds, then extraction of the bases with ether. This extract, after concentration, is injected into a flame ionisation gas chromatograph equipped with a 2% polyethylene glycol 6000, alkaline treated, column at 165°. Linear detector response between 5 and 100 μg base/ml urine was observed (peak height with respect to an internal marker) and replicate analyses indicated good reproducibility. No interfering compounds have been encountered nor any "on column" decomposition. The amines were further identified by chromatography of their acetone derivatives.

METHODS have been described for the identification and determination of ephedrine and norephedrine in biological materials (Richter, 1938; Axelrod, 1953; Heimlich, MacDonnell, Flanagan & O'Brien, 1961), but they lack sensitivity and specificity and are tedious for routine analysis. Gas chromatography of ephedrine, norephedrine and pseudoephedrine has been reported, but primarily from a qualitative aspect (Parker, Fontan & Kirk, 1962, 1963; Brochmann-Hansson & Svendsen, 1962). The adaptation of this technique to a specific quantitative determination of ephedrine and related compounds in the presence of each other in biological fluids, is described herein.

Experimental

Reagents. Analar diethyl ether, freshly distilled. Hydrochloric acid, 5 N. Sodium hydroxide solution, 5 N. Internal marker solution: (2,6-dimethylphenoxy)ethylamine hydrochloride; a solution equivalent to 10 μg base/ml in 0·1 N hydrochloric acid.

Apparatus. A Perkin Elmer F 11 chromatograph equipped with a flame ionisation detector and a 0 to 5 mv Leeds and Northrup Speedomax G recorder, Model S were employed. The chromatographic column was stainless steel tubing $\frac{1}{8}$ inch o.d., 1 metre in length, and packed with 80–100 mesh Chromosorb G, acid-washed and treated with dimethyldichlorosilane. This was coated with 5% w/w potassium hydroxide and 2% w/w polyethyleneglycol 6000. It was conditioned for 24 hr under the operating conditions: oven temperature, 165°; injection block temperature about 250°; hydrogen pressure, 14 lb/sq. in; air pressure, 25 lbs/sq. in; nitrogen flow rate, 40 ml/min; stream split ratio, 1 : 5. The column was silanized *in situ* with 2 × 5 μl hexamethyldisilazane before use.

PROCEDURE

To internal marker solution (1·0 ml), in a glass stoppered centrifuge tube, was added 1 ml to 5 ml urine and 0·1 ml 5 N hydrochloric acid.

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The solution was diluted to 6·1 ml and extracted with 3 × 2·5 ml diethyl ether, centrifuging to break any emulsion. The ether extract was rejected and urine made alkaline with 5 N sodium hydroxide (0·5 ml). A further 3 × 2·5 ml diethyl ether extracts were combined in a 15 ml Quickfit test tube, finely tapered at the base, and concentrated to about 50 μl at 42°; 3·0 to 5·0 μl of this solution was injected onto the column using a 10 μl Hamilton syringe. The concentration of the sympathomimetic amine was obtained by calculating the ratio of peak heights of the amine to that of the internal marker and relating this to a previously constructed calibration curve of the amine in urine.

The acetone derivatives of the amines (Brochmann-Hanssen & Svendsen, 1962), were formed by adding acetone (1 ml) to the ether concentrate in the tapered test tube; the solution was allowed to stand for 2 hr, concentrated to about 50 μl at 60° and 3·0 to 5·0 μl injected onto the column.

The amines in urine at various pH values were stored at 4° and the amine content determined periodically. The amines in the ether extract and concentrate were also determined before and after storage at 4° for 24 hr.

Results and discussion

Well resolved symmetrical peaks were obtained for the amines (Fig. 1), however, it was not possible to separate ephedrine and pseudoephedrine under the described operating conditions. These two amines were differentiated and identified, as were the other amines, by their retention times and by the characteristic peak-shift on formation of the respective acetone derivatives (Table 1). Methylephedrine being a tertiary amine

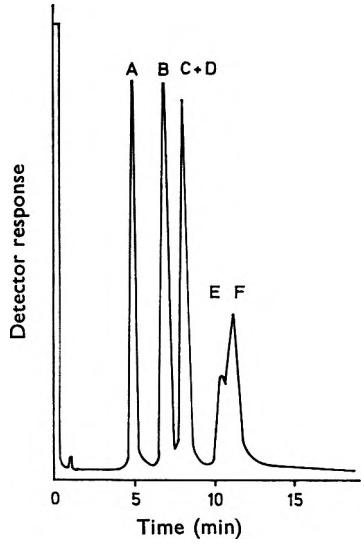

FIG. 1. Separation of a mixture containing A, internal marker; B, methylephedrine; C, ephedrine; D, pseudoephedrine; E, norpseudoephedrine; and F, norephedrine extracted from urine.

did not form an acetone derivative. Conversion of the other amines to their acetone derivatives did not go to completion under the described conditions.

TABLE 1. RETENTION TIMES OF SOME EPHEDRINE CONGENERS

Compound	Retention time in min	
	Base	Acetone deriv.
Internal marker, (2,6-dimethylphenoxy)ethylamine	4·9	8·0
Methylephedrine	6·8	Not formed
Pseudoephedrine	8·2	3·6
Ephedrine	8·2	4·0
Norpseudoephedrine	10·5	4·2
Norephedrine	11·3	4·0

A consistent linear detector response was obtained for all the individual amines over the range 5–100 μg base/ml urine. Sixteen analyses of a urine sample containing 20 μg amines/ml gave a standard deviation of 0·64.

No interfering substances were found in the many samples of urine analysed and no deterioration of the amines occurred when they were stored (4 days at 4°) in urine. Both the ether extract and concentrate were stable at 4° for 24 hr. Alkaline treatment of the column support was necessary for symmetrical peaks and "on column" decomposition of the amines was not observed; this can probably be attributed to the silanized steel tubing used (cf. Vessman & Schill, 1962; Vessman, 1964). The decomposition of ephedrine-type amines reported by Parker & others (1962) is probably an artifact caused by the use of acetone solutions of the amines.

The advantages of this method over those previously reported are its specificity as shown by the absence of interference from normal urinary constituents, the short analysis time (16 samples may be analysed in about 6 hours), and the ease of determination of a mixture of the amines. Furthermore, although 5 μg/ml was the lowest concentration determined in the present study, by the use of larger volumes of urine and smaller amounts of internal marker much smaller concentrations may be estimated.

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SHORT COMMUNICATION

Urinary excretion of (−)-methylephedrine, (−)-ephedrine and (−)-norephedrine in man

A. H. BECKETT AND G. R. WILKINSON

DESPITE the long and extensive clinical use of ephedrine and related compounds, little is known of their metabolism and excretion in man. It has been reported that the metabolism of (−)-ephedrine in various animals shows considerable species differences (Axelrod, 1953). Thus demethylation to norephedrine is a major route of metabolism in the dog and guinea-pig, but only a minor one in the rat; hydroxylation is of minor importance in the dog but a major pathway in the rat. Williams (1959) suggests that demethylation of ephedrine is likely in man although Richter (1938) claimed that about 100% of the dose was excreted unchanged in 24 hr. It must be noted, however, that the analytical method used by the latter author did not differentiate between ephedrine and norephedrine.

The development of a specific and sensitive gas-liquid chromatographic assay for ephedrine and its congeners in urine, (Beckett & Wilkinson, 1965b), permits detailed investigation into the metabolism and excretion of these compounds. Table 1 shows the results obtained when aqueous solutions of (−)-methylephedrine, (−)-ephedrine, and (−)-norephedrine were administered orally in the form of their hydrochlorides to a male volunteer.

TABLE 1. EXCRETION OF (−)-METHYLEPHEDRINE, (−)-EPHEDRINE AND (−)-NOREPHEDRINE IN MAN (pH OF URINE NOT CONTROLLED)

Drug	Dose, mg base	% Dose excreted in 24 hr		
		Methylephedrine	Ephedrine	Norephedrine
(−)-Methylephedrine	10·0	31·8	8·1	—
(−)-Ephedrine	27·3	—	79·3	4·3
(−)-Norephedrine	25·0	—	—	90·8

The results indicate that demethylation of (−)-methylephedrine and (−)-ephedrine is not a major pathway of metabolism in man and that the pharmacological activity of these drugs probably resides in the parent structure. The rate of excretion of (−)-methylephedrine (pK_a 9·20) and its metabolite, ephedrine (pK_a 9·47) fluctuated with changes in urinary pH. These results are consistent with the concept of reabsorption of the unionised drug moiety in the kidney tubules (Weiner & Mudge, 1964). Thus the excretory behaviour of the ephedrine-type compounds in man shows many similarities with the results already reported for methylamphetamine and its metabolite amphetamine (Beckett & Rowland, 1965) and also with chlorpheniramine (Beckett & Wilkinson, 1965a).

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Urinary excretion kinetics of methylamphetamine in man

A. H. BECKETT AND M. ROWLAND

The urinary excretion of methylamphetamine and its metabolite, amphetamine, was studied after oral administration of (+)- and (−)-methylamphetamine hydrochloride to three male subjects. Fluctuations in the excretion rate of both amines occurred and were associated with changes in urinary pH. The fluctuations were abolished when the urine was maintained either acid or alkaline, by administration of ammonium chloride and sodium bicarbonate respectively. The total amount of both amines excreted was lower under alkaline than acid urine conditions. *N*-Demethylation of methylamphetamine was small, but greater for the (+)- than the (−)-isomer.

SIGNIFICANT amounts of methylamphetamine are reported to be excreted in urine in man. After oral administration of (−)-methylamphetamine, Richter (1938) recovered 56% in 48 hr, while Utena, Ezoe & Kato (1955) gave values of 70–90% recovery for the (+)-isomer. The analytical procedures were non-specific and would not differentiate between methylamphetamine and its *N*-demethylation product amphetamine, so that the observed results were probably higher than the true values. Cartoni & de Stefano (1963), using a specific method of assay, reported 50% of the drug was excreted unchanged and 17% as amphetamine.

The urinary excretion of amphetamine (pK_a 9·77, Leffler, Spencer & Burger, 1951; 9·93, Lewis, 1954) is influenced by urinary pH (Beckett & Rowland, 1964; Asatoor, Galman, Johnson & Milne, 1965). Since methylamphetamine has a similar pK_a value of 9·87 (Leffler & others, 1951) the effect of urinary pH on the excretion of methylamphetamine has been examined. Preliminary findings have been reported elsewhere (Beckett & Rowland, 1965b).

Experimental

URINE EXCRETION TRIAL

Three male subjects were given an oral dose of 11·0 mg methylamphetamine base in the form of 13·7 mg hydrochloride in water (50–100 ml). When the urinary pH was not controlled, the (+)- and (−)-isomers were given on separate occasions. In acid and alkaline urine trials, only (+)-methylamphetamine was administered.

The regimen of administration of drug, times of urination, measurement of urinary pH and dosage for ammonium chloride and sodium bicarbonate was as described for amphetamine (Beckett & Rowland, 1965c).

Any subjective effects experienced by the subjects were noted.

Plasma study. Blood was collected 1½ hr after an oral dose of 11·0 mg (+)-methylamphetamine had been given to three subjects.

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DETERMINATION OF METHYLAMPHETAMINE AND AMPHETAMINE IN URINE AND PLASMA

Methylamphetamine and amphetamine were determined by gas-chromatography as previously reported, and in a number of experiments amphetamine was further identified as its acetone derivative (Beckett & Rowland, 1965a). Methylamphetamine (0·1–10 μg/ml urine; 1 μg/ml plasma) and amphetamine (0·1–10 μg/ml urine) were added to the urine or plasma of subjects who had not received any drug. These solutions and others in which no drug had been added were analysed. Methylamphetamine (1 μg/ml) was added to acid and alkaline urine and stored at 4°. The methylamphetamine content was determined daily for 4 days.

Results

Linear calibration curves for both amine components in urine within the range 0·1–10 μg/ml were obtained. The presence of methylamphetamine interfered with the determination of amphetamine, to the extent of 1% of the methylamphetamine peak at the t_R value for amphetamine, and any appropriate correction was made. No substance which interferes with the determination of the amines was found in the urine or plasma of subjects who had not taken methylamphetamine. Methylamphetamine was stable for at least 4 days in acid or alkaline urine stored at 4°.

URINARY EXCRETION

pH of urine not controlled. The results are shown in Table 1. The excretion rate of methylamphetamine and amphetamine varied with urinary pH but not with urine output (Fig. 1). The urinary pH fluctuated within the range 5·2–7·6, whether or not the drug was given.

TABLE 1. URINARY EXCRETION OF METHYLAMPHETAMINE AFTER ORAL ADMINISTRATION OF 13·7 MG (+)- AND (−)-METHYLAMPHETAMINE HYDROCHLORIDE. URINARY pH NOT CONTROLLED

Subject	Isomer	% of the methylamphetamine dose excreted					
		As methylamphetamine			As amphetamine*		
		16 hr	24 hr	48 hr	16 hr	24 hr	48 hr
M.R. ..	(+)	14·0	22·1	26·7	1·7	3·8	6·0
	(−)	17·3	27·3	38·3	0·8	1·7	3·1
N.B. ..	(+)	15·3	21·3	28·9	2·5	4·5	9·2
	(−)	15·1	25·8	43·5	0·5	1·4	4·0
E.J.T. ..	(+)	31·5	42·7	54·9	5·2	6·5	9·6
	(−)	30·6	48·5	67·5	1·0	2·0	3·6

* Calculated as the equivalent amount of methylamphetamine

Urinary pH alkaline. The amount of methylamphetamine excreted in 16 hr in alkaline urine (pH 8·0 ± 0·2) was 0·6 to 2·0% (mean 1·5%) of the dose administered; negligible amounts of its metabolite amphetamine were excreted. Urine collected in the period 40–48 hr after drug administration, by which time the urine had become acid (pH 5·5–6·0) (sodium

bicarbonate administration was discontinued at the 14th hr), yielded significant amounts of methylamphetamine (4·3%) and its metabolite amphetamine (2·6%) (Fig. 2).

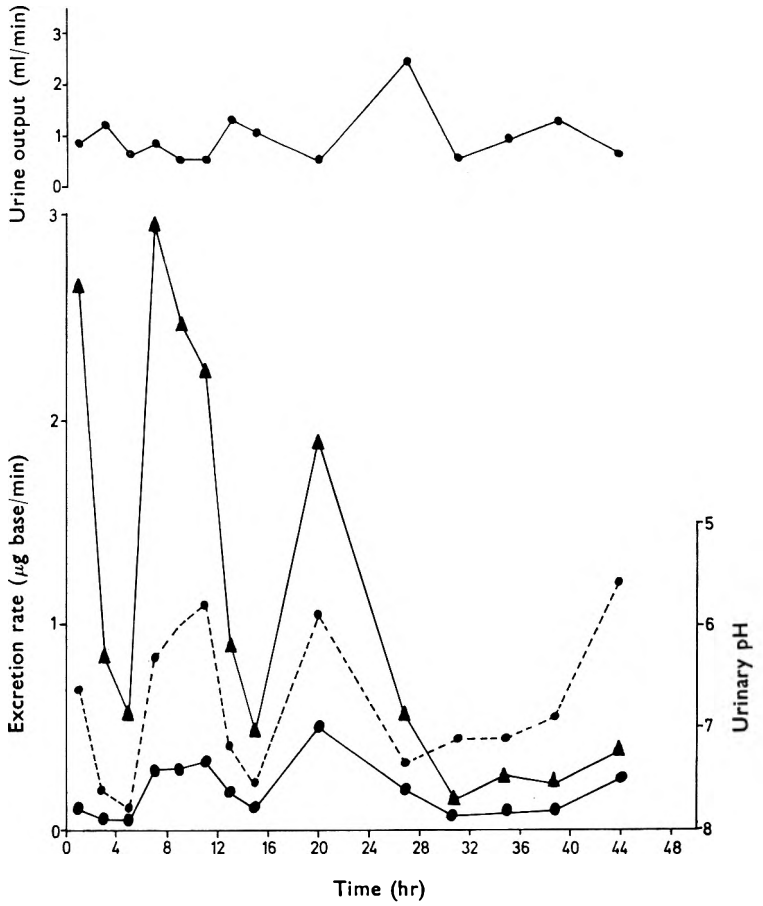

FIG. 1. The influence of urinary pH and urine output on the urinary excretion of methylamphetamine (and its metabolite) in man, after oral administration of 11·0 mg (+)-methylamphetamine. Subject M. R. ---●--- = Urinary pH. —▲— = Methylamphetamine. —●— = Amphetamine. (Similar patterns were obtained in other subjects).

Urinary pH acid. Table 2 gives the 16 hr urinary excretion of unchanged methylamphetamine (mean 63%) and amphetamine (mean 6·6%), as a percentage of the dose administered, after oral administration of (+)-methylamphetamine to subjects whose urine was maintained acid. Under these constant acidic conditions, the observed fluctuations in the excretion rate of methylamphetamine and amphetamine were abolished (see Fig. 1). Also, changes in urine output had little effect on the

excretion of either of these amines. The excretion rate of methylamphetamine reached a maximum about 2½ hr after administration of the drug and then fell exponentially (Fig. 3). Amphetamine excretion rate reached a maximum 5–7 hr after administration of methylamphetamine.

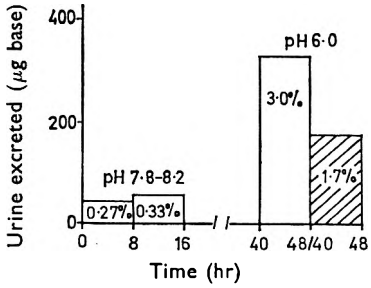

FIG. 2. Urinary excretion of methylamphetamine and its metabolite amphetamine, after a single oral dose of 13·7 mg (+)-methylamphetamine hydrochloride under alkaline and normal urinary pH conditions. Methylamphetamine unshaded, amphetamine shaded. Subject M.R.

PLASMA LEVELS

Not more than 0·03 μg/ml methylamphetamine could be detected in the plasma after an oral dose of 11 mg (+)-methylamphetamine.

TABLE 2. URINARY EXCRETION OF METHYLAMPHETAMINE AND AMPHETAMINE AFTER ORAL ADMINISTRATION OF 13·7 MG (+)-METHYLAMPHETAMINE HYDROCHLORIDE; URINE MAINTAINED ACIDIC THROUGHOUT THE TRIAL

	% of the methylamphetamine dose excreted						Biological half-life, hr	
	As methylamphetamine			As amphetamine				
Subject	16 hr	24 hr	Total	16 hr	24 hr	Urinary pH	Methylamphetamine	Amphetamine*
M.R.	63·2	—	70·6	7·0	—	5·0 ± 0·2	4·3	5·0
N.B.	57·4 (15 hr)	58·2 (19 hr)	61·0	6·5 (15 hr)	7·6 (19 hr)	5·1 ± 0·2	4·0	4·75
E.J.T.	69·4	75·5	77·5	5·8	7·2	4·95 ± 0·2	4·6	4·90

* Beckett & Rowland (1965c)

CLINICAL EFFECTS

Central nervous stimulation and dryness of the mouth were more pronounced after the administration of (+)-methylamphetamine when the urine was maintained alkaline than when the urinary pH was not controlled or the urine maintained at an acid pH. No effects were observed with the (–)-isomer.

Discussion

The marked fluctuations in the excretion of methylamphetamine and its metabolite amphetamine may be explained by non-ionic diffusion of bases in the kidney (Milne, Scribner & Crawford, 1958; Weiner & Mudge, 1964). This is indicated by the following evidence.

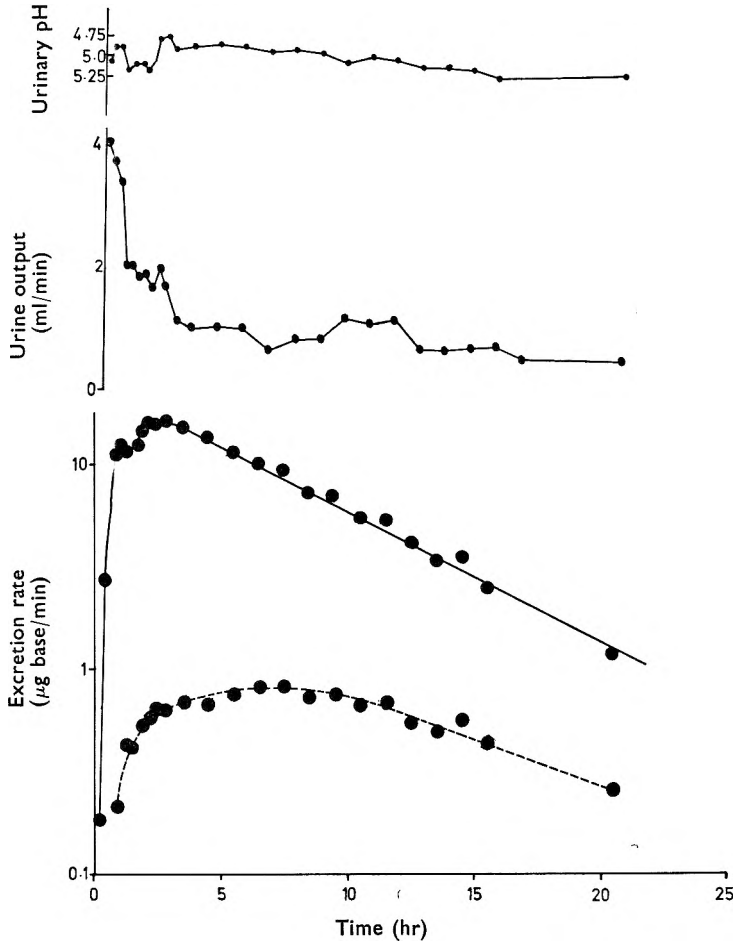

FIG. 3. Urinary excretion of methylamphetamine and amphetamine, urinary pH and urine output after oral administration of 11·0 mg (+)-methylamphetamine; urine maintained acidic throughout the trial. —●— = Methylamphetamine. - - -●- - - = Amphetamine. Subject E. J. T.

(*a*) Fluctuations in the excretion rate of these amines appear to depend upon urinary pH changes.

(*b*) There is a forty fold increase in the 16 hr excretion of methylamphetamine in acid urine over that found in alkaline urine.

(*c*) The excretion patterns of (+)- and (−)-methylamphetamine are similar.

(*d*) Under alkaline urine conditions, subjective effects were more prolonged indicating reabsorption and longer retention of the drug in the body; when sodium bicarbonate treatment was stopped at the 14th hr, significant amounts of methylamphetamine were excreted 40–48 hr after a dose of the drug. Even though there is only 0·5% unionised drug in

alkaline urine, reabsorption of methylamphetamine from the kidney is almost 100%.

When the urine is maintained at an acid pH, excretion of unchanged drug is the major route of elimination of methylamphetamine from the body. The total expected excretion of this drug (Table 2), calculated by the same procedure as for amphetamine (Beckett & Rowland, 1965c), indicates that excretion of unchanged drug is essentially complete within 24 hr of the dose. Under such conditions the biological half-lives of (+)-methylamphetamine and (+)-amphetamine are similar (Table 2). This result could be explained by a passive excretion process predominating, or by assuming that the rate constants for secretion of these bases into the kidney tubules are similar (Weiner & Mudge, 1964).

Under normal conditions, most of the methylamphetamine is excreted unchanged, and only a small amount of *N*-demethylation occurs, we suggest therefore that the pharmacological activity of the drug probably resides in the unchanged molecule. Some degree of stereospecific *N*-demethylation of methylamphetamine is indicated by the difference in the amounts of amphetamine excreted after administration of each isomer (+) or (−) of methylamphetamine; this cannot be attributed to differences in the metabolism and excretion of (+)- and (−)-amphetamine (Beckett & Rowland, 1965c). Since methylamphetamine is relatively stable and yet only low plasma levels were found, it is concluded that the drug is concentrated extravascularly.

The clinical implications of these results are the same as for amphetamine (Beckett, Rowland & Turner, 1965). These present findings further illustrate the need to record urinary pH changes when studying the urinary excretion of bases.

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SHORT COMMUNICATION

The urinary excretion of aminoglutethimide in man

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AMINOGLUTETHIMIDE (Elipten, α-ethyl-α-*p*-aminophenylglutarimide) is used clinically as an anticonvulsant drug and it is therefore of interest to determine its metabolic fate in man. It is chemically related to the hypnotic drug glutethimide (α-ethyl-α-phenylglutarimide) which is extensively metabolised *in vivo* (Kebrle, Schmid, Hoffmann, Vuilleumier & Bernhard, 1959).

Experimental

Aminoglutethimide forms a coloured Schiff's base with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) and this reaction has been utilised in estimating the glutarimide in solution. Various amounts of aminoglutethimide in absolute ethanol (9 ml) were treated with Ehrlich's reagent, 1 ml (Werner, 1939), and the intensity of the yellow colour formed was measured at 440 mμ, this wavelength being the absorption maximum of the Schiff's base formed between aminoglutethimide and the reagent. A linear relationship between colour intensity and concentration of drug was obtained for the range 0–15 μg aminoglutethimide per ml of the final solution.

To devise a method for extracting aminoglutethimide from urine, the partition of the drug between aqueous and organic phases was investigated. This was done by shaking equal volumes of various organic solvents with an equal volume of distilled water containing aminoglutethimide (50 μg/ml) for 30 min at room temperature. The two phases were each saturated with the opposite phase before their use in partition experiments. Aliquots of the organic layers were evaporated to dryness and the residues of aminoglutethimide were determined by the procedure described above. Dichloromethane extracted the aminoglutethimide almost completely. The partition was pH dependent and when the aqueous solution had pH 6 was entirely in favour of the dichloromethane.

pH of aqueous phase	2·2	3·05	3·95	5·1	6·1	7·4	8·9	13·1
% of aminoglutethimide remaining in dichloromethane (mean of 4 values)	4·2	37·1	72·1	98	99·8	99·2	98·8	8·7

For the estimation of aminoglutethimide in human urine, samples were adjusted to pH 7 and shaken (30 min) with 50 ml and then with 25 ml dichloromethane. The combined organic layers were dried (30 min) over

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anhydrous sodium sulphate (20 g). The desiccant was filtered off and washed twice with dichloromethane (50 ml). Combined washings and extract were evaporated to dryness and the residue dissolved in absolute ethanol (5 ml). Suitable aliquots of this solution were diluted to 9 ml with ethanol and assayed for aminoglutethimide as previously described. As a control, a 50 ml sample of urine containing no aminoglutethimide was treated in an identical manner. At the dilutions employed in the assay itself, this produced no measurable colour with Ehrlich's reagent. The recovery of aminoglutethimide added to urine at the levels of 2 and 4 mg/50 ml was 86% (s.d. 1·6%).

TABLE 1. CHROMATOGRAPHY OF EXTRACTS OF URINE FROM MEN DOSED WITH AMINOGLUTETHIMIDE (250 mg ORALLY)

Solvent system	Rf value		
	Extract of test Urine	Aminoglutethimide	Urea
n-Butanol : acetic acid : water 12 : 3 : 5 v/v	0·44 0·72	0·73	0·44
Chloroform : methanol .. .. 1 : 1 v/v	0·29 0·93	0·93	0·29
Carbon tetrachloride : acetic acid : water (lower layer) .. .. 1 : 2 : 1 v/v	0·06 0·34	0·34	0·06
Aqueous sodium chloride 10% w/v	0·60 0·74	0·74	0·59

Chromatograms run on Whatman No. 1 paper by the ascending technique. Compounds detected with *p-NN*-dimethylaminocinnamaldehyde.

Finely powdered aminoglutethimide (250 mg and 500 mg) was administered orally to two healthy men on three separate occasions. All urine passed 24 hr before and 24 and 48 hr after administration of the drug was collected. Aliquots (50 ml) of the urine were adjusted to pH 7, extracted

TABLE 2. REACTION OF CHROMATOGRAMS OF EXTRACTS OF URINE FROM MEN DOSED WITH AMINOGLUTETHIMIDE (250 mg ORALLY) TO VARIOUS PROCEDURES

Procedure	Reaction		
	Extract of test urine	Aminoglutethimide	Urea
Ultraviolet light (350 mμ) ..	Rf 0·72 absorbs	absorbs	not detected
Ehrlich's reagent	Rf 0·44 yellow colour Rf 0·72 yellow colour	yellow colour	yellow colour
Nitrous acid-(naphthyl)ethylene-diamine (Bridges & Williams, 1963)	Rf 0·72 purple colour	purple colour	not detected
p-NN-dimethylaminocinnamaldehyde HCl (Bridges & Williams, 1963)	Rf 0·72 permanent red colour Rf 0·44 red colour turning to yellow	Permanent red colour	red colour fading to yellow
Sodium hypochlorite-potassium iodide-starch (Jackson & Moss, 1960)	Rf 0·44 Blue black colour Rf 0·72 blue black colour	blue black colour	blue black colour

All reagents were applied to the paper chromatograms as sprays. Solvent system was n-butanol : acetic acid : water 12 : 3 : 5 v/v.

and assayed for aminoglutethimide as described previously. Chromatography of the urine extracts on Whatman No. 1 paper showed the presence of two Ehrlich-positive spots, one of which was urea. The other spot had the same mobility as aminoglutethimide in several solvent systems (Table 1) and gave similar colour reactions with various reagents (Table 2). This spot could not be demonstrated in extracts of control urine. All Ehrlich-positive material in the extracts was therefore estimated in terms of aminoglutethimide.

TABLE 3. URINARY EXCRETION OF AMINOGLUTETHIMIDE* AFTER ORAL ADMINISTRATION TO TWO MEN

Dose administered (mg)	Day	mg Aminoglutethimide excreted in urine in 24 hr	
		Subject A†	Subject B†
250	1	87·7	85·4
	2	33·7	12·4
250	1	74·8	84·7
	2	15·1	—
500	1	221·9	222·5
	2	32·7	59·9

* As Ehrlich-positive material estimated in dichloromethane extracts of urine.
Results corrected for 100% extraction. †Means of 3 determinations on each sample.

The results (Table 3) show that most of the aminoglutethimide excretion occurred in the first 24 hr. The average total excretion after 48 hr represents 39% of the 250 mg dose and 54% of the 500 mg dose.

This evidence suggests that after an oral dose of 250 or 500 mg of aminoglutethimide, the drug is absorbed and excreted unchanged to a considerable extent in the urine. The metabolic fate of α-ethyl-α-*p*-aminophenylglutarimide thus appears to differ significantly from that of α-ethyl-α-phenylglutarimide.

Acknowledgement. Grateful acknowledgement is made for a generous gift of aminoglutethimide from Ciba Laboratories, Horsham, Sussex.

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The seed haemagglutinins of some *Phaseolus vulgaris* L. cultivars

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The haemagglutinating properties of *Phaseolus vulgaris* L. vars Canadian Wonder, Masterpiece, Mont D'or, Navy Pea and Red Kidney seed extracts against erythrocytes of the A_1A_2BO and $D(Rh_0)$ human blood groups have been investigated. Under some conditions, Navy Pea extract reacted preferentially with erythrocytes possessing the A_1 and B antigens, but the remaining extracts showed no blood group selectivity. The agglutination reactions are discussed, and it is suggested that Navy Pea seed contains a non-specific agglutinin and an anti-A_1 + B agglutinin.

THE haemagglutinin of *Phaseolus vulgaris* L. seed, first extracted by Landsteiner & Raubitschek (1908), was shown to be a mucoprotein by Renkonen (1950) and Rigas, Li & Osgood (1951). It is produced commercially in both mucoprotein and protein forms by the method of Rigas & Osgood (1955), and used for the agglutination of erythrocytes in the isolation of leucocytes from whole blood (Skoog & Beck, 1956; Seabright, 1957). It finds use as a mitogenic additive to leucocyte cultures (Nowell, 1960; Moorhead, Nowell, Mellman, Battips & Hungerford, 1960).

Renkonen (1948) discovered that seed agglutinins of some species react selectively with red cells of certain human blood groups. Although subsequent investigators (Boyd & Reguera, 1949; Cazal & Lalaurie, 1952; Krupe, 1953; Ottensooser & Silberschmidt, 1953; Makela & Makela, 1956; Makela, 1957; Bird, 1951, 1956, 1957) found further sources of "specific" phytagglutinins (lectins), some of which are used in blood group diagnosis, *P. vulgaris* L. agglutinin has been reported to be non-selective for human red cell antigens (Renkonen, 1948; Makela, 1957; Bird, 1959a). Cultivars of this species have been mentioned in connection with experiments on blood group specificity by Boyd & Reguera (1949) and Maron (1951), and it was the object of the present study to determine if the agglutinating properties of extracts of certain *P. vulgaris* seed cultivars differed.

Experimental

MATERIALS

Seeds of the following *P. vulgaris* L. cultivars were supplied by Brook, Parker & Co., Ltd., Bradford: Canadian Wonder, Masterpiece, Mont D'or, Navy Pea, Red Kidney.

First and second generation plants grown from these seeds possessed the botanical characters of *P. vulgaris* L., which are well documented (Robbins, 1917; anonymous 1920; Fawcett & Rendle, 1920; Bailey, 1949; Mclean & Ivimey-Cook, 1952; Ministry of Agriculture, 1962). Cultivars were authenticated by comparing the characters of the plant, flower, pod and seed with those recorded by Steinmetz & Arny (1932) or North & Squibbs (1953).

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Seeds of *Sophora japonica* L. and *Ulex europaeus* L. were supplied by Thompson & Morgan, Ltd., Ipswich.

METHODS

Seed extracts were titrated with human erythrocytes of the A_1A_2BO and $D(Rh_o)$ groups as follows: in 0·9% saline and 20% bovine albumin solution at 4° increments between 8° and 20° and at 2° increments between 20° and 56°. They were also examined at 20° in undiluted human AB serum. Titrations were also made at 20° in media varying from pH 4·4 to pH 9·75. The extracts were subjected to absorption, elution and sugar-inhibition tests. Controls for spontaneous agglutination were set up in each experiment and consisted of mixtures in which the seed extract was replaced by 0·9% saline.

Preparation of seed extracts, blood samples and erythrocyte suspensions. Intact seeds (50 g) were washed in 70% ethanol, soaked (16 hr) in sterile 0·9% saline (200 ml) in a Paladin Blender, and macerated for 5 min. The macerate was slowly stirred in the blender for 3 hr, centrifuged twice (715 × *g* for 30 min followed by 4,277 × *g* for 30 min) and the supernatant was clarified by membrane filtration in a sterile unit. 2 ml aliquots of the filtrate were freeze-dried in sterile, tared, wide-necked ampoules fitted with lint caps (modified from Greaves, 1946). After drying (16 hr) over P_2O_5, the weight of dry extract in each ampoule was accurately determined; ampoule necks were fused to exclude micro-organisms. Preparations were stored at −25°.

Before use, each extract was reconstituted with sterile water to produce a 5% w/v solution. Such samples from each batch were sterile when tested in aerobic and anaerobic media.

Extracts of *Sophora japonica* L. seed and *Ulex europaeus* L. seed prepared as above had comparable titres to those reported by Morgan & Watkins (1953) and Boyd & Sharpleigh (1954) respectively.

Human red cells of the following blood groups were used:

O(D+), O(D negative), A_1, A_2, B, A_1B, A_2B (all D+).

Venous blood (10 ml–15 ml) was mixed with sterile ACD* solution (2 ml) and stored at 4° for a maximum period of two weeks.

Erythrocytes were washed three times with 0·9% saline and suspended as follows:

For titration at various pH values:

pH 4·40–pH 7·95, 2% v/v in McIlvaine's Buffer (Documenta Geigy, 1962) of double-strength.

pH 8·33–pH 8·75, 2% v/v in Kolthoff's Buffer (Documenta Geigy, 1962) of double-strength.

pH 9·15–pH 9·75, 2% v/v in boric acid/sodium hydroxide buffer (Palmer, 1946) of double-strength.

*5 volumes of 2% disodium hydrogen citrate solution + 1 volume of 15% dextrose solution.

For other titrations: 5% v/v in 0·9% saline, in 20% bovine albumin solution, and in human AB serum.

For absorption and elution tests: 5% v/v in 0·9% saline and in 20% bovine albumin solution.

For inhibition tests: 5% v/v in 0·9% saline.

Titration. The method of two-fold serial dilutions was used. Equal volumes (0·05 ml) of extract dilution and erythrocyte suspension were mixed in a stoppered glass tube (50 mm × 6 mm) and allowed to stand for 2 hr in a temperature-controlled water-bath. The deposited corpuscles were re-suspended and examined for macroscopic agglutination, and, if necessary, for microscopic agglutination at ×80. The titre of an extract is the reciprocal of the greatest dilution which causes agglutination, i.e. the dilution which existed before admixture with erythrocyte suspension. pH values quoted with titres are those of the final mixtures showing agglutination.

Inhibition tests. Equal volumes (0·05 ml) of a 1/16 dilution of seed extract and 8% sugar solution were mixed, erythrocyte suspension (0·10 ml) added and the mixture allowed to stand (2 hr at 20°) before it was examined for agglutination. Controls were mixtures in which the sugar solution was replaced by 0·9% saline.

Using this method with two-fold serial dilutions of sugar solution, the minimum concentrations of D-galactose and β-lactose required to prevent agglutination by a 10% w/v extract of *Sophora japonica* L. seed were identical with those reported by Morgan & Watkins (1953).

Absorption and elution tests. For absorption, equal volumes (1 ml) of undiluted seed extract and washed packed red cells of the required group were mixed, allowed to stand overnight at 4°, and centrifuged at 2,000 rpm for 3 min. The supernatant was pipetted off and reabsorbed with half its volume of fresh, packed erythrocytes of the same group for 2 hr. The process was repeated with fresh cells of the same group until agglutination ceased to occur, after which the supernatant was tested in saline and bovine albumin media for agglutinins against red cells of other blood groups.

For elution, red corpuscles (1 ml) which had been agglutinated by seed extract (1 ml) were washed three times with 0·9% saline at room temperature and resuspended in 1 ml fresh saline. The suspension was placed in a water-bath at 56° for 15 min and the supernatant pipetted off from the cell sediment. After clarification by centrifugation (2,000 rpm for 3 min) the solution was tested for agglutinins in saline and bovine albumin media.

Results

In both saline and bovine albumin media, all extracts gave an identical variation in titre with temperature, irrespective of the phenotype of the test erythrocytes (Fig. 1). In human AB serum, Navy Pea seed extract reacted differently from the other extracts, and titres in saline, albumin and AB serum at 20° are compared in Table 1.

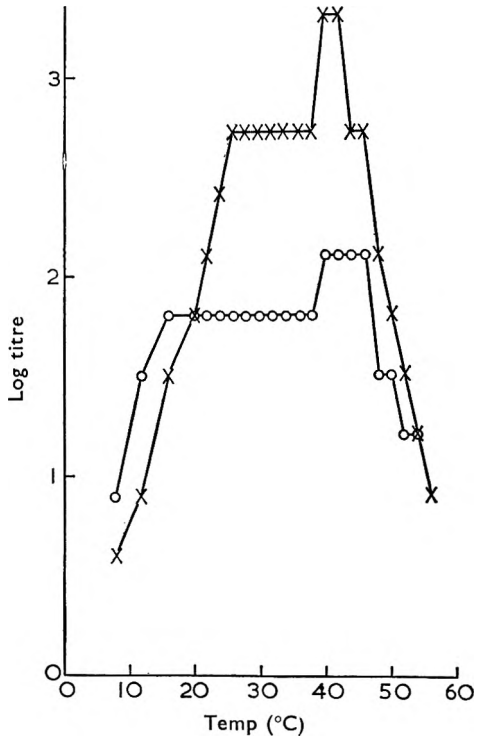

FIG. 1. Effect of temperature on the titre of *Phaseolus vulgaris* L. vars Canadian Wonder, Masterpiece, Mont D'or, Navy Pea or Red Kidney seed extract with erythrocytes of any A_1A_2BO blood group. ○ = 0·9% saline medium. × = 10% bovine albumin medium. Titres are the reciprocals of the greatest dilutions causing agglutination. Each point represents the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

TABLE 1. TITRES OF *Phaseolus vulgaris* L. SEED EXTRACTS AGAINST HUMAN ERYTHROCYTES SUSPENDED IN SALINE, BOVINE ALBUMIN AND HUMAN AB SERUM MEDIA AT 20°

Extract of	Medium	Blood group						
		O(D+)	O(D negative)	A_1(D+)	A_2(D+)	B(D+)	A_1B(D+)	A_2B(D+)
Canadian Wonder	0·9% saline	64	64	64	64	64	64	64
	10% bovine albumin	64	64	64	64	64	64	64
	human AB Serum	16	16	16	16	16	16	16
Navy Pea	0·9% saline	64	64	64	64	64	64	64
	10% bovine albumin	64	64	64	64	64	64	64
	human AB serum	16	16	64	16	32	64	32

Titres are recorded as the reciprocals of the greatest dilutions causing agglutination, and represent the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

Masterpiece, Mont D'or, and Red Kidney seed extracts reacted in the same way as Canadian Wonder seed extract.

The pH values of reconstituted extracts were: Canadian Wonder, 6·00; Masterpiece, 6·00; Mont D'or 6·10; Navy Pea, 6·10; Red Kidney, 5·00. In buffered media, four of the preparations reacted identically with erythrocytes of all A_1A_2BO groups, but between pH 6·03 and pH 7·35 the titre of Navy Pea seed extract varied with erythrocytes of different A_1A_2BO groups (Fig. 2).

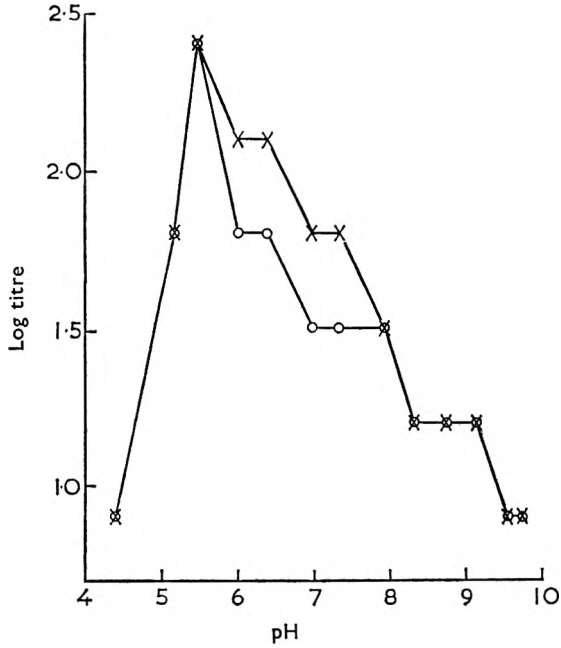

FIG. 2. Effect of pH on the titre of *Phaseolus vulgaris* L. seed extract. ○ = Canadian Wonder, Masterpiece, Mont D'or, or Red Kidney extract against erythrocytes of any A_1A_2BO blood group, or Navy Pea seed extract against O(D+), O(D negative) or A_2(D+) erythrocytes. × = Navy Pea extract against A_1, B, A_1B or A_2B (all D+) erythrocytes. Titres are the reciprocals of the greatest dilutions causing agglutination. Each point represents the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

Absorption of Canadian Wonder, Masterpiece, Mont D'or or Red Kidney seed extract with erythrocytes of any A_1A_2BO group, left supernatants devoid of haemagglutinating activity. This was also true for Navy Pea seed extract absorbed with A_1, B, A_1B, or A_2B (all D+) cells, but absorption with O(D+ and D negative) or A_2(D+) cells left a supernatant active only against cells possessing the A_1 and/or B antigen. The titres of this supernatant are recorded in Table 2.

The following 27 sugars and sugar-derivatives were tested for their capacity to inhibit agglutination: aldopentoses (D-ribose; D-arabinose; L-arabinose; D-(+)-xylose; α-L-xylose; D-lyxose), aldohexoses (D-(+)-glucose; α-D-glucosamine hydrochloride; *N*-acetyl-D-glucosamine; D-galactose; D-galactosamine hydrochloride; *N*-acetyl-D-galactosamine;

D-mannose), 6-deoxy-hexoses (L-(+)-rhamnose; D-fucose; L-fucose), ketohexoses (fructose; L-sorbose; D-tagatose), disaccharides (sucrose; maltose; β-lactose; cellobiose; D-(+)-trehalose; D-(+)-turanose), and trisaccharides (D-raffinose (pentahydrate); D-(+)-melizitose). None of these compounds inhibited agglutination.

In all experiments the cell controls behaved normally.

TABLE 2. TITRES OF SUPERNATANT FROM NAVY PEA SEED EXTRACT ABSORBED WITH O(D+), O(D NEGATIVE) OR A_2(D+) ERYTHROCYTES

Blood group	Medium	Titre
O(D+) or O(D negative) or A_2(D+)	0·9% saline	—
	10% bovine albumin	—
A_1(D+) or A_1B(D+)	saline	8
	albumin	32
B(D+)	saline	2
	albumin	16
A_2B(D+)	saline	1
	albumin	8

"—" = no reaction.
Titres are recorded as the reciprocals of the greatest dilutions causing agglutination, and represent the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

Discussion

In each experiment the extracts of Canadian Wonder, Masterpiece, Mont D'or and Red Kidney seeds reacted identically with erythrocytes of all A_1A_2BO groups.

The titres of Navy Pea seed extract with red cells of different phenotypes under various conditions are compared in Table 3, which shows that it reacted more strongly with cells possessing the A_1 and/or B antigen than with cells of groups A_2 and O, in which respect it conformed to the general pattern of the tribe Phaseoleae (Bird, 1959b) and not to the species pattern. It reacted identically with D+ and D negative erythrocytes of group O in all tests, showing that D(Rh_0) specificity was absent.

None of the extracts showed blood group selectivity in saline or bovine albumin solution at any temperature between 8° and 56°. Above 56°, agglutinin and red cells would not react. Titres in albumin were higher

TABLE 3. TITRES OF NAVY PEA SEED EXTRACT WITH HUMAN ERYTHROCYTES UNDER VARIOUS CONDITIONS

Test	Blood group					
	A_1	A_1B	B	A_2B	A_2	O
Reaction medium = human AB serum	64	64	32	32	16	16
pH 6·03–pH 6·40..	128	128	128	128	64	64
Extract absorbed with O or A_2 erythrocytes and titrated in:						
saline:	8	8	2	1	—	—
bovine albumin:	32	32	16	8	—	—

"—" = no reaction.
Titres are recorded as the reciprocals of the greatest dilutions causing agglutination, and represent the constant results from five titrations with the erythrocytes of one person of each group, and two titrations with the erythrocytes of not less than two other persons of each group.

than those in saline between 22° and 52°, and there was a difference of 1,920 between the maximum titres in the two media (Fig. 1). The marked change in titre between 8° and 40° (Fig. 1) is of interest since Makela (1957) reported that the activity of *P. vulgaris* haemagglutinin altered little over this temperature range.

None of the 27 sugars or sugar derivatives tested had any effect on the agglutination reaction. Although the action of many phytohaemagglutinins is selectively inhibited by simple sugars, *P. vulgaris* seed extract is not unique in being unaffected by these compounds. For example, Morgan & Watkins (1953) reported that the O cell agglutinins of *Laburnum alpinum* J. Presl and *Cytisus sessilifolius* L. seeds were unaffected by 2% concentrations of twelve sugars, all of which prevented the reaction of *Lotus tetragonolobus* seed extract with group O erythrocytes.

The titre of Navy Pea seed extract from pH 6·03–pH 7·35 was higher with A_1, B, A_1B or A_2B cells than with A_2 or O cells (Fig. 2). This cannot be attributed solely to pH, since that of the undiluted extract was 6·10, and blood group selectivity did not occur in saline. It has been shown that anions have no effect on phytohaemagglutination (Makela, 1957; Liener, 1958) and so the reaction was unlikely to have been modified by the chemical natures of the buffer constituents, all of which were sodium salts. A slight turbidity was observed when Navy Pea seed extract and buffered cell suspension were mixed, and erythrocytes suspended in the buffer solutions were found on microscopical examination to be crenated, which suggests that a change in the condition of the red cell membrane and salting-out of a seed protein fraction played some part in this blood group selectivity.

The latter conclusion is supported by the results of absorption and elution tests, for whereas all haemagglutinating activity was removed from Navy Pea seed extract by absorption with A_1, B, A_1B or A_2B erythrocytes, absorption with A_2 or O erythrocytes left a supernatant reactive only with cells having the A_1 and/or B antigen. It reacted more strongly in albumin than in saline, and was of lower titre than the original extract (Table 3). Eluates from erythrocytes of each group which had been agglutinated by fresh extract contained a non-specific agglutinin. These phenomena can be explained by assuming the extract to contain a non-specific agglutinin and a separate anti$-A_1+B$ agglutinin.

In human AB serum, the agglutination of erythrocytes of all A_1A_2BO groups by Canadian Wonder, Masterpiece, Mont D'or and Red Kidney seed extracts was equally inhibited. Agglutination by Navy Pea seed extract of O and A_2 cells was more strongly inhibited than that of B and A_2B cells, and agglutination of A_1 and A_1B cells was not inhibited. The fact that Navy Pea seed extract differentiated between erythrocytes of different A_1A_2BO groups in AB serum may have been due to partial inhibition of the non-specific agglutinin by normal serum proteins, and partial inhibition of the anti $-A_1+B$ agglutinin by A and B substances.

Northrop & Liener (1959) found that a number of mucoproteins containing sialic acid prevented agglutination of papainised rabbit erythrocytes by Wax-Bean (*P. vulgaris* L.) agglutinin, and it is possible

that one or more proteins may selectively inhibit the action of Navy Pea agglutinin in such a way as to render it specific for erythrocytes of one human blood group.

Previously no importance has been attached to the cultivar of *P. vulgaris* L. seed used in the preparation of haemagglutinin, and there is no evidence to suggest that cultivars used have been systematically examined and identified. The present study has shown that the haemagglutinating properties of seed extracts from a number of authenticated *P. vulgaris* cultivars are not identical. Since one cultivar has been shown to exhibit blood group selectivity under certain experimental conditions, it is probable that other cultivars may also do so.

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SHORT COMMUNICATION

Structure-activity studies for the teratogenic effects of disazo dyes

J. B. LLOYD, F. BECK AND A. GRIFFITHS

ADMINISTRATION of trypan blue to pregnant rats leads to the appearance at term of offspring exhibiting a variety of congenital malformations. The defects induced are similar in nature to common spontaneous malformations (e.g. anencephaly, hydrocephalus, spina bifida) and consequently the dye is widely used today in experimental work on the genesis of deformities. Investigations into the relationship between chemical structure and teratogenic activity of dyes of the trypan blue group have been made (Gillman, Gilbert, Spence & Gillman, 1951; Wilson, 1955; Beaudoin & Pickering, 1960) which indicate that apart from trypan blue, only an isomeric dye (azovan blue) has significant activity. Two aspects of this earlier work suggest the need for a re-evaluation of the problem, namely the widespread use of dyestuffs of unconfirmed identity and purity and the restriction of biological testing to a single dose level. Our experience has shown that these may constitute serious sources of error (Lloyd & Beck, 1963; Beck & Lloyd, 1964).

METHODS AND RESULTS

Commercial samples of Niagara blue 2B, Niagara blue 4B, Afridol blue and azovan blue (Evans blue) were confirmed as authentic (see Lloyd & Beck, 1964a for formulae and method), then freed from salt and converted into the free acid form as has been described previously (Lloyd & Beck, 1964b). Inbred Wistar rats were injected subcutaneously at 8·5 days pregnancy (the time of maximum sensitivity to trypan blue; Wilson, Beaudoin & Free, 1959) with 1% aqueous solutions of the dyes at doses from 50 to 200 mg/kg. At 20·5 days the mothers were killed, the foetal resorption sites counted and the live foetuses examined for evidence of external malformation. The results show that, in addition to trypan blue, Niagara blue 2B and Afridol blue are potent teratogens in the rat, the difference being that the first is optimally active at 50 mg/kg and the other two at 150 mg/kg. Neither Niagara blue 4B nor azovan blue has more than marginal teratogenic activity, although both caused foetal death at high doses.

As a possible aid to explaining these results a study was made of the plasma levels of the dyes at various times after subcutaneous injection of 50 mg/kg. To date, these experiments have been made on the dyes trypan blue, Niagara blue 4B, Afridol blue and azovan blue, and the most striking feature observed has been the behaviour of trypan blue, which

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reaches and maintains plasma levels of 15–18 mg %, some 3–4 times those maintained by the other dyes. Differences between the remaining dyes are also apparent, azovan blue reaching the blood only slowly (maximum concentration at ca. 36 hr) but Niagara blue 4B very quickly (maximum at 4 hr). These findings suggest an explanation for the differences in teratogenic behaviour of the dyes. Trypan blue at 50 mg/kg and Afridol blue at 150 mg/kg are teratogens of similar potency, and the plasma dye concentration experiment suggests that injection at these doses would result in similar levels being reached. This was confirmed by determining plasma levels in a further series of rats injected with Afridol blue at 150 mg/kg, and it would appear, therefore, that both dyes are teratogenic if plasma concentrations of 15–18 mg % are maintained for some hours on about the 9th day of gestation. With Niagara blue 4B the observed plasma levels lead to the expectation that teratogenicity would be manifest at doses of 150–200 mg/kg, but at these doses the dye is toxic to the mother, resulting in a high mortality before term (Beck & Lloyd, 1965). In surviving mothers a high resorption rate masks the teratogenic effects of the dye, the resorptions being due to a direct toxic effect upon the embryo (Beck & Lloyd, 1965), no doubt related to the maternal toxicity. Azovan blue reaches the bloodstream much more slowly than the other dyes, suggesting that, to obtain 15 mg% on the 9th day of gestation, azovan blue would have to be administered at 100 mg/kg at 7·5 days. A series of rats was therefore subjected to this régime and a high teratogenic potency observed. Five mothers yielded 43 implantations of which 19 were resorbed and 7 (16·3%) exhibited external malformation.

DISCUSSION

The mechanism of teratogenic action of disazo dyes is at present unknown and a number of hypotheses have been advanced (see review by Beck & Lloyd, 1965). In discussing the question many authors have placed much emphasis upon differences in ability to elicit a variety of biological responses between "teratogenic" and "non-teratogenic" dyes. The present report shows that many disazo dyes are active teratogens; indeed no disazo dye has yet been investigated over a wide dosage range and at different stages of pregnancy and found to be without activity. The dosage levels at which activity is observed, and the degree of response, are both subject to wide variation. These differences are in part, at least, explained by differences between the dyes in the rate of release from the subcutaneous injection site and in the rate of removal from the circulation.

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Molecular interactions of caffeine with benzoic acid, *o*- and *p*- methoxybenzoic acids and *o*- and *p*- nitrobenzoic acids

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The solubility of the complexes formed by caffeine with benzoic acid and substituted benzoic acids has been studied. An insoluble complex is formed by *o*-methoxybenzoic acid, whereas *p*-methoxy-, *o*- and *p*-nitrobenzoic acids and benzoic acid itself form soluble complexes. The phase diagrams have been analysed to evaluate the approximate stability constants for 1 : 1 interaction. The stabilities increase as the electron-withdrawing power of the benzoic acid substituent decreases, and there is a correlation between log K values and pK_a values (or σ constants) of the acids. This indicates that the binding strength is related either to the polarity of the carboxyl group or to the electron density of the aromatic ring. The implications of these results are discussed in terms of the mechanism of complex-formation.

A LARGE variety of complexes of caffeine have been examined in an attempt to elucidate the mechanism of complex-formation. Both qualitative and quantitative methods have been employed and stability constants have been reported by Labes (1930) and by Higuchi & Zuck (1952, 1953) and Higuchi & Lack (1954). Of the numerous mechanisms proposed, no single one accounts for the phenomena observed, and in the extensive studies made by Higuchi & others, dipole-dipole interactions, hydrogen bonding and the "squeezing-out" effect of water on the hydrophobic group have been proposed, to explain the constants obtained. Results have been difficult to correlate because of differences in solubility and in the stoichiometric ratios among the complexes, and because mixtures of complexes are formed. Quantitative methods of study have been restricted, since the usual spectroscopic and electrochemical methods are not applicable although the molar ratios may be determined refractometrically (Donbrow & Jan, 1963).

We report initial solubility studies made on a closely-related series of complex-forming molecules.

Experimental

SOLUBILITY STUDIES

An excess quantity of the organic acid was shaken at 15° ± 0·05 with varying weighed quantities of caffeine in water or in mineral acid solution (to suppress ionisation of the organic acid). The time of shaking varied between 8 hr and several days according to the supersaturation tendencies of the system and an intermediate storage period of 12 hr at 5° was interposed.

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An aliquot portion of the solution was removed by suction through a filter stick and its content of organic acid determined either titrimetrically or spectrophotometrically. The method of calculation has been described by Higuchi & Zuck (1953).

TABLE 1. SOLUBILITY OF *o*-METHOXYBENZOIC ACID IN AQUEOUS SOLUTIONS OF CAFFEINE AT 15° AND STABILITY CONSTANTS FOR INTERACTION OF *o*-METHOXYBENZOIC ACID AND CAFFEINE

Caffeine conc. moles × 10^3	Acid conc. moles × 10^3	Complex moles × 10^3	Free caffeine moles × 10^3	K (moles^{-1} litre)
0·000	2·05	—	—	—
0·503	2·27	0·22	0·283	37·92
0·979	2·46	0·41	0·569	35·15
1·497	2·66	0·61	0·887	33·54
1·980	2·87	0·82	1·110	36·03
2·980	3·28	1·23	1·750	34·29
3·48	3·52	1·47	2·010	35·67
3·98	3·70	1·65	2·330	34·50
4·50	3·87	1·82	2·680	33·13
4·98	4·08			
6·02	3·90			
7·98	3·90			
9·03	3·88			
10·06	4·00			
10·99	3·76			
12·03	3·66			
13·50	3·39			
14·83	2·92			

Average K = 34·9 moles^{-1} litre: K value from slope = 34·7 moles^{-1} litre

Quantities of acids used were as follows:

o-*Methoxybenzoic acid* (500 mg) in water (50 ml). Titration indicator, α-naphtholphthalein. The results are listed in Table 1.

p-*Methoxybenzoic acid* (100 and 200 mg) in water (50 ml). Titration indicator as above (Table 2).

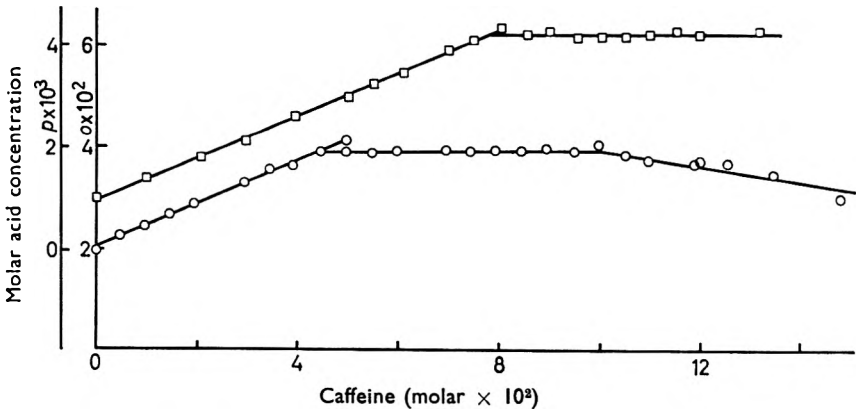

FIG. 1. Solubility of *ortho-* and *para-* methoxybenzoic acids in caffeine solutions. —□— = *para.* —○— = *ortho.*

o-*Nitrobenzoic acid* (500 mg) in 0·005N hydrochloric acid (50 ml). Titration indicator, bromothymol blue (Table 3).

p-*Nitrobenzoic acid* (100 mg) in 0·001N hydrochloric acid (50 ml). Titration indicator as above (Table 4).

Benzoic acid (500 mg) in 50 ml of either water or 0·001N hydrochloric acid. Titration indicator, phenol red (Tables 5 and 6).

TABLE 2. SOLUBILITY OF *p*-METHOXYBENZOIC ACID IN AQUEOUS SOLUTIONS OF CAFFEINE AT 15° AND STABILITY CONSTANTS FOR INTERACTION OF *p*-METHOXYBENZOIC ACID AND CAFFEINE

Caffeine conc. moles × 10^2	Acid conc. moles × 10^3	Complex moles × 10^3	Free caffeine moles × 10^2	K ($moles^{-1}$ litre)
0·00	1·00	—	—	—
0·97	1·40	0·40	0·930	43·0
2·06	1·80	0·80	1·970	40·6
2·95	2·10	1·10	2·840	38·7
3·95	2·60	1·60	3·79	42·2
4·99	2·95	1·95	4·80	40·6
5·57	3·20	2·20	5·35	41·1
6·08	3·40	2·40	5·83	41·1
6·97	3·85	2·85	6·68	42·6
7·50	4·05	3·05	7·20	42·3
8·02	4·30			
9·01	4·25			
10·02	4·10			
11·53	4·20			
13·15	4·20			

Average K = 41·3 $moles^{-1}$ litre: K value from slope = 42·3 $moles^{-1}$ litre

TABLE 3. SOLUBILITY OF *o*-NITROBENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·005N HYDROCHLORIC ACID AT 15° AND STABILITY CONSTANTS FOR INTERACTION OF *o*-NITROBENZOIC ACID AND CAFFEINE

Caffeine conc. moles × 10^2	Acid conc. moles × 10^2	Complex moles × 10^2	Free caffeine moles × 10^2	K ($moles^{-1}$ litre)
0·00	2·84	—	—	—
0·94	2·93	0·09	0·850	3·73
1·46	3·28	0·44	1·020	15·18
2·20	3·28	0·44	1·764	8·78
3·09	3·65	0·81	2·280	12·50
4·17	3·75	0·91	3·26	9·82
4·98	3·97	1·13	3·85	10·33
6·02	4·15	1·31	4·71	9·79
7·01	4·44	1·60	5·41	10·41
8·01	4·53	1·69	6·32	9·41
8·99	4·78	1·94	7·05	9·69
10·20	5·06	2·22	7·98	9·79
12·00	5·42	2·58	9·42	9·64
13·01	5·38			
14·01	5·40			
15·00	5·48			
16·69	5·48			

Average K = 9·92 $moles^{-1}$ litre: K value from slope = 9·60 $moles^{-1}$ litre

TABLE 4. SOLUBILITY OF *p*-NITROBENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·001N HYDROCHLORIC ACID AT 15° AND STABILITY CONSTANTS FOR INTERACTION OF *p*-NITROBENZOIC ACID AND CAFFEINE

Caffeine conc. moles × 10^2	Acid conc. moles × 10^3	Complex moles × 10^3	Free caffeine moles × 10^2	K ($moles^{-1}$ litre)
0·00	0·55	—	—	—
1·08	0·70	0·15	1·07	25·6
2·12	0·80	0·25	2·10	21·7
3·17	0·95	0·40	3·13	23·2
3·96	1·05	0·50	3·91	23·2
4·98	1·20	0·65	4·92	24·0
6·08	1·25	0·70	6·01	21·2
7·04	1·35	0·80	6·96	21·0
7·55	1·50			
8·01	1·40			
9·06	1·50			
10·01	1·45			

Average K = 22·8 $moles^{-1}$ litre: K value from slope = 23·0 $moles^{-1}$ litre

TABLE 5. SOLUBILITY OF BENZOIC ACID IN CAFFEINE SOLUTIONS CONTAINING 0·001N HYDROCHLORIC ACID AT 15° AND STABILITY CONSTANTS FOR INTERACTION OF BENZOIC ACID AND CAFFEINE IN 0·001N HYDROCHLORIC ACID

Caffeine conc. moles × 10^2	Acid conc. moles × 10^2	Complex moles × 10^2	Free caffeine moles × 10^2	K (moles^{-1} litre)
0·00	2·06	—	—	—
1·02	2·52	0·46	0·561	39·8
2·66	3·09	1·03	1·630	30·6
4·01	3·63	1·57	2·436	31·2
4·55	3·78	1·72	2·830	29·5
6·01	4·41	2·35	3·660	31·1
7·46	4·78			
8·50	4·80			
9·03	4·91			
10·32	4·80			
11·09	4·89			

Average K = 30·6 moles^{-1} litre (first value disregarded). K value from slope = 30·4 moles^{-1} litre

TABLE 6. SOLUBILITY OF BENZOIC ACID IN AQUEOUS SOLUTIONS OF CAFFEINE AND STABILITY CONSTANTS FOR INTERACTION OF BENZOIC ACID AND CAFFEINE IN WATER

Caffeine conc. moles × 10^2	Acid conc. moles × 10^2	Complex moles × 10^2	Free caffeine moles × 10^2	K (moles^{-1} litre)
0·00	2·05	—	—	—
0·50	2·23	0·18	0·320	27·4
0·97	2·42	0·37	0·604	29·8
1·97	2·80	0·75	1·220	30·0
2·93	3·12	1·07	1·860	28·1
3·48	3·29	1·24	2·240	27·0
3·99	3·51	1·46	2·530	28·1
5·01	3·79	1·74	3·270	26·0
5·96	4·31	2·26	3·700	29·7
6·54	4·49	2·44	4·098	29·0
7·48	4·73	2·68	4·800	27·2
8·96	5·04			
10·02	4·94			
11·02	4·82			
12·01	4·83			
16·33	4·79			

Average K = 28·2 moles^{-1} litre: K value from slope = 27·4 moles^{-1} litre

Discussion

PHASE DIAGRAMS

o-*Methoxybenzoic acid.* The solubility diagram is shown in Fig. 1. The initial rise is due to formation of a soluble complex and the plateau to the precipitation of a complex. During the latter process the composition of the solution is invariant until the solid acid is exhausted. The length of the plateau is determined by the quantity of excess solid acid in the system, and the stoichiometric ratio of the complex is given by the ratio of the excess solid acid present at the onset of precipitation to the caffeine consumed over the plateau length. The subsequent decrease in solubility is caused by the conversion of free acid in solution into a precipitated complex as further caffeine is added. Chemical analysis of precipitates separating in this plateau region indicated stoichiometric ratios of 2·6 : 1 and 2·9 : 1 (caffeine : acid), whilst the value calculated from the plateau of the phase diagram was 2·45 : 1. However, the linearity of the initial part of the diagram is positive evidence *against* the formation of a complex with more than one molecular proportion of caffeine, and

a constant K value (average 34·9) was only obtained on the assumption of a 1 : 1 reaction in solution. The stability constant K was also calculated from the slope of the rising portion in the following manner:

For the reaction: caffeine (C) + acid (A) $\rightleftharpoons$ complex (CA),

$$K = \frac{[CA]}{[C] \times [A]}$$

If A_o is the solubility of the acid in the solvent

A_t is the stoichiometric concentration of acid, i.e., [CA] + [A]

C_t is the stoichiometric concentration of caffeine, i.e., [CA] + [C]

$$\text{then } K = \frac{A_t - A_o}{(C_t - A_t + A_o)\,A_o} \qquad (1)$$

$$KA_o\,(C_t - A_t + A_o) = A_t - A_o$$

$$A_t + KA_oA_t = KA_oC_t + KA_o^2 + A_o$$

$$A_t(1 + KA_o) = C_t(KA_o) + A_o(1 + KA_o)$$

$$A_t = \frac{KA_o}{1 + KA_o}\,C_t + A_o$$

If A_t is plotted against C_t, the slope $S = KA_o/1 + KA_o$

$$\text{hence } K = S/(1 - S)A_o \qquad (2)$$

The value thus obtained 34·7 moles^{-1} litre is in good agreement with the mean calculated K.

The divergence between the 3 : 1 ratio of the solid and the 1 : 1 ratio from equilibrium calculations may be explained either by the formation of mixed crystals or by pseudo "salting-out" of the caffeine by the acid. It is interesting to note that salting-out effects have been observed by Chambon (1937) and Gusyakov (1959) when certain salts were added to caffeine. In the present case, the concentration of acid is probably too low for such an effect to operate, nor indeed would salting-out effects give definite stoichiometric ratios. In view of the relative constancy of these, the theory of mixed crystal formation is preferred.

p-*Methoxybenzoic acid.* The phase diagram shown in Fig. 1 differs from that of the *ortho* isomer in that the plateau is caused by saturation of the solution with respect to caffeine. From this point, the solid phase is a mixture of excess acid with increasing quantities of solid caffeine. The initial linear slope implies the formation of a soluble complex of caffeine with acid mononuclear with respect to the caffeine (Rossotti & Rossotti, 1961). From the phase diagram we may calculate the stoichiometric ratio as the ratio of the complexed acid in solution to the complexed caffeine in solution (i.e. the difference between the total caffeine in solution at the precipitation point and the solubility of caffeine at 15°). By this method the caffeine : acid ratio is 2·88. This ratio did not yield a constant K value, whereas on a 1 : 1 basis, good agreement was obtained between the average calculated value and the above slope value (41·3, 42·3 moles^{-1} litre respectively).

o- *and* p-*nitrobenzoic acids.* The phase diagrams for the interaction of these with caffeine are shown in Fig. 2. The increase in solubility of

these is linearly related to the amount of caffeine added. The break in the curve is due to the saturation of the solution with respect to caffeine and the acid concentration beyond this point is invariant.

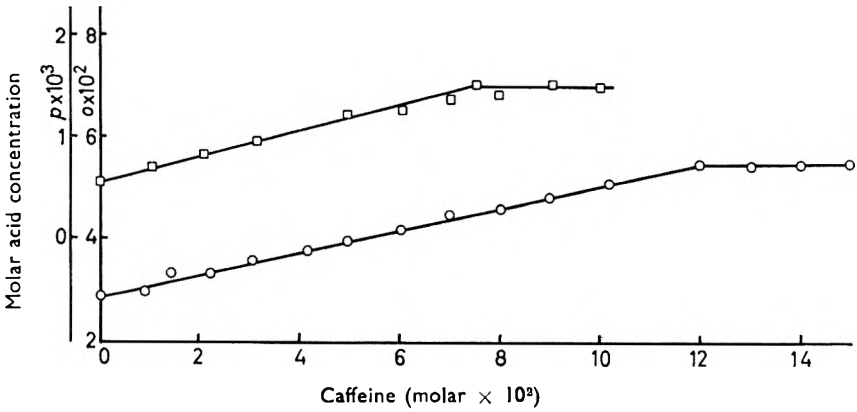

FIG. 2. Influence of caffeine on the solubilities of *ortho-* and *para-* nitrobenzoic acids. —○— = *ortho* in 0·0025 N HCl. —□— = *para* in 0·001 N HCl.

As in the previous case where the interaction product had a high solubility, the stoichiometric ratios could not be obtained from the phase diagrams, and the method used by Higuchi & Lack (1954) may yield misleading results in such systems. In the present study with *o-* and *p*-nitrobenzoic acids, the ratio (caffeine : acid) obtained corresponded to 1·9 and 6·02, respectively. The stability constant for the interaction of *o*-nitrobenzoic acid and caffeine calculated on a 2 : 1 (caffeine : acid) basis

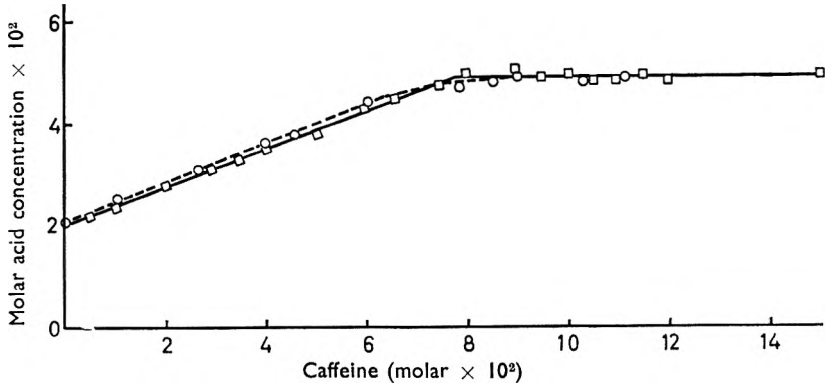

FIG. 3. Effect of caffeine on the solubility of benzoic acid at 15° C. - - -○- - - = solvent, 0·001 N HCl. —□— = solvent, water.

did not yield a constant value, but the assumption of a 1 : 1 reaction in both *ortho* and *para* acid-caffeine systems yielded K values which were in good agreement (*ortho*-acid, average K 9·92; slope method, 9·60; *para*-acid, average K 22·8; slope method 23·0, all values being in moles^{-1} litre).

A possible cause of the discrepancy may lie in the assumptions made, namely:

(i) The concentration of free acid is assumed to remain constant throughout and to be unaffected by the presence of the complexing agent.

(ii) The solubility of caffeine is assumed to remain unaltered in the presence of acid and complex.

Unfortunately a test of the accuracy of these assumptions is difficult to make although we have obtained some evidence that the solubility of caffeine is unaffected. Stoichiometric ratios have not been calculated for a number of soluble complexes by Higuchi & others, probably for the same reason.

Thermal analysis on *o*- and *p*-nitrobenzoic acid–caffeine systems has revealed no interaction (Sekiguchi, 1961).

Benzoic acid. The phase diagrams (Fig. 3) resemble those of the nitrobenzoic acids, the plateau being due to the precipitation of caffeine.

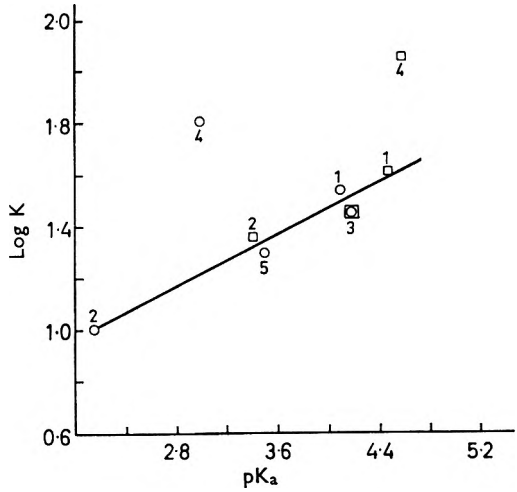

FIG. 4. Relation between log K and pK_a for substituted benzoic acids: 1. Methoxy. 2. Nitro. 3. Hydrogen. 4. Hydroxy. 5. Acetyloxy. ○ = *ortho* acids. □ = *para* acids.

The initial solubility of the organic acid in 0·001N hydrochloric is slightly higher than in water probably due to the greater polarity of the former solvent. With the addition of caffeine the solubility increase of benzoic acid in acid and in water is not identical, the slopes of the lines being 0·380 and 0·353, respectively. However, the maximum concentration attained in both the cases is the same.

The true stoichiometric ratio of the complex cannot be calculated from the phase diagram, but since the solubility increase is linear, a 1:1 caffeine:acid complex may be formed. K values in both solvents are reasonably constant on a 1:1 basis and the average values of 28·2 and 30·6 moles^{-1} litre in water and 0·001N hydrochloric acid respectively are

in good agreement with the values of K calculated from the slopes of the solubility phase diagrams (27·4 and 30·4 moles^{-1} litre, respectively).

This system has been studied by Higuchi & Zuck (1952, 1953) by the distribution method; two complexes 1:1 and 1:2 (caffeine:acid) were postulated. A 2:1 complex has also been detected in addition to 1:1 and 1:2 complexes by means of the interferometric technique (Donbrow & Jan, 1963). With solubility studies, a 1:2 complex is not easily detected if formed in small amount in solution in the presence of the 1:1 complex. It is interesting to note that the depression of ionisation results in non-linearity at high caffeine concentration when acid is used as a solvent; the solubility increase becomes less pronounced (Fig. 3).

Sekiguchi's (1961) studies of the melting point–composition diagram of the mixtures of benzoic acid and caffeine did not indicate any interaction.

STABILITY CONSTANTS AND STRUCTURE

Taken in conjunction with earlier data, the stability constants reported in this work enable a qualitative assessment to be made of the relative tendencies of substituted benzoic acids to form complexes with caffeine. Stability constants for 1:1 complexes are listed in Table 7. The values for the hydroxybenzoic acids were estimated from Higuchi's solubility data, and are approximate. It is apparent that the binding strength decreases in the order *p*-OH > *p*-OMe > H > *p*-NO_2, and *o*-OH > *o*-OMe > H > *o*-Ac > *o*-NO_2. The trend is in the order of increasing electronegativity of the substituent, and follows the order of the Hammett σ values of the *para* compounds (see Table 6; Jaffé, 1953).

TABLE 7. STABILITY CONSTANTS (K) FOR COMPLEXES OF CAFFEINE WITH SUBSTITUTED BENZOIC ACIDS R.C_6H_4·COOH (CALCULATED ON 1:1 BASIS AT 15°)

R	(moles^{-1} litre) K (mean) 1:1	log K	pK_a‡
o-OMe ..	34·9	1·54	4·09
p-OMe ..	41·3	1·62	4·47
o-NO_2 ..	9·9	1·00	2·17
p-NO_2 ..	22·8	1·36	3·44
H	28·2	1·45	4·20
o-OH ..	64*†	1·81	2·98
p-OH ..	115*†	2·06	4·58
o-OCOMe ..	19·8†	1·30	3·49

* Values estimated by present authors. † Higuchi & Zuck (1953). ‡ Braude & Nachod (1955).

The polar effects of substituents in benzoic acids is reflected in the pK_a values of the acids, and a plot of pK_a against log K indicates that there is an approximately linear relationship between these functions for all the acids with the exception of the hydroxybenzoic acids (Fig. 4). It is therefore probable that the same polar effects (i.e. a summation of inductive, resonance and steric effects) operate in complex formation as in ionisation, though without necessarily involving proton release. The linear trend implies a common mechanism throughout the series, and there are two main possibilities to be considered. One involves the carboxyl

group, which may interact with a particular part of the caffeine molecule either by means of electrostatic (dipole-dipole or dipole-induced dipole) forces or by a donor-acceptor mechanism. The second is concerned with the aromatic ring electrons, which may be involved in the formation of a donor-acceptor complex.

Direct involvement of the substituent group in the interaction is improbable however. If there were a direct substituent-caffeine link, one would expect to find a simple correlation between binding strength and a property of the substituent group such as dipole moment or polarisability; this is not the case. Indeed, the varying geometry and differing polar properties of the substituent groups would make a common specific mechanism involving these groups in the *ortho-* and *para-*positions highly improbable. The same argument militates against two-point attachment, which would not give a linear pK_a–logK plot. However, the deviation of the hydroxybenzoic acids is such that, for these acids, it is reasonable to postulate hydrogen-bonding as an additional binding mechanism.

Electron donor-acceptor mechanisms should cause changes in spectroscopic properties. Eckert (1962) has postulated such a mechanism, with the caffeine as acceptor, to account for new ultraviolet "charge-transfer" bands which he obtained in caffeine-procaine hydrochloride mixtures by differential methods. We have repeated his work and obtained similar bands using his and our own systems, but consider the bands to be spurious and experimental artifacts caused by excessive diminution of energy in the reference beam. From quantum mechanical calculations, Pullman (1958) considered that caffeine should be a good electron donor. The stability constants reported above would on the contrary support electron-acceptance by the caffeine and there is in fact an approximate correlation between the ionisation potentials of the monosubstituted benzenes (no data are available for the acids) and the log K values of the complexes. However, since the electron charge density in the carboxyl group may be similarly related to the ionisation potentials, the data do not distinguish between the mechanisms.

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Adsorption studies on steroid powders

J. H. CHAPMAN AND E. L. NEUSTADTER

The adsorption of hydroxyethylcellulose, sodium carboxymethylcellulose, polysorbate 80, glucose and sucrose, by steroid powders has been examined. Hydroxyethylcellulose and polysorbate 80 were adsorbed, but glucose, sucrose and sodium carboxymethylcellulose were not. Hydroxyethylcellulose, which can cross-link with the sedimented particles, caused most caking of the sediment.

CELLULOSE derivatives, sometimes in combination with surfactants, are used extensively in the formulation of pharmaceutical aqueous suspensions. They delay settling of the suspension before administration. Some workers hold the opinion that they are also of value in preventing caking.

It has previously been noted (Steiger-Trippi, 1958) that high molecular weight cellulose derivatives can adversely affect the redispersibility of zinc oxide, talc and titanium dioxide suspensions. Blackadder (1964) also pointed out that polymeric materials capable of linking particles can have a compacting effect on settled suspensions.

The present communication describes a study of the adsorption of certain carbohydrates by the steroid powders in the presence of surfactants. The effect of two cellulose derivatives on steroid suspensions has been examined: hydroxyethylcellulose enhanced the tendency of the settled suspensions to caking.

Experimental and results

The specific surface areas of the hydrocortisone acetate and betamethasone alcohol used were determined by *p*-nitrophenol adsorption from water containing 10% ethanol (Giles, 1962). The specific surface area of hydrocortisone acetate was also determined by BET nitrogen adsorption (at the Paint Research Station) and by air-permeability using the Fisher Sub-sieve Sizer. Adsorption of hydroxyethylcellulose, sodium carboxymethylcellulose, glucose and sucrose by the steroid powders was measured by the anthrone method devised for the assay of carbohydrates by Trevelyan (1952). The adsorption of polysorbate (Tween) 80 was followed by the gravimetric technique of Oliver (1949) (Table 1).

The state of flocculation of the steroid powders in aqueous suspensions containing the various additives was examined by suspending the powders in 2 inch × $\frac{1}{4}$ inch sample tubes and ascertaining the settling volume, and also by measuring how readily the powders moved from the bottom of the tilted tubes. The results are not precise and serve only as an approximate measure of the effect of the additive (Table 2).

Caking of the suspensions was measured by the number of tilting motions required to remove the sediment from a betamethasone suspension from the bottom of a container 3·3 inches high × 1·4

From the Physical Chemistry Unit, Glaxo Research Ltd., Greenford, Middlesex.

inches diameter. The tilting apparatus was as described by Steiger-Trippi (1961). Suspensions were filled into the containers, which were then allowed to stand upright at room temperature for 30 days before the caking tests were made.

TABLE 1. ADSORPTION BY STEROIDS FROM SOLUTIONS (μg/g)

Steroid		Adsorbate [strength of solution (w/v) in brackets]					
Substance	Pre-treatment	P80 (0·5)%	HEC(0·4%) (+ P80, 0·5%)	HEC (0·4%)	NaCMC (0·5%)	Glucose (0·4%)	Sucrose (0·4%)
Hydrocortisone	No surfactant	3,700	0	17,000	0	0	0
Cortisone acetate	No surfactant	6,900	0	8,800	0	0	0
Betamethasone alcohol	Polysorbate 80	0*	0	9,000	0	0	0
Prednisolone acetate	Cetrimide	1,180	0	6,000	0	0	0

P = polysorbate. HEC = hydroxyethylcellulose. NaCMC = sodium carboxymethylcellulose
* See text.
Specific surface area values
Hydrocortisone acetate: *p*-nitrophenol 4·4 m²/g; N_2 3·3 m²/g; Fisher 1·3 m²/g.
Betamethasone alcohol: *p*-nitrophenol 1·6 m²/g.

Betamethasone suspension with the cellulose derivative required 12 tilting operations (mean of 15 estimations) to clear the sediment. Without the cellulose derivative, 7 operations (mean of 6 estimations) were needed. Two samples of the suspension prepared without the cellulose derivative but with polysorbate 80 required 6 and 4 operations.

TABLE 2. STATE OF FLOCCULATION OF STEROID POWDERS IN AQUEOUS SUSPENSIONS CONTAINING ADDITIVES

	Treatment (conditions—see Table 1)						
Steroid	Water only	P80	HEC/ P80	HEC	NaCMC	Glucose	Sucrose
Cortisone acetate ..	Flocculated free flowing	Low volume	Low volume	Low volume	Flocculated free flowing	Flocculated free flowing	Flocculated free flowing
Prednisolone acetate ..	Low volume	Low volume	Low volume	Low volume	Low volume	Low volume	Low volume
Betamethasone alcohol ..	Low volume	Low volume	Low volume	Low volume	Low volume	Low volume	Low volume
Hydrocortisone acetate ..	Flocculated free flowing	Low volume	Low volume	Low volume	Flocculated free flowing	Flocculated free flowing	Flocculated free flowing

A distribution-free test showed the first two groups to be significantly different at the 5% level. Although there are not sufficient data to place the issue beyond all doubt, there is an indication that the presence of hydroxyethylcellulose produces caking.

Discussion

All the steroid powders adsorb hydroxyethylcellulose from aqueous solution.

For hydrocortisone acetate there are three independent methods of measurement of the specific surface area. The agreement between nitrogen and *p*-nitrophenol adsorption is reasonable, though the Fisher air-permeability method produces a rather lower result.

According to the manufacturer, the hydroxyethylcellulose used has a "repeating unit" with a molecular weight of 442. The projected area of this segment as measured with Catalin models is as follows: Face down = 90Å^2. Edge on = 20Å^2. Table 1 shows that for hydrocortisone acetate 0·17 g hydroxyethylcellulose is adsorbed per g steroid, thus the area that would be occupied if the hydroxyethylcellulose were adsorbed flat is 21 m^2, or if adsorbed edge-on, 4·6 m^2. By the *p*-nitrophenol method the experimentally observed value of the specific surface area was 4·4 m^{2}g^{-1}.

We have measured the adsorption of hydroxyethylcellulose by the steroid powder at one concentration only, and this is the concentration at which the cellulose derivative is used in the suspension vehicle employed to prepare a typical betamethasone intra-articular injection.

It is obvious that the molecule cannot be adsorbed "flat" in the form of a monolayer. It is also impossible for each segment of the molecule to be attached to the surface, though it would appear that they could be accommodated on the surface.

One of the characteristics of polymer adsorption at solid surfaces is that apparently much more material adheres than corresponds to a flat monolayer (Eirich, 1959). It is generally recognised that the adsorption occurs at only a fraction of the active groups of the polymer molecule. The unadsorbed segments penetrate deeply into the surrounding solution, but are attached to the surface by other units of the same molecule. It would appear that the adsorption of hydroxyethylcellulose on hydrocortisone acetate also occurs in the manner described, since we have shown that a flat monolayer of the cellulose derivative would occupy about four times as much space as is available at the surface of the particles.

For betamethasone alcohol we find the specific surface area by *p*-nitrophenol adsorption to be 1·6 m^2/g and that the hydroxyethylcellulose adsorbed per g of powder is 0·009 g. These figures give areas for monolayers of 11 m^2 if the derivative is adsorbed flat and 2·4 m^2 if it is adsorbed edge-on. Again, the adsorption is in excess of the area available on the particle surface and the mode of attachment of the derivative to the betamethasone particle surface is probably the same as it is for hydrocortisone acetate.

We note that polysorbate 80 is adsorbed by all the steroid powders, except, as would be expected, the betamethasone alcohol, which had been pre-treated with polysorbate 80. Prednisolone acetate, which had been precipitated in the presence of cetrimide, also gave a low value.

In the presence of polysorbate 80, hydroxyethylcellulose does not appear to be adsorbed (less than 2μg/g was absorbed from a 0·2% w/v solution). It may well be that polysorbate 80 is preferentially adsorbed by the steroids, thus preventing the adsorption of the cellulose derivative.

Sodium carboxymethylcellulose was not adsorbed by any of the powders (limits as above) and at present we can offer no explanation for this.

A long-chain polymer molecule is adsorbed at many segments along its chain. In order to be desorbed, it must be released from the adsorbing surface simultaneously at each of these points of attachment. This is one of the reasons for the adsorption of long chain polymers, such as hydroxyethylcellulose and the failure of substances such as glucose and sucrose to be adsorbed.

In Table 2 we compare the state of sedimentation of the aqueous suspension with different additives. From column 2, which shows the suspension of the powders in water, we see that only cortisone acetate and hydrocortisone acetate were flocculated and free-flowing after two weeks standing, whereas betamethasone alcohol and prednisolone acetate formed low-volume sediments and were not free-flowing. This is further borne out by data in column 3, which show the effect of polysorbate 80. On the addition of this surfactant, all four steroid powders formed low-volume sediments. The same was true for the addition of hydroxyethyl cellulose alone and also with polysorbate 80.

Sodium carboxymethylcellulose, which was not adsorbed, had no effect on the flocculated sediments of the acetates of cortisone and hydrocortisone, neither did glucose nor sucrose which also had no effect on the two steroids not already deflocculated.

The results of the caking tests support the contention that the presence of a polymer adsorbed by betamethasone enhances the tendency to cake. The caking of a suspension can have many causes, and therefore the omission of hydroxyethylcellulose would not be expected to eliminate caking altogether.

There are indications that when polysorbate 80 is substituted for hydroxyethylcellulose the caking of the suspension is similar to that of the suspension without either of the additives. Both substances produce low-volume sediments, but the hydroxyethylcellulose has a stronger tendency to link the sedimented particles together. It can vary in molecular weight between 40,000 and 350,000, which approximates to molecules of between 600 and 4800 Å in length, while the approximate diameter of a non-ionic surfactant micelle is 100 Å: hence the greater ability of the cellulose derivative to cross-link particles. It should be noted that omission of hydroxyethylcellulose and polysorbate 80 also produces a low volume sediment. As previously indicated (Table 1), the betamethasone alcohol used in these experiments had been pre-treated with the surfactant.

We have thus shown that the presence of the polymer has enhanced the caking tendency of the low-volume sediment.

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The temperature coefficient of some bactericides in oil : water dispersions

H. S. BEAN AND S. M. HEMAN-ACKAH*

The temperature coefficient of a bactericide in oil : water dispersion changes with the nature of the oil and the phase : volume ratio. The variations in the temperature coefficient are due to temperature effects on the availability of the bactericide in the aqueous phase, and at the oil : water interface of the dispersions. Failures in the preservation of oil : water formulated products encountered during storage in different climates may be due to such underlying phenomena which are not considered in the initial stages of formulation.

THE manner in which temperature influences antibacterial activity is important theoretically and practically. Information is available about temperature coefficients of commonly used antimicrobial agents in aqueous solutions but cannot be used to predict preservative performance in oil-water formulations because of the interaction of many factors (Kabelik, 1947, Galloway, 1952; de Navarre, 1962.)

Some of the factors which control the activity of a bactericide in oil : water dispersions have been reported elsewhere (Bean, Richards & Thomas, 1962; Bean & Heman-Ackah, 1964; Bean, Heman-Ackah & Thomas, 1965a, 1965b). The overall activity is dependent largely on the concentration of the bactericide partitioned to the aqueous phase of the dispersion, but it is enhanced by an effect due to the oil : water interface. A rise in temperature may shift the oil : water partition coefficient (K_w^o) and hence alter the concentration of the bactericide available in the aqueous phase and at the oil : water interface. Consequently, the temperature coefficient of the bactericide in the dispersion (θ_s) differs from that in aqueous solution (θ_A), and it is dependent on the nature of the oil and the ratio of the phases (Bean & Heman-Ackah, 1963).

The present communication reports a correlation between the temperature coefficient of a bactericide in the dispersion (θ_s) and the rate of change of the oil : water partition coefficient with temperature which may be used for the primary evaluation of preservatives in oil : water systems.

Experimental

MATERIALS AND METHODS

The test organism (*Escherichia coli* NCTC 5933), oils, bactericides and experimental techniques, were as described previously (Bean & Heman-Ackah, 1963, 1964). A spectrophotometric method was used in assaying the aqueous phase concentrations of bactericides in oil : water systems when determining K_w^o; an extinction time method was employed for the evaluation of bactericidal activity.

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LINEAR RELATIONSHIP BETWEEN THE OIL : WATER PARTITION COEFFICIENT (K_w^o) AND TEMPERATURE (T)

The oil : water partition coefficients, K_w^o, of bactericides were determined at 5, 15, 25, 35 and 45° respectively. Table 1 shows that a rise in temperature may cause an increase in K_w^o (e.g. phenol in liquid paraffin : water), a slight increase in K_w^o (e.g. chlorocresol in arachis oil : water) or a decrease

TABLE 1. INFLUENCE OF TEMPERATURE ON THE PARTITION COEFFICIENT (K_w^o) OF BACTERICIDES IN OIL : WATER SYSTEMS

Temperature °C	Phenol in paraffin : water	Phenol in liquid paraffin : water containing 5% propylene glycol in aqueous phase	Phenol in liquid paraffin : water containing 0·9% sodium chloride in aqueous phase	Chlorocresol in liquid : paraffin water	Chlorocresol in arachis oil : water	Phenylmercuric acetate in liquid paraffin : water
5	0·0592	0·0351	0·0389	1·192	113·4	0·1405
15	0·0620	0·0488	0·0586	1·341	116·7	0·1203
25	0·0688	0·0594	0·0992	1·528	116·7	0·0953
35	0·1167	0·0801	0·1360	1·782	116·0	0·0847
45	0·1535	0·1104	0·1787	2·003	117·2	0·0700
$\frac{K_w^o \text{ at } 45°}{K_w^o \text{ at } 5°}$	2·59	3·14	4·59	1·68	1·03	0·50

in K_w^o (e.g. phenylmercuric acetate in liquid paraffin : water). The presence of 5% propylene glycol in the aqueous phase of liquid paraffin : water decreases K_w^o for phenol at all the test temperatures. On the other hand, 0·9% sodium chloride in the aqueous phase of liquid paraffin : water increases the value of K_w^o for phenol at 25–45°, causes no change at 15°, and decreases K_w^o at 5–15°.

The relationship between log K_w^o and T is linear for all bactericide : oil : water systems (Fig. 1) and is summarised by the expression :

$$\log K_w^o = \epsilon T + \text{constant.}$$

ϵ is defined as the *temperature coefficient of the partition coefficient* (Heman-Ackah, 1965) and it is the appropriate increase in K_w^o per °C rise in temperature. Its numerical value may be positive or negative depending upon whether the K_w^o increases or decreases with rise in temperature (Table 3).

INFLUENCE OF TEMPERATURE ON THE ACTIVITY OF BACTERIDES IN OIL : WATER DISPERSIONS

Temperature influences the activity of bactericides in oil : water dispersions in a complex manner. Temperatures above 50° cause the death of vegetative cells by protein coagulation, enzyme inactivation or both. There are additionally, the indirect effects of temperature on: (i) the distribution of the bactericide between oil and water phases (Table 1) which produces differences in the availability of the bactericide in the

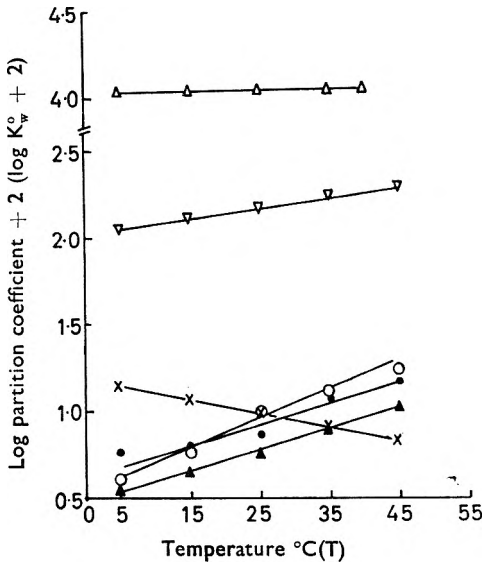

FIG. 1. The linear relationship between partition coefficient and temperature. ● = Phenol : liquid paraffin : water. ▲ = phenol : liquid paraffin : water containing 5% propylene glycol in aqueous phase. ○ = Phenol : liquid paraffin : water containing 0·9% sodium chloride in aqueous phase. ▽ = chlorocresol in liquid paraffin : water. △ = chlorocresol in arachis oil : water. × = Phenylmercuric acetate in liquid paraffin : water.

aqueous phase of the dispersion, and hence in the activity (Bean & Heman-Ackah, 1963); (ii) the oil : water interfacial activity which diminishes with rise in temperature (Heman-Ackah, 1965); (iii) the velocity of the bactericidal action which increases with rise in temperature (Madsen & Nyman, 1907; Chick, 1908; Phelps, 1911).

Table 2 shows the variation of the log of temperature coefficient (θ) of bactericides with the oil : water ratio (ϕ) of the dispersions. One of two phenomena may occur.

1. K_w^o *increases with rise in temperature.* The normal effect of temperature on the bactericidal activity is offset by an effect due to a progressive depletion of the bactericidal content of the aqueous phase and hence of the oil : water interface. In this instance, the temperature coefficient of the bactericide in the dispersion (θ_s) is less than that in aqueous solution (θ_A) containing the same overall concentration of the bactericide (i.e. the aqueous reference solution), and it decreases with increase in the oil : water ratio (ϕ). A previously reported relationship (Bean & Heman-Ackah, 1963; Heman-Ackah, 1965) applies:

$$\frac{\log \theta_A - \log \theta_s}{\phi} = k \text{ (where k = constant)}$$

At a unique oil : water ratio, the activity of the dispersion becomes independent of temperature (i.e. $\theta_s = 1$). Under such a condition, the appropriate increase in the bactericidal activity per °C rise in temperature

is offset by a parallel depletion of the bactericidal content of the aqueous phase and by a smaller effect of the interface on the activity.

2. K_w^o *decreases with rise in temperature.* The normal effect of temperature on the bactericidal activity is enhanced by an effect due to increased concentration of the bactericide in the aqueous phase and at the oil : water interface. Consequently, the temperature coefficient of the bactericide in the dispersion (θ_s) is greater than that of the aqueous reference solution (θ_A). The following relationship applies (Heman-Ackah, 1965)

$$\frac{\log \theta_A - \log \theta_s}{\sqrt{\phi}} = k' \text{ (where } k' = \text{constant).}$$

Therefore there cannot be a dispersion in which the activity is independent of temperature.

The constant k (or k') is a measure of the change in activity of a bactericide in the dispersion per °C rise in temperature as compared with that of an aqueous reference solution. The concentration of the bactericide

TABLE 2. LOG TEMPERATURE COEFFICIENT OF BACTERICIDES IN AQUEOUS SOLUTIONS AND IN OIL/WATER DISPERSIONS (5–45°)

	K_w^o increase with rise in temperature					K_w^o decrease with rise in temperature
Oil : water ratio (ϕ)	0·5% Phenol in liquid paraffin : water	0·5% Phenol in liquid paraffin : containing 5% propylene glycol in aqueous phase	0·5% Phenol in liquid paraffin : water containing 0·9% sodium chloride in aqueous phase	0·075% Chlorocresol in liquid paraffin : water	4·0% Chlorocresol in arachis oil : water	0·002% Phenylmercuric acetate in liquid paraffin : water
0 (Aqueous reference) solution	0·0588	0·0557	0·0680	0·0396	—*	0·0219
0·2	0·0547	0·0508	0·0646	0·0374	—	0·0246
0·5	—	—	—	—	0·1019	—
0·6	—	—	—	—	0·0873	—
0·7	—	—	—	—	0·0837	—
0·8	—	—	—	—	0·0698	—
1·0	0·0402	0·0309	0·0503	0·0283	0·0656	0·0285
1·5	0·0266	0·0156	0·0419	—	—	—
2·0	0·0166	—	0·0278	—	—	0·0322
3·0	—	—	—	—	—	0·0351
3·5	—	—	—	0·0030	—	—
5·0	—	—	—	—	—	0·0367
$k = \frac{\log\theta_A - \log\theta_s}{\phi}$	+0·020	+0·025	+0·018	+0·012	—	—
$k' = \frac{\log\theta_A - \log\theta_s}{\sqrt{\phi}}$	—	—	—	—	—	−0·007

* Impracticable to determine

in the aqueous phase of a dispersion changes with rise in temperature because the K_w^o does not remain constant, whereas the concentration of the bactericide in the aqueous reference solution is maintained the same over the range of temperature studied. Consequently, the constant k (or k′) reflects the availability of the bactericide in the aqueous phase and at the oil : water interface, and therefore, reflects also the rate of change of the oil : water partition coefficient with temperature.

RELATIONSHIP BETWEEN THE CONSTANT k (OR k′) AND THE TEMPERATURE COEFFICIENT OF THE PARTITION CONSTANT, ϵ

Table 3 shows the relationship between the constant, k (or k′) and ϵ the temperature coefficient of the partition constant. For systems where k_w^o increases with temperature rise, k is approximately twice the value of ϵ (Mean Ratio $k/\epsilon = 2{\cdot}0$) whereas for systems where K_w^o decreases with temperature rise k′ is about equal to the value of ϵ (Ratio $k/\epsilon = 0{\cdot}9$).

TABLE 3. RELATIONSHIP BETWEEN THE CONSTANT k (OR k′) AND THE TEMPERATURE COEFFICIENT OF THE PARTITION CONSTANT (ϵ)

System (K_w^o increase with rise in temp.)	$k = \frac{\log\theta_A - \log\theta_B}{\phi}$	$\varepsilon = \frac{\Delta \log K_w^o}{\Delta T}$	Ratio $\frac{k}{\varepsilon}$
Phenol in liquid paraffin : water	+ 0·020	+ 0·011	1·8
Phenol in liquid paraffin : water containing 5% propylene glycol in aqueous phase	+ 0·026	+ 0·012	2·2
Phenol in liquid paraffin : water containing 0·9% sodium chloride in aqueous phase	+ 0·018	+ 0·010	1·8
Chlorocresol in liquid paraffin : water	+ 0·012	+ 0·005	2·4
Chlorocresol in arachis oil/water	—*	+ 0·0003	—
System (K_w^o decreases with rise in temp.)	$k' = \frac{\log\theta_A - \log\theta_B}{\sqrt{\phi}}$	$\varepsilon = \frac{\Delta \log K_w^o}{\Delta T}$	Ratio $\frac{k'}{\varepsilon}$
Phenylmercuric acetate in liquid paraffin : water	− 0·007	− 0·0076	0·9

* $\log\theta_A$ for 4·0% chlorocresol in aqueous solution was impracticable to determine.

There is, therefore, a direct correlation between the temperature coefficient of the bactericide in the dispersion and the rate of change of the oil : water partition coefficient with temperature. This indicates that the toxic concentration of the bactericide in the dispersion is the available concentration in the aqueous phase and at the oil : water interface. It is inferred that the variation of the temperature coefficient of the bactericide with the oil : water ratio is purely a physical phenomenon and that the fundamental bactericidal action remains unaltered.

Discussion

The numerical value of the temperature coefficient (θ) is dependent on the disinfectant and test organism but it varies with (i) the range of temperature used for the determination (Tilley, 1942; Ames & Smith, 1944; Jordan & Jacobs, 1946); (ii) the concentration of disinfectant employed

for the determination (Chick, 1908; Jordan & Jacobs, 1944; Berry & Michaels, 1950).

In oil : water dispersions it is also shown that θ varies with the oil : water ratio even for the same overall concentration of bactericide, test organism, and range of temperature employed for the experiments. This fact, though inherent in published data, has not been previously commented upon.

Beléhrádek (1935) has said that "when one and the same biological process gives two or more different temperature coefficients under various conditions, three different explanations are possible: (i) the formula used does not adequately express the relationship between temperature and reaction velocity in that particular case, and another type of formula should be tested; (ii) if the formula holds good, then the value of the temperature coefficient is modified by the conditions at which the reaction takes place; (iii) that there are two or more distinct reactions of a catenary series on which the whole process is based under varying conditions." The explanation given in (ii) would account for the variations in the temperature coefficient of bactericides in oil : water dispersions, the modifying conditions being the changes in the concentration of the available bactericide in the aqueous phase and at the oil : water interface, both of which are controlled by the oil : water ratio and temperature.

Failures in preservation have been encountered in oil : water formulated products stored under tropical conditions and it has been claimed that these are readily explained on grounds of chance contamination by different organisms "insensitive" to the preservative (de Navarre, 1962). It is more likely that the underlying phenomenon is the variation in the temperature coefficient of the preservatives in the oil : water systems (θ_s). This means that when a rise in temperature takes place, preservative may pass from the aqueous to the oily phase where it is no longer available to exert its activity. The present work has shown that for any given overall concentration of a bactericide in the dispersion, the activity can be estimated, from a knowledge of the rate of change of K_w^o with temperature and the temperature coefficient of the bactericide in aqueous solution (θ_A). No bacteriological evaluation is necessary.

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