Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

British Pharmaceutical Conference
One Hundred and Fifth Meeting
Birmingham, September 1968

Volume 20: Supplement
December 1968

British Pharmaceutical Conference

Birmingham
September 9-13, 1968

CHAIRMAN

H. Burlinson

Journal of Pharmacy and Pharmacology
17 Bloomsbury Square, London, W.C.1

Journal of Pharmacy and Pharmacology

British Pharmaceutical Conference

Supplement

Editor: D. W. Mathieson, B.Sc., Ph.D., F.R.I.C.
Press Editor: J. R. Fowler, B.Pharm., F.P.S.

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Science Papers

SHORT COMMUNICATION

The bactericidal effect of silver ions on *Pseudomonas aeruginosa*

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

THERE are frequent reports of *Pseudomonas aeruginosa* infections in hospitals, with contamination of hospital equipment and pharmaceutical preparations (Editorial, 1967). Cason, Jackson & others (1966) report a high incidence of this organism in burn patients and found silver nitrate compresses to be an effective prophylactic measure.

Little information is available on the action of silver ions on *Ps. aeruginosa*, and the present communication reports the initial stages of such a study. Using cultures whose growth was eventually limited by the magnesium concentration in the culture medium, Brown & Melling (1968) found that *Ps. aeruginosa* resistance to EDTA was related to the degree of magnesium-limitation. We have examined the effect of magnesium-limitation on sensitivity to silver.

EXPERIMENTAL

Ps. aeruginosa NCTC 6750 was grown in the following medium: D-(+)-Glucose 0·001M, $(NH_4)_2HPO_4$ 0·01M, $(NH_4)_2SO_4$ 0·01M, NaCl 0·0005M, KCl 0·0005M with either 1×10^{-6} or 2×10^{-6} or 1×10^{-4}M $MgSO_4$ as described by Brown & Melling (1968). After 24 hr at 37·5°, cultures were allowed to cool slowly to 25°, and were then centrifuged and washed three times with water at 25°; this procedure resulted in no loss of viability. The cell concentration was adjusted to give an absorbance of 0·20 measured in a 10 mm cell at 470 mμ using a Unicam SP 600 spectrophotometer. This corresponded to a viable count of about 4×10^8 cells/ml. 1 ml of a washed suspension was added to 99 ml of a test solution containing silver nitrate in acetate buffer at 25° to give a final concentration of 1×10^{-5}M silver, ionic strength 2×10^{-4}, pH 5·85 or 6·25, and containing initially about 4×10^6 cells/ml. Viable counts were made at 2·5 min intervals by adding 0·5 ml test suspension to 19·5 ml of $1{\cdot}5 \times 10^{-2}$M thioglycollate in Oxoid nutrient broth No. 1 at 25°. After standing for 35 min at room temperature (20–25°), further dilutions were made in nutrient broth and appropriate volumes spread on over-dried nutrient agar plates. Colonies were counted after 20 to 40 hr at

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37·5°. Quenching in $1{\cdot}5 \times 10^{-2}$M thioglycollate in broth for 35 min before dilution was shown to give optimal recovery of organisms which had been exposed to 1×10^{-5}M silver at pH 6·25 at 25° for 10 min. Preliminary experiments showed that nutrient broth with and without additional sodium chloride or polysorbate 80, or both, effectively neutralized much higher silver concentrations (e.g., 0·5 ml 10^{-3}M of silver to 19·5 ml of neutralizer) when undamaged cells were tested, but these were much less effective than thioglycollate in broth for the recovery of silver-damaged cells. This is consistent with the suggestion of Richards & El Khouly (1967) that penetration of thioglycollate into cells damaged by phenylmercuric nitrate is required for optimal recovery.

RESULTS AND DISCUSSION

Cells obtained from the medium containing 1×10^{-4}M (i.e., excess) magnesium abruptly stopped dividing when the medium became depleted of glucose and will be referred to as "glucose-limited" cells. In the media containing the lower magnesium concentrations, cell division did not cease abruptly but became slower as growth proceeded and as the magnesium levels in the media fell. Cells grown in the medium containing 2×10^{-6}M magnesium had ceased dividing after 24 hr at 37·5° because of glucose depletion (glucose-magnesium-limited cells). Cells grown in 1×10^{-6}M magnesium were still dividing slowly after 24 hr because the glucose had not been completely used, they were thus magnesium-limited but not glucose-limited.

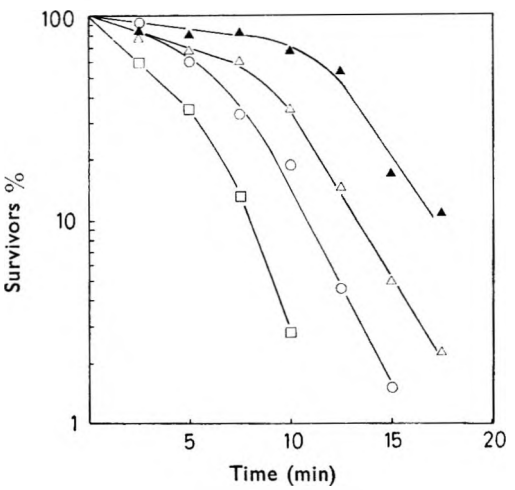

FIG. 1. Rate of kill of *Ps. aeruginosa* in 1×10^{-5}M silver nitrate at 25°. ▲ Glucose-limited cells at pH 5·85. △ Glucose-limited cells at pH 6·25. ○ Glucose-magnesium limited cells at pH 6·25. □ Magnesium-limited cells at pH 6·25.

Representative results given in Fig. 1 show that the glucose-limited cells were more resistant to silver ions than the glucose-magnesium-limited cells. This suggests that cell walls of magnesium-limited cells provide a less effective barrier first to the adsorption by the cell and later

perhaps to entry into the cell of the silver ions. The even lower resistance of cells grown in the medium containing 1×10^{-6}M magnesium may be due to more extensive changes in the cell wall but may be due to differences in glucose-limitation. The difference in sensitivity between magnesium-limited and non magnesium-limited cells may reflect a difference in cell wall composition. Differences in cell wall composition of magnesium limited and non-magnesium limited cells have been shown with *Aerobacter aerogenes* (Ellwood & Tempest, 1967).

Fig. 1 also shows that silver ions are less effective at the lower pH suggesting competition between metal cations and hydrogen ions for anionic sites on the bacteria. Variation of effectiveness with pH may explain the differences in activity of different silver salts reported by Foord, McOmie & Salle (1938).

None of the survivor curves are linear and a shoulder seems to be characteristic of silver-damaged organisms recovered in thioglycollate. These shoulders were not found when test systems (containing glucose-limited cells) were neutralized in nutrient broth; in this case linear survivor curves suggesting much more rapid kill were obtained. It seems likely that although silver is taken up by the cell very quickly, reaction with a vital centre occurs more slowly so that if thioglycollate becomes available during this time it combines with the silver and permits the cell to recover and multiply.

Acknowledgement. We thank Miss C. Copson for excellent technical assistance.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 4S–7S Received April 1, 1968

The effect of a non-ionic surfactant upon the antifungal activity of benzoic acid

K. J. HUMPHREYS*, G. RICHARDSON AND C. T. RHODES

The effect of a non-ionic polyoxyethylene surfactant upon the fungicidal activity of benzoic acid has been investigated by a viable count method. The results show that theories equating antimicrobial activity to the concentration of non-micellar preservative are inadequate. The significance of these findings is discussed and some possible additional factors involved in the fungicidal process indicated.

PRESERVATION of creams from microbial spoilage has attracted considerable attention; Bean, Heman-Ackah & Thomas (1965) have emphasized that both the physical and microbiological properties of the system must be considered.

In any L_1 isotropic, aqueous liquid, system containing a surfactant and drug there exists an equilibrium between the micellar and non-micellar species of cosolute.

$$[D_w] \overset{K}{\rightleftharpoons} [D_m] \qquad (1)$$

where $[D_w]$ represents the concentration of free, unbound drug and $[D_m]$ the concentration of micellar drug.

It has been suggested (Allawala & Riegelman, 1953) that in solubilized systems the preservative activity is a function of $[D_w]$, $[D_m]$ acting as an inert reserve of drug. This concept has been extended by a number of workers, for example Mitchell (1964) and Mitchell & Brown (1966) who postulated that the activity of a drug in a solubilized system was governed by an R value or Saturation Ratio:

$$R = C/C_s \qquad (2)$$

where C is the drug concentration and C_s its saturation solubility.

By means of equilibrium dialysis and solubility techniques Humphreys & Rhodes (1968) have shown that the solubilization of benzoic acid by a series of n-alkyl polyoxyethylene surfactants is governed by a form of the Distribution Law (3) and the distribution constant, K_d, has been evaluated for these systems.

$$K_d = [D_m^o]/[D_w^o] \qquad (3)$$

where $[D_m^o]$ is the saturation solubility of drug in a hypothetical micellar bulk phase and $[D_w^o]$ is the saturation solubility of drug in the aqueous phase.

From the data collected by Humphreys & Rhodes it is possible to estimate the $[D_w]$ and $[D_m]$ values in the surfactant systems studied within the temperature range, 18–45°. Thus it is possible to test quantitatively the validity of the theory outlined above concerning the biological activity of cosolute in the presence of surfactant. This theory suggests

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that the antimicrobial activity of benzoic acid in systems having the same $[D_w]$ value should be identical regardless of the $[D_m]$ value or the total concentration of the drug in the system $[D_t]$.

In the present paper we report studies of the correlation between $[D_w]$ and the fungicidal activity of benzoic acid in a number of systems with and without surfactant.

Experimental

Materials. Analar benzoic acid; a commercial sample of an n-alkyl-polyoxyethylene surfactant (Texofor, Glover) of mean molecular formula $Me[CH_2]_{15}\cdot O[CH_2\cdot CH_2\cdot O]_{30}\cdot OH$, characterized according to Rhodes (1967).

The Test organism was a 24 hr culture of *Schizosaccharomyces pombé* washed from agar slopes and resuspended in water.

Method of count. Samples of reaction mixture (1 ml), appropriately diluted, were plated in roll tubes, each containing 4 ml of Wort Agar (Oxoid), and these were incubated inverted (bungs removed) for four days at 27°.

Experimental design. Controls were used to establish (a) The acid tolerance of the organism, (b) The absence of fungicidal activity of the surfactant, (c) The absence of activity from "carry-over" of reaction mixture into the roll tubes.

Fungicidal studies. The fungicidal activity of benzoic acid was determined in the presence and absence of 2% w/v Texofor. Comparison was made between the activity of (A) solutions of benzoic acid, (B) solutions of benzoic acid in 2% surfactant of the same total benzoic acid content and (C) solutions of benzoic acid, of concentration equivalent to that of the free acid in the surfactant solution. All solutions were adjusted to pH 3·0 with hydrochloric acid.

TABLE 1. COMPARISON OF FUNGICIDAL ACTIVITY OF EQUAL AMOUNTS OF BENZOIC ACID IN THE PRESENCE AND ABSENCE OF SURFACTANT

Reaction mixture	% survival in replicate determinations				
	Exposure time (hr)	1½	1¾	1¾	3½
	$[D_t] = [D_w]$	15·0 mM	26·0 mM	26·0 mM	26·0 mM
A Benzoic acid alone		2·4	0·1	0·014	0
		2·9	0·03	0·000	0
		3·0	0·2	0·007	0
		2·9	0·1	0·2	0
				0·007	0
	Mean	2·8	0·1	0·01	<0·0001
	$[D_t]$	15·0 mM	26·0 mM	26·0 mM	26·0 mM
	$[D_w]$	7·5 mM	13·5 mM	13·5 mM	13·5 mM
B Benzoic acid + surfactant		105	3·2	12·8	0·8
		96	3·2	12·7	0·7
		109	4·6	13·1	0·8
		97	3·5	11·2	0·9
		87		15·3	
	Mean	99	3·6	13·0	0·8

Saturation solubility, C_s (benzoic acid) in *N*/1000 HCl = 26·5 mM. C_s (benzoic acid) in 2% w/v surfactant = 51·0 mM.

TABLE 2. COMPARISON OF FUNGICIDAL ACTIVITY OF BENZOIC ACID SYSTEMS, WITH AND WITHOUT SURFACTANT, OF THE SAME [D_w] CONTENT

Reaction mixture	% survival in replicate determinations					
	Exposure time (hr)	1¾	3½	3½	1½	1¾
	[Dt]	26·0 mM	26·0 mM	26·0 mM	29·0 mM	29·0 mM
	[D_w]	13·5 mM	13·5 mM	13·5 mM	15·0 mM	15·0 mM
B Benzoic acid + surfactant		12·8	0·8	0·1	0·04	0·09
		12·7	0·7	0·1	0·07	0·02
		13·1	0·8	0·2	0·00	0·1
		11·2	0·9	0·1	0·03	0·09
		15·3			0·00	0·07
	Mean	13·0	0·8	0·1	0·03	0·07
	[Dt] = [D_w]	13·5 mM	13·5 mM	13·5 mM	15·0 mM	15·0 mM
C Benzoic acid alone		24·9	5·3	0·2	0·6	2·8
		23·4	7·5	0·2	0·9	3·2
		27·2	6·3	0·2	0·4	3·0
		26·1	7·5		0·4	2·5
			3·8		0·5	2·8
	Mean	25·4	6·1	0·2	0·6	2·9

Saturation solubility, C_s (benzoic acid) in N/1000 HCl = 26·5 mM. C_s (benzoic acid) in 2% w/v surfactant 51·0 mM.

Results

At pH 3·0, no lethal effect upon the organism was detected over a period of 21 hr. Change in pH from 2·0 to 3·0 was found to have little effect (less than 2%) upon the ionization of the unbound acid or the micellar solubilization. The controls showed that solutions of surfactant up to 5% w/v possessed no intrinsic antifungal activity and confirmed that the degree of preparing dilution was sufficient to inactivate reaction mixtures carried over into the counting medium.

The effects of the surfactant upon viable counts are summarized in Tables 1 and 2.

Discussion

Table 1 shows that the fungicidal activity of benzoic acid is reduced by the presence of surfactant. Reference to the values, in each vertical column, all determined under the same experimental conditions, shows that there is a significant increase in the percentage survival when surfactant is added.

Table 2 shows the results of several sets of comparisons of fungicidal activity of a series of benzoic acid systems of the same [D_w] value but differing in [D_t] because of the presence of surfactant. Comparison, within each vertical column, of data obtained under the same experimental conditions, clearly shows significant increases of percentage survival in those systems containing surfactant. From the results it must be concluded that the simple theory as outlined in the introduction, is insufficient to rationalize the fungicidal activity of benzoic acid in a system containing surfactant. From the data presented in Tables 1 and 2 the R, saturation ratio, values may also be calculated and it can be seen that fungicidal activity is not a simple function of this term as has been previously

suggested (Mitchell, 1964; Mitchell & Brown, 1966). There must, therefore, be additional factors controlling the extent of fungicidal activity.

The first possibility is that the non-micellar surfactant may exert a synergistic effect upon the fungicidal activity of $[D_w]$. Since it has been shown by Gershenfeld & Stedman (1949) that synergism occurs with submicellar concentrations of non-ionic surfactants, this is a likely explanation of the results.

Secondly, the micelles may act as a reservoir of drug so that if $[D_w]$ becomes significantly depleted some of the bound drug is released to restore the equilibrium shown in (1). Experiments with solubilized chloroxylenol by Bean & Berry (1951, 1953) suggest the possibility of this mechanism.

A third explanation is that some form of "mixed micelle" is formed between the organism, the surfactant molecules and the benzoic acid, so that the organism is brought into intimate contact with the cosolute. This postulated mechanism appears analogous to that suggested by Bean & Dempsey (1967), who obtained results indicating that phenols exhibited greater antibacterial activity if first adsorbed on to small quantities of carbon. It seems that the organism was also adsorbed on to the treated carbon so that the effective concentration of phenol adjacent to the organism was increased.

The results presented in this paper indicate that, if formulation of L_1 surfactant systems with bactericides is based upon the simple two phase theory, a more than adequate degree of protection will be conferred.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 8S–13S Received May 1, 1968

Cell size changes during the growth of *Escherichia coli* partially inhibited by some antibacterial agents

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The effects of tetracycline, phenol, chloramphenicol and ampicillin on the rates of division and mass increase in growing cultures of *Escherichia coli* have been examined by comparing the number, carbon content and the size of the cells from partially inhibited cultures with those of control cultures. In cultures treated with low concentrations of tetracycline, phenol or chloramphenicol the increase in cell mass is inhibited more than cell division, thus causing a decrease in the cell size. At higher concentrations of these agents and at all concentrations of ampicillin, cell division is inhibited more than increase in cell mass so causing an increase in cell size.

THE growth of bacteria in the presence of low concentrations of antibacterial agents can result in cell size changes. Increases in cell size resulting from cellular division being inhibited to a greater extent than cellular growth have frequently been observed (Dean & Hinshelwood, 1966). This paper describes the results of studies of the relative inhibition of growth and division in *Escherichia coli*, produced by different concentrations of phenol, tetracycline, chloramphenicol and ampicillin; all results were obtained during the early stages of growth when complications due to breakdown of the antibacterial agents or adaptation by the cells were unlikely to have occurred.

Experimental

Escherichia coli (NCTC 1013) was cultivated in a mineral salts medium containing 2mg/ml of uniformly labelled [^{14}C]glucose (specific activity 0·01μc/mg). The media, conditions of culture and methods for measuring radioactivity and absorbance have been described previously (Rye & Wiseman, 1966).

Total cell counts and size (volume) distributions were determined using a model B Coulter electronic particle counter fitted with a 30μ orifice tube. The electrolyte solution and methods for obtaining total cell counts and size distributions have already been described (Rye & Wiseman, 1967).

Antibacterial agents. Freshly prepared solutions of the following compounds in a glucose-free medium were used; tetracycline B.P., phenol B.P., chloramphenicol B.P. and ampicillin B.P. Preliminary experiments were made to determine the concentrations of these agents required to partially inhibit the growth of *E. coli*.

Inhibition of cultures. The absorbance of exponentially growing cultures of *E. coli* was measured at intervals and when this reached a value of 0·200, 10ml volumes were mixed with equal volumes of solutions of the antibacterial agents at 37°. Incubation of these partially inhibited cultures, together with control cultures diluted with glucose-free medium alone, was continued with shaking; growth was followed by means of absorbance measurements. When the absorbance of each culture reached

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0·200, the time taken was noted, duplicate samples were diluted with electrolyte solution for determining total cell counts and size distributions; cells from duplicate 2ml samples were collected on membrane filters for radioactivity measurements.

Results

Fig. 1a shows the results of measurements made during the growth of *E. coli* in the presence of concentrations of tetracycline between 0 and 1·5μg/ml. The abscissa represents the growth rate of the tetracycline-treated cultures relative to that of the uninhibited control; the values were calculated for each culture by dividing the time required for the absorbance to increase from 0·100 to 0·200 in the control by the time required for the same increase to occur in the treated cultures. The ordinate shows the ratios of (a) the total cell count and (b) the total cellular carbon ^{14}C-content of each of the treated cultures to those of the control.

The total cellular carbon ^{14}C-content is approximately the same in all the treated cultures and the control suggesting that an absorbance of 0·200 represents the same total cell mass in all cases. In the treated

TABLE 1. THE PERCENTAGE OF CELLS OF *E. coli* FALLING WITHIN DIFFERENT SIZE RANGES DURING UNINHIBITED AND PARTIALLY INHIBITED GROWTH IN THE PRESENCE OF TETRACYCLINE, PHENOL, CHLORAMPHENICOL AND AMPICILLIN

Antibacterial agent (μg/ml)	Size range (μ³)			
	0–0·53	0·53–1·06	1·06–2·12	> 2·12
Tetracycline				
0·00	1·1	54·4	37·7	6·7
0·075	4·1	63·6	29·0	3·3
0·15	6·8	64·7	25·6	2·9
0·25	5·4	65·0	26·9	2·7
0·375	2·1	62·3	32·2	3·5
0·50	0·9	58·1	37·1	4·0
1·00	0·1	33·7	58·0	8·3
1·50	0·3	27·0	62·7	10·1
Phenol				
0·00	1·1	57·0	36·1	5·8
125	2·3	57·7	34·4	5·6
250	4·3	59·9	31·0	4·8
375	4·7	60·4	30·3	4·6
500	4·0	58·2	32·1	5·6
625	3·6	57·8	32·7	5·9
750	2·7	58·3	32·9	6·1
1,000	2·3	52·4	37·9	7·4
1,250	0·6	32·6	52·5	14·4
Chloramphenicol				
0·00	4·3	68·2	25·9	1·7
0·25	5·1	69·1	24·2	1·5
0·50	5·2	69·3	24·0	1·5
0·75	4·2	68·2	26·2	1·5
1·00	2·3	66·0	29·9	1·8
1·50	0·0	61·1	36·6	2·3
2·00	0·0	50·3	46·6	3·5
3·00	0·2	35·4	57·7	6·7
Ampicillin				
0·00	10·1	67·3	21·1	1·5
0·125	8·4	66·3	23·8	1·6
0·25	8·1	67·7	22·7	1·5
0·50	6·4	63·3	28·1	2·2
1·00	0·5	51·9	42·9	4·6
1·50	0·6	42·0	50·7	6·8
2·00	0·0	43·1	49·6	7·3
4·00	0·6	40·6	51·1	7·7
10·00	0·6	38·5	52·6	8·3

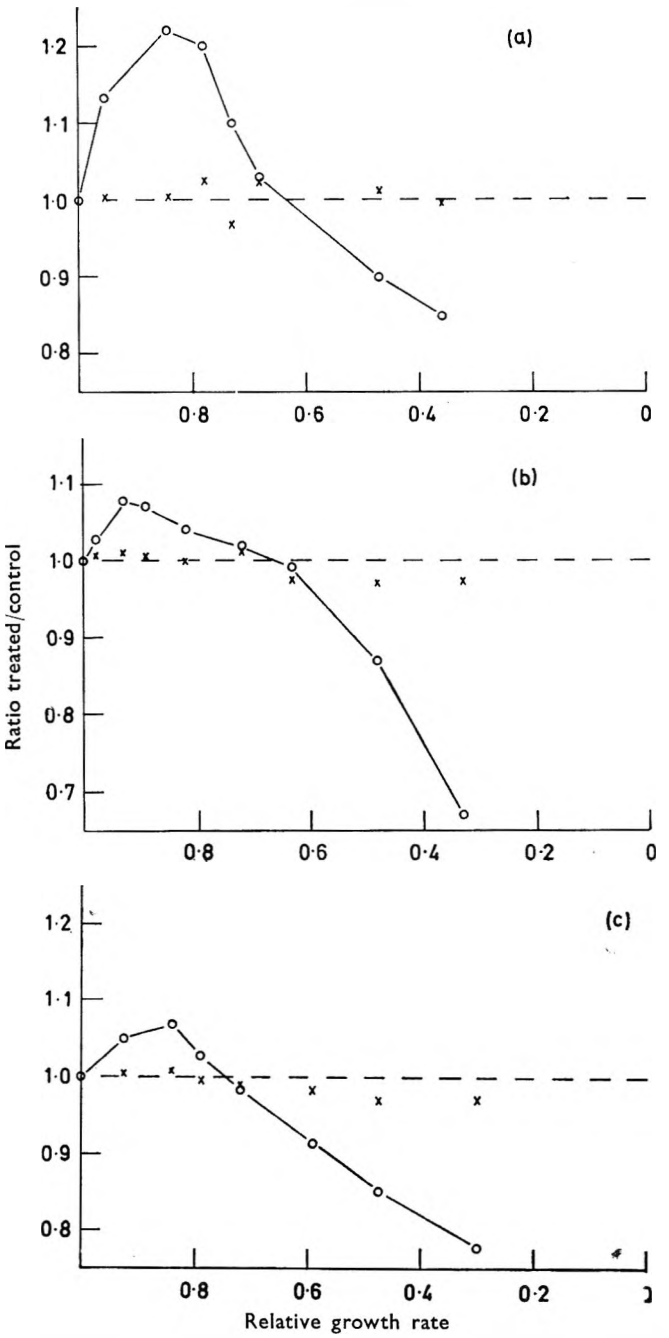

FIG. 1. The ratios of the total cellular ^{14}C-content and total cell counts of (a) tetracycline, (b) phenol and (c) chloramphenicol treated cultures to those of untreated controls. All measurements were made at an absorbance of 0·200. × Carbon content, ○ total cell counts.

cultures with relative growth rates between 0·65 and 1·0, however, the total cell count is greater than in the control thus indicating a decrease in the ^{14}C-content/cell. At slower growth rates (relative growth rate $<0{\cdot}65$) the total cell count in the treated suspensions is less than in the control and the ^{14}C-content/cell is consequently greater.

Table 1 shows the proportion of cells in different size ranges in these treated and untreated cultures and the actual concentrations of tetracycline used to partially inhibit growth. These results show that the variations in ^{14}C-content/cell are accompanied by corresponding changes in cell size. Thus in the presence of low concentrations of tetracycline the cells are smaller than in the control whilst in the higher concentrations they are larger.

Fig. 1b and c and Table 1 show that when cells are treated with phenol and chloramphenicol the results are qualitatively similar to those obtained with tetracycline but the decrease in cell size and ^{14}C-content/cell at the higher relative growth rates is not as marked.

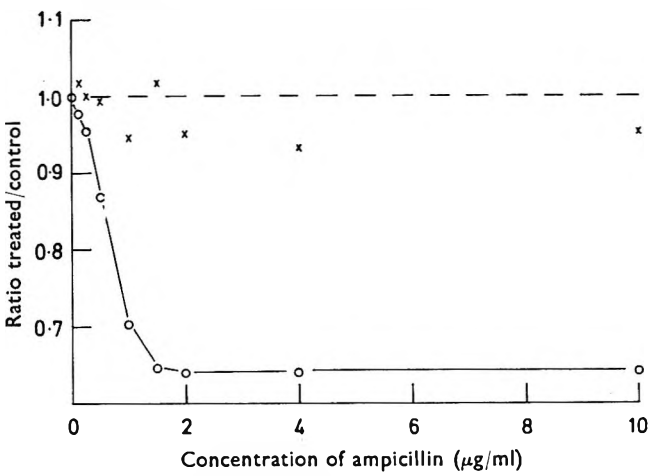

FIG. 2. The ratios of the total cellular ^{14}C-content and total cell counts of ampicillin treated cultures to those of an untreated control. All measurements were made at an absorbance of 0·200. × Carbon content, ○ total cell counts.

Fig. 2 and Table 1 show the results obtained when cells were treated with ampicillin. No change in the rate of increase in absorbance occurred with any of the ampicillin concentrations used and as the relative growth rate was consequently unaltered, the results are expressed as a function of ampicillin concentration. The total cell counts in the treated cultures were lower than in the control, decreasing rapidly with increasing concentrations of ampicillin, reaching a minimum at a concentration of 1·5μg/ml and then remaining constant. These results were reflected by changes in cell size and ^{14}C-content/cell, both increased with increasing concentrations of ampicillin reaching a maximum at 1·5μg/ml. At no concentration was any decrease in their values observed.

Microscopical examination of partially inhibited cultures revealed no evidence of cell clumping with any of the antibacterial agents used.

Discussion

During the normal exponential growth of bacteria the rates of cellular division and of increase in cell mass are approximately equal and the mean cell size consequently remains virtually constant. Although it has been shown that the average size of cells is dependent on their rate of growth (Schaechter, Maaloe & Kjeldgaard, 1958) and that changes in the environment can lead to changes in cell size (Kjeldgaard, Maaloe & Schaechter, 1958), the mechanisms by which the balance between division and increase in mass is maintained are not yet understood. Several antibacterial agents have been shown to produce morphological changes in growing cultures (Duguid & Wilkinson, 1961; Dean & Hinshelwood, 1966) but studies of their relative effects upon division and growth have been almost entirely restricted to observations of swelling or of filament formation as evidence of preferential inhibition of division. Few, if any, reports of a decrease in cell size have previously been made.

The magnitude of any size changes which occur during imbalanced growth will depend not only upon the relative rates of division and mass increase, but also upon the total increase in cell mass which occurs. Thus in any quantitative investigation of size changes in partially inhibited cultures, a constant increase in total cell mass should be allowed after the agent has been added. In this paper a doubling in absorbance from 0·100 to 0·200 has been used as a measure of this constant amount of growth. The validity of absorbance measurements for determining total cell mass has been shown from theoretical considerations by Koch (1961) and is apparently confirmed by our observations that both partially inhibited and uninhibited cultures of the same absorbance contain the same amounts of cellular carbon. The results presented in Figs 1–3 are expressed using relative growth rate as the abscissa. This scale enables all rates of growth from that characteristic of a normal uninhibited culture, to no growth at all, to be shown: it also allows results from antibacterial agents acting at widely different concentrations to be directly compared.

All four antibacterial agents examined upset the balance between division and mass increase when added to cultures of *E. coli*. At low concentrations of tetracycline, and to a lesser extent phenol and chloramphenicol, the observed decrease in cell size indicates that the increase in mass is being inhibited more than cellular division. At higher concentrations the opposite effects occur. The remarkable similarity in the inhibitory patterns produced by these different agents suggests that some common factor must exist in the mechanism by which they exert their effects upon division and growth. Possibly at low concentrations, when there is little penetration of these agents into the cells, the decrease in cell size results from a slowing of growth due to some inhibition of enzymes at the cell surface, or other accessible sites, with no corresponding inhibition at the internal sites of crosswall formation. At the higher concentrations when more penetration will have occurred, the increase in size may be due either to a particularly high sensitivity of crosswall synthesis to inhibition or may simply result from the fact that to maintain a balanced

supply of nutrients at a decreased rate of growth, a lower surface area to volume ratio can be tolerated. Ampicillin has virtually no effect upon the rate of increase in mass even at concentrations six times as high as those required for maximal effect upon cell division. With this agent, which has a highly specific action upon cell wall synthesis and particularly upon crosswall formation, no decrease in cell size was expected or observed even in the lowest concentrations used.

If the imbalance between division and mass increase, which can occur in partially inhibited cultures, continued indefinitely, the changes in cell size would become increasingly marked with the passage of time. In the case of cells which are becoming larger, such a continual change in size would be possible although perhaps unlikely as there is no theoretical limit to the maximum size of an individual cell. In conditions where the cell size is decreasing, however, a limit must be imposed upon the extent of change which can occur as a minimum possible cell size must exist! When this stage is reached a new balance between division and mass increase may be attained maintaining the cell size at this minimum value. In any studies using antibacterial agents which can cause size changes such as those described above, great caution should be exercised in converting results obtained in terms of cell number into terms of cell mass as any conclusions drawn after such a procedure may well be invalid.

Acknowledgement. We are grateful to Mrs. Sheena Kaye for technical assistance.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 14S–17S Received May 1, 1968

SHORT COMMUNICATION

The uptake of cetyltrimethylammonium bromide by *Escherichia coli*

W. G. SALT AND DAVID WISEMAN

THE uptake of cetyltrimethylammonium bromide (CTAB) by bacteria has previously been examined by Salton (1951) and by McQuillen (1950). Both workers determined its concentration in solutions by titration with sodium cetyl sulphate using pinacyanol bromide as indicator, and reported that uptake was in the form of a typical adsorption isotherm with saturation of the cells at an uptake equivalent to several theoretical close packed monolayers.

This communication reports on studies of the uptake of ^{14}C-labelled CTAB by cells of *Escherichia coli* and the concurrent release of phosphorus compounds from their metabolic pool.

EXPERIMENTAL

NNN-Trimethyl-[cetyl-1-^{14}C]ammonium bromide was obtained from the Radiochemical Centre, Amersham and unlabelled cetyltrimethylammonium bromide was kindly prepared by J. E. Adderson using the method of Adderson & Taylor (1964).

The organism used was *E. coli* NCTC 1013; the culture and suspending media, conditions of cultivation and methods of measuring absorbance were as described by Rye & Wiseman (1966).

Cell suspensions. Cultures in the exponential phase of growth at an absorbance between 0·500 and 0·700 were harvested by membrane filtration, washed with and resuspended in sufficient glucose-free medium at 25° to give an absorbance of 0·600, equivalent to 0·25 mg and $6{\cdot}4 \times 10^8$ cells/ml.

Similar suspensions with the metabolic pool of the cells preferentially labelled with ^{32}P were prepared by the method of Rye & Wiseman (1966).

Uptake of [^{14}C]CTAB. Volumes (5 ml) of unlabelled cell suspensions were added to 5 ml samples of solutions containing known amounts of [^{14}C]CTAB in glucose-free medium, rapidly mixed, and maintained at 25° for 15 min. Samples of the reaction mixtures (0·2 ml) were then evaporated to dryness on lens tissue in 5 cm planchets and the remainder of the mixtures centrifuged at 5000 rev/min for 10 min to remove the cells. Samples of the supernatant fluids (0·2 ml) were dried on planchets as above and the radioactivity of the samples measured in a Beckman Lowbeta automatic planchet counter.

Release of metabolic pool material. Equal volumes of ^{32}P pulse labelled cell suspensions and of unlabelled CTAB solutions or of glucose-free medium were mixed and maintained at 25°. After 15 min the total cellular and non metabolic pool ^{32}P contents of the cells were determined by the method of Rye & Wiseman (1966).

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RESULTS AND DISCUSSION

Fig. 1 a,b shows the amount of CTAB taken up by cells of *E. coli* as a function of the concentration remaining in solution and Fig. 2 the amount of metabolic pool phosphorus remaining in the cells as a function of the amount of CTAB adsorbed.

The mean dimensions of the cells used were length 3·2 μ, diameter 0·6 μ and volume 0·85 μ^3. Assuming the area occupied by a CTAB molecule in a close packed monolayer to be 45 Å^2 (Salton, 1951), and the cells to be cylinders with hemispherical ends, an uptake of 2·3 μg/ml of suspension is equivalent to a single close packed monolayer at the surface of the cells.

The adsorption isotherm (Fig. 1 a,b) which corresponds to the S4 type in the classification system of Giles, MacEwan & others (1960) suggests that uptake occurs in two distinct phases. The primary phase is characterized initially by an increase in the proportion of CTAB adsorbed with increase in the added concentration, followed at higher concentrations by a progressive decrease in the proportion adsorbed. Such an S type isotherm is reported by Giles & others to be characteristic of the uptake of monofunctional polar solutes at polar surfaces and to indicate that the molecules are orientated perpendicularly to the cell surface. The point of inflexion A in Fig. 1b marks the completion of this primary adsorption and corresponds to an uptake equivalent to two close packed monolayers of CTAB molecules. The point equivalent to the uptake of a single monolayer is not clearly defined indicating that the first molecules are so orientated on the cell that the newly formed surface favours the uptake of a second layer.

This suggests that the molecules in the primary monolayer have their polar groups adjacent to the cell so that the new surface presented to the solution is hydrophobic, allowing the adsorption of a second layer with their polar groups distal to the cell. The surface formed by such a double layer would be positively charged. Further uptake of CTAB cations would not therefore be facilitated and an extended plateau in the isotherm would be expected.

The primary uptake however is followed at still higher concentrations by an increase in the proportion of CTAB adsorbed giving a secondary isotherm which is approximately linear over much of its range. Taken in isolation this secondary isotherm is similar to the type C of Giles & others who suggest that such an uptake results from the penetration of solute molecules into regions inaccesible to the solvent. It is therefore probable that this corresponds to penetration of CTAB into the cells possibly into the hydrophobic lipid layers of the cell membranes. Adsorption finally terminates at an uptake equivalent to approximately 20 theoretical monolayers due either to saturation of the cells or possibly to competition from association of the solute molecules in the bulk of the solution.

Resting cells of *E. coli* with their metabolic pool preferentially labelled with ^{32}P slowly leak radioactive material into the suspending medium.

In the presence of CTAB concentrations below that required for the

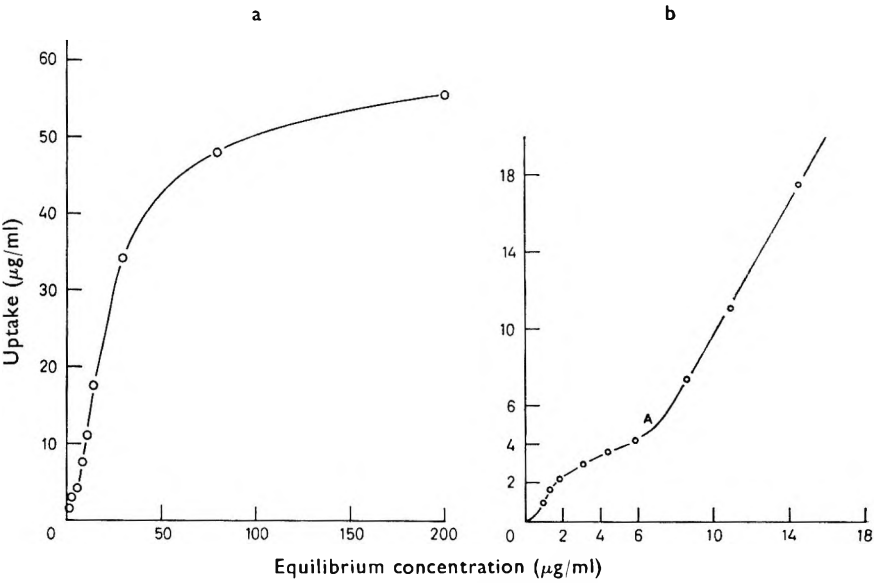

FIG. 1. a, b. Uptake of [^{14}C] CTAB by *E. coli* suspended in glucose-free medium pH 7·7 plotted as a function of the [^{14}C] CTAB concentration in the supernatant fluid. Temp 25°. Cell concentrat on 0·125 mg/ml.

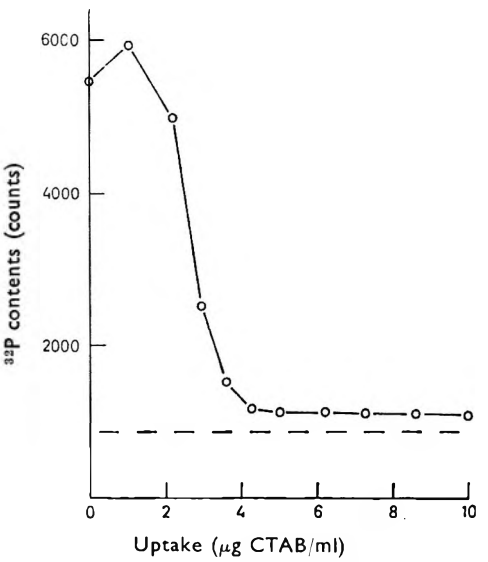

FIG. 2. The effect of treatment with CTAB on the ^{32}P content of pulse labelled cells of *E. coli* suspended in glucose free medium pH 7·7 at 25°. Contact time 15 min. Cell concentration 0·125 mg/ml. ○ Total cellular radioactivity. — — Radioactivity remaining after treatment with 5% trichloroacetic acid for 30 min at 4°.

uptake of a monolayer, the amount of ^{32}P released within 15 min is less than from untreated cells. This is probably caused by mechanical blockage due to the partially formed monolayer which is effectively hydrophobic. When cells are treated with concentrations of CTAB higher than that required for the uptake of a monolayer, the release after 15 min is greater than from untreated cells. The extent of release increases as the CTAB concentration increases and reaches a maximum value, equivalent to the loss of the entire metabolic pool. This occurs at a CTAB concentration equivalent to the uptake of a double layer.

It has been reported that the individual cells in bacterial suspensions treated with low concentrations of CTAB, are not all affected to the same extent. McQuillen (1950) observed such an effect when studying electrophoretic mobility whilst Rye & Wiseman (1968) have shown that in cultures of *E. coli* partially inhibited with CTAB, the growth of some of the cells is completely arrested whilst the remainder grow at the same rate as in untreated cultures.

If the adsorbed CTAB were uniformly distributed over the available cell surface a classical Langmuir adsorption isotherm would be expected. Giles & others (1960) suggest that an S type isotherm results from a mutually stabilizing effect of molecules adsorbed at adjacent sites on a surface. Such an effect would result in the formation of localized concentrations of molecules at the surface and thus probably in an uneven distribution amongst the cells. If this non-uniform distribution continued throughout the adsorption process some cells would take up a damaging amount of CTAB even from the lowest concentrations. The existence of a threshold concentration for cellular damage equivalent to that required for the uptake of a monolayer by all the cells, suggests therefore that some degree of uniformity is reattained at this point. Further uptake towards the formation of a double layer would then result in new localized concentrations and the reappearance of a non-uniform distribution.

It thus seems likely that in an individual cell, increase in permeability, and probably loss of viability (Salton, 1951), coincides with the completion of a double layer of CTAB molecules at its surface and that in a bacterial population the number of cells damaged corresponds directly with the number on which such a complete double layer has formed.

Acknowledgement. We wish to thank Mr. R. M. Rye for valuable advice and one of us (W. G. S.) is indebted to the Science Research Council for a research studentship.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 18S–22S Received May 1, 1968

Binding of crystal violet by nucleic acids of *Escherichia coli*

E. ADAMS

The absorption isotherms of DNA and RNA from *E. coli* for crystal violet are sigmoid. Binding of dye was determined i) by difference in its absorbance before and after addition of nucleic acids and ii) by measuring the amount of dye dialysing through a membrane containing dye and nucleic acid. pH change caused no significant change in the degree of binding.

IN the presence of nucleic acids, basic dyes have been observed to undergo changes in their ultraviolet visible spectrum (Michaelis, 1947).

A non-linear relation between the extent of binding by DNA and the equilibrium concentration of rosaniline has been reported by Cavalieri & Angelos (1950), the process being reversible. Peacocke & Skerrett (1956) observed strong interaction between amino-acridine dyes and nucleic acids from herring sperm and *Aerobacter aerogenes*, the binding being independent of pH over the range 3·7 to 7·4.

The present paper reports work on the binding of crystal violet by nucleic acids of *Escherichia coli*.

Experimental

REAGENTS

Saline–EDTA, 0·15M sodium chloride and 0·1M EDTA, adjusted to pH 7 with sodium hydroxide. Acetate–EDTA, 3M sodium acetate and 0·001M EDTA. Concentrated saline-citrate, 1·5M sodium chloride and 0·15M sodium citrate. For dilute saline-citrate this was diluted 1 in 100. All chemicals were of laboratory reagent quality. Crystal violet B.P. was recrystallized from ethanol and a 0·01M aqueous solution prepared.

EXTRACTION OF NUCLEIC ACIDS FROM *E. coli*

Several methods have been described for the extraction of nucleic acids from bacteria (Marmur, 1961; Smith & Burton, 1965; Kirby, Fox-Carter & Guest, 1967). The method of Marmur, primarily devised for the extraction of DNA, was modified as follows.

E. coli 1 was grown in bottles (37°; 24 hr) on the surface of a nutrient agar layer (Oxoid blood agar base, No. 2, CM 271, with additional agar, Oxoid No. 1 L 11). The growth was washed off with quarter-strength Ringer solution, the organisms were centrifuged off, then washed with saline–EDTA to remove metal ions (Mg) which activate nucleases. They were suspended in saline–EDTA to form a suspension free from clumps.

Sodium lauryl sulphate (25% in water; 1 ml/g moist weight organisms) was added and the mixture heated (10 min) at 60° to cause lysis. The suspension increased in viscosity and showed partial clearing; it was

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cooled to room temperature, and one fifth volume of 2·5M sodium chloride solution added. The mixture was shaken (30 min) with an equal volume of chloroform–amyl alcohol (24 : 1), and centrifuged (5 min) at 5,000 *g* when three layers were formed. The uppermost (aqueous) layer was carefully withdrawn and an equal volume of absolute ethanol added with stirring, when the nucleo-proteins "spooled" on to the glass rod.

The nucleoproteins were lightly pressed against the side of the beaker to remove excess ethanol, and quickly dissolved in dilute saline–citrate (1 ml/g moist weight organisms). One tenth volume of concentrated saline–citrate was added to the solution, which was shaken (15 min) with an equal volume of chloroform–amyl alcohol to remove protein. The mixture was centrifuged (5 min at 5,000 *g*) and the upper aqueous layer carefully removed, shaken with further aliquots of chloroform–amyl alcohol until no protein was visible as a whitish precipitate at the interface. The upper layer was carefully removed and solid sodium chloride added to produce a 4M solution to precipitate the RNA. This was removed by centrifuging (10 min at 5,000 *g*), washed with 4M sodium chloride solution, and dissolved in dilute saline–citrate solution (0·2 ml/g moist weight organisms). The RNA was precipitated therefrom with an equal volume of ethanol, as a light flocculent precipitate and then re-dissolved in dilute saline–citrate solution (0·2 ml/g moist weight organisms). All the above 4M sodium chloride solutions which contained the DNA were reserved and treated as follows.

Addition of absolute ethanol gave a DNA precipitate, which was dissolved in dilute saline–citrate solution (0·5 ml/g moist weight organisms). One tenth volume of acetate–EDTA solution was added, a small glass mechanical stirrer fitted and 0·6 volume isopropanol added slowly to the vortex, when DNA spooled on to the stirrer. This was dissolved in dilute saline–citrate and re-precipitated as above. The precipitate was washed with 70% aqueous ethanol, then with 90% aqueous ethanol, and dissolved in dilute saline–citrate, and adjusted to pH 8·0 with sodium hydroxide solution.

A few drops of chloroform were added to the nucleic acid solutions and these were stored at 4°, or the solutions were freeze-dried. Nitrogen and phosphorus determinations* were made. A sample of the DNA solution was heated to 60° and the absorbance at 260 mμ noted, to detect the presence, if any, of single-stranded DNA or of RNA.

DETERMINATION OF BINDING OF DYE BY NUCLEIC ACIDS

Difference-in-absorbance method. The absorption spectrum of an aqueous crystal violet solution was determined on a spectrophotometer between 450 and 650 mμ. This was repeated with the addition of varying amounts of DNA and RNA separately.

The absorbance of crystal violet solution at 591 mμ was determined

* By Mr. G. S. Crouch, School of Pharmacy, University of London, Brunswick Square, E.C.1. Nitrogen by a modified Schöniger method, phosphorus by perchloric acid digestion and molybdenum blue colorimetric finish.

at different pH values over the range 4 to 9. This was repeated after the addition of the nucleic acids.

Equilibrium dialysis method. Pieces of Visking dialysis tubing (4 in $\times$ $\frac{5}{8}$ inch diam.) were soaked in distilled water and tied at one end with thread to form a bag. A plastic ring was inserted into the open end, and they were allowed to dry. Glass bottles (3 in $\times$ 1 in diam.) were filled with dilute saline-citrate solution (8 ml) and the dialysis bags placed therein. The bags were then filled with known amounts of crystal violet and nucleic acid solutions, the volume being made up to 8 ml with dilute saline-citrate. The bottles were placed in an incubator (2 days; 25°) with occasional agitation. The concentration of crystal violet in the bottles was then determined by measurement of the absorbance at 591 mμ. The amount of dye bound by the nucleic acids was then calculated, allowance being made for the uptake of dye by the dialysis tubing and the bottles. The solution in the bottles was then replaced by an equal volume of fresh saline–citrate and the bags replaced. Dialysis was repeated, the concentration of dye in the bottles again determined, and the release of dye from combination with the nucleic acids calculated.

The experiment was repeated at different pH values using a constant dye concentration and buffer solutions. Dialysis tubing is permeable to crystal violet but not to nucleic acids.

Results

The yields of nucleic acids obtained by extraction of *E. coli* were as follows: DNA 4·1 mg dry weight/g moist weight organisms, $e(_p)_{260} = 9350$. Phosphorus 6·7%, nitrogen 12·7% (both based on dry weight). RNA 2·9 mg dry weight/g moist weight organisms, $e(_p)_{260} = 8040$. Phosphorus 6·8%, nitrogen 12·1%. $e(_p)_{260}$ is the molar absorptivity based on 1 g atom of P per litre.

DNA samples showed a slight increase in absorbance when heated at 60°, indicating the presence of a small amount of RNA or single-stranded DNA (Kirby & others, 1967).

Fig. 1 shows the absorption spectrum of crystal violet alone and in the presence of different amounts of DNA; an essentially similar picture was presented by RNA. Small amounts of nucleic acids reduce the absorbance of crystal violet, large amounts raise the absorbance, and the absorption peak is displaced from 591 to 598 mμ, except for small amounts of RNA.

Fig. 2 shows the absorbance at 591 mμ of crystal violet alone and with the addition of DNA or RNA at various pH values. The difference reflects the binding of the dye with nucleic acid.

Using the equilibrium dialysis method at different pH values the amount of dye bound showed little change with change in pH over the range 4 to 9·2.

Fig. 3 shows the amount of dye bound by the nucleic acids against the equilibrium concentration of dye. A specimen calculation is given in Table 1. A precipitate was produced by equilibrium concentrations above 50×10^{-6}M crystal violet. Retention curves followed approximately the binding curves.

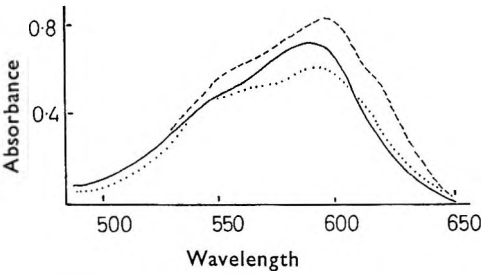

FIG. 1. Absorption spectrum of crystal violet alone, and in the presence of DNA. Full line, 2×10^{-5}M crystal violet; dotted line, dye with 8×10^{-6}M DNA; broken line, dye with $2{\cdot}7 \times 10^{-3}$M DNA.

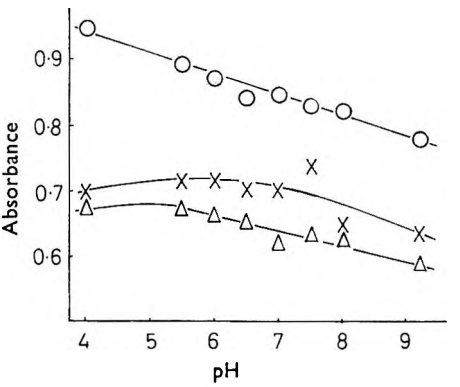

FIG. 2. Effect of pH on absorbance at 591 mμ of crystal violet alone and in the presence of nucleic acids of *E. coli*. ○ dye alone; × dye with DNA; △ dye with RNA shortly after mixing.

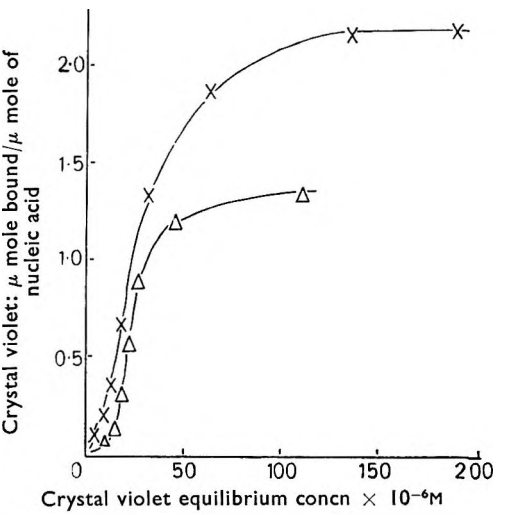

FIG. 3. Amount of crystal violet bound by nucleic acids plotted against equilibrium concentration of dye. × DNA, Δ RNA.

TABLE 1. SPECIMEN CALCULATION OF BINDING OF CRYSTAL VIOLET BY NUCLEIC ACIDS

a Tube	b Theoretical concentration of dye if no binding occurred ($\times 10^{-6}$M)	c Amount DNA (μmole)	d Equilibrium concentration of dye ($\times 10^{-6}$M)	e Difference between theoretical and equilibrium concentration of dye (b − d)	f Amount of dye taken up by bag and bottle (μmole)*	g Amount of dye bound by DNA (μmole)	h Amount of dye bound per μmole DNA (μmole) (g/c)
A	12·5	2·65	3·4	9·1	0·0025	$\frac{(9{\cdot}1 \times 16)}{1000} - 0{\cdot}0025$	0·054
D	125	2·65	19·6	105·4	0·0110	$\frac{(105{\cdot}4 \times 16)}{1000} - 0{\cdot}0110$	0·632

* Separately calibrated.
Note. 1 ml of 10^{-6}M contains $1{\cdot}1000 \times 10^{-6}$ mole.

Discussion

The displacement of the absorption peak of crystal violet to longer wavelengths in the presence of nucleic acids indicates a more polar environment, and suggests complexing between crystal violet and the nucleic acids. A similar phenomenon has been observed with proflavine and herring sperm DNA (Peacocke & Skerrett, 1956), and with crystal violet and bovine serum albumin (Blei, 1957). Like the sorption of crystal violet by whole *E. coli* organisms (Adams, 1967), the absorption isotherms of crystal violet for the nucleic acids were sigmoid, suggesting that Langmuir's law did not apply. Similar results are reported by Peacocke & Skerrett (1956) for proflavine.

Binding of crystal violet approaches a maximum at *ca* 2 mmole dye/mmole DNA, and at *ca* 1·2 mmole/mmole RNA, and precipitates are formed. Experiments showed the binding to be largely irreversible, and to be unaffected by changes of pH over the range 4 to 9.

Peacocke & Skerrett (1956) found little effect of pH over the range 4·7 to 7·4 in the binding of proflavine by the nucleic acids of *Aerobacter aerogenes*.

Since no change in the binding of dye to nucleic acid at pH values between 4 and 9 can be demonstrated, the increase in antibacterial activity at pH 8 and above must be ascribed to other causes.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 23S–25S Received May 1, 1968

SHORT COMMUNICATION

Some observations on the use of absorbance measurements in bacteriology

R. M. RYE AND DAVID WISEMAN

ABSORBANCE measurements are commonly used in bacteriology to follow the growth of cultures and for standardizing cell suspensions. This communication reports some investigations into the relation between absorbance and dry weight of washed cell suspensions and growing cultures of *Escherichia coli*.

EXPERIMENTAL

The organism used was *Escherichia coli* (NCTC 1013). The media, conditions of cultivation and methods of measuring absorbance and radioactivity have previously been described (Rye & Wiseman, 1966). All experiments were made on cultures obtained by diluting exponentially growing cells with medium at 37° to an absorbance of about 0·050 (650 mμ). Uniformly labelled cells were obtained by replacing the glucose in the growth medium with ^{14}C-labelled glucose (specific activity 0·01 μc/mg) and allowing growth through at least eight generations.

Dry weight determinations. Membrane filters (8 cm, Oxoid) were washed with water, dried to constant weight by heating at 95° for 10 min and stored in a calcium chloride desiccator. 100 ml volumes of bacterial suspensions were filtered through these membranes, the cells washed with 10 ml distilled water and their weight determined by difference after redrying the membranes as above.

RESULTS AND DISCUSSION

Fig. 1A shows the absorbance of dilutions of a washed cell suspension of *E. coli* in glucose-free medium as a function of relative bacterial concentration. The relation is almost linear showing that in these washed cell suspensions where growth has been arrested an approximate proportionality exists between absorbance and cell concentration, whether this is measured in terms of cell number or total cell mass. The correction for deviation from linearity, calculated using the Longsworth equation (Longsworth, 1936; Kavanagh, 1963) is only 2% for an observed absorbance of 0·500.

With exponentially growing cells the relation between absorbance and total cell mass was investigated by making dry weight measurements on samples from exponentially growing cultures after different periods of growth when the absorbances were between 0·100 and 0·700.

The graph of these weights against absorbance was markedly curved showing that absorbance measurements made on growing cultures are

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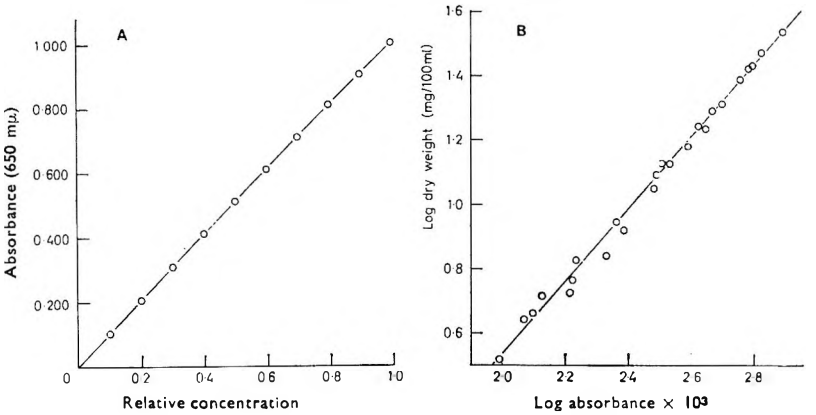

FIG. 1A. The absorbance of dilutions of a washed cell suspension of *E. coli*.

B. The relation between the absorbance and dry weight of exponentially growing cultures of *E. coli*.

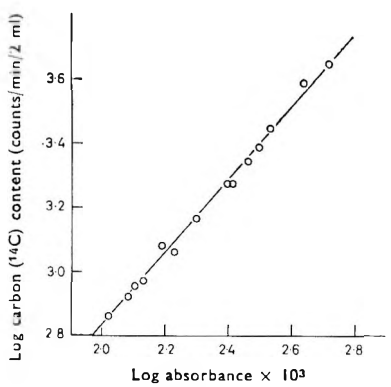

FIG. 2. The relation between absorbance and total cellular carbon[^{14}C] content of cultures of *E. coli*. The line has been drawn with a slope of 1·12 which is equivalent to the equation derived from the results of Fig. 1A.

not proportional to total cell mass. These results are presented in Fig. 1B as a log-log plot and the relation observed can be represented by the equation

$$M = 0{\cdot}0195A^{1{\cdot}12} \quad .. \quad .. \quad .. \quad (1)$$

where M is dry weight mg/100 ml and A is absorbance $\times 10^3$.

From this equation the dry wt/ml of a growing culture at an absorbance of 0·500 will be 20% greater than would be expected from its dry weight when at an absorbance of 0·100 if the relation shown in Fig. 1A applied during growth.

Fig. 2 shows a log-log plot of absorbance with the total cell carbon [^{14}C] content for exponentially growing cultures of *E. coli* together with a line of slope 1·12 representing the relation between dry weight and absorbance given by equation (1). The total cell carbon content increases

at the same rate as dry weight showing that the cell carbon/dry weight ratio remains constant during growth and that measurements of carbon content can be used as a measure of relative cell mass.

The apparent contradiction between the results obtained using washed cells with those using exponentially growing cells may be attributed to changes in cell size, shape, density or relative refractive index occurring during growth. Koch (1961) discussed the effects of such changes and suggested that "absorbancy measurements are more nearly a measure of bacterial mass than of bacterial numbers". A direct proportionality between dry weight and absorbance which was independent of variations in cell size and shape has been observed with *Salmonella typhimurium* (Schaechter, Maaloe and Kjeldgaard, 1958) and similar results have been reported for a species of lactobacillus (Burns, 1959).

The conclusions to be drawn from our results are (*a*) that suspensions of equal absorbance prepared by harvesting cells from growing cultures of *E. coli* will not necessarily contain the same total cell mass, and (*b*) that the dry weights of suspensions prepared during the early stages of exponential growth will be lower than those of suspensions of the same absorbance prepared from cells harvested at a later time. These conclusions are confirmed by the results of measurements of the carbon [^{14}C] content of four suspensions of equal absorbance prepared from cells harvested at different times during exponential growth (Table 1).

TABLE 1. THE CARBON [^{14}C] CONTENT OF WASHED CELL SUSPENSIONS PREPARED BY HARVESTING CELLS AT DIFFERENT TIMES FROM AN EXPONENTIALLY GROWING CULTURE AND ADJUSTING THE ABSORBANCE TO 0·100

Time of harvesting (min)	81	119	137	153
Absorbance of culture at harvesting	0·142	0·225	0·281	0·340
^{14}C content as counts/min/2 ml	646 ± 2·2	690 ± 2·4	703 ± 2·4	741 ± 2·5

Clearly in quantitative investigations where suspensions of *E. coli* are standardized by absorbance measurements, good correlation between replicate experiments cannot be expected unless the cells are harvested after the same extent of exponential growth has occurred.

Acknowledgement. We are grateful to Mrs. Sheena Kaye for technical assistance.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 26S–31S Received April 29, 1968

Water-in-oil solubilized vaccine adjuvants

C. L. J. COLES, J. R. HEPPLE, MARJORIE L. HILTON AND C. A. WALTON

The solubilization of water and toxoid solutions in oils by non-ionic surface-active agents has been examined. Water-in-oil solubilized adjuvant formulations of vaccines containing *Clostridium welchii* type D toxoid as antigen have been tested in laboratory animals. The antitoxin titres in rabbit serum induced by the most successful formulation were as high 14 weeks after a single dose, as the peak titres after two doses of a simple aluminium hydroxide adsorbed vaccine. The vaccines are clear and of low viscosity which facilitates accurate measurement and handling by syringe.

APART from the early observations by Rabinowitsh (1897), the experimental results of Freund & Bonato (1944) first demonstrated the elevation and prolongation of antitoxin levels obtained by dispersing diphtheria toxoid in oily vehicles before injection. Water-in-oil emulsions have been used as adjuvants for a number of antigens, and the literature has been reviewed by Hilleman (1966). The adjuvant action of oil-in-water emulsions (Brit. Pat., 1963), multiple emulsions (Herbert, 1965, 1967), water emulsified in vegetable oils gelled with aluminium monostearate (Stokes, Weibel & others, 1964) and dried antigens dispersed in paraffin oil gelled with aluminium monostearate (Coles, Heath & others, 1965) has also been described. Although elevated and persistent antibody levels have been reported for all these adjuvants, they suffer from the practical disadvantages of high viscosity which limits their use in a syringe. They also require critical control of processing to ensure their physical stability and to allow their immunological advantages to be realized.

The solubilization of water in oil has been reported in the literature (Winsor, 1954; McBain & Hutchinson, 1955) and Higuchi & Misra (1962) reported studies on the solubilization of water in paraffin by cationic surfactants. Applications for such systems include dry cleaning (Fulton, Alexander & others, 1953), preparation of amphiphilic salts (Brit. Pat., 1941) and preparation of clear solutions of hydrated ephedrine alkaloid in paraffin (Bellafiore, 1965).

This paper describes water-in-oil solubilized adjuvant systems, which are effective, easily prepared, suitable for use by syringe and physically stable under practical storage conditions.

Experimental and results

Mixtures of commercial non-ionic surfactants and light mineral oil were examined, and formulae identified which permitted solubilization of water, or *Clostridium welchii* type D toxoid solution: clarity was used as the criterion of solubilization. Water contents above and below those which yielded clear products produced slightly hazy, birefringent gels which could be changed to thin clear solutions by gentle agitation and for this reason the use of the "Staggered Walk" technique (Boffey,

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Collison & Lawrence, 1959) for identification of phase boundaries was rejected.

To identify phase boundaries, accurately measured aliquots of solutions of surfactant in oil were distributed into neutral glass ampoules, and accurately measured incremental volumes of water were added. The ampoules were sealed, packed into cylinders and rolled at approximately 30 rev/min for 16 hr. The ampoules were then allowed to stand for 2 hr before examination.

A surfactant hydrophile : lipophile balance (HLB) of 10 (Griffin, 1949) is the near optimum value for the solubilization of water in paraffin oil. In general the use of mixtures of surfactants permitted the highest concentrations of water to be solubilized. The addition of a small proportion of a predominantly lipophilic surfactant to a system containing a single surfactant of HLB 10 enabled a markedly increased quantity of water to be solubilized. Fig. 1 shows the effect of the addition of Arlacel 80

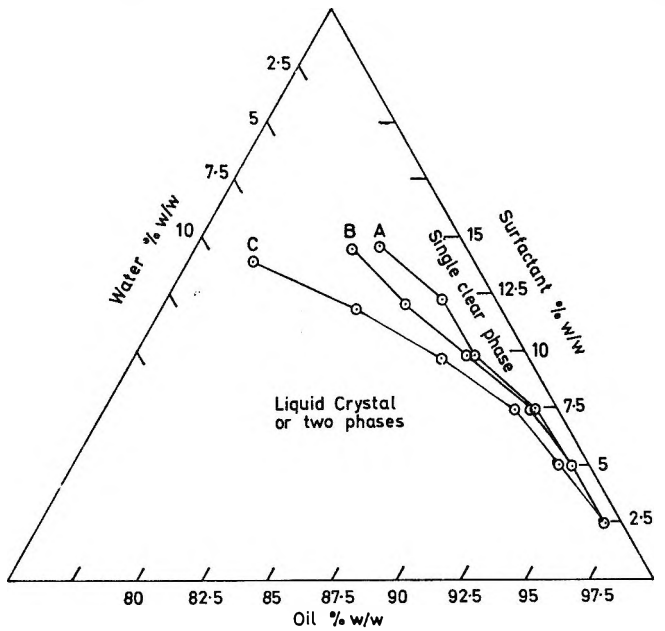

FIG. 1. Phase boundaries in the system light paraffin oil, surfactant, water. Surfactants: A. Tween 81. B. 5% Arlacel 80 in Tween 81. C. 10% Arlacel 80 in Tween 81.

(sorbitan mono-oleate) to a system of Tween 81 [polyoxyethylene (5) sorbitan mono-oleate], light paraffin oil and water. When toxoid solution was substituted for water, the volume which could be solubilized was, in contrast, decreased in the presence of small proportions of lipophilic surfactant.

Water could be solubilized in paraffin oils and pure hydrocarbons, both straight chain and branched; in fatty alcohols and their fatty acid

esters in significantly lower concentrations, and in extremely low concentrations in vegetable oils, pure triglycerides and fatty alcohol ethers.

A series of vaccines was prepared by sterilization of solutions of the surfactants in oil by membrane filtration, followed by aseptic addition of antigen solution, with gentle agitation. The formulae of the vaccines thus prepared are shown in Table 1.

TABLE 1. FORMULAE OF VACCINES TESTED

No	Antigen (Lf/ml)	Surfactants*		Surfactant conc. % w/v	Oil
1	CWD 65	AA/T 20	65:35	8·75	Puremor**
2	CWD 65	A 80/T 20	60:40	8·25	Puremor
3	CWD 65	AA/T 80	55:45	9·50	Puremor
4	CWD 50	AA/T 20	60:40	8·25	Puremor
5	CWD 50	T 81		10·00	Puremor
6	CWD 50	T 81		10·00	Tridecyl myristate
7	CWD 50	A 80/T 80	10:90	10·00	Squalane
8	CWD 250	Tn x-100/Tn x-15	50:50	18·00	Puremor
9	CWD 50	T 81		10·00	Puremor
10	CWD 50	T 81		10·00	Puremor
11	CWD 50	T 81		10·00	Puremor

*Abbreviations:
T = Tween (Honeywill Atlas Ltd.)
A = Arlacel (Honeywill Atlas Ltd.)
Tn = Tritons (Charles Lennig & Co.)
** Puremor extra light white oil (Burmah Oil Co.).

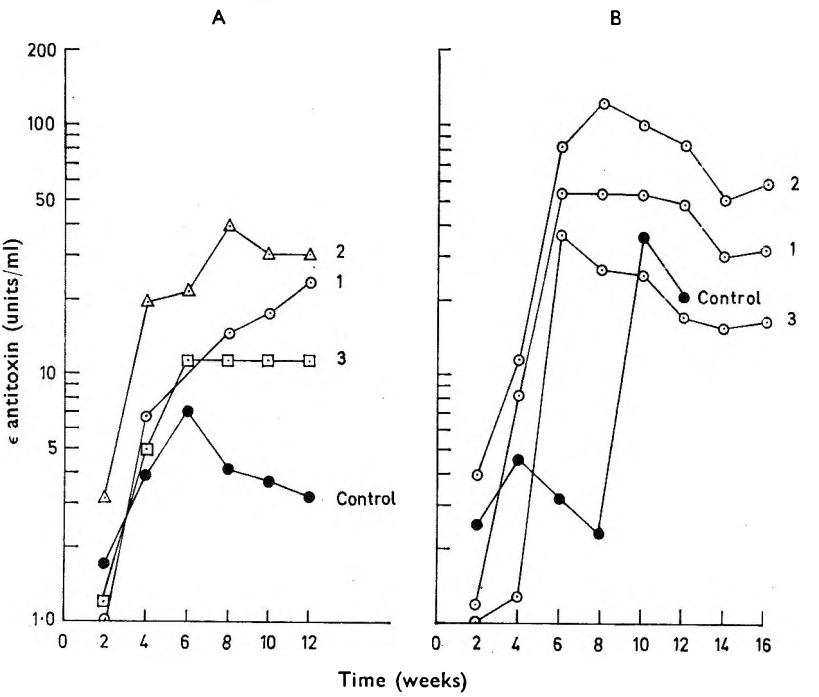

FIG. 2. ϵ-Antitoxin titres produced in rabbit serum by: (A), a 2 ml subcutaneous injection of *Clostridium welchii* type D vaccine, and (B), a second dose after 4 weeks. Formulae 1, 2 and 3. Control, 65 Lf/ml aluminium hydroxide adsorbed toxoid.

Vaccines were injected subcutaneously into laboratory animals. Groups of six rabbits or six guinea-pigs were used, and serum samples were titrated for their content of *Clostridium welchii* ϵ antitoxin. Results for rabbits are the mean titres from six animals; those for guinea-pigs are from pooled sera of six animals.

Vaccines 1–3, containing 65 Lf/ml of *Clostridium welchii* type D toxoid, were injected subcutaneously into rabbits and guinea-pigs. Dose schedules and serum antibody titres are shown in Fig. 2 and Table 2.

Vaccine 4 was prepared to the same formula as the most successful vaccine above (No. 2) but with the toxoid content reduced to 50 Lf/ml. Antibody levels measured in rabbits are shown in Fig. 3.

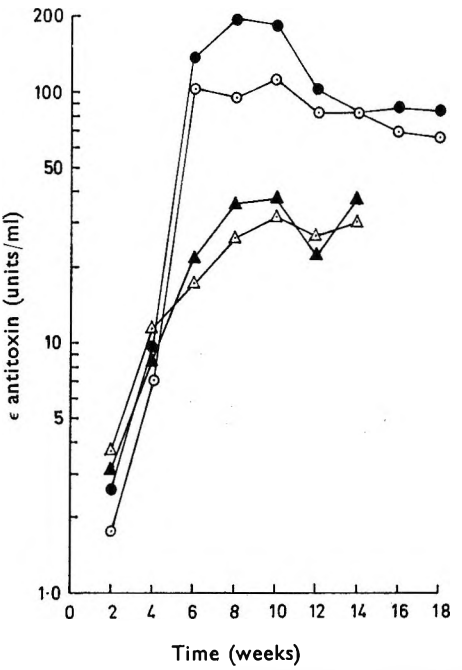

FIG. 3. ϵ-Antitoxin titres produced in rabbit serum after subcutaneous doses of vaccine 4. ● 1 × 2 ml followed by 1 × 2 ml after 4 weeks; ⊙ 1 × 1 ml followed by 1 × 1 ml after 4 weeks; ▲ 1 × 2 ml; △ 1 × 1 ml.

Vaccines 5–8 were prepared using a range of oils and types of surfactant. Antibody levels measured in guinea-pigs are shown in Fig. 4.

One vaccine, to the same formula as vaccine 5, was prepared by three different methods, namely, addition of antigen solution to a solution of surfactant in oil (vaccine 9), addition of surfactant to a crude emulsion of antigen solution in oil (vaccine 10) and addition of oil to a blend of antigen solution and surfactant (vaccine 11). All these products were stable and physically indistinguishable. Tests made in guinea-pigs showed negligible differences in antibody levels induced.

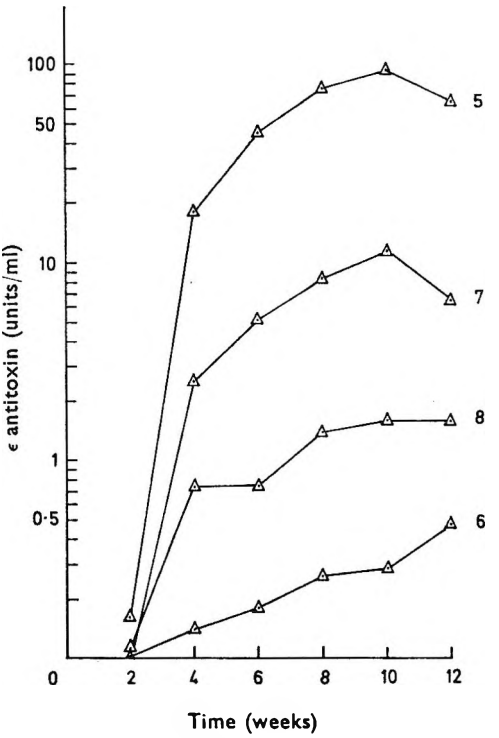

FIG. 4. ϵ-Antitoxin titres in guinea-pig serum after 1 ml subcutaneous doses of vaccines 5–7 and a 0·2 ml dose of vaccine 8.

Discussion

All but two formulations tested were good adjuvants. Levels of circulating antibodies in experimental animals were much elevated compared with those in control animals vaccinated with the same toxoid dose adsorbed on aluminium hydroxide gel before injection, and there is some evidence of prolongation of elevated levels (Figs 2 and 3 and Table 2). Neither reduction of the toxoid dose (Figs 3 and 4) nor

TABLE 2. ϵ-ANTITOXIN TITRES IN GUINEA-PIG SERUM AFTER 1 ML SUBCUTANEOUS INJECTION OF VACCINES 1–3
Controls 1, single 1 ml dose; 2, second 1 ml dose after 4 weeks of 65 Lf/ml aluminium hydroxide adsorbed toxoid

Time (weeks)	Formula 1	Formula 2	Formula 3	Control 1	Control 2
2	0·8	0·4	1·4	0·28	0·28
4	14	14	14	5·6	—
6	14	28	14	5·6	80
8	14	28	—	5·6	14
10	28	28	28	5·6	14
13	28	28	28	5·6	14

halving of the dose volume (Fig. 4) had significant effects on the responses.

Of the two vaccines that failed, that prepared in tridecyl myristate (No. 6) was physically unstable *in vitro* at 37°. The other vaccine that failed (No. 8) was in a dose volume of 0·2 ml, the phase equilibrium of the formulation requiring this vaccine to be prepared containing 250 Lf/ml. The small dose volume may have contributed to the poor result obtained.

All the vaccines reported contained *Clostridium welchii* type D toxoid as antigen, but *Cl. welchii* type B and C, *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* toxoids have also been examined; similar results were obtained.

In contrast to emulsified vaccines, the vaccines described are physically stable, easily prepared, and their clarity and low viscosity permit accurate measurement and convenient administration with a hypodermic syringe.

These vaccine formulations are the subject of patent applications (B.P. Application No. 25,595/66).

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 32S–33S Received May 1, 1968

SHORT COMMUNICATION

An impurity in polyethylene glycol 300

P. F. G. BOON AND A. W. MACE

DURING routine examination of a non-aqueous formulation containing 1% tripelennamine hydrochloride in a vehicle composed principally of polyethylene glycol 300 (liquid macrogol BPC), large losses (up to 40%) of active agent were noted in some instances. Such losses were occasioned only by some batches of polyethylene glycol 300 and occurred after autoclaving for 30 min at 15 psi, or after several days at room temperature.

Gas chromatography of available batches revealed that all contained ethylene oxide (0·01–0·2%); loss of tripelennamine was associated with batches containing more than about 0·1% of this impurity. The ethylene oxide content of polyethylene glycol 300 was determined by direct addition of the sample (3 μl) to a column (9 ft) of Carbowax 20M (20%) on Gas Chrom P: gas flow rate 45 ml/min (N_2), temperature 30° (injection block 80°); retention time 2 min. Tripelennamine may normally be recovered from formulations of this kind by basifying then extracting with chloroform. In batches containing ethylene oxide, only a proportion of added tripelennamine may be recovered. Examination of the residual aqueous phase revealed a substance with a modified ultraviolet spectrum (Fig. 1). Windmueller, Ackerman & others (1959) have

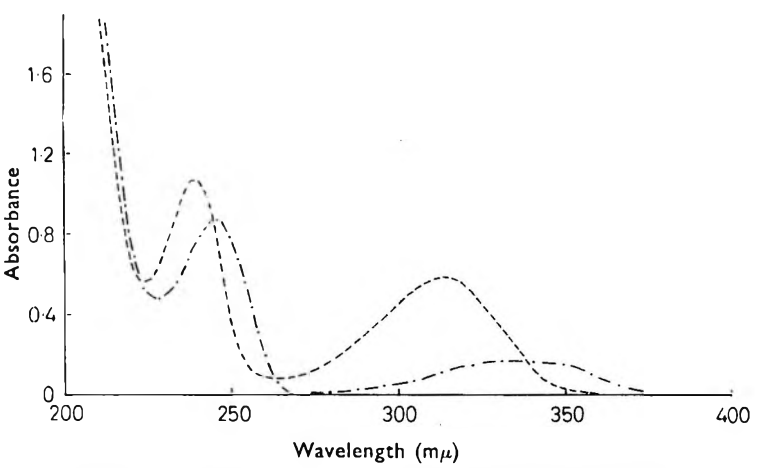

FIG. 1. Ultraviolet absorption spectra in 0·01N-hydrochloric acid of – – – – – – 0·002% tripelennamine hydrochloride – · – · – · – 0·002% tripelennamine hydrochloride-ethylene oxide reaction product.

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shown that ethylene oxide reacts with nicotinamide to form a quaternary ammonium compound and a similar reaction would seem indicated in the present instance. This is supported by our inability to extract the reaction product with chloroform from the formulation.

Analysis of the dipicrate (m.p. 147°, tripelennamine dipicrate 192°) formed by the reaction product gave C, 46·7; H, 4·15; N, 16·4%. The dipicrate of a product formed by addition of one molecule of ethylene oxide to one molecule of tripelennamine would contain C, 46·5; H, 4·3; N, 16·3%.

The high reactivity of ethylene oxide raises the possibility of interaction with other drugs and we have observed a bathochromic shift (268 to 260 mμ) in the spectrum (in 3N HCl) of clioquinol after autoclaving a 0·1% solution in polyethylene glycol containing 0·1% ethylene oxide. O'Leary & Guess (1968) have shown ethylene oxide to be cytotoxic, in addition the reaction of ethylene oxide with inorganic chloride to give the toxic ethylene chlorohydrin has been reported (Wesley, Rourke & Darbishire, 1965). Deficiencies of inorganic chloride in our degraded samples support the belief that chlorohydrin formation proceeds simultaneously with quaternization.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 34S–42S Received May 1, 1968

The anomalous chromatographic behaviour of dichlorophen and *p*-chlorophenol

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The variation of Rf value of *p*-chlorophenol and di-(5-chloro-2-hydroxyphenyl) methane when run alone or together on thin layer chromatograms using toluene and silica gel is attributable to adsorption rather than partition processes. This conclusion is based on a quantitative study of Rf value variation, adsorption and partition behaviour. The adsorption isotherms are used to calculate the Rf values. The latter were of similar magnitude to those obtained directly from the chromatograms.

THIN-LAYER chromatography is used to detect *p*-chlorophenol, an impurity in di-(5-chloro-2-hydroxyphenyl)methane (Dichlorophen) (B.P. 1963, addendum 1964). Mr. C. A. Johnson (personal communication) reported that the Rf values of these substances show anomalous behaviour when toluene and silica gel are used as the solvent and stationary phase respectively. In particular each substance affects the Rf value of the other. Variations in Rf value are explored in terms of the partition coefficients and adsorption isotherms of these two substances.

THEORY

The equation relating the Rf value of a solute to the partition coefficient (K) and the effective cross-sectional areas of the mobile (a) and stationary (b) phases has been derived by Consden, Gordon & Martin (1944).

$$Rf = \frac{1}{\left(1 + K\frac{b}{a}\right)} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$\text{where } K = \frac{\text{Concentration of solute in stationary phase}}{\text{Concentration of solute in mobile phase}} \quad .. \quad (2)$$

It is generally accepted that the surface of silica gel particles is covered by several layers of chemisorbed and physically adsorbed water molecules, and that such layers are firmly held up to temperatures of 200°. The relevant partition coefficient is that for water and toluene.

From the adsorption aspect, it is convenient to modify equations (1) and (2) so as to apply to unit mass or length of adsorbent or stationary phase used in the chromatography apparatus. If g_s is the amount of solute in the stationary phase associated with one unit of adsorbent, and g_m that in the corresponding amount of mobile phase, then equation 2 becomes

$$K = \frac{g_s}{g_m} \cdot \frac{a}{b} \quad .. \quad .. \quad .. \quad .. \quad (3)$$

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and equation 1,

$$Rf = \frac{1}{\left(1 + \frac{g_s}{g_m}\right)} \quad .. \quad .. \quad .. \quad .. \quad (4)$$

The volume of the mobile phase may be identified as the void volume (V_v) associated with a unit of adsorbent; then $g_m = cV_v$, where c is the solute concentration. Equation 4 may be rewritten

$$Rf = \frac{1}{\left(1 + \frac{gs}{cV_v}\right)} \quad .. \quad .. \quad .. \quad (5)$$

which expresses the dependence of the Rf value on void volume. Any variation in V_v from plate to plate will introduce variations in the apparent Rf value. Although the volume of the stationary phase can be calculated precisely for a chromatographic system in which the support is coated with a known amount of stationary phase, in the toluene and silica gel system this is not possible. The void volume may be identified, however, with the volume occupied by the adsorbate at maximum coverage (V_m), a value which can sometimes be derived from the appropriate isotherm equation. For example if the adsorption is represented by the Langmuir isotherm, then

$$\frac{\rho g_s}{V_m} = \frac{Bc}{1 + Bc} \quad .. \quad .. \quad .. \quad (6)$$

in which ρ is the density of the adsorbate layer and B the Langmuir constant. Equations (5) and (6) may then be combined to eliminate g_s which shows the relation between the Rf value and the mobile phase concentration of the solute in the region of the chromatogram spot.

$$\frac{1}{Rf} = 1 + \frac{BV_m}{\rho V_v (1 + Bc)} \quad .. \quad .. \quad .. \quad (7)$$

This concentration is a function of the mean concentration in the spot, since diffusional processes are operating radially. The Rf value is concentration dependent, since the Langmuir equation is non-linear with respect to g_s and c; it is indirectly time dependent because of diffusion. Given the spot area, the total quantity of solute, together with the mass and void volume of the silica gel associated with this area, then in principle the Rf value can be calculated.

The following factors must therefore be determined experimentally if the quantitative aspects of the investigation are to be realized: (*a*) Variation of Rf values with load and duration of run. (*b*) Area and total solute concentration of chromatogram spots. (*c*) Water–toluene partition coefficients. (*d*) Density and bulk density of silica gel.

Experimental

Commercially available dichlorophen was crystallized once from dichloroethane and four times from toluene: m.p. 175–176° *E* (1%, 1 cm) at 304 mμ in 0·1N sodium hydroxide solution, 276·2. A sample thus

purified contained no *p*-chlorophenol when examined by thin-layer chromatography. Its purity was probably better than 99·9%.

p-Chlorophenol was recrystallized from toluene to a constant melting point of 41° (uncorr.): $\bar{E}$ (1%, 1 cm) at 297 mμ in 0·1N sodium hydroxide, 188·0. Its purity was probably better than 99·9%.

Merck silica gel G was extracted with toluene to remove substances absorbing in the ultraviolet region. It was then dried for 1 hr at 105°, a treatment corresponding to that employed in the activation of thin-layer silica gel plates.

Toluene was fractionated on a Vigreux column of effective length 43 cm, that fraction distilling at 110·8° was used.

Equal volumes of a 20% w/v solution of ferric chloride and 1% w/v solution of potassium ferricyanide were sprayed on to the chromatograms to detect the spots. The method and materials complied with the requirements of the B.P. (1963), Addendum (1964).

CHROMATOGRAPHIC PROCEDURE

The chromatographic procedure described in the last reference was employed, plates were always used on the day in which they were prepared. The depth of the silica gel was standardized at 250μ ± 7μ, solutions of dichlorophen and *p*-chlorophenol of known concentrations in absolute ethanol were applied to the plates using a calibrated Agla microsyringe.

Chromatograms were run at 25° ± 2° using toluene in glass tanks fitted with grooved glass lids and presaturated with toluene vapour. After running, the plates were removed, allowed to stand for 10 min to remove the toluene, and sprayed with reagent to reveal the spots. Two min later the chromatograms were traced on to semi-transparent paper.

The centre of each spot was assumed to lie at the intersection of the diagonals of a rectangle formed by drawing perpendicular and horizontal tangents at the periphery of the spot. The diameter of the spot was measured with vernier calipers and the spot area calculated. The mean Rf value from five separate determinations on different plates is reported. The coefficient of variation of the Rf values ranged from 2 to 16%. The smaller the Rf value the higher was the coefficient of variation.

ADSORPTION MEASUREMENTS

Solutions of dichlorophen and *p*-chlorophenol of known, but variable, composition in toluene were allowed to equilibrate (24 hr)* at 25° ± 2° enclosed and in contact with weighed amounts of silica gel. Aliquots of each of the solutions and also identical solutions which had not been in contact with silica gel, were then assayed. The amount adsorbed was calculated from each pair of assays. All experiments were made at least

* Dichlorophen attains equilibrium in 1 hr but more than 4 hr are required for *p*-chlorophenol. It was convenient, however, to allow 24 hr for this process.

in duplicate. This procedure revealed 17% decomposition of *p*-chlorophenol in 24 hr, and this was allowed for in determining the equilibration concentration.

Solutions of dichlorophen and *p*-chlorophenol used alone or in admixture were analysed by extracting the toluene four times with 0·1N sodium hydroxide and making up to an appropriate volume. The absorbance at 297 mμ (*p*-chlorophenol) and 304 mμ (dichlorophen) in 1 cm cells were used for assay. Single component solutions were optically regular at both wavelengths up to 25 μg/ml exhibiting, at the above wavelengths respectively, E (1%, 1 cm) values of 242·0 and 276·2 for dichlorophen; and 188·0 and 168·1 for *p*-chlorophenol. Control cells were filled with 0·1N sodium hydroxide that had been shaken with toluene. Single component solutions were assayed using the wavelength of greater absorbance. Both wavelengths were used for mixtures.

TABLE 1. ANALYSIS OF KNOWN MIXTURES

Dichlorophen μg/ml			*p*-Chlorophenol μg/ml		
Theory	Found	% error	Theory	Found	% error
5·00	5·04	+0·74	15·42	15·40	−0·13
5·10	5·01	−1·80	15·42	15·53	+0·70
5·00	4·82	−3·60	15·00	15·45	+3·00
10·00	10·17	+1·70	10·28	10·05	−2·10
10·20	9·81	−3·70	10·28	11·20	+8·90
10·00	10·15	+1·50	10·00	10·04	+0·90
15·00	15·28	+1·80	5·14	4·87	−5·60
15·30	15·27	−1·60	5·14	5·27	+2·50
15·00	15·29	+2·10	5·00	4·66	−6·70

Assays for nine known mixtures are given in Table 1 and, to a first approximation, the error is not random, since the return tends to be low for the lower concentrations of each component. The error is, however, generally small compared with the effects measured.

BULK, TRUE DENSITY AND VOID VOLUME OF SILICA GEL

The true density (ρ_1) of silica gel, determined in a specific gravity bottle with toluene by displacement was 2·21 g/ml. The bulk density (ρ_2), determined by the three tap method (Martin, 1960), was 0·56 g/ml. If the weight and mean thickness of silica gel from known areas of thin-layer plates was used, the bulk density obtained was 0·57 g/ml. Void volume (V_v) was calculated from the formula: $V_v = 1/\rho_2 - 1/\rho_1$. Values of 1·33 ml/g and 1·30 ml/g were obtained. The void volume of a silica gel layer in a plate is thus significantly lower than that for the dry material.

Density of p-*chlorophenol and dichlorophen* were determined in a similar manner and are as follows at 25°, *p*-chlorophenol 1·31 g/ml, dichlorophen 1·46 g/ml.

PARTITION COEFFICIENTS

Toluene solutions of each component were shaken with known volumes of water until equilibrium was attained. Toluene solutions were assayed

by extraction with 0·1N sodium hydroxide, aqueous solutions by the addition of sodium hydroxide to give an 0·1N solution. Each was then assayed spectrophotometrically. No dichlorophen was detected in the aqueous layer.

The coefficient for *p*-chlorophenol is concentration dependent. The following values for the ratio concentration in water/concentration in toluene, were obtained; 0·69, 0·70 and 0·82 at, respectively, concentrations of 2·6, 5·1 and 8·8 μg/ml in the toluene layer.

RF VALUES

These show an almost linear decrease as the load on the plate is decreased (Table 2). They also decrease with time (Table 3); with dichlorophen the effect is more pronounced as the load is decreased, with *p*-chlorophenol the effect is greater at the higher loading.

The spot diameter increases with time, though that of dichlorophen at low loads apparently decreases (Table 4). The latter phenomenon is ascribed to the failure of the spray reagent to show the small concentration at the periphery of the spot, a defect which must operate at all loads but which may be masked by the more rapid increase in spot area with time for higher loads.

When the plates are loaded with mixtures of the two components each appears to increase the Rf value of the other (Table 5). In general the increase is greater for dichlorophen.

TABLE 2. VARIATION OF RF VALUE WITH LOAD

Load (μg)	Dichlorophen	*p*-Chlorophenol
200	0·062	0·140
100	0·029	0·155
50	0·022	0·137
25	0·021	0·140
10	0·021	0·136
5	0·019	0·116
1	0·017	0·098
0·25	0·017	0·098
0·1	0·013	0·087

The solvent front was allowed to advance 10 cm from the origin.

TABLE 3. VARIATION OF RF VALUE WITH TIME OF RUN

Time (min)	Load (μg)					
	100	50	25	10	5	1
	Dichlorophen Rf values					
5	0·035	0·036	0·025	0·042	0·042	0·041
10	0·039	0·036	0·026	0·025	0·031	0·021
30	0·034	0·030	0·018	0·018	0·021	0·015
60	0·031	0·025	0·012	0·017	0·018	0·010
	p-Chlorophenol Rf values					
5	0·170	0·170	0·170	0·170	0·128	0·112
10	0·139	0·132	0·134	0·126	0·122	0·111
30	0·131	0·120	0·127	0·144	0·108	0·100
60	0·125	0·113	0·120	0·105	0·094	0·091

The advance of the solvent front for these runs is variable.

TABLE 4. VARIATION OF SPOT DIAMETER WITH TIME AND LOAD

Time (min)	Load (μg)					
	100	50	25	10	5	1
	Dichlorophen spot diameter (mm)					
5	6·6	6·5	6·5	6·3	6·1	5·6
10	6·6	6·4	6·0	5·7	5·9	5·0
30	7·6	6·6	6·6	5·9	6·0	5·1
60	8·5	7·3	7·1	6·6	5·8	5·2
	p-Chlorophenol spot diameter (mm)					
5	10·0	9·0	9·0	9·2	7·3	6·6
10	11·4	10·2	10·2	10·4	7·4	7·3
30	12·7	11·8	11·2	11·7	8·7	7·2
60	13·1	12·7	12·3	11·5	9·3	7·4

TABLE 5. RF VALUES OF MIXTURES OF COMPONENTS

Ratio in mixture Dichlorophen-*p*-chlorophenol	Rf values			
	Mixed components		Components alone at same load	
	Dichlorophen	*p*-Chlorophenol	Dichlorophen	*p*-Chlorophenol
4	0·032	0·134	0·022	0·119
3	0·042	0·146	0·032	0·124
1	0·032	0·162	0·022	0·144

TABLE 6. ADSORPTION OF DICHLOROPHEN

Concentration of solution (C) μg/ml	Amount adsorbed (X) μg/g	Amount adsorbed calculated from best fitting Langmuir equation μg/g
0·71	190	207
1·79	486	427
2·64	580	574
3·95	768	800
5·88	1030	1067
7·60	1231	1266
10·32	1492	1500

Langmuir equation $\frac{X}{Xm} = \frac{BC}{1 + BC}$

B = constant = 0·51.

Xm = maximum X = 2844.

TABLE 7. ADSORPTION OF *p*-CHLOROPHENOL

Concentration of solution (C) μg/ml	Amount adsorbed (X) μg/g	Amount adsorbed calculated from best fitting Fowler–Guggenheim equation μg/g
4·1	31	22
7·6	40	43
11·4	51	71
13·8	123	113
18·2	236	238

Fowler–Guggenheim equation: $\frac{C}{C_0} = \frac{1}{BC_0} \cdot \frac{X/Xm}{1 - X/Xm} \exp\left(\frac{WN^2}{MRT}\right) \cdot \frac{X}{Xm}$

where B = constant = 1·34; C_0 = saturated soln. conc; W = interaction term; M = mol weight; R = Gas const; T = Temp °K; N = Avogadro's Number; Xm* = maximum X = 2718.

* This value has been calculated from the value of Xm for dichlorophen (Table 6) using the densities of dichlorophen and *p*-chlorophenol on the assumption that two molecules of *p*-chlorophenol occupy approximately the same volume as one molecule of dichlorophen.

Adsorption measurements for dichlorophen and *p*-chlorophenol alone are given in Tables 6 and 7 respectively and must be interpreted bearing in mind the accuracy of the assay procedure. The results for the former are best represented by a Langmuir isotherm equation, whilst those for the latter appear to conform best to a Fowler–Guggenheim isotherm. (Fowler & Guggenheim, 1939.) The same maximum adsorption space is assumed to be available in each case. This conclusion is based on a detailed statistical examination of the results (Scartsiaris, 1966) in which conformity with the Henry, Langmuir, BET, Freundlich and Fowler–Guggenheim isotherm equations was investigated. For each component the amounts adsorbed are given with those calculated from the best fitting isotherm in the adjacent column of Tables 6 and 7. The fit is less satisfactory in the case of *p*-chlorophenol.

TABLE 8. MIXED ADSORPTION

Dichlorophen			*p*-Chlorophenol		
Solution conc µg/ml	Amount adsorbed µg/g	Single adsorption calculated µg/g	Solution conc µg/ml	Amount adsorbed µg/g	Single adsorption calculated µg/g
8·53	1172	1360	34·3	46·3	—
19·24	1773	—	18·75	76·2	—
5·30	941	987	23·6	80·1	—
12·27	1362	—	12·18	17·6	79·2
2·29	372	520	4·86	5·86	26·5
12·16	1226	—	5·62	18·2	30·8
3·69	746	754	9·32	17·5	54·2
4·08	693	814	8·85	23·4	50·0

Table 8 shows the adsorption when mixtures of the two components are examined. Where possible the anticipated adsorption of each component, had it been alone, has also been calculated from the appropriate isotherms; the results are given in the third and sixth columns of Table 8. Each component depresses the adsorption of the other but *p*-chlorophenol clearly has a disproportionately greater effect on dichlorophen despite the smaller affinity of the former for the surface. The fact that the molecular weight of *p*-chlorophenol is half the molecular weight of dichlorophen is not sufficient to account for this effect.

Discussion

The Rf values of each component clearly vary with the load and the duration of the run: both these factors involve the concentration of the component in the area of the spot. The relation is given by equation (4) in which the term g_s/g_m occurs. This term is a simple function of the partition coefficient or the slope of the adsorption isotherm. Only *p*-chlorophenol is partitioned between water and toluene and its partition coefficient increases with concentration. Hence by equation (1) the Rf value should decrease with increasing load whereas the reverse has been shown to be the case. Moreover dichlorophen has no measurable water/toluene partition coefficient, so that any interpretation of the observed chromatographic behaviour based on a partition coefficient theory is clearly inadequate.

The slope of the adsorption isotherm for dichlorophen decreases as the concentration increases, giving a predictable increase in Rf value as the load is increased. Diffusional processes tend to decrease the concentration and the system would thus move to a position of the isotherm with a greater slope. Therefore the Rf value will also decrease with increasing duration of run: both these trends have been observed.

For *p*-chlorophenol the increase in Rf value is less marked but this could be predicted from the generally smaller slope of the isotherm, since this component is less strongly adsorbed.

In mixtures, each component depresses the adsorption of the other and this will effectively decrease the slope of the isotherm for each component; *p*-chlorophenol has a disproportionately greater effect than dichlorophen. Not only should each increase the Rf value of the other, but also that for dichlorophen should be the more affected. This is in accord with the experimental results.

The Fowler–Guggenheim isotherm equation, to which *p*-chlorophenol appears to conform, allows for adsorbate - adsorbate interaction. The shape of the experimentally determined isotherm suggests attractive interaction between *p*-chlorophenol molecules. A repulsive interaction between dichlorophen and *p*-chlorophenol could explain the phenomenon, in which case the adsorption isotherm for dichlorophen in the presence of *p*-chlorophenol should exhibit Fowler–Guggenheim characteristics. Initial examination of the results fails to reveal this. Nevertheless an explanation of the anomalous behaviour of the two components based on their adsorption behaviour on silica gel would appear more valid. On thin-layer plates the separation of the components is effected by adsorption rather than partition processes.

The load on the chromatography plate and the spot area (Table 4) together with the bulk density, void volume and thickness of the layer may be used with the adsorption data to calculate values for g_s/g_m. These are then inserted in equation (4) to obtain the Rf value. The calculated Rf values of dichlorophen for loads of 1, 5 and 10 μg are, respectively, 0·004, 0·005 and 0·007 and the corresponding experimentally determined values are 0·017, 0·021 and 0·022. Similarly, the calculated Rf values of *p*-chlorophenol are 0·192, 0·145 and 0·096 and the corresponding experimentally determined values are 0·108, 0·116 and 0·137. The partition coefficient, K, for *p*-chlorophenol may be used in equation (1) to calculate the Rf value if the ratio b/a is known. The ratio

$$b/a = \frac{X_m \text{ (}p\text{-chlorophenol)}}{V_v \times \text{density of } p\text{-chlorophenol}} = 1{\cdot}55 \times 10^{-3}.$$

Calculated values based on the partition coefficient thus yield Rf values slightly less than unity. This tends to support the contention that adsorption is the main factor for the system studied. However the values show that whilst the Rf values calculated for *p*-chlorophenol are of the right order of magnitude, their trend with load is opposite to the experimentally found values. The trend for dichlorophen is in the right direction but the calculated values are a third of those experimentally determined.

Acknowledgements. One of us (A.S.) is grateful to the Greek Government for a fellowship, and for the encouragement accorded by Remek S.A. towards the end of the studies. Thanks are also due to Dr. E. J. Shellard and Mr. M. M. Ahmed for their help and valuable comments. Samples of dichlorophen and *p*-chlorophenol were kindly supplied by British Drug Houses Ltd.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 43S–44S Received April 30, 1968

SHORT COMMUNICATION

The cathode ray polarography of phenylmercuric nitrate

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EXPERIMENTS on the absorption of phenylmercuric nitrate by rubber teats required a simple, rapid and sensitive assay for concentrations of phenylmercuric nitrate from 0·5 to 20·0 μg/ml. Both ultraviolet spectrometry (Eldridge & Sweet, 1956) and chemical methods (British Pharmacopoeia, 1963; Department of Pharmaceutical Sciences Report, 1962) are relatively insensitive, the spectrometric method also being subject to interference by extractive from rubber teats. Ordinary polarographic methods have been used for higher concentrations of phenylmercuric nitrate, two cathodic waves being observed at half-wave potentials of about −0·2 V and −0·9 V respectively. These have been observed in a number of supporting electrolytes, including hydrochloric acid (Page & Waller, 1949), Britton-Robinson buffers (Benesch & Benesch, 1951) and a glycerol-triethanolamine mixture with gelatin (Osborne, 1950).

The cathode ray polarography of phenylmercuric nitrate (0·4–20·0 μg/ml) was therefore investigated in hydrochloric acid (0·1N), lithium chloride (0·2N) and buffer solutions (Britton-Robinson and Sorensen) in the pH range 1·8 to 12·3. In all cases only one wave of analytical importance was seen, the peak voltage being about −0·3 V. Since good linearity, wave shape and sensitivity were found with both 0·2 N lithium chloride and 0·1 N hydrochloric acid, the latter was chosen as supporting electrolyte.

EXPERIMENTAL AND RESULTS

Apparatus. A Davis differential cathode ray polarograph was used, and peak voltages were recorded with reference to the mercury pool anode.

Operation. Trace impurities in the supporting electrolyte became apparent at high instrument sensitivities. Phenylmercuric nitrate concentrations in the range of 0·4–2·0 μg/ml were therefore examined by comparing 50 mS derivative operation with the twin cell subtractive mode. This enabled subsequent adoption of the more rapid and convenient 50 mS derivative working for lower concentrations. Direct operation was used in the concentration range 2·0–20·0 μg/ml. Determinations were made at 25°, all solutions being previously flushed with nitrogen until dissolved oxygen no longer affected wave shape or peak height (5 min).

Calibration graph. To solutions (5 ml) containing 0·4–20·0 μg/ml of phenylmercuric nitrate (B.D.H.) in a polarographic cell, 0·6 N hydrochloric acid (1 ml) was added and the solution polarographed (starting potential −0·[illegible] V). A ten point calibration graph was linear ($r = 0{\cdot}997$, $P > 0{\cdot}999$), each point representing the mean of six readings from duplicate determinations on each of three solutions prepared from solid

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phenylmercuric nitrate. Limits of error at the 1·0 μg/ml level were ±0·04 μg/ml.

Wave characteristics. The wave is irreversible since on reverse sweep an altered pattern was obtained. With increase in pH from 1·8 to 12·3 the peak voltage showed little change, but became less negative with increased depolarizer concentration. Peak currents fell by about 30% with increase in pH from 1·8 to 12·3; in hydrochloric acid (0·1N) the peak current temperature coefficient was negative (−0·24% per degree) in the range 25–50°.

Concentration effects could not be examined above 40 μg/ml in hydrochloric acid due to precipitation of phenylmercuric chloride. Using a support electrolyte of pH 2·0 however (0·1 M sodium acetate, M acetic acid, 0·1 M hydrochloric acid), concentrations of phenyl mercuric nitrate (20·0–160·0 μg/ml) were examined and one wave only observed. The peak current did not increase directly with concentration but reached a maximum, with distortion of the wave peak, at about 120 μg/ml, indicating an adsorption effect. If lead ion (10·0 μg/ml) and phenylmercuric nitrate (120·0 μg/ml) were present in the same solution, the more negative lead wave (peak voltage —0·5) was unaffected by the presence of phenylmercuric nitrate.

DISCUSSION

The electrode reactions suggested by Benesch & Benesch (1951) for the two polarographic waves observed during the reduction of phenylmercuric nitrate are as follows. For the less negative wave, a single electron reduction to the phenylmercury radical, which is adsorbed on the mercury drop surface (Benesch & Benesch, 1952):

$$PhHgNO_3 + e \rightarrow PhHg\cdot + NO_3^-$$

The more negative wave corresponds to removal of the mercury atom from the aromatic ring, with uptake of one electron:

$$PhHg\cdot + e + H_3O^+ \rightarrow PhH + Hg + H_2O$$

The single wave observed during cathode ray polarography is probably produced by the rapid consecutive occurrence of the above reactions. Were it due only to the former reaction, adsorption of the radical at high concentrations would be expected to reduce the effective drop area with consequent distortion or diminution of the wave produced by included lead. At lower depolarizer concentrations (0·4–20·0 μg/ml), any adsorption effect is slight, as shown by peak current/concentration linearity.

The method described for concentrations of phenylmercuric nitrate from 0·4–20·0 μg/ml is simple and rapid. A single determination takes 6–7 min and a ten point calibration graph 40 min.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 45S–47S Received May 1, 1968

SHORT COMMUNICATION

The polarography of cephalosporin C derivatives

I. F. JONES, J. E. PAGE* AND C. T. RHODES

WE report some preliminary measurements on the polarographic reduction of cephalosporin C, cephalothin [7-(2-thienylacetamido)-cephalosporanic acid] and cephaloridine [7-(2-thienylacetamido)-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine].

Green, Page & Staniforth (1965) have shown that cephalosporin C derivatives give well-defined infrared and nuclear magnetic resonance spectra that can be used for their qualitative identification. Martin & Shaw (1965) have reviewed the application of methods such as ultraviolet spectrophotometry, paper chromatography and paper electrophoresis for cephaloridine, while Chapman, Page & others (1968) have described infrared and X-ray powder measurements for this compound. The application of mass spectroscopy to the structural elucidation of cephalosporin derivatives has been reported by Richter & Biemann (1965).

EXPERIMENTAL

Polarograms were obtained using a Tinsley Mark 19 pen-recording polarograph and an Electrochemical Laboratories manual polarograph. A dropping mercury electrode was used as cathode and a saturated calomel electrode as external anode; the latter was connected to the cell solution by means of a potassium chloride salt-agar bridge. The polarographic cell which held about 10 ml of solution, was maintained in a thermostat at 25° (±0·1°). All solutions were deoxygenated at 25° with solvent-saturated, oxygen-free nitrogen for 10 min before electrolysis. Solutions of Britton–Robinson buffer pH 3–7 and 0·1M hydrochloric acid were used as supporting electrolytes.

The half-wave potentials ($E_{0.5}$) reported (Table 1) are averaged values from replicate polarograms recorded on the manual apparatus. Capillary drop times were recorded at the appropriate half-wave potential. The diffusion currents reported refer to the maximum diffusion currents registered on the recording polarograph without condenser damping.

RESULTS AND DISCUSSION

Derivatives of cephalosporin C differ from those of benzylpenicillin (Brezina & Zuman, 1958) in showing reduction waves, the shapes and half-wave potentials of which are pH dependent. Typical polarograms of cephalosporin C, cephalothin and cephaloridine are shown in Fig. 1 and the change in the wave form of cephaloridine with pH in Fig. 2. We have used the polarograms of cephaloridine for quantitative analysis, those for the other compounds are reported for qualitative comparison.

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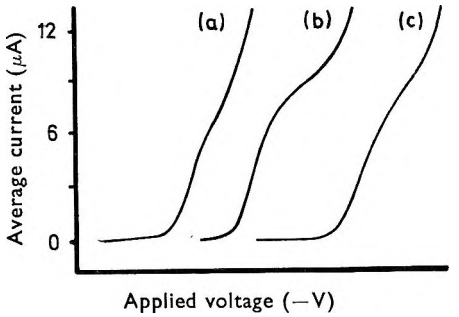

FIG. 1. Polarographic waves for (a) 1·0 mM cephalosporin C, (b) 1·0 mM cephaloridine and (c) cephalothin, in Britton–Robinson buffer pH 3·05. Each wave starts at 0·85 V. Scale 10 mm = 0·25 V.

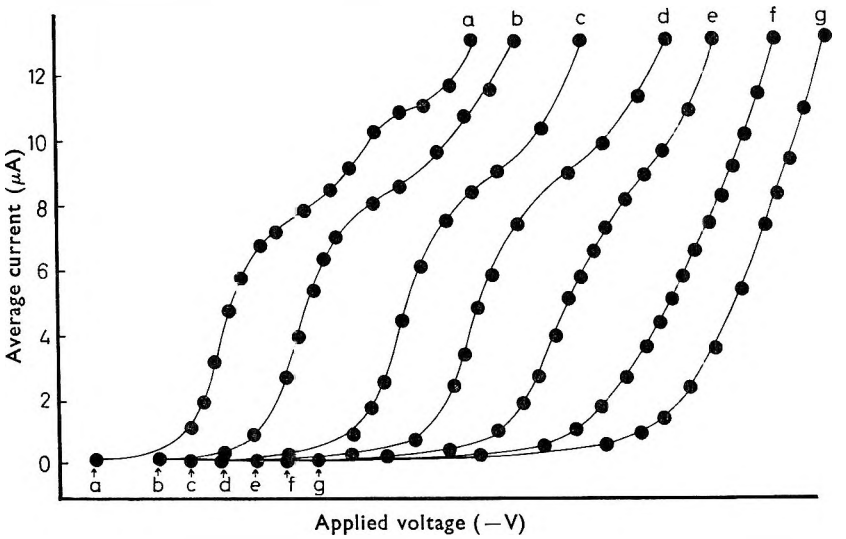

FIG. 2. Effect of pH on wave form of 1·0 mM cephaloridine in hydrochloric acid (a) 1·0 M, (b) 0·1 M. Britton–Robinson buffer (c) pH 3·05. (d) pH 3·98. (e) pH 4·96. (f) pH 6·03. (g) pH 7·02. Each wave starts at 0·70 V. Scale, 10 mm = 0·12 V.

The waves produced in acid solution appear to be diffusion controlled, the diffusion current (i_d) of cephaloridine being a linear function of the height of the mercury reservoir, i.e. $i_d = k\sqrt{h_{corr.}}$, where $h_{corr.}$ is the height of the mercury reservoir corrected for back pressure of mercury. The diffusion current of cephaloridine is linearly related to the capillary characteristics as predicted by the Ilkovic equation, for diffusion controlled waves. The relation between i_d and $m^{2/3}t^{1/6}$ is given by the equation $i_d = 3{\cdot}67m^{2/3}t^{1/6} + 0{\cdot}03$ where m and t have the usual significance (correlation coefficient for four settings of the mercury reservoir height between 43 and 79 cm = 0·99). Preliminary studies on the measurement of the diffusion coefficient suggest that the wave produced by cephaloridine in acidic solution is a two electron step.

The shape of the cathodic waves hindered the accurate measurement of diffusion current. This was overcome by drawing a tangent at the foot of the wave parallel to the limiting current, the diffusion current then being taken as the vertical distance between the two parallel lines.

The measured diffusion current is proportional to the concentration of the depolarizer as predicted by the Ilkovic equation. A linear relation, $i_d = 6{\cdot}59C + 0{\cdot}02$, was obtained for levels of cephaloridine between 2×10^{-3} and 1×10^{-5}M in 0·1M hydrochloric acid (correlation coefficient for 9 values = 0·99). Concentrations as low as 2×10^{-6}M cephaloridine could be detected on the recording polarograph.

TABLE 1

MEAN HALF-WAVE POTENTIALS AND DIFFUSION CURRENTS FOR CEPHALOSPORIN DERIVATIVES AT 25° IN 0·1M HYDROCHLORIC ACID. m=4·41 mg/sec.

Conc. mM	Cephalosporin C			Cephalothin*			Cephaloridine		
	$-E_{0\cdot5}$ (V)	i_d(μA)	$t_{-E_{0\cdot5}}$ (sec)	$-E_{0\cdot5}$(V)	i_d(μA)	$t_{-E_{0\cdot5}}$ (sec)	$-E_{0\cdot5}$(V)	i_d(μA)	$t_{-E_{0\cdot5}}$ (sec)
1·00	1·05	4·31	1·91	1·08	5·62	1·80	0·93	7·28	1·81
0·50	1·01	2·17	1·94	—	—	—	0·90	3·56	1·83
0·10	0·97	0·46	1·96	—	—	—	0·85	0·77	1·86

*Recorded in 0·1M hydrochloric acid containing 50% v/v ethanol.

The half-wave potentials for cephalosporin C, cephalothin and cephaloridine (Table 1) depend on the concentration of the depolarizer, the half-wave potentials becoming more negative with increasing concentration as shown for cephalosporin C and cephaloridine. All $E_{0\cdot5}$ values were adjusted for the potential required to overcome the internal resistance of the cell ("iR drop"). Experiments using a potassium nitrate salt-agar bridge to connect the cell solution to the saturated calomel electrode showed that cephaloridine at potentials up to +0·3V yielded no anodic step at the dropping mercury electrode in the supporting electrolytes examined.

The sensitivity of the polarographic method for cephaloridine is similar to that of the ultraviolet spectrophotometric method (Martin & Shaw, 1965).

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 48S–50S Received May 2, 1968

SHORT COMMUNICATION

Routine detection and identification in urine of stimulants, analgesics, antihistamines, local anaesthetics and other drugs, some of which may be used to modify performance in sport

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MANY sporting authorities throughout the world proscribe, in addition to the stimulants, other classes of drugs, e.g. the narcotics and phenothiazine tranquillizers. Present gas-liquid chromatographic procedures for the routine detection in urine of all these classes of drugs suffer from the disadvantage of using a variety of operating conditions (see for example Kazyak & Knoblock, 1963, who listed retention time data for 59 compounds using a single SE-30 column and temperatures from 115–250°, and also Street, 1967).

The work of Beckett, Tucker & Moffat (1967) on stimulants is therefore now extended, using two isothermal GLC systems, to obtain retention data for 74 additional compounds. This allows the analysis of 116 compounds to be made using only three isothermal GLC systems. Drugs, other than those which may be used to modify performance in sport, are included since sportsmen may use them for medical reasons, e.g. phenacetin in analgesic preparations or a local anaesthetic for injury, and these may interfere with the dope control test.

METHOD

The method previously reported is used with the following modifications.

Extraction procedure A is carried out twice, on the same 5 ml of urine, using (*a*) diethyl ether and (*b*) methylene dichloride. The extracts are concentrated on water baths at 40° and 55° respectively.

1 μl of the ethereal concentrate is injected into System C at 210° and, as before, 5 μl into System B at 140°. 1 μl of the dichloromethane concentrate is also injected into System C at 210°.

Table 1 lists the retention times of the compounds screened using System C at 210° (and also at 120° for comparison) along with System B at 140° for some compounds. A composite chromatogram is represented in Fig. 1, each peak represents approximately 1 μg free base injected in 1 μl solvent.

DISCUSSION

The detection of the metabolite(s) of a drug in urine affords additional proof of the ingestion of the parent compound. Some drug metabolites

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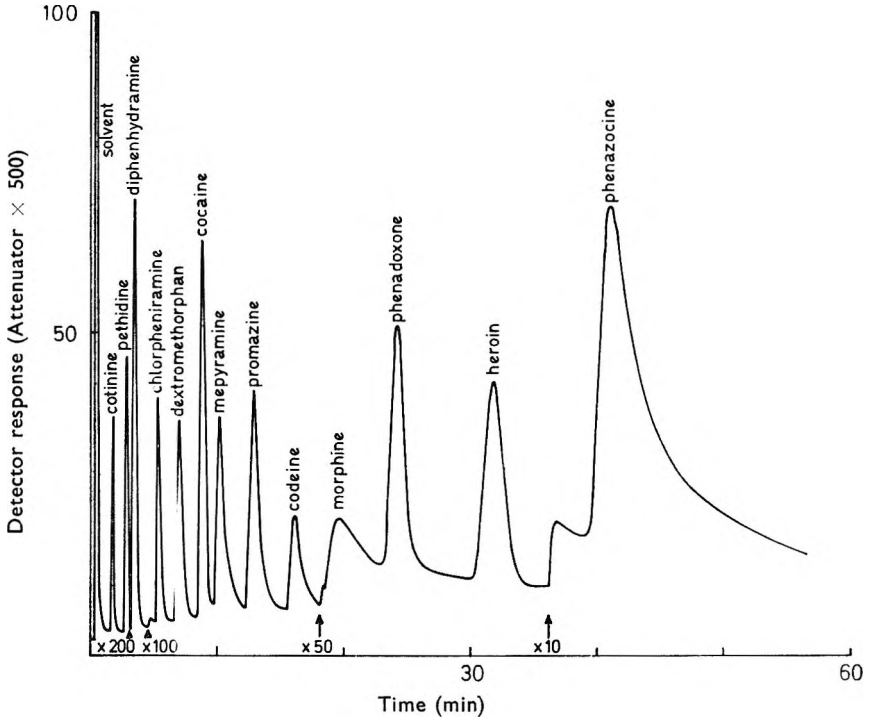

FIG. 1. Composite chromatogram of some pharmaceutical compounds on Column "C" at 210°.

have therefore been included in Table 1, e.g. the cyclic metabolite of methadone (1,5-dimethyl-3,3-diphenyl-2-ethyl-1,2-dehydropyrrolidine). However, there are some classes of metabolites that this method will not detect, e.g. ring hydroxylated compounds, and in these cases modification of the extraction technique is necessary. Glucuronides, and other conjugates of the parent drug, can be hydrolysed to the drug and extracted as usual. Of the compounds examined 34 were shown to be excreted as parent drug after oral administration.

The dual extraction used is necessary because heroin, morphine, cotinine, phenacetin, caffeine, cinchonine, and cinchonidine are relatively insoluble in ether.

All the compounds studied gave single peaks which were nearly symmetrical (Fig. 1) with the exceptions of tetrahydroziline, xylometazoline. morphine and cinchonine (System C) and meprobamate (System B), which gave broad diffuse peaks and propoxyphene which gave two peaks (System C). The use of System C is sufficient to detect and identify any of the 74 compounds in Table 1. Further chromatography on System B (at 140°, or at a higher temperature), combined with derivative formation and chromatography, is sufficient to establish unequivocally the identity of the compound.

TABLE 1. GLC DATA FOR SOME PHARMACEUTICAL COMPOUNDS

	Retention time (min) for compounds using systems						
	C	C	B	C		C	
	120°	210°	140°		210°		210°
Benzocaine	18·5	1·2	62·1	*Chlorpheniramine	5·2	Trimeprazine	12·0
Amylocaine	20·9	1·4	4·7	Cyclic Metabolite of Methadone	5·3	*Promazine	12·7
*Phenacetin	36·6	1·6	—			*Antazoline	13·6
Cotinine	33·7	1·7	50·6	Normethadone	6·2	Dexoxadrol	14·2
*Meprobamate	—	2·2	6·3†	Dextromethorphan	7·0	Dihydrocodeine	15·4
				Isomethadone	7·2	*Codeine	16·1
Fenmetramide	—	2·3	—	Xylometazoline	7·3†	Pyrrobutamine	17·3
Norpethidine	—	2·3	25·8	Brompheniramine	7·3	*Morphine**	19·6†
Tryptamine	—	2·4	—	Methaqualone	7·4	Ethylisobutrazine	20·1
Pethidine	—	2·6	13·6	Pipradrol**	7·5	Dipipanone	21·0
*Glutethimide	—	2·7	—	Phenindamine	7·8	Methdilazine	21·1
Prilocaine	—	2·8	46·3	*Propoxyphene	8·4a	*Chlorpromazine	21·9
*Caffeine**	—	2·9	—	*Methadone	8·4	Desmethylchlor-promazine	21·9
Ethoheptazine	—	2·9	—	Amitriptyline	8·7		
*Pheniramine	—	3·0	22·1	Cocaine**	8·8	Didesmethylchlor-promazine	21·9
Etryptamine	—	3·0	—	Primidone	9·3		
Tymazoline	—	3·3	—	*Chlorcyclizine	9·6	Phenadoxone	24·1
*Diphenhydramine	—	3·4	26·8	Triflupromazine	9·7	Methotrimeprazine	24·8
*Lignocaine	—	3·5	43·1	Imipramine	9·9	Pyrathiazine	25·4
*Amidopyrine	—	3·7	—	Desmethylimipramine	10·2	*Heroin	31·6
Orphenadrine	—	3·8	32·3	Mepyramine	10·2	*Chloroquine	31·9
Tetrahydroziline	—	4·2†	—	Triprolidine	10·6	*Cinchonidine	37·6
*Tripelennamine	—	4·3	54·0	Isothipendyl	10·7	*Cinchonine	39·0†
*Methapyrilene	—	4·3	—	Promethazine	11·2	Trifluoroperazine	40·5
*Procaine	—	4·8	—	Pentazocine	11·3	Phenazocine	41·3
Cyclizine	—	5·2	—	Bupivacaine	11·3	Acepromazine	42·9

* Drugs examined by Kazyak & Knoblock (1963).
** Drugs most likely to be used as doping agents
† Broad diffused peak
— No peak observed in 60 min.
Major peak

Acknowledgements. We wish to thank the various pharmaceutical companies who supplied many of the drugs: Arthur H. Cox & Co. Ltd., for Orphenadrine HCl; CIBA Laboratories Ltd., for Xylometazoline HCl; McNeil Laboratories Inc.,for Fenmetramide; Wm. S. Merrell Co., for Pipradrol HCl; Pfizer Ltd., for Tetrahydroziline HCl; Laboratoires Robert & Carrière, for Tymazoline HCl; Roche Products Ltd., for Phenindamine; Upjohn Co., for Dexoxadrol HCl and Etryptamine acetate.

A.C.M. also thanks the Science Research Council for a research studentship.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 51S–53S Received May 1, 1968

SHORT COMMUNICATION

The determination of diamorphine by thin-layer chromatography and spectrophotometry

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THE assay of diamorphine in Diamorphine Injection B.P. is based on its hydrolysis to morphine followed by a colorimetric determination (Allport & Jones, 1942; Stephens, 1951; Hooper, Shaw & Tims, 1967). Stored solutions of diamorphine may contain O^3- and O^6-acetylmorphines as well as diamorphine and morphine (Davey, Murray & Rogers, 1967); all are converted into morphine in the first stage of the British Pharmacopoeial assay of the injection and thus contribute to the final morphine content.

Separation of opium alkaloids and their acetylated derivatives by thin-layer chromatography has already been reported (Mary & Brochmann-Hanssen, 1963; Vignoli, Guillot & others, 1965, 1966a, b) and the application of this technique to diamorphine, the acetylmorphines and morphine is discussed below.

EXPERIMENTAL

Aluminiumoxid DS-5 (Camag), pH 7·5–7·8, was heated at 100° (30 min) with distilled water. It was filtered through glass, then washed repeatedly with distilled water. This procedure removed absorbing impurities and reduced the "blank" absorbances at both wavelengths (see below) to 0·05 or less. Plates (20 × 20 cm) were spread with this material in the usual way to a thickness of 0·25 mm and were air-dried then activated at 120° for 1 hr.

The assay is carried out as follows.

Apply the sample in aqueous solution (10 μl containing 250–750 μg of diamorphine hydrochloride) by means of an Agla micrometer syringe, leaving about 2·5 cm on each side of the plate to eliminate edge effects; this allows room for the development of two sample solutions, a standard solution and a blank. Tanks lined with Whatman No. 1 paper impregnated with the running solvent are used and chromatograms are developed (ascending technique) with benzene–methanol–aqueous ammonia (0·880) (90 : 10 : 0·2 by vol.).

Solvent is allowed to ascend 15 cm from the starting line (45 min) then plates are air-dried (5 min). Alkaloids are located by viewing in ultraviolet light (254 mμ). Diamorphine appears as a faint yellow fluorescent spot at Rf 0·63; O^3-acetylmorphine, O^6-acetylmorphine and morphine, if present, appear at Rf 0·53, 0·35 and 0·06, respectively.

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Scrape the alkaloid spot into water (5 ml), mix (2 min) and centrifuge. Filter the solution through a MF Millipore membrane filter (no diamorphine was adsorbed on the filter) of mean pore size 0·22 μ in a Swinny filter holder. Elute similarly a blank area of alumina from the same plate, equal in size to that of the sample spot, and at a location on the plate with identical Rf. Measure the absorbance of the sample filtrate against the blank at 278·5 and 252 mμ, which are wavelengths of maximum and minimum absorption of diamorphine.

Calculate the concentration of diamorphine in the sample solution by comparison with the results obtained from a standard solution run on the same plate.

$$C_{sample} = C_{standard} \frac{(A_{278\cdot5} - A_{252})_{sample}}{(A_{278\cdot5} - A_{252})_{standard}}$$

where C represents the concentration of diamorphine hydrochloride, and $A_{278\cdot5}$ and A_{252} are the absorbances at 278·5 and 252 mμ, respectively.

RESULTS

The procedure has been applied to solutions containing 10 mg of diamorphine hydrochloride per ml. Sample A, of pH 4·02, was sterilized by filtration through a MF Millipore filter. Sample B was sterilized by heating with a bactericide (0·002% phenylmercuric nitrate); its pH was 4·45 before and 2·65 after sterilization. The chromatograms from sample A showed only one spot, at Rf 0·6, whereas sample B showed two spots, at Rf 0·6 and 0·3, corresponding to diamorphine and O^6-acetylmorphine. Neither phenylmercuric nitrate nor sodium metabisulphite caused any interference. The results of six replicate assays of each sample are given in Table 1. The absorbance difference, $A_{278\cdot5} - A_{252}$, was directly proportional to the concentration of diamorphine in the range of concentrations studied.

TABLE 1. RESULTS FOR DIAMORPHINE INJECTION
(mg of diamorphine hydrochloride per ml)

	Sample A	Sample B
Plate	Sterilized by filtration	Sterilized by heat
I	9·97	9·37
II	10·09	9·46
III	9·75	9·25
IV	9·90	9·55
V	10·05	9·42
VI	10·05	9·60

The concentrations before sterilization were 10·00 mg/ml.
The coefficient of variation, based on 12 determinations of degraded and undegraded solutions, was 1·3%.

The percentage recovery of diamorphine from the chromatograms varied with each plate and with different batches of alumina, but it was always in the range 93–98%.

Acknowledgement. We thank Macfarlan Smith Limited for samples of diamorphine hydrochloride.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 54S–60S Received May 23, 1968

Pharmaceutical applications of vapour-controlled* thin-layer chromatography

ROKUS A. DE ZEEUW

In thin-layer chromatography, solvent vapour plays an important role. To obtain full benefit of the influence of vapour in the technique, a new chamber has been developed, providing full vapour control over the entire plate. This allows better separations of chemically related compounds. The properties of the vapour-control chamber are shown in separations of barbiturates and local anaesthetics.

RECENT work (de Zeeuw, 1968a) has shown that solvent vapour plays an important role in thin-layer chromatography, particularly with multicomponent solvents. Depending on the affinity of the vapour components for the adsorbent, varying amounts of solvent vapour may be adsorbed on the dry plate and separation of the components of any mixture is affected by this phenomenon. It has been shown (de Zeeuw, 1968b) that if the development is begun before the atmosphere in the tank is in equilibrium with the running solvent vapour (so-called unsaturated chambers), then separations with multicomponent solvents are more efficient than in the situation where equilibrium is established before commencing development (so-called saturated chambers). Because of ascending solvent the lower parts of the plate will adsorb less vapour than the upper parts, thus a concentration gradient of adsorbed vapour is formed from the bottom to the top of the plate. With multicomponent solvents this adsorbate will consist mainly of the more polar components. During development, the faster running spots will pass into plate areas enriched in the more polar components of the solvent, thus producing an acceleration in the rate of migration. At the same time the slower running spots pass through areas with a lower concentration of the polar components. The migration rate of these will thus be affected to a smaller extent and hence a better separation will result. In saturated chambers a concentration gradient is less easily formed, because when the plate is placed in a tank saturated with respect to the vapour of the running solvent the maximum amount of vapour will be adsorbed almost immediately. I therefore suggest that unsaturated chambers are preferable to saturated chambers when multicomponent solvents are used.

Little or no control can be exercised of course on the extent to which any gradient is developed on the thin-layer plate. This will depend mainly on the rate of evaporation of the solvent components and on their affinity for the adsorbent used. Thus, although the suggested technique with unsaturated chambers yields improved separations, the conditions are not necessarily optimum for every case.

The apparatus described below, however, does allow full vapour control over the entire plate, thus making it possible to affect the migration rate of each individual spot.

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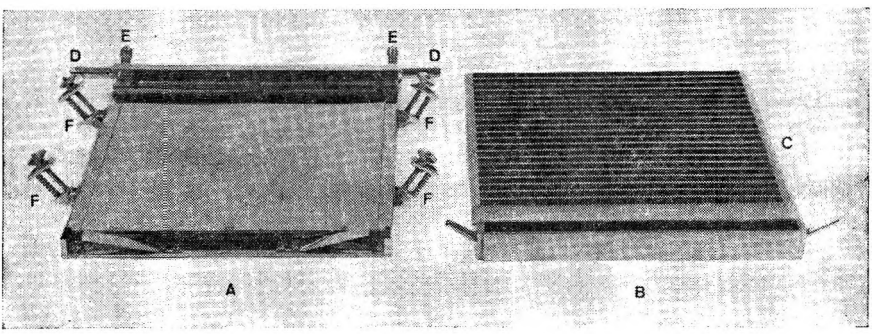
* Recently the name Vapor-Programmed TLC was introduced for this technique.

APPARATUS

The vapour-control chamber (de Zeeuw, 1968c) (VC-chamber) consists of three parts, a ground plate (A) 20 × 20 × 1 cm on which is placed a solvent reservoir (B) 20 × 1 × 2 cm, and a trough chamber (C) 20 × 17 × 1·5 cm, containing 21 troughs 19 × 0·6 × 1·3 cm, all of chromium-plated brass (Fig. 1a, b). In use the troughs are filled with mixtures of

a



b

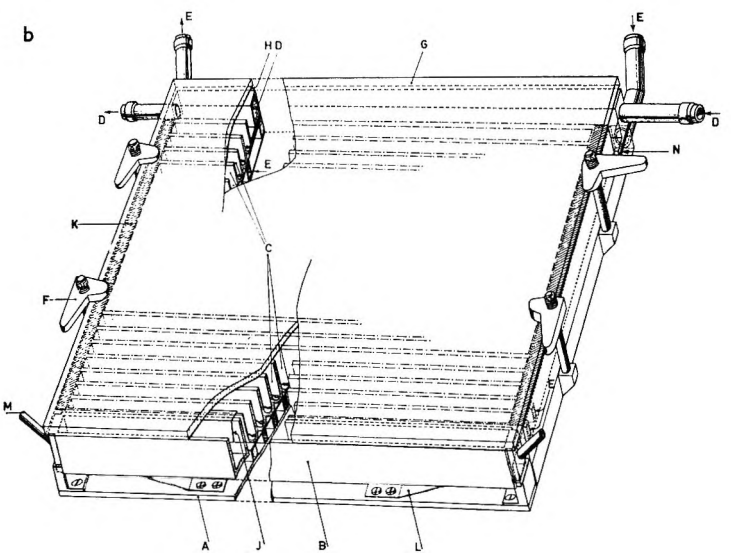


FIG. 1a. The components of the VC-chamber. A: ground plate, B: solvent reservoir, C: trough chamber with 21 troughs. The ground plate is equipped with a warm water tube (D), an internal tube system for water-thermostating, the inlet and outlet being visible at E, and fixation clamps (F).

b. Schematic view of the VC-chamber. A: groundplate, B: solvent reservoir, C: trough chamber with 21 troughs, D: warm water tube, E: water circulating system for cooling purposes (the arrows at D and E indicate the direction of water flow), F: fixation clamps, G: TLC plate, H: adsorbent, J: filter paper strip, K: spacer, L: metal springs, M: solvent inlet, N: asbestos layer for warm water tube insulation.

polar and non-polar solvents increasing in polarity from one to the other. The vapour from such solvent mixtures is used to equilibrate with the area of adsorbent directly over the particular trough concerned and thus to form the concentration gradient referred to above. Running solvent is placed in the reservoir (B), development of the plate, which takes place in a horizontal position with the adsorbent immediately facing the troughs, may be continued for an unlimited time, since excess solvent can evaporate from the end of the plate. For this purpose the end of the plate extends 0·5 cm over a warm water tube (D), which is attached to the groundplate (A) but insulated from it by asbestos. Running solvent is led on to the plate from the reservoir (B) by means of a strip of filter paper. The solvent reservoir is pressed gently to the plate by two metal springs. Adsorbent on the plates (20 × 20 cm) is removed from three sides to a width of 0·5 cm. During development the plate rests on two Teflon spacers of 0·5 mm placed on opposite edges. Thus, the space between the plate and the trough chamber is small enough to prevent vapour currents without the layer touching the troughs. Development of the thin-layer takes place with the ground plate thermostatted; inlet and outlet tubes of the water circulation system are shown at E. Four clamps (F) fix the plate to the chamber.

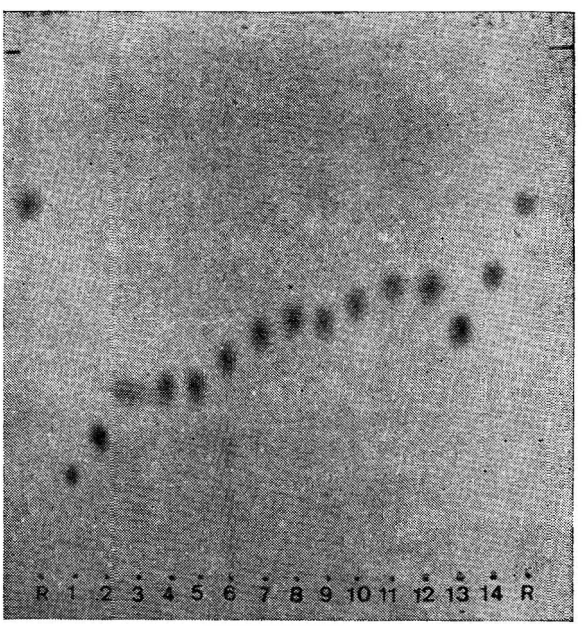


FIG. 2. Separation of 14 commonly used barbiturates with chloroform–isopropanol–25% ammonia (45:45:10) in saturated normal chambers on silica gel GF 254, Temperature 20·6°, relative humidity 39%, saturation 60 min, development 75 min., load 10 μg. 1 = heptobarbitone, 2 = phenobarbitone, 3 = bralloba:bital, 4 = barbitone, 5 = allobarbitone, 6 = cyclobarbitone, 7 = aprobarbitone, 8 = butalbital, 9 = butobarbitone, 10 = amylobarbitone, 11 = pentobarbitone, 12 = quinalbarbitone, 13 = methylphenobarbitone, 14 = hexobarbitone, R = reference 4-nitroaniline.

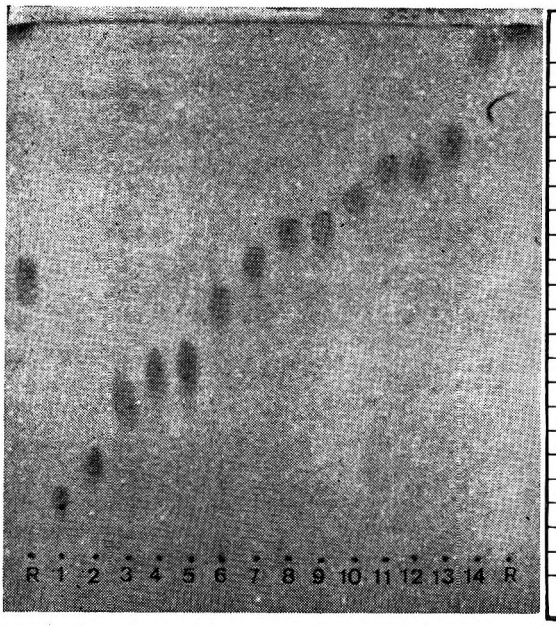


FIG. 3. The same substances as in Fig. 2, now separated in the VC-chamber with chloroform–isopropanol (92·5 : 7·5) (saturated with 25% ammonia) and a chloroform–isopropanol–methanol–ammonia vapour gradient. The position of the troughs and the liquid composition therein during development are shown at the right. Temperature 21·6°, relative humidity 40%, saturation 10 min, development 110 min, strips 1 mm, cooling ground plate 19°, Code: C = chloroform, saturated with 25% ammonia, I = isopropanol, M = methanol, A = 25% ammonia. Numbering as in Fig. 2. Note: the left-hand reference contains an impurity.

Because the solute spots are finite in size, migration of the upper parts of the spot will tend to be more rapid than the lower parts and tailing will consequently result. This may be prevented by interspersing troughs with solvents of low polarity between troughs containing the more polar mixtures. Troughs thus interspersed will exert a decelerating effect on the migration rate of the spots. When working with a chloroform–ether gradient for example, the following would suitably be used: troughs 1 and 2, chloroform; trough 3, chloroform–ether 90 : 10; trough 4, chloroform; trough 5, chloroform–ether 75 : 25; trough 6, chloroform; trough 7, chloroform–ether 60 : 40; and so on. In this way compact spots can be obtained.

Experimental

Solvents used were of reagent grade (Merck). All substances examined were 99·0% chromatographically pure, except cyclobarbitone which showed slight decomposition. Solvent compositions are given by volume.

Silica gel GF 254 (Merck) was used as adsorbent, in layers 0·25 mm on glass plates 20 × 20 cm. After spreading, the plates were air dried

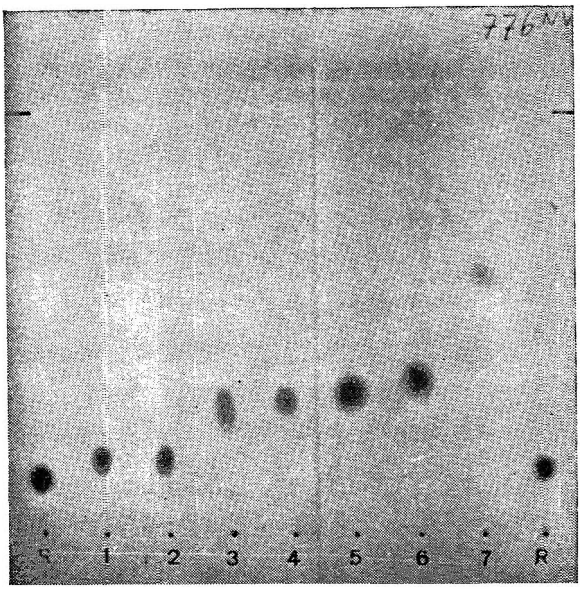


FIG. 4. Separation of chemically related local anaesthetics with hexane–chloroform–methanol (60 : 35 : 5) in saturated normal chambers on sodium hydroxide impregnated silica gel GF 254. Temperature 22·0°, relative humidity 40%, saturation 45 min, development 25 min, load 5 and 6: 10 μg, 1, 2 and 4: 15 μg, 3: 30 μg, 7: 50 μg. 1 = procaine, 2 = tutocaine, 3 = tetracaine, 4 = butacaine, 5 = ethylaminobenzoate, 6 = butylaminobenzoate, 7 = lignocaine.

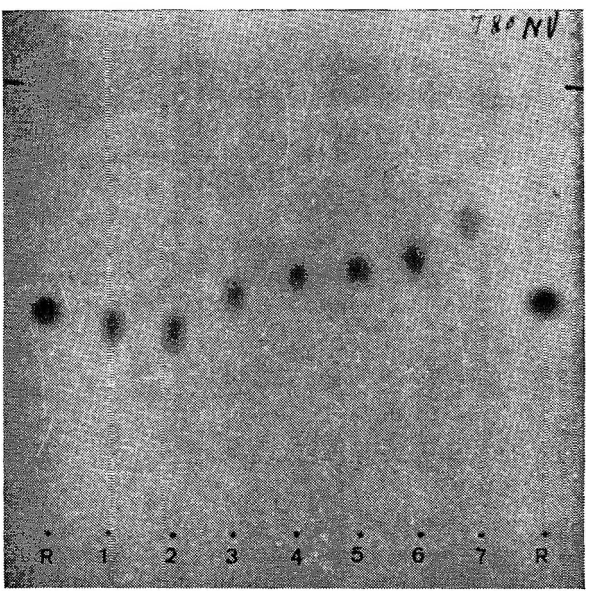


FIG. 5. The same substances and technique as in Fig. 4, with chloroform–methanol (95 : 5) as solvent. Temperature 22·0°, relative humidity 44%, saturation 45 min, development 30 min. Numbering as in Fig. 4.

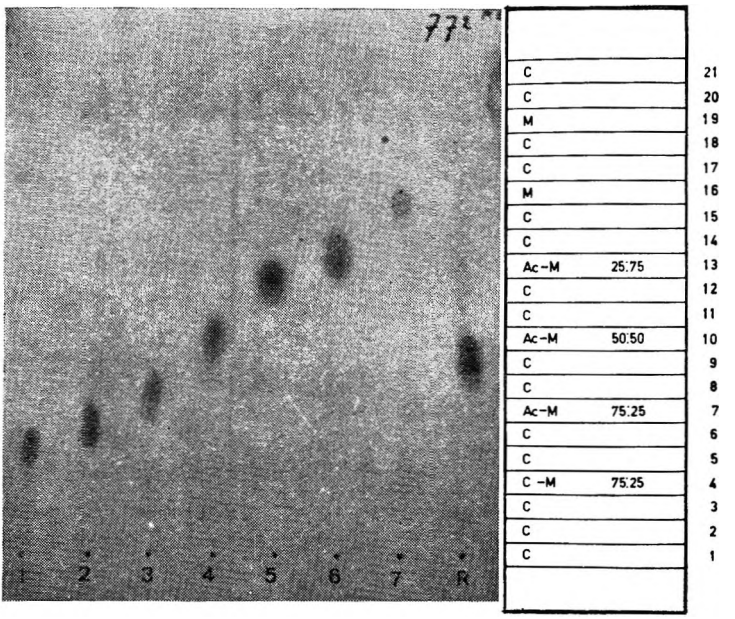


FIG. 6. The same substances as in Figs 4 and 5, now separated in the VC-chamber with hexane–chloroform–methanol (60 : 35 : 5) as solvent and a chloroform–acetone–methanol vapour gradient. The position of the troughs and the liquid composition therein during development are given at the right. Temperature 22°, relative humidity 45%, saturation 10 min, development 32 min, strips 0·5 mm, cooling ground plate 20°. Code: C = chloroform, M = methanol, Ac = acetone. Numbering as in Fig. 4.

(15 min), heated (30 min) at 110° in an oven with a fan, then cooled and stored in a desiccator.

Solutions (5 μl) in chloroform (barbiturates) or ethanol (anaesthetics), were applied with 10 μl micropipettes, 2·5 cm from the bottom edge of the plate, 1·5–2 cm apart.

Troughs were filled with about 5 ml each of the appropriate liquid mixtures, the plate fixed in position, and after allowing time for equilibration (10 min) the solvent reservoir was filled with 25 ml of running solvent.

All experiments were made at 20–22° and a relative humidity of 39–45%. Within these ranges reproducibility of the chromatogram runs was observed.

Normal chromatograph tanks, 21 × 21 × 9 cm, were used as controls. These contained 100 ml of solvent and were saturated with solvent vapour by lining the inner walls with filter paper. After 45–60 min the thin-layer plate was introduced and development started. Solvent was allowed to run 15–17 cm.

Spots were visualized under ultraviolet light of 254 mμ (Camag), chromatograms where photographed under two such light sources on Agfacolor CT 18 Diapositive film with an Asahi-Pentax camera, type SV,

with 49 mm ultraviolet "ghostless" filter; exposure time 15 sec, distance 70 cm, aperture 5·6.

On all plates, 4-nitroaniline reference (R), was run as a control substance.

Results and discussion

The separation of 14 commonly used barbiturates in the normal tank using chloroform–isopropanol–25% ammonia (45 : 45 : 10) is shown in Fig. 2. The spread of the spots is poor using about one-third of the plate only. Changing the ratio of solvent composition does not materially improve the separation or the spread. Fig. 3 shows the results obtained on the same compounds in a VC-chamber. The running solvent was chloroform–isopropanol (92·5 : 7·5), saturated with 25% ammonia used in conjunction with mixtures of chloroform–isopropanol–methanol–ammonia to form a vapour gradient. For the interspersed troughs of low polarity, solvent chloroform saturated with 25% ammonia was used. The spread of spots almost extends over the entire plate; the size of the spots is not significantly increased. Most of the barbiturates are now clearly separated, although there remain a few critical pairs. Such pairs can often be separated by application of slightly different gradients. The overall time required for this separation in the VC-chamber is almost the same as in the normal tanks. The time for development in the former is longer but this is compensated for by the fact that the saturation time can be reduced to 10 min due to the small space between the plate and the troughs.

A similar comparison of methods can be made with local anaesthetics. Seven chemically related substances chromatographed by the classical method are poorly separated using hexane–chloroform–methanol (60 : 35:5) and silica gel impregnated with sodium hydroxide as adsorbent* (Fig. 4). With a more polar solvent like chloroform–methanol (95 : 5) the spots run faster and separation is worse (Fig. 5). The results with the VC-chamber and the systems used in Fig. 4 are shown in Fig. 6. Chloroform–acetone–methanol was used for the vapour gradients. Separation is complete and can be used for identification purposes, with a spread of spots over the plate of about 50%. The time required for the separation of the anaesthetics in the VC-chamber is about 30% less than in the normal tank.

Acknowledgements. The author is indebted to Mr. J. Wijsbeek for skillful assistance, to Prof. Dr. J. S. Faber and Dr. D. A. Doornbos for critical comments and to Mr. A. Oosterhoff and Mr. G. P. Rijskamp for constructing the VC-chamber.

* 0·1N sodium hydroxide is used to prevent retention of the basic substances at the starting point.

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Pharm. Pharmac., 1968, **20**, *Suppl.*, 61S–64S Received May 1, 1968

SHORT COMMUNICATION

ɛamination of fennel fruits by gas chromatography ithout preliminary distillation

J. BETTS

Γ has been observed that growing fruits of fennel, *Foeniculum vulgare* Mill., of both the bitter (var. *vulgare* (Mill.) Thelung) and sweet (var. *lce*) forms grown in the same garden develop approximately the same ıount of anethole per 100 fruits during their maturation (Betts, 1968). irteen dried fennel fruits of various origins have now been examined by s-liquid chromatography to see how the content and proportion of the ential oil constituents relate to the values previously recorded. Anethole ıtents differed widely, and a fennel virtually free of anethole was found. tragole was present instead of anethole, its presence being confirmed by rared spectroscopy. This fennel has not been reported in commerce ore.

ERIMENTAL

Drug material and its extraction. Specimens of dried fennel fruit were ained from the sources indicated in Table 1, where they are also cribed. 20 to 50 larger mericarps of each specimen, excluding stunted ıbnormal fruits, were crushed under n-hexane (0·5 ml) and the solvent anted into a 2 ml volumetric flask; the fruit residue was triturated in with further small quantities of n-hexane and the extracts used to ke the contents of the flask up to volume. (This n-hexane contained -carvone as an internal standard, together with sufficient dry ethanol ield a clear solution. Carvone was selected as having a retention time ween anethole and estragole.)

as chromatography was as previously described (Betts, 1968), three mns being used under the operating conditions detailed in Table 2, hydrogen supplied to the flame ionization detector at half the column rate, thus giving maximum detector sensitivity. 1 μl aliquots of the el extracts were injected onto the columns, reference solutions of hole, estragole, fenchone and limonene being used to identify and uate peaks.

olation of fennel fruit constituent for infrared spectroscopy. Crushed el fruit J (0·8 g) was co-distilled with water (100 ml) and n-hexane l) for 3 hr in the B.P. apparatus for Determination of Volatile Oil in gs. The oil in hexane condensate was dried and passed through a mn of silicagel MFC (2·5 g), eluting with n-hexane. After removal of ne hydrocarbons, the eluate of the next constituent was evaporated on

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TABLE 1. ORIGIN AND DESCRIPTION OF FENNEL SPECIMENS

Reference letter and source	Size (length × width)	Colour	Form	Av. wt. i mg of 10 cremocarp
A Museum specimen "Roman"	16–8 mm × 3 mm	Pale brown	Mostly obovoid lanceolate cremocarps	1670
B Essen Botanical Garden, Germany. Recent	10–9 mm × 3 mm	Dark green with buff ridges	Mostly flat or recurved mericarps, well ridged	1555
C Museum specimen "Saxon"	9–6 mm × 3 mm	Dark brown with buff ridges	Mixed cremocarps and mericarps	1460
D Museum specimen "Indian" presented 1939	8–6 mm × 2·5 mm	Pale buff	Mostly cremocarps	1190
E Stock drug at School of Pharmacy	9–5 mm × 2·5 mm	Pale buff -greenish	Mixed cremocarps and mericarps	1165
F Myddelton House, 1967. "Sweet" variety grown in School Drug Garden	6 mm × 3 mm	Brownish buff	Mostly ovoid arcuate mericarps, well ridged	1130
G Retail grocer's proprietary spice pack	9–6 mm × 2·5 mm	Pale buff -greenish	Mostly cremocarps	1090
H Museum specimen, un-named	8–6 mm × 3 mm	Pale buff -greenish	Mixed cremocarps and mericarps	785
J Istanbul Botanical Garden, Turkey. Recent	6 mm × 1·5 mm	Grey brown -greenish	Mostly narrow mericarps, some recurved, not well ridged	710
K Nsukka, Nigeria, Africa. Recent	7–5 mm × 1·5 mm	Grey or brown	Mostly narrow cremocarps with very fine wavy ridges	560
L Broadstairs, Kent, England. Wild, sea cliffs, 1965	4–3 mm × 1·5 mm	Dark grey	All ovoid arcuate mericarps	540
M Myddelton House, 1967. "Bitter" variety grown in School Drug Garden	4·5 mm × 1·5 mm	Dark grey -brownish	Mostly ovoid arcuate mericarps	530
N Myddelton House, 1966. "Bitter" variety grown in School Drug Garden	4·5 mm × 1 mm	Brown with lighter ridges	Mostly ovoid arcuate mericarps	475

TABLE 2. RETENTION TIMES RELATIVE TO (—)-CARVONE OF FENNEL FRUIT STITUENTS ON THE GAS CHROMATOGRAPHIC COLUMNS

Column		10% polyethylene glycol adipate on 100–120 mesh Celite, purged at 215°	10% silicone elastomer E 301 on 100–120 mesh Chromosorb W, purged at 325°
Operating temperature		140°	125°
Theoretical plates (approx.):	fenchone	1200	550
	estragole	2000	1000
	anethole	2350	1600
t_r rel.:	limonene	0·08	0·32
	fenchone	0·25	0·42
	estragole	0·69	0·72
(standard)	carvone	1·00	1·00
	anethole	1·33	1·16
	anisaldehyde	2·8	1·6

Mobile phase nitrogen, with flow rate at column exit 40 ml/min. The Carbowax column pre used (Betts, 1968) gave very similar results to those above with polyethylene glycol adipate.

sodium chloride discs for infrared spectroscopy. The spectrum obta (Perkin-Elmer 237) was that of estragole, not anethole, and the constit gave a gas chromatographic peak corresponding to estragole.

RESULTS AND DISCUSSION

Wide variations were noted in the content of the constituents of the essential oils of different fennel fruits (Table 3). This bore no relation to the weight of the fruits. In Museum specimens A, C, D (Table 1), as well as specimen G, the anethole had partly decomposed to anisaldehyde on prolonged storage. Calculations from the peak area of the latter substance were made, based on the relative molecular hydrocarbon content of the two molecules, to deduce the amount of anethole that had decomposed, these values being added to the anethole figures in Table 3. In the Indian fennel D, most of the anethole had decomposed.

TABLE 3. ESSENTIAL OIL CONSTITUENTS OF FENNEL SPECIMENS

Reference (see Table 1 for details)	mg per 100 cremocarps (and % of total)					Oil yield % w/w
	Anethole + *anisal*[1]	Estragole	Fenchone	Limonene etc.[2]	Total	
Bitter (approx. 60% anethole, 30% fenchone)						
N	19·9 (63)	0·7	10·2 (32)	0·8	31·6	6·66
M	19·1 (64)	0·7	9·1 (31)	0·9	29·8	5·62
C	13·9 + *3·3* (55)	1·9	10·5 (34)	1·5	31·1	2·13
A	1·3 + *1·4* (59)	0·5	1·1 (24)	0·3	4·6	0·28
D	0·1 + *0·9* (56)	0·2	0·5 (28)	0·1	1·8	0·15
Sweet (approx. 80% anethole, 10% fenchone)						
B	42·6 (81)	1·8	5·7 (11)	2·3	52·4	3·37
F	19·5 (82)	0·8	3·2 (13)	0·3	23·8	2·11
K	6·3 (79)	0·3	0·9 (11)	0·5	8·0	1·43
G	5·8 + *0·6* (80)	0·3	0·8 (10)	0·5	8·0	0·73
Anethole-free (approx. 80% estragole, 15% fenchone)						
L	nil	14·1 (86)	2·4 (14)	trace	16·5	3·06
J	trace	13·9 (84)	2·7 (16)	trace	16·6	2·34
H	trace	11·8 (79)	2·2 (15)	0·9	14·9	1·90
Anomalous						
E	4·1 (46)	3·4 (38)	0·7 (8)	0·7	8·9	0·76

[1] Anisaldehyde figures have been converted to anethole equivalents and added to the anethole to give the percentage of this in the total oil, as anisaldehyde is a decomposition product.
[2] Limonene figures include total terpene hydrocarbons.

With the exception of specimen E, all fennel fruits examined fell into three groups as indicated in Table 3. The classification has been based on the percentage of anethole and fenchone present in the essential oil. The first and second groups probably represent the bitter and sweet varieties respectively, whilst the third group may be *Foeniculum piperitum* Presl., in which estragole replaces anethole as chief essential oil component (Pellini, 1923). Figures for the anethole and fenchone content of the dried Myddelton House grown fruits F, M, N corresponded fairly well with those obtained from fresh, ripe fruits (Betts, 1968). The slightly lower anethole content observed may represent some oil loss on drying and

storing the fruits (in thick polythene bags), for Tóth (1967b) observed a decrease during two years' storage in brown paper bags. Specimen K, for which Osisiogu (1967) found 2·2% oil, only yielded 1·4% here. Such losses may account for the low oil content of some Museum fennels, although specimen C contained as much oil as recent collection N.

Results here confirm the previously observed disagreement (Betts, 1968) with the analyses of Tóth (1967a). Of the thirteen fennels examined, only one contained (slightly) less than 10% fenchone. Either two forms of bitter fennel were examined here, or Tóth suffered preferential loss of fenchone during steam distillation to isolate the oils. Osisiogu (1967) could not detect fenchone in his distilled oil although it is present in fennel K.

The highest proportion of estragole previously recorded for fennel fruit oil is 20% by Naves & Tucakov (1959). Estragole (allyl-*p*-methoxybenzene) shows distinctive infrared spectral peaks at wavenumbers greater than 3020 cm^{-1} and in the region of 1640, 990 and 910 cm^{-1}, these not being shown by its propenyl isomer anethole. These and other matching features could even be detected in the spectrum of the unfractionated oil obtained by steam distillation from anethole-free fennel. Its presence in a Museum specimen and in drug stock E (see below) indicates that this non-B.P.C. fennel occurs in commerce. Such fruits have no distinctive morphological or sensory character, although estragole has a less intensely spicy odour than anethole. The two isomers have the same Rf value and give similar response to detection methods on thin-layer chromatograms (Tóth, 1967b; Betts, 1968). Gas chromatographic examination by direct solvent extraction of the fruits, as used here, is the best method for detecting the anethole-free fennel. The technique is sensitive enough to work with single cremocarps using a concentrated extract. Atypical fennel E was thus found to be a mixed specimen; the larger, narrower fruits being a sweet fennel contributing the anethole and fenchone content of the bulk, whilst the smaller, wider fruits were an estragole fennel, unusually almost devoid of fenchone as well as anethole. On cultivation, the anethole-free fruits grow into plants bearing leaves devoid of anethole and rich in estragole, so the distinction is maintained at other stages of plant growth.

Acknowledgements are due, as in the previous paper (Betts, 1968) to Mr. L. Guglielmi, Mr. M. Humphrey and Dr. I. U. W. Osisiogu for providing some fruits or materials. Thanks also to Mr. J. W. Adcock for help with spectroscopy.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 65S–73S Received April 26, 1968

Isothermal diffusion measurement on the system water–sodium salicylate–sodium chloride at 25° at one composition

A. DESHMUKH AND R. FLEMING

The main and cross-term diffusion coefficients for the system water–sodium-salicylate–sodium chloride have been measured at the compositon 0·05 g cc^{-1}, and the results indicate that sodium chloride is effective in producing a flow of sodium salicylate at this concentration. Density, refractive index, partial specific volumes and gravitational data are also reported. The system has been assumed to be 3-component.

THE Goüy interferometric technique has been used to measure the main and cross-term diffusion coefficients for the system water–sodium salicylate–sodium chloride. The initial results indicate that the flow interaction of both solutes is taking place. Such studies provide basic information about diffusional processes in the system and may be of use in describing the flow of these substances in a biological environment.

Theory

In an isothermal system where there are more than two components and where the components do not diffuse independently, the solute flows can be described by the expression (Baldwin, Dunlop & Gosting, 1955)

$$(J_i)_v = -\sum_{j=1}^{r} (D_{ij})_v \frac{\partial \rho_i}{\partial x} \qquad (i = 1, 2, \ldots, r) \quad \ldots \quad \ldots \quad (1)$$

in which ρ_i is the concentration of component i in g cc^{-1}, $(D_{ij})_v$ are the diffusion coefficients corresponding to the volume fixed reference frame and have the units of cm sec^{-1}, and J_i is the flow of component i in units of g cm^2 sec^{-1}. The summation is taken over r solutes.

Thus the flow of sodium salicylate which in this paper has been given the subscript 1 is described by the equation

$$(J_1)_v = -D_{11}\frac{\partial \rho_1}{\partial x} - D_{12}\frac{\partial \rho_2}{\partial x} \quad \ldots \quad \ldots \quad (2)$$

and the flow of sodium chloride, which has been given subscript 2

$$(J_2)_v = -D_{21}\frac{\partial \rho_1}{\partial x} - D_{22}\frac{\partial \rho_2}{\partial x} \quad \ldots \quad \ldots \quad (3)$$

For the small concentration differences used in these differential diffusion experiments, the volume fixed reference frame can be considered to be identical with the cell (or apparatus)—fixed reference frame (Kirkwood, Baldwin & others, 1960). Because of its greater ionic radius, the salicylate ion would be expected to diffuse slower than the smaller sodium and

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chloride ions. Furthermore, from *a priori* considerations of ionic mobilities the flow of salicylate $(J_1)_v$ produced by the flow of sodium chloride will be large and the cross-term diffusion coefficient $(D_{12})_v$ finite. Conversely, the flow of sodium chloride $(J_2)_v$ due to the salicylate gradient will be small and the cross-term diffusion coefficient $(D_{21})_v$ correspondingly small but non-zero.

APPARATUS

The optical beam of the diffusiometer consists of two rigid cast-iron beds, of uniform box-like construction 3·2 m and 1·8 m in length, purchased from Hilger and Watts. These kinematically mounted beds rest on a platform of braced iron girders whose weight is evenly distributed on eight Barrymount type SM4–8 spring mountings. This method of support ensures that the rigid substructure has a minimal sensitivity to extraneous vibration. Light from a water-cooled 100 watt mercury-vapour G.E.C. lamp passes through a Wratten No. 77A filter which isolates the mercury green line at 5460·7 Å before being brought to a focus on a Hilger and Watts source slit (1 division = 0·005 mm). The image of the slit is focussed on a photographic plate by a 101·5 cm focal length acromatic doublet objective lens, 11 cm in diameter and corrected to $\frac{1}{8}$th wavelength at 5460·7 Å.

The water bath has a capacity of 100 litres and is controlled to better than (25 $\pm$ 0·01°). It has four optical flat windows 2 cm thick, 11 cm in diameter and corrected to $\frac{1}{20}$th of a wavelength at 5460·7 Å. The frame holding the 11 cm Tiselius cell is similar to that described by Gosting, Hanson & others (1949), and by Dunlop & Gosting (1953; 1955).

Experimental

A rough boundary was formed in the Tiselius cell between the upper solution A and the lower more dense solution B. This crude boundary was then brought down through the cell and sharpened at the optic axis position by siphoning off the liquids through a stainless-steel capillary at a rate of approximately 1·4 to 1·6 ml/min. When 80 ml of liquid had been collected the flow was stopped, the capillary was withdrawn and the top and bottom sections of the Tiselius cell were closed. Photographs of the interference pattern were taken at times t′ after stopping the flow from the stainless-steel capillary. Usually 10 to 12 photographs were taken on Kodak Ortho 800 plates. Measurements of the distance of the fringe minima from the optic axis were made to 0·001 mm using a Gaertner M2001P toolmakers' microscope, and C_t, the maximum displacement of light according to ray optics at time t′ is found by the customary extrapolation procedure. For each photograph a preliminary value of D_a', the reduced height–area ratio, was calculated using the relation

$$D_a' = \frac{J_m^2 \lambda^2 b^2}{C_t^2 t' 4\pi} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (4)$$

where J_m is the total number of fringes in the pattern, $\lambda = 5460{\cdot}7 \times 10^{-8}$ and b is the optical lever distance from the centre of the Tiselius cell to the photographic plate emulsion (b = 260·1973 cm).

The integral number of fringes was counted directly from an early pattern, the fractional part being measured using horizontal Rayleigh slits. A plot of D'_a versus $\frac{1}{t'} = 0$ was made using a least-squares procedure to obtain a value of D_a the reduced-height area ratio, corrected for any initial imperfections in the boundary. Values of the starting time correction Δt, obtained from the slope of this graph varied between 6·7 and 14·5 sec. A fringe-deviation graph was plotted of Ω_j the average value of the reduced fringe deviation against $f(\zeta)$ the reduced fringe number, where

$$\Omega_j = e^{-(\zeta)2} - Y_j/C_t \quad .. \quad .. \quad .. \quad (5)$$

in which Y_j is the fringe displacement from the optic axis and

$$f(\zeta) = \frac{j + 0{\cdot}75}{J_m} \quad .. \quad .. \quad .. \quad (6)$$

in which j has an integral value greater than 6.

The area of the fringe deviation graph Q where

$$Q = \int_0^1 \Omega_j df(\zeta) \quad .. \quad .. \quad .. \quad .. \quad (7)$$

was calculated using Simpson's $\frac{1}{3}$rd rule. Mean values of Ω_j were read at intervals of 0·025 along the $f(\zeta)$ axis from a large scale fringe-deviation graph and calculation of the four diffusion coefficients $(D_{1j})_v$ was then made by the method of Fujita & Gosting (1960).

A set of four experiments were made with α_1 equal to approximately 0·0, 0·2, 0·8 and 1·0 where α_1 the refractive index fraction is defined by

$$\alpha_1 = \frac{R_i \Delta\rho_1}{\sum_{j=1}^{2} R_j \Delta\rho_j} \quad (i = 1, 2) \quad .. \quad .. \quad .. \quad (8)$$

and where R_1 and R_2, the refractive index increments are given by

$$n(\rho_1, \rho_2) = n(\tilde{\rho}_1, \tilde{\rho}_2) + R_1(\rho_1 - \tilde{\rho}_1) + R_2(\rho_2 - \tilde{\rho}_2) \quad .. \quad (9)$$

In equation (9), $n(\rho_1, \rho_2)$ is the refractive index of the solutions with the concentrations of the solutes ρ_1, ρ_2; $n(\tilde{\rho}_1, \tilde{\rho}_2)$ is the refractive index of the solution when $\rho_1 = \tilde{\rho}_1$ and $\rho_2 = \tilde{\rho}_2$. In all experiments $\Delta\rho_1 + \Delta\rho_2 = 0{\cdot}01$ g cc^{-1} which gave a convenient number of fringes ($J \simeq 90$).

The concentration increment between upper and lower solutions

$$\Delta\rho_i = [(\rho_i)_B - (\rho_i)_A] \quad (i = 1, 2) \quad .. \quad .. \quad (10)$$

The first two experiments were made with α_1 equal to zero and unity respectively. Sufficient data were then available to solve the equation

$$\frac{\lambda}{a}\left(\frac{J_m}{\Delta\rho_1 + \Delta\rho_2}\right) = \left(\frac{\Delta\rho_1}{\Delta\rho_1 + \Delta\rho_2}\right)(R_1 - R_2) + R_2 \quad .. \quad (11)$$

simultaneously for R_1 and R_2. In equation (11), the internal width, a, of the diffusion cell, is 2·4979 cm. These values of the refractive index increments were then used to calculate the concentrations required to yield solutions for which $\alpha_1 = 0{\cdot}2$ and $\alpha_1 = 0{\cdot}8$.

By inserting the four experimentally determined values of α_1 and D_a in equation (12), and applying a least squaring procedure, values for the coefficients I_a and S_a were obtained together with a smoothed value for D_a.

$$\frac{1}{\sqrt{D_a}} = I_a + S_a\alpha_1 \qquad .. \qquad .. \qquad .. \qquad (12)$$

Fujita & Gosting (1960) have shown that the term $Q/\sqrt{D_a}$ can be related to the four diffusion coefficients and α_1 by the following expression

$$E = \frac{Q}{\sqrt{D_a}} = E_0 + E_1\alpha_1 - E_2\alpha_1^2 \qquad .. \qquad .. \qquad (13)$$

Since I_a and S_a are themselves functions of $(D_{ij})_v$, it is necessary to use a method of successive approximations to derive accurate values for E_0, E_1 and E_2, from which the four $(D_{ij})_v$ values can be calculated.

By measuring the slope of the graph $\dfrac{E - E_0}{\alpha_1}$ a first approximation for E_2 is obtained, the preliminary value for E_0 being taken as the experimental value of $Q/\sqrt{D_a}$ at $\alpha_1 = 0$.

After rearrangement of terms, equation (13) will yield first approximations for the ratios E_0/E_2 and E_1/E_2, which can be smoothed by a least squaring procedure and from which approximate values of $(D_{ij})_v$ can be derived. Since $E_2 \propto S_a^2$ (Fujita & Gosting, 1960) an improved value of E_2 can then be computed together with subsequent values of E_0/E_2 and E_1/E_2, until the values of $(D_{ij})_v$ from two successive calculations agree to within $(0{\cdot}0001 \times 10^{-5})$. These calculations were carried out using the Atlas computer of the University of London Institute of Computer Science.

MATERIALS

Sodium salicylate and Analar sodium chloride were purchased from British Drug Houses Ltd. The molecular weights were taken to be 160·11 and 58·45 respectively.

SOLUTIONS

To prepare these solutions at the appropriate concentrations it was necessary to predict the densities of the solutions within a few parts in a hundred thousand. Previous experiments on the binary system water–sodium salicylate (unpublished) showed that the densities could be predicted in the region of $\bar{\rho}_1 = 0{\cdot}05$ g cc^{-1} by the equation

$$d = 0{\cdot}99724_3 + 0{\cdot}4056_5\rho_1 - 0{\cdot}00049_3\rho_1^2 + 0{\cdot}00021_3\rho_1^3 \qquad .. \qquad (14)$$

which was obtained by the method of least squares.

The density of a 0·05 g cc^{-1} solution of sodium chloride was estimated using the relation (Woolf, Miller & Gosting, 1962)

$$d = d_0 + \frac{C_2}{1000}(M_2 - d_0\phi_2) \quad .. \quad .. \quad (15)$$

in which d_0 the density of pure water at 25° was taken as 0·997048 g cc^{-1}, c_2 is the molar concentration and M_2 the molecular weight of sodium chloride. ϕ_2, the partial molal volume was calculated from the formula (Harned & Owen, 1958)

$$\phi_2 = 16{\cdot}50 + 2{\cdot}034\ C_2^{1/2} + 0{\cdot}0121\ C_2^{3/2} \quad .. \quad .. \quad (16)$$

In this way the contributions of sodium chloride and sodium salicylate to the density of a solution containing 0·05 g cc^{-1} of each solute could be estimated. The predicted value was tested experimentally and the differences between the two values was noted, and for solutions at other concentrations ($\alpha_1 = 0$, $\alpha_1 = 1$) the difference was subtracted from the predicted densities using equations (15) and (16).

The density of each ternary mixture was measured in triplicate at 25°C ± 0·01 using matched, single stem, Pyrex glass pycnometers which were weighed against a calibrated, sealed tare of similar shape filled with air-free double distilled water, whose density was taken as 0·997048 g cc^{-1}.

By applying multilinear regression analysis to the density data obtained from the measurement of the two solutions in which $\alpha_1 = 0$ and $\alpha_1 = 1$ respectively the expression

$$d = 0{\cdot}99854_8 + 0{\cdot}39713_8\rho_1 + 0{\cdot}65696_0\rho_2 \quad .. \quad .. \quad (17)$$

was obtained for the density of a ternary water–sodium salicylate–sodium chloride solution. This relationship was used to predict the densities of solutions in which $\alpha_1 = 0{\cdot}2$ and $\alpha_1 = 0{\cdot}8$. Analysing the density data from experiments (10–12) in a similar way produced a refined expression

$$d = 0{\cdot}99855_0 + 0{\cdot}39341_3\rho_1 + 0{\cdot}66044_0\rho_2 \quad .. \quad .. \quad .. \quad (18)$$

for the density of the solution in terms of ρ_1 and ρ_2. The average deviation of values predicted by equation (18) and the experimentally obtained density measurements is ±0·0018%.

All solutions were prepared by weighing in air and were subsequently corrected for weight *in vacuo*.

The partial specific volumes of solvent and solutes were calculated using equations (Dunlop & Gosting, 1959)

$$\bar{v}_i = \frac{1 - H_i}{d - (H_1\rho_1 + H_2\rho_2)} \qquad (i = 1, 2) \quad .. \quad (19)$$

$$d = \rho_0 + \rho_1 + \rho_2 \quad .. \quad .. \quad .. \quad .. \quad (20)$$

$$\bar{v}_0\rho_0 + \bar{v}_1\rho_1 + \bar{v}_2\rho_2 = 1 \quad .. \quad .. \quad .. \quad (21)$$

where d is the density of the solution in g cc^{-1}, H_i is a density derivative

and the subscripts 0, 1 and 2 refer to water, sodium salicylate and sodium chloride respectively. The partial specific volume and density results are reported in Table 1.

TABLE 1. PARTIAL SPECIFIC VOLUME AND DENSITY DATA FOR THE SYSTEM: WATER–SODIUM SALICYLATE–SODIUM CHLORIDE AT 25°C

Experiment No.	9	10	11	12
$(\rho_1)_A$	$0{\cdot}044864_3$	$0{\cdot}046198_6$	$0{\cdot}049167_8$	$0{\cdot}050005_8$
$(\rho_2)_A$	$0{\cdot}049845_7$	$0{\cdot}048802_6$	$0{\cdot}045829_0$	$0{\cdot}045023_4$
d_A	$1{\cdot}04910_8$	$1{\cdot}04896_9$	$1{\cdot}04813_7$	$1{\cdot}04796_6$
$(\rho_1)_B$	$0{\cdot}054833_8$	$0{\cdot}053801_7$	$0{\cdot}050826_9$	$0{\cdot}050000_2$
$(\rho_2)_B$	$0{\cdot}049886_7$	$0{\cdot}051197_7$	$0{\cdot}054165_6$	$0{\cdot}055025_9$
d_B	$1{\cdot}05309_4$	$1{\cdot}05354_4$	$1{\cdot}05428_8$	$1{\cdot}05457_8$
H_1	$0{\cdot}393_4$			
H_2	$0{\cdot}660_4$			
$\bar{v}_0$	$1{\cdot}0014_4$			
$\bar{v}_1$	$0{\cdot}607_5$			
$\bar{v}_2$	$0{\cdot}340_1$			

0 = H_2O
1 = Salicylate
2 = NaCl

A = upper solution
B = lower solution

Units: concentrations (ρ_1, ρ_2), g cc^{-1};
partial specific volumes ($\bar{v}_i$), cc g^{-1}
density (d_A, d_B), g cc^{-1}.

Discussion

The values for the refractive index derivatives R_1 and R_2, $(D_{1j})_V$ and areas of the fringe deviation graphs are reported in Table 2. (Lines 8–9, 10–13, and 4 respectively.)

TABLE 2. DIFFUSION DATA FOR TERNARY SYSTEM WATER–SODIUM SALICYLATE–SODIUM CHLORIDE AT 25° AT ONE COMPOSITION

1	Experiment No.	9	10	11	12
2	$J_{expt.}$	90·13	85·78	75·90	71·80
3	$J_{calc.}$	90·16	85·77	74·95	71·92
4	$Q_{expt.} \times 10^4$	$12{\cdot}_{24}$	$25{\cdot}_{63}$	$10{\cdot}_{66}$	$-44{\cdot}_{60}$
5	$D_a \times 10^5$	$0{\cdot}7860_0$	$0{\cdot}8673_0$	$1{\cdot}2358_0$	$1{\cdot}4060_0$
6	$D_{a\ calc.} \times 10^5$	$0{\cdot}7845_7$	$0{\cdot}8697_1$	$1{\cdot}2334_0$	$1{\cdot}4072_6$
7	α_1	0·9967	0·7991	0·1996	− 0·0007
8	R_1	0·19706			
9	R_2	0·15731			
10	$(D_{11})_V \times 10^5$	$0{\cdot}768_6 \mp 0{\cdot}001_9$			
11	$(D_{12})_V \times 10^5$	$0{\cdot}076_7 \mp 0{\cdot}001_8$			
12	$(D_{21})_V \times 10^5$	$0{\cdot}029_0 \pm 0{\cdot}003_8$			
13	$(D_{22})_V \times 10^5$	$1{\cdot}258_4 \pm 0{\cdot}003_4$			

0 = H_2O
1 = Salicylate
2 = NaCl

$\tilde{\rho}_1$ = 0·05
$\tilde{\rho}_2$ = 0·05

Units: Densities ρ_i, g cc^{-1};
Reduced height: Area ratio D_a, g cm^2 sec^{-1};
Diffusion coefficients $(D_{ij})_V$, g cm^2 sec^{-1} corresponding to a volume-fixed frame of reference;
Refractive index derivatives R_i, cc g^{-1}.

The Tiselius cell was found to have a small positive area Q′ when fringe deviation graphs were plotted for data obtained from the binary system water–sodium salicylate (unpublished). Values of Q measured for the ternary system were then adjusted by subtracting $0{\cdot}2 \times 10^{-4}$ from each observed value.

The range of accuracy indicated for the values of $(D_{ij})_v$ were calculated by changing the value of Q by its anticipated error of $\pm 1 \times 10^{-4}$ and evaluating the corresponding values for the diffusion coefficients. As $\rho_2 \to 0$ at constant ρ_1, the main diffusion coefficient $(D_{11})_v$ approaches $(D_1)_m$, the binary mutual diffusion coefficient for water–sodium salicylate. Similarly, as $\rho_1 \to 0$ at constant ρ_2, $(D_{22})_v$ approaches $(D_2)_m$, the binary mutual diffusion coefficient for water–sodium chloride. Measurement of these limiting values of $(D_{11})_v$ and $(D_{22})_v$ will involve further experiments on this system at other compositions combined with data obtained from tracer diffusion studies. The literature value (Vitagliano & Lyons, 1955) for $(D_2)_m$ at this concentration is $= 1{\cdot}456_0 \times 10^{-5}$ cm^2 sec^{-1}, whilst the result obtained from preliminary experiments carried out on the binary system water–sodium salicylate for a mean concentration of $0{\cdot}05$ g cc^{-1} is $0{\cdot}913_9 \times 10^{-5}$ cm^2 sec^{-1}. These values may be compared with lines 13 and 10 respectively of Table 2.

Since $(D_{12})_v$ and $(D_{21})_v$ are both positive and significantly different from zero, a concentration gradient of either solute contributes significantly to the flow of the other solute. In particular, at solute concentrations of $0{\cdot}05$ g cc^{-1} a concentration gradient of sodium chloride is about $\frac{1}{10}$th as effective in producing a flow of salicylate as the concentration gradient of sodium salicylate.

Clearly, because of the difference in the ionic mobilities of the ions involved, electrostatic potential gradients are set up in the system due to the flows. In experiment (12) the Cl^- ions tend to move faster than the Na^+ ions and in this process some Na^+ ions were transported with salicylate$^-$ ions. Similarly, some Cl^- ions were transported with Na^+ ions in experiment (9).

In ternary systems the condition

$$\rho_B > \rho_A \qquad .. \quad .. \quad .. \quad .. \qquad (22)$$

does not by itself ensure that

$$\left(\frac{\partial \rho}{\partial x}\right)_t > 0 \qquad .. \quad .. \quad .. \quad .. \qquad (23)$$

The latter inequality is a necessary and sufficient condition for the gravitational stability (G_α, G_β) of the diffusing columns of liquid.

G_α and G_β have been calculated using equations (24–30) derived by Wendt (1962).

$$G_\alpha = H_1K_1^- + H_2K_2^- \qquad .. \quad .. \quad .. \quad .. \qquad (24)$$

$$G_\beta = H_1K_1^+ + H_2K_2^+ + \sqrt{\sigma_-/\sigma_+}\,(H_1K_1^- + H_2K_2^-) \qquad (25)$$

where

$$K_1^{\pm} = \pm \frac{[(D_{22} - D_{11} \pm U)\Delta\rho_1 - 2\,D_{12}\Delta\rho_2]}{4U} \quad .. \quad (26)$$

$$K_2^{\pm} = \pm \frac{[(D_{11} - D_{22} \pm U)\Delta\rho_2 - 2D_{21}\Delta\rho_1]}{4U} \quad .. \quad .. \quad (27)$$

$$\sigma\pm = \frac{(D_{11} + D_{22} \pm U)}{2S^2} \quad .. \quad .. \quad .. \quad .. \quad (28)$$

and

$$U = [(D_{22} - D_{11})^2 + 4D_{12}D_{21}]^{1/2} \quad .. \quad .. \quad .. \quad (29)$$

$$S = (D_{11}D_{22} - D_{12}D_{21})^{1/2} \quad .. \quad .. \quad .. \quad .. \quad (30)$$

The values obtained are summarized in Table 3.

Because $G_\alpha \geqslant 0$ .. .. (31)

$G_\beta > 0$ .. .. (32)

these diffusion columns are gravitationally stable.

G_α decreases rapidly with increase in $\Delta\rho_1$ and decrease in $\Delta\rho_2$, and since $\Delta\rho_1 + \Delta\rho_2 \simeq 0{\cdot}01$ convective mixing would occur if $\Delta\rho_2$ were only slightly less than the lowest figure reported. In contrast, G_β decreases less rapidly with decrease in $\Delta\rho_2$. Thus, for the condition $G_\beta < 0$ to obtain, $\Delta\rho_2$ would have to be considerably smaller than its lowest value in Table 3.

TABLE 3. DATA FOR THE GRAVITATIONAL STABILITY FOR THE EXPERIMENTS REPORTED

Experiment No.	9	10	11	12
$\Delta\rho_1$	$0{\cdot}009969_6$	$0{\cdot}007603_1$	$0{\cdot}001659_1$	$-0{\cdot}000005_8$
$\Delta\rho_2$	$0{\cdot}000041_0$	$0{\cdot}002395_1$	$0{\cdot}008336_6$	$0{\cdot}010001_7$
G_α	$0{\cdot}00022_4$	$0{\cdot}00101_4$	$0{\cdot}00301_8$	$0{\cdot}00357_6$
G_β	$0{\cdot}00192_8$	$0{\cdot}00206_1$	$0{\cdot}00241_6$	$0{\cdot}00250_7$

Units: concentration ρ_i, g cc^{-1}; Gα and Gβ, g cc^{-1} 1 = Salicylate 2 = NaCl $\tilde{\rho}_1 = 0{\cdot}05$ $\tilde{\rho}_2 = 0{\cdot}05$

A non-Gaussian refractive-index curve could arise from hydrolysis of the sodium salicylate producing a multicomponent system, from the concentration dependence of the solute diffusion coefficients or because refractive index and concentration are not linearly related. Unfortunately, the Goüy fringes are not markedly sensitive to skewed distributions, but it is hoped that further investigation may enable more definite conclusions to be reached about the physical state of the system.

Acknowledgements. The instrument was built with grants received from The Royal Society (Government grant), the Science Research Council and the Central Research Fund of the University of London.

The authors are deeply indebted to Professor Gosting for drawings of the Tiselius cell-holder and for his advice on the apparatus optics. One of us (A.D.) is grateful to the S.R.C. for the award of a research studentship. Calculations were carried out on the Atlas computer, Institute of Computer Science, University of London and on an Olivetti 101 Programma desk computer.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 74S–81S Received May 2 1968

Gas-liquid chromatography of heteroyohimbine alkaloids: the effect of methoxy substitution and of configuration

A. H. BECKETT AND D. DWUMA-BADU

The gas–liquid chromatographic retention times of heteroyohimbine alkaloids on a 1% SE-30 column are in the order pseudo < epiallo < allo < normal. The introduction of one methoxy group into the indole nucleus doubles the retention time while two methoxy groups increase it by a factor of four. Binding of the indole nucleus reinforced by the suitably orientated lone pair electrons of the basic nitrogen to the liquid phase of the column in the important conformers of the various configurations, is used to explain the results.

AMONG the heteroyohimbine alkaloids stereochemical features have been shown to influence their behaviour on thin-layer chromatograms (Phillipson & Shellard, 1967).

The present work describes the use of gas–liquid chromatography for the separation and characterization of heteroyohimbine alkaloids of known stereochemistry (Wenkert & Bringi, 1959; Wenkert, Wickberg & Leicht, 1961a, b; Shamma & Moss, 1961; Joshi, Raymond-Hamet & Taylor, 1963; Beckett, Shellard & Tackie, 1965; Lee, Trager & Beckett, 1967; Trager, Lee & Beckett, unpublished observations). The retention times of these alkaloids are interpreted in terms of stereochemical, conformational and electronic factors.

Experimental

Alkaloids. Tetrahydroalstonine, aricine, reserpinine, tetraphylline, isoreserpinine, reserpiline, rauniticine, raunitidine, corynantheidine, mitragynine (Smith Kline and French Laboratories, Philadelphia, U.S.A.); raumitorine and epi-3-rauvanine (Dr. J. Poisson); rauvanine (Dr. M. M. Janot); isoraunitidine (Dr. M. Shamma); dihydrocorynantheine (S. B. Penick & Co.); speciogynine, speciociliatine, mitraciliatine and paynantheine (Dr. J. D. Phillipson); akuammigine, hirsutine and mitrajavine (Dr. E. J. Shellard); 3-isocorynantheidine and iso-paynantheine were prepared from corynantheidine and paynantheine respectively (unpublished).

APPARATUS

A Perkin Elmer Model F.11 Gas Chromatograph with hydrogen flame ionization detector was used under the following conditions: 1% S.E.30 on Gas-Chrom. P, 80–100 mesh, acid washed and treated with dimethyl dichlorosilane, oven temperature 215°, injection temperature 330°, hydrogen pressure 20 lb/inch^2, air pressure 25 lb/inch^2, nitrogen pressure 10 lb/inch^2, column length 1 metre. The packed column was conditioned at 190° under continuous nitrogen flow for four days.

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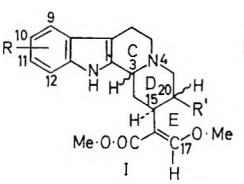
ALKALOIDAL SOLUTIONS

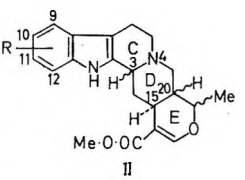
0·1% Solutions of the alkaloids in ethyl acetate were used and 3 μl injected until a constant retention time was obtained. The 0·1% solution was diluted to 0·05 and 0·025% and injections made to check whether retention times changed with dilution. The retention times of all the alkaloids were determined relative to ajmalicine which was maintained at constant retention time throughout the experiment by suitable adjustment of the nitrogen gas flow through the column.

Results and discussion

The retention times of the heteroyohimbine alkaloids possessing the closed ring E are shown in Table 1 and those of the open ring E in Table 2.

The alkaloids are of two main types: (I) heteroyohimbine alkaloid with open ring E (I) in which R=H or OMe and R′=Et or $-CH=CH_2$ and (2) heteroyohimbine alkaloids with closed ring E (II) in which R=H, mono or di-OMe groups.





The alkaloids of type I have the same stereochemistry at C-15 and about the double bond; four diastereoisomers are then possible as follows (Trager, Lee & Beckett, 1967):

Configuration	C-3H	C-15H	C-20H
Normal	α	α	β
Pseudo	β	α	β
Allo	α	α	α
Epiallo	β	α	α

The closed ring E compounds have an extra asymmetric centre at C-19 and the methyl group is designated α or β respectively when below or above ring E.

Tables 1 and 2 show that the retention times of these alkaloids are influenced principally by (*a*) the introduction of methoxy substituents into the indole nucleus and (*b*) the overall geometry of the molecule, i.e., whether normal, pseudo, allo or epiallo. Differences in the location of the methoxy substituents have only a slight influence on retention times [Table 1: cf. compounds 9 with 8 (Rt 20·3, 19·7) and 2 with 3 (Rt 22·5, 22·7)]; this effect is also shown in simple indoles where retention times (at 105° on 1% SE-30, N_2 7 lb/inch², H_2 20 lb/inch², air 20 lb/inch²; injection temp. 160°) are: indole 3·1, 4-OMe indole 9·3, 5-OMe indole 10·1 and 5-OHMe indole 10·3. Table 1 shows that one methoxy group approximately doubles the retention time of the compound with none (cf. 1 with 2, 6 with 7, 11 with 12, 15 with 16) whilst two methoxy groups

TABLE 1. RETENTION TIMES AND CONFIGURATIONS OF CLOSED RING E HETERO-YOHIMBINE ALKALOIDS

Alkaloid	Configuration[1] R		Configuration of C-19-Me	Retention time (min)	pKa[2]
1 Ajmalicine	Normal	H	α	10·5	6·31
2 Tetraphylline	Normal	11-OMe	α	22·5	6·39
3 Raumitorine	Normal	10-OMe	β	22·7	
4 Rauvanine	Normal	10,11-di-OMe	β	40·1	
5 Rauniticine	Allo	H	β	7·1	6·24
6 Tetrahydroalstonine	Allo	H	α	8·9	5·83
7 Raunitidine	Allo	11-OMe	β	16·3	6·20
8 Aricine	Allo	10-OMe	α	19·7	5·75
9 Reserpinine	Allo	11-OMe	α	20·3	6·01
10 Iso-reserpiline	Allo	10,11-di-OMe	α	34·0	6·07
11 Akuammigine	Epiallo	H	α	7·1	
12 Iso-reserpinine	Epiallo	11-OMe	α	15·6	6·49
13 Iso-raunitidine	Epiallo	11-OMe	β	17·9	6·42
14 Reserpiline	Epiallo	10,11-di-OMe	α	26·3	6·20
15 Iso-ajmalicine	Pseudo	H	α	5·3	
16 Mitrajavine	Pseudo	9-OMe	α	9·9	
17 Epirauvanine	Pseudo	10,11-di-OMe	β	23·6	

[1] Wenkert & others (1961a, b); Shamma & Moss (1961).
[2] Moss (1962).

TABLE 2. RETENTION TIMES AND CONFIGURATION OF OPEN RING E HETEROYOHIMBINE ALKALOIDS

Alkaloid	Configuration[1] R		R′	Retention time (min)	pKa[2]
18 Dihydrocorynantheine	Normal	H	–Et	9·9	7·47
19 Speciogynine	Normal	9-OMe	–Et	21·3	7·40
20 Paynantheine	Normal	9-OMe	$-CH=CH_2$	21·0	7·42
21 Corynantheidine	Allo	H	–Et	8·7	7·15
22 Mitragynine	Allo	9-OMe	–Et	16·7	7·06
23 3-Isocorynantheidine	Epiallo	H	–Et	4·2	7·45
24 Speciociliatine	Epiallo	9-OMe	–Et	16·3	7·44
25 Hirsutine	Pseudo	H	–Et	6·3	7·89
26 Mitraciliatine	Pseudo	9-OMe	–Et	12·1	7·95
27 Isopaynantheine	Pseudo	9-OMe	$-CH=CH_2$	12·0	

[1] Tamelin, Aldrich & Katz (1956); Wenkert & Bringi (1959); Joshi & others (1963); Bartlett, Sklar & others (1962); Shamma & Moss (1962); Weisbach, Kirkpatrick & others (1965); Lee & others (1967); Trager, Phillipson & Beckett (1968); Trager, Lee & others (1967).
[2] Beckett & Morton (1967).

increase it by a factor of 4 (cf. 1 with 4, 5 with 10, 11 with 14, 15 with 17).

Because of conformational changes (see later) the introduction of one methoxy group into the epiallo open ring E alkaloids has a greater effect than the other substitution. The change from open ring E to closed ring E compounds with an α-C-19-methyl group produces minor effects on retention time (Tables 1 and 2: cf. 18 with 1, 21 with 6, 25 with 15, 19 with 2) provided of course that major conformational changes are also not involved (see below). Replacement of the C-20 ethyl group by the C-20 vinyl group in the open ring E alkaloids has little effect on the retention time (Table 2: cf. 19 with 20, 26 with 27).

THE EFFECT OF CONFIGURATION ON RETENTION TIMES

From Tables 1 and 2 it can be seen that there is a progressive increase in retention time in the order pseudo < epiallo < allo < normal except

that the retention times of the epiallo open ring E compounds are apparently anomalous; the retention time of the non-methoxy compound (23) showing a fall compared with the pseudo compound (25) (Rt 4·2, 6·3) while that of the monomethoxy compound (24), instead of being less, is similar to the corresponding allo compound (22) (Rt 16·3, 16·7).

It has been established (Trager, Lee & Beckett, 1967), that in the open ring E alkaloids, the normal, pseudo and allo configurations exist almost exclusively in the conformations shown in Fig. 1. In non-polar solvents, the epiallo configuration exists as an equilibrium between DI and DIII conformations (see Fig. 1) with DI predominating.

In the closed ring E alkaloids with the C-19-α-methyl group, the normal, pseudo and allo configurations exist almost exclusively in conformations analagous to those of the open ring E alkaloids for rings A, B, C, D (see Fig. 2), whereas the epiallo configuration exists as an equilibrium between

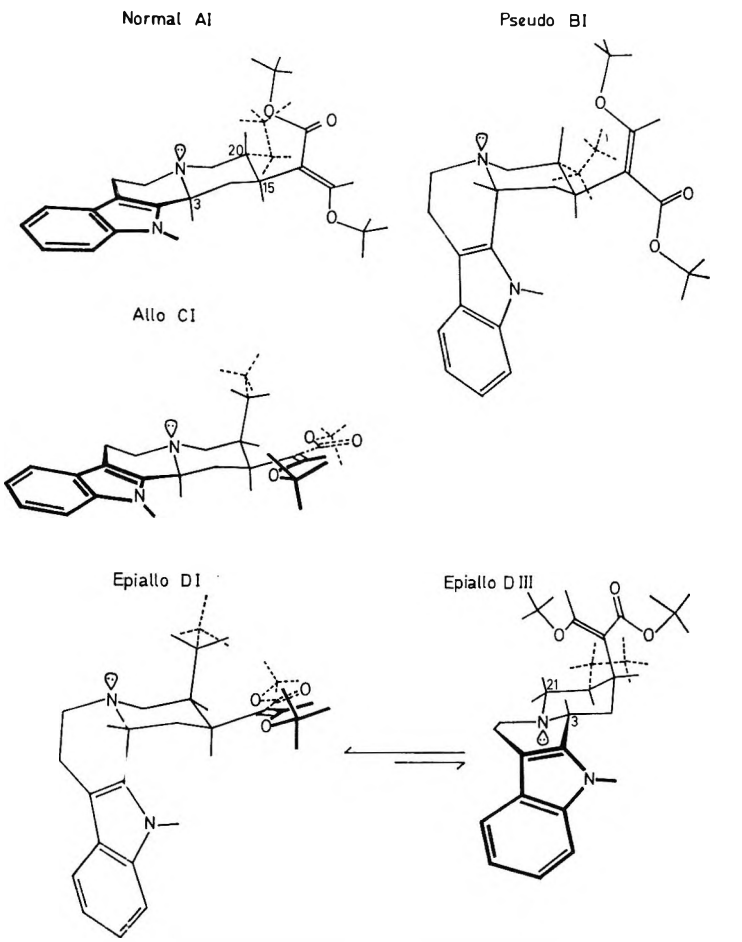


FIG. 1. The preferred conformations of open ring E alkaloids (Trager & others, 1967) AI, normal; BI, pseudo; CI, allo; DI and DIII epiallo.

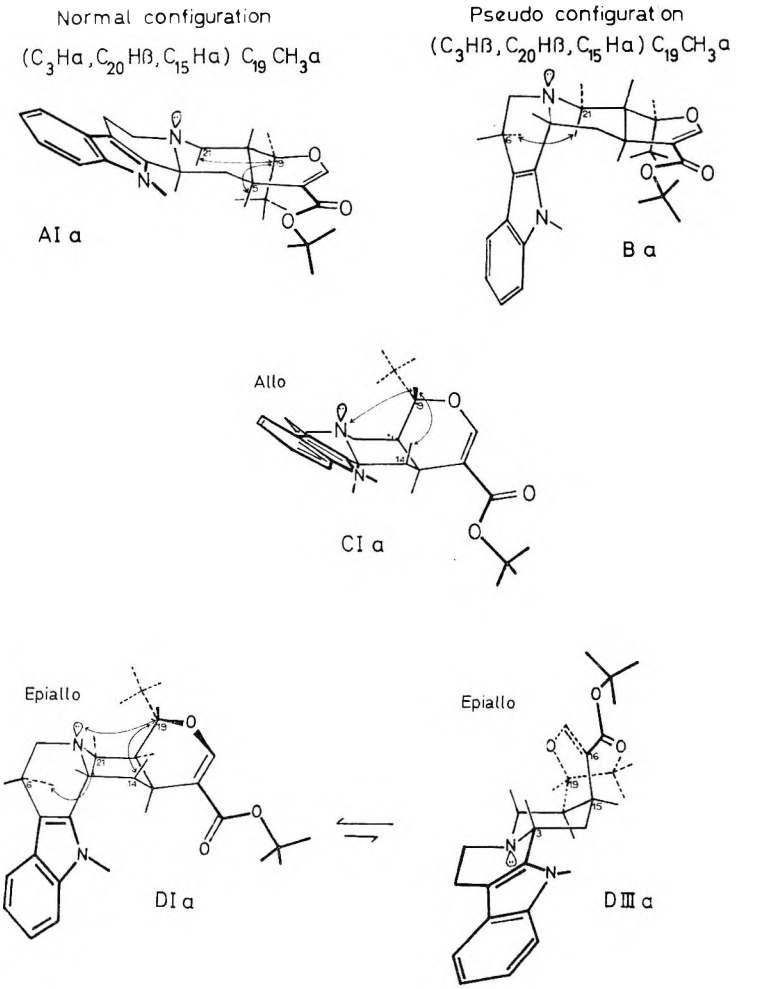


FIG. 2. The preferred conformations of closed E-ring $C_{19}-CH_3\alpha$ heteroyohimbine alkaloids (Trager, Lee & Beckett, unpublished observations) AIα, normal; Bα, pseudo; CIα, allo; DIα and DIIIα, epiallo.

DIα and DIIIα in roughly equal amounts. Models suggest that the change from C-19-α-methyl to C-19-β-methyl does not alter the preferred conformation of the normal and pseudo configurations (Fig. 3). Such a change in C-19 geometry, however, would be expected to cause the allo configuration to exist as an equilibrium mixture of about equal contributions from CIβ and CIIIβ (Fig. 3). This change in the C-19-methyl would also result in the epiallo equilibrium between DIβ and DIIIβ being displaced more in the direction of DIIIβ (Fig. 3).

INTERPRETATION OF RETENTION TIMES IN TERMS OF PHYSICO-ORGANIC CHARACTERISTICS

We first consider the gas–liquid partition characteristics of those

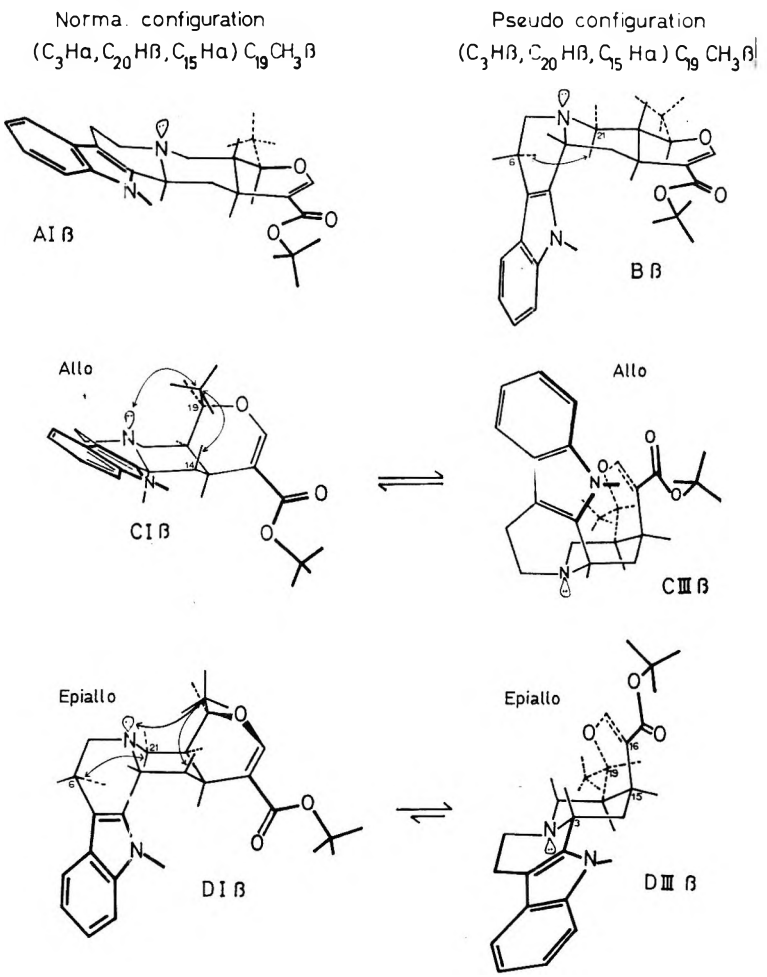


FIG. 3. The preferred conformations of closed ring E C-19-Meβ heteroyohimbine alkaloids (Trager, Lee & Beckett, unpublished observations). AIβ, normal; Bβ, pseudo; CIβ and CIIIβ allo; DIβ and DIIIβ, epiallo.

configurations which exist substantially as one conformer, i.e., normal, pseudo and allo configurations of open ring E alkaloids and closed ring E alkaloids with the C-19-α-methyl group.

Since the change from the open to the closed ring E makes only small changes in the retention times of these alkaloids (Tables 1 and 2: cf. 18 with 1, 25 with 15, 21 with 6), the ring E probably plays little part in the equilibrium between stationary phase and carrier gas despite the variety of conformations it adopts in these various alkaloids (see Figs 1 and 2).

On the other hand, methoxy groups in the indole nucleus displace the equilibrium in favour of the stationary phase in a consistent manner and play a major role. In the normal isomers, rings A, B, C and D are in

the same plane whilst in the pseudo isomers (see Figs 1 and 2) ring D is at right angles to the co-planar A, B and C. Such a change reduces the retention times significantly (Table 2: cf. 18 with 25, 19 with 26, 20 with 27; Table 1: cf. 1 with 15, 4 with 17), although this effect is not so great as that introduced by methoxy groups. In the normal configuration, the lone pair of the basic nitrogen atom is suitably orientated to reinforce the binding of the indole nucleus to a surface whereas its direction in the pseudo isomer (Figs 1 and 2) is in the plane of rings A, B and C rather than at right angles to this plane.

We suggest (*a*) that displacement of the equilibrium in favour of the SE-30 stationary phase is strongest in those isomers in which the binding of the indole nucleus to the stationary phase is reinforced by the optimally orientated lone pair of the basic nitrogen atom and (*b*) that introduction of the methoxy groups into the indole nucleus has an even greater effect than the optimally orientated lone pair. Changes in configuration and conformation, in so far as they may influence the closeness of approach to the stationary phase may then be used to interpret the results obtained for the other heteroyohimbine alkaloids.

In the open ring E allo alkaloids, the axial C-20-ethyl group (Fig. 1) constitutes more of a barrier to nitrogen lone-pair reinforcement than does the equatorial orientation in the corresponding normal alkaloids; retention times are thus reduced (Table 2: cf. 21 with 18, 22 with 19); the change from normal to allo geometry in the closed ring E alkaloids with the C-19-α-methyl group (Table 1: cf. 1 with 6, 2 with 9) has the same effect. This steric factor has less influence than the change in orientation of the lone pair relative to the plane of the indole nucleus. Thus allo compounds in the open ring E and C-19-α-methyl closed ring E series have shorter retention times than their corresponding normal isomers but longer than the corresponding pseudo compounds.

When more than one conformation is present in major amounts in the equilibrium mixtures for a particular configuration, as in the open ring E and closed ring E epiallo alkaloids and in the closed ring E allo alkaloids with C-19-β-methyl groups, changes in conformation may produce apparent anomalies to the above generalizations.

The retention time of the epiallo open ring E alkaloid containing a methoxy group (24) is longer than that of a corresponding pseudo alkaloid (26; Rt 16·3, 12·1) but shorter when this group is absent (cf. 23 with 25; Rt 4·2, 6·3). This may be explained by the substantial contribution of DIII in the methoxy epiallo configuration in which the nitrogen lone pair can reinforce the inherent binding of the methoxy substituted indole nucleus at a surface. In the absence of this strongly binding methoxy group, DI (Fig. 1), will be the preferred epiallo conformation; this conformation is similar to the pseudo BI conformation but possesses an axial C-20-ethyl rather than the equitorial one in BI. Despite the fact that in BI and DI the lone pair is not correctly orientated to substantially reinforce the indole nucleus binding, it will be expected that an axial ethyl will reduce binding more than the equitorial one.

Consideration of the conformation of normal ring E (Fig. 1, AI) and the

normal closed ring E alkaloids with C-19-methyl-α (Fig. 2, AIα) or β (Fig. 3, AIβ) indicates that corresponding alkaloids of these series should have the same retention times; results are in accord with this view (Tables 1 and 2; cf. 18 with 1, 19 with 2 and 3). Also, the closed ring E alkaloids with C-19-methyl α or β in the normal series should have similar retention times to those of the allo series with C-19-α-methyl since this group represents the same steric hindrance to the nitrogen lone pair, in the conformations AIα, AIβ and CIα (Figs 2 and 3); results are in accord with this deduction (Table 1: cf. 1 with 6, 2 with 3 and 9). Corresponding allo alkaloids with C-19-methyl-β, because of the conformational equilibrium between CIβ and CIIIβ (Fig 3), should have shorter retention times than the C-19-methyl-α isomers since in CIIIβ the nitrogen lone pair orientation cannot reinforce the binding of the indole nucleus; results support this conclusion (Table 1: cf. 5 with 6, 7 with 9). In the closed ring E epiallo configurations, the greater contribution of DIIIβ in the C-19-β-methyl isomer (13) with the nitrogen lone pair suitably orientated to the indole nucleus to reinforce binding (Fig. 3) should have a slightly longer retention time than the corresponding C-19-α-methyl isomer (12) in which DIIIα (Fig. 2) is less important in the equilibrium mixtures (Table 1: cf. 13 with 12). Also a consideration of the conformational contributions of CIβ and CIIIβ in the allo C-19-β-methyl configuration and the DIα and DIIα conformation in the epiallo C-19-α-methyl configuration leads to the conclusion that allo-β and epiallo-α alkaloids should have similar retention times and this is borne out by results (Table 1: cf. 5 with 11, 7 with 12).

Acknowledgements. We thank Smith, Kline and French Laboratories, Philadelphia, Dr. J. Poisson, Dr. M. M. Janot, Dr. M. Shamma, S. B. Penick & Co., Dr. E. J. Shellard, and Dr. J. D. Phillipson for generously supplying alkaloids. The authors are grateful to Mr. Brown for preparing the figures; Mr. R. E. Haddock, Dr. C. M. Lee and Mr. A. C. Moffat for helpful discussions.

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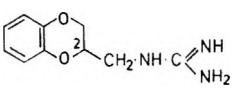
J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 82S–93S Received April 30, 1968

The preparation and properties of (+)- and (−)-guanoxan

J. B. STENLAKE*, D. D. BREIMER§, G. A. SMAIL*, ANNE STAFFORD** AND W. C. BOWMAN†

(±)-2-Aminomethyl-1,4-benzodioxan has been resolved into its optically active isomers: (+)- and (—)-guanoxan have been synthesized from these. The pharmacological effects of racemic-guanoxan and of the two optical isomers have been compared on the isolated central ear artery of the rabbit, on the pithed rat in which pressor responses were evoked by stimulation of the thoraco-lumbar sympathetic outflow, and on the pre- and post-ganglionically stimulated nictitating membrane of the cat. The two isomers were equipotent in producing adrenergic neuron blockade. Initial catecholamine release was weak in the cat, but occurred more powerfully in the rabbit ear artery and in the rat. Ability to produce this effect resided mainly with the (+)-isomer. α-Adrenoreceptor blocking activity was detectable in the rat and was produced mainly by the (+)-isomer suggesting that its stereochemical configuration corresponds to that of D-(—)-noradrenaline. Ganglion blockade was an unimportant action of the compounds, but both isomers possessed weak atropine-like activity in the rat.

GUANOXAN, (±)-guanidinomethyl-1,4-benzodioxan (I) is an antihypertensive agent (Augstein & Green, 1964; Peart & MacMahon, 1964), which acts primarily by preventing the release of noradrenaline from adrenergic nerve endings (Davey & Reinert, 1965; Augstein, Green & others, 1965; Baines, Cobb & others, 1965), although it also has some α-adrenoreceptor blocking activity, especially in the dog (Davey & Reinert, 1965). Its action resembles that of guanethidine (II) in that it depletes peripheral adrenergic neurons of their stores of noradrenaline. In contrast to guanethidine, however, it also depletes noradrenaline stores in the hypothalamus (Augstein & others, 1965; Davey & Reinert, 1965).



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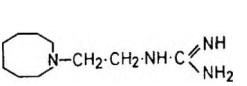

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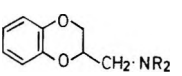

III

Guanoxan (I) possesses an asymmetric centre at the 2-position, and hitherto has not been resolved into its optical isomers. The stereoisomers of adrenaline, noradrenaline and other sympathomimetic amines show marked differences in pharmacological action, and similar differences

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have been observed in the potency of isomeric pairs of various anti-adrenaline drugs, including the benzodioxan (III R = Et) (Trefouel, Trefouel & Dunant, 1935), of which the (−)-isomer is about six times as effective as the (+)-isomer in blocking the pressor actions of adrenaline in the cat (Bovet & Simon, 1935). Since guanoxan also possesses an asymmetric centre at position 2, it was considered of interest to isolate the two optical isomers of (±)-guanoxan for pharmacological evaluation.

Chemistry

Although guanoxan readily forms salts with many of the optically active acids used as resolving agents, the salts are insoluble in most organic solvents. For this reason direct resolution of guanoxan proved impossible; 2-methylamino-1,4-dioxan (III, R = H), however, was successfully resolved.

Salts with several acids, including D-tartaric acid, D-dibenzoyltartaric acid, D-camphorsulphonic acid, D-quinic acid and L-mandelic acid, were prepared by the general procedure of Greenstein & Winitz (1961). Of these, the (−)-2-aminomethyl-1,4-benzodioxan L-(+)-mandelate crystallized preferentially from ethyl acetate-absolute ethanol (3:1), whilst the (−)-2-aminomethyl-1,4-benzodioxan D-(−)-dibenzoyltartrate crystallized from methanol.

Decomposition of these purified salts as described in the experimental procedures gave (+)- and (−)-2-aminomethyl-1,4-benzodioxans; the rotations of the derived hydrochlorides were $[\alpha]_D^{21}$ + 72·2° (*c*, 0·483 MeOH) and $[\alpha]_D^{21}$ − 72·9° (*c*, 0·461 MeOH) respectively, thus confirming their optical purity.

(+)- and (−)-Guanoxan sulphates were synthesized by treating the corresponding (+)- and (−)-2-aminomethyl-1,4-benzodioxans with *S*-methylisothiourea sulphate. The chemical purity of the products was confirmed by elementary analysis and thin-layer chromatography in two separate solvent systems. Both isomers showed complete absence of traces of the respective (+)- and (−)-2-aminomethyl-1,4-benzodioxans, and *S*-methylthiourea sulphate, and also had the same Rf values as authentic (±)-guanoxan sulphate. The optical purity follows from the specific rotations of the two isomeric guanoxan sulphates which were $[\alpha]_D^{22}$ + 54·8° (*c*, 0·485 water) and $[\alpha]_D^{22}$ − 55·0° (*c*, 0·487 water) respectively.

EXPERIMENTAL

(±)-2-*Aminomethyl*-1,4-*benzodioxan* (III, R = H) may be obtained from guanoxan (I) as follows. The method used is that described by Carter, Clarke & others (1946) for the degradation of streptidine to streptamine. Guanoxan sulphate (54 g) was refluxed (30 hr) with sodium hydroxide solution (6N; 200 ml). Ether extraction in the usual way yielded (±)-2-aminomethyl-1,4-benzodioxan (20·9 g; 60·4%), b.p. 105°/0·5 mm; n_D^{17} 1·5512. Augstein & others (1965) quote

b.p. 92–94°/0·4 mm; n_D^{25} 1·5583. Hydrochloride, m.p. 219° (decomp.). Augstein & others (1965) give m.p. 220–222°.

(+)-2-*Aminomethyl*-1,4-*benzodioxan* L-(+)-*mandelate*. (±)-2-Aminomethyl-1,4-benzodioxan (17·5 g; 0·106 mole) in a mixture of ethyl acetate and ethanol (3:1; 400 ml) at 50° was added to L-(+)-mandelic acid (8·09 g; 0·053 mole) in the same solvent (350 ml) also at 50°. Crystallization (64 hr) at room temperature yielded a crude product (10·5 g), $[\alpha]_D^{21}$ + 80·5° (*c*, 0·796 MeOH), which after three re-crystallizations from the same solvent gave (+)-2-*aminomethyl*-1,4-*benzodioxan* L-(+)-*mandelate* (5·2 g; 31·4%), m.p. 167–168°, $[\alpha]_D^{22}$ + 90·5° (*c*, 0·772 MeOH). Found: C, 64·3; H, 5·7; N, 4·45. $C_{17}H_{19}NO_5$ requires C, 64·3; H, 6·0; N, 4·4%.

The mother liquors, cooled in the refrigerator for 24 hr, yielded further amine mandelate (1·3 g), $[\alpha]_D^{22}$ + 73·2° (*c*, 0·786 MeOH). Concentration of the remaining solution to 25 ml *in vacuo* gave a further crop of amine mandelate (3·5 g), $[\alpha]_D^{21}$ + 41·2° (*c*, 0·785 MeOH).

(−)-2-*Aminomethyl*-1,4-*benzodioxan* D-(−)-*dibenzoyltartrate*. The concentrated collected mother liquors from the preparation of (+)-2-aminomethyl-1,4-benzodioxan L-(+)-mandelate were diluted with methanol (25 ml), warmed to 50°, and mixed with a solution of D-(−)-dibenzoyltartaric acid (9·46 g; 0·265 mole) in methanol (40 ml) also at 50°. Crystallization (12 hr) at 0° yielded a crude product (12·3 g), $[\alpha]_D^{22}$ − 98·0° (*c*, 0·55 MeOH), which after three re-crystallizations from the same solvent gave (−)-2-*aminomethyl*-1,4-*benzodioxan* D-(−)-*dibenzoyltartrate* (5·6 g; 31·2%), m.p. 175–176°, $[\alpha]_D^{21}$ − 108·2° (*c*, 0·621 MeOH). Found: C, 62·6; H, 5·0; N, 4·1. $C_{36}H_{36}O_{12}N_2$ requires C, 62·8; H, 5·3; N, 4·1%.

(+)-2-*Aminomethyl*-1,4-*benzodioxan hydrochloride*. (+)-2-Aminomethyl-1,4-benzodioxan L-(+)-mandelate was treated with sodium hydroxide solution (2%), the liberated amine extracted with ether (3 × 25 ml). Treatment with dry HCl, gave (+)-2-*aminomethyl*-1,4-*benzodioxan hydrochloride*, m.p. 251° (decomp.), $[\alpha]_D^{21}$ + 72·2° (*c*, 0·483 MeOH). Found: C, 54·2; H, 6·2; N, 6·9%. $C_9H_{12}ClNO_2$ requires C, 53·6; H, 6·0; N, 7·0%.

(−)-2-*Aminomethyl*-1,4-*benzodioxan hydrochloride* similarly obtained from the dibenzoyltartrate had m.p. 251° (decomp.), $[\alpha]_D^{21}$ − 72·9° (*c*, 0·461 MeOH). Found: C, 53·9; H, 6·0; N, 7·2. $C_9H_{12}ClNO_2$ requires C, 53·6; H, 6·0; N, 7·0%.

(+)-*Guanoxan sulphate*. (+)-2-Aminomethyl-1,4-benzodioxan (2·46 g; 0·0149 mole) was stirred with a solution of *S*-methylisothiourea sulphate (Shildneck & Windus, 1943) (2·07 g; 0·0075 mole) in water (18 ml) at room temperature during three days. The product obtained by adding acetone was recrystallized from aqueous acetone, and dried *in vacuo* over phosphorus pentoxide. (+)-*Guanoxan sulphate* resulted (2·52 g; 63·4%), m.p. 225°, $[\alpha]_D^{22}$ + 54·8° (*c*, 0·485 water). Found: C, 47·1; H, 5·6; N, 16·5%. $C_{20}H_{28}N_6O_8S$ requires C, 46·9; H, 5·5; N, 16·4%.

(−)-*Guanoxan sulphate*. (−)-2-Aminomethyl-1,4-benzodioxan (2·3 g) treated with *S*-methylisothiourea sulphate as above gave, after three

recrystallizations, (—)-guanoxan sulphate (1·68 g; 47·2%), m.p. 224–224·5°, $[\alpha]_D^{22}$ — 55·0° (*c*, 0·487 water). Found: C, 47·0; H, 5·5; N, 16·4%. $C_{20}H_{28}N_6O_8S$ requires C, 46·9; H, 5·5; N, 16·4%.

Thin-layer chromatography on silica gel in chloroform–methanol–0·88 ammonia (75:20:5) gave Rf values for (+)- and (—)-guanoxan sulphate, 0·15 and 0·17; (+)- and (—)-2-aminomethyl-1,4-benzodioxan, 0·95; *S*-methylisothiourea sulphate, 0·64. Iodine vapour was used for visualization. n-Propanol–aqueous ammonium carbonate (M) (40:30) gave Rf values for (+)- and (—)-guanoxan sulphate, 0·40; for (+)- and (—)-2-aminomethyl-1,4-benzodioxan, 0·54. (Concentrated sulphuric acid was used as a spray reagent).

Pharmacology

EXPERIMENTAL

The effects of the two isomers and of the racemate were examined on three preparations.

1. *The isolated central artery preparation of the rabbit's ear* (de la Lande & Rand, 1965). Isolated arteries from 45 ears were perfused at a constant rate of 8 ml/min, with McEwen (1956) solution warmed to 36–37° and gassed with 5% carbon dioxide in oxygen. Sympathetic nerve endings within the artery wall were stimulated through periarterial electrodes with rectangular pulses of 1 or 2 msec duration at a frequency of 2/sec for 10 sec in every 3 min. The stimulus strength was greater than that required to produce maximal responses at this frequency. The preparations were left for 1–1½ hr until responses to nerve stimulation were constant. Injections of noradrenaline were made into the perfusion fluid just before it entered the lumen of the artery. (±)-, (+)- or (—)-Guanoxan sulphate was added to the reservoir of perfusion fluid, the final concentrations ranging from 0·07–0·2 μg/ml.

In a further series of experiments on this preparation, the paired ear arteries from 7 rabbits were perfused simultaneously. One artery of each pair was perfused successively with 3 concentrations of the (+)-isomer while the other was similarly and simultaneously perfused with the same 3 concentrations of the (—)-isomer to compare the ability of the two isomers to cause catecholamine release. The concentrations of the isomers used were 0·2, 0·5 and 1·0 μg/ml and each concentration was perfused for 20 min.

2. *The blood pressure of the rat.* Ten rats were pithed and prepared to record pressor responses from a carotid artery evoked by stimulation of the thoraco-lumbar sympathetic outflow by the method described by Gillespie & Muir (1967). Pressor responses were elicited every 5 min by stimulating for 10 sec at a frequency of 10/sec with pulses of 2 msec duration. The voltage output from the stimulator was 50 V and at this strength the pressor responses were submaximal. Drugs were injected through a cannula in a jugular vein.

The α-adrenoreceptor blocking action of guanoxan and of the isomers

was tested on similar pithed preparations in which the sympathetic outflow was not stimulated. Two of these rats were pretreated with reserpine (1·5 mg/kg intraperitoneally) on the evening before the experiment.

Three rats were anaesthetized with urethane (0·55 ml of a 25% solution per 100 g body weight) injected intraperitoneally, and the ECG was recorded. At 2 min intervals the left vagus nerve was stimulated for 5 sec with trains of rectangular pulses of 100 μsec duration. The frequency of the trains was such as to produce complete heart block and was constant in each experiment. Every 6 min, instead of vagal stimulation, acetylcholine (15 μg) was injected intravenously. (±)-Guanoxan sulphate and the two isomers were injected intravenously in doses of 0·5 to 2·0 mg/kg.

3. *Nictitating membrane of the cat.* Two cats were anaesthetized with a mixture of chloralose (80 mg/kg) and sodium pentobarbitone (6 mg/kg) injected intravenously. Contractions of both nictitating membranes were recorded simultaneously on smoked paper by means of isotonic frontal writing levers which magnified the contractions about 12 times. One nictitating membrane was excited by stimulation of the pre-ganglionic cervical sympathetic trunk after sectioning the nerve centrally to the electrodes, while the other was excited by stimulating the post-ganglionic trunk after crushing the superior cervical ganglion. Both stimulating electrodes were connected to the same output of the stimulator and contractions were evoked by stimulation at a frequency of 5/sec for 10 sec in every 2 min with rectangular pulses of 0·5 msec duration and of a strength greater than that necessary to produce maximal contractions at this frequency. Blood pressure was recorded from a femoral artery. Drugs were injected intravenously. The effects of (+)-guanoxan were studied in one cat and the effects of the (−)-isomer in the other.

The drugs used were: (−)-adrenaline (British Drug Houses), (−)-noradrenaline bitartrate (Koch-Light), acetylcholine chloride (Roche), (±)-guanoxan sulphate (Pfizer) and the two isomers of guanoxan prepared as described above. The doses quoted refer to the sulphates in the case of guanoxan and its isomers, and to the base or cation for the other drugs.

Results

Adrenergic neuron blockade. Since the adrenergic neuron blocking action of this type of compound is not readily reversed, only one of the isomers or the racemate was examined in each preparation. Fig. 1 expresses the mean results of experiments on 45 isolated rabbit ear arteries in which the time taken for each compound to reduce the constrictor response to nerve stimulation by 50% was noted. These experiments showed that there was little if any difference between the potencies of (±)-, (+)- and (−)-guanoxan as adrenergic neuron blocking drugs in this preparation, and similar results were obtained in the rat and the cat.

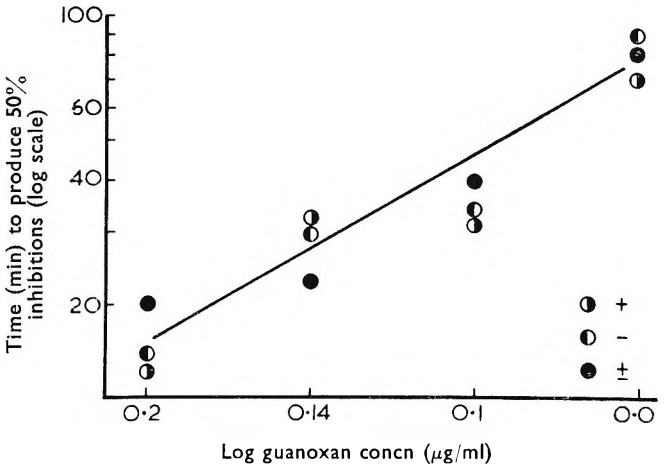

FIG. 1. Potencies of (+)-, (−)- and (±)-guanoxan in blocking responses of the isolated central artery of the rabbit's ear to periarterial nerve stimulation.

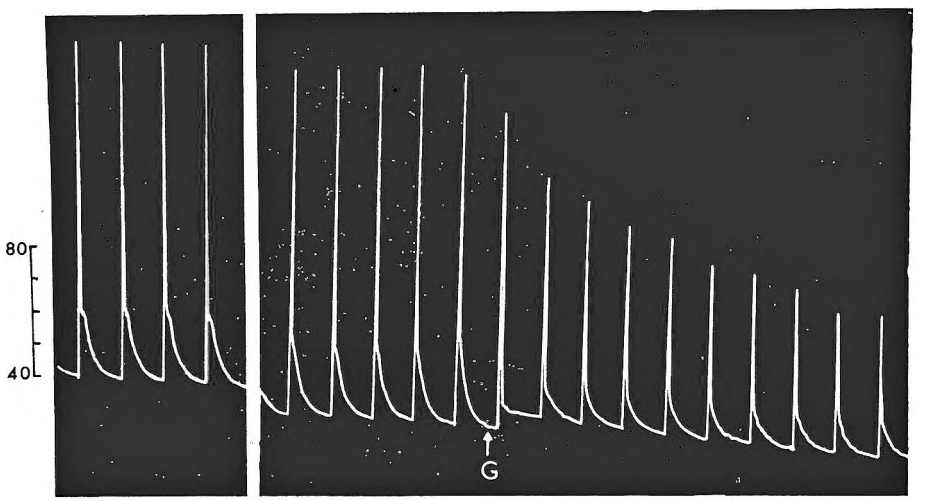

FIG. 2. Presso- responses (mm Hg) in the pithed rat to stimulation of the thoraco-lumbar sympathetic outflow (10/sec for 10 sec every 5 min). The gap in the record corresponds to 1 hr. At G, 0·5 mg/kg (±)-guanoxan intravenously.

In the pithed rat, pressor responses evoked by spinal cord stimulation usually increased in size during the first 30–60 min and then remained constant for several hours (Fig. 2). In each experiment, (±)-guanoxan, or one of the isomers, was initially injected in a dose of 0·25 mg/kg, and this dose reduced the pressor response in only 1 rat out of 10, and then only by 10%. Twenty min later, a dose of 0·5 mg/kg was injected and 20 min after that, a dose of 1 mg/kg was injected. The decrease in pressor response produced was noted 15–18 min after each injection and the results obtained for the racemate and the two isomers are expressed

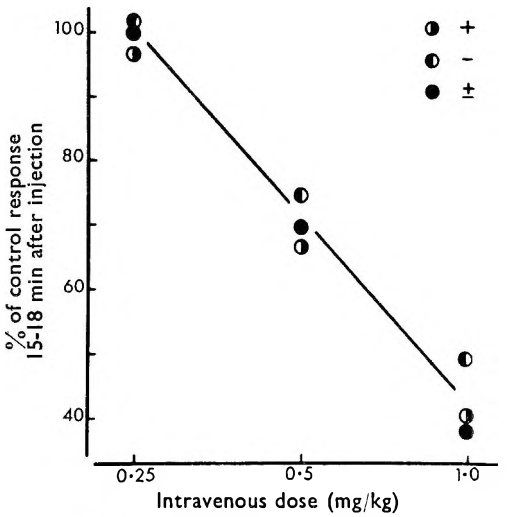

FIG. 3. Potencies of (−)-, (−)- and (±)-guanoxan in blocking pressor responses to spinal cord stimulation in the pithed rat.

graphically in Fig. 3. No account of cumulative effects could be taken and the graphs are therefore not meant to represent dose-response lines, but merely to illustrate that, as with the rabbit ear artery, there was little or no difference in the potencies of the three compounds.

In the two cats, the (+)- or the (−)- isomer were each injected at a dose level of 5 mg/kg intravenously. Responses of the nictitating membrane to both pre- and post-ganglionic stimulation were depressed at a similar rate and to a similar extent. In the dose used, both isomers produced about 80% depression of the nictitating membrane contractions within 20 min after injection. Fig. 4 illustrates the effects of the (+) isomer.

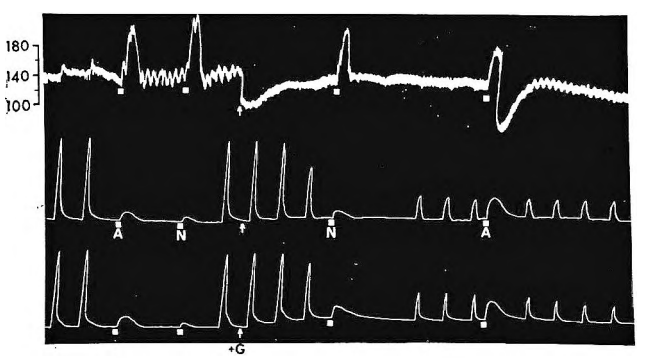

FIG. 4. Cat, 3·4 kg. Upper record, blood pressure (calibration in mm Hg); middle record, contractions of the right nictitating membrane in response to post-ganglionic stimulation; lowest record, contractions of the left nictitating membrane in response to pre-ganglionic stimulation (5/sec for 10 sec every 2 min). At A, 30 μg adrenaline; at N, 30 μg noradrenaline and at +G, 5 mg/kg (+)-guanoxan. All injections intravenously.

Davey & Reinert (1965) reported a similar potency of the racemic compound on the contractions of the cat nictitating membrane.

Catecholamine release. In concentrations of 0·2 μg/ml and above, (±)-guanoxan or the isomers usually produced constriction of the isolated rabbit ear artery. This effect of the (+)-isomer is illustrated in Fig. 8. Experiments on paired ear arteries showed that the (+)-isomer was more potent than the (−)-isomer in producing this effect (Figs 5 and 6) and the activity of the racemate is therefore primarily due to the (+)-isomer.

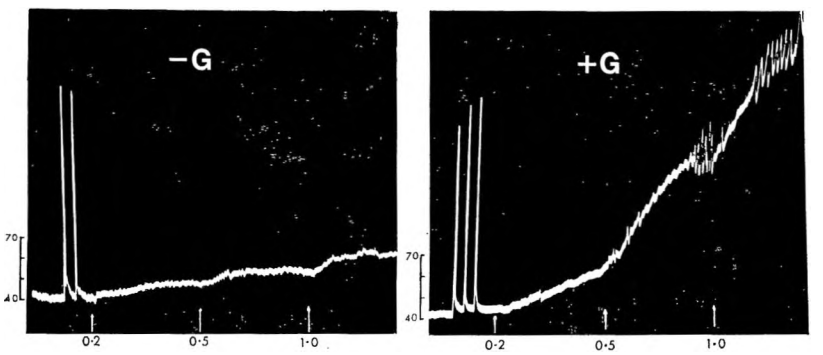

FIG. 5. Paired isolated central ear arteries of a rabbit. Responses on the left are to periarterial nerve stimulation. The left panel shows the effect of perfusion with (−)-guanoxan and the right with (+)-guanoxan. The concentrations, in μg/ml, are given below the arrows. Each concentration was perfused for 20 min. Calibration in mm Hg.

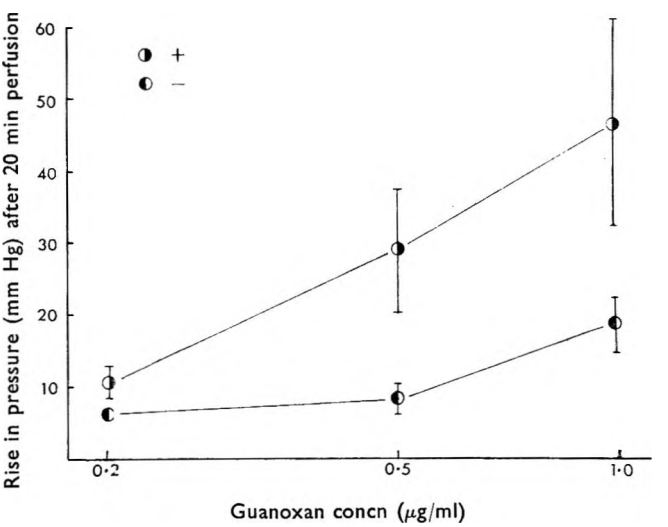

FIG. 6. Results of perfusion with (+)- or (−)-guanoxan obtained on 7 pairs of isolated central ear arteries of rabbits. The vertical lines are the standard errors

In the pithed rat, a transient rise (5–10 min) in blood pressure was produced immediately after intravenous injection of the compounds (Fig. 7A). With repeated injections, tachyphylaxis to this pressor effect was evident (Fig. 7A) but the results showed that the order of potency of the compounds in producing this effect was again (+) > (±) > (−). In pithed rats that had been pretreated with reserpine to deplete the stores of catecholamine, the pressor response to the compounds was weak or absent (Fig. 7B) indicating that it was probably the result of catecholamine release.

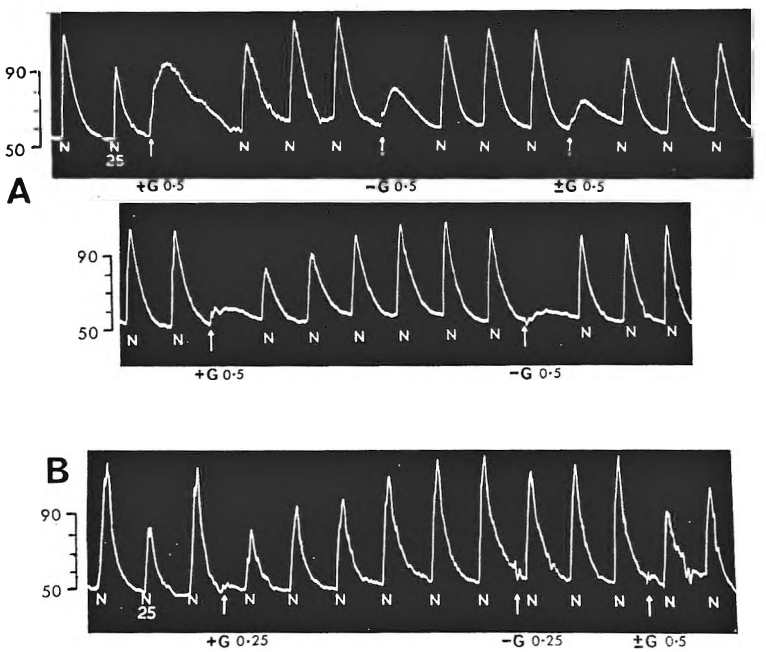

FIG. 7. Blood pressure of pithed rats (calibration in mm Hg); the record labelled B was obtained from a rat pretreated with reserpine. At N, 50 ng of noradrenaline; at N/25, 25 ng of noradrenaline; at +G, −G and ±G, (+)-, (−)- and (±)-guanoxan respectively. The doses of guanoxan and its isomers were as illustrated, in mg/kg. All injections intravenously. Noradrenaline was injected every 5 min except after guanoxan. The gap between the two panels of Fig. 7A corresponds to 30 min.

Catecholamine release by the (+)-isomer in a cat was evidenced by a small and gradually developing increase in the resting tone of the nictitating membrane (Fig. 4). This effect was not produced in the cat treated with the (−)-isomer, and neither isomer produced an initial rise in blood pressure. In fact, the immediate blood pressure response to the isomers in the two cats was a small and transient depressor effect (Fig. 4). No evidence of an initial pressor effect or of contraction of the nictitating membrane in the cat was reported to follow intravenous injection of the racemate by Davey & Reinert (1965), and in general the results confirm that catecholamine release following intravenous injection

in the cat is very much less than that following intravenous injection of guanethidine.

α-Adrenoreceptor blocking action. Davey & Reinert (1965) described a marked α-receptor blocking action of the racemate in the dog, but this effect was very weak in the cat. The present experiments on two cats confirmed that the α-receptor blocking action of the isomers is weak in this species. Fig. 4 illustrates a slight reduction in the pressor response to noradrenaline and the unmasking of a secondary depressor component of the response to adrenaline after injection of 5 mg/kg of the (+)-isomer. At the same time, however, the contractions of the nictitating membrane produced by the amines were enhanced. In the same cat, a further dose of 10 mg/kg of the (+)-isomer did not produce a stronger blocking action. This dose was more than sufficient to abolish the responses of the membrane to nerve stimulation. In the other cat no evidence of α-receptor block by the (−)-isomer was obtained.

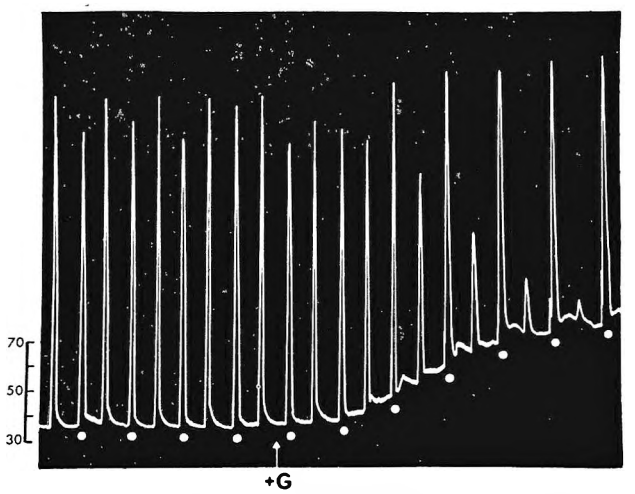

FIG. 8. Responses (calibration in mm Hg) of the isolated central ear artery of the rabbit to periarterial nerve stimulation (unlabelled responses) and to 30 ng noradrenaline (at the dots). Responses to one or the other were evoked every 2 min. At +G, perfusion with (+)-guanoxan (0·2 μg/ml) was commenced and continued throughout the rest of the record. Note that responses to noradrenaline were not depressed whereas those to nerve stimulation were blocked. Guanoxan caused an increase in the background resistance to perfusion.

In the isolated ear artery of the rabbit no evidence of α-receptor blockade by racemic guanoxan or either of its isomers was obtained. Fig. 8 illustrates persisting responses to noradrenaline during abolition of the responses to nerve stimulation by perfusion with 0·2 μg/ml of (+)-guanoxan. Even in concentrations up to 2 μg/ml of the compounds, responses to noradrenaline were never reduced but were increased in most experiments.

Of the preparations from the three species studied, definite evidence of α-receptor block was produced only in the rat. (±)-Guanoxan in a

dose of 0·5 mg/kg intravenously reduced the pressor response to noradrenaline in the pithed rat by 40–60%. Full recovery occurred within 20–30 min. Most, but not all, of the α-receptor blocking action of the racemate was displayed by the (+)-isomer, the (−)-isomer having a relatively weak effect. Fig. 7 illustrates the antagonistic action of the compounds against the pressor responses to noradrenaline in the pithed rat. In the experiment illustrated by Fig. 7B, the rat had been pretreated with reserpine to avoid the complication due to the pressor action of the compounds resulting from catecholamine release. It is of interest that the (+)-isomer was the most potent both in producing an initial pressor effect and in producing α-receptor block. Since these two actions are antagonistic, the catecholamine release is probably greater than that reflected by the pressor response.

α-Receptor block probably did not contribute greatly to the reduction in response to stimulation of the sympathetic outflow in the pithed rat. The time courses of the two actions were different; maximum α-receptor block was evident immediately after injection of guanoxan (Fig. 7) but the effect wore off quickly. Maximum adrenergic neuron block took about an hour to develop (Fig. 2) and was irreversible.

Ganglion blockade and atropine-like action. Davey & Reinert (1965) recorded only a weak and transient depressant effect of guanoxan, compared with that of guanethidine, on the evoked ganglionic action potential recorded from the superior cervical ganglion of the cat, and it is possible that ganglion block was responsible for the initial transient depressor effect of the isomers in the present experiments on cats. However, no difference could be detected between the rate of onset of block of contractions of the nictitating membrane evoked by pre- or post-ganglionic stimulation (Fig. 4) showing that any contribution by ganglion block is unimportant in the cat.

Spinal stimulation of the pithed rat excites pre-ganglionic sympathetic fibres (Gillespie & Muir, 1967) and the possibility that ganglion block was a contributory factor in this species was investigated by recording the heart block produced by vagal stimulation or by acetylcholine. Doses of (±)-, (+)- or (−)-guanoxan ranging from 0·5–2·0 mg/kg intravenously reduced, but did not abolish, the response to vagal stimulation. However, the response to acetylcholine was always reduced more than that to nerve stimulation, suggesting that a weak atropine-like action was mainly responsible. This effect persisted for up to 15–60 min depending on the dose. There appeared to be no difference in the potencies of the two isomers in producing this atropine-like effect but insufficient experiments were done to detect small differences.

Discussion

The results showed that in the preparations from the three species studied, (±)-guanoxan and its two isomers are equipotent in producing adrenergic neuron block, indicating that the stereochemical configuration at the 2 position of the compound is unimportant in this action.

Species difference is evident in the α-adrenoreceptor blocking action of guanoxan. Of the species studied, it is most pronounced in the dog (Davey & Reinert, 1967) and the rat, weakly evident in the cat, and undetectable in the rabbit. It is of interest that both the α-receptor blocking action and the ability to cause catecholamine release reside mainly in the (+)-isomer. The absolute stereochemical configuration of the compounds has not yet been elucidated.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 94S–103S Received May 1, 1968

The crystallization of aspirin from ethanol

J. GLASBY AND K. RIDGWAY

The rate of growth of aspirin crystals has been examined in a circulatory crystallizer in which the crystals are held in suspension as a fluidized bed. The deposition rate was measured, as a function of both the degree of supersaturation and the solution temperature, by observing the rate of weight increase and the change in particle size distribution of the crystals. The dependence of the mass transfer coefficient for the process upon the temperature was of the Arrhenius type, the activation energy being 21·8 kcal/mole. This indicates that the surface reaction step is rate-controlling, diffusional transport to the crystal surface being rapid. The density of aspirin solutions in alcohol has also been measured as a function of temperature and concentration.

ALTHOUGH the crystal form of many substances has been determined, and the molecular arrangement within the unit cell characterized, there is a dearth of experimental work on the rate at which crystals are formed by deposition from solution onto seed crystals. This is true for pharmaceutical materials in general and for aspirin in particular. Although much must be known within industry about its crystallization, little has been published.

In crystallization systems in general, Ting & McCabe (1934) showed that the Ostwald concept of a metastable region bounded by the solubility line and a "supersolubility line" was an oversimplification, the position of the supersolubility line being a function of the rates of cooling and of agitation as well as of the presence and amount of seed material. However, the Ostwald picture is of great practical importance and is widely used in crystallizer design. Rumford & Bain (1959) pointed out that the rate of formation of nuclei was an exponential function of supersaturation: the supersolubility line is therefore not a boundary, but merely a region where the ease and rapidity of formation of nuclei suddenly increases.

Two theories have been advanced to explain the mechanism and rate of crystal growth, that is the deposition of material onto crystals previously produced by nucleation or added as seed crystals.

The Noyes & Whitney (1897) diffusional theory assumed that the amount of material deposited was proportional to the concentration difference between the crystal surface and the bulk of the solution, the limit on the rate of deposition being the rate of diffusion through a laminar boundary layer. It was also suggested that the rate-controlling step was some form of surface reaction.

Valeton (1923) suggested that the overall process involved two stages, namely diffusion from the solution to the crystal surface, followed by a surface reaction in which the molecules arranged themselves into the crystal lattice. The two stages can be represented by the equations (1) and (2) which refer to diffusion and surface reaction respectively.

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$$\frac{dM}{dt} = K_D (C_1 - C_2) \quad (1)$$

$$\frac{dM}{dt} = K_R (C_2 - C_3) \quad (2)$$

where dM/dt is the mass rate of deposition per unit area of the crystal surface, C_1 and C_2 are the concentrations in the bulk of the solution, and at the crystal surface-solution interface; C_3 is the equilibrium saturation concentration, and K_D and K_R are the mass transfer coefficients for the diffusion and surface reaction steps.

As is usually the case in mass transfer, only an overall coefficient is determinable, and the interface concentration C_2 is inaccessible to measurement. The overall mass transfer obeys the equation, formed by adding equations (1) and (2).

$$\frac{dM}{dt} = K (C_1 - C_3) \quad (3)$$

where $1/K = 1/K_D + 1/K_R$. Either K_D or K_R may be the limiting factor on the deposition rate, but a single rate measurement will not disclose which it is. Knowledge of the diffusivity of the solute, or experiments covering a range of temperature, will enable a decision to be made. In this paper, the temperature variation of K leads to the view that the activation energy of the process is such that the surface reaction is the rate-controlling step.

Experimental

MATERIALS

The aspirin used was acetylsalicylic acid B.P. (Laporte Industries Ltd).

The ethanol was absolute ethanol, R. R. grade, (James Burrough Ltd.). This had a strength of 99·7% v/v and a maximum water content of 0·3% v/v.

APPARATUS

The apparatus used was a continuously circulating crystallizer of the type designed and reported by Mullin & Garside (1968). It is shown diagrammatically in Fig. 1. Solution is circulated by the stainless steel pump A. It passes through a variable-area flowmeter B, which is a more convenient method of flow measurement than is the orifice plate of the original design, then through a heating section C where any small crystal nuclei are dissolved. This heating section is in two parts, a main section rated at 2·5 kW and a small section for fine control of temperature rated at 100 W. The hot solution is then cooled by the water condenser D to the temperature required for the experimental section E in which the crystals are grown. Control of the temperature governs the extent of supersaturation, and a contact thermometer F allows the temperature to be held to within 0·05°.

Crystals for growth are contained in the vertical tube E, either freely or in a small cage G to prevent loss and to enable them to be removed easily.

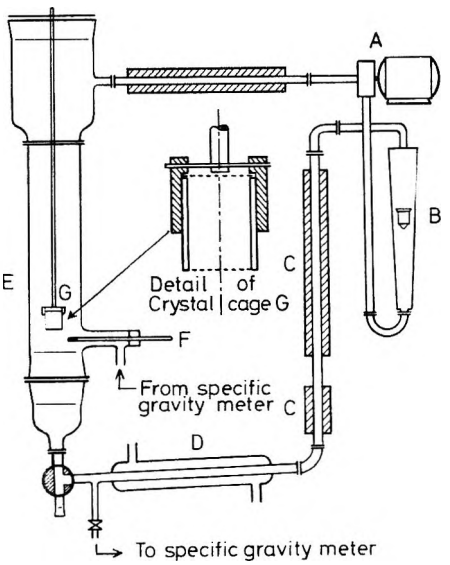

FIG. 1. The continuous circulation crystallizer. The pump A sends solution through flowmeter B, heater C and cooler D to the experimental section E, where crystals are grown as a fluidized bed, at a temperature controlled by contact thermometer F, in sample cage G. Sample cage G is shown in detail in the centre of the figure.

In either case the solution circulation velocity is maintained at a rate sufficient to ensure that the crystals are supported by the liquid to form a small fluidized bed, each crystal then being separate from its neighbours. The solution is taken from the top of the experimental section E through a heated tube back to the pump and round the circuit again.

The solution was continuously sampled, being bled off just before the experimental section and passed to a specific gravity meter (Sangamo Ltd.) connected to a chart recorder. The density measurement allowed the concentration of acetylsalicylic acid to be calculated to within better than 0·01%.

In the earlier crystallization runs, the initial seed crystals were added to the wider bore calming region at the top of the experimental section; fluid velocity was then reduced to allow them to fall to the crystallization region and then increased sufficiently once more to support them. As the seed crystals increased in size, the specific gravity meter showed a decrease in solution concentration. Sufficient warm concentrated aspirin solution was then added to bring the concentration back to the starting value. Small quantities of the seed crystals were removed at 5 min intervals over about 1½ hr, dried, and sieve analyses made.

For temperatures at the higher values within the range examined, difficulty was experienced in maintaining a constant concentration without affecting the stability of the apparatus; consequently a different method was used for some of the later experiments. A small weight of 30/40 seed

crystals (0·2 g) was contained in a crystal cage detailed in the centre of Fig. 1. This was a Perspex tube with a stainless steel mesh at each end. The cage was placed in the crystallizing region for intervals of time between 1 and 30 min, using a stainless steel rod to which the cage could be attached by a stainless steel pin. On removal from the crystallizer, the crystals were quickly drained, dried and placed in a desiccator until required for sieve analyses. After each product removal the solution in the crystallizer was heated to dissolve any solid material, the concentration adjusted, the solution cooled to the required crystallization temperature and a fresh batch of identical seed crystals immersed for a different time. During any run the product crystal weight did not increase to beyond 0·8 g, which caused a depletion of 0·6 g of aspirin from the 10 litres of solution contained in the crystallizer.

Crystallization runs were made at various degrees of supersaturation in the temperature range 27–50°.

Sieve analyses were made using a nest of 2 in diameter Endecott test sieves, with precautions as outlined by Mullin (1961). Each sieve fraction was examined to ensure that clumping had not taken place, then weighed and, in selected cases, photographed. Results were plotted in various ways to show any particle size distribution changes occurring. Cumulative weights of crystals retained by each sieve were plotted against mean equivalent particle diameter retained by each sieve on log probability paper, so that the mean equivalent particle diameter for each product batch could be found.

The density of solid aspirin was found by the usual specific gravity bottle technique and the shape of the crystals found from photomicrographs. These two factors enabled the weight and surface area of a crystal to be estimated from the mean equivalent particle diameter. Aspirin crystallizes in the monoclinic system, with unit cell dimensions $a = 11{\cdot}446$, $b = 6{\cdot}596$ and $C = 11{\cdot}388$ Å. The axis angle is 95° 33′ (Wheatley, 1964). After inspection of actual crystals, it was decided to take the crystal shape, for calibration purposes, to be a hexagonal prism with a length to width ratio of 1·3. The volume is $0{\cdot}843\ d^3$, where d is the diameter of the crystal measured between opposing apices of the hexagonal cross-section. The density of crystalline aspirin was 1·2 g/cm³, so that the weight of a crystal of nominal width d is $1{\cdot}098\ d^3$ g. The surface area of the crystal is $5{\cdot}198\ d^2$. Since the crystal diameter d is also the mean equivalent particle diameter for sieve analysis, the rate of deposition of aspirin per unit area of exposed surface can be calculated from measurements of the initial and final particle size distribution and from the change in weight of the sample. Since the solution concentration is known, the mass transfer coefficient for the crystallization process at a particular temperature can be calculated, provided the equilibrium solubility for aspirin in ethanol is known as a function of temperature.

The solubility of aspirin in ethanol was therefore measured. Saturated aspirin solutions at 20 and 50° were shaken (48 hr) with solid aspirin at various temperatures between 20 and 50° in an apparatus developed for the purpose. This consisted of two round-bottomed flasks connected

neck to neck by a tube containing a quantity of glass wool to act as a filter. The saturated solution and solid particles were contained in one flask. After the required time the apparatus was inverted, still in the water bath at the required temperature, so that solid particles were held back by the glass wool. Approximately 20 ml samples of the filtered solution were removed and weighed. The ethanol was evaporated off by heating in an oven for 4 hr, and the solid material weighed. In other experiments the samples were weighed and assayed for aspirin by the official B.P. method.

During crystallization experiments, conditions were sometimes attained where neither growth nor dissolution of seed crystals occurred over a 20-min period. These conditions were taken as the equilibrium conditions and, using them, a much better solubility line was obtained. Further confirmation was obtained by extrapolating plots of growth rate against concentration back to zero growth rate.

The metastable limit for aspirin in ethanol was investigated by cooling different strength solutions at a constant rate, with a constant circulation rate in the crystallizer and noting the temperature at which the solution became opaque. Although this is not strictly the point at which spontaneous nucleation takes place, it is the point at which the nuclei have formed and grown to a size sufficient to produce opacity. It was taken as the nucleation temperature.

The density of a range of solutions of different concentrations was measured by the specific gravity meter at a number of controlled temperatures. The results of these experiments were used to set the meter temperature-compensating device; they enabled the density of aspirin solutions in ethanol to be plotted as a function of both concentration and temperature.

Results and discussion

The relation between the density of solutions of aspirin in ethanol and concentration and temperature was obtained. The measured data were plotted in the form of graphs of density against temperature, with concentration as a parameter. A series of parallel straight lines was obtained. They are represented by the equation;

Density $(g/cm^3) = 0{\cdot}8066 - 0{\cdot}00082T + 0{\cdot}0033C$ where T is the temperature (°C) and C is the concentration (% w/w).

From the nucleation observations and the determinations of concentration and temperature at which no crystal growth or dissolution took place, a solubility-supersolubility diagram can be obtained (Fig. 2). The metastable region lies between the solubility line, which is well-defined, and the spontaneous nucleation line, which cannot be fixed to the same degree of precision. The results from the equilibrium solubility determinations by evaporation and by the B.P. assay for aspirin are also shown. These lie in the metastable region, indicating that in this system the attainment of equilibrium is difficult unless there is pronounced relative motion of crystals and mother liquor: such motion occurs in the

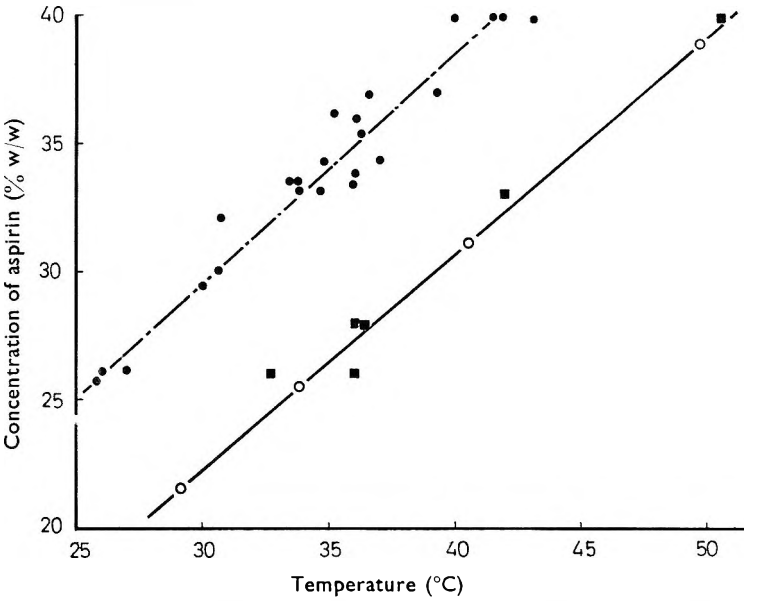

FIG. 2. Solubility-supersolubility diagram for the aspirin-ethanol system. ○ Determined in the crystallizer. ■ Determined in the solubility apparatus. ● Nucleation.

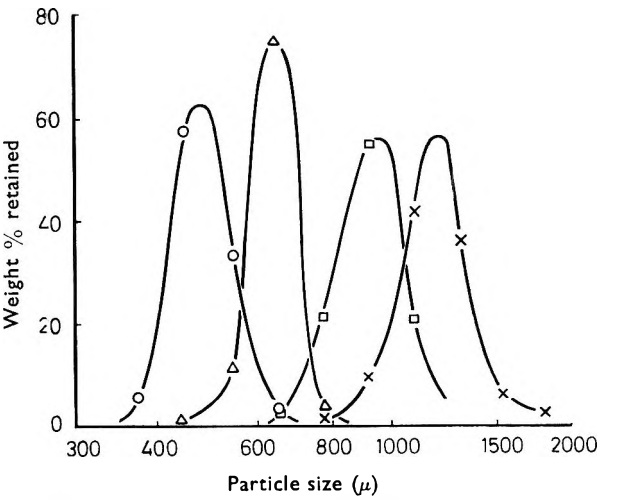

FIG. 3. Seed and product crystal size distribution. ○ Seed. △ 23 min. □ 58 min. × 102 min.

circulatory crystallizer used, but not in the normal solubility-measuring apparatus.

Repeated temperature cycling of the same solution did not change the nucleation point. This behaviour is in contrast to that found for potassium sulphate by Gaska (1966), where temperature cycling appeared to destroy some form of heterogeneous catalytic agent for nucleation.

Storage of the solution for periods of more than 12 hr in the crystallizer caused a slight fall in the nucleation temperature, suggesting that some decomposition had occurred, although it has been stated (Wing, 1956) that aspirin is stable for at least 2 years in ethanol. The two cases are not strictly comparable since our solutions are both heated and agitated in contact with glass, plastic and stainless steel, whereas those of Wing were presumably stored in closed all-glass containers. Another possibility is that the ethanol had picked up some water from the atmosphere.

These preliminary experiments defined the limits between which aspirin can be deposited onto seed crystals. A number of runs were made to measure the rate of deposition onto seed crystals at various temperatures and degrees of supersaturation. The supersaturation unit chosen is the

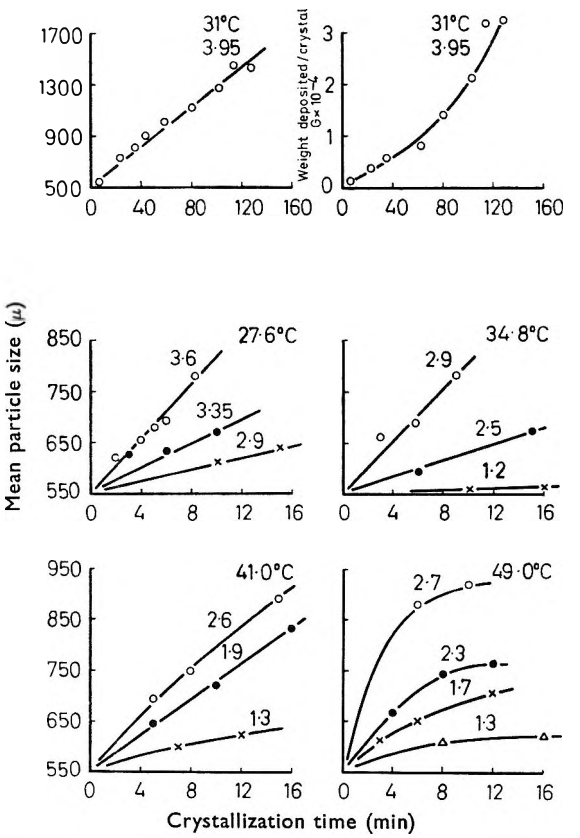

FIG. 4. Change of mean crystal size with time at various conditions of temperature and supersaturation. The figures on the lines represent Δc. The upper curves are for a 20 g batch of crystals grown at 31°, keeping the concentration in the system constant by adding strong aspirin solution. The curves for 27·6°, 34·8°, 41·0° and 49·0° were obtained by growing small samples of seed crystals, about 0·2 g in weight, for different times.

excess percentage, w/w, of aspirin over and above the equilibrium solubility, i.e.

(% of aspirin in solution contacting the crystals)
— (% of aspirin in equilibrium solution at the same temperature).

This quantity is given the symbol $\triangle c$.

Fig. 3 shows the particle size distribution for the seed crystals, which were identical for all the crystallization runs made. Also shown are some typical product crystal size distributions. The size range tended to broaden with increasing crystallization time, probably because of stratification in the bed of crystals. Larger crystals tend to spend more time at the bottom of the bed, where they meet fresh mother liquor and therefore tend to grow faster. If the seed size distribution and the product size distributions are plotted on log-normal probability paper, straight lines are obtained, parallel to the seed line, showing that the size distributions are all log-normal and similar. This means that valid conclusions may be drawn from the change in mean particle size of a batch of crystals as they grow. This simplifies the analysis of the results. Fig. 4 shows a plot of mean particle size as a function of crystallization time for various temperatures and degrees of supersaturation. Most experiments up to 40° produced a series of straight lines with slopes depending on the concentration difference present. These graphs could be compared with linear growth rate results, if in our experiments the relative growth rate of each face remained the same. Since no great crystal shape change occurs in the range of concentration difference studied, this is probably valid.

TABLE 1. MEASURED MASS TRANSFER COEFFICIENTS FOR THE CRYSTALLIZATION OF ASPIRIN FROM ETHANOL AT VARIOUS TEMPERATURES AND DEGREES OF SUPERSATURATION

Experiment No.	Temperature, °C	Solution concentration, % w/w	$\triangle c$ % w/w	Mean K, 10^{-2}g/cm² min %
1	31·0	27·0	3·95	0·122
2	37·5	35·5	6·9	0·281
3	50·0	40·5	1·3	0·923
	40·5		2·2	
4	41·2	33·3	1·6	0·386
	39·2		3·3	
	33·4		2·9	
5	33·8	28·0	2·5	0·177
	35·4		1·2	
	27·2		3·6	
6	27·5	23·5	3·4	0·120
	28·1		2·9	
	28·8		1·9	
7	29·7	40·0	1·1	0·434
	50·5		0·4	
	29·1		3·1	
8	29·7	24·6	2·6	0·093
	48·5		2·3	
9	49·2	40·2	1·7	0·823
	49·7		1·3	
	40·0		2·6	
10	41·0	33·4	1·9	0·368
	41·7		1·3	

Table 1 shows the parameters for each of the nine major experiments carried out. From each experiment, a linear relation was obtained between the weight deposited per unit area of crystal surface per unit time,

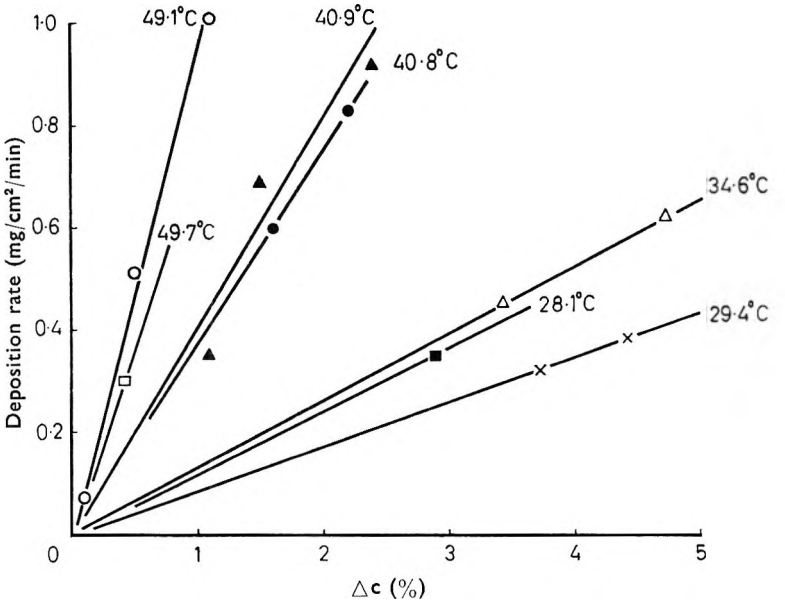

FIG. 5. Weight of aspirin deposited per cm² of mean area of crystal per min as a function of concentration driving force, △c. The slope of each line is the mass transfer coefficient, K, measured in grams deposited/cm²/min/unit percentage concentration difference. The figures on the lines represent the temperatures of crystallization.

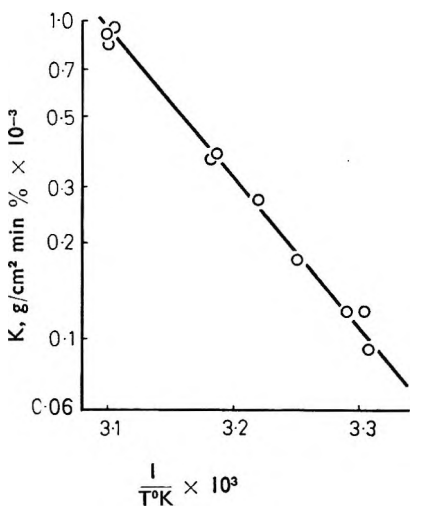

FIG. 6. Arrhenius plot of log K against 1/T, leading to a value of 21·8 kcal/mole for the activation energy.

and the concentration driving force, $\triangle c$, for the deposition process. This is shown in Fig. 5. The slope of the line is the mass transfer coefficient, K, usually expressed as grams deposited per cm^2 per sec per unit concentration difference. The linear relation between rate of deposition per unit area and the concentration difference $\triangle c$ is an indication that K is constant for any given experiment, and is therefore a function only of temperature.

The variation of K with temperature was found to follow an Arrhenius type law. Fig. 6 shows a plot of log K against 1/T where T is the temperature in °K. The equation of the straight line is

$$\log K = \frac{-4760}{T} + \text{constant.}$$

The Arrhenius equation is $K = A \exp(-E/RT)$, or $\log K = \frac{-E}{2{\cdot}303\ RT}$ + constant. Thus we have $\frac{E}{2{\cdot}303\ R} = 4760$ or $E = 21{\cdot}8$ kcal/mole.

This value may be compared with van Hook's (1944) values for sucrose, which are 22 kcal/mole at 0° C falling to 6·5 kcal/mole at 70° C. This was for crystallization from water. A value of 21·8 kcal/mole is indicative of the rate-determining step being the incorporation of a molecule onto the crystal lattice, rather than the rate at which the molecules are able to diffuse to the crystal surface. Diffusion-controlled mechanisms usually give activation energy values nearer to 5 kcal/mole, (cf. the crystallization of sodium chloride from water, Rumford & Bain, 1959).

Since it is the step of incorporation into the lattice which is rate-limiting in the growth of aspirin under the conditions of these experiments, it might be expected that where incorporation is rapid, a larger number of imperfections would be built into the crystal. The hardness of a crystal which had been grown more rapidly would then be expected to be less, and its ultimate compressive strength would be reduced compared to a more slowly-grown specimen. Preliminary measurements of microhardness and tensile strength support this conclusion.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 104S–107S Received May 1, 1968

SHORT COMMUNICATION

Solubilities of testosterone propionate in non-polar solvents at 100°

D. B. BOWEN AND K. C. JAMES

THE solubilities of the formate to valerate esters of testosterone in non-polar solvents at 25° were determined by James & Roberts (1968) who also compared them with ideal mole fraction solubilities (X_2), calculated from the equation,

$$-\ln.x_2 = \frac{\Delta H^F}{R}\left[\frac{T_M - 298}{298T_M}\right] \quad (1)$$

(Hildebrand & Scott 1962). ΔH^F is the heat of fusion of the solute and T_M the melting point. Changes in solubility as the homologous series is ascended were predicted by equation (1), but the individual experimental results did not agree with the calculated values. ΔH^F was calculated from the heat of fusion at the melting point, ΔH^F_M, by correcting for the differences in heat capacity of the solid and the supercooled liquid between T_M and T. The correction was estimated with a differential scanning calorimeter by extrapolating the liquid enthalpy line back to 25° and measuring the area between the extrapolation and the enthalpy line of the solid. The method was considered questionable, however, because it assumed that the enthalpy line of the supercooled liquid decreased linearly over the whole range of temperature. This theory is tested below by comparing the measured and calculated solubilities of testosterone propionate at a temperature just below its melting point, where the heat capacity correction is small and ΔH^F_M can be used for ΔH^F.

The solvents examined by James & Roberts (1968) had smaller molar volumes than the testosterone esters, and it was suggested that the difference in molar volume between solute and solvent could prevent the random distribution assumed by regular solution theory. Prediction of solubility would thus improve as the molar volume of the solvent approached that of the solute. The test is applied below by determining the solubility of testosterone propionate in a range of solvents.

EXPERIMENTAL

Volume fraction solubilities (ϕ_2) were calculated from the equation,

$$\phi_2 = \frac{X_2V_2}{X_1V_1 + X_2V_2} \quad (2)$$

where X is mole fraction solubility and V molar volume. The suffix 1 represents solvent, and 2 solute.

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Calculated mole fraction solubilities were obtained from the equation

$$-\ln X_2 = \frac{\Delta H^F}{R}\,\frac{(T_M - 373)}{373T_M} + \frac{V_2\phi_1^2(\delta_1 - \delta_2)^2}{RT} \qquad (3)$$

where δ is solubility parameter (Hildebrand & Scott, 1962). ΔH_M^F (5·29 kcal mole^{-1}) taken from James & Roberts (1968), was substituted for ΔH_F.

Measured solubilities were determined using a method which has been described by Gordon & Scott (1952). Weighed quantities of solute and solvent were sealed in glass tubes, and the temperature at which solution occurred noted. Solubility at 100° was obtained from the plot of log solubility against log temperature by interpolation.

Solubility parameters and molar volumes were mainly from Hildebrand & Scott (1962). Molar volumes not quoted in this reference were determined from density measurements. The corresponding solubility parameters were calculated as $\left[\frac{\Delta H_v - RT}{V}\right]^{\frac{1}{2}}$ using molar heats of vaporisation (ΔH_v) from the equation,

$$\Delta H_V = -2950 + 23{\cdot}7\,T_b + 0{\cdot}020\,T_b^2 \qquad (4)$$

(Hildebrand & Scott, 1950). Boiling points (T_b) were measured using a Perkin Elmer differential scanning calorimeter. Molar volume and solubility parameter of testosterone propionate were taken from James & Roberts (1968).

RESULTS AND DISCUSSION

Experimental solubilities are compared in Table 1 with those calculated from equation (3). The lack of agreement indicates that even when the value for heat of fusion is reliable, equation (3) does not predict the solubilities of testosterone esters in non-polar solvents. It also suggests, however, that even if the heat capacity correction were in error, it was only partly responsible for the poor agreement between experimental and calculated solubilities observed by James & Roberts (1968).

TABLE 1. COMPARISON OF EXPERIMENTAL AND CALCULATED SOLUBILITIES OF TESTOSTERONE PROPIONATE IN VARIOUS SOLVENTS

	Molar volume of solvent (cm^3)	Solubility parameter of solvent ($cal^{\frac{1}{2}}\ cm^{3/2}$)	Mole fraction solubility at 100°		Measured volume fraction solubility at 100°
			Measured	Calculated	
Benzene	89	9·2	0·33	0·70	0·62
Cyclohexane	109	8·2	0·38	0·63	0·62
Naphthalene	123	9·9	0·40	0·69	0·61
Phenanthrene	158	9·8	0·56	0·69	0·70
Dekalin	156	7·9	0·41	0·54	0·57
Tetralin	135	9·5	0·47	0·68	0·66
Carbon disulphide	61	10·0	0·51	0·69	0·83
Chloroform	81	9·2	0·63	0·70	0·86
Carbon tetrachloride	97	8·6	0·56	0·69	0·79
Carbon tetrabromide	105	10·7	0·63	0·68	0·83

In regular solutions, forces of attraction between like molecules are overcome by thermal agitation, resulting in completely random distribution. When the difference between the molar volumes of solute and

solvent is large, however, geometrical considerations may demand a more ordered arrangement, resulting in a finite entropy of mixing, and a lower solubility than that predicted. If the observed solubility is low because of the entropy of mixing, arising in turn from the differences in molar volume of solute and solvent, theoretical prediction of solubility should improve as the molecular shape and size of the solvent approach those of the solute. Ratios of measured to calculated solubility of testosterone propionate in several solvents are plotted in Fig. 1, against molar volume of solvent, and show the anticipated improvement in prediction of solbility as the molar volume of the solvent increases. The hydrocarbon solvents gave a linear relation, extrapolating to theoretical solubility at a molar volume of 205 cc, while carbon disulphide, carbon tetrabromide, carbon tetrachloride and chloroform appeared to belong to a different series.

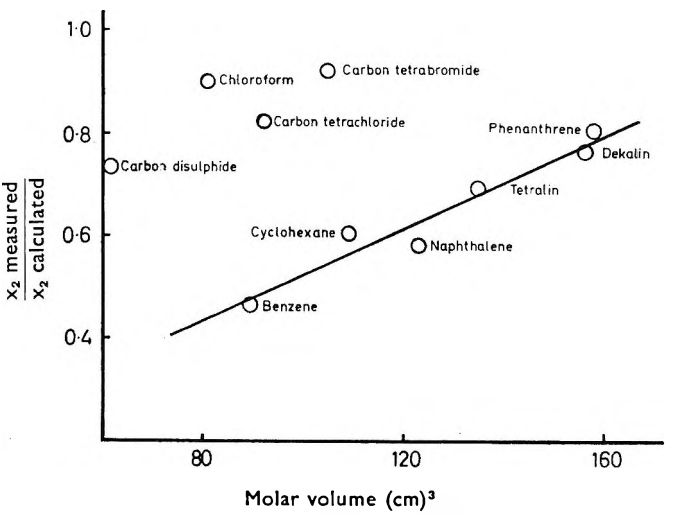

FIG. 1. Effect of molar volume of solvent on prediction of solubility.

Since there is a definite relation between molar volume of solvent and the accuracy with which the solubility of testosterone propionate can be predicted, the solubility parameter of 9·5 cal$^{\frac{1}{2}}$ cm$^{3/2}$, because it was determined from solubilities in solvents of varying molar volumes, is suspect. If the solubility parameter is in fact not 9·5 cal$^{\frac{1}{2}}$ cm$^{3/2}$, the correct value could bring all the results onto the same line. The solubility parameter giving the best straight line, fitting all the results, was calculated using an Elliott 803 electronic computer. The value obtained for all the results was 9·67 cal$^{\frac{1}{2}}$ cm$^{3/2}$ with a correlation coefficient of $-0{\cdot}19$ while that for the hydrocarbon solvents alone was 9·50 cal$^{\frac{1}{2}}$ cm$^{3/2}$ with a correlation coefficient of 0·98. The two series must therefore be different, and the solubility parameter of 9·5 cal$^{\frac{1}{2}}$ cm$^{3/2}$ for testosterone propionate is confirmed.

Volume fraction solubilities are shown in Table 1. The values for the three halogen compounds and carbon disulphide are very similar, and those for the hydrocarbon solvents are also reasonably constant. It appears from this that the molecular proportions of solute and solvent which have been recorded as observed solubility at 100° are actually the limit at which the volume of solvent is no longer sufficient to maintain the system in the liquid state. The difference between the volume fractions for the two series suggests that, in the solvents examined, there are two types of orientation of solute molecules with respect to solvent molecules, one being more compact than the other.

Acknowledgements. We are grateful to Mr. D. M. Ellis, from the Department of Mathematics of this Institute, for the computer results, and to the Science Research Council for a grant to D.B.B.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 108S–113S Received May 1, 1968

The effect of pH and sodium metabisulphite on the stability of physostigmine sulphate solutions to heat and ionizing radiation

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A method which assays the alkaloid in the presence of its breakdown products has been used to investigate the effects of heating and exposure to ionizing radiations on the degradation kinetics of aqueous physostigmine solutions. Maximum stability at 90° is between pH 2·2 and 3·0. Sodium metabisulphite retards the degradation of physostigmine when exposed to gamma irradiation, but has no effect on the degradation by heat.

A MAJOR difficulty in investigating the stability of physostigmine in aqueous solution has been the lack of a specific assay technique whereby physostigmine may be assayed in the presence of its degradation products. Haugas (personal communication) has devised a method based on the reaction of physostigmine with sodium nitrite in acid solution to form a yellow nitroso-compound which, after stabilizing with ammonium sulphamate, is extracted with chloroform, and the intensity of the colour measured at 417 mμ. A modification of Haugas' method (Fletcher, 1968) has enabled physostigmine to be successfully assayed in the presence of its breakdown products and has afforded a fresh opportunity to assess the chemical stability of physostigmine solutions. In this paper a comparison of the influence of pH and sodium metabisulphite on the effects of heat or exposure to ionizing radiation on the degradation kinetics of aqueous physostigmine systems is reported.

Experimental

Materials. Ammonium sulphamate (Laboratory reagent, B.D.H.); citric acid, chloroform, disodium phosphate, lactic acid, sodium chloride and sodium nitrite were AR grade, glycine (Biochemical grade, B.D.H.) and physostigmine sulphate B.P.C. (suppl. 1966).

Reagent solutions. Lactic acid 20% w/v in distilled water. Sodium nitrite 1% w/v in distilled water.

Buffer solutions. (a) For aqueous solutions of physostigmine sulphate in the *absence* of sodium metabisulphite, Sorensen's glycine–sodium chloride–0·1N hydrochloric acid buffer (pH range 1·2 to 3·6) was used. For the range 2·2 to 8·0 McIlvaine's citric acid–disodium phosphate buffer was used.

(b) For aqueous solutions of physostigmine sulphate at pH 7·0 to contain sodium metabisulphate the formulae used were: (i) sodium metabisulphite 0·2 g, citric acid (0·1M) 12·0 ml, disodium phosphate (0·2M) to 100·0 ml; (ii) sodium metabisulphite 0·5 g, citric acid (0·1M)

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5·0 ml, disodium phosphate (0·2M) to 100·0 ml. Unlike the buffers described in Documenta Geigy (p. 315) these proved satisfactory in controlling the pH.

ASSAY METHOD

Physostigmine sulphate solution (0·3 ml), lactic acid solution (10 ml) and sodium nitrite solution (1 ml) were shaken for 30 sec. After 30 min to allow development of the yellow nitroso-compound, ammonium sulphamate (1 g) was added and the yellow compound extracted with 8, 4, 4 and 4 ml aliquots of chloroform. The combined chloroform layers were made up to 25 ml with chloroform. The absorbance of the chloroform solution was determined at 417 mμ in a Unicam SP 600 spectrophotometer using 1 cm glass cells. Each determination was in duplicate, the results averaged and used to calculate the percentage of physostigmine in the sample by reference to a Beer-Lambert plot. The ratios of the standard errors of the slopes to the slopes were better than 0·02. Because of batch to batch variation Beer-Lambert plots were constructed for each fresh batch of physostigmine sulphate used.

HEATING EXPERIMENTS

The effect of pH on the degradation rate of aqueous physostigmine sulphate solutions at 90°. To 2 ml, of an approximately 10% w/v solution of physostigmine sulphate were added 38·0 ml of the required buffer solution, previously heated to 90°, to provide a final concentration of 0·5% w/v. This solution was shaken continuously in an oil bath at 90° $\pm$ 0·5°, 1 0 ml samples being withdrawn at suitable intervals of time and 0·3 ml aliquots assayed. Solutions of physostigmine sulphate at pH 8·0, 7·0, 6·0, 5·1, 4·05, 3·0, 2·2 and 1·5 were used and the degradation rate constants at each of these pH values were calculated. The results are shown in Fig. 2.

The effect of sodium metabisulphite on the degradation rate of aqueous physostigmine sulphate solutions. Solutions of physostigmine sulphate 0·5% w/v containing 0·2 or 0·5% w/v sodium metabisulphite were buffered to pH 7·0 as previously described and 2 ml of each solution was placed in 5 ml neutral glass ampoules and heated at 90° $\pm$ 0·5°. Samples were removed at the requisite time intervals, 0·3 ml aliquots assayed and the degradation rate constants for the two solutions calculated.

The rate constant (k sec^{-1}) for physostigmine sulphate solutions containing sodium metabisulphite 0·2, 0·5 and 0·5 (but irradiated with 2·5 megarads γ-radiation) (% w/v) are respectively $3{\cdot}9650 \times 10^{-4}$, $3{\cdot}6580 \times 10^{-4}$ and $3{\cdot}2973 \times 10^{-4}$.

IRRADIATION EXPERIMENTS

Physostigmine sulphate solution (40 ml) at a known pH was placed in a "Gravmer Gravatom 1½ in Fixed Cobalt 60 Source" whose characteristics have been described elsewhere (Fletcher, 1968). At the requisite time intervals for the required dose, 1 ml samples were withdrawn and 0·3 ml aliquots assayed. The following systems were investigated and

degradation rate constants obtained. Physostigmine sulphate solutions at pH 1·5, 5·1 and 7·0 and at pH 7·0 containing 0·2 or 0·5% w/v sodium metabisulphite.

The results for physostigmine alone at pH 1·5, 5·1 and 7·0 are in Table 1. The degradation rate constants of the physostigmine solutions containing sodium metabisulphate at 0·2 and 0·5% w/v are 0·0580 and 0·0475. The value without the metabisulphite is 0·1029.

PRE-IRRADIATION AND SUBSEQUENT HEATING EXPERIMENTS

Quantities of 40 ml of two 0·5% w/v physostigmine sulphate solutions, one containing 0·5% w/v sodium metabisulphite, were irradiated with a sterilizing dose of 2·5 megarads. Aliquots of 2 ml of the solutions were then placed in 5 ml neutral glass ampoules and heated at 90° ± 0·5°. Samples were removed at suitable time intervals and 0·3 ml aliquots assayed as before and the degradation rate constants obtained.

Results and discussion

On all occasions when solutions of physostigmine were heated or irradiated, plots of log percentage residual concentration against time or dose of radiation were rectilinear. Thus the initial rate of disappearance of physostigmine follows first-order kinetics.

The curves obtained when solutions of different pH were heated at a constant temperature are shown in Fig. 1 and a plot of log k (specific rate constant) against pH is in Fig. 2. These show that physostigmine is more stable at acid pH than at alkaline pH and that the pH of maximum stability exists between pH 2·2 and 3·0.

The curves obtained by irradiation of solutions of different pH were superimposable indicating that the specific rate constant does not change over the pH range of 1·5 to 7·0. The observed rate constant for irradiation will include a temperature effect and the true radiation constant is

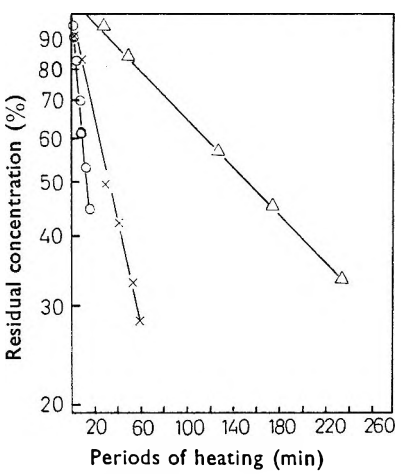


FIG. 1. Plot of "percentage residual concentration" against "period of heating" for solutions of physostigmine sulphate at pH 8·0 ○ —— ○; pH 7·0 × —— ×; and pH 6·0 △ —— △, all at 90° C.

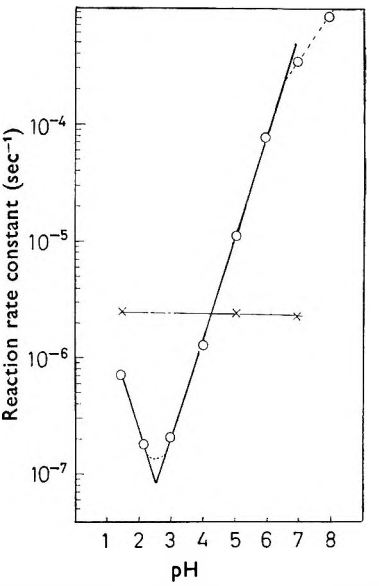


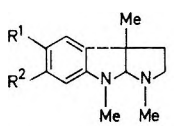
FIG. 2. Plot of "reaction rate constant" against pH for solutions of physostigmine sulphate heated at 90° C ○ —— ○; and for solutions irradiated × —— ×.

given by $k_{irr} = k_{exp} - k_{heat}$. The temperature of the Cobalt 60 source used was about 23° and Table 1 shows the calculated values for k_{irr} in units of time and units of radiation dose. Fig. 2 clearly shows that in the plot of log k_{irr} against pH, the rate of degradation caused by radiation is almost independent of pH.

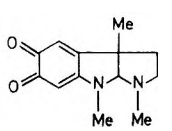
TABLE 1. DEGRADATION RATE CONSTANTS $K_{exp.}$, K_{heat} AND $K_{irr.}$ FOR THE IRRADIATION OF PHYSOSTIGMINE SULPHATE SOLUTIONS AT pH 1·5, 5·1 AND 7·0

	$k_{exp.}$		k_{heat}	$k_{irr.}$	
pH	Mega-rad^{-1}	sec^{-1}	sec^{-}	sec^{-1}	Mega-rad^{-1}
1·5	0·1119	$2{\cdot}551 \times 10^{-6}$	$1{\cdot}068 \times 10^{-11}$	$2{\cdot}551 \times 10^{-6}$	0·1119
5·1	0·1078	$2{\cdot}458 \times 10^{-6}$	$6{\cdot}267 \times 10^{-10}$	$2{\cdot}458 \times 10^{-6}$	0·1078
7·0	0·1029	$2{\cdot}345 \times 10^{-6}$	$1{\cdot}933 \times 10^{-8}$	$2{\cdot}326 \times 10^{-6}$	0·1021

It is therefore apparent that the mechanism of breakdown of physostigmine sulphate in these two processes is different. When the alkaloid is heated in aqueous solution it undergoes hydrolytic cleavage of the methylurethane side chain.



Physostigmine: $R^1 = MeNHCO_2^-$; $R^2 = H$
Eseroline: $R^1 = OH$; $R^2 = H$
Leuco-rubreserine: $R^1 = R^2 = OH$



Rubreserine

The irradiated alkaloid may decompose by two possible mechanisms: (a) a direct ionizing effect on the molecule leading to disruption, and (b) an indirect oxidative chain reaction effect due to attack of such agents as $OH^{\cdot}$, $HO_2^{\cdot}$, $H^{\cdot}$ and H_2O_2 resulting from the irradiation of water.

Sodium metabisulphite has little effect on the degradation rate of physostigmine sulphate, the plot of % residual concentration against period of heating being superimposable in solution with and without metabisulphite. This confirms the reports of Hellberg (1949) and Riegelman & Vaughan (1958) that the presence of an antioxidant does not stabilize physostigmine solutions but simply masks the development of colour resulting from further degradation. This prevention of colour development is probably due to reduction of rubreserine to the colourless leuco-rubreserine (Heacock, 1959).

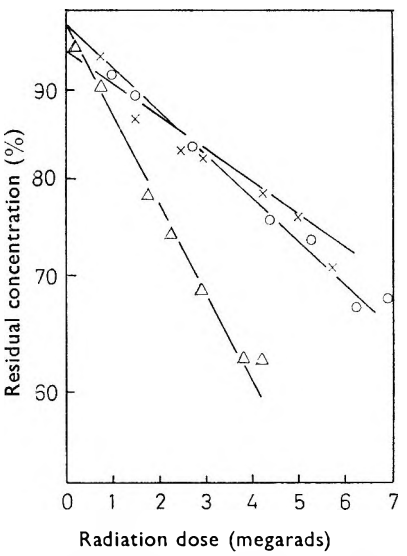


FIG. 3. Plot of "percentage residual concentration" against "dose of radiation" for 0% w/v △ —— △; 0·2% w/v ○ —— ○; 0·5% w/v × —— × sodium metabisulphite in physostigmine sulphate solutions at pH 7·0.

Fig. 3 shows that the presence of sodium metabisulphite in solutions of physostigmine improves the stability of the alkaloid towards irradiation under the conditions described: 0·5% w/v solutions are more effective than 0·2% w/v. The mechanism of degradation is probably oxidative.

Irradiation with 2·5 megarads has no effect on the degradation rate of the solution when subsequently heated at 90°. Since the plots of % residual concentration against dose of irradiation and subsequent period of heating and period of heating with no irradiation are superimposable, it may be concluded that a solution, when stored after irradiating, will degrade at the normal rate for the storage temperature. The presence of 0·5% w/v sodium metabisulphite does not affect the rate

of degradation of irradiated solutions when subsequently heated, but does affect the amount of degradation that occurs during the exposure to 2·5 megarads. Knowing the amount of degradation resulting from 2·5 megarads, it is possible to formulate eye-drops to include the required excess of drug. Thus the solutions could be presented sterile and containing the full amount of the stated amount of drug in the preparation.

Another aspect of the results is that the only effective method of stabilizing physostigmine solutions to heat degradation is to reduce the pH of the solution. For maximum stability, a pH of 3·0 is required and this is generally considered too low for ophthalmic solutions. Schradie & Miller (1959), however, have suggested that provided a solution is buffered with a low capacity system such that, on administration, it quickly adjusts to the normal pH of the eye, a solution at pH 3·0 is quite satisfactory. Using ionizing radiation where the degradation of the alkaloid is independent of the pH of the solution, it is possible, however, to formulate the eye-drops at pH values more acceptable for administration.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 114S–121S Received April 29, 1968

The isolated expansor secundariorum—a smooth muscle preparation from the wing of the domestic fowl

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Some aspects of the pharmacology of an isolated smooth muscle, the expansor secundariorum, of the domestic fowl have been investigated. Adrenaline, isoprenaline, tyramine and 5-hydroxytryptamine caused the muscle to contract. The responses to adrenaline and noradrenaline were blocked by phentolamine but not by propranolol at a concentration of $1{\cdot}7 \times 10^{-4}$M. Tyramine was without effect on the muscle of reserpine treated birds. Cocaine potentiated the effect of noradrenaline but blocked the effects of tyramine. Acetylcholine and histamine had no effect and did not alter the responses of the muscle to noradrenaline. The response to noradrenaline was greater at temperatures below 23° and less at 38°. The muscle contracted rapidly on the addition of Tyrode cooled to 18°. It is concluded that the muscle is wholly innervated by adrenergic postganglionic fibres.

THE attention of pharmacologists was drawn to the expansor secundariorum muscle of birds by George & Berger (1966). This muscle is present in most birds (Berger 1956) although the extent to which it is developed varies in different species. The muscles responsible for the movement of the feathers are innervated by sympathetic fibres (Langley, 1904) and since the analagous pilomotor muscles of the cat are unaffected by acetylcholine it was considered that the expansor secundariorum might provide a large smooth muscle with a purely sympathetic innervation. This report describes some of the properties of this muscle preparation.

Experimental

SETTING UP THE TISSUES

Domestic fowls (Cox 404) between 8–12 weeks and 500 to 1300 g were killed by ether inhalation and the expansor secundariorum muscles dissected immediately. The anatomy of this muscle in the domestic fowl is similar to that described by Berger (1956) for the pigeon (*Columba livia*). The long tendon of the muscle runs beneath the skin on the posterior edge of the wing, from the elbow to the axilla. When the skin in the elbow region is incised and reflected, the expansor secundariorum can be seen embedded in connective tissue. The tendon was separated from the skin and a cotton thread tied around the tendon 2 cm medial to the muscle. A piece of cotton was threaded through the expansor secundariorum so that it passed through the bases of the attached feathers. These were then trimmed level with the muscle and all excess skin removed before detaching the muscle from the surrounding connective tissue. To ensure that the isolated muscle gives consistent contractions, it is essential that the cotton passes through the bases of the feathers.

The muscle preparation was set up in a 10 ml tissue bath containing Tyrode solution gassed with a mixture 5% carbon dioxide in oxygen.

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Muscle contractions were recorded on a smoked drum by means of an isometric lever. This consisted of a short length of watch spring which could be adjusted to allow the muscle a maximum shortening of less than 10% of the resting length. A light lever arm was then attached so as to give a 25x magnification of the contractions. The initial tension in the tissue was about 1 g and the lever was calibrated at the conclusion of each experiment. Except where the temperature was varied as a part of the experimental procedure all experiments were at 23°.

MATERIALS

The Tyrode solution was made up as follows (g/litre): NaCl 8·0, KCl 0·2, $MgCl_2$ 0·1, $NaHCO_3$ 1·0, NaH_2PO_4 0·05, glucose 1·0. The weights refer to anhydrous salts.

Solutions of the following compounds were used freshly prepared in Tyrode solution: acetylcholine chloride, adrenaline hydrochloride, cocaine hydrochloride, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, isoprenaline sulphate, nicotine acid tartrate, phentolamine mesylate, propranolol hydrochloride, tyramine hydrochloride. All concentrations are expressed as the final molar concentration in the tissue bath.

Results

Spontaneous activity. When first placed in the tissue bath, the muscle usually showed some relaxation of tension followed by small spontaneous contractions. The degree of activity varied greatly; some preparations developed a tension of 0·7 g (Fig. 1), whereas others were completely quiescent. Some preparations showed markedly decreased spontaneous activity after the addition of several doses of one of the catecholamines (Fig. 1), whereas others maintained spontaneous contractions throughout experiments lasting 7 hr. The results with phentolamine (q.v.) suggest that the muscle retained some inherent tone and that the muscle fibres were not at their true resting length. Muscle tone was less at 38° than at 23°.

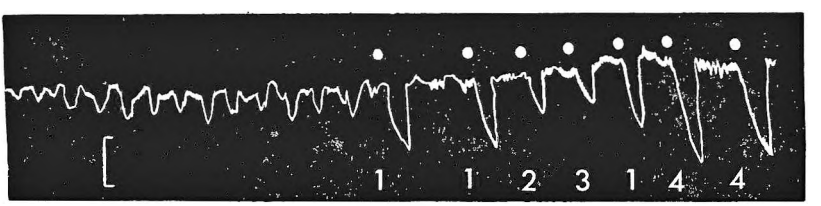

FIG. 1. Spontaneous contractions and the response after addition of tyramine. Four concentrations of tyramine added at 0 to give (1) $5·8 \times 10^{-5}$M. (2) $5·8 \times 10^{-6}$M. (3) $5·8 \times 10^{-7}$M. (4) $2·0 \times 10^{-4}$M. Scale 1 g. Contractions are downwards.

Catecholamines. As would be expected from the innervation of the muscles moving the feathers (Langley, 1904), adrenaline, noradrenaline and isoprenaline caused a contraction of the expansor secundariorum. Adrenaline was the most potent of the three catecholamines used, causing

a contraction at $1{\cdot}6 \times 10^{-8}$M. Noradrenaline was approximately 5 times less potent than adrenaline, but was sixty times more potent than isoprenaline. The time to reach the maximum tension for all these compounds at 23° was 3–4 min. It was not possible in most instances to obtain dose response curves for more than one catecholamine on one preparation because the high concentrations caused the muscle to fatigue and the time taken for relaxation was prolonged (15 min). Fig. 2 shows dose-response curves for adrenaline and noradrenaline.

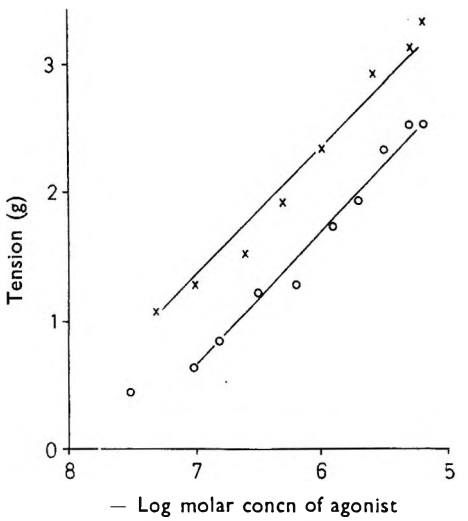

FIG. 2. Dose-response curves for adrenaline (×) and noradrenaline (○).

Tyramine. The expansor secundariorum preparation contracted in the presence of 5×10^{-7}M tyramine and gave greater contractions as the concentration of tyramine was increased (Fig. 1). The effects of reserpine and cocaine on the tyramine-induced contractions are presented under their separate headings.

5-Hydroxytryptamine. The sensitivity of the preparation to this substance was similar to that of noradrenaline. 5-HT did not affect the contractions caused by noradrenaline.

Nicotine. Three out of five preparations tested with nicotine gave contractions at concentrations between 5×10^{-4} and $2{\cdot}5 \times 10^{-3}$M. Fig. 3 shows that more than one contraction can be obtained from a preparation but the response is variable and is not always proportional to dose. Fig. 3A shows that $2{\cdot}5 \times 10^{-4}$M elicited a response but 5×10^{-4}M caused less tension development whilst $2{\cdot}5 \times 10^{-3}$M had the same effect as $2{\cdot}5 \times 10^{-4}$M. The preparation in Fig. 3B gave only two responses to nicotine (5×10^{-5} and $2{\cdot}5 \times 10^{-4}$M) and the preparation in Fig. 3C failed to respond to nicotine. In all instances the addition of nicotine had no effect on the responses to subsequent doses of noradrenaline.

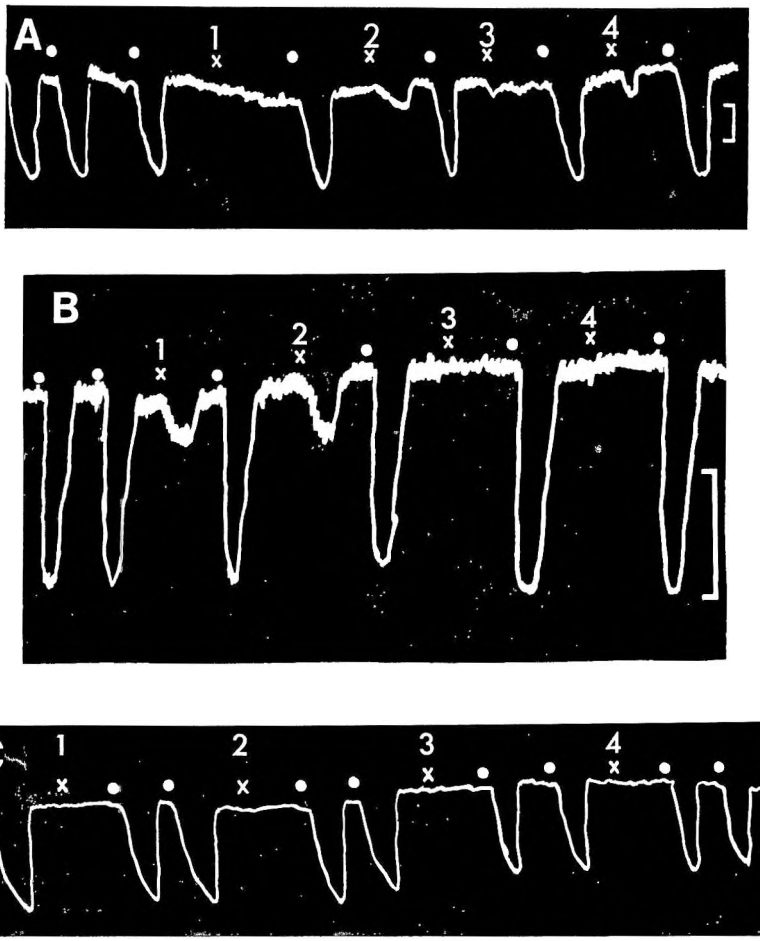

FIG. 3. The response of three preparations to nicotine. Noradrenaline was added at (O) to give a concentration of $3{\cdot}1 \times 10^{-6}$M. Nicotine added at (×) to give the concentrations (1) 5×10^{-5}M. (2) $2{\cdot}5 \times 10^{-4}$M. (3) 5×10^{-4}M. (4) $2{\cdot}5 \times 10^{-3}$M. Scale 1 g. Contractions are downwards.

Antagonists of catecholamines. Phentolamine and propranolol were used in an attempt to determine the nature of the adrenergic receptors in the expansor secundariorum. In these experinents the antagonist was added to the bath 3 min before the addition of the noradrenaline or adrenaline. Propranolol up to a concentration of $1{\cdot}7 \times 10^{-4}$M had no effect and did not influence the contractions produced by adrenaline and noradrenaline. Phentolamine at a concentration of $1{\cdot}3 \times 10^{-6}$M caused some relaxation of the preparation and inhibited the actions of adrenaline and noradrenaline. At a concentration of $1{\cdot}3 \times 10^{-5}$M, phentolamine abolished the response of the preparation to $6{\cdot}3 \times 10^{-6}$M noradrenaline.

Acetylcholine. At concentration $5{\cdot}4 \times 10^{-8}$ to $5{\cdot}4 \times 10^{-3}$M acetylcholine had no effect and did not influence the contractions produced by

adrenaline when this was added to the bath already containing acetylcholine.

Reserpine. Fowls were pretreated by injecting reserpine intramuscularly 1 mg/kg on the first day and on the following day 0·65 mg/kg. After each injection the birds became lethargic and unresponsive to changes in their environment. Expansor secundariorum muscles were used one or two days after the second injection of resperpine. When tyramine was added to the bath at concentrations from $5{\cdot}8 \times 10^{-7}$ to $2{\cdot}9 \times 10^{-4}$M, no effect was observed. Sensitivity to noradrenaline was not different from that of muscles from untreated birds. After the addition of noradrenaline tyramine caused contractions when added in concentrations within the range which caused contractions of muscles from normal birds. Fig. 4 shows the declining effect of successive doses of $5{\cdot}8 \times 10^{-5}$ tyramine to a muscle from a reserpinzied fowl which had been exposed to a noradrenaline at a concentration of $3{\cdot}1 \times 10^{-5}$M. When the response to tyramine had declined to 13% of that produced by the first dose immediately following the noradrenaline, a further dose of noradrenaline ($3{\cdot}1 \times 10^{-5}$M) elicited a normal contraction and restored the ability to respond to further addition of tyramine.

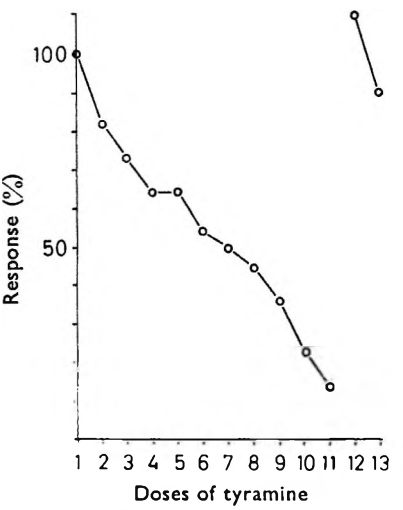

FIG. 4. The response of a muscle from a reserpinized bird to successive doses of tyramine. The muscle was unresponsive to tyramine until the tissue had been exposed to a direct-acting amine. The concentration of tyramine was $5{\cdot}8 \times 10^{-6}$M in each instance, and the response is expressed as a percentage of the response to the first dose of tyramine after obtaining contractions with noradrenaline. A normal response to noradrenaline was obtained between tyramine doses 11 and 12.

Cocaine. Cocaine $2{\cdot}9 \times 10^{-5}$M produced an immediate contraction. After washing, the muscle relaxed and the sensitivity to tyramine was decreased whereas the sensitivity to noradrenaline was increased. In concentrations of $5{\cdot}9 \times 10^{-6}$M cocaine did not cause a contraction but the response of the muscle to tyramine was decreased and the response to noradrenaline was increased. Cocaine did not have an immediate effect

on the responses to tyramine and noradrenaline but required a period of contact of about 10 min before its maximum effect was produced.

Histamine. In concentrations ranging from $3{\cdot}3 \times 10^{-8}$ to $3{\cdot}3 \times 10^{-5}$M histamine neither produced a contraction nor affected the responses to adrenaline.

The Effect of alterations of temperature. The temperature was changed by adding Tyrode solution at the required temperature to the bath. A thermometer in the tissue bath was used to monitor the temperatures. At 38° the response to adrenaline was approximately one third of that produced by the same concentration at 23°. At 17° the response of the muscle to adrenaline was slightly potentiated. The time taken to reach peak tension at 38° and 17° was greater than at 23°. At 18° and below the preparation gave a marked spontaneous contraction reversed quickly by raising the temperature to 23°. When the muscle was partially relaxed from a "cold contraction" it still responded normally to noradrenaline. The maximum tension developed in a "cold contraction" was also developed at 23° when sufficient noradrenaline was added. The amount of tension developed in a cold contraction was related to the temperature inducing the contraction over the range 18–12°. Each cold contraction was followed by a normal response to noradrenaline at 23°. Phentolamine ($1{\cdot}3 \times 10^{-4}$M) did not inhibit the "cold contractions" when added with the cold Tyrode solution.

Discussion

Although there is an overall similarity of pharmacological properties between the pilomotor muscles of the cat (Hellman, 1963a, 1963b) and the expansor secundariorum of the fowl, there are important differences. The most obvious of these is the spontaneous activity present in the expansor secundariorum but absent in the isolated pilomotor muscles of the cat (Hellman, 1963a). It is of interest that cooling can produce spontaneous pilomotor activity in man (Lewis & Marvin, 1927). The spontaneous contractions, and the contraction occasioned by cooling which are properties of the expansor secundariorum, are difficult to ascribe to a physiological function. According to Jollie (1957) the function of this muscle is control of the secondaries and tertiaries during flight. Since phentolamine does not block the cold contraction it is likely that this contraction is not mediated by the nerve endings.

In general, any sudden alteration of temperature can act as a stimulus and provoke a contraction in muscles (Evans, 1926). In the case of the expansor secundariorum this applies only for a fall in temperature, a rise producing a small degree of relaxation. This is in contrast to the guinea-pig ileum which gives a long-lasting contraction on both cooling and rewarming (Innes, Kosterlitz & Robinson, 1957) and the cat isolated iris which develops increased tonus as the temperature falls to 12° and develops further tonus as it is raised again to 20° (Verbitzky, 1923).

Cooling potentiates the actions of catecholamines on the isolated pilomotor muscles of the cat (Hellman, 1963b) and the expansor secundariorum. The rate of catecholamine-induced contraction of the expansor secundariorum at 38° and 15°C is slower than at 23°. The pilomotor muscles contract more slowly as the temperature is raised above 20° but in contrast to the expansor secundariorum the rate is not altered below this temperature (Hellman, 1963b).

The sensitivity of the expansor secundariorum to noradrenaline isoprenaline, phentolamine and propanolol is indicative of α-adrenergic receptors in the tissue. Whilst this is analagous to the suggestion of Hellman (1963a) with respect to the pilomotor muscles, there is a difference between these two muscles in their relative sensitivities to isoprenaline. In the pilomotor muscles of the cat the ratio adrenaline: isoprenaline sensitivity is 1:10,000 (Hellman 1963a) whereas in the expansor secundariorum the ratio is 1:20. The sensivity to phentolamine is similar in the two tissues.

The effects of reserpine and tyramine agree well with the effects of these compounds observed on other tissues. This evidence provides strong support for the tentative conclusion that adrenergic nerve terminals are present in the expansor secundariorum. The lack of effect of nicotine on some of our muscle preparations is not unusual since Bell (1968) and Hellman (1963a) present similar data for the vas deferens and pilomotor muscles.

Potentiation of the action of noradrenaline by cocaine can be explained by an effect of cocaine on the postsynaptic receptors (Reiffenstein, 1968) or by an effect of blocking the uptake of noradrenaline into the nerve terminals (Trendelenburg, 1966). The decreased response of the expansor secundariorum to tyramine in the presence of cocaine is difficult to explain in terms of a postsynaptic effect if tyramine acts on the stores of noradrenaline, as indicated by the lack of its effect in reserpinized muscles. A presynaptic action of cocaine can only potentiate the action of noradrenaline if uptake into the nerve terminals is a factor limiting the action of noradrenaline. In the tissue bath where the amount of noradrenaline is great compared to the capacity of the nerve terminals to take up noradrenaline, it is unlikely that uptake would be important. It thus seems possible that the blocking of the effects of tyramine by cocaine is produced by a blockade of uptake into nerve terminals, whereas the potentiation of the response to noradrenaline is produced by a direct action on the adrenergic receptors.

The lack of effect of acetylcholine provides strong support for the assumption that there is no cholinergic innervation of the expansor secundariorum. The absence of any actions of acetylcholine when added in a wide range of concentrations does not support the hypothesis of Burn & Rand (1965) which requires that acetylcholine releases noradrenaline from sympathetic nerve endings.

The expansor secundariorum is an interesting preparation because it appears to be innervated wholly by postganglionic sympathetic fibres and is completely unresponsive to acetylcholine. The use of this muscle

preparation should make it possible to study the effects of drugs on the assumption that whatever their effects they are unlikely to be mediated via cholinergic receptors or parasympathetic fibres.

Acknowledgements. We are grateful to Professor A. S. King, Department of Veterinary Anatomy for helpful advice on some parts of this work. The birds were provided by Thornber's Hatcheries, Mytholmroyd. Part of the expenses of this investigation were defrayed by a grant from the Ministry of Defence.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 122S–125S Received April 29, 1968

SHORT COMMUNICATION

Effect of pretreatment with monoamine oxidase inhibitors or (+)-amphetamine on leptazol convulsions in mice and rats

T. A. R. TURNER AND P. S. J. SPENCER

IT has been claimed that monoamine oxidase inhibitors inhibit the actions of leptazol in experimental animals (Chow & Hendley, 1959; Prockop, Shore & Brodie, 1959; Yen, Salvatore & others, 1962). But other workers have failed to confirm this anticonvulsant action (Kobinger, 1958; Lessin & Parkes, 1959) and some have claimed a proconvulsant effect (Sansome & Dell'Omodarme, 1963; Spoerlein & Ellman, 1961). Reports of the effect of (+)-amphetamine on leptazol convulsions are equally conflicting. Small doses capable of antagonizing electroshock convulsions are described as ineffective against leptazol according to Wolff & Stock (1966), whilst Friebel & Klatt (1959) demonstrated a proconvulsant action. Reserpine enhances the effect of leptazol in animals, the greatest effect appearing to coincide with maximal depletion of tissue amines. If the animals are pretreated with a monoamine oxidase inhibitor before receiving reserpine, however, the subsequent sensitivity of the animal to leptazol is reduced (Pfeifer & Galambos, 1967) or is unaffected (Chen & Bohner, 1961; Spoerlein & Ellman, 1961).

We report initial observations during a re-examination of the interaction between leptazol and five representative monoamine oxidase inhibitors.

EXPERIMENTAL

Animals. Adult male TO albino mice, weighing 18–25 g, and adult male Wistar albino rats, weighing 150–200 g, were used. They were maintained on a 41 B cube diet and water until 2 hr before experiment. Twenty-four hr before experiment, all animals were transferred to a temperature controlled room at 20 ± 0·5°, relative humidity 60%, where the experiments were made.

Leptazol convulsions. Convulsions were produced in groups of 10 mice by intraperitoneal injection of leptazol (80 mg/kg) in 0·9% saline (0·2 ml/20 g weight). In groups of 10 rats convulsions were produced by the intraperitoneal injection of leptazol (55 mg/kg) dissolved in 0·9% saline (0·5 ml/100 g weight). Saline injection of the same volumes were used as controls in both species. The *mortality ratio* was the proportion of mice dead in the test group divided by the proportion dead in the control group, 15 min after the leptazol injection. The *convulsive ratio* was the number of convulsive episodes in a group of test rats divided by the number of episodes in a control group of equal size, during the 15 min period after leptazol injection. Mortality or convulsive ratios

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greater than one indicate a pro-convulsant effect, and ratios less than one indicate an anti-convulsant effect. The results in Figs 1 and 3 are the means from at least three duplicate experiments; significance of difference was calculated by Student's *t*-test.

Locomotor activity. Groups of 4 mice received intraperitoneal injections of (+)-amphetamine, 5 mg/kg, or its vehicle, 0·9% saline. Locomotor activity was determined for successive 30 min intervals during the next 6 hr, using a Faraday Animal Activity Recorder (Hawkesley & Sons, Lancing, Sussex). By previously acclimatizing the mice to the procedure of intraperitoneal injection, and making activity counts in their home cages, the contributions of fear and exploration to the total activity counts were reduced to a minimum.

Body temperature. Using the method of Brittain & Spencer (1964), the oesophageal temperatures of groups of ten mice were determined at 30 min intervals after the intraperitoneal injection of (+)-amphetamine, 5 mg/kg, or its vehicle, 0·9% saline (0·2 ml/20g).

Administration of drugs. The six compounds studied were: iproniazid phosphate, phenelzine hydrogen sulphate, nialamide, tranylcypromine sulphate, pargyline hydrochloride and (+)-amphetamine sulphate. Each drug was dissolved in 0·9% saline and administered intraperitoneally. All doses in the text have been expressed in terms of the free base.

RESULTS AND DISCUSSION

The intraperitoneal injection of 0·9% saline alone, or the intraperitoneal injection of any of the five monoamine oxidase inhibitors or (+)-amphetamine alone (at the doses in Fig. 1), did not induce convulsions or cause death in mice. The results of the pretreatment at varying times before leptazol are shown in Fig. 1.

A transient but significant proconvulsant effect was observed in mice pretreated with iproniazid and phenelzine (substituted hydrazines), and also with tranylcypromine which does not belong to this chemical group. By contrast, the hydrazine monoamine oxidase inhibitor nialamide, and the non-hydrazine monoamine oxidase inhibitor pargyline were devoid of this proconvulsant effect. A definite anticonvulsant action was not observed with any of the five monoamine oxidase inhibitors at the doses used.

The doses quoted in Fig. 1 all produce effective inhibition of monoamine oxidase activity. This was verified using the method of Corne, Pickering & Warner (1963). Groups of 10 mice were pretreated with a monoamine oxidase inhibitor for 1½ or 4 hr before the intraperitoneal injection of 5-hydroxytryptophan at 50 mg/kg. The proportion of control mice eliciting the characteristic head-twitch response varied between 0 and 10% when examined 25 min later. In contrast, the proportion of mice giving this response after pretreatment with a monoamine oxidase inhibitor was consistently 60% or greater. This potentiation of the head-twitch response is considered to indicate an effective inhibition of monoamine oxidase activity by these doses of inhibitor drugs.

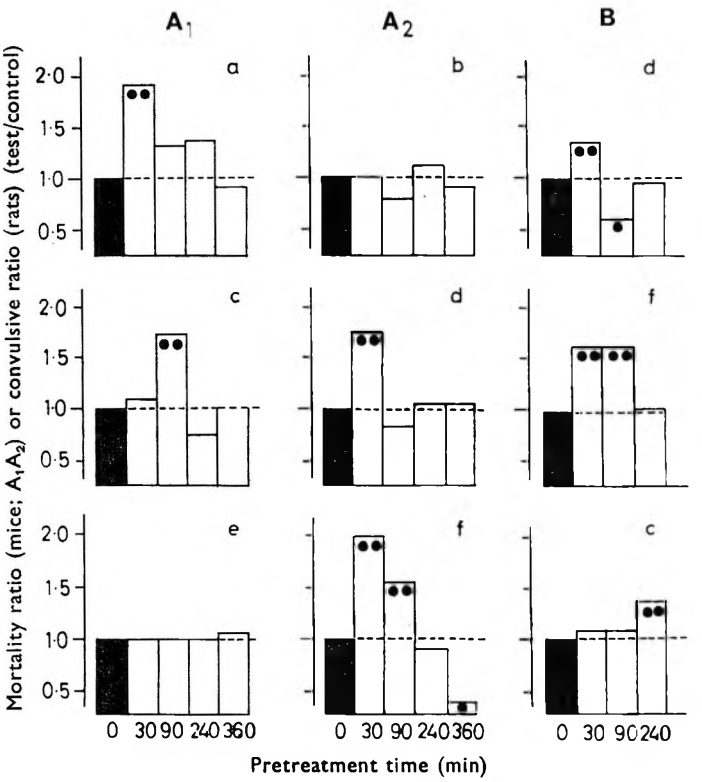

FIG. 1A. Effects of (a) iproniazid (65 mg/kg), (b) nialamide (10), (c) phenelzine (6), (d) tranylcypromine (2), (e) pargyline (50) and (f) (+)-amphetamine (5) on leptazol-induced mortality in mice. Each drug was injected at various times before administration of leptazol; group mortality was assessed 15 min later. (● ● indicates significant proconvulsant effect, and ● indicates significant anticonvulsant effect $P = <0{\cdot}05$).

B. Effects of phenelzine, tranylcypromine and (+)-amphetamine on leptazol-induced convulsions in rats. Pargyline was also tested but did not differ from the control. Each drug was injected at various times before administration of leptazol; the number of convulsive episodes in test and control groups was counted during the next 15 min (● ● indicates significant proconvulsant effect, and ● indicates significant anticonvulsant effect, $P = <0{\cdot}05$).

These results indicate that a proconvulsant action by a monoamine oxidase inhibitor is not related to its ability to inhibit this enzyme, nor to the presence or absence of the hydrazine moiety.

A feature common to the three compounds showing proconvulsant activity is their inherent sympathomimetic activity (Goldberg, 1964) and therefore, (+)-amphetamine should also exert a proconvulsant action with leptazol, and this was investigated (see Fig. 1). At a dose of 5 mg/kg, this produced a marked and slightly more prolonged potentiation of the convulsant and lethal effects of leptazol than was observed with iproniazid, phenelzine or tranylcypramine, yet, 6 hr after its administration, a definite anticonvulsant effect was observed which may be a true

postictal effect related to the earlier intense central adrenergic stimulation produced by (+)-amphetamine.

(+)-Amphetamine (5 mg/kg) did not increase the body temperature of mice but it did cause an elevation of motor activity, 30 min counts being $\approx 3 \times 10^3$ from 0–3 hr while controls were $< 0{\cdot}25 \times 10^3$.

The effects of phenelzine, tranylcypromine and (+)-amphetamine on leptazol convulsions in rats (see Fig. 1B) support the conclusions from the mice experiment, that monoamine oxidase inhibition *per se* was not associated with a potentiation of the convulsive effects of leptazol. But like (+)-amphetamine, inhibition of monoamine oxidase by inhibitors with inherent sympathomimetic activity potentiated the effects of leptazol in rats.

It is concluded that leptazol convulsions and deaths due to leptazol may be potentiated in rats and mice by pretreatment with (+)-amphetamine or monoamine oxidase inhibitors possessing inherent sympathomimetic activity. The effect is transient; for (+)-amphetamine it appears to coincide roughly with the elevation of motor activity by the drug and thus may be due to an effect on the central rather than the peripheral adrenergic nervous system. Nevertheless, an effect on the animals' ability to inactivate leptazol cannot be excluded. Drugs which simply inhibit monoamine oxidase, whether of the hydrazine group or not, possess neither proconvulsant nor anticonvulsant activity.

Acknowledgement. One of us (T.A.R.T.) is grateful to the Medical Research Council for a training scholarship.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 126S–129S Received May 1, 1968

SHORT COMMUNICATION

The effect of alloxan diabetes upon adjuvant-induced arthritis in the rat

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THE relation between inflammation and carbohydrate metabolism has been the subject of several investigations using acute inflammatory models such as the anaphylactoid reaction in the rat (Adamkiewicz & Adamkiewicz, 1959; 1960) and true anaphylaxis in the rat and mouse (Kraus, 1964; Gulbenkian, Yanell Grasso & Tabachnick, 1967; Dhar, Sanyal & West, 1967). These acute inflammatory reactions are depressed by the hyperglycaemia produced by alloxan (Adamkiewicz & Adamkiewicz, 1959; 1960), by 2-deoxyglucose (Goth, 1959), by glucose loading (Dhar & others, 1967) and by glucagon (Lefebvre & Van Cauwenberge, 1962).

Using the model of "adjuvant arthritis" in the rat, produced by the injection of Freund's adjuvant (Pearson, 1956), Kellett (1965) showed that this chronic arthritic condition was also suppressed when alloxan diabetes was induced in rats before an injection of adjuvant. The syndrome of adjuvant arthritis occurs in two phases, firstly an acute inflammatory oedema at the injection site which is followed after a latent period of about ten days by a secondary, chronic polyarthritis involving all limbs and the tail.

Because of the biphasic course of the inflammation, and the possible dependence of the chronic phase on the prior appearance of an adequate initial phase, we decided to investigate the effect of alloxan diabetes induced at various times during the development of the adjuvant arthritic syndrome.

EXPERIMENTAL

Groups of seven male Wistar rats (Scientific Products Farm Ltd.), weight 150–200 g were used. Arthritis was produced by the intradermal injection of 0·03 ml of a 5 mg/ml suspension of dead tubercle bacilli in liquid paraffin, into the plantar surface of the left hind paw. The volume of both hind paws and the animals' body weights were determined at intervals during the next 28 days.

Paw volume was measured by immersion in a mercury manometer, the rise in the level of mercury being detected by a Devices pressure transducer and recorded using a calibrated Devices DC 2c amplifier and pen recorder. The paw volume is expressed as ml/kg body weight; standard errors of the means were calculated, and significance of difference between groups calculated using Student's *t*-test.

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To produce diabetes, rats were starved for 24 hr and, under halothane anaesthesia, given 35 mg/kg of alloxan in 0·9% saline into the right femoral vein. All animals so treated had a glucosuria of at least 0·5% within 48 hr of the injection. Non-diabetic control animals were similarly treated receiving intravenously only the appropriate volume of saline.

Urine from both diabetic animals and non-diabetic controls was examined daily for glucose using "Clinistix" (Ames and Co.). The diabetic rats consistently showed a glucosuria in excess of 0·5%. In contrast, glucose was never detected in the urine of the control animals of this strain.

RESULTS AND DISCUSSION

The course of the arthritis was followed in control animals and animals made diabetic at three different stages: (*a*) alloxan was administered four days before the injection of adjuvant; (*b*) alternatively, alloxan was given on the fourth day *after* the adjuvant injection, i.e. after the acute inflammation had become maximal, but before the onset of the secondary phase; (*c*) alloxan was administered on the fifteenth day after adjuvant injection, i.e. when the second chronic arthritic phase had been established.

In the first experiment, when diabetes was produced before the injection of adjuvant, our results agree with those obtained by Kellett (1965) in a similar experiment; the diabetes significantly attenuated both the acute and secondary chronic reactions to the adjuvant (Fig. 1A). This effect of diabetes on the initial acute local response can be related to other work on acute inflammation and hyperglycaemia in the rat (Adamkiewicz & Adamkiewicz, 1959; Goth, 1959). It has been pointed out (Goth, 1959) that dextran and egg-white both cause inflammation and both contain a carbohydrate moiety. Lack of insulin may inhibit the participation of these carbohydrate-containing molecules in inflammatory reactions. A similar situation may arise with Freund's adjuvant since it has been shown (Tanaka, Ishibashi & Sugiyama, 1967) that the antigenic component of Wax D from *Mycobacterium tuberculosis* also contains carbohydrate. With respect to the secondary reaction, Waksman, Pearson & Sharp (1960) maintain that the chronic reaction to adjuvant in rats is probably a delayed hypersensitivity to the tubercular antigen. Consequently the diabetic state may interfere with the antibody formation fundamental to the development of the chronic response.

Newbould (1964) demonstrated that immunologically competent lymphocytes are first released from lymph nodes between the fifth and seventh days after adjuvant injection. In the second experiment (Fig. 1B) alloxan given on the fourth day after adjuvant reduced the secondary response, although the attenuation of the chronic reaction was less marked than in the first experiment. This difference could be explained on the assumption that antibody formation was markedly suppressed in the first experiment but not in the second, in which essentially normal levels of antibody would be present at the time the animal developed diabetes.

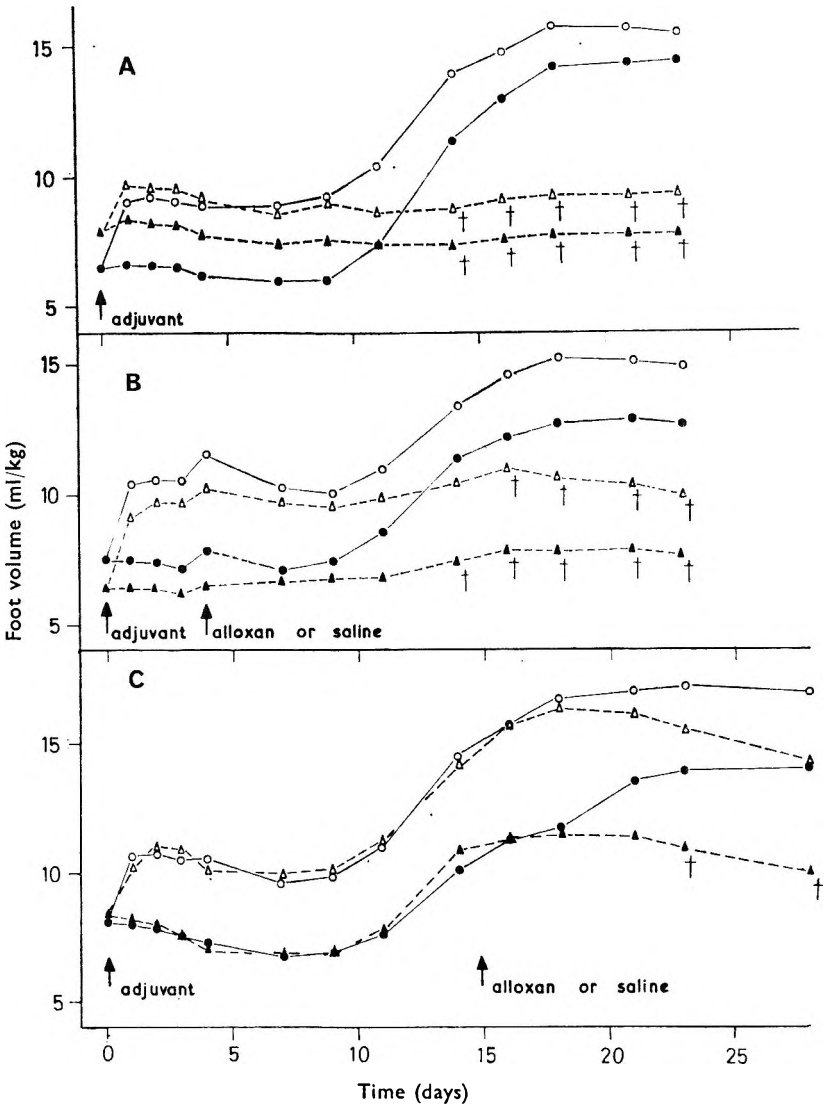

FIG. 1. Changes in hind foot volume in control rats and rats made diabetic A, 4 days before, B, 4 days, C, 15 days after the injection of adjuvant. The symbol † indicates a significant difference ($P = <0{\cdot}05$) between the corresponding feet of diabetic and control groups. Controls: ●—● right foot; ○—○ left foot. Diabetic: ▲--▲ right foot; △—△ left foot.

When rats were injected with alloxan fifteen days after adjuvant injection, the hind paw oedema already present (chronic arthritic phase) slowly declined from day fifteen to day twenty-eight (Fig. 1C). Such reversal of an established chronic arthritis by diabetes may be a direct effect on the inflammatory events subsequent to antibody formation; alternatively, there might be a further reduction in the circulating antibody

necessary to maintain the inflammatory reaction. Clinically, hyperglycaemia has been shown to reduce the severity of asthmatic attacks (Abrahamson, 1941) and rheumatoid arthritis (Helmer, Kirtley & Ridolfo, 1957). Consequently, there may be a place for induced hyperglycaemia in the treatment of inflammatory disease in man.

Acknowledgements. The technique for measuring paw volume was suggested by Dr. R. T. Brittain, of Allen & Hanburys, Ware, Hertfordshire. We are also indebted to the Ministry of Agriculture Veterinary Laboratories, Weybridge, Surrey, for a supply of dead tubercle bacilli.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 130S–134S Received May 1, 1968

An adrenergic neuron blocking action of propranolol in isolated tissues

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Propranolol was tested for adrenergic neuron blocking activity in three isolated sympathetically-innervated smooth muscle preparations; the rat vas deferens, rabbit ileum and rabbit ear artery. In each preparation propranolol impaired the responses to sympathetic stimulation without reducing the responses to added noradrenaline. This blocking action of propranolol resembled that of guanethidine in time of onset and persistence of blocking activity but, unlike blocking by guanethidine, was not reversed by (+)-amphetamine. Desipramine and noradrenaline also failed to reverse the blocking action of propranolol. In the rat vas deferens preparation lignocaine had a weaker and more transient sympathetic blocking action than propranolol. It is suggested that the sympathetic blocking action of propranolol may contribute to its antihypertensive effect in man.

PROPRANOLOL is a potent and specific β-adrenergic receptor blocking agent with little intrinsic sympathomimetic activity (Black, Crowther & others, 1964). Propranolol also has potent local anaesthetic activity (Morales-Aguilerá & Vaughan-Williams, 1965) and clinically has been shown to exhibit antifibrillatory (Rowlands, Howitt & Markman, 1965), anti-anginal (Gillam & Prichard, 1965) and antihypertensive properties (Prichard & Gillam, 1964).

It has been suggested that propranolol lowers arterial blood pressure by impairing cardiac sympathetic tone and thus reducing cardiac output (Prichard, 1968). An antihypertensive agent with this mode of action is of particular interest since it might be free from many side-effects caused by non-selective sympathetic blockade such as occurs with the adrenergic neuron-blocking drugs (Green, 1962). The adrenergic neuron-blocking drugs xylocholine, bretylium and guanethidine have antihypertensive properties in common with propranolol and are potent local anaesthetics (Green, 1962). Propranolol was therefore tested for a possible pre-synaptic blocking action on peripheral adrenergic neurons.

Experimental

Rat isolated vas deferens. Both vasa deferentia removed from recently killed rats were threaded through bipolar platinum electrodes and were set up in organ baths containing aerated Tyrode solution at 32° in separate but simultaneous experiments. Electrical stimulation of the intramural sympathetic nerve endings was with pulses of supramaximal strength (usually 20 V) of 2 msec duration and at a frequency of 5 to 20 pulses/sec delivered from a constant voltage electronic stimulator for periods of 15 sec repeated every 5 min.

Finkleman preparation of rabbit ileum. Preparations were set up and electrically stimulated as described by Day & Rand (1961) except that the Ringer solution was replaced by aerated Tyrode at 37°.

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Rabbit isolated ear artery preparation. This preparation was set up and electrically stimulated as described by De la Lande & Rand (1965).

Results

Rat isolated vas deferens. In this preparation propranolol (1 to 5 μg/ml) caused a progressive impairment of the responses to sympathetic nerve stimulation whilst the responses to added noradrenaline were either unaffected, or more usually, increased. The result of an experiment in which the sympathetic nerve blocking action of propranolol was compared with that of guanethidine is shown in Fig. 1. In this experiment propranolol (3 μg/ml) caused a similar degree of impairment of the responses to sympathetic stimulation as did guanethidine (1 μg/ml). In each experiment the response to added noradrenaline (2 μg/ml) was slightly increased after establishment of the block. Whereas the adrenergic neuron blocking action of guanethidine was reversed 1 hr after adding (+)-amphetamine (0·05 μg/ml) to the bath (Fig. 1B), this treatment did not restore the responses to sympathetic stimulation after propranolol

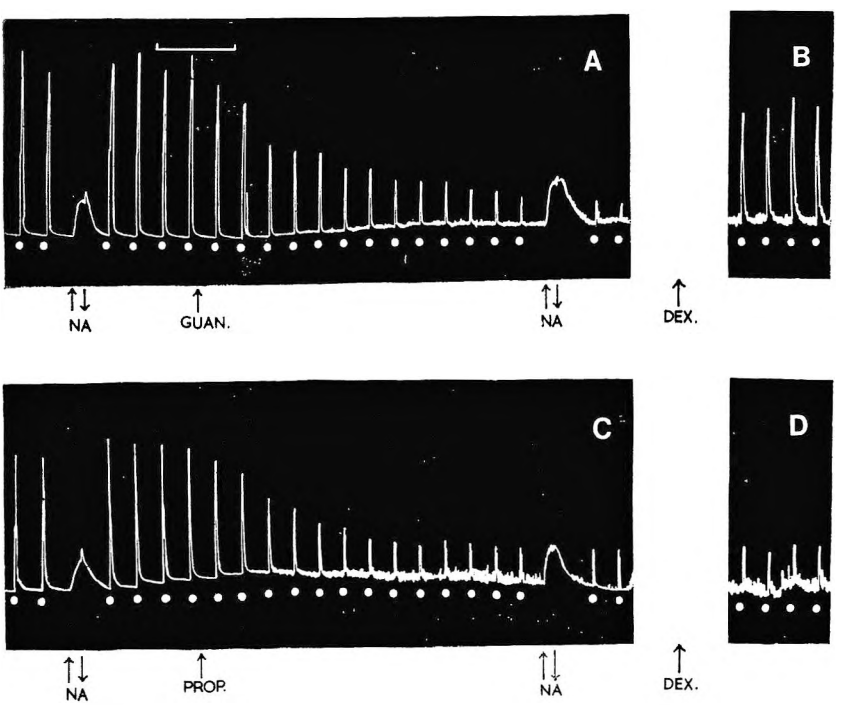

FIG. 1. Rat vas deferens preparations. At white dots stimulation of intramural sympathetic nerves with 2 msec 20 V pulses at frequency of 10 pulses/sec. 2 μg/ml noradrenaline (NA) added to bath at arrows and left in contact with the preparations for 2 min. Upper record: 1 μg/ml guanethidine caused sympathetic block which was partly reversed in B 60 min after adding (+)-amphetamine (DEX) (0·05 μg/ml) to the bath. Lower record: contralateral preparation from same rat sympathetic blockade produced by 3 μg/ml propranolol was not reversed (in D) 60 min after adding (+)-amphetamine to the bath.

(Fig. 1D). The adrenergic neuron blocking action of propranolol was persistent and was only very slowly reversed by repeated washing of the preparation over several hours.

In other experiments, attempts were made to reverse the blocking action of propranolol with either noradrenaline (1 to 2 μg/ml) or desipramine (0·1 to 0·5 μg/ml). These concentrations of noradrenaline initially contracted the tissue but caused no increase in the sympathetic responses after propranolol left in contact for up to 45 min. Desipramine caused a large increase in the sensitivity to added noradrenaline but had no effect on the response to sympathetic stimulation when added before or after the establishment of a propranolol block.

In a few preparations pronethalol was used instead of propranolol and was found to have a similar action in blocking nervously-mediated responses without reducing the responses to added noradrenaline. Pronethalol was approximately half as potent as propranolol in producing nerve block and was more readily reversed by washing.

Finkleman preparation. This preparation was chosen to test the effects of propranolol on inhibitory sympathetic responses because the responses are mediated by an action of neuronal noradrenaline on both α- and β-adrenergic receptors (Furchgott, 1960). The results using this preparation were essentially the same as those obtained using the isolated vas deferens preparation. Thus, propranolol (3 μg/ml) produced a similar impairment of the responses to sympathetic nerve stimulation as did guanethidine (1 μg/ml). Fig. 2 illustrates an experiment in which propranolol (3 μg/ml) produced a rapidly developing impairment of the responses to sympathetic stimulation although the inhibitory responses to added noradrenaline were virtually unaffected. As in the vas deferens preparation, the blocking action of propranolol was not reversed by (+)-amphetamine (0·1 to 0·5 μg/ml) and was only slowly reversed by repeated washing of the preparation. The blocking action of guanethidine was even more persistent after washing the preparation but was readily

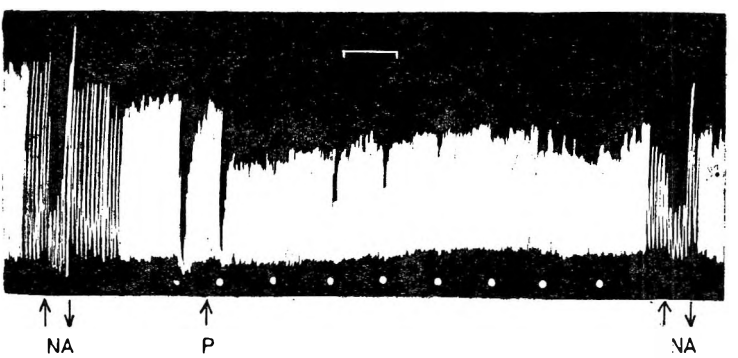

FIG. 2. Finkleman preparation of rabbit ileum. At white dots periarterial sympathetic nerves stimulated with 2 msec 10 V pulses at frequency of 50 pulses/sec. Noradrenaline 0·05 μg/ml added to bath (at NA) and left in contact with preparation 30 sec. Propranolol 3 μg/ml (at P) added to bath. Drum speed increased during noradrenaline responses.

reversed by (+)-amphetamine. Pronethalol had a similar effect in this preparation to propranolol but again was less potent, was more easily reversed, and itself inhibited the spontaneous activity of the preparation.

Rabbit isolated ear artery preparation. This preparation was chosen to determine whether propranolol had a similar adrenergic neuron blocking action on sympathetically innervated vascular smooth muscle as it did in other smooth muscle preparations tested, since this may have some bearing on its use as an antihypertensive agent. It was found that propranolol (0·25 to 1 μg/ml) produced a slowly-developing but persistent impairment of the constrictor responses to sympathetic stimulation whereas the responses to injected noradrenaline were enhanced. In this preparation, unlike the other preparations tested propranolol was at least as potent as guanethidine in producing adrenergic neuron blockade.

Comparison of the nerve blocking actions of propranolol and lignocaine. Propranolol has similar local anaesthetic potency to lignocaine (Morales-Aguilerá & Vaughan-Williams, 1965) and it was thought possible that this action could explain its effects on adrenergic neurons. For this reason the blocking action of propranolol was compared with that of lignocaine in the Finkleman preparation of rabbit ileum and in the rat isolated vas deferens. In the rabbit ileum preparation lignocaine usually caused impairment of the pendular movements of the preparation in concentrations (10 to 30 μg/ml) which did not significantly affect the responses to sympathetic stimulation. Propranolol on the other hand caused a complete abolition of the nervously mediated responses at a concentration of 1 to 3 μg/ml which did not affect the spontaneous activity of the preparation.

In the isolated vas deferens preparation lignocaine did not affect the responses to sympathetic stimulation at a concentration (30 μg/ml) ten times higher than that of propranolol needed to cause an almost complete block of the responses. At a concentration of 50 to 100 μg/ml, lignocaine caused a partial nerve blockade which unlike the propranolol block was readily reversed by washing.

Discussion

The results described indicate that propranolol has a potent blocking action on adrenergic sympathetic neurons in isolated smooth muscle preparations. The adrenergic neuron blocking action of propranolol appears to be pre-synaptic and independent of its post-synaptic effect on β-adrenergic receptors. Thus, at a time when the block was at a maximum the responses to exogenous noradrenaline were either unaffected or increased; in addition the block occurred in tissues such as the rat vas deferens and rabbit ear artery in which only α-adrenergic receptors are involved.

The potency of propranolol in blocking adrenergic neurons was only slightly less than that of guanethidine to which it has a similar time of onset and was almost equally persistent in its blocking action after changing the bath fluid. However, the blocking action of propranolol could be distinguished from that of guanethidine by the fact that only that of

guanethidine was reversed by (+)-amphetamine. Antagonism occurs with (+)-amphetamine and other adrenergic neuron blocking agents and is probably competitive in nature (Day, 1962; Day & Rand, 1963). Similarly it is unlikely that the blocking action of propranolol is caused by depletion of noradrenaline from the sympathetic nerves, as occurs with reserpine, since the block was not reversed by noradrenaline. Desipramine was tested as a potential propranolol antagonist because of the recent report that it partially antagonized the action of propranolol in preventing the increase in rate of beating of isolated atria in response to sympathetic stimulation (Shimamoto & Toda, 1968). No such antagonism was found in the rat vas deferens preparation despite a large increase in sensitivity of the preparation to added noradrenaline caused by desipramine.

Thus the most likely explanation of the blocking action of propranolol is to be found in its potent local anaesthetic property. However, in a direct comparison with lignocaine, with which it has been reported to be approximately equipotent as a local anaesthetic (Morales-Aguilerá & Vaughan-Williams, 1965), propranolol was found to be much more potent and persistent in its blocking action on adrenergic neurons. We cannot preclude the possibility that the sympathetic blocking action of propranolol is a consequence of its local anaesthetic activity since it may be that it exerts this action on sympathetic nerve endings more effectively than lignocaine possibly as a result of more complete penetration into the tissue.

The antihypertensive effect of propranolol in man is of slow onset (Prichard & Gillam, 1964) and this is consistent with the hypothesis that the drug is slowly accumulated in peripheral adrenergic neurons thus causing a reduction in sympathetic vasomotor tone which would tend to reinforce its better known β-blocking action on cardiac receptors in lowering arterial blood pressure.

Acknowledgements. We wish to thank I.C.I. Ltd. for generous gifts of propranolol and pronethalol, the Medical Research Council for a grant, and Mr. A. Richardson for technical assistance.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 135S–141S Received May 1, 1968

Micro-colorimetric determination of adenosinetriphosphatase activity in freeze-dried sections of rat diaphragm muscle

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A technique is described for determination of ATPase activity in 3–30 μg samples dissected from freeze-dried sections of rat diaphragm muscle. Tissue samples are incubated at room temperature with optimal concentrations of ATP (10 mM) and $MgSO_4$ (5 mM) in 0·1 M tris/HCl buffer at pH 7·4. Inorganic phosphate is measured spectrophotometrically. Mean activity for 20 rats was 0·22 ± 0·06 (s.d.) mole-Pi/kg/wet tissue/15 min. Results did not depend on plane of section or section thickness. 5 mM Mg^{2+}, 10 mM Ca^{2+} and 1 mM 2,4-dinitrophenol produced maximal ATPase activation and inhibition was obtained with *p*-chloromercuribenzoic acid (pI_{50}3·9) but not chlorpromazine (0·01–0·1 mM). There was no significant evidence of Na^+ plus K^+ activated ATPase or inhibition with ouabain (5 mM). ATPase activity was uncharacterized and relative contributions of specified muscle ATPase systems were unknown. As an insoluble enzyme system was involved and photomicrographs showed characteristic muscle features in freeze-dried sections, ATPase activity appeared due to enzymic function *in situ*. Results are discussed in relation to mutual availability of enzymic sites and reagents.

THE importance of studying enzymes in the milieu in which they normally have their function was emphasized by Linderstrøm-Lang & Holter (1931). However, this cannot be achieved with any degree of certainty in the case of adenosinetriphosphatase (ATPase) systems in muscle due to limitations of the techniques available. For example, homogenization and differential centrifugation are far from ideal because the enzymes are removed from their cellular sites. With histochemical staining procedures the problems are enzyme diffusion and quantitation. To minimize experimental artefacts, the quantitative histochemical procedure described by Lowry, Roberts & others (1954) for determination of ATPase activity in freeze-dried sections of central nervous tissue was applied to muscle. Modifications included technical refinements, different incubation conditions, and the use of cupric ions to catalyse the formation of reduced phosphomolybdate during the colorimetric determination of inorganic phosphate (Peel & Loughman, 1957). The technique was developed as part of an investigation on the pharmacological significance of enzymes in voluntary muscle (Buckley & Nowell, 1966).

Experimental

Buffer solution. N hydrochloric acid (125 ml) was mixed with 1·2 M tris base [2-amino-2-(hydroxy-methyl)-propane-1,3-diol, BDH] (125 ml) and the required volume of stock magnesium sulphate solution added. The pH was adjusted to 7·4 with hydrochloric acid and the volume made up to 500 ml with distilled water to give a 0·3 M tris pH 7·4 buffer/15 mM $MgSO_4$ mixture. When required, other cations were added as chloride

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salts. Calcium chloride solution was prepared by dissolving weighed calcium carbonate in N hydrochloric acid.

ATP solution. A 30 mM solution of adenosine 5-triphosphate, disodium salt (Sigma) in 0·045 N sodium hydroxide.

Colour reagent. Prepared by mixing 1 ml 1% ammonium molybdate 1 ml 1% ascorbic acid, 1 ml 0·3 mM cupric sulphate and 22 ml 0·1 M acetic acid/0·065 M sodium acetate buffer.

Activators and inhibitors. Solutions of 2,4-dinitrophenol, sodium salt (BDH), *p*-chloromercuribenzoic acid (Sigma), chlorpromazine hydrochloride ("Largactil", May and Baker) and ouabain (stropanthin-G, BDH) were prepared in buffer solution at the concentrations required.

METHOD

Male Wistar rats (100–250 g) were decapitated and the diaphragms removed. Pieces of diaphragm muscle, approximately 4 × 4 × 1 mm, were snap frozen on to a microtome chuck using liquid nitrogen. 17·5 μ sections were cut using a refrigerated microtome ("Slee" cryostat) and freeze-dried at −18° (Lowry, 1953) with the aid of a liquid nitrogen cold finger fitted in the vacuum line (1 cm bore tubing). The sections still under vacuum were warmed to room temperature and then removed from the bottle. Approximately 1 mm² samples of freeze-dried muscle were dissected from the sections under the dissecting microscope, at 32 × magnification, taking care to exclude tendon, fat, connective tissue and blood vessels. The tissue samples were weighed on a quartz "fish pole" balance (Lowry, 1953), transferred with a hair point to tapered "Pyrex" reaction tubes (30 mm × 4 mm) and pressed to the bottom by means of a glass rod with a round tip. Such fixation was necessary to prevent the samples rising up the sides of the tubes when solutions were added. Buffer solution (20 μl) and distilled water (20 μl) were added to each tube followed by ATP solution (20 μl) 20 min later. Blanks (20 μl buffer, 20 μl H_2O, 20 μl ATP) and standards (20 μl buffer, 20 μl 1 mM KH_2PO_4, 20 μl ATP) were prepared similarly. Activators and/or inhibitors were added in the buffer solution, a Hamilton micro-syringe being used for all additions. The tubes were incubated (15 min) at room temperature and the reaction stopped by the addition of 0·5 ml colour reagent. The tubes were then inverted twice and 30 min later the absorbance at 870 mμ determined in a 1 cm microcuvette. Results were obtained from not less than five tissue samples taken from each diaphragm. A previously determined factor of five was used for the ratio of wet weight/dry weight of tissue. Since the rate of inorganic phosphate release from ATP is linear with time over the period of determination, ATPase activities were calculated for each tissue sample as moles of inorganic phosphate (Pi) produced per kg of wet tissue per 15 min (mole Pi/kg/15 min).

Results

The mean ATPase activity of 3–30 μg samples of freeze-dried diaphragm muscle taken from twenty rats was 0·22 ± 0·06 (s.d.) mole-Pi/kg/15 min. Incubation of tissue samples in buffer for 35 min at room temperature

produced no demonstable activity in the supernatants; all the activity remained in the tissue samples. This implied that the enzyme system was insoluble. Further results were obtained under varying conditions; each point and bar shown on the graphs represent the mean result from at least six animals ± the standard deviation of the mean.

SUBSTRATE CONCENTRATION

Maximal activity was observed with 9–12 mM ATP (Fig. 1). When 10 mM ATP was used there was a reasonable correlation between phosphate

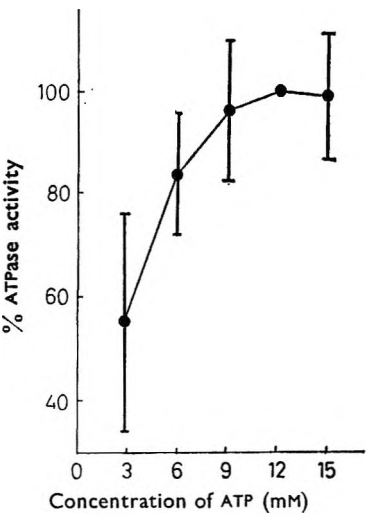

FIG. 1. A plot of ATPase activity against subtrate concentration for freeze-dried muscle sections.

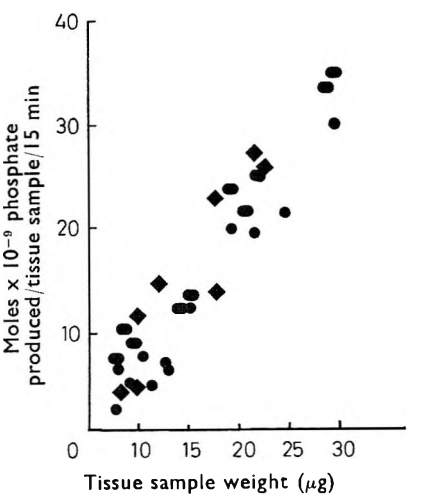

FIG. 2. Relation between tissue sample weight and phosphate measured in determination of ATPase activity of freeze-dried muscle sections. Rat 1 ◆. Rat 2 ⬬. Rat 3 ●.

determined and tissue sample weight (Fig. 2). Optimal working conditions were thus obtained.

ACTIVATORS AND INHIBITORS

There was negligible activity in the absence of added magnesium or calcium ions and similar maximal activities were produced by approximately 5 mM $MgSO_4$ and 10 mM $CaCl_2$ (Fig. 3). Sodium and potassium ions in various concentrations caused no significant activation in the presence or absence of magnesium ions using disodium or tris ATP.

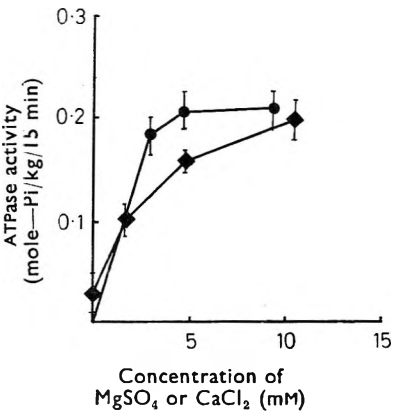

FIG. 3. Variations of ATPase activity of freeze-dried muscle sections with magnesium and calcium ion concentration. ● $MgSO_4$. ◆ $CaCl_2$.

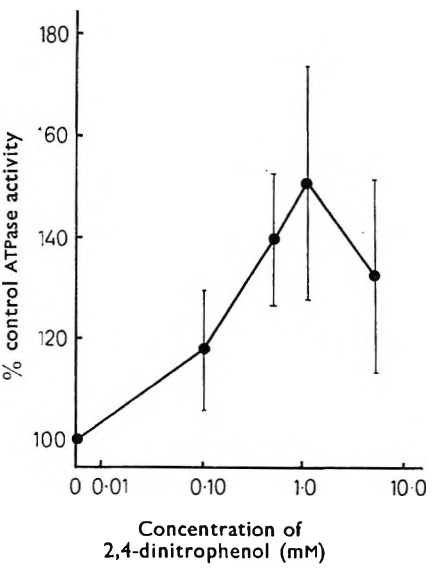

FIG. 4. Plot of 2,4-dinitrophenol activated ATPase activity of freeze-dried muscle sections. Log scale abscissa.

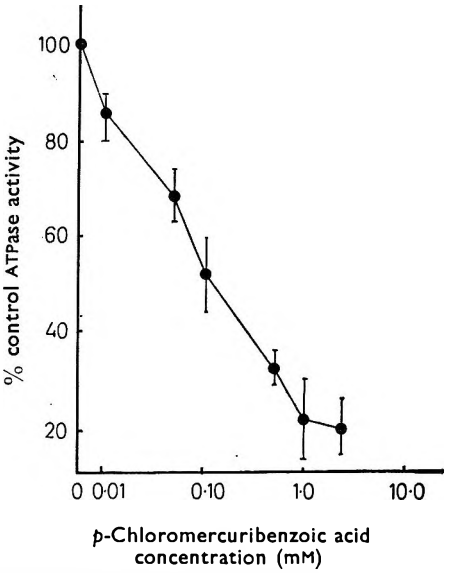

FIG. 5. Plot of *p*-chloromercuribenzoic acid inhibition of ATPase activity of freeze-dried muscle sections. Log scale abscissa.

A peak of 50% activation (Fig. 4) was shown with 1 mM 2,4-dinitrophenol whereas *p*-chloromercuribenzoic acid produced inhibition (pI_{50} 3·9) (Fig. 5). Nc significant effects were observed with 0·01–0·1 mM chlorpromazine (higher concentrations of chlorpromazine could not be used because of its low solubility). Ouabain in concentrations up to 5 mM, in the presence or absence of sodium and potassium ions, also had no significant effect.

HISTOLOGICAL FACTORS

Photomicrographs of frozen and freeze-dried sections showed striations and nuclei which were characteristic of normal muscle.

When ten carefully selected tissue samples were prepared from longitudinal and transverse muscle sections, taken from each of five animals, the activities were 0·19 $\pm$ 0·04 (s.d.) and 0·25 $\pm$ 0·05 (s.d.) mole-Pi/kg/15 min respectively. This suggested that any areas of transverse section which were present in the longitudinal sections generally used (due to curling of the tissue during freezing) did not influence activity. When the section thickness was varied from 5·6–35·5 μ no corresponding effects on activity were obtained (Table 1). Thus any variation in results was not due to occasional inconsistencies in section width experienced with the cryostat.

A comparison of results for twelve tissue samples fixed to the reaction tubes, with twelve not fixed showed no significant difference in the respective activities (0·20 $\pm$ 0·04 (s.d.) and 0·18 $\pm$ 0·07 (s.d.) mole-Pi/kg/15 min). Thus the procedure did not cause an irregular liberation of activity appreciable enough to significantly influence the results.

TABLE 1. THE EFFECT OF SECTION THICKNESS OF ATPASE ACTIVITY OF FREEZE-DRIED MUSCLE

Rat	Section thickness (μ)	Mean ATPase activity* (mole-Pi/kg/15 min)	Standard deviation
1	5·6	0·22	0·04
1	9·4	0·18	0·07
1	16·1	0·18	0·02
1	17·5	0·18	0·02
2	17·5	0·20	0·04
1	18·9	0·20	0·02
1	21·7	0·20	0·02
2	22·0	0·18	0·02
2	26·5	0·18	0·02
2	31·0	0·20	0·02
2	35·5	0·18	0·02

* 10 determinations; respective serial sections from same muscle block.

Discussion

Measurements represent net phosphate release from ATP rather than true ATPase activity, since no steps were taken to eliminate myokinase, pyrophosphatase and apyrase. Many muscle enzymes behave as ATPases and the results refer to uncharacterized ATPase activity in the presence of magnesium ions at pH 7·4. Thus no conclusions can be drawn about the relative contributions of ATPase systems in the myofibrils, mitochondria, sarcoplasm, nuclei, sarcolemma and microsomes. The last two mentioned are probably of minor importance because there was little evidence of inhibition with ouabain or Na^+ plus K^+ activated ATPase activity which is generally associated with ion transport systems in cell membranes and cytomembranes (Albers, 1967). Such a view would agree with the reported that the Na^+ plus K^+-activated ATPase activity of muscle (ouabain sensitive) is low compared with other tissues (Bonting, Simon & Hawkins, 1961).

Since the freeze-dried tissue used was reasonably intact it is suggested that the results observed depend on enzymic function *in situ*: this is supported by the observation that no ATPase leaked into the incubation medium. An increase in activity was obtained when freeze-dried tissue was homogenized, thus the activity of the enzyme system in the tissue may have been limited by the availability of substrate or of enzymic sites. But homogenates of fresh tissue were five times more active than freeze-dried sections and the respective activity/substrate curves were similar, so the availability of substrate was concluded not to be a major problem (Hopsu & McMillan, 1964). This is supported by the observation that activity was not influenced by section thickness.

The effects of inhibitors were smaller in freeze-dried sections than in homogenates of fresh tissue. Chlorpromazine for example although inactive in freeze-dried sections, was an effective inhibitor in homogenates. *p*-Chloromercuribenzoic acid was 2–3 times more potent an inhibitor in homogenates than in freeze-dried sections. In these cases there was no evidence whether the small effects in freeze-dried sections were due to lack of access of the inhibitor to the enzyme sites or to a lack of the sites themselves. Although concentrations of inhibitor lower than those

of substrate were used, the inhibitors have higher lipid solubility so that presumably they may penetrate the tissue relatively easily.

Acknowledgements. This work was supported financially by the Ministry of Defence. Pure chlorpromazine hydrochloride was obtained by the courtesy of Mr. A. W. Nineham, Products Research Group, May & Baker Ltd., Dagenham, Essex. We are also grateful to Professor Andrew Wilson, Head of the Department of Pharmacology University of Liverpool, for helpful advice and encouragement throughout the work which was part of a project on the development of ultramicrobiochemical techniques for exploring the mode of action of drugs.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 142S–149S Received April 25, 1968

The metabolism of 16α-ethylprogesterone by rat liver *in vitro*

J. B. STENLAKE, RICHARD TEMPLETON AND D. C. TAYLOR

The metabolism of 16α-ethylprogesterone by rat liver homogenate at different steroid to tissue ratios has been examined. The products, identified by thin-layer chromatography, gas-liquid chromatography and combined gas-liquid chromatography-mass spectrometry were 16α-ethyl-5α-pregnane-3,20-dione and 16α-ethyl-3α-hydroxy-5α-pregnan-20-one. The yield of these metabolites was 14 and 48% respectively. The results are discussed in relation to the liver *in vitro* metabolism of progesterone and 16α-hydroxyprogesterone.

PARTIAL regression of carcinoma of the endometrium has been obtained in some patients treated with progesterone (Kelley & Baker, 1961) or with the synthetic progesterone analogues 17α-hydroxyprogesterone caproate (Kistner, Griffiths & Craig, 1965; Mussey & Malkesian, 1966), 6α-methyl-17α-hydroxyprogesterone acetate (medroxyprogesterone acetate) or 6α,17α-dimethyl-6-dehydroprogesterone (Smith, Rutledge & Soffar, 1966). The latter authors noted that progesterone analogues showed a higher degree of endometrial activity than progesterone itself. Substituents present in such analogues also confer on the progesterone molecule an increased resistance to enzymic attack (Cooke & Vallance, 1965). Thus the increased biological stability of synthetic progesterone derivatives may be correlated with their increased endometrial activity and wider use in the management of endometrial carcinoma. Where regression is observed on administration of these synthetic steroids only biologically slow growing tumours are affected; tumours of rapid growth remain largely unaffected (Kennedy, 1963; Frick II, 1965). The mechanism of such compounds is unknown.

The metabolism of progesterone by liver preparations has been examined by several authors. In a study with human liver Atherden (1959) identified 5α- and 5β-pregnane-3,20-dione, 3α-hydroxy- and 3β-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3α,20α-diol. With the exception of 5β-pregnane-3,20-dione, Taylor (1955) isolated a similar range of products from the metabolism of progesterone by rabbit liver preparations. In contrast, the same author (Taylor, 1954) isolated only 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnan-20-one from the incubation of progesterone with rat liver. From the metabolism of 16α-hydroxyprogesterone by rat liver preprations, however, Wettstein, Neher & Urech (1959) identified 3α,16α-dihydroxy-5β-pregnan-20-one and 3α,16α-dihydroxy- and 3β,16α-dihydroxy-5α-pregnan-20-one. Thus progesterone and 16α-hydroxyprogesterone appear to be metabolized by different routes in rat liver. The results of a study of the metabolism of 16α-ethylprogesterone by female rat liver homogenate are presented here.

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Experimental

MATERIALS AND APPARATUS

Solvents were of A.R. grade or were redistilled.

Absorbance measurements were made on a Unicam SP500 spectrophotometer.

Thin-layer chromatography plates were prepared, 0·25 mm thick, from Merck silica gel G to which was added zinc silicate (2%) as a phosphor. The plates were activated by heating for 0·5 hr at 110°, and stored over silica gel. As the method of analysis was "internally compensating", the use of either non-treated silica gel or that which had been refluxed with methanol was without effect on the results. The plates were examined under light of 254 and 350 mμ wavelength.

Gas-liquid chromatography was performed on two instruments. A Pye Panchromatograph equipped with an argon ionization detector contained a 5 ft × 4 mm i.d. glass column packed with 1% cyclohexane dimethanol succinate plus 1% polyvinyl pyrrolidone on chromosorb G (60–80 mesh); column temperature 225° and argon flow rate 50 ml/min. A Pye 104 series instrument, equipped with flame ionization detectors, contained two 5 ft × 4 mm i.d. glass columns packed with (*a*) 3% SE30 on Celite (85–100 mesh); column temperature 224° and nitrogen flow rate 50 ml/min: and (*b*) 3% QF1 on Celite (85–100 mesh); column temperature 224° for free steroids and 209° for steroid trimethylsilyl ethers, nitrogen flow rate in both cases was 60 ml/min.

Mass spectral data was recorded on an LKB 9000 gas chromatograph-mass spectrometer. Two 5 ft × ¼ o.d. glass columns were used in this instrument, (*a*) 1% SE30 on gas chrom Q (100–120 mesh); column temperature 230°, helium flow rate 30 ml/min: and (*b*) 1% QF1 on gas chrom Q (100–120 mesh); column temperature 200°, helium flow rate 30 ml/min. Mass spectrometer ionization voltage 70 eV.

METHODS

Incubation media. Female Wistar rats (120 to 150 g weight) were stunned, exsanguinated, and the livers were removed and placed in ice-cold phosphate buffer (0·1M, pH 7·0). Connective tissue was removed and liver (approximately 5 ml by displacement) was homogenized with two volumes of phosphate buffer (0·1M, pH 7·0) for 2 min at full speed by an MSE homogenizer. The homogenate was filtered through four layers of muslin. All operations were at 0° to 2°, and the homogenate was stored briefly at this temperature till used. Incubation media consisted of glucose-6-phosphate (10 μmole), NADP (0·1 μmole), nicotinamide (15 μmole), magnesium chloride (20 μmole), phosphate buffer (0·2M, pH 7·4, 0·5 ml) and homogenate (1 ml) in a total volume of 3 ml. Substrate was added in solution (7·24 μg/μl) in propylene glycol. Incubation was effected aerobically with shaking for 2 hr at 37°.

Determination of the extent of metabolism with different substrate to tissue ratios. For each substrate concentration three incubation media were prepared: (1) *active metabolism*—incubation of substrate plus media;

(2) *maximum recovery determination*—incubation of media alone with addition of substrate after incubation and immediately before protein precipitation; (3) *background value*—incubation of media plus the relevant aliquot of propylene glycol.

The last determination (3) was used as a spectrophotometric blank in determining the absorbance of (1) and (2). Loss of substrate as given by the difference between values for (2) and (1) was taken as the amount of 16α-ethylprogesterone metabolized.

Immediately following incubation, protein was precipitated with methylene chloride–methanol (2:1; 12 ml) (McKerns & Nordstrand, 1965). The mixture was shaken (45 min) then centrifuged at 2000 *g* (5 min); the liquid phases were removed by aspiration, and the residual pellicle washed with methylene chloride–methanol (2:1; 12 ml). The combined liquid phases were evaporated to dryness under vacuum at a temperature not greater than 55°, and the residue was dried over phosphorus pentoxide. Acetone (1 ml) was added, and an aliquot (100 μl) of the acetone solution was placed on a thin-layer plate, which was developed with chloroform-acetone (95:5) for a distance of 15 cm. The area corresponding to 16α-ethylprogesterone, defined by a dark spot when it was irradiated at 254 mμ, was scraped from the plate. An equal area was taken from the chromatogram of the *background value*—defined as (3) above. The silica gel was extracted with ether (1 × 6 ml and 1 × 2 ml) the ether extract evaporated to dryness under a stream of nitrogen, and the residue was dissolved in ethanol (5 ml; increased to 10 ml in the more concentrated substrate determinations). The absorbance was measured at 240 mμ.

Identification of metabolites by thin-layer chromatography. Aliquots (20 μl) of the acetone solutions of the crude extracts from the incubation media were run on thin-layer plates in the solvent systems chloroform-acetone (95:5) and benzene–ethyl acetate (60:40) (Neher & Wettstein, 1960). 16α-Ethylprogesterone was located on the plate by irradiating it at 254 mμ: other steroids were visualized by heating the plate at 110° (3 min) then spraying either with sulphuric acid (sp.gr. 1·84)–water (1:1) or sulphuric acid (sp.gr. 1·84)–ethanol (1:1) (Neher, 1964). The plates were further heated at 110° (10 min) and the Rf values of the spots noted together with their colours when viewed in daylight or ultraviolet light (350 mμ). Reference steroids were run on the same plates as the extracts.

Identification of metabolites by gas-liquid chromatography. By direct injection: aliquots (1–2 μl) of the acetone solutions of the crude extracts were used.

By trimethylsilyl ether formation: an aliquot (0·9 ml) of the acetone solution of the crude extracts was evaporated to dryness under a stream of nitrogen. Pyridine (1·0 ml), hexamethyldisilazane (0·2 ml) and trimethylchlorosilane (100 μl), were added to the residue, and the mixture left at room temperature (1 hr) before evaporation to dryness under a stream of nitrogen. The residue was dissolved in n-hexane (100 μl), and aliquots (1–2 μl) were injected. Trimethylsilyl ethers of reference steroids (1 mg) were prepared and injected in a similar manner.

By combined thin-layer and gas-liquid chromatography: an aliquot (100 μl) of the acetone solution of the crude extracts and appropriate reference steroids were subjected to thin-layer chromatography as described above. After development, the reference steroids only were visualized and those areas from the extract which corresponded to the reference steroids were removed. The silica gel was extracted with ether (1 × 6 ml and 1 × 2 ml), and the ether extract was evaporated to dryness under nitrogen. The residue *per se* or in the form of the trimethylsilyl ethers (see above) was analysed by gas-liquid chromatography alone and in combination with mass spectrometry.

Quantitative determination of metabolites. Acetone solutions of the extracts from the active metabolism determination and from the maximum recovery determination of the same experiment were analysed by gas-liquid chromatography. Peak areas corresponding to 16α-ethylprogesterone (peak height × width at half height), were compared for equivalent amounts of the solutions injected. The difference in peak area between the maximum recovery determination and the active metabolism determination indicated the loss of 16α-ethylprogesterone resulting from metabolism. The areas of the remaining two peaks corresponding to metabolites in the active metabolism extract (the cholesterol peak was disregarded) were compared with that of the 16α-ethylprogesterone peak, and the percentage of each metabolite relative to recovered 16α-ethyl progesterone calculated. The calculation requires a similar molar response of the flame ionisation detector to the three steroids concerned. Application of the method of Sternberg, Gallaway & Jones (1962) indicates a maximum difference in molar response of 3%. The observed differences in molar response over a range of steroid concentrations were within the limits of experimental error.

Results

Metabolism of 16α-ethylprogesterone by female rat liver homogenate fortified with NADP, nicotinamide, glucose-6-phosphate and magnesium chloride was observed to an extent of about 60% over the range of steroid to tissue ratios studied (Table 1). These results are based on measurement of the absorption of the Δ^4-3-keto-chromophore at 240 mμ, and represent only metabolism resulting in loss of this chromophore. Identification of the metabolites (below) proved this to be a valid assessment of

TABLE 1. PERCENTAGE METABOLISM OF 16α-ETHYLPROGESTERONE BY RAT LIVER HOMOGENATE AT DIFFERENT STEROID TO TISSUE RATIOS

Experiment	Amount of steroid in incubation medium (μg)	Steroid : tissue ratio	Metabolism %		
			Series I*	Series II*	Mean value
1	180	1 : 1854	64	61	62·5
2	288·3	1 : 1156	67	60	63·5
3	370·4	1 : 900	59	47	53
4	520·0	1 : 641	72	64	68
5	1333·3	1 : 250	62	63	62·5

* Each value mean of three determinations.

total metabolism. Recovery of 16α-ethylprogesterone from an incubation medium in the absence of metabolism is 68%. No interference with the spectrophotometric determination (240 mμ) was observed, and Beer's law was obeyed over the concentration range 0 to 0·18 μmole of steroid per 5 ml ethanol.

Extracts from the experiments with higher steroid to tissue ratios (3, 4 and 5, Table 1) were separately analysed for metabolites. Relative retention values on thin-layer chromatograms and colour development with sulphuric acid–ethanol of reference 16α-ethyl-steroids are shown in Table 2. The limit of detectability of 16α-ethylprogesterone on fluorescent silica gel when irradiated with light of 254 mμ is 0·25 μg; that of 16α-ethyl-3α-hydroxy-5α-pregnan-20-one is 0·05 μg when a sulphuric acid–ethanol spray is used for visualisation. Cholesterol was chosen as an internal standard as it was an endogenous constituent of the extracts. Reference steroids were chromatographed on the same plates as the extracts, or were developed separately with the addition of cholesterol as reference standard. Table 2 shows that in addition to starting material

TABLE 2. THIN-LAYER CHROMATOGRAPHY RELATIVE RETENTION VALUES (CHOLESTEROL = 1·0) OF REFERENCE STEROIDS AND OF COMPONENTS PRESENT IN EXTRACTS OF INCUBATION MEDIA

Reference steroids		Relative retention in solvent system 1	Relative retention in solvent system 2	Colour in daylight (a)	Colour at 350 mμ (a)
16α-Ethylprogesterone		1·33	1·20	Light brown	Absorbs at 254 mμ (b)
16α-Ethyl-5α-pregnane-3,20-dione		1·39	1·23	Light brown	Pale blue
16α-Ethyl-5β-pregnane-3,20-dione		1·35	1·20	Light brown	Grey
16α-Ethyl-3α-hydroxy-5α-pregnan-20-one		0·98	0·96	Red-brown	Brown
16α-Ethyl-3β-hydroxy-5α-pregnan-20-one		0·83	0·93	Pink-brown	Brown
16α-Ethyl-3α-hydroxy-5β-pregnan-20-one		0·88	0·95	Red-brown	Brown
16α-Ethyl-5α-pregnane-3α,20β-diol		0·61	0·64	Pink-brown	Blue-grey
16α-Ethyl-20β-hydroxy-pregn-4-ene-3-one		0·76	0·78	Yellow	Yellow
16α-Ethyl-20β-hydroxy-5β-pregnan-3-one		0·93	0·96	Yellow-green	Blue
Cholesterol (reference standard)		1·00	1·00	Purple	Red
Components detected on chromatograms of extracts of incubation media	1	0·96	0·95	Red-brown	Brown
	2	1·00	1·00	Red-purple	Red
	3	1·30	1·18	Light brown	Absorbs at 254 mμ (b)
	4	1·38	1·23	Brown	Blue

Solvent system 1. Chloroform–acetone (95 : 5); cholesterol Rf = 0·54.
Solvent system 2. Benzene–ethyl acetate (60 : 40); cholesterol Rf = 0·56.
(a) Colours observed after spraying with sulphuric acid-ethanol.
(b) Absorption at 254 mμ determined before spraying.

(16α-ethylprogesterone), 16α-ethyl-5ε-pregnane-3,20-dione, 16α-ethyl-3ε-hydroxy-5ε-pregnan-20-one, and endogenous cholesterol were the only components present in extracts of incubation media. The relative retention data from both solvent systems suggested that the configuration at C-5 of the pregnanedione present (component 4) was alpha rather than beta: this was supported by the distinctive colour reactions of the two isomers. The epimeric 3-hydroxy-5α-pregnanones were clearly separated by the chloroform-acetone solvent system, as were the 3α-hydroxy-5α- and 5β-pregnanones. Thus component (1) of the incubation extract was probably 16α-ethyl-3α-hydroxy-5α-pregnan-20-one.

This assignment was confirmed by gas-liquid chromatographic analysis

(Table 3) which also excludes the 3β-hydroxy-5α- and 3α-hydroxy-5β-isomers. Table 3 also indicates that the relative retention values of components (2) and (3) of the extracts correspond with those of 16α-ethyl-5α-pregnane-3,20-dione and 16α-ethylprogesterone, respectively, again confirming thin-layer chromatography evidence. Retention data of the steroid trimethylsilyl-ethers gave further proof of the presence of the 3α-hydroxy-5α-pregnan-20-one, in the incubation extract.

Final confirmation of the structures of the isolated steroids was by subjecting the extracts to combined gas chromatography-mass spectrometry after a preliminary "clean-up" by thin-layer chromatography. The m/e values of the main fragment ions of components 3, 2 and 1 (Table 3) of the extract coincided with those of 16α-ethylprogesterone, 16α-ethyl-5α-pregnane-3,20-dione and 16α-ethyl-3α-hydroxy-5α-pregnan-20-one, respectively (Table 4). Minor differences in the percentage abundance figures can probably be attributed to extract-derived background in the effluent from gas-liquid chromatography. Where possible the mass spectrum of the background adjacent to the relevant peak was determined and subtracted from that of the peak. No major peak was present in the spectrum of any extract component for which there was not a corresponding peak in the spectrum of the relevant reference steroid.

Quantitative comparison of the 16α-ethylprogesterone peak areas obtained from gas-liquid chromatography of both the maximum recovery

TABLE 3. GAS-LIQUID CHROMATOGRAPHY RELATIVE RETENTION VALUES (CHOLESTEROL = 1·00) OF REFERENCE STEROIDS AND OF COMPONENTS PRESENT IN EXTRACTS OF INCUBATION MEDIA, AND OF THEIR TMS DERIVATIVES

Reference steroids		Relative retention in system 1	Relative retention in system 2A	Relative retention in system 3
16α-Ethylprogesterone		0·57	3·40	2·89
16α-Ethyl-5α-pregnane-3,20-dione		0·49	2·12	1·89
16α-Ethyl-5β-pregnane-3,20-dione		0·43	1·94	—
16α-Ethyl-3α-hydroxy-5α-pregnan-20-one		0·43	1·01	0·93
16α-Ethyl-3β-hydroxy-5α-pregnan-20-one		0·45	1·29	1·03
16α-Ethyl-3α-hydroxy-5β-pregnan-20-one		0·39	1·06	—
16α-Ethyl-5α-pregnane-3α,20β-diol		0·65	0·89	—
Cholesterol (reference standard)		1·00	1·00	1·00
Peaks observed on chromatograms of extracts of incubation media	1	0·43	1·01	0·92
	2	0·49	2·10	1·92
	3	0·56	3·50	2·86
	4	1·00	1·00	1·00
TMS Ethers of reference hydroxy steroids		Relative retention in system 1	Relative retention in system 2B	
16α-Ethyl-3α-hydroxy-5α-pregnan-20-one		0·33	0·81	
16α-Ethyl-3β-hydroxy-5α-pregnan-20-one		0·43	1·16	
16α-Ethyl-3α-hydroxy-5β-pregnan-20-one		0·34	0·83	
16α-Ethyl-5α-pregnane-3α,20β-diol		0·47	0·44	
Cholesterol		1·00	1·00	
Peaks observed on chromatograms of extracts of incubation media subject to TMS derivitization	1	0·34	0·81	
	2	1·00	1·00	

System 1. 3% SE30, column temp. 222°; Cholesterol Rt = 36·40 min; Cholesterol TMS ether Rt = 43·70 min.
System 2. 3% QFI, A. column temp. 224°; Cholesterol Rt = 12·30 min.
B. column temp. 209°; Cholesterol TMS ether Rt = 15·20 min.
System 3. 1% CDMS + 1% PVP, column temp. 225°; Cholesterol Rt = 9·20 min.

TABLE 4. MASS SPECTRAL DATA OF REFERENCE STEROIDS AND OF COMPONENTS PRESENT IN EXTRACTS OF INCUBATION MEDIA

m/e value	% of base peak: 16α-Ethyl progesterone	% of base peak: GLC peak 3 Table 3	m/e value	% of base peak: 16α-Ethyl-5α-pregnane-3,20-dione	% of base peak: GLC peak 2 Table 3	m/e value	% of base peak: 16α-Ethyl-3α-OH-5α-pregnan-20-one	% of base peak: GLC peak 1 Table 3
342[1]	56·2	53·3	344[1]	36·5	35·1	346[1]	36·8	35·0
327	17·6	17·6	329	33·2	34·8	331	38·7	32·4
300	29·1	22·8	315	63·3	64·2	317	62·3	55·2
257	17·9	20·2	301	16·8	21·6	288	32·1	29·5
244	30·3	29·9	286	37·9	36·1	285	21·7	21·8
219	20·2	17·5	246	59·0	56·5	248	52·8	54·8
175	16·7	18·8	231	53·1	53·7	233	54·7	51·9
161	16·8	20·5	147	27·0	26·9	147	24·9	24·7
135	19·7	21·1	135	23·8	26·6	135	29·6	25·3
124[2]	100·0	100·0	124	19·7	21·9	133	30·2	23·3
123	26·3	32·9	123	53·0	59·5	123	41·5	44·6
121	30·8	38·9	121	41·1	48·0	122	54·3	57·9
199	19·3	27·2	119	26·7	39·8	121	45·3	41·4
112	17·8	20·1	112[2]	100·0	100·0	112[2]	100·0	100·0

[1] Molecular ion. [2] Base peak of spectrum.

determination and active metabolism determination in experiment 5 (Table 1) confirmed the extent of metabolism as 62%. Comparison of the peak areas of the metabolites with that of recovered 16α-ethylprogesterone on the same chromatogram showed the amount of each metabolite formed to be 14% for 16α-ethyl-5α-pregnane-3,20-dione and 48% for 16α-ethyl-3α-hydroxy-5α-pregnan-20-one.

Discussion

A species difference is apparent in the nature of the products identified from the catabolism of progesterone by liver preparations (Table 5). Human liver and rabbit liver possess enzyme systems which catalyse the reduction of the C-4 double bond, yielding pregnanes of the 5α- and 5β-series, as well as reduction of the C-3 carbonyl function, giving both epimeric C-3 hydroxyl groups. On the other hand, reduction of the C-20

TABLE 5. *In vitro* LIVER METABOLISM OF PROGESTERONE, 16α-HYDROXYPROGESTERONE AND 16α-ETHYLPROGESTERONE

Metabolites identified				
Pregnane derivatives			16α-HO-pregnane derivatives	16α-Ethylpregnane derivatives
Human liver Progesterone (1)	Rabbit liver Progesterone (2)	Rat liver Progesterone (3)	Rat liver 16α-hydroxy-Progesterone (4)	Rat liver 16α-ethyl-Progesterone
3-one, 5α, 20-one	3-one, 5α, 20-one	3-one, 5α, 20-one	—	3-one, 5α, 20-one (a)
3-one, 5β, 20-one	—	—	—	—
3α-OH, 5α, 20-one	3α-OH, 5α, 20-one	3α-OH, 5α, 20-one	3α-OH, 5α, 20-one (a)	3α-OH, 5α,20-one (b)
3β-OH, 5α, 20-one	3β-OH, 5α. 20-one	—	3β-OH, 5α, 20-one (b)	—
3α-OH, 5β, 20-one	3α-OH, 5β. 20-one	—	3α-OH, 5β, 20-one (c)	—
3α-OH, 5β, 20α-OH	3α-OH, 5β. 20α-OH	—	—	—
Major metabolites of 5β-Series	Major metabolites of 5β-Series	Above metabolites plus recovered progesterone in 28% yield	% yield of metabolites (a) 39·3% (b) 11·4% (c) 15·9%	% yield of metabolites (a) 14% (b) 48%

(1) Atherden (1959). (2) Taylor (1955).
(3) Taylor (1954). (4) Wettstein & others (1959).

carbonyl function yielded only the C-20α-hydroxyl group. Pregnanes of the 5α- and 5β- series have also been reported as metabolic products of 16α-hydroxyprogesterone by rat liver *in vitro* (Wettstein & others, 1959). Reduction of the C-3 carbonyl function gave both epimeric hydroxyl groups, although attack on the C-20 carbonyl function was not observed.

In the present investigation only 3α-hydroxy and 5α-steroids resulted, no evidence being obtained for either a 3β-hydroxy group or 5β-pregnanes. These observations are similar to those of Taylor (1954), who found that 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnan-20-one were the only products of the catabolism of progesterone by rat liver preparations. In this tissue therefore 16α-ethylprogesterone and progesterone are metabolized by similar enzyme systems, whereas the 16α-hydroxy-analogue is subject further to reductase systems which produce β-isomerism at C-3 and C-5. Thus the metabolism of the progesterone molecule by rat liver homogenate would appear to be profoundly influenced by a 16α-hydroxyl substituent although the substituent itself is not metabolized. In addition in presence of a 16α-hydroxy-group but not a 16α-ethyl-group, the progesterone molecule is subject to enzymic change more similar to that encountered in rabbit liver than in rat liver.

Note added in proof. Recent evidence (Shirley & Cooke, 1968) indicates that 3β-hydroxy-5α- pregnan-20-one (5·8%) is a product of the metabolism of progesterone by female rat liver homogenate. A difference is therefore apparent in the metabolism of progesterone and 16α-ethylprogesterone by this tissue, as in the metabolism of the latter no product having the 3β-hydroxy-configuration was identified. The 16α-ethyl group may thus be significant in suppressing metabolism by this route. 5β-Pregnane-derivatives were not observed as metabolites (Shirley, I. M. & Cooke, B. A., 1968, *J. Endocr.*, **40** (4), 477–483).

Acknowledgements. The authors wish to thank Dr. C. J. W. Brooks of Glasgow University for use of the LKB 9000 mass spectrometer, Dr. G. F. Woods of Organon Laboratories for donating the 16α-ethyl steroids and the Marie Curie Foundation for financial support from the Draffen Bequest to one of us (D.C.T.).

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 150S–156S Received April 25, 1968

Protein-binding of small molecules: new gel filtration method

P. F. COOPER AND G. C. WOOD

The use of frontal analysis chromatography on Sephadex columns, previously described for protein-protein interactions (Nichol & Winzor, 1964) has been extended to the treatment of protein-small molecule interaction. Chromatograms obtained with bovine serum albumin and three different sulphonamides (sulphanilamide, sulphapyridine and sulphamethoxypyridazine) were found to conform to the theoretical patterns, and the values for the fraction of drug bound were in good agreement with those obtained by equilibrium dialysis.

THE binding of drugs to plasma and tissue proteins is an important factor affecting their distribution (Brodie, 1965) and rate of metabolism (Newbould & Kilpatrick, 1960; Anton & Boyle, 1964). As part of a program aimed at exploring any quantitative relation between protein-binding and the rate of drug metabolism, the use of gel filtration as a measure of protein-binding has been investigated. The widely used equilibrium dialysis method (Klotz, Walker & Pivan, 1946) requires prolonged equilibration times, with consequent risk of deterioration of protein in experiments at physiological temperature. Ultrafiltration (see, for example, Rehberg, 1943) is suspect because of possible changes in protein concentration during the experiment which would disturb binding equilibrium, although the use of small aliquots of filtrate overcomes this objection (Bennett & Kirby, 1965). Differential sedimentation in preparative or analytical ultracentrifuges (Büttner & Portwich, 1961; Cummings, Kuff & Sober, 1968; Steinberg & Schachman, 1966) although theoretically sound is not well adapted to making many routine measurements.

The use of gel filtration with Sephadex as partitioning medium has been explored by several workers. A frequently used procedure (Hardy & Mansford, 1962; Doe, Fernandez & Seal, 1962; Quincey & Gray, 1963) is to apply a small volume of protein ligand mixture to a column of Sephadex G-25 and to elute with buffer. The pore size of the stationary phase is such as to exclude proteins together with bound ligand, which therefore pass rapidly down the column, while admitting free ligand which slowly migrates as a separate zone. Since the two zones are completely separated this method is reliable only if the protein-ligand complex dissociates at a rate which is low compared with the rate of elution. Failure to appreciate this can lead to misleading results (De Moor, Heirwegh & others, 1962). This difficulty may be met (Hummel & Dryer, 1962) by applying protein-ligand mixture to a Sephadex column previously equilibrated with a solution containing the same ligand concentration as the mixture, elution being with the same solution. While results from this method have not been compared with those from equilibrium dialysis, it is theoretically sound, but extravagant of drug (Clausen, 1966). Several workers (Scholtan, 1964; Ashworth & Heard, 1966; Souleil & Nisonoff, 1968)

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have reported the use of Sephadex and similar gels in a batchwise manner, a known volume of solution containing known concentrations of ligand and protein being equilibrated with a known weight of dry Sephadex of suitable porosity. From the change of ligand concentration in the external (protein containing) phase the extent of protein-binding can be calculated provided the solvent uptake by the Sephadex is known. The method is equivalent to equilibrium dialysis but the composition of only one compartment, that containing protein, can be assayed with any precision. Together with the necessity to correct for adsorption of ligand onto Sephadex, this fact renders the method imprecise, particularly at low degrees of binding. We were therefore led to consider an alternative chromatographic method which appears to have none of the above disadvantages.

FRONTAL ANALYSIS OF PROTEIN LIGAND MIXTURES ON SEPHADEX COLUMNS

When a large volume of solution containing a reacting system, of the type $A + B \rightleftharpoons C$ is passed through a Sephadex column and then eluted

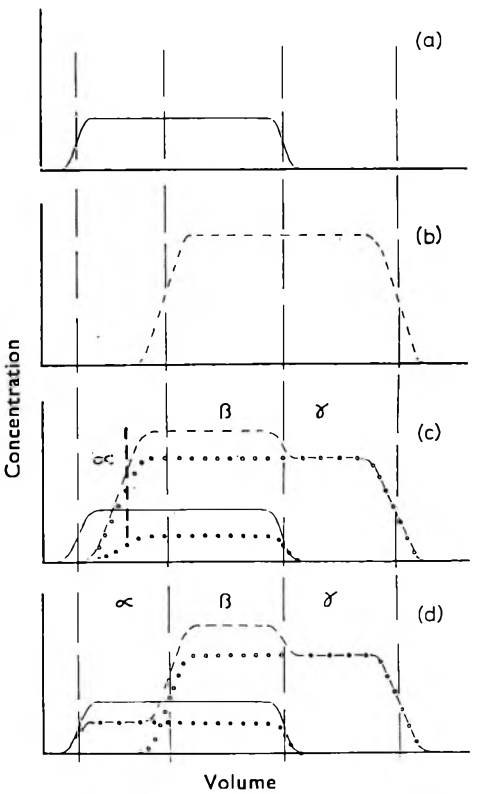

FIG. 1. Diagrammatic Sephadex chromatograms. (a) and (b) Pure reactants A and B respectively. (c) Equilibrium mixture of the rapidly reversible type. (d) Equilibrium mixture of the slowly reversible type. ——— total concentration of A; – – – total concentration of B; ○ ○ ○ ○ concentration of free B; ● ● ● ● concentration of C. Vertical lines indicate boundary positions.

with solvent the forms of the leading and trailing boundaries of the elution pattern depend on (*a*) the velocities, V_x, with which the various components move down the column, (*b*) the proportions of the three components and (*c*) the rates of the forward and reverse reactions.

When $V_A = V_C > V_B$ and the equilibrium is established rapidly compared with the rates of migration the form of the elution profiles is as shown diagramatically in Fig. 1c. Provided the velocities of the components are independent of the composition of the solution the positions of the various boundaries are related to those of pure reactants (Fig. 1a and b). It has been shown (Nichol & Winzor, 1964; Nichol, Ogston & Winzor, 1967) that under these conditions

$$C_B^\gamma = C_B^\beta,\ C_A^\alpha = \bar{C}_A^\beta \text{ and } C_C^\beta = \bar{C}_B^\beta - C_B^\gamma$$

where $\bar{C}_A^\beta = C_A^\beta + C_C^\beta,\ \bar{C}_B^\beta = C_B^\beta + C_C^\beta$

and C_A^α, C_A^β, C_B^β, C_B^γ, C_C^β are the molar concentrations of components A, B and C in the plateau regions α, β and γ of the chromatogram.*

If component A is a protein, component B a small molecular species and component C the protein-ligand complex, the fraction of ligand bound to protein in zone β (whose composition is identical to that of the starting solution) is

$$f = C_C^\beta/\bar{C}_B^\beta = (\bar{C}_B^\beta - C_B^\gamma)/\bar{C}_B^\beta \quad \ldots\ldots\ldots (1)$$

Many proteins form higher complexes with small molecules, of the type AB_2, AB_3 . . . . AB_n. Provided all these complexes migrate with the same velocity as C (independent of solution composition), and provided all the equilibria are rapidly attained, the above considerations are still valid. The number of moles of ligand bound per mole of protein in zone β is then

$$r = (\bar{C}_B^\beta - C_B^\gamma)/\bar{C}_A^\beta \quad \ldots\ldots\ldots\ldots\ldots (2)$$

The pore size of Sephadex G-25 is sufficient to exclude all proteins and drug-protein complexes (which would be expected to move with equal velocities) while admitting and consequently retarding small drug molecules. Some small organic molecules, particularly aromatic and basic substances, are reversibly adsorbed on Sephadex (Gelotte, 1960). Adsorption of proteins on the other hand appears to be negligible except at low salt concentration (Glazer & Wellner, 1962). Provided adsorption is reversible such factors will not affect concentrations in the plateau regions though they will affect elution volumes. Concentration dependence of adsorption coefficients will affect boundary shape. If the velocity of migration of one or more of the reacting components depended on the presence of the other, equations 1 and 2 would be invalid.

To test the validity of this approach to binding measurements the following experiments were made with serum albumin and several sulphonamides chosen to cover a wide range of extent of binding (Anton & Boyle, 1964).

*If the rates of the forward and reverse reactions are very slow compared with the rate of column flow the elution profile will approximate to that expected for three separate components (Fig. 1d) and $\bar{C}_B^\beta = C_B^\gamma + C_C^\alpha$.

Experimental

MATERIALS

Crystalline bovine serum albumin was obtained from Sigma, U.K. Ltd. Sulphanilamide (B.D.H. Ltd), sulphamethoxypyridazine (Lederkyn, Cyanamid of Great Britain, Ltd), and sulphapyridine (May and Baker Ltd), were used without further purification. Sephadex G-25 (fine grade) was obtained from Pharmacia Ltd. All other reagents were Analar or laboratory reagent grade.

SEPHADEX CHROMATOGRAPHY

Sephadex was allowed to swell in Sorensen's M/15 phosphate buffer, pH 7·0, at room temperature for 16 hr and packed into jacketed precision bore tubes (internal diameter 5 mm) to give columns 15 or 30 cm long. These were equilibrated for at least 4 hr with buffer at the required temperature (usually 37°) supplied at 12 ml/hr by a Buchler peristaltic pump. Sample solution (12 ml) was introduced through the pump and 5-drop fractions (approx. 0·3 ml) collected during sample introduction and subsequent elution with 12–15 ml buffer. Alternate fractions were assayed for protein and sulphonamide, correction being made for variation of drop size during the run.

EQUILIBRIUM DIALYSIS

Visking dialysis tubing (36/32 inch inflated diameter) was heated to 70° twice in distilled water and once in phosphate buffer and stored in buffer at 3° until required. Sample solution (20 ml) containing protein and sulphonamide was placed inside the dialysis tubing which, after closure, was immersed in buffer (80 ml) containing an amount of sulphonamide calculated to be approximately that expected in the external solution at equilibrium. The system was kept in a closed vessel at 3° for 48 hr. Equilibration was completed by agitating the vessel in a shaking thermostat bath at the required temperature (usually 37°) for 6 hr. Protein and sulphonamide were assayed in the internal and external solutions. To compare results from equilibrium dialysis and chromatography, samples of the equilibrated internal solution were passed through Sephadex columns.

Suitable control experiments showed that no protein passed through the Visking tubing during dialysis and that no material leached out of the tubing itself to interfere with protein or sulphonamide determinations.

Protein was estimated by the biuret method (Gornall, Bardawill & David, 1949) using bovine serum albumin as the standard: sulphonamides were assayed by a modification of the method of Bratton & Marshall (1939).

Results and discussion

Fig. 2 shows the results of a typical experiment in which the elution patterns of a number of mixtures of bovine serum albumin and sulphamethoxypyridazine are compared with the patterns obtained with the

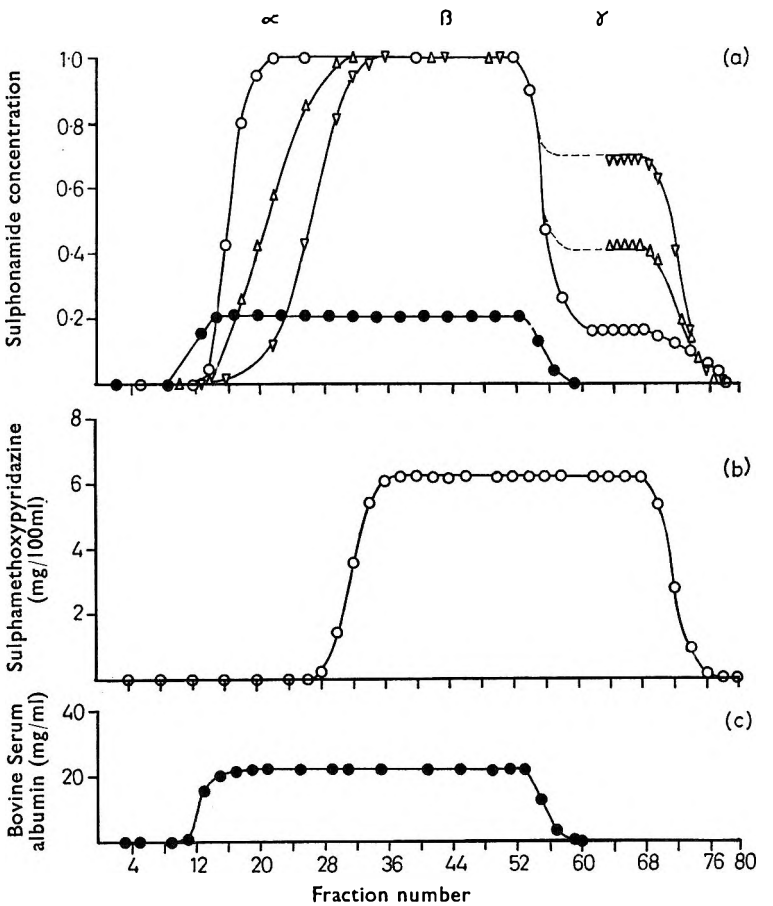

FIG. 2. Typical experimental Sephadex chromatograms. (a) Mixtures of sulphamethoxypyridazine and different concentrations of bovine serum albumin (○ 84%, △ 58% and ▽ 32% of drug bound), ● position of protein fronts in all three runs; in order to compare the results, the sulphonamide concentration for each run is expressed as a fraction of its value in the central plateau region β. (b) Pure sulphamethoxypyridazine. (c) Pure bovine serum albumin.

protein and sulphonamide separately. Particular features to note are:

(*a*) The positions of the leading and trailing boundaries of the protein zone are not affected by the sulphonamide. (In separate experiments they were also shown to be independent of protein concentration).

(*b*) The position of the slowest sulphonamide boundary is not affected by the presence of protein. (It was also shown to be independent of sulphonamide concentration).

(*c*) The sulphonamide boundary between zones β and γ coincides approximately with the trailing protein boundary but the leading sulphonamide boundary is in advance of the leading boundary for sulphonamide alone to an extent which depends on the composition of the mixture.

(*d*) The protein concentration is the same in zones α and β.

Similar results were obtained with sulphanilamide and sulphapyridine.

Thus the form of the elution boundaries for these drug protein mixtures conforms to the simple theory for rapidly reversible equilibria and since in addition the composition of the solution in zone β was found to be identical with that of the original mixture (see also Table 1) the conditions necessary for the validity of equations 1 and 2 are satisfied.

Comparison of results obtained by equilibrium dialysis and chromatography (Table 1) shows that the concentration of sulphonamide in zone γ (C_B^{γ}) is the same as the free sulphonamide concentration estimated from dialysis and that values of r and f calculated from both sets of results are in good agreement. Frontal analysis thus provides a valid method for measurement of protein-binding, at least in cases such as the sulphonamides which, while showing some reversible adsorption on the Sephadex, have adsorption coefficients which are independent of concentration.

TABLE 1. COMPARISON OF BINDING RESULTS FROM DIALYSIS AND CHROMATOGRAPHY (for definition of symbols—see text)

Sulphonamide	Protein conc * (mM)	Total sulphonamide conc. (mM)	Free sulphonamide conc. (mM) from dialysis	Chromatogram plateau conc. (mM)			r		f	
				$\bar{C}_A^{\beta}$	$\bar{C}_B^{\beta}$	C_B^{γ}	Dial.	Chromat.	Dial.	Chromat.
Sulphamethoxypyridazine	6·6	23·5	16·0	6·8	23·6	16·1	1·13	1·11	0·321	0·319
	6·5	23·3	16·2	6·5	23·2	16·0	1·09	1·11	0·302	0·309
	14·0	19·8	8·1	14·2	21·1	8·7	0·84	0·87	0·590	0·584
	14·0	20·0	8·1	14·5	21·4	8·7	0·85	0·87	0·595	0·594
	29·7	28·7	7·5	30·0	28·3	7·2	0·71	0·71	0·739	0·748
	28·5	28·9	7·8	28·7	27·8	7·0	0·74	0·73	0·733	0·749
	29·7	27·9	7·4	28·7	28·5	7·2	0·68	0·74	0·735	0·750
Sulphapyridine	43·7	35·6	28·3	42·2	36·2	28·3	0·17	0·19	0·209	0·219
	44·1	35·2	28·1	41·8	35·2	28·1	0·16	0·17	0·207	0·209
Sulphanilamide	20·2	15·6	14·8	19·8	15·8	15·0	0·040	0·040	0·055	0·054
	19·9	16·2	15·5	19·8	16·3	15·7	0·035	0·030	0·048	0·041

* Molecular weight assumed—68,000.

In the present work the boundary and plateau regions of the chromatograms were evaluated in detail in order to validate the method but for routine purposes the procedure could be much simplified since the only information required is the concentration of the drug in the plateau regions. This fact would also make the method particularly suitable for continuous automatic monitoring of column eluate. It could also be scaled down considerably to reduce the minimum volume of sample required; this must always be sufficient, however, to give an overlap of protein and drug zones.

The method thus combines the advantages of speed, simplicity, economy of sample and reasonable precision. The possibility that analysis of the leading drug boundary can yield information about rates of formation and breakdown of drug-protein complexes (see footnote to p. 152 S), information which is not available from the other methods of measuring drug-protein equilibria, is being explored.

Acknowledgements. Gifts of sulphonamides from Cyanamid, Great Britain, Ltd. and May and Baker, Ltd., are gratefully acknowledged. This work was carried out during the tenure by one of us (P.F.C.) of a Medical Research Council Scholarship. We are also indebted to the Nuffield Foundation for financial support.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 157S–167S Received April 25, 1968

Some limitations of continuous shear methods for the study of pharmaceutical semi-solids

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The limitations of the Ferranti Shirley viscometer are reviewed and discussed. An experimental study of four pharmaceutical materials indicates that the apparatus is restricted to a qualitative use for semi-solids because of effects due to evaporation, sweep time selection and shear fracture. When the above effects are gross, the rheograms demonstrate obvious peculiarities. If they are slight, they could escape unnoticed physically and yet lead to incorrect interpretation. The rheograms obtained for such materials are not reproducible for a once sheared sample and hence their quantitative interpretation would be meaningless. Transient tests or oscillatory studies are suggested as alternative methods of examination.

A WIDE range of gels and ointment bases (Schulte & Kassem, 1963, 1964) as well as aqueous solutions of surfactants (Gohlke & Hoffman, 1967) have been examined by continuous shear methods. In this field, the Ferranti Shirley cone and plate viscometer, available with an automatic program unit, enables continuous rheograms to be plotted at varying sweep times and maximum shear rates. This instrument has been used to study a range of ointment bases (Boylan, 1966, 1967), creams stabilized by complex films (Groves, 1967; Talman, Davies & Rowan, 1967) and soap, water amphiphile mixtures (Barry & Shotton, 1967a).

All these workers reported rheograms of the hysteresis loop type of varying degrees of complexity, including maximum and minimum shear stresses, which indicated that the systems studied were complex. The loop test method of analysis using the Ferranti Shirley viscometer, however, has a number of disadvantages and limitations. These are as follows:

(*a*) Continuous shear methods do not provide any fundamental rheological parameters (Warburton & Barry, 1968). These authors considered it more valuable, both practically and theoretically, to examine a system in its rheological ground state. Creep analysis, based on theories of linear viscoelasticity (Ferry, 1961) was suggested as an alternative.

(*b*) With the Ferranti Shirley instrument different combinations of sweep times and maximum shear rates will affect the shape of the resultant hysteresis loop and make comparisons difficult:

Reference	Sweep time (sec)	Maximum shear rate (sec^{-1})
Boylan (1966)	120	1074
Groves (1967)	600	188
Barry & Shotton (1967a) ..	600	1632

(*c*) Slippage effects have been considered by Jefferies (1965), and by Boylan (1967) who recommended that long sweep times should be used

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with semi-solids to avoid "serious slippage artefacts", the cause of these was not explained.

(*d*) At high shearing speeds viscous heating of the sample can become considerable (McKennell, 1956) and as a result of this, a hysteresis loop occurs under cyclic testing even for non-time dependent materials. To reduce the heating effect Cheng (1965) recommends that the time of shearing should be as small as possible.

(*e*) When the Ferranti Shirley viscometer is used with the large (3·5 cm radius) cone at short sweep times the large peripheral skirt can produce a "fly wheel" inertia effect resulting in a hysteresis loop even for Newtonian materials. This loop differs from that due to time effects in that the down curve lies closer to the torque axis than the up curve. Cheng (1965, 1967) has pointed out that the cone is driven by the motor via a torsion spring and that at small sweep times unsteady conditions will result in which the cone is subjected to accelerating forces; thus the Y plot will not be a true measure of shear rate. The X plot also is not a true measure of shear stress. Such errors can be corrected by procedures that involve first and second derivatives of the X–Y curve and by taking into account whether the material has a yield value or not.

(*f*) The placing of the sample in the viscometer disturbs the structure and it is generally not practicable to leave the sample between the cone and plate long enough before examination so that the structure may be built up once more (See (*g*) below).

(*g*) Difficulty arises in the determination of reversibility for aqueous systems which have a thixotropic structure that builds up slowly after cessation of shear, as evaporation effects limit the time a sample can be left in the viscometer.

(*h*) Acceleration variations of up to 10% during the up and down sweeps have recently been reported by Barry & Shotton (1968).

(*i*) In some materials fracture of the sample can occur at certain shear rates with an attendant fall in shear stress due to a decrease in the effective shearing radius. Such an effect can be erroneously interpreted as thixotropy as the fractures can heal (stress recovery) unless the shear rate has been such that material is ejected from the shearing zone (Hutton, 1963).

The phenomenon, also known as cavitation or tensile failure, can occur in liquids that are nearly Newtonian although is more common in pigment suspensions and materials with high elastic component (Hutton, 1963, Lammiman & Roberts, 1961). An optical method has been suggested for observing the phenomenon (Stiggles, 1965), whilst Lenk (1965) avoids spurious effects due to fracture by a preliminary determination of the shear rate at which it first occurs.

(*j*) For viscoelastic materials containing particulate matter the particles migrate to the outside of the cone due to normal force effects; this leads to a fall in viscosity (Highgate & Whorlow, 1967).

In the present work a critical evaluation of the Ferranti Shirley viscometer is made using a range of pharmaceutical materials.

Experimental and results

Materials. White soft paraffin, lanolin and paraffin ointment (white) were B.P. ointment bases. The emulsion system was a 60% dispersion of liquid paraffin stabilized by potassium laurate (2·5%) prepared as described by Shotton & Davis (1968).

Apparatus. A Ferranti-Shirley cone and plate viscometer with automatic flow curve recorder unit* was used according to Barry (1967). The manufacturers' anti-evaporation unit, consisting of a solvent trough and vapour hood, was fitted in an attempt to prevent evaporation effects with aqueous systems. Cone sizes of 3·5 cm, 2·0 cm and 1·0 cm radius with cone angles, 0·0062 radians, 0·0064 radians and 0·0061 radians respectively were used. A 200 g cm torque spring was fitted; this had a constant of 549 dyne cm/division.

A wide ended pipette was used for the fluid samples and a small spatula for semi-solids; samples were disturbed as little as possible. The calibration of the viscometer was checked using a N.P.L. viscometry standard (liquid paraffin B.P.—1·572 poise at 25·00°).

Hysteresis loop areas were measured by planimeter.

RHEOLOGICAL ANALYSIS

Evaporation effects. Rheological structure can be studied by following the change in a rheological parameter, usually relative viscosity, with temperature (Reiner, 1960). This has been done with the Ferranti Shirley viscometer by Lenk (1965) and by Barry & Shotton (1967a). Davis (1967) found a slight increase in viscosity, apparently due to evaporation, even though the apparatus was fitted with an anti-evaporation unit. The effect of evaporation, caused by increases in temperature, or long sweep time, on an emulsion of known rheological properties using the 3·5 cm radius cone has therefore been examined. The results (Fig. 1) show that at 40° and with a 60 sec sweep time, a simple pseudoplastic flow curve is obtained. A similar curve was obtained at the same temperature using a Couette viscometer where evaporation effects are negligible. Use of a 600 sec sweep time gave rise to a large hysteresis loop that differed from that normally found with time-dependent systems in that the down curve lay nearer the torque axis than the up curve. For the long sweep time, evaporation effects at 40° become evident after the sample has been in the viscometer for about 3·5 min. The lack of an evaporation loop at the 60 sec sweep time is thus explained.

Sweep time effect. The effect of sweep time on the shape of the hysteresis loop was investigated using white soft paraffin B.P. The eight sweep times available on the autoplotter (from 10 to 600 sec up curve sweep) were used and the maximum shear rate was 1692 sec^{-1} in each case. Fig. 2 shows that at low sweep times the shape of the loop is markedly dependent on sweep time. As the sweep time increases, the static yield value (Levy, 1962) and maximum shear stress both decrease as does the loop area and the shear stress at the maximum shear rate. A plot of the

* Ferranti Ltd., Moston, Manchester.

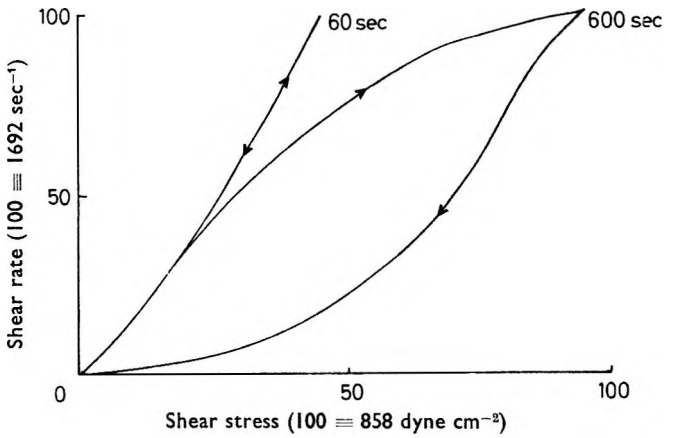

FIG. 1. The effect of temperature on the flow curve of an emulsion of liquid paraffin in potassium laurate (40°). Figures on curves are sweep times.

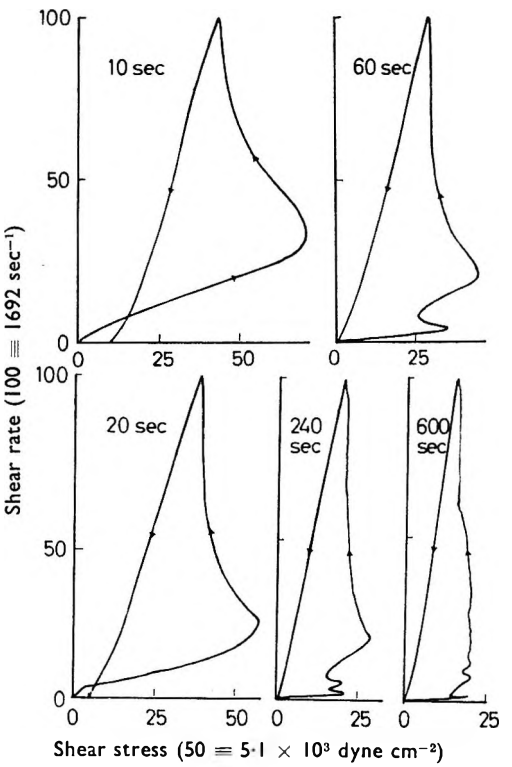

FIG. 2. The effect of sweep time on the shape of the hysteresis loop for white soft paraffin.

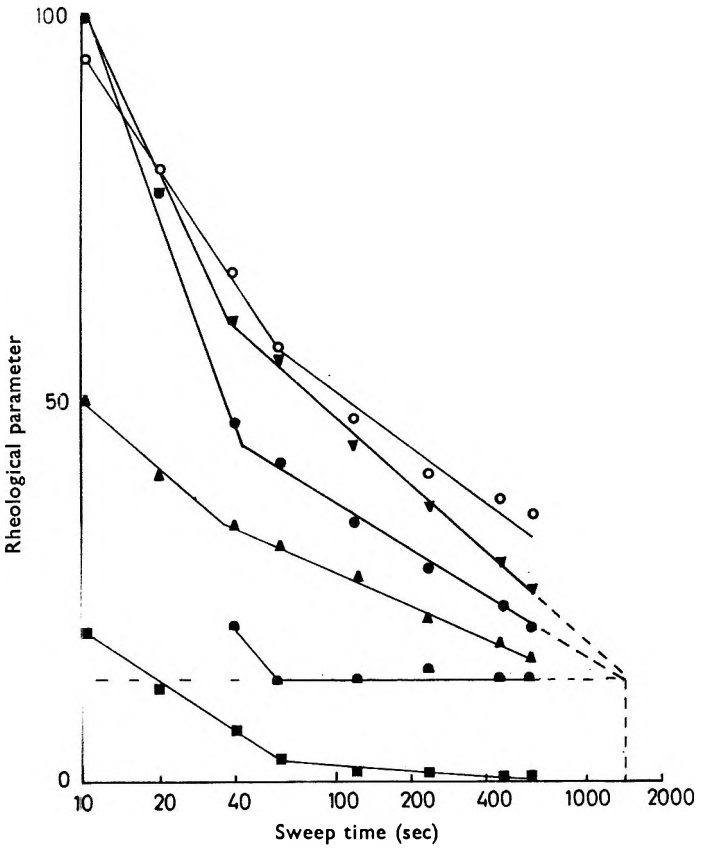

FIG. 3. The change in rheological parameter with sweep time for white soft paraffin. ● Static yield value. ▼ Shear stress maximum. ■ Dynamic yield value. ◓ Extrapolated static yield value. For all: 100 ≡ $7{\cdot}5 \times 10^3$ dyne cm^{-2}. ▲ Loop area (100 ≡ 600 cm^2). ○ Apparent viscosity (100 ≡ 3·0 poise).

selected rheological parameter against log of sweep time (up curve) indicates that a discontinuity exists in the region of 60 sec (Fig. 3). The lowest static yield value (at 600 sec/sweep) is in the region of 1700 dyne cm^{-2}, whereas the extrapolated static yield value at sweep times greater than 40 sec has a value that is independent of sweep time and is in the region of 10^6 dyne cm^{-2}. This is close to a yield value of 995 dyne cm^{-2} found by the successive addition of weights to two scale pans attached to the inner cylinder of the concentric cylinder viscometer described by Warburton & Barry (1968). An extrapolation of shear stress maximum, static yield value and extrapolated static yield value to higher sweep times indicates that all these have an identical value at 1400 sec sweep (up curve).

Shear fracture. The up curve of the rheogram for lanolin B.P. at 25° showed a sudden fall in shear stress at a critical shear rate whilst for paraffin ointment B.P. at 25°, once the static yield value had been reached,

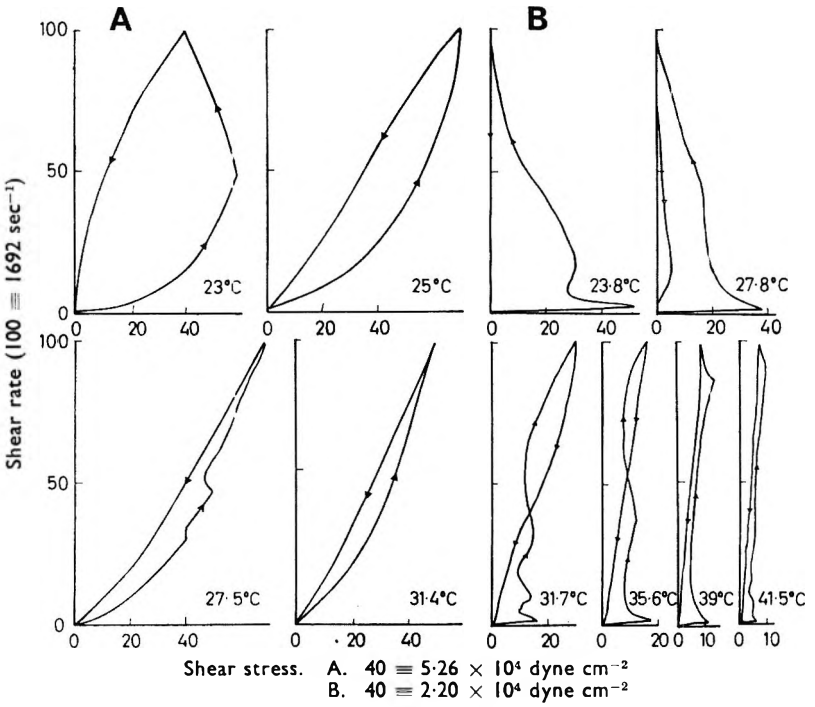

FIG. 4. The effect of temperature on the hysteresis loop of A. Lanolin, B. Paraffin ointment. Sweep time = 600 sec.

the shear stress decayed rapidly to almost zero. On separating the cone and plate, after the materials had been sheared, very little of the sample remained in the gap. This effect is due to fracture of the sample and its subsequent ejection from the gap. No loss was observed with white soft paraffin.

The effect of temperature within the range 20–42° on the loss of shear stress due to fracture was investigated for lanolin, paraffin ointment and white soft paraffin (Fig. 4A and B). Changes in loop area, and apparent viscosity at maximum shear rate, with temperature, for lanolin and paraffin ointment are different from the changes shown by white soft paraffin (Figs 5 and 6). The three materials all show a linear relation between yield value, or shear stress at low shear rate, and temperature.

Thixotropy. A test for thixotropy was made on white soft paraffin (Fig. 7). A sample was subjected to the loop test at 25° (curve 3) and then resheared after a 10 min rest period (curve 2). The up and down curves in the second cycle are close to the down curve in the first cycle indicating negligible build up of structure. A third cycle after a two day period (curve 1) demonstrated some build up of structure, but the initial loop was not completely recovered.

Creep analysis. The three ointment bases were examined by the creep method using the apparatus of Warburton & Barry (1968). The applied

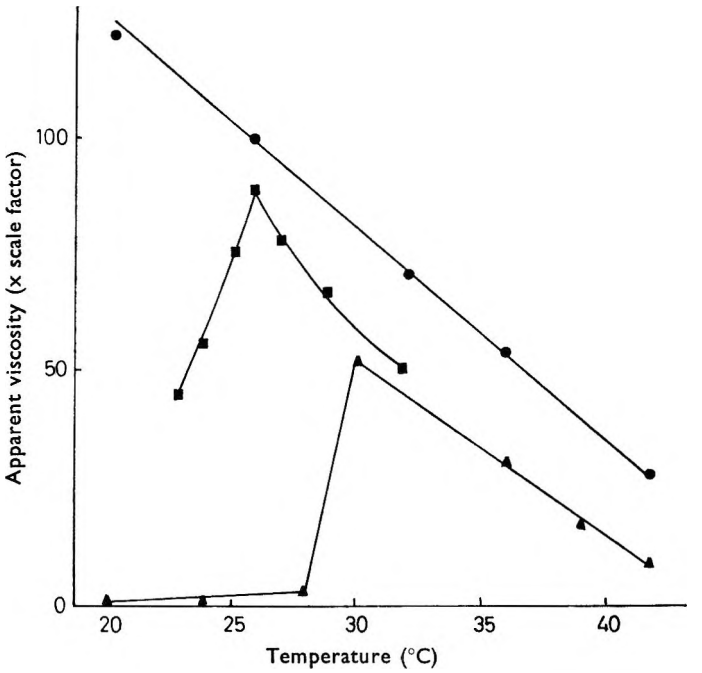

FIG. 5. The change in apparent viscosity with temperature for three ointment bases. Sweep time = 600 sec. ● White soft paraffin (100 = 1·2 poise). ■ Lanolin (100 = 76·5 poise). ▲ Paraffin ointment (100 = 19 poise).

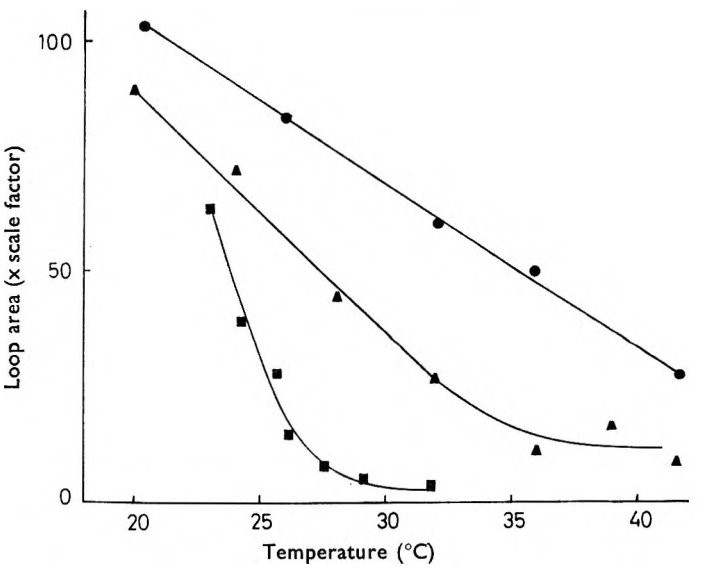

FIG. 6. The change in hysteresis loop area with temperature for three ointment bases. Sweep time = 600 sec. ● White soft paraffin. ▲ Paraffin ointment (100 = 180 cm²). ■ Lanolin (100 = 1400 cm²).

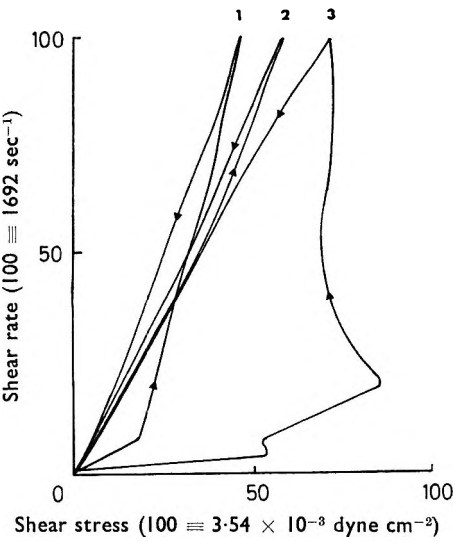

FIG. 7. White soft paraffin thixotropy test. Sweep time = 600 sec. (1) After two days rest. (2) After 600 sec rest. (3) Original curve.

stress was adjusted so that the strain response was in the linear region (Ferry, 1961). The results were analysed by the method of Barry & Shotton (1967b) and Warburton & Barry (1968) in which shear behaviour is described in terms of series Voigt model elements. The creep curves for lanolin and paraffin ointment were typical of viscoelastic behaviour and may be represented as a Maxwell unit in series with two Voigt units. The values of the individual components are given in Table 1. White soft paraffin gave a curve typical of an elastic (Hookean) solid with the strain independent of time after the instantaneous shear component. For all three materials, removal of the stress gave immediate recovery of the instantaneous shear component.

TABLE 1. THE VISCOELASTIC PARAMETERS OF THREE SEMI-SOLIDS

System	Voigt unit	Values of viscoelastic parameters		
		τ (sec)	G dyne cm^{-2}	η poise
White soft paraffin ..	0	—	$1{\cdot}37 \times 10^{7}$	—
Lanolin	0	—	$6{\cdot}72 \times 10^{4}$	$7{\cdot}87 \times 10^{7}$
	1	$5{\cdot}98 \times 10^{3}$	$4{\cdot}80 \times 10^{4}$	$2{\cdot}87 \times 10^{7}$
	2	$5{\cdot}91 \times 10$	$1{\cdot}08 \times 10^{6}$	$6{\cdot}40 \times 10^{6}$
Paraffin ointment ..	0	—	$7{\cdot}28 \times 10^{5}$	$6{\cdot}42 \times 10^{8}$
	1	$4{\cdot}46 \times 10^{2}$	$2{\cdot}40 \times 10^{6}$	$1{\cdot}07 \times 10^{8}$
	2	$5{\cdot}65 \times 10$	$5{\cdot}16 \times 10^{6}$	$2{\cdot}92 \times 10^{7}$

τ = retardation time
G = shear modulus of Voigt model
η = shear viscosity of Voigt model ($\eta = G\tau$)

Discussion

The use of the Ferranti Shirley viscometer for aqueous systems can lead to spurious results due to evaporation at elevated temperatures, even when

the viscometer is fitted with the manufacturers' anti-evaporation unit. For a simple emulsion system of known rheological properties, the use of lower sweep times provides a correct rheogram. With time dependent materials however, the breakdown of the material due to thixotropy or irreversible shear thinning will be opposed by the effect of evaporation. Lower sweep times may result in a radical change in the shape and area of the hysteresis loop and in the values of rheological parameters. Although evaporation effects are greatly reduced in concentric cylinder geometry, this apparatus is limited to non time-dependent materials, since no apparatus of this geometry is available with the automatic programming facilities of the Ferranti Shirley viscometer.

The choice of sweep time greatly affects the values of the rheological parameters that can be calculated from the hysteresis loop. A decrease in all the measured parameters occurs with increase in sweep time (Fig. 3) and more detail in the up curve is resolved. An extrapolation of the static yield values and shear stress maximum to higher sweep times shows they have an identical value of 10^3 dyne cm^{-2} at a sweep time of 1400 sec (up curve). This value is very close to that obtained by a successive loading test in concentric cylinder geometry and it may well be that at a sweep time of 1400 sec white soft paraffin will give the up curve of a typical plastic (Bingham) material without shear stress minima or maxima.

The acceleration of the cone at different sweep times will affect the loop size and shape by several mechanisms. (1) At low sweep times the apparatus will constitute an oscillatory system (Cheng, 1965) introducing effects due to inertia and the torsional stiffness of the spring (Ferry, 1961, Van Wazer, 1963). (2) The yield value of a material is not independent of time (Houwink, 1958). It has been found (McVean & Mattocks, 1961) that the static yield value is a function of the rate of strain and is associated with viscoelasticity and retardation behaviour. With a cone and plate apparatus, Bauer, Shuster & Wiberley (1960) found that the yield stress was affected by the rate of increase of stress. Fischer, Bauer & Wiberley (1961) have pointed out that the measurement of yield stress may give values dependent on the apparatus design, as well as the nature of the test material and the time scale of the test procedure.

The cause of shear stress minima in flow curves for concentric cylinder geometry was suggested by Enneking (1958) to be a combination of slippage and variation of shear rate across the gap. Although the shear rate is independent of radius for cone and plate, a similar explanation can be advanced if it is assumed that there are regions in the material which act as "stress raising points". We suggest that at the static yield value the material flows only in the region of the cone surface and not throughout the whole gap because of the formation of a slippage plane of the type described by Wood, Giles & Catacalos (1964). As the cone accelerates, further structure breakdown occurs and the effective cone angle will increase. Since the shear rate is proportional to the reciprocal of cone angle, the actual shear rate will decrease even though the speed of rotation of the cone increases. Because shear stress is related to shear rate it shows a decrease and the process continues until flow occurs in the

whole gap and the stress minimum is reached. When the whole of the sample is sheared, the effective cone angle will be the true cone angle and the shear rate and rev/min (given by the Y axis of the autoplotter) will be directly proportional. The true flow curve will thus be obtained. In support of this theory it has been found for white soft paraffin that if the up curve after the stress minimum is extrapolated back to the shear stress axis, the yield value so obtained is close to that obtained by the loading test method, i.e. 10^3 dynes cm^{-2}.

Lanolin and paraffin ointment both demonstrate loss in shear stress due to fracture and subsequent expulsion of material from the shearing zone of the instrument due to the viscoelastic nature of these materials. This is characterized by a *fall* in shear stress with increase in shear rate. An increase in temperature causes viscoelastic structure to be broken down, resulting in less expulsion from the gap and a rise in apparent viscosity. The rheograms become simpler until eventually the classical type of loop, with shear stress increasing with shear rate, is obtained. Fracture of fluids in shear has been linked with elasticity (Hutton, 1963). A viscoelastic liquid in a state of flow will possess elastic energy so that when shear is stopped the liquid will recoil due to release of this energy. Hutton has postulated that when the total elastic energy exceeds a critical value the liquid will fracture. Initially such breakdown is believed to be localized as a conical fracture surface that starts at the periphery and grows radially inwards. The shear stress falls as the effective shearing radius decreases as the fracture grows. On reduction of shear the fracture can heal and the whole effect may be erroneously interpreted as some form of thixotropy or structural breakdown. Gross fracture, i.e. loss from the gap, is clearly shown in the present work by a negative gradient in the shear stress/shear rate relation. Difficulty will still arise however, even if lower shear rates are used, in ascertaining whether a fall in consistency is due to thixotropy or shear failure and subsequent stress recovery.

White soft paraffin, which appears to be a Hookean solid below its yield point, does not exhibit noticeable shear fracture, and the selected rheological parameters decrease linearly with temperature (Figs 5 and 6). The final part of the up curve for white soft paraffin shows an abrupt change in gradient and is then almost vertical (Fig. 7). Some form of shear fracture or even slight ejection may be occurring. In the test for thixotropy the incomplete build up of structure after two days may be a real effect but equally well it could be due to slight loss of material from the gap during the initial shearing cycle. This of course cannot be recovered on resting.

CONCLUSIONS

It is concluded that the value of continuous shear methods using the Ferranti Shirley apparatus with pharmaceutical semi-solids is strictly limited. Problems arise due to evaporation, sweep time selection, shear fracture and wind up of the torque sensing spring, and at best it should be used as a *qualitative* measurement of rheological structure. Analysis

methods where the material is kept in its rheological ground state, such as creep (Warburton & Barry, 1968) or oscillatory testing (Warburton & Davis, 1968) are more suitable for semi-solid materials.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 168S–175S Received May 1, 1968

Thixotropy and rheopexy of propyliodone suspensions in arachis oil: effect of median particle size

B. S. CHAWLA

Propyliodone oily injection B.P. exhibits thixotropy under high shear rates and rheopexy under low shear rates. The rate and the degree of rheopectic build-up of structure are influenced by the particle size, the effect increasing as the median particle size is reduced.

A RARE rheological phenomenon has been observed in propyliodone oily injection B.P. Although, in common with many suspensions, this preparation exhibits thixotropy or shear thinning at high rates of shear, it shows shear thickening at low rates of shear. This phenomenon is known as rheopexy and has not hitherto been reported in pharmaceutical preparations.

Thixotropy and rheopexy are referred to as non-Newtonian and time-dependent properties. In both cases, the changes in apparent viscosity are thought to be associated with the building or breaking of structure arising from intermolecular forces as well as from interaction between particles.

Thixotropic materials, as characterized by Green & Weltmann (1943, 1944, 1946), Green (1949) and Wilkinson (1960), suffer structural breakdown with a consequent decrease in apparent viscosity when sheared: the degree of breakdown depends on both the duration and the rate of shearing. The rate of breakdown during shearing at any given rate depends on the number of structural linkages that can be broken by the given shearing force. On cessation of shearing, the original structure reforms and the initial apparent viscosity is regained at a rate depending on the availability of possible linkages. During shearing at a constant rate, structural breakdown and reformation proceed simultaneously and a dynamic equilibrium is reached when the internal forces tending to rebuild the structure are equal to the applied shearing forces. The apparent viscosity at equilibrium is dependent on the shear rate.

On the other hand rheopectic materials exhibit a gradual formation of structure when gently sheared. Freundlich & Juliusburger (1935) were first to observe this phenomenon in thixotropic sols (V_2O_5) of anisometric particles, the time of "solidification" i.e., build-up of structure, being shortened by gentle and regular motion of the sol. It is rare to find both phenomena of thixotropy and rheopexy exhibited by one material. Other materials in which rheopexy has been reported are: gypsum, kaolin and Solnhoten slate (Burger & Sollner, 1936); mixtures of glycerol, water and wheat starch (Bon, 1936); bentonite gels (Hauser & Reed, 1936); clay suspensions (Gurvich, 1956); some polyesters and their concentrated solutions in toluene and ethyl benzene (Steg & Katz, 1965); concentrated suspension of the explosive hexahydro-1,3,5-trinitro-s-triazine (RDX; cyclonite) in molten trinitrotoluene (Williamson, 1959).

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In this paper the effect of particle size on the rheopectic properties of suspensions of propyliodone in arachis oil is reported.

Experimental

APPARATUS

Rheological measurements were made with a Ferranti-Shirley cone-plate viscometer equipped with control unit and X-Y recorder (McKennell, 1954, 1956, 1960; Van Wazer, Lyons & others, 1963). The apparatus was set up and used in accordance with the instrument manual, an automatic cone-plate setting unit being used throughout the operation, to maintain a constant gap-width between the cone and plate of the instrument.

Two modes of operation were used:

1. To record shearing stress versus rate of shear, the controls were set to give a uniform acceleration of the cone from zero to maximum rate of shear (1,730 sec^{-1}) in a sweep time of 60 sec and then to decelerate uniformly to zero.

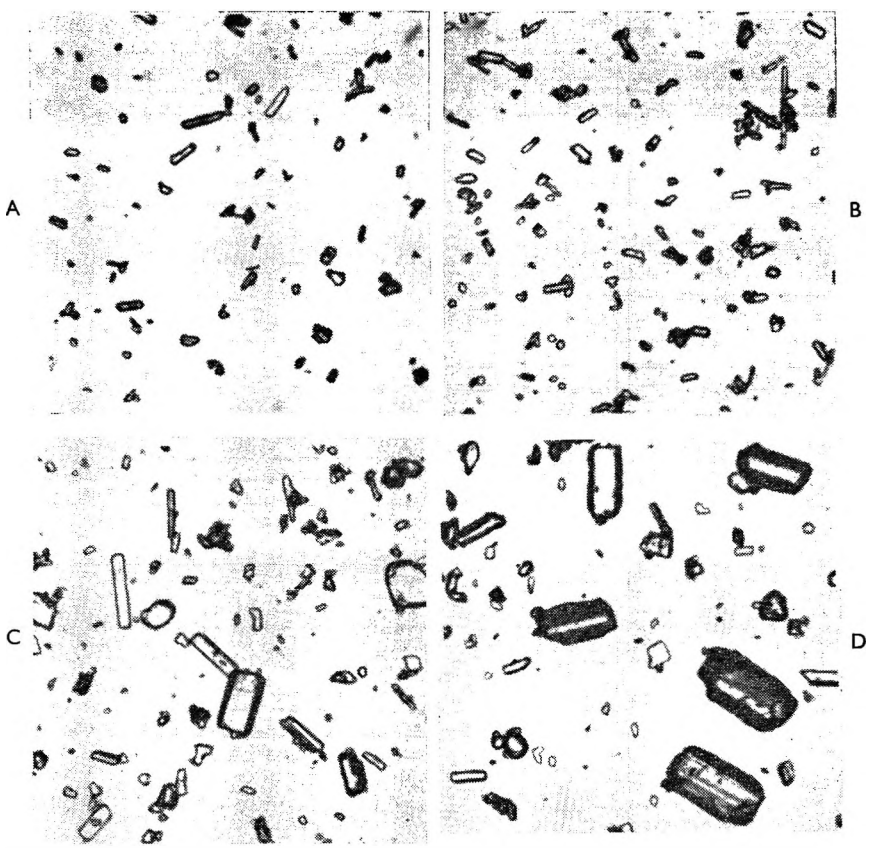

FIG. 1. Crystal shape and size distribution of propyliodone samples A, B, C and D (× 285).

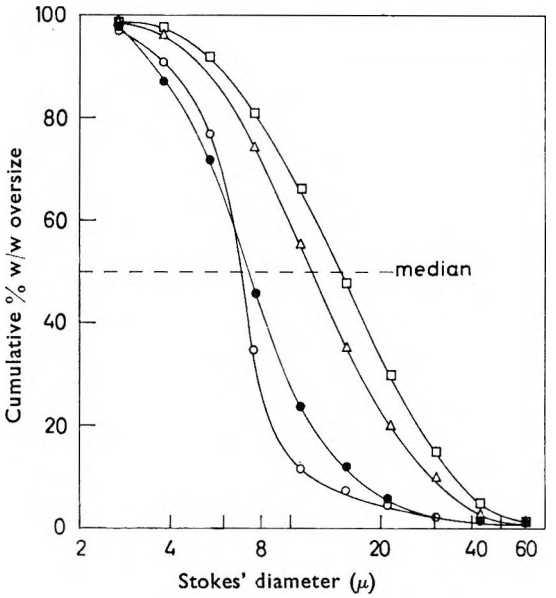

FIG. 2. Particle size analysis of propyliodone crystals. ○ Sample A, median diameter 6·7 μ. ● Sample B, median diameter 7·3 μ. △ Sample C, median diameter 12·0 μ. □ Sample D, median diameter 14·8 μ.

2. To record shearing stress versus time at constant rates of shear, the controls were set to give a constant rate of shear by rotating the cone at a fixed speed; the Y axis of the recorder was used to record time. These recordings are shown in Figs 5 and 6. Due to the limitation in the length of the Y-axis on the recorder, the curve is folded on itself, by reversing the direction of the pen along the time axis at the preselected sweep time (40 sec). Coincidence of the up and down traces indicates attainment of an equilibrium stress reading. Cone angle = 20′ 45·5″; cone radius = 3·5 cm; shear stress constant = 24·59; rate of shear constant = 17·30.

MATERIALS

Arachis oil B.P. (Viscosity of 0·74 poise, at 25°.) Propyliodone B.P. Four samples were used: they are characterized by photomicrographs (Fig. 1) and the particle size distribution shown in Fig. 2.

PARTICLE SIZE ANALYSIS

The Sartorius sedimentation balance was used to determine the particle size distibution of propyliodone. The apparatus is an Odén-type balance which gives a continuous record of sediment weight against time. Details of operation and setting up of the apparatus are described by Edmundson (1967). The samples (0·2% w/v) were dispersed in distilled water containing 1·0% w/v polyethylene glycol 600 mono-oleate.

The sedimentation curves were differentiated by an arithmetic method (Stairmand, 1947: British Standards Institution, 1963, Part 2).

PREPARATION OF SUSPENSIONS

Suspensions of propyliodone 60% w/v (equivalent to 27·9% v/v) were prepared by gradually adding the powder to arachis oil whilst stirring with a 1 inch diameter three-bladed marine-type propeller, the rate of stirring being regulated to minimize the incorporation of air. When all the powder was dispersed the suspension was stirred for 1 min at 4000 rev/min.

Entrapped air was removed from the suspension at a pressure equivalent to a few mm of Hg till no further air bubbles were visible. Samples were stored in 360 ml wide mouth bottles (internal diameter 6·5 cm) in a horizontal position and after 20 hr the suspensions were stirred gently with a spatula to ensure homogeneity before rheological tests.

RHEOLOGICAL TESTS

Samples for testing on the Ferranti-Shirley viscometer were removed from the bottles with a small scoop and held between the cone and plate (3 min) to reach temperature equilibrium (25° ± 0·1). A fresh scoopful was taken for each test and each suspension was tested in the two modes of operation of the viscometer. The resulting flow curves are designated 1 in Fig. 3. The records of shearing stress versus time at constant rate of shear are shown in Fig. 5.

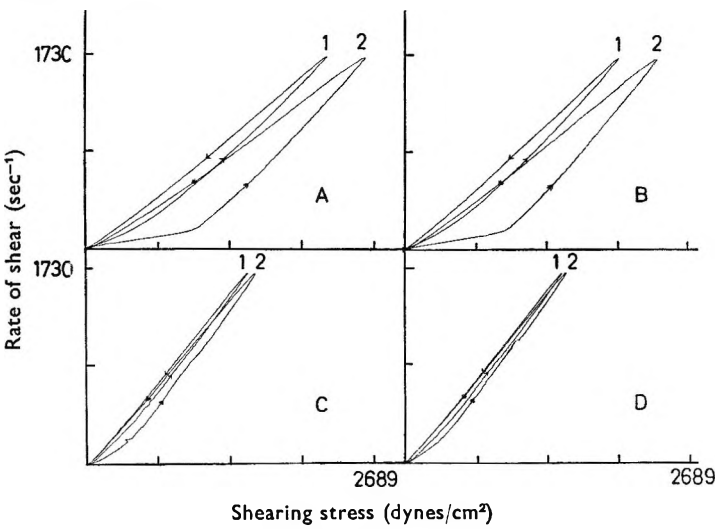

FIG. 3. Flow curves of suspensions A, B, C and D. 1. Before systematic rolling of suspensions. 2. After maximum "rheopectic" build-up of structure by gentle systematic rolling of suspensions.

The bottles of suspension were then rolled at 18 rev/min with their axes horizontal. Periodically during the rolling, scoopfuls were removed and flow curves were recorded. When the suspensions had attained maximum build-up of structure as shown by the superimposition of consecutive flow curves, the suspensions were tested at a constant rate of shear of 1730 sec^{-1}. The resulting traces are shown in Fig. 5, Series I.

Results

All the suspensions showed similar rheological behaviour, structural build-up when gently rolled, and structural break-down of the system when subjected to a higher rate of shear.

The flow curves of the suspensions determined at various intervals of time of rolling showed an increase in the apparent viscosity and in the degree of thixotropy in the system (judged by the area within the hysteresis loop). Flow curves of the suspensions before rolling and after maximum build-up of structure by rolling are shown in Fig. 3. To illustrate the rheological changes in the suspension during the course of rolling treatment, shearing stress readings versus time of rolling are plotted in Fig. 4. The shearing stress readings were taken from flow curves determined, after various periods of rolling, in the region of rate of sheer which showed the widest portion of the hysteresis loop (567 sec^{-1}). In Fig. 4 the graphs show: (*a*) that the rate and the degree of build-up of structural viscosity increases as the median particle size of the system decreases (graphs with closed symbols); (*b*) that the degree of thixotropy increases

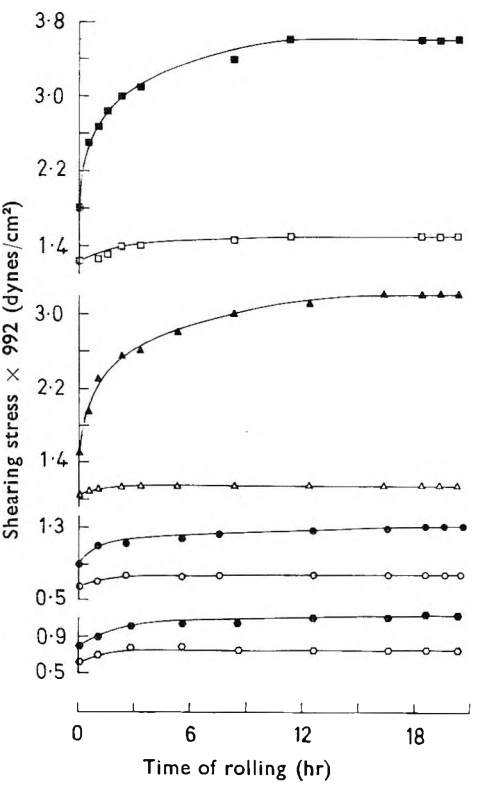

FIG. 4. Demonstrating progressive build-up of "rheopectic" structure with time of rolling. Squares, Susp. A; triangles, Susp. B; circles, Susp. C; hexagons, Susp. D; open symbols, stress reading of the up curve; closed symbols, stress reading of the down curve. Data obtained from flow-curves at 576 sec^{-1} rate of shear.

as the median particle size of the system decreases, this is indicated by the vertical distance between the up and down curves, characterized by closed and open symbols respectively; (*c*) that the shearing stress readings of the down curves (open symbols) are higher after rolling.

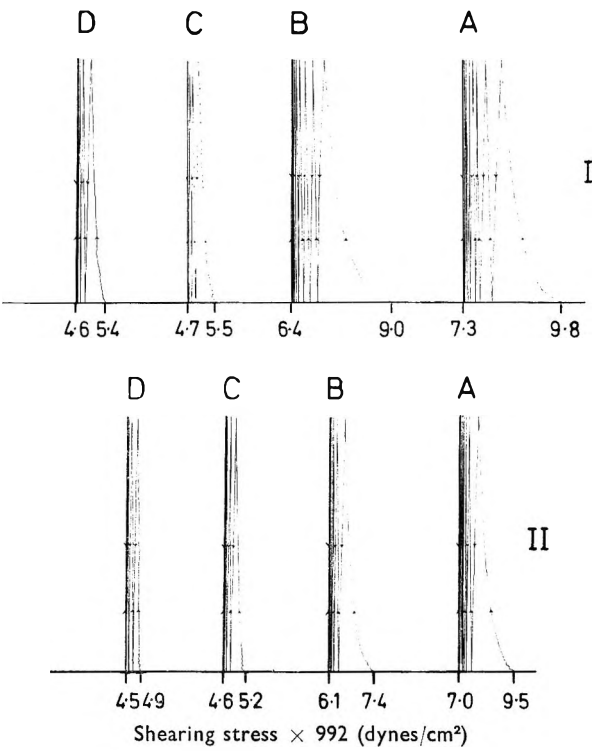

FIG. 5. Plots of shearing stress versus time* at constant rate of shear (1730 sec^{-1}). Series I. After rheopectic build-up of structure by systematic rolling of suspension. Series II. Before rolling of suspension. * One sweep cycle up and down = 80 sec.

The records of shearing stress versus time at 1730 sec^{-1} rate of shear, (Fig. 5, Series I and II) show the strength, the rate of breakdown and the degree of breakdown of structure of the unrolled suspensions and of rolled suspensions; the latter were tested only after maximum build-up of rheopectic structure. The traces show that the structure in the rolled samples breaks down more rapidly under shear than the structure in the unrolled samples, and also that the rate of breakdown is greater with suspensions of smaller median particle size. All suspensions give higher equilibrium stress readings after rolling than before, which suggests that the structure induced by rolling is not only more extensive but also stronger than that existing before rolling.

Although no systematic study of the retention of rheopectic structure has been made, sample A was retested after 4 weeks of storage; the flow curves showed only a slight change in apparent viscosity and thixotropy.

It is difficult to calculate the order of rate of shear that applied in the

rolling experiment. However, the effect of low and high shear rates on structural changes was studied on that suspension which proved most sensitive to shear treatment (Suspension A). This was done by recording shearing stress versus time at various constant rates of shear. Below 104 sec^{-1} rate of shear the system showed structural build-up. The rate and degree of build-up of structure increased rapidly as the shear rate decreased, whereas above 104 sec^{-1}, the rate and the degree of breakdown increased as the rate of shear increased. This is shown in Fig. 6.

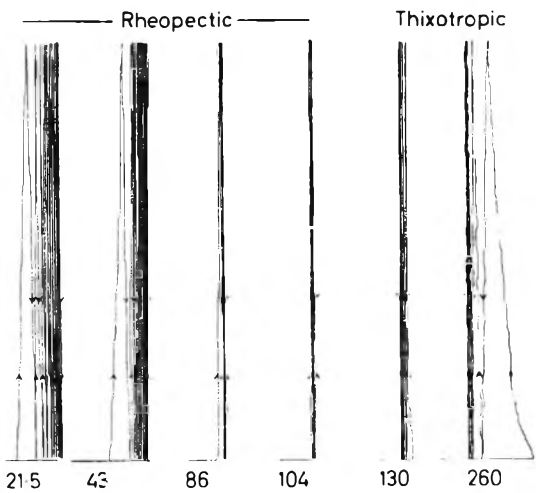

FIG. 6. Demonstrating "rheopectic" and "thixotropic" properties of propyliodone suspension in oil (Sample A). Recording of shearing stress (horizontal axis) versus time* (vertical axis) at various constant rates of shear (indicated below each tracing, sec^{-1}). * One up and down cycle = 80 sec.

Discussion

The propyliodone suspensions studied here show typical rheopectic properties as defined by Freundlich & Juliusburger (1935).

The terms rheopexy, dilatancy and antithixotropy have sometimes been confused in the literature. Although these phenomena all involve what may be called shear thickening they differ in several respects. Dilatancy is dependent on rate of shear only and occurs when there is a high concentration and close packing of highly dispersed particles; the system dilates under shear, appears dry and stiff, but reverts spontaneously to the more liquid state when the stress is removed. Antithixotropy (Eliassaf, Silberberg & Katchalsky, 1955; Chong, Eriksen & Swintosky, 1960) is dependent on the duration as well as the rate of shear. It is a reversible isothermal increase in consistency during shearing with the system returning spontaneously to a sol state on standing; note that this is the exact reverse of normal thixotropy. Antithixotropic systems are characterized by flow curves that are similar to thixotropic curves but with the direction of the hysteresis loops reversed (Chong & others, 1960; Chawla, 1967).

With rheopexy, however, the structural build-up and the increase in consistency produced by slow shearing are retained, at least for some time, after shearing ceases. A system with rheopectic structure is in a state of equilibrium.

The influence of median particle size on the rate of build-up, the degree of build-up and the strength of the rheopectic structure has been demonstrated. However, the exact mechanism which causes rheopexy in arachis oil suspensions of propyliodone is uncertain. According to Freundlich & Juliusburger (1935), an increase in consistency is associated with "orientation of the particles; the regularity of the stirring applied inhibits movements in a vertical axis, and rod-shaped particles are therefore orientated entirely with their axes lying horizontally". Although our propyliodone crystals were strongly anisometric (Fig. 1), it is difficult to visualize a mechanism whereby such perfect orientation of the particles in the field of shear, would cause an increase in consistency of the suspensions. Our system is relatively simple, propyliodone crystals in arachis oil, uncomplicated by ionic or hydrogen bond mechanisms. Some form of orientation may take place with propyliodone crystals under slow shear. The fact that rheopexy is exhibited more readily with smaller median particle size suggests that flocculation or disorientation may be induced by slow rolling.

Acknowledgement. I thank Mr. I. C. Edmundson for helpful discussions.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 176S–181S Received May 1, 1968

Energy utilization in a fluid energy mill

K. RIDGWAY AND C. A. WALTON*

A "Gem" fluid energy mill was built into an assembly which allowed the temperature, pressure and quantity of air flowing through it to be measured. The feed rate of powder to be ground was also controlled. The increase in surface area of sodium chloride was determined as a function of air pressure and feed rate in order to establish the conditions for optimum performance.

THE history of fluid energy milling may be traced from approximately 1880, through a range of devices designed to accelerate particles to high velocities in fluid jets, particle reduction being achieved either by impact with fixed objects (Auger, 1936), or by attrition in opposing streams (Rowley & McCabe, 1948) or by mutual attrition in converging streams and turbulent flow as in the "Micronizer" (Andrews, 1936) or "Jetomiser" (Stephanoff, 1946).

As fluid energy mills became commercially available the suggestion arose that their energy consumption is excessive, and this was examined by Stephanoff (1949) and by Pendleton (1963). Dotson (1962) determined the energy requirements of both fluid energy and ball mills. All three authors found that for similar degrees of particle size reduction, the energy requirements were comparable. Dotson, however, tested three sizes of fluid energy mill on silicon metal powder and obtained results which indicated that smaller mills were less efficient, in terms of surface area produced per unit of power expended, than larger ones. The present work was started in order to determine whether this was in fact the case.

Experimental

The mill used was a research model "Gem" fluid energy mill (George W. Helme Inc., Trost Jet Mill Division). Its construction is shown in Fig. 1, together with details of how probes were inserted to measure conditions inside it. The probes could be inserted at various positions, the holes not in use being closed off by means of Duralumin plugs. Piezometric and total heads were measured by tubes flush with the wall and by a small pitot tube respectively. The pitot tube carried a copper-constantan thermocouple within it.

The mill was clamped during measurements to the end of a 6 inch diameter brass cylinder as shown in Fig. 2. All the air leaving the mill through the filter bag which accumulated the milled product, was thus collected and made to pass through the rotameter.

To obtain a constant controllable rate of powder feeding, a feed apparatus was devised as shown in Fig. 3. The principle is that of a rotary tabletting machine. A rotating circular upper plate with a ring of holes near its outer edge was driven by a geared-down electric motor. The motor speed was set and maintained constant by a Pye smooth speed controller. A polythene funnel acted as a feed hopper, filling the holes

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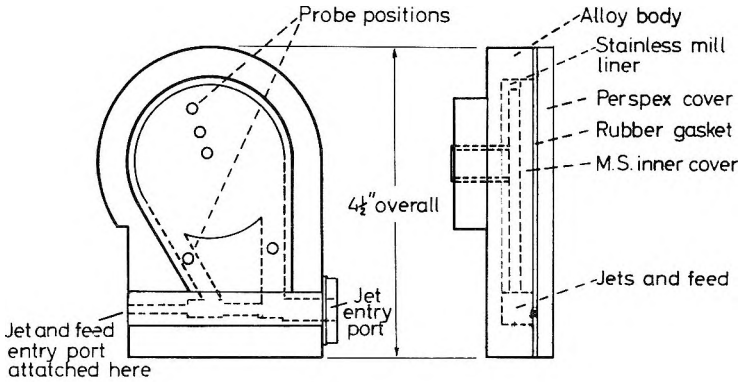
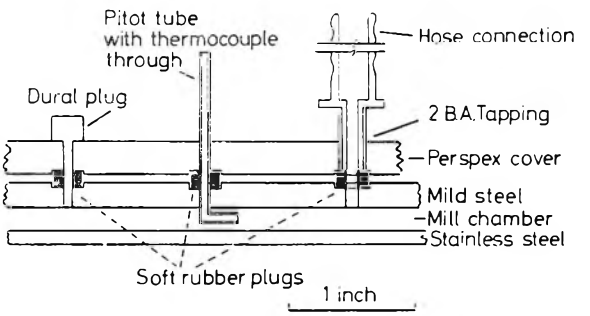


(a)



(b)

FIG. 1. (a) The fluid energy mill and (b) detail of the attachment of the probes to the mill face.

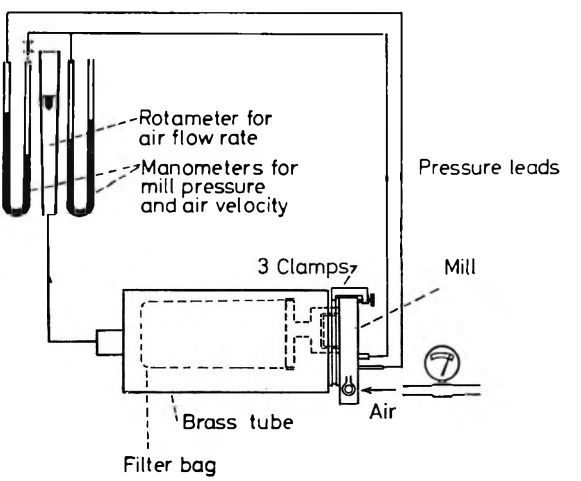


FIG. 2. The mill clamped to the air flow measuring assembly.

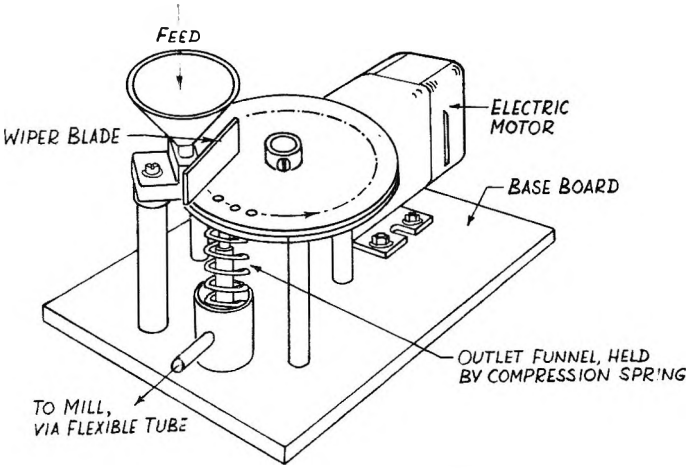


FIG. 3. The constant-rate powder feed apparatus.

as they passed beneath it. The powder was carried round over the stationary lower plate until the hole passed over a hole in the lower plate, to which the mill feed suction pipe was attached. The powder was then drawn into the mill by the feed Venturi.

The feed to the mill was Analar sodium chloride. 2 kg of this material was coned and quartered to 2 g and a sample of this examined microscopically. 300 particles were measured by a calibrated eyepiece vernier indicator. The mean particle size by area was 330μ. The milled product was examined by a Fisher sub-sieve sizer to obtain surface area and hence mean particle size.

A typical run was carried out as follows: the mill bag was weighed, attached to the mill and the mill apparatus assembled. The feed device was switched on and its delivery rate determined by collecting and weighing over a few minutes. Compressed air was then fed to the mill with the powder feed tube closed and after settling down, the air flow-rate, mill pressure, velocity head thermocouple readings were taken. These readings were taken again with the feed tube open, and then powder supply was commenced and continued for 20 min, measuring all parameters at intervals. After shutdown, the bag was reweighed. 10 g of the product was dried at 150° for 1 hr and examined on the Fisher sub-sieve sizer. All the data for the evaluation of mill performance are thus easily available, with the exception of the energy supplied by the air.

The energy supplied by unit mass of gas in expanding from pressure p_2 to pressure p_1 and specific volume V_1 is given by

$$W = \frac{k}{k-1} p_1 V_1 \left[\left(\frac{p_2}{p_1} \right)^{\frac{k-1}{k}} - 1 \right]$$

where k varies from the ratio of specific heats (= 1·403 for air) for an adiabatic expansion to unity for isothermal conditions. The ideal

maximum energy would be that obtained by adiabatic expansion, and it is this value which has been used to calculate the energy required to create unit surface area in Table 1. Although the expansion of the compressed air through the jet must be almost adiabatic, the measured mill temperatures show no great divergence from ambient. The temperature given by the mill thermocouple must be corrected for the effect of the approach velocity of the gas, since this velocity is high, by the method of Hottel & Kalitinsky (1945). Velocities in the mill of 6–12,000 cm/sec were obtained, and the temperature corrections involved are from 2–10°. Because of the complex and repetitive nature of the calculations, a program was written for the calculation of results, which was run on the London University Atlas computer.

TABLE 1. DEGREE OF COMMINUTION OF SODIUM CHLORIDE AT FEED RATES BETWEEN 150–700 G/HR AS A FUNCTION OF THE APPLIED AIR PRESSURE OVER THE RANGE 40–80 PSIG

Feed rate g/hr	Product particle size, μ	Area created m^2/hr	Air supply pressures psig	Mill pressure cm Hg	Mill temp. °C	Air velocity m/sec	Energy supplied 10^5 joules/hr	Energy to create unit area 10^3 joules/m^2
170	5·0	93	40	82·9	18·2	83·5	4·80	5·17
155	1·7	248	60	93·2	17·6	94·7	10·50	4·22
159	1·7	266	70	97·5	16·6	84·5	11·97	4·50
180	1·5	331	80	101·9	20·5	108·6	14·93	4·51
248	7·6	88	40	83·1	21·3	84·0	4·82	5·47
226	2·0	308	60	90·1	14·1	93·7	9·81	3·18
267	2·8	262	70	94·9	15·0	120·9	12·30	4·70
247	1·5	443	80	102·9	15·6	102·0	14·51	3·28
346	12·0	77	40	82·2	20·9	79·3	4·91	6·37
328	5·2	281	60	88·1	15·0	92·1	9·50	3·54
380	2·3	454	70	94·1	23·8	91·9	12·78	2·81
337	2·1	442	80	101·6	15·2	102·3	14·76	3·32
443	9·0	133	40	81·8	25·6	80·1	5·01	3·78
420	3·6	320	60	87·3	18·0	66·1	10·14	3·17
432	2·8	432	70	91·1	29·1	82·3	13·27	3·07
450	2·9	427	80	97·5	14·4	105·1	15·33	3·60
527	24·2	56	40	82·0	32·0	63·5	5·11	9·12
545	10·0	144	60	86·0	28·8	79·8	10·71	7·44
537	12·0	120	70	87·9	31·7	90·7	14·14	11·82
561	6·4	283	80	93·6	26·9	90·2	16·70	7·01
701	11·5	163	60	87·1	26·6	73·9	10·48	6·43
698	4·8	397	70	89·0	23·6	103·3	13·59	3·42
711	9·1	211	80	93·3	23·0	102·6	16·55	7·86
381	24·6	40	40	82·2	26·8	82·6	4·99	12·58
388	4·8	218	60	86·3	24·7	85·0	10·54	4·83
381	3·9	264	70	92·1	22·5	114·4	13·03	4·94
378	3·8	277	80	94·3	23·1	126·0	16·37	5·92

Results and discussion

The degree of comminution achieved at feed rates between 150 and 700 g/hr is given in Table 1 as a function of the applied air pressure over the range 40–80 psig. If a graph is plotted of new surface area created against energy supplied, at constant feed rate, a straight line is obtained. One typical line is shown in Fig. 4 for a feed rate of about 170 g/hr. Similar lines may be obtained for other feed rates. The slope of any such line is a measure of the efficiency of utilization of the energy in the air supplied to the mill, since it represents the new surface area created per unit of energy input. Thus firstly it appears that Rittinger's law applies, since the energy required is proportional to the new surface area created. Secondly, the slope of the line varies with feed rate, at first

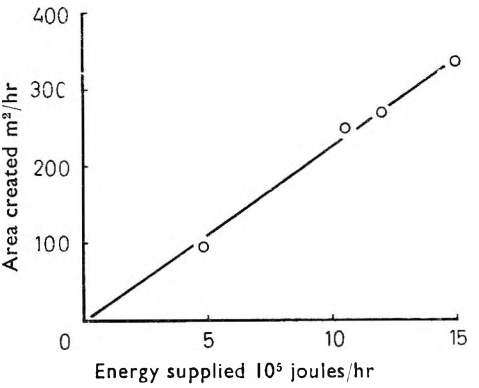


FIG. 4. New surface area created as a function of energy supplied. The feed rate for this plot is approximately 170 g/hr.

increasing and then decreasing sharply, so that there is an optimum feed rate at which the mill is operating at maximum efficiency. This is shown in Fig. 5, where the energy required to create unit area of new surface is plotted as a function of feed rate. The optimum, where this energy is a minimum, is the result of two opposing effects. At low feed rates the particle concentration is low in the grinding chamber, and there are relatively few inter-particle collisions. At high feed rates, the particle concentration is so high that particles have no time to accelerate up to a high enough velocity in between collisions to enable fracture to occur. Somewhere between these two extreme conditions there must be an optimum.

The scatter on the optimum plot is large. Some of this is due to there being no control over the degree of comminution achieved. Once the feed rate and air supply are set, the product size is not controllable. There must be a difference between creating the type of surface area needed to

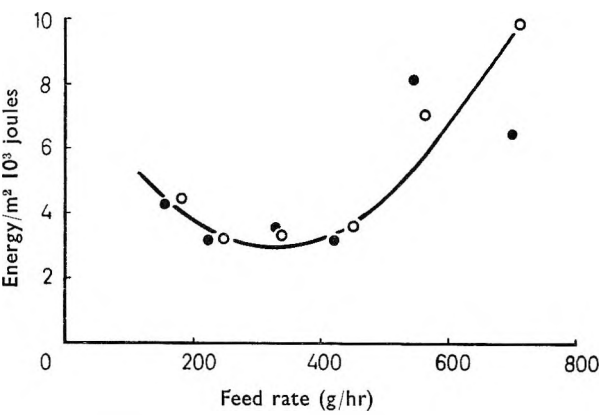


FIG. 5. Energy required to create unit surface area as a function of feed rate, showing the optimum operating conditions. ● 60 psig. ○ 80 psig.

reduce the particle size from the feed size, 330μ, down to say 15μ, and creating the area needed to go from 15 to 3μ.

The energy required for reduction of particle size in this small mill compares well with the crushing energy for sodium chloride quoted by Schellinger (1952) determined by calorimetric measurements using a ball mill, and as observed by Piret (1953) the required energy is far in excess of that predicted from surface energy considerations.

Comparing the performance of the mill, operating at or near its optimum, with Dotson's figures shows that the small size of the mill does not reduce its performance. For example, taking the fourth line of Table 1, 331 m^2/hr of surface are created by feeding air at 80 psig at a rate of almost 130 litres/min, measured at atmospheric pressure, to the mill. This represents an energy input rate of 0·42 h.p. Since 331 m^2 is approximately 3000 ft^2, the mill is creating surface at the rate of about 7,000 ft^2/h.p. hr, which may be compared with Dotson's figures of 2,000 ft^2/h.p. hr for a 2 inch elliptical mill, ranging up to 12,000 ft^2/h.p. hr for a ball mill. Part of the difference is probably due to Dotson's working with silicon, whereas the present authors have used the rather more easily-fractured sodium chloride, but some of the difference is likely to reflect a genuine improvement in efficiency. The improvement may be due to the double jet principle of the Gem mill, which causes direct impaction of the ingoing feed with oversize returning from the mill chamber to the opposing jet.

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SHORT COMMUNICATION

A modified pressure wheel for the instrumentation of rotary tabletting machines

J. J. DEER, K. RIDGWAY, P. H. ROSSER AND E. SHOTTON

IN any research into tabletting processes, it is necessary to know the force applied at the punch so that the tabletting pressure may be calculated. Much work has been done using single punch machines having strain gauges bonded to the shank of the punch (Higuchi, Nelson & Busse, 1954; Shotton & Ganderton, 1960). High-speed rotary machines require additional sophistication to enable useful results to be obtained, and there are two methods known. The first is that used by Shotton, Deer & Ganderton (1963). The resistance of strain gauges near the punch tips was used to control the frequency of an oscillator, and the signal was transmitted from the rotary part of the machine to a stationary aerial by radio emission. Thus the difficulty of providing a slip ring connection to the strain gauges was circumvented. However, because the radio transmitter took up space normally occupied by punches, it was difficult to obtain readings from more than one pair of punches, and impossible to obtain readings from all. In the second method (Knoechel, Sperry & others, 1967), strain gauges were placed on the compression screws interposed between the arm holding the movable axis of the pressure wheel and the spring used to adjust the compression force applied. Deflection, measured by the strain gauges, was proportional to the force applied to the punch. Since the gauges were stationary, their output could easily be displayed on a cathode ray oscilloscope, and the pressure to each punch recorded as its head passed under the pressure wheel.

The radio-link method allows the strain gauge to be on the punch itself, so that the recorded stress is in the actual machine component by which the compression force is applied: this makes for accuracy. The compression screw method gives a pressure record for every station, but the strain gauge is attached at a point remote from the place at which the force is actually being exerted. Between the punch head and the gauge are the pressure wheel axle, two linkages and a bar in torsion, all of which cause a reduction of, and introduce a possible non-linearity into, the strain to be measured. There is also a direct reduction due to the difference in length between the effective lever length of the arm carrying the pressure wheel axle and that communicating with the compression screw.

Accordingly, a modified pressure wheel* has been designed, which enables the applied force at all stations of a rotary machine to be measured; it also enables the measuring position to be kept in line with, and only 3 inches from, the head of the punch. The principle is to make the normally solid pressure wheel into one with two spokes, so that the periphery

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* Subject of British Patent Application No. 13309/68.

of the wheel can move relative to the hub, when the compression force is applied to the punch. The amount of movement produced by a given force is governed by the length and thickness of the spokes. A few thousandths of an inch per ton of applied force is a typical figure. The movement obtained may be measured in a number of ways, but two are described. The wheel (Fig. 1). consists of a central hub with a hole in

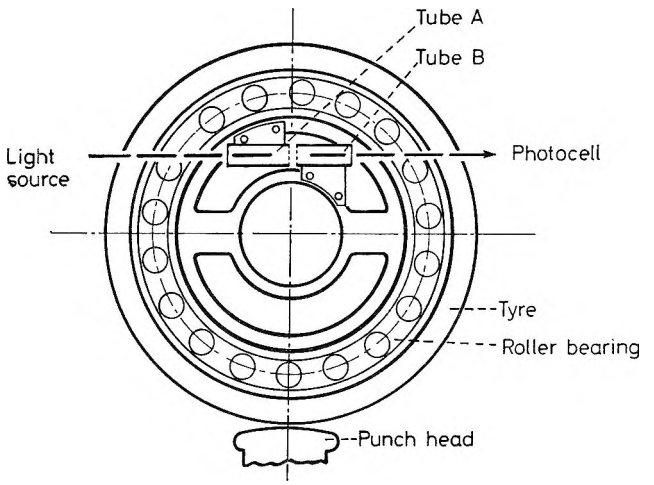

FIG. 1. A diagrammatic view of the pressure wheel, showing the tubes A and B which carry the moiré fringe plates.

its centre to fit the axle which normally runs in the machine bearings. The axle and the hub do not rotate: both are fixed relatively to the tabletting machine by means of grub screws. The hub carries two spokes, which carry a rim. Hub, spokes and rim are machined from a single piece of steel. The rim is a push fit into a $7\frac{1}{2}$ inch outside diameter Skefco roller bearing of conventional design. A hardened and ground outer steel tyre is shrunk onto the outside of the roller bearing. The tyre is impacted by the punch heads as they come round, and acts to spread the force of the impact onto several of the rollers and prevent the overloading which could result from a localized blow.

The horizontal tubes A and B (Fig. 1) are fitted at their ends with small matching moiré fringe plates made of glass. The black lines ruled thereon are equal in width to the spaces between them, and 100 lines to the inch proved a useful ruling interval. The moiré plates are adjusted so that the lines of the plate in tube A are opposite the spaces in the plate of tube B, both sets of lines running horizontally. The field of view through the tubes is then dark. Since A is fixed to the wheel rim and B to the hub, any applied force moves the moiré plates relative to one another, and light from a small projector bulb can pass down the tubes and fall onto the sensitive area of the phototransistor mounted in the end of tube B. The output of the transistor, a Mullard OCP 71, is displayed on an oscilloscope.

Two of these pressure wheels have been made, and one of them operated as the upper wheel of a Manesty D3 rotary tabletting machine. A commercial aspirin granulation ("Asagran", made by Monsanto Ltd.) was tabletted at the normal operating speed of about 500 tablets/min. An output of about 200 mV was available using the 100-line moiré fringe plates, the pressure wheel having a load sensitivity of 0·0025 inch per ton of applied load, as determined by a static test in a hydraulic press. There were no problems of inertia: the instrumentation system was capable of following the progress of a single compression by increasing the scanning rate of the oscilloscope. Alternatively, all 16 compressions occurring in one revolution of the rotary table could be inspected for peak height by displaying them on the screen simultaneously.

The second method of measuring the displacement of the wheel rim relative to the hub is to use strain gauges on the wheel spokes. By using four gauges, two on each spoke, it is possible to make the change of resistance a function only of vertical loading. The strain gauge is followed in the normal way by a bridge/amplifier.

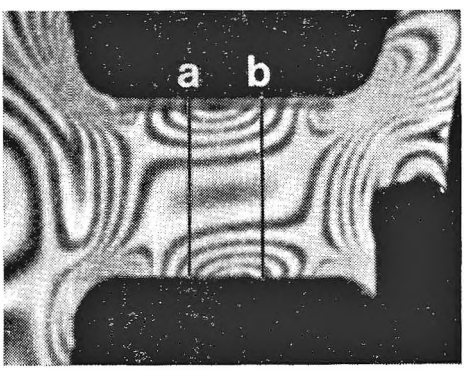

FIG. 2. The photoelastic fringe pattern produced by stressing a Perspex model of the hub, spokes and rim of the wheel. The fringe pattern characteristic of pure bending, parallel bands, appears between lines a and b, showing that a strain gauge in this position should give a response which is linear with applied force.

Strain gauges have not as yet been fitted to the wheel, but a Perspex model has been made. The model was stressed and viewed by polarized light; a typical fringe pattern is shown in Fig. 2. Between the lines marked on this photograph, the fringes are horizontal and parallel, indicating that in this small region the spokes are free from end effects and are subject to a pure bending moment. The stress in the spokes in this region is thus a linear function of the applied load, and if a strain gauge is fixed to a spoke in this region, it too will have a linear response.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 185S–193S Received May 1, 1968

The mixing of powders flowing down an inclined plane

K. RIDGWAY, R. RUPP AND A. SANCHEZ*

An apparatus has been constructed in which a pair of hoppers feed powders at controlled rates to the top end of a chute in two moving layers, one on top of the other. The layers move down the chute and at the lower end are separated again by an adjustable knife edge. Subsequent analysis of the fractions obtained enables the amount of mixing which has occurred to be determined. Since the mixing takes place under controlled conditions through a known area of contact between the powders, the apparatus measures "powder miscibility". Two photoelectric cells enabled the powder velocity to be measured. Powder velocities and bed structure are reported for the flow of single layers of sand of various thicknesses. Mixing results are presented, in terms of an effective diffusion coefficient, for the flow of 30/36 mesh sand.

THE rate of flow of particulate material through orifices of various shapes and sizes at the base of hoppers has been studied by a number of workers e.g. Brown & Richards (1965) and Pilpel (1966), who have demonstrated that the rate of outflow is independent of the head of powder above the orifice. Such conclusions apply to powder in free fall after passing through the discharge orifice. Although studies have been reported on the flow of powder along inclined chutes, (Harris, 1963) the chutes used had a porous base, being fed with compressed air so that the powder was fluidized and lifted clear of the base. Material was thus enabled to flow along the chute at quite small angles of tilt. Little work has been reported on the gravity-induced flow of powders down inclined planes, although it is a common phenomenon in equipment handling industrial solids. The flow of particulate material down an inclined plane also takes place in rotary drum mixers, in rotary coating pans and in ball mills where the sloping face of the charge in the rotating drum is made up of particles moving down in a combination of rolling and sliding.

Work described below with an inclined chute was aimed at simulating such a sloping face of tumbling particles. Parameters investigated include the angle of the face, the rate of feeding the powder which controls the depth of the layer, the effect of feeding one layer on top of another which gives a measure of the amount of mixing, and finally the roughness of the chute which gives some degree of control of the amount of shear imposed on the particles.

Experimental

The apparatus, shown diagrammatically in Fig. 1, consists of a pair of hoppers which feed two streams of powder, one on top of the other, to the upper end of an inclined polished brass chute, 6·3 cm wide, 50 cm long, with sides 1·5 cm high. The two layers of powder slide or roll down the chute, and at its lower end are separated again by a horizontal knife edge,

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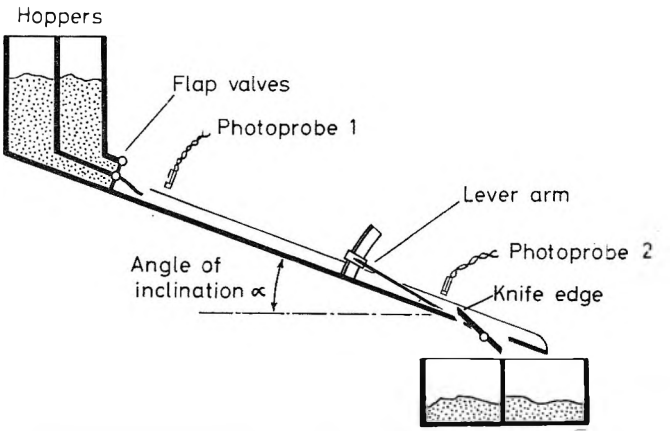

FIG. 1. Diagram of the apparatus. The hoppers feed sand through the (preset) openings of the flap valves onto the chute, set at the required angle α. The knife, adjusted by the lever arm, splits the bed at the lower end of the chute. The photocells measure the time which the material takes to pass from one to the other.

each layer going into a separate container. Any mixing which has taken place between the two layers is assessed by analysing the contents of the two containers.

The powder outflow from each hopper is controlled by a flap valve, operated by a long lever so that it can be opened rapidly. An adjustable stop enables the degree of opening to be set in advance so that the outflow rate is predetermined. The normal requirement is that the flowrates from the two hoppers shall be identical. The hoppers and the chute are integral with one another, so that the chute angle may be adjusted without effect on the hopper delivery. A contoured plate leads the powder smoothly from the upper hopper into the upper layer. Without this plate, the upper powder stream seriously affects the lower.

After falling down the chute, the layers are separated by an adjustable knife edge, the lower layer going beneath the knife into one Perspex box, whilst the upper layer goes over the knife into another. Preliminary trials are required at any given chute angle to ensure that equal amounts of powder are delivered in equal times from the hoppers, and that the knife edge splits the travelling bed in half so that equal amounts are collected, during a run, in each sample box.

The apparatus exposes a known interfacial area of one powder to another, under controlled and reproducible conditions of flow and shear. The amount of diffusional mixing taking place across this plane area is determined by analysis of the collected samples: it is a procedure analogous to the classical method of measuring the diffusion coefficient of a solute in a liquid. The mathematical relation between diffusional transport thus determined and the diffusion coefficient of the solute (Stefan-Kawalki data) has been conveniently tabulated by Jost (1960).

For the measurement of powder velocity, two small probes were constructed, each containing a Mullard ORP 11 photoconductive cell. Using a double-beam storage oscilloscope in the externally-triggered,

single-sweep storage mode, it was possible to record a voltage change from each probe as the powder bed passed it, together with a measurement of the elapsed time interval. A typical trace is shown in Fig. 2. The velocity was also checked by taking photographs of the moving sand bed at exposures of 1/100 and 1/500 sec. The lengths of the streaks formed by individual particles could be measured with sufficient precision to confirm not only that the photoelectric method was yielding correct results but also that particle velocities much greater or much less than the mean bed velocity did not occur.

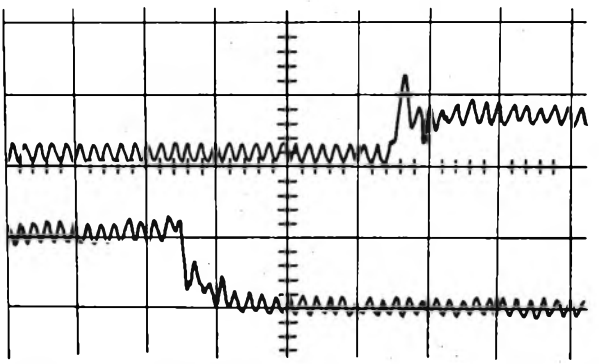

FIG. 2. A typical pair of traces obtained from the photoprobes. In the horizontal direction 1 division = 50 m sec, and in the vertical direction 1 division = 5 V.

To determine the bed thickness, two methods were used. For the first, the small knife-edged plate at the lower end of the chute was used. This plate pivots on an axle and may be tilted by moving an arm at the side of the chute. When in the retracted position, the plate fits neatly into the chute and particles pass undisturbed over it. As it is raised, it splits the descending bed, some particles passing below it and some above. Thus it was possible to determine the weight of particles in the bed travelling at any height above the chute surface.

For the second method, a knife edge was lowered onto the flowing bed by means of a micrometer screw and the position was noted at which the edge caused particles to accumulate on its upstream side. This was determined with a reproducibility of 0·05 mm.

The material used to form the flowing beds was Leighton Buzzard sand (George Garside Ltd.) which has uniform properties and rounded grains. The fraction used was that between 30 and 36 mesh, a size range of 422 to 500 μ. Where colour was important, as in the mixing experiments, it was dyed green with Naphthol Green B. This had no effect on the flow properties, but analysis by a light reflection method was rendered easy and rapid.

Mixing determinations were made as follows. Each hopper was loaded with 300 g of sand. The flap valves were opened simultaneously, and the sand flowed as two layers down the chute, the upper layer being directed smoothly onto the lower layer by the fairing installed for this

purpose. The particle velocity was measured by the two photoprobes and the layers were split by the knife edge and directed into twin Perspex collecting boxes. The two collected fractions were separately homogenized by shaking and stirring before being analysed by means of a photosensitive transformer ratio-arm bridge, (Deer, Ridgway & Rupp, 1968). The bridge measured the amount of light reflected by the mixed sand by comparing it with unmixed sand, either white or green. Analysis was rapid, taking about 30 sec per sample, and accurate to better than 0·5%.

The chief difficulties encountered were in the setting-up. Preliminary runs with mono-coloured sand samples were necessary to ensure firstly that the two hoppers emptied in exactly the same time, so that both layers ceased flowing together, and secondly to ensure that the adjustable knife edge was correctly positioned to direct an equal quantity of sand into each collecting box. These adjustments became easier as operating experience was accumulated. The adjustment of the hopper flap valves was the more important, since if one layer flowed for a shorter time than the other, the last of the sand, whether from the upper or the lower hopper, would flow as a layer of half the normal thickness and would all pass beneath the knife edge, falsifying the results. Failure of the knife edge to split the bed into two 300 g portions was less serious, because the error thus introduced is second-order.

Results and discussion

FLOW OF A SINGLE LAYER

As a preliminary experiment, the time taken for the front of the sand bed to travel a distance of 35 cm was determined as a function of chute angle, measured from the horizontal, and as a function of flap valve opening, using the lower hopper opening only. The upper photoprobe was positioned as close as possible to the flap valve, and the lower was 35 cm from it. The results are shown in Fig. 3. As would be expected, increasing the slope of the chute decreases the time of travel. There is also a tendency for the sand velocity to increase with increasing valve opening, i.e. increasing layer thickness.

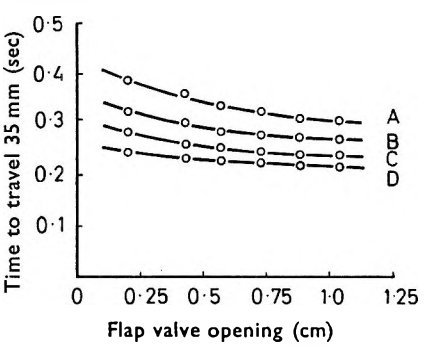

FIG. 3. Time to travel a distance of 35 cm down the chute, as a function of flap valve opening. The letters on the curves refer to the chute angles. A = 60°, B = 50°, C = 40° and D = 32·5°.

TABLE 1. BED VELOCITIES AS MEASURED BY THE PHOTOPROBES

Angle of chute degrees	Time (sec) to traverse a distance of			
	15 cm	25 cm	35 cm	45 cm
24·5	0·181	0·285	0·383	0·475
32·5	0·156	0·244	0·338	0·390
37·5	0·143	0·222	0·288	0·360
40	0·142	0·211	0·281	0·340
45	0·133	0·205	0·261	0·320
50	0·126	0·194	0·251	0·306
61	0·117	0·176	0·229	0·271

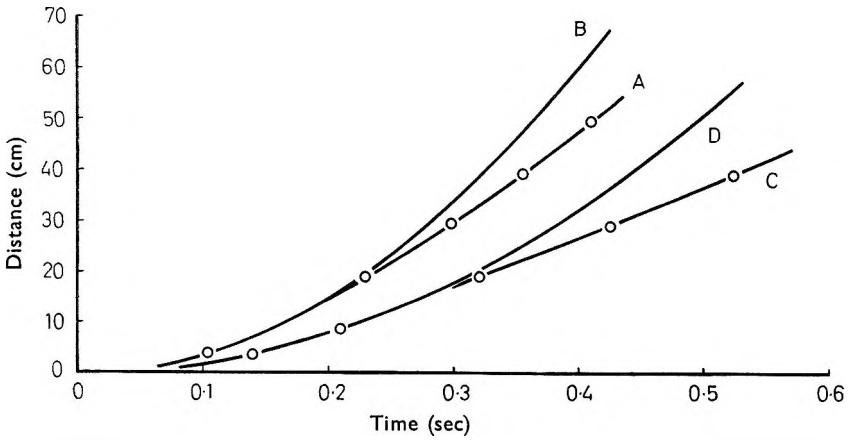

FIG. 4. Distance versus time plots, from the data of Table 1 (Zero shifted by 4 cm). The ideal curves are of the form distance $= \frac{1}{2} g \sin\alpha$ (time)^2. Curve A is for a chute angle of 50°, and curve B is the corresponding ideal curve. Curve C is for a chute angle of 24·5°, and curve D is the corresponding ideal curve.

The main body of the experimental data for flow of a single layer are given in Table 1, where the time intervals are listed for travel over distances of 15, 25, 35 and 45 cm between photoprobes, for various slopes of the chute. In each case the upper probe was 2·8 cm from the flap valve. Fig. 4 shows two distance-time plots derived from the data of Table 1. Also shown are the "ideal" curves which would be obtained for frictionless powder sliding down an inclined plane at the particular angle. These are easily calculated, since the acceleration is $g \sin\alpha$ where g is the acceleration due to gravity and α is the angle of inclination. In Table 1, zero time is the instant at which the powder passes the first photoprobe (2·8 cm from the flap valve). Initial agreement with the first part of the ideal curves in all cases is best when the time zero is taken to be 4 cm before the first photoprobe, i.e. 1·2 cm inside the hopper. This is effectively the point at which the powder starts from rest. The experimental curves, with this zero correction, follow the ideal curve but then fall below it as frictional forces come into play. It would seem reasonable that a constant, terminal velocity would be attained eventually in all cases if the chute were long enough. At an inclination of 24·5° constant velocity is practically

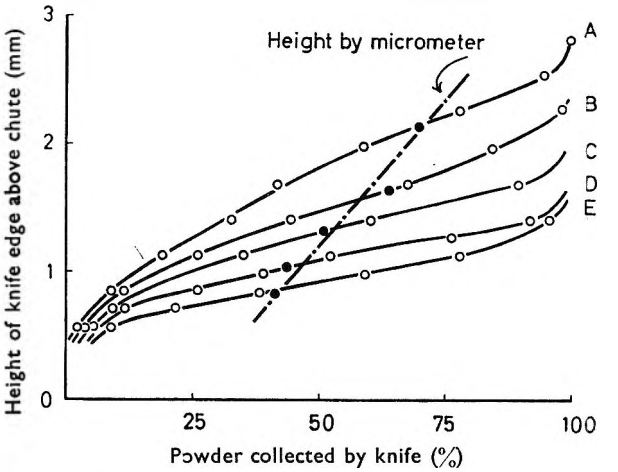

FIG. 5. Percentage of the flowing layer caught by the knife as a function of height of the knife edge above the chute surface, at a chute angle of 32·5°. The letters on the curves refer to the flap valve opening: A = 3·4 mm, B = 5·0 mm, C = 6·5 mm, D = 8·0 mm, E = 9·6 mm.

achieved, but at greater angles than this the sand is still accelerating even at 47·8 cm from the flap valve.

Fig. 5 gives the results of measurements of the percentage of the flowing layer diverted by raising the adjustable knife edge to various heights above the surface of the chute. The flowrate increases by a factor of more than ten between the lower and the upper curve, whereas the ordinate, the layer thickness, is only doubled.

If the velocity of all particles in the bed is approximately the same, and equal to the velocity measured by the photoprobes, then the density variation through the depth of the flowing layer may be calculated. This is done by finding the increase in weight of collected powder for each increase of height of the knife edge. Since the chute width is known, the area of cross-section through which a known weight of sand passes, at a known velocity, is also known. The density may be calculated from the simple relation—

$$\text{mass flow-rate} = \text{density} \times \text{linear velocity} \times \text{area perpendicular to flow direction}$$

Density profiles through layers of various thicknesses are shown in Fig. 6. In all cases there is a layer of low density, or high voidage, near the chute surface. This is to be expected, as any drag force applied to the bed will increase its voidage.

In the thinner beds, where the bed depth approaches one particle diameter, the drag produces rolling of the particles, and probably some saltation occurs too, so that some particles leap over the knife edge. In the thicker beds this type of motion will be prevented by the upper layers of the bed: shear will occur and voidage will increase.

The density increases throughout most of the depth of any bed, and the

maximum density achieved increases with overall bed thickness. The maxima of the density curves themselves lie along a curve which is tending asymptotically to a value of almost 1·3 g/ml as thickness increases. This may be compared with the bulk density of the stationary sand, which is 1·6 g/ml.

It should be stressed that the density profiles as drawn are based upon the assumption that the linear velocity is constant throughout the depth of the bed. This is an approximation; to determine the velocity within the bed would require more sophisticated techniques than have been used in the present work. If the opposite assumption, constant density through the bed, were adopted, the curves of Fig. 6 would be velocity profiles. This assumption, however is less likely to be correct.

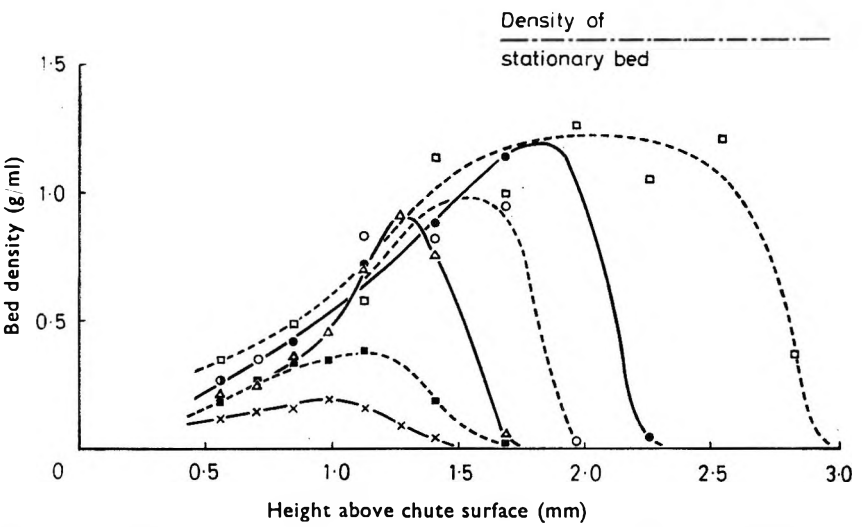

FIG. 6. Profiles of density at any particular level in the bed as a function of distance from the surface of the chute. Flap valve openings ×, 2·0 mm. ■, 3·4 mm. △, 5·0 mm. ○, 6·5 mm. ●, 8·0 mm. □, 9·6 mm.

THE MIXING OF TWO LAYERS OF FLOWING PARTICLES

Runs were made in groups, each group being characterized by flap valve opening. Having set the valves, the chute angle could be changed without further adjustment being needed. Two runs were made at each chute angle, one with green sand as the upper layer and one with white, in case any difference in flow properties had been caused by the dyeing process. The efflux time of the sand from both hoppers was noted. The results are presented in Table 2. There was no regular trend according to which colour of sand formed the upper layer of the flowing bed, and the percentages given in the Table are each the mean of at least two measurements, one with each colour on top. From the observed compositions of the two layers separated by the knife edge, the value of the quantity $h/2\sqrt{Dt}$ may be found from the Stefan-Kawalki tables (Jost, 1960).

TABLE 2. MIXING RESULTS FOR SUPERPOSED FLOWING LAYERS OF GREEN AND WHITE SAND

Chute angle degrees	Time of contact (sec)	Depth of lower layer (cm)	Green sand %		Diffusion coefficient cm²/sec
			Upper layer	Lower layer	
32·5	0·342	0·217	87	13	0·303
32·5	0·342	0·157	78·9	21·1	0·391
32·5	0·342	0·110	80·7	19·3	0·165
32·5	0·342	0·079	73·4	26·6	0·0163
32·5	0·345	0·059	60·8	39·2	0·0219
32·5	0·348	0·048	49·4	50·6	0·0467
40	0·295	0·217	87·6	12·4	0·338
40	0·295	0·157	75·1	24·9	0·652
40	0·295	0·110	80·6	19·4	0·199
40	0·295	0·079	71·3	28·7	0·0219
40	0·297	0·059	54·4	45·6	0·0425
40	0·299	0·048	49·2	50·8	0·0488
50	0·256	0·217	88·8	11·2	0·309
50	0·256	0·157	77·8	22·2	0·588
50	0·256	0·110	82·2	17·8	0·246
50	0·256	0·079	67·6	32·4	0·0327
50	0·257	0·59	56·7	43·3	0·040
50	0·258	0·48	49·3	50·7	0·0591

D is the diffusion coefficient, t the time and h the thickness of each layer. The time of contact is obtained from the photoprobe measurements, and the setting height of the knife edge necessary to collect half the bed is known. This gives h for the lower layer, and it is assumed to have a similar value for the upper layer. Thus D may be calculated.

Variation of diffusion coefficient with increasing layer thickness, i.e. with increasing rates of powder flow, is shown in Fig. 7. The curves all start from a low value, increase to a maximum and then decline. The low values occur at small bed heights, where visual observation shows that the voidage is high and the particles in the upper layer have ample opportunity to fall through into the lower layer. The Stefan-Kawalki tables provide the minimum uniform value of D necessary to achieve the observed amount of mixing, and at small bed thicknesses this is a small value.

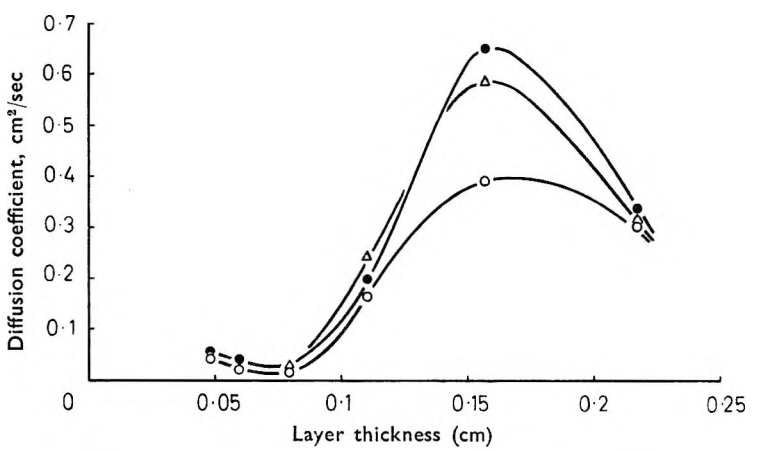

FIG. 7. Diffusion coefficient as a function of half-depth of the flowing layer, and hence of the rate of flow. ○, 32·5°. ●, 40°. △, 50°.

The possibility of a higher value at one part of the flow path and a lower value elsewhere is not excluded. As the layer thickness increases, the amount of diffusive mixing increases (although the percentage composition change actually achieved decreases). This is presumably due to the increasing amount of shear between the upper and lower layers.

At still greater layer thicknesses the increase in density causes the bed to become more resistant to shear as the amount of void space becomes smaller (Fig. 6). Under these conditions the bed is sliding as a whole and tending to act as a rigid body. Hence the value of D decreases.

Further development of the apparatus is possible. For example, it would be advantageous to increase the chute length to allow different contact times to be studied at the same angle. At present, contact time can only be changed by changing the angle of the chute. However, it has been shown that the miscibility of granular powders can be examined by the method described, and that the technique has potential usefulness in development work. In this connection it could be used to assess the size segregation of powders in movement.

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The use of diffusion theory for the assessment of mixing in a rotary coating pan

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The effectiveness of a coating pan in treating all the contained tablets identically depends upon its being a good mixing device; the mechanics of particle motion within such a pan are therefore of interest. One measure of the mixing rate in rotary drum mixers is the coefficient of self diffusion, a parameter difficult to measure in a coating pan. Three methods of measuring such a coefficient are reported and compared: linear sampling, total compartmenting of the bed, and high speed cinéphotography. The third method is the best, and a technique is described for treating the pictorial data to obtain quantitative results.

ONE of the more readily usable parameters in the kinetics of mixing processes in rotary drum mixers is the effective diffusion coefficient. To measure this, the mixer is initially loaded with two, usually equal, batches of particles, the batches being distinguished from each other by any property which can be assumed to have no influence on the mixing rate. The progressive interchange of the particles of the two halves of the initial charge is followed, and by the application of known solutions for the differential equation expressing Fick's second law of diffusion,

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2},$$

under the appropriate boundary conditions, the diffusion coefficient D for the mixing process can be calculated. Here c is the concentration, t is the time, and x is the linear measure of distance from the initial position of the interface between the two halves of the initial batch.

Such an approach has been used by Kaye & Sparrow (1964), who examined the sideways mixing of streams of particles as they flowed down an inclined plane. This was an attempt to isolate the surface diffusion mechanism in a rotary drum mixer from the convective mixing and shear mixing which, according to Lacey (1954), all contribute simultaneously to the mixing process in a rotary drum.

The rate of mixing in the axial direction in a long rotating cylinder was studied by Hogg, Cahn, & others (1966). These authors showed that the concentration along the mixer axis closely followed the appropriate solution of Fick's equation. In a later paper, Cahn, Fuerstenau, & others (1966) considered the mechanism by which random axial motion is generated in the shear zone of a drum mixer. Such random motion is a necessary presupposition if the use of diffusion theory is to be appropriate. They considered this a sufficiently important question to justify the simulation of the diffusion process by a Monte Carlo method on a computer (Cahn & Fuerstenau, 1967).

To study the mechanics of particle movement within a coating pan, and relate them to the overall performance, a measure of the prevailing rate

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of particle mixing is needed. Although the diffusion coefficient is an obvious choice, it is subject to some restrictions and disadvantages. The solution to the case of diffusion across a boundary from one semi-infinite cylinder to another is readily available from standard texts, e.g., Jost (1960).

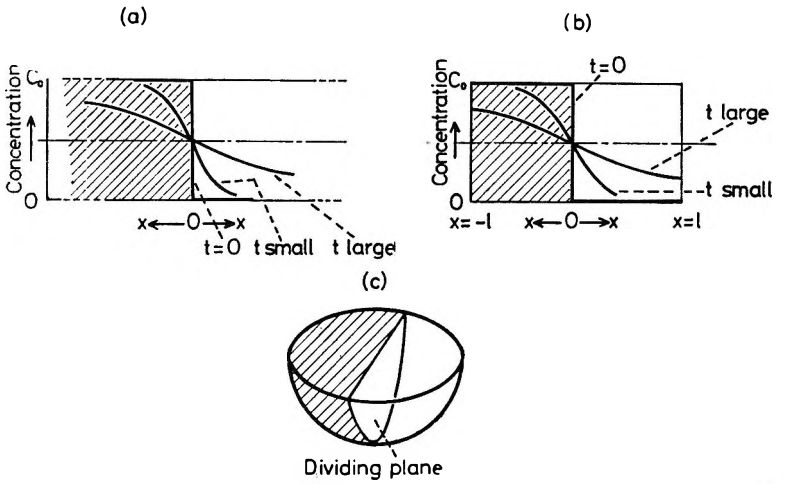

FIG. 1. (a) diffusive mixing in a semi-infinite system. Eqn: $c(x,t) = \frac{1}{2}c_o \mathrm{erfc} \frac{x}{2\sqrt{Dt}}$. (b) diffusive mixing in a system of finite length. Eqn: $c(x,t) = \frac{1}{2} c_o \sum_{\text{all n}} \left\{ \frac{\mathrm{erf}(2n-l) \pm x}{2\sqrt{Dt}} \right\}$. (c) the geometry of the mixing system formed by the two-coloured bed in a coating pan.

Fig. 1 (a) shows the initial conditions and the progress of diffusive mixing with time for this case. Where the diffusional transfer is between cylinders of finite length, the solution becomes more complex, but still exists and is tractable. Because the diffusion equation is linear, any linear combination of solutions is a solution, which allows "reflection" at the plane barrier at the end of a cylinder to be used as a mathematical device to satisfy the condition of zero diffusion rate at such a boundary. Fig. 1 (b) shows the curves for this situation. The solution is a rapidly-converging series, especially for small values of t. The summation represents the reflection and super-positioning of the concentration wave.

The coating pan, however, is represented by Fig. 1 (c), and the explicit solution will be so complex that the best approach is probably a successive approximation type of numerical analysis by computer for any specific case. The question then arises whether the simple equation of Fig. 1 (a) will be applicable to conditions within the split load of a coating pan during times which are short compared with the time taken for mixing to progress through the entire charge. Experimentally, this paper shows that it is applicable, and that a value of the effective diffusion coefficient can be obtained. As soon as reflection occurs from the non-plane boundary, the simple theory breaks down.

Experimental

THE COATING PAN AND ACCESSORIES

The apparatus initially used consisted of a standard enamelled coating pan, 25 cm in diameter, mounted on and driven by an Erweka unit type KU 1 (Erweka Apparatebau G.m.b.H; Frankfurt). The speed and axial tilt of the pan could be controlled. In later experiments a transparent pan became necessary, and one was constructed from a 10 litre single-necked flask (Q.V.F. Ltd., Stone, Staffs). A large circular hole was cut in the base of the flask, which was then fastened by its buttress-ended neck to a plate which could be mounted in the rotary drive unit. The inside of the neck was filled with Polyfilla so that the inside surface of the flask was uninterruptedly spherical.

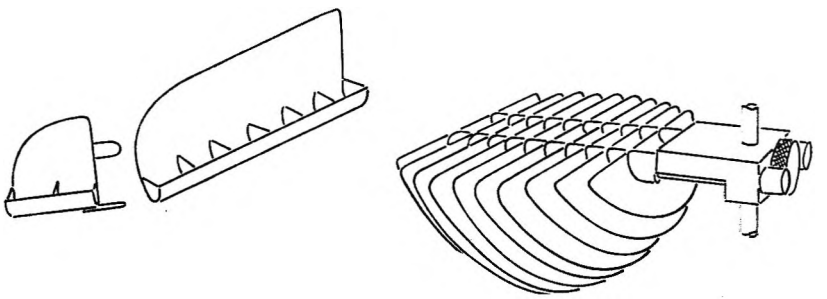

FIG. 2. The linear sampling device and the pan-dividing vanes.

Ancillary equipment included a linear compartmental sampling device and a set of pan-dividing vanes, both shown in Fig. 2. The sampler was made by bending a sheet of brass into a J-shape. Small semicircular pieces of brass were then soldered into the hook of the J to divide it into eight small compartments. A sample of the pan contents could then be taken along a straight line. The set of pan-dividing vanes was cut from thin stainless steel sheet; the completed set consisted of 12 plates located 14·5 mm apart, each one of a profile to fit the pan closely in the position in which it was fixed. The vanes enabled multicoloured beds of particles to be set up, and also enabled mixing rate determinations to be made.

The vanes were supported from outside the pan and observations could be made of particle transfer from one zone to another as the pan was rotated with the vanes held stationary. A second set was made to fit the glass pan.

Some of the ciné-photography was done with a Bolex H 16 reflex camera (Paillard S.A., St. Croix, Switzerland). This was an excellent camera for normal speed work, but it had a maximum speed restriction of 64 frames/sec. The higher speed work was done with a Fastax 16 mm high speed camera, Model WF 3 (Revere-Wollensak Div., Minnesota Mining and Manufacturing Co., Rochester, N.Y.) which could operate at speeds between 150 and 8000 frames/sec. Illumination was provided by two or more Phillips Photolita 500 W lamps, and for colour work, Ektachrome EF type 7242 film was used, which had a speed of 125 ASA. A zoom lens

was used to vary the field of view without changing any other variable, and this gave the incidental advantage of a great reduction in perspective effects. Thus the view of the moving surface of the particles was practically an orthographic projection.

MANUFACTURE OF UNIFORM PARTICLES

Lactose B.P. (2 kg), of mean particle size 94 μ, and a size range of 30 to 170 μ, was placed in the pan which was rotated at 30 rev./min with its axis at 30° to the horizontal. A 12% aqueous solution of polyvinylpyrrolidone (Plasdone) was sprayed in with a hand atomizer until the critical liquid content was attained, and granule growth began. Rotation was continued until spherical particles of diameter about 7·5 mm formed the bulk of the pan contents, when hot air was blown in to dry the surfaces of the particles. The pan contents were then sieved into three fractions: (*a*) <6·7 mm (*b*) 6·7–8·0 mm and (*c*) >8·0 mm. Fraction (*b*) was dried overnight at 35° and stored. Fraction (*a*) was grown to larger size by adding lactose powder and more Plasdone solution. Fraction (*c*) was ground and re-used. The spherical particles of the correct size were coated with 12% Plasdone in chloroform solution to give them resistance to abrasion; dyes were incorporated in the final coat to give spheres which differed from one another only in colour.

The bulk density of about 8 kg of the particles was determined. The value obtained was 0·722 g/ml.

MEASUREMENT OF DIFFUSION COEFFICIENT

Three methods have been used to measure the amount of mixing taking place in the pan in terms of an effective diffusion coefficient.

(*a*) Half the pan was charged with granules of one colour, and the other half with granules of another but of the same diameter. The division between the charges was vertical and generally at right angles to the axis of rotation of the pan, i.e., the division was between the front and the back of the pan looking in through the pan mouth. The pan was rotated and after a number of revolutions was stopped. Eight samples of about 20 granules were taken, with the linear sample device, along a central line from back to front of the pan. The number of each colour of granule in each compartment was determined and the rotation of the pan restarted.

(*b*) The pan was operated with the dividing vanes in position and held stationary, starting with a bed divided in two colours as before. Mixing on the rolling surface of the bed still took place under these conditions, but without impactive mixing at the toe of the bed because the granules did not converge there. Pan rotation was stopped periodically, the pan was rotated backwards about 45° so that the bed surface returned to the horizontal; the vanes then divided the bed completely into sections. Each section was withdrawn by suction into a conical flask connected to a vacuum pump, counted and returned to the pan into the section from which it had been withdrawn.

(*c*) Both the above methods interfered to some extent with the mixing process within the pan, since it was necessary to stop the pan movement to remove and to return the sample of the granules. In the third method

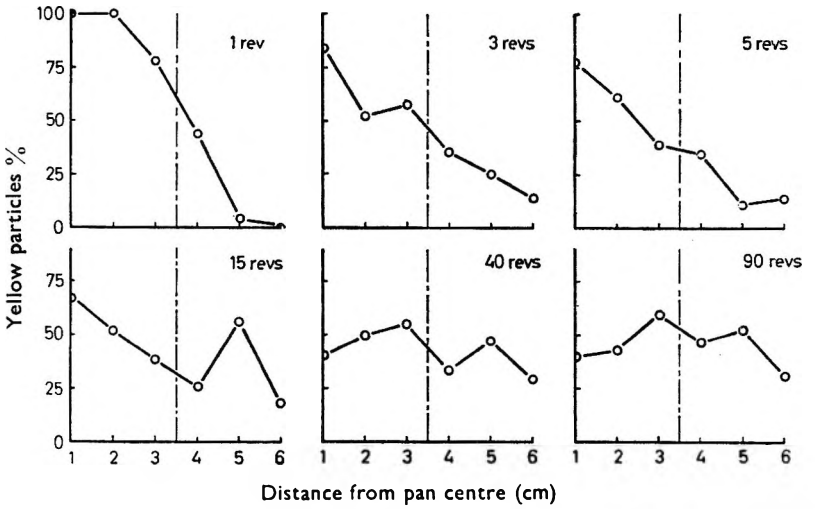

FIG. 3. Diffusive mixing results for method (a): periodically arresting the pan to take a linear sample.

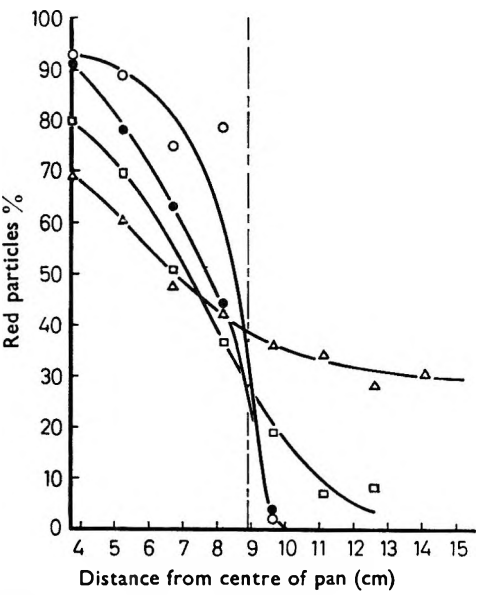

FIG. 4. Diffusive mixing results for method (b): running the pan with the dividing vanes in position, counting all the particles in each compartment periodically. ○ 5 revolutions, ● 10 revolutions, □ 40 revolutions, △ 100 revolutions.

a two coloured bed was prepared and mixed by rotation of the pan. The process was followed by ciné-photography. Subsequently, frames of the film were projected and a tracing made of the convoluted interface between the granules of the two colours. The percentage of each colour at any

distance from the mean interface position was then determined by measuring the lengths of the intercepts made by a line parallel to the mean interface position, and at the required distance from it.

All three methods give a measure of the composition, as a colour percentage, along a line from the back to the front of the pan, as a function of number of revolutions. The composition distribution starts as a sudden discontinuity and gradually progresses through a series of sigmoid curves to a straight line at the 50% level.

Results and discussion

Method (*a*). The results are shown in Fig. 3, in which the graphs are similar in form to those of Fig. 1 (b). The scatter of the points is quite large, $\pm$ 10% would be a conservative estimate, because of the sampling error. One compartment of the linear sampling device takes a sample of about 20 particles from the bulk. If the true proportion of one colour, p, in the bulk is 50%, the variance to be expected in a sample of n particles, where $n = 20$ is $np(1-p) = 5$ with a standard deviation $\sqrt{5} = 2{\cdot}23$. Since the proportion of one colour is estimated from a sample containing about 10 particles of that colour, this represents an error of about $\pm$20%, a severe limitation on the method. Previous authors have not encountered this problem because they worked with ballotini of such small particle diameter that samples of 500 particles were taken by suction through a hypodermic needle. Any attempt to increase the sample size in a coating pan means that quantities of particles are being withdrawn large enough to be a significant proportion of the total available.

Method (*b*) overcomes the sampling difficulty since the entire bed is divided and each section counted individually. The results for this type of experiment are shown in Fig. 4. The difficulty of this technique is that

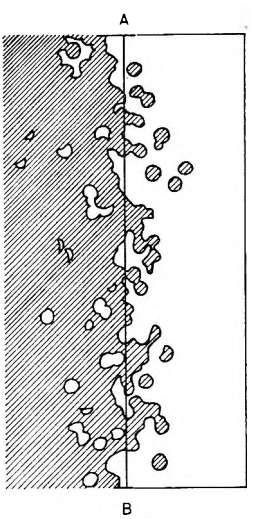

FIG. 5. Method (c): tracing of one frame of the ciné film. AB is a typical line on which the intercepts are measured and summed.

the pan must be operated with the vanes in position: any attempt to insert them to split the bed into slices causes more mixing. Thus the bed is mixing under rather different conditions. However, the effect of the vanes should be minimal because coating pans operate within the speed range classified as "low" by Carley-Macauly & Donald (1962). Mixing in this range occurs only in the cascading surface layer: particles in the bulk of the bed are held by their neighbours and are carried round in an arc of a circle by the pan rotation. With the vanes in position, the movement of most of the bed surface was not impeded. But it is still true that in attempting to remove the sampling errors, the mechanism of particle movement is modified.

Method (*c*) ensures that there is no interference with the mixing mechanism, since all observation is photographic. Sampling presents a difficulty, in that only the surface layer of the bed is visible. Were a group of 20 particles counted, they would cover an area so large that the concentration change across it would be large. The method used was to trace a few frames of the film at convenient times from the start of the experiment, by projecting the picture onto tracing paper (cf. Fig. 5). The mean interface position was established by planimeter measurement. Lines were drawn at 2 mm intervals parallel to the mean interface, and the intercepts on each line made by regions of each colour were determined. The concentration of each colour as a function of distance from the interface was then plotted for each of a number of times of mixing. Some of these curves are shown in Fig. 6, and their quality is better than that of the curves of Figs 3 and 4. The points on the curves from the first two methods are so scattered that any form of derived plot is of doubtful value. In Fig. 7 the curves of Fig. 6 are plotted on probability paper; as expected, they give straight lines, since they are of the general form e^{-x^2}. Because the equation relating concentration to distance is:

$$C = \frac{c_0}{2}\left[1 - \operatorname{erf}\left(\frac{x}{2\sqrt{Dt}}\right)\right]$$

where c_0 is the initial concentration difference between the two halves of the system, then

$$\frac{dc}{dx} = -\frac{c_0}{2} \cdot \frac{2}{\sqrt{\pi}} \cdot \exp\left[-\frac{x^2}{4Dt}\right] \cdot \frac{1}{2\sqrt{Dt}}$$

and for $x = 0$,

$$\frac{dc}{dx} = \frac{c_0}{2\sqrt{\pi Dt}}$$

This gives the slope of the curve of concentration against distance, at the initial interface position, as a function of time. This slope is unaffected by plotting on probability paper, and the slope of the entire line, if it is straight, on a probability plot will be $c_0/2\sqrt{\pi Dt}$. Thus, if the slope is measured at successive intervals of time, a plot of the slope against $1/\sqrt{t}$, or alternatively of $(\text{slope})^2$ against $1/t$, should be a straight line so long as D is constant. Constancy of D is a test of whether the simple diffusion law is applicable.

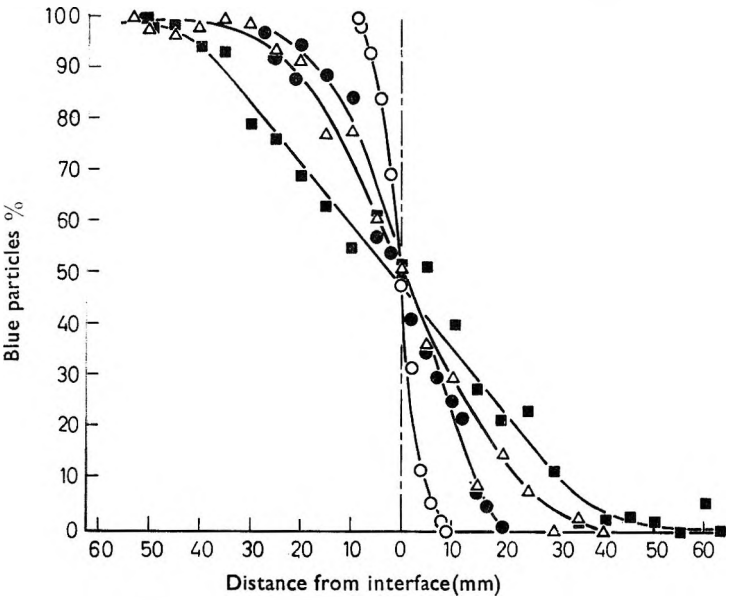

FIG. 6. Diffusive mixing results for method (c), calculated from a number of frames similar to Fig 5. ○ 2, ● 4, △ 8, ■ 12 sec.

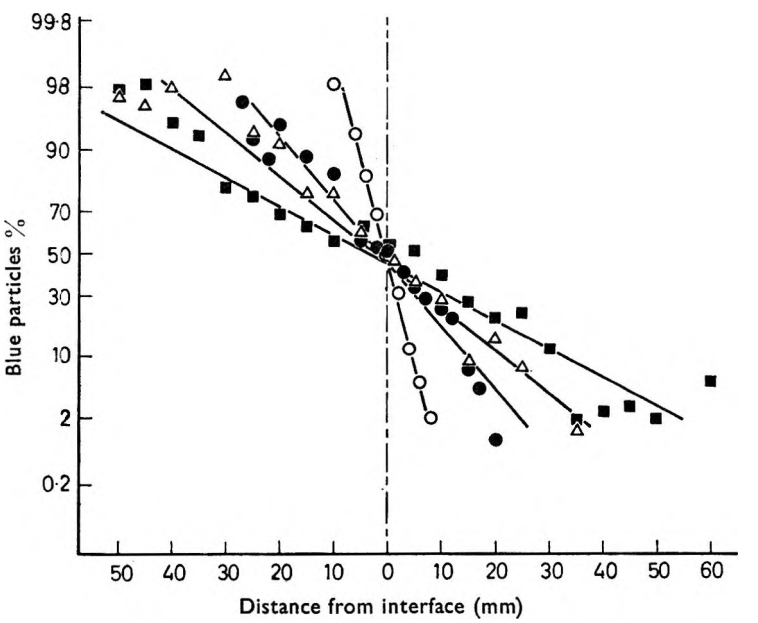

FIG. 7. The curves of the type shown in Fig. 6, plotted on probability paper to gi a series of straight lines of slope $1/2\sqrt{\pi Dt}$. ○ 2, ● 4, △ 8, ■ 12 sec.

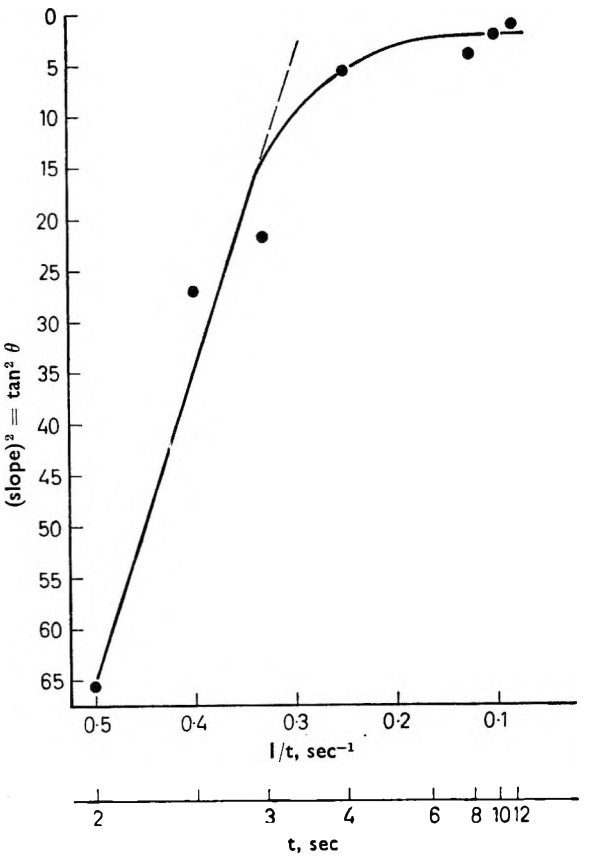

FIG. 8. A plot of the (slope)² from curves of the type shown in Fig. 7 against the reciprocal of the time. The linear portion of the graph gives the time over which the simple equation of Fig. 1(a) is applicable.

Such a plot is made in Fig. 8, and it is seen that for short times there is a straight line relation. The value of D for the straight part of the curve is 0·9 cm^2 sec^{-1}. The main criticism of the third method is that it is based only upon observation of the surface layer of the bed, and it is the surface value of D that is being measured. The entire series of measurements for the third method occupied only about half a revolution of the pan, and represents the time taken for the initial dividing plane between the two colours of the charge to be completely destroyed. It is thus the period over which the top end of the bed surface is being supplied with unmixed particles.

CONCLUSION

Three methods of determining the effective diffusion coefficient between the two differently-coloured halves of a batch of particles in a rotary coating pan have been tested. The values obtained are independent of

the boundary conditions which must be applied in solving the diffusion equation for any particular physical situation, provided that the elapsed time, measured from the start of the mixing process, is short.

Acknowledgements. The authors wish to thank the National University of Mexico for the award of a Research Studentship to E. S.

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Coagulation and flocculation in suspensions of griseofulvin and polystyrene latex

B. A. MATTHEWS AND C. T. RHODES

Coagulation and flocculation mechanisms have been examined in suspensions of the drug griseofulvin containing anionic surfactants. It has been shown that both mechanisms can give suspensions which are readily redispersible but that flocculation may produce pharmaceutically undesirable effects. The differences between the two phenomena are discussed.

THE need to distinguish between coagulation and flocculation, in colloidal dispersions has been stressed by La Mer (1964). He suggested that the term "coagulation" should be restricted to aggregation involving the reduction of the repulsive potential at the double layer and that "flocculation" should be reserved for aggregation mechanisms where chemical bridging between the particles is involved.

It has been suggested (Ecanow Grundman & Wilson, 1966) that the same distinction must be applied to aggregation phenomena in pharmaceutical suspensions. They studied the physical stability of suspensions of sulphamerazine, wetted with dioctyl sodium sulphosuccinate in the presence of aluminium chloride. This system was previously examined by Haines & Martin (1961), whose conclusions had been criticized by Wilson & Ecanow (1963), because of complications due to electrolyte/surfactant precipitation.

The present paper continues earlier work, Matthews and Rhodes, (1968a, b, c) on flocculation and coagulation in pharmaceutical and model suspensions: the conclusions reached are compared with those of earlier workers.

Experimental

MATERIALS

Griseofulvin fine particle (Glaxo Laboratories, Greenford, Middlesex). m.p. 221°. $\lambda_{max}^{EtOH} = 291$ mμ, E (1%, 1 cm) = 686. Density = 1·440 (determined by sp.gr. bottle). (B.P. 1963 cites m.p. 218–224°; λ_{max} 291 mμ; E (1%, 1 cm) 686.) The particle size distribution of this material has been described by Matthews & Rhodes (1967). It has a modal particle diameter, determined on the Coulter Counter, of 3–4 μ.

Surfactants. The surfactants selected were sodium dodecyl sulphate (SDS, $C_{12}H_{25}SO_4Na$) and sodium dioxyethylated dodecyl sulphate (SDDS, $C_{12}H_{25}\cdot[O\cdot CH_2\cdot CH_2]_2\cdot SO_4Na$), which has only two oxyethylene groups more than SDS. Their chemical similarity therefore allows a direct comparison to be made between their respective properties. Matthews & Rhodes (1968b) have previously shown that SDS is precipitated in the presence of aluminium salts and is analogous to dioctyl sodium sulphosuccinate.

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SDS (Marchon Products Ltd., Whitehaven) had the following manufacturer's analysis: SDS 98·00%, free lauryl alcohol 0·66%, water content 0·72%, sodium sulphate 0·13% and sodium chloride 0·40%. This same batch of material has been shown by Barry & Shotton (1967) to contain 98·6% C_{12}, 1·0% C_{10} and 0·4% C_{14}.

SDDS (pure) was synthesized from dioxyethylated dodecanol which was shown by gas-liquid chromatography to be 98·4% pure. The ether alcohol had a hydroxyl value of 6·08% (theoretical = 6·20%); $n_D^{60} = 1{\cdot}4332$, f.p. 19°. The sodium salt of the sulphate had a sodium content of 5·90% (theoretical 6·12%) and a critical micelle concentration (determined by penacyanole titration), of $2{\cdot}58 \times 10^{-3}$M.

SDDS *commercial* (Empicol ESB3, Marchon Products Ltd). Prepared from a narrow cut lauryl alcohol, was supplied as an aqueous solution which was assayed by titration with M/500 cetrimide using methylene blue as an indicator and the pure SDS as standard. It contained 27·2% w/w calculated as $C_{12}H_{25}{\cdot}[O{\cdot}C_2H_4]_2{\cdot}SO_4Na$ (manufacturers' claim 27·6% w/w).

Proton magnetic resonance spectra of the pure surfactants and of the commercial sample after freeze drying, were consistent with the structures assigned.

Polystyrene Latex. A "Dow" polystyrene latex (Serva Entwicklungslabor, Heidelberg, Germany) was used as a model system. It had a quoted mean diameter of 0·714 μm (electron microscopy gave a mean diameter 0·71 μm).

Water. Distilled water was freshly redistilled from an all-glass still.

Aluminium chloride (British Drug Houses Ltd., Poole): reagent grade, $AlCl_3{\cdot}6H_2O$. This was assayed before use by the B.P. 1963 method for alum.

Sodium chloride, *barium chloride* (British Drug Houses Ltd., Poole): Analar.

METHODS

Flocculation and coagulation in suspensions of griseofulvin and polystyrene latex have been examined by: surfactant/electrolyte compatibility using visual inspection for opalescence and precipitation; and speed of coagulation and flocculation by recording the change of the ratio of settled height to original height with time (Matthews & Rhodes, 1968b); redispersibility and coagulation rates in terms of stability ratios were determined as previously described (Matthews & Rhodes, 1968a,b).

The particle size of the griseofulvin was determined by the method of Matthews & Rhodes (1967), using a two-tube technique with 30 μm and 50 μm apertures.

Results and discussion

SURFACTANT/ELECTROLYTE COMPATABILITY

With 10^{-1}M aluminium chloride, SDS gave a definite immediate opalescence at 10^{-3}M and a flocculant precipitate at 10^{-2} and 10^{-1}M. Both samples of SDDS at the same three concentrations remained clear after one week as did controls containing surfactant and electrolyte alone.

SURFACTANT WETTING PROPERTIES

Suspensions of griseofulvin at a concentration of 2·5% w/v were prepared with the wetting agents alone to determine the optimum concentrations to use. Both samples of SDDS wetted the drug at a concentration of 10^{-3}M but SDS did not. A concentration of 10^{-2}M was effective and was used for both surfactants.

The suspensions were highly deflocculated and the particles sedimented slowly leaving an opalescent supernatant due to very small discrete particles. After storage for 1 week at 25° the sediment volume was 3·5% in both bases. More than 500 revolutions in the redispersing machine (Matthews & Rhodes, 1968b) were necessary to achieve redispersion.

SPEED OF COAGULATION AND FLOCCULATION

It has been claimed (Ecanow & others, 1966) that flocculation involving the precipitation of a surfactant by an electrolyte is a rapid reaction comparable to that of an inorganic reaction in which a precipitate results. These authors observed also that the settling rate of the suspension is *reduced* whereas when coagulation occurs, settling rates are increased and the process leads to caking of the suspension. Since sodium and potassium ions were used to induce coagulation and aluminium ions to induce flocculation, it is difficult to compare the two mechanisms absolutely.

To compare these two mechanisms in essentially similar systems, 2·5% suspensions of griseofulvin were prepared in 10^{-2}M SDS and 10^{-2}M SDDS: (since preliminary coagulation tests had shown identical results with the pure and commercial grades, the latter was used). The suspensions were made up to 90% of the final volume. Volumes of 10 ml of aluminium chloride solution at a tenfold concentration were added at zero time and the suspensions mixed by gentle inversion. Photographs of the suspensions were taken at intervals up to 1 hr and sedimentation volumes were obtained from the photographs, enabling accurate readings to be obtained at short intervals in the initial period. The results are shown in Fig. 1, together with similar readings taken on the suspensions without electrolyte. These were identical for both surfactants.

The results of the test with SDS confirm the statement by Ecanow & others (1966) that flocculation is relatively rapid but contrary to these authors, we found, with griseofulvin, an *increased* rate of settling. The reduced rate noted by Ecanow & others may be due to air entrapment and the presence of glycerol in their suspensions (see later). We found the coagulation of griseofulvin to be slower than the flocculation but only by a factor of approximately two. The aggregates in the coagulated suspension were less granular and the supernatant more opalescent. After 1 hr the sediment volumes were almost identical and only the supernatant served to distinguish them. After storage for 1 week at 25° the suspensions were almost identical apart from the slightly more granular appearance of the flocculated sediment. The sediment volumes were 14% compared with 3·5% in the absence of electrolyte.

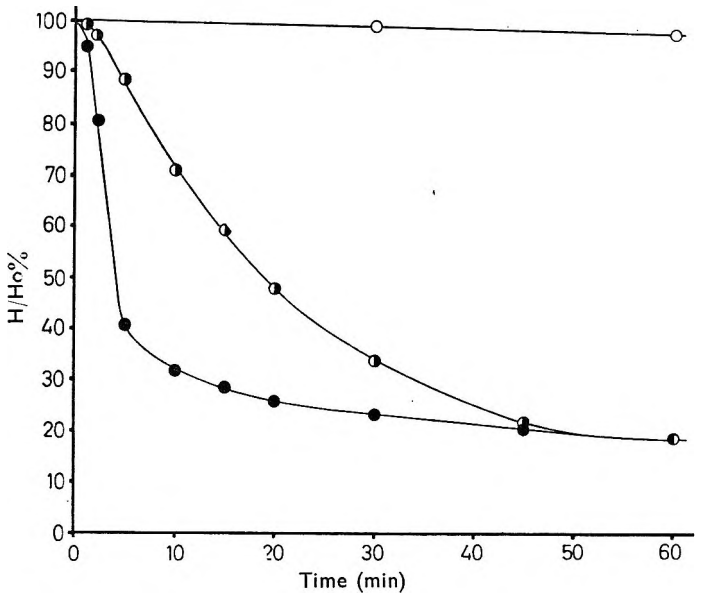

FIG. 1. The ratio of settled height/original height (H/Ho) expressed as a percentage, as a function of time during coagulation and flocculation of 2·5% griseofulvin suspensions. ● 10^{-2}M SDS + 10^{-1}M aluminium chloride. ◑ 10^{-2}M SDDS (commercial) + 10^{-1}M aluminium chloride. ○ 10^{-2}M SDS. ◐ common point.

REDISPERSIBILITY OF COAGULATED AND FLOCCULATED SUSPENSIONS

After storage for 1 week at 25°, the coagulated suspension redispersed completely after 3 revolutions and produced a smooth suspension; the flocculated sample required 14 revolutions and left 2–5 mm aggregates of the drug at the base of the container. Although Ecanow & others (1966) have postulated "the pharmaceutical necessity" of preparing flocculated suspensions, our experiments have suggested that such an approach could lead to pharmaceutically inelegant suspensions containing large aggregates. The inclusion of materials such as methyl cellulose, glycerol or sorbitol may partially obviate this. These aggregates are probably caused by chemical bridging between adjacent particles coated with surfactant, arising as a result of the aluminium ions reacting with the surfactant to give insoluble aluminium lauryl sulphate.

The present experiments suggest that the coagulation mechanism may provide a more suitable basis for producing redispersible pharmaceutical suspensions and it has been previously shown, Matthews & Rhodes (1968b) that this coagulation can be interpreted by zeta potential changes. The statement by Ecanow & others (1966) that coagulation leads to caking has been shown not to apply in this system. However, it has been previously found (Matthews & Rhodes 1968b), that when the modal particle diameter of the drug griseofulvin is increased from 4 to 15 μ, the effect of coagulation on sedimentation volume is less pronounced and may not be possible to apply the coagulation principle to very coarse or dense powders.

LONG-TERM STORAGE OF COAGULATED SUSPENSIONS

The maximum period of storage in the redispersibility experiments was 1 week. Samples of suspensions described previously (Matthews & Rhodes, 1968b) have now been on storage for 9 months at laboratory temperature. These suspensions have experienced a change of sedimentation volume of less than 1% during storage and redispersed to give smooth uniform suspensions after only 5 revolutions in the redispersing machine. This is good evidence that such systems are stable over long periods.

THE COAGULATION MECHANISM

Coagulation in colloidal dispersions is a diffusion phenomenon caused by Brownian motion of the particles, von Smoluchowski (1917). Calculations were made using Stokes law and the equation for the displacement due to Brownian motion, to compare the displacements for different particle sizes and to determine the size ranges in which sedimentation and Brownian motion predominate. These calculations have been made for griseofulvin on the assumption that the particles are spherical although Matthews & Rhodes (1967) have shown that the material is acicular. The results are shown in Fig. 2. The displacements have been calculated

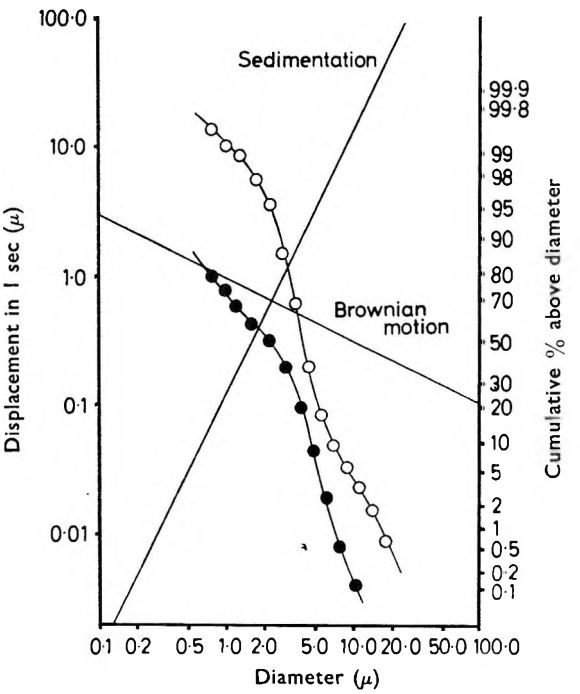

FIG. 2. Theoretical predictions of displacement due to sedimentation and Brownian motion in 1 sec, as a function of diameter for fine particle griseofulvin. Superimposed are cumulative weight % oversize and cumulative number % oversize distributions as a function of diameter. ○ Cumulative weight % oversize. ● Cumulative number % oversize.

for a period of 1 sec which is suggested as a suitable value by Burton (1926). Also shown in Fig. 2 are particle size distributions of fine particle griseofulvin.

The calculations indicate that for a spherical particle of griseofulvin of diameter 2 μ suspended in water, the displacements due to Brownian motion and sedimentation, in 1 sec, are identical. Below this size, Brownian motion predominates, above it, sedimentation. Burton (1926) has suggested that this is the critical diameter for a particle and is the largest diameter which will not settle under sedimentation. Since Brownian motion is random and sedimentation is unidirectional we suggest that at this diameter, sedimentation can still be superimposed on Brownian motion and that the critical diameter for no settling is probably slightly lower than 2 μ. The sample of griseofulvin has 50% by number and 6% by weight of particles with a diameter smaller than 2 μ and so Brownian motion can still be postulated as a major cause of coagulation in this system since it is dependent on the number of particles. Smoluchowski's theory was developed for a monodisperse system and it has been shown by Matthews & Rhodes (1968c) and by Ho & Higuchi (1968) that heterodispersity accelerates coagulation due to Brownian motion. Heterodispersity is also likely to assist coagulation caused by sedimentation since the differential sedimentation velocities will cause particle collisions.

Ecanow & others (1966) do not state the particle size of the sulphamerazine used in their experiments and for reasons which are not stated, they add 50% of glycerol to their suspensions. It has been pointed out by Martin (1960) that glycerol can slow down or halt Brownian motion completely, and we suggest that the inclusion of this material at such a concentration, in an experiment to compare coagulation and flocculation may obscure differences caused by the two phenomena. Ecanow & others point out that in the absence of electrolyte, no sedimentation was observed over a period of seven days.

In a colloidal system, particle collisions result in permanent aggregates because of the van der Waals forces of attraction. Kruyt (1952a) has referred to the theoretical existence of long-range London/van der Waals forces in hydrophobic suspensions in the range 2–5 μ and has stated that coagulation is possible in the secondary minimum that occurs in the energy of interaction curves at about 100–200 mμ distance. We suggest that sedimentation and Brownian motion can cause particles to approach one another to this distance in low electrolyte concentrations and to the primary minimum in high concentrations and thus yield a loosely coagulated structure.

THE EFFECT OF VALENCY ON COAGULATION

Ecanow & others (1966) have observed that Na^+ and K^+ are less effective than Al^{+++} in particle aggregation although the concentrations used are not stated. The Schultze-Hardy rule, Kruyt (1952b), states that the colloidal coagulation values for mono-, di- and tri-valent ions are in the

ratio 100: 1·6: 0·13. If similar principles apply to coagulation of supra-colloidal suspensions, one would expect Na^+ to coagulate a suspension to the same extent as Al^{+++}, only if the Na^+ are present in very high concentration.

In order to test whether the Schultze-Hardy rule applies to supra-colloidal suspensions, the coagulation rate of a 0·7 μ Dow polystyrene latex was determined in the presence of Na^+, Ba^{++} and Al^{+++}. The chlorides of each were chosen to minimize any opposing charge effect. Sedimentation of the 0·7 μ latex did not occur during the experiments.

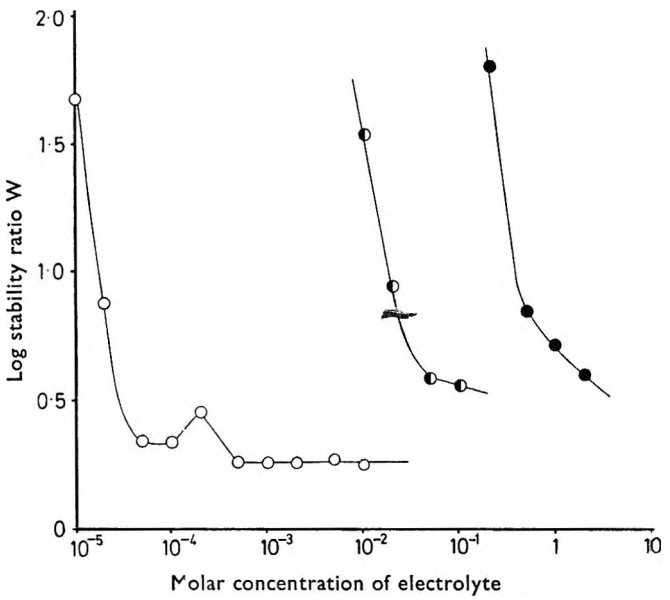

FIG. 3. Log stability ratio W, as a function of electrolyte concentration for a 0·714 μ "Dow" polystyrene latex. ○ Aluminium chloride (from Matthews & Rhodes, 1968a). ◐ Barium chloride. ● Sodium chloride.

The results are shown in Fig. 3. The experimental values of the stability ratios do not reach the theoretical value of unity (log W = O) even in high electrolyte concentrations and the reasons for this kind of situation have been discussed earlier (Matthews & Rhodes 1968a). There is also evidence of curvature in the graphs obtained with the two lower valency electrolytes. This may be a feature of the particle size since the *absence* of curvature was commented on by Ottewill & Shaw (1966) who used polystyrene latices of diameter 0·06–0·42 μ. Despite the curvature we observed, the vertical portions of the graphs were extrapolated to give an estimated coagulation value. These are given in Table 1 together with values for a negatively charged arsenious sulphide sol taken from Kruyt (1952c).

TABLE 1. COAGULATION VALUES OF A 0·714 μ "DOW" POLYSTYRENE LATEX IN DIFFERENT ELECTROLYTES

Electrolyte	Coagulation Value (mmoles/litre)	
	Dow latex	As_2S_3* sol.
NaCl	1000	51
$BaCl_2$	60	0·69
$AlCl_3$	0·05	0·093

* Kruyt (1950c).

These results show that aluminium chloride is a very much more powerful coagulating agent for polystyrene latex suspensions than either barium or sodium chloride even allowing for the Schultze-Hardy rule. The coagulation value for aluminium chloride in this system was even lower than that quoted by Kruyt for the arsenious sulphide sol but within the range of 0·003–0·096 mmole/litre given by this author for various aluminium salts and negatively charged colloids. The results for barium and sodium chloride indicate a considerable variation from those expected and this may be due to the small proportion of sulphonate stabilizer present in these latex polymers, (Higuchi, Okada & others, 1963). This may precipitate in the presence of the trivalent cation but not with the mono-or divalent. In these experiments the coagulation rate in 2M NaCl (11·7% w/v) was not as great as in much lower concentrations of aluminium chloride, and this is further evidence that a direct comparison of aggregation phenomena in electrolytes of different valencies may give different results from those obtained in identical electrolyte systems.

CONCLUSIONS

In devising experiments to distinguish between flocculation and coagulation it is important to limit the number of variables and to exclude secondary phenomena which may obscure important differences. It has been demonstrated that both coagulation and flocculation can produce suspensions of griseofulvin which are readily redispersible after storage and it has been shown that for powders containing particles in the range 0·5–20 μ, coagulation can produce suspensions which are more pharmaceutically elegant than those produced by flocculation. This contrasts with the advice of some other workers.

It has been shown that SDDS is a very suitable wetting agent, despite a foaming tendency: it can be used as a charge conferring agent for subsequent coagulation by aluminium salts. It is suggested that provided it is suitably non-toxic, it would be a useful material for the pharmaceutical formulator.

Acknowledgements. We thank Mr. K. A. Lees of Glaxo Laboratories Ltd., Greenford, Middlesex, for the griseofulvin, Dr. B. W. Barry of the School of Pharmacy, Portsmouth, for the sodium dodecyl sulphate and Dr. J. K. Weil of Eastern Regional Research Laboratories, Philadelphia, Pa., U.S.A., for the sodium dioxyethylated dodecyl sulphate.

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The anti-acetylcholinesterase activities of the alkaloids of *Physostigma venenosum* seeds*

B. ROBINSON AND J. B. ROBINSON

Anti-acetylcholinesterase activities of physostigmine, physovenine, N_a-norphysostigmine, geneserine and eseramine have been investigated *in vitro* using erythrocyte acetylcholinesterase. The former three bases show comparable activities whereas geneserine and eseramine are inactive under the same experimental conditions. The biologically active form of these bases is discussed in light of these enzyme inhibitory studies.

PHYSOSTIGMINE (eserine) (I, R = Me, X = N-Me), the major alkaloid of *Physostigma venenosum* seeds, and a large number of synthetic analogues have been evaluated for anti-acetylcholinesterase activity and the chemical features essential for such activity have been established (Stempel & Aeschlimann, 1956). The structures of the minor alkaloids physovenine (I, R = Me, X = O), N_a-norphysostigmine (I, R = H, X = N-Me), geneserine [I, R = Me, X = (N→O)Me] and eseramine (I, R = Me, X = N-CONHMe) have been established and they have been shown to have the same absolute configurations as physostigmine about the B/C ring junction, which must be *cis*-fused (Robinson, 1968).

The structure-action relations in this related group of compounds have been examined using erythrocyte acetylcholinesterase.

Experimental

Substrate. Acetylcholine perchlorate (B.D.H.—biochemical grade).

Inhibitors. A stock solution of each alkaloid was prepared by dissolving an accurately-weighed quantity (approximately 10 mg) of the alkaloid in 95% ethanol (100 ml). Test solutions were prepared by diluting (1 in 100) with phosphate buffer (pH 7·4; 1×10^{-3}M) (Documenta Geigy, 1962).

Enzyme solution. Erythrocyte acetylcholinesterase (Sigma Chemical Co., London) (250 μM units) was dissolved in a 1% solution of partially-hydrolysed gelatin containing magnesium chloride (0·04M) and sodium chloride (0·05M) (200 ml); this solution was stored at 0° in the dark until required. The enzyme is stable for 3–4 weeks under these conditions.

HYDROLYSIS RATES

Acetylcholine hydrolysis rates were measured by the pH-stat method (Alles & Hawes, 1940) using a Radiometer Automatic Titrator (Type TTT 1c) equipped with a recorder (SBR 2c) and syringe burette (SBU 1a) (Radiometer, Copenhagen). The reactions were carried out at pH 7·4 in a 50 ml jacketed vessel at 25 ± 0·1°, the reaction mixture being stirred and bubbled with carbon dioxide-free nitrogen. Sodium hydroxide (0·02N) was used as the titrant.

The reaction mixture was made up to 0·04M in magnesium chloride

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* For the preceding paper in this series see Longmore & Robinson (1966).

and to 0·05M in sodium chloride and contained enzyme, inhibitor and substrate in a total volume of 25 ml of solution.

Reaction mixtures (less inhibitor and substrate) were pre-incubated for 10 min before adjusting the solution to pH 7·4. Inhibitor was added and allowed to equilibrate with the enzyme for varying times (see Table 1) before initiating the hydrolytic reaction by addition of the substrate.

The velocity of the reaction was calculated from the slope of the recording of volume of sodium hydroxide added against time, obtained during the second and third minute of the incubation. The results were plotted in the manner of Lineweaver & Burk (1934) and the values of K_I calculated from the gradients of the resulting plots using the equation:

$$\text{Gradient} = \frac{K_m (1 + [I]/K_I)}{V_{max}}$$

where K_m is the Michaelis constant, K_I is the enzyme-inhibitor dissociation constant, [I] is the concentration of inhibitor and V_{max} is the maximum velocity of the reaction.

The activity of the enzyme was checked at intervals during the experiments and gave a consistent value for K_m of $4{\cdot}4 \times 10^{-4}$ with a V_{max} of $2{\cdot}1 \times 10^{-6}$ M/min.

The calculated values of K_I are shown in Table 1 and a typical set of experimental results is given in Fig. 1a,b.

Discussion

Table 1 shows that physostigmine, physovenine and N_a-norphysostigmine are highly active inhibitors of the enzyme acetylcholinesterase whereas geneserine and eseramine are inactive under the experimental conditions used. These conclusions are in agreement with the results of previous pharmacological studies on the rat diaphragm-phrenic nerve and frog rectus abdominus muscle. In these preparations the activity of physovenine in potentiating the action of acetylcholine is of the same order as physostigmine, whereas that of eseramine is much lower (Robinson, 1968).

TABLE 1. ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF THE ALKALOIDS OF *Physostigma venenosum* SEEDS

Alkaloid	Molar conc. ($\times 10^7$)	Dissociation constants ($\times 10^5$) after pre-incubation of the inhibitor with acetylcholinesterase for:		
		1 min	3 min	10 min
Physostigmine	1·438	5·5*	2·3†	2·5‡
	2·876	—	2·9†	—
Physovenine	1·534	5·4*	4·2†	2·4‡
	3·068	—	3·3‡	—
N_a-Norphysostigmine ..	1·463	4·1*	3·3†	1·9‡
	2·926	—	2·9‡	—
Eseramine	1·25 and 2·50	Inactive at both these molar concentrations		
Geneserine	1·433 and 2·866	Inactive at both these molar concentrations		

* Competitive inhibition. † Mixed inhibition. ‡ Non-competitive inhibition.

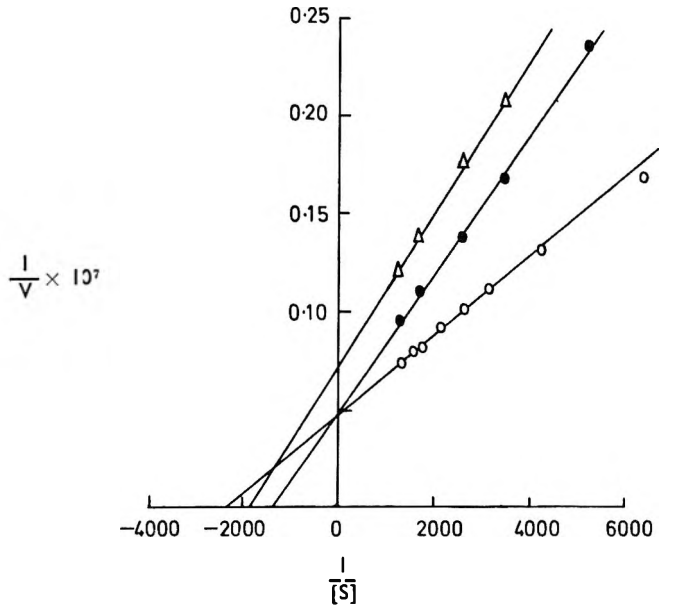


FIG. 1a. Effect of concentration on the kinetics of physostigmine inhibition of acetylcholinesterase, 3 min pre-incubation. —○—○— acetylcholine. —●—●— $1{\cdot}438 \times 10^{-7}$ molar physostigmine. —△—△— $2{\cdot}876 \times 10^{-7}$ molar physostigmine.

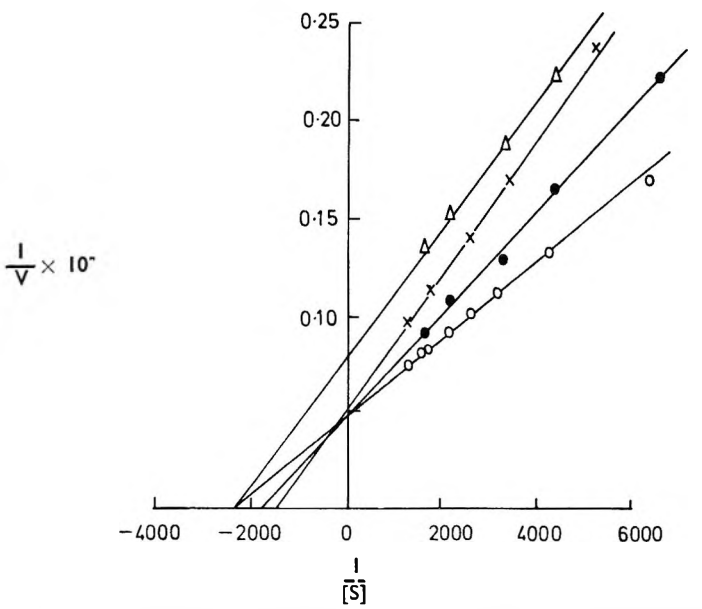


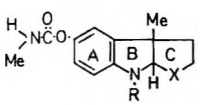
FIG. 1b. Effect of time of pre-incubation on the kinetics of physostigmine inhibition of acetylcholinesterase. Concentration of physostigmine = $1{\cdot}438 \times 10^{-7}$ molar. —●—●— 1 min pre-incubation. —×—×— 3 min pre-incubation. —△—△— 10 min pre-incubation. —○—○— acetylcholine.

Since a decrease in the value of K_I can be taken as an index of increasing inhibitory strength then our results show that the intensity of the inhibition depends on the time of pre-incubation of the inhibitor with the enzyme. Coupled with the increase in inhibitory strength, the kinetics of the inhibition change from competitive to non-competitive, and this may be explained by the formation of a carbamoylated enzyme by transfer of a carbamyl group from the inhibitor as shown below (cf. Main & Hastings, 1966).

$$E + AcCh \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E.AcCh] \xrightarrow{k_2} E.Ac + choline \xrightarrow{k_3} E + AcOH \text{ (acetic acid)}$$

$$E + I \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} [E.I] \xrightarrow{k'_2} E - N\text{-Methylcarbamoyl} \xrightarrow{k'_3} E + N\text{-Methylcarbamic acid}$$

Thus if k'_3 is much smaller than k_3, we may expect to observe a non-competitive component in the inhibition (Wilson, 1963). Table 1 shows that the three very active alkaloids act initially as competitive inhibitors but during pre-incubation of the inhibitor with the enzyme, some carbamoylation of the enzyme occurs (i.e. these compounds are acting as very poor substrates for the enzyme). It is interesting to note that earlier studies (Nachmansohn, Rothenburg & Feld, 1948; Augustinsson & Nachmansohn, 1949) on the inhibition of acetylcholinesterase by physostigmine and neostigmine (II) report only a competitive inhibition. However, the pre-incubation periods reported by these authors (using enzyme



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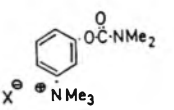

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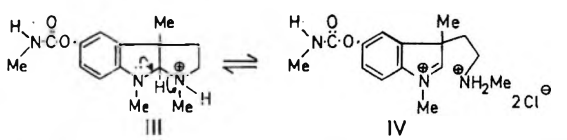
isolated from electric eel) were up to 150 min, and since later work (Wilson, Harrison & Ginsburg, 1961) showed that the half-life of the methylcarbamoylated acetylcholinesterase is about 38 min, the carbamoylated enzyme would have undergone considerable hydrolytic recovery under these prolonged pre-incubation conditions.

Comparative studies on the effect of the pH on the inhibition of acetylcholinesterase by physostigmine and neostigmine (II) (Wilson & Bergmann, 1950) led to the suggestion that the protonated form (III)



III IV

of physostigmine is the enzyme inhibitor (Nachmansohn & Wilson, 1951). Such a hypothesis will also account for the high anti-acetylcholinesterase activity exhibited by N_a-norphysostigmine and the inactivity (under the

present experimental conditions) of eseramine and geneserine, in which the nitrogen atoms corresponding to N_b (protonated in physostigmine) are non-basic. It does not account, however, for the high inhibitory activity shown by physovenine, in which the physostigmine N_b-Me group is replaced by a relatively non-basic oxygen atom. This may be explained by considering the acid-catalysed opening of ring C which occurs in physostigmine and related compounds. It has been shown on the basis of ultraviolet absorption and proton magnetic resonance studies (Jackson & Smith, 1964) that opening of ring C in physostigmine as shown in (III) takes place in 6N hydrochloric acid to give (IV). We find that both N_a-norphysostigmine and physovenine behave similarly and in 6N-hydrochloric acid undergo opening of ring C to yield the corresponding 3*H*-indolium cations as in (IV), λ_{max} 236, 298, λ_{inf} 241 mμ (ϵ = 6,400, 5,300 and 6,000) and 233, 239 and 284 mμ (ϵ = 6,600, 6,100 and 5,400) respectively. In 6N hydrochloric acid the ultraviolet spectrum of geneserine is indicative of a mixture of 3*H*-indolium cation and indoline chromophores whereas eseramine is fully protonated on N_a under such conditions (indicated by a typical benzenoid absorption). Ring C in both geneserine and eseramine is, however, opened in 11N hydrochloric acid, both alkaloids showing typical 3*H*-indolium cation absorption under these conditions with λ_{max} 238, 242 and 294–5 mμ (ϵ = 5,800, 5,750 and 5,500) and λ_{max} 236, 293–4, λ_{inf} 240 mμ (ϵ = 6,650, 5,500 and 6,300) respectively.

Although the pH values at which such cleavages of ring C in physostigmine, physovenine and N_a-norphysostigmine have been effected are too low to be considered applicable to a biological system, the possibility that analogous enzyme-catalysed reactions occur at the acetylcholinesterase surface is not precluded. It is therefore suggested that the reactive species responsible for the anti-acetylcholinesterase activity of physostigmine is the ring C-opened 3*H*-indolium cation (IV), analogous cations also being the active forms of physovenine and N_a-norphysostigmine.

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Synthesis and pharmacological action of some *N*-alkyl morpholines and their salts

A. H. BECKETT, W. H. HUNTER AND P. KOUROUNAKIS

The preparation is described of some 2-hydroxy-2- and 4-alkylmorpholines by ring closure of the corresponding phenacyl hydroxyalkylamines. The influence of structure upon the ring closure reaction is examined and the weak pharmacological action of the title compounds on leptazol convulsions, the autonomic nervous system and in the mouse hot-plate test is discussed in relation to the reversed esters of pethidine.

INTRODUCTION of an oxygen atom into the heterocyclic ring structure of the reversed esters of pethidine (I) has been investigated by Lutz & Jordan (1949) and the effect of this change upon the differences in pK_a between piperidines and morpholines has been discussed (Beckett, 1956). The reduction in pK_a is about 2·6 and should result in a much greater proportion of compound in the unionized form at physiological pH values.

The 2,2-substituted morpholines do not have the same relation between the phenyl group and the nitrogen atom as do the reversed esters of pethidine but they are analogues of the 3,3-substituted piperidine compounds (II) whose analgesic action has been reported (McDonald, Woolfe & others, 1946; Bergel, Hindley & others, 1944). We therefore investigated the synthesis of morpholines of structure (III, R^1 R^2 R^3 = H, alkyl, aryl, aralkyl) and of their salts. The general method used for the synthesis was the reaction of a phenacyl bromide with the appropriately substituted amino-alcohol (Lutz & Jordan, 1949; Cromwell & Tsou, 1949): the phenacylamino-alcohols (IV) formed initially, cyclized spontaneously to the hemi-ketal structures (III). Compounds prepared by this method are shown in Table 1. Structures III and IV are isomeric and the allocation of structure to the products was made by examination of the infrared spectra in the solid state (Nujol mull). Structures such as III, showed sharp bands around 3350–3400 cm^{-1} arising from the tertiary hydroxyl group and did not show any characteristic carbonyl absorption band. In one case (IV, R^1=Ph, R^2=R^3=H) the open-chain compound only was isolated.

The structures proposed for the compounds were supported also by their ultraviolet absorption spectra (Fig. 1). Ring closure of the phenacyl amines, to morpholines, was accompanied by a large reduction of ϵ_{max} due to the loss of the carbonyl chromophore. Values of ϵ_{max} in ethanol are recorded for salts of the ring-closed compounds in Table 2. The cyclic morpholine structures proposed for the compounds in the solid state are therefore retained in alcoholic solutions of the bases and their salts.

2-Hydroxymorpholine compounds were formed from the phenacylamines irrespective of the nature of the group R^1 in (IV): the electronic effect of R^1 had therefore little influence on the ring closure reaction. The influence of the nitrogen substituent was more important since it was

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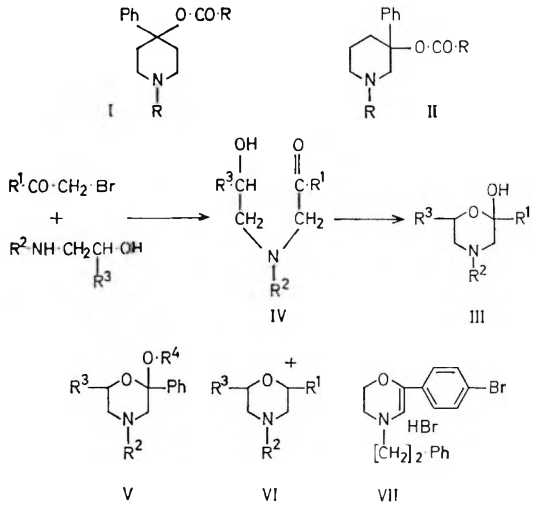


not possible to effect ring closure of the secondary amine (IV, R^1=Ph, R^2=R^3=H) and only the open-chain compound could be isolated. This hydroxyketone could not be converted to a morpholine compound by treatment with acid in anhydrous media, conditions normally used for the formation of ketals. We suggest that the ring closure reaction depends upon the adoption of a favourable conformation by the hydroxyalkyl side-chain. This conformation could be assisted by the steric effect of an additional substituent on the nitrogen atom. This hypothesis is supported by the ready ring closure of quaternary salts of bases such as

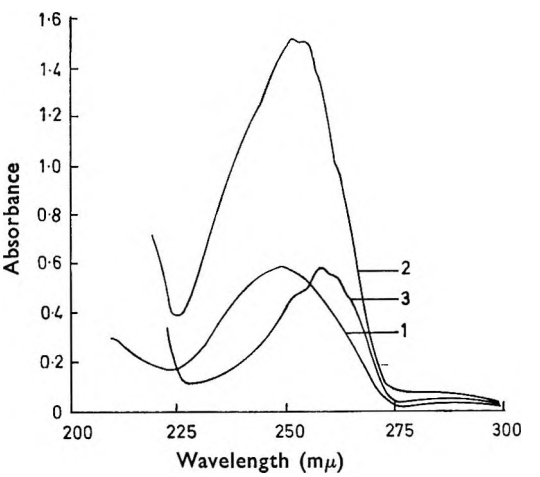
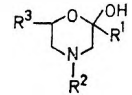


FIG. 1. Ultraviolet spectra of 1. Phenacyl bromide (9 mg/litre). 2. 2-Hydroxy-2-phenyl-4-phenylethylmorpholine hydrochloride (575 mg/litre). 3. 2-Ethoxy-2 phenyl-4-phenylethylmorpholine hydrochloride (555 mg/litre).

TABLE 1. PREPARATION AND PROPERTIES OF 2-HYDROXY-2,4,6-SUBSTITUTED MORPHOLINES

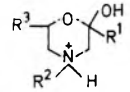


R^1	R^2	R^3	Starting material: Amino-alcohol	Starting material: α-Halo ketone	Yield %	M.p.	Empirical formula	Found: C	Found: H	Found: Br	Found: N	Required: C	Required: H	Required: Br	Required: N
-Ph	-$[CH_2]_2$·Ph	H	2-phenylethyl-aminoethanol	Phenacyl bromide	69	90°	$C_{16}H_{17}NO_2$	76·6	7·7		5·2	76·3	7·5		4·9
-*p*-C_6H_4Br ..	-$[CH_2]_2$·Ph	H	2-phenylethyl-aminoethanol	*p*-Bromo-Phenacyl bromide	66	61°	$C_{16}H_{16}BrNO_2$	59·3	5·7	21·8	4·0	59·6	5·6	22·1	3·9
-Ph	-$[CH_2]_2$·Ph	-Me	1-(2-phenylethyl-amino)-2-propanol	Phenacyl bromide	71	75°	$C_{17}H_{19}NO_2$	77·0	7·9	Equiv. wt 296·3		76·8	7·8	Equiv. wt 297·4	
-Ph	-C_6H_{11} (cyclohexyl)	H	2-cyclohexyl-aminoethanol	Phenacyl bromide	77	86°	$C_{16}H_{23}NO_2$	73·5	8·7		5·5	73·8	8·5		5·4
-Ph	-Me	H	2-methyl-aminoethanol	Phenacyl bromide	66	52–53°*	$C_{11}H_{13}NO_2$			Equiv. wt 192·7		Equiv. wt 193·3			
-Ph	-Et	H	2-ethylamino-ethanol	Phenacyl bromide	77	54°*	$C_{12}H_{15}NO_2$			Equiv. wt 206·8		Equiv. wt 207·4			
-Ph	-CH_2·Ph	H	2-benzylamino-ethanol	Phenacyl bromide	66	61·5°*	$C_{17}H_{19}NO_2$			Equiv. wt 268·9		Equiv. wt 269·4			
-Me	-$[CH_2]_2$·Ph	H	2-phenylethyl-aminoethanol	Chloro-acetone	46	liquid n_D^{23} 1·5178	$C_{13}H_{19}NO_2$			Equiv. wt 220·4		Equiv. wt 221·3			

Infrared spectra (Nujol mull) of all compounds showed bands at 3200–3400 cm^{-1} (ν OH), 1030–1140 cm^{-1} ν C–O. Bands at 1620–1720 cm^{-1}, characteristic of ν C=O were not observed.

* Cromwell & Tsou (1949).

TABLE 2. PROPERTIES OF 2-HYDROXY-2,4,6-SUBSTITUTED MORPHOLINES HYDROHALIDES



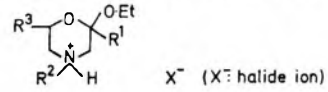
Salt	R¹	R²	R³	M.p.	Empirical formula	Found C	Found H	Found Br	Found Cl	Found N	Required C	Required H	Required Br	Required Cl	Required N
Hydrochloride* ..	Ph	-[CH₂]₂·Ph	H	150°	$C_{18}H_{22}ClNO_2$	67·4	6·9		11·0	4·3	67·6	6·9		11·1	4·4
Hydrochloride** ..	*p*-C_6H_4Br	-[CH₂]₂·Ph	H	149–150°	$C_{18}H_{21}BrClNO_2$	54·2	5·5	20·0	9·2	3·5	54·2	5·3	20·0	8·9	3·5
Hydrobromide	Ph	-[CH₂]₂·Ph	-CH_3	178°	$C_{19}H_{24}BrNO_2$	58·8	6·7	22·0		4·2	60·3	6·4	21·1		3·7
Hydrobromide† ..	Ph	C_6H_{11} (cyclohexyl)	H	194°	$C_{18}H_{24}BrNO_2$	56·3	6·9	23·2		4·1	56·1	7·1	23·3		4·1
Hydrobromide	Ph	Me	H	139°§	$C_{11}H_{16}BrNO_2$	Equiv. wt 273·0					Equiv. wt 274·2				
Hydrobromide	Ph	-CH_2·Ph	H	152°	$C_{17}H_{20}BrNO_2$	Equiv. wt 348·0					Equiv. wt 350·3				
Hydrochloride	Me	-[CH₂]₂·Ph	H	159°	$C_{13}H_{20}ClNO_2$	62·5	8·2		13·9	4·7	60·6	7·8		13·8	5·4

* λ_{max} 251 mμ, ε_{max} = 900 (methanol)
** λ_{max} 260 mμ, ε_{max} = 950 (methanol)
† λ_{max} 250 mμ, ε_{max} = 700 (methanol)

Infrared spectra (Nujol mull) of all compounds showed bands at 3200–3400 cm^{-1} (ν OH), 1040–1140 cm^{-1} (ν C–O) and 2500–2700 cm^{-1} (ν NH, protonated tertiary amino group).

§ Cromwell & Tsou (1949).

TABLE 3. PROPERTIES OF 2-ETHOXY-2,4,6-SUBSTITUTED MORPHOLINES HYDROHALIDES



Salts	R¹	R²	R³	M.p.	Empirical formula	Found C	Found H	Found Br	Found Cl	Found N	Required C	Required H	Required Br	Required Cl	Required N
Hydrochloride* ..	Ph	-$[CH_2]_2$·Ph	H	157–158°	$C_{20}H_{26}ClNO_2$	69·1	7·5		10·3	4·1	69·0	7·5		10·2	4·0
Hydrochloride	*p*-C_6H_4Br	-$[CH_2]_2$·Ph	H	184°	$C_{20}H_{25}BrClNO_2$	56·1	5·9	Total halogen as Cl 16·4		3·4	56·3	5·9	Total halogen as Cl 16·6		3·3
Hydrobromide	Ph	-$[CH_2]_2$·Ph	Me	175–176·5°	$C_{21}H_{28}BrNO_2$	62·4	7·0	20·0		3·6	64·6	7·2	20·4		3·6
Hydrobromide	Ph	C_6H_{11} (cyclohexyl)	H	198–199°	$C_{18}H_{28}BrNO_2$	58·7	7·6	21·9		3·9	58·5	7·4	21·6		3·8
Hydrobromide	Ph	Me	H	151°	$C_{13}H_{20}BrNO_2$	51·7	6·8	26·2		4·8	51·7	6·7	26·4		4·6
Hydrobromide	Ph	-CH_2·Ph	H	165°†	$C_{19}H_{24}BrNO_2$	Equiv. wt 377·5					Equiv. wt 378·4				
Hydrobromide	Me	-$[CH_2]_2$·Ph	H	158°	$C_{15}H_{24}BrNO_2$	54·5	7·3	24·3		4·2	54·6	7·3	24·0		4·4
Hydrobromide** ..	Ph	C_6H_{11} (cyclohexyl)	H	197°	$C_{19}H_{30}BrNO_3$	59·2	7·6	21·0		3·7	59·4	7·8	20·8		3·6
Hydrobromide** ..	Me	$[CH_2]_2$·Ph	H	149°	$C_{16}H_{26}BrNO_2$	55·2	7·7			4·2	55·8	7·6			4·0

* λ_{max} 257 mμ ε_{max} = 400 (in ethanol)
** 2-isopropoxy compounds

Infrared spectra (Nujol mull) of all compounds showed bands at 2500–2800 cm^{-1} (ν N–H, protonated tertiary amino-group) and 1020–1150 cm^{-1} (ν C–O). Bands at 3200–3800 cm^{-1} characteristic of ν O–H were not observed.

† Cromwell & Tsou (1949).

(IV) (Long & Schueler, 1954). The failure of secondary amines to ring close even in strongly acidic media indicates also that the −I effect of the protonated amino-group or of the quaternary group is relatively unimportant when compared with the steric effect of the extra substitution on the nitrogen atom.

The expected morpholine compound from *N*-phenacylaminoethanol (IV, R^1=Ph, R^2=R^3=H) was prepared as its ethyl ketal (V, R^2=R^3=H, R^4=Et) by hydrogenolysis of the corresponding benzyl compound (V, R^2=$-CH_2Ph$, R^3=H, R^4=Et) which was easily formed by ring closure of the open chain compound (IV, R^1=Ph, R^2=$-CH_2{\cdot}Ph$, R^3=H) followed by treatment with acidified ethanol. The ethyl ketal (V, R^2=R^3=H, R^4=Et) was stable as the hydrobromide, but hydrolysis with dilute acid removed the ethyl group to give the open-chain compound (IV, R^1=Ph, R^2=R^3=H). This reversion to the open-chain form agrees with the results of attempts to synthesize this compound from the secondary amine. Hydrolysis of this ketal was surprisingly slow and the final value of ϵ_{248} = 12,700 for the product was reached only after 16 hr. 2-Hydroxy-2-phenyl morpholine is therefore fairly stable, though it could not be made directly.

The 2-hydroxymorpholine compounds that were prepared reacted readily with alcohols in presence of traces of acid to form the alkyl ketals (Table 3). Ketal formation occurred quite readily when the bulky isopropyl group was being introduced and when the other 2-substituent was methyl, as in (III, R^1=Me, R^2=$CH_2{\cdot}CH_2{\cdot}Ph$, R^3=H) or phenyl, as in (III, R^1=Ph, R^2=cyclohexyl, R^3=H) but none of the other hydroxymorpholines of Table 2 formed an isopropyl ketal. The formation of ketals was shown by the disappearance of the tertiary hydroxyl adsorption band at around 3400 cm^{-1} in the infrared spectra. Since formation of acetals and ketals is acid-catalysed (Bell & Norris, 1941) and probably proceeds through carbonium ions, the hemiketals under discussion must readily form carbonium ions (VI) even when R^1 is methyl. The dehydration of hemi ketals such as (III, R^1=*p*$Br.C_6H_4$, R^2=$[CH_2]_2{\cdot}Ph$, R^3=H) to give the 2,3-dehydromorpholine derivative (VII), could also be interpreted in this way.

The possibility of diastereoisomer formation during the ring closure reaction was investigated by the preparation of (III, R^1=Ph, R^2=$[CH_2]_2{\cdot}Ph$, R^3=Me) but chromatography in a variety of systems using silica-gel or alumina plates gave only a single spot and no separation into diastereoisomeric pairs could be demonstrated. We suggest that either the ring closure reaction occurs by attack of the hydroxyl group on the carbonyl group in (IV) from one direction only, or, more probably, the ring-closed structure (III) is in equilibrium, in solution, with a small proportion of the open chain form (IV).

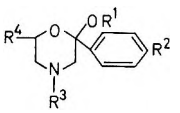
In the case of the homologous seven membered ring compound only the open-chain form could be isolated. Similarly, we could not prepare a five-membered ring compound by cyclization of *N*-2-hydroxyethyl-*N*-phenylethyl-*p*-nitro-benzamide. The presence of the *p*-nitro-group might be expected to increase the electrophilic character of the amide carbonyl

group but the product existed entirely in the open-chain form. Amide carbonyl groups are not usually susceptible to nucleophilic attack (in this case by –OH) and the failure of this ring closure is probably due to electronic effects rather than steric factors.

PHARMACOLOGICAL RESULTS

The compounds screened are shown in Table 4 and were administered as single doses of 100 mg/kg, orally to female mice. Administration of compounds 6, 7 and 8 produced convulsions at the dose levels used but compounds 1 and 2 had slight anti-convulsant action against leptazol. Compounds 3, 4 and 5 had no apparent effect at this dose level. The remaining compounds, 6, 7 and 8, showed some feeble action on the autonomic nervous system by producing blockade at ganglia and at post-ganglionic sites as shown by the inhibition of the responses to nicotine and to acetylcholine.

TABLE 4. MORPHOLINE DERIVATIVES TESTED PHARMACOLOGICALLY



Compound	R^1	R^2	R^3	R^4
1	H	H	$[CH_2]_2$·Ph	H
2	Et	H	$[CH_2]_2$·Ph	H
3	H	Br	$[CH_2]_2$·Ph	H
4	Et	Br	$[CH_2]_2$·Ph	H
5	H	H	$[CH_2]_2$·Ph	Me
6	Et	H	C_6H_{11}*	H
7	$CHMe_2$	H	C_6H_{11}*	H
8	Et	H	H	H

* Cyclohexyl

None of the compounds showed any analgesic activity in mice at this dose level when examined by the hot-plate method. The analogy between the 2,2-substituted morpholines and the 3,3-substituted piperidines is therefore not valid, nor does the analgesic activity of compounds of this type depend solely upon the relative pK_a values of the corresponding piperidines and morpholines (Hunter & Kourounakis, unpublished observations).

Experimental

Melting points were recorded on an Electrothermal capillary melting point apparatus and are uncorrected. Ultraviolet spectra were recorded using a Beckmann DK2 spectrophotometer, infrared spectra on a Unicam SP200 instrument and nuclear magnetic resonance spectra on a Perkin-Elmer R-10, 60 megacycle instrument with tetramethylsilane as internal reference in deuterated dimethylsulphoxide as solvent.

The amino-alcohols used as starting materials were prepared by published methods as follows, 2-phenylethylaminoethanol (Barbier, 1940),

2-(1-phenyl-2-propylamino)ethanol (Rapp & Karnov, 1958), 1-(1-phenyl-2-propylamino)-2-propanol (Kiprianov & Khrapal, 1950), 2-benzylaminoethanol and 2-cyclohexylaminoethanol (Cope & Hancock, 1942), 3-(2-phenylethylamino)propanol (Hromatika, 1942) and *N*-phenacylethanolamine (IV, R^1=Ph, R^2=R^3=H) (Brighton & Reid, 1945).

1-(2-*Phenylethylamino*)-2-*propanol* was prepared by heating (1·5 hr at 100°) phenylethylamine (41·7 g), 1,2-epoxypropane (15 g) in water (5 ml). The product (26 g), a colourless liquid, had b.p. 96°–100°/0·2 mm η_D^{23} 1·5223. Equiv. wt: found 182, $C_{11}H_{17}NO$ requires 179.

The *hydrobromide*, crystallized in colourless needles from alcohol-ether, m.p. 88°. Found: C, 50·7; H 6·8; Br, 30·5; N, 5·2. $C_{11}H_{18}BrNO$ requires C, 50·7; H, 7·0; Br, 30·7; N, 5·4.

GENERAL METHOD FOR PREPARATION OF 2-HYDROXY-2,4-DISUBSTITUTED MORPHOLINES

Method I. A dry ethereal solution of the appropriate α-haloketone (1 mole in 200 ml) was added slowly to a similar solution of the amino-alcohol (2·2 mole in 500 ml) and the mixture kept 16 hr at 20°. The ether layer decanted from the precipitated salt was washed twice with saturated sodium chloride solution, dried and evaporated. The semi-solid residue was crystallized from ether–light petroleum (b.p. 40°–60°). The yields obtained and physical properties of the compounds prepared are shown in Table 1.

Method II. A 20% w/v solution of the haloketone (1 mole) in dimethylsulphoxide was added to a solution of the amino-alcohol (1·1 mole) in the same solvent (700 ml) at 45–50°. After 0·5 hr, triethylamine (1·0 mole) was added and stirring continued for 1 hr. The solution was poured into water and the product isolated by ether extraction as before.

Salts of the substituted morpholines (Table 2) were prepared by adding the calculated amounts of hydrogen bromide in isopropanol or hydrogen chloride in ethanol to a solution of the base in isopropanol. Crystallization was induced by the addition of ether and the salts were recrystallized from ethyl acetate-ethanol.

GENERAL METHOD FOR PREPARATION OF THE 2-ALKOXY 2,4-SUBSTITUTED MORPHOLINES

A salt of the appropriate 2-hydroxy-2,4-substituted morpholine was refluxed (4 hr) in absolute ethanol or isopropanol containing a few drops of a solution of hydrogen bromide or hydrogen chloride in the same alcohol. The solvent was evaporated to one-third volume and dry ether added to precipitate the salt of the ethoxy or isopropoxy ketal in quantitative yield. Ketals thus prepared are shown in Table 3.

The alkoxy group could be exchanged in (V, R^2=$-CH_2{\cdot}CH_2{\cdot}Ph$, R^3=H, R^4=$-CHMe_2$) and (V, R^2=$-C_6H_{11}$, R^3=H, R^4=$-CHMe_2$). The isopropoxy ketal hydrobromides were refluxed (6 hr) in ethanol containing a few drops of ethanolic hydrogen bromide solution. The salts of the ethoxy ketals were precipitated with ether as described above and recrystallized from ethanol-ethyl acetate in about 80% yield.

2-*Ethoxy*-2-*phenyl*-4-*methyl morpholine*. The free base was liberated from the ketal hydrobromide (V, R^2=Me, R^3=H, R^4=Et, hydrobromide) by treating it with saturated potassium carbonate solution followed by extraction with ether. Evaporation of the ether gave the product m.p. 57–58° (from ether–light petroleum, 40–60°). (Found, C, 70·7; H, 8·8. $C_{13}H_{19}NO_2$ requires C, 70·5; H, 8·7. Equiv. wt; found 220·7, required 221·3).

2-p-*Bromophenyl*-4-*phenylethyl*-2,3-*dehydromorpholine hydrobromide* (VII). 2-Hydroxy-2-*p*-bromophenyl-4-phenylethylmorpholine (III, R^1=*p*-Br·C_6H_4, R^2=$[CH_2]_2$·Ph, R^3=H) as the hydrobromide (1 g) was added to a mixture of isopropanol and ethyl acetate (5 ml, 1 : 1), a few drops of a 10% solution of hydrogen bromide in isopropanol added and the solution refluxed for 6 hr. Ether (20 ml) was added to the cooled solution and the precipitated salt filtered off and crystallized from isopropanol-ethyl acetate to give (VII) (0·4 g), m.p. 190°, ν_{max} (Nujol mull) 1650 cm^{-1} (C=C); τ 3·80 (D_6-DMSO) (olefinic proton); λ_{max} (ethanol) 320 mμ (ϵ_{max} 14,000). (Found, C, 51·0; H, 4·6; Br, 34·7; N, 3·4. $C_{18}H_{19}Br_2NO$ requires C, 50·8; H, 4·5; Br, 35·2; N, 3·3).

2-*Ethoxy*-2-*phenylmorpholine hydrobromide* (V, R^2=R^3=H, R^4=Et). 2-Ethoxy-2-phenyl-4-benzylmorpholine hydrobromide (V, R^1=CH_2·Ph, R^3=H, R^4=Et) (2·7 g) in absolute ethanol (150 ml) was shaken with hydrogen in presence of palladium-charcoal (0·2 g; 10%) at room temperature and pressure. When the calculated amount of hydrogen had been taken up the mixture was filtered and the solution concentrated to 20 ml. Addition of ether-ethyl acetate (20 ml, 1 :1) gave the product (1·9 g), m.p. 127·5°. Found: C, 50·1; H, 6·1; Br, 29·9; N, 5·1; $C_{12}H_{18}BrNO_2$ requires C, 50·0; H, 6·3; Br, 27·7; N, 4·9.

Phenacyl-(1-*phenyl*-2-*propyl*)*ammonium nitrate*. Phenacyl bromide (2 g), (±)-amphetamine (2·8 g) in ether (20 ml) were allowed to stand 24 hr, when a semi-solid precipitate formed. The ether was decanted and the residue well washed with further quantities of ether. From the combined ether extracts an oily layer separated and was removed. The residual ether solution was washed with water (2 × 15 ml), dilute acetic acid (2 × 15 ml of 2·5%) and finally with water (2 × 10 ml). Dilute nitric acid (60 ml, 0·25N) was added and the viscous phenacyl-(1-phenyl-2-propyl)ammonium nitrate layer separated from the resulting three-phase mixture. Trituration of the viscous oil with ethyl acetate–acetone (2 : 1) produced the pure *nitrate* (0·36 g) as colourless crystals, m.p. 126°. Found: C, 64·0; H, 6·1; N, 8·0; $C_{17}H_{20}N_2O_4$ requires C, 64·5; H, 6·4; N, 8·8. λ_{max} 248 mμ, ϵ_{max} = 13,000 (in ethanol).

N-2-*Hydroxyethyl*-N-*phenylethyl* p-*nitrobenzamide*. *p*-Nitrobenzoyl chloride (0·9 g) in dry tetrahydrofuran (10 ml) was added slowly to a solution of 2-phenylethylaminoethanol (2·7 g) in dry tetrahydrofuran (10 ml) and shaken occasionally over 2 hr at room temperature. The solvent was removed and the resulting oil treated with a mixture of ice and dilute hydrochloric acid (1 : 1). The solid mass obtained was well washed with saturated sodium bicarbonate solution and finally with water.

Crystallization from aqueous ethanol gave N-2-*hydroxyethyl*-N-*phenylethyl* p-*nitrobenzamide* (1·1g), m.p. 70°. Found: C, 64·7; H, 6·0; N, 8·9. $C_{17}H_{18}N_2O_4$ requires C, 64·9; H, 5·8; N, 8·9.

Acknowledgements. We thank the Greek 'State Scholarship Foundation' for the award of a scholarship to one of us (P. K.). We are indebted to Dr. G. A. Schmeidler of Camden Chemical Co. Ltd, for the pharmacological results.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 228S–231S Received May 1, 1968

SHORT COMMUNICATION

The dissolution of paracetamol tablets and the *in vitro* transfer of paracetamol with and without sorbitol

V. WALTERS

MANY adjuvants such as sorbitol, glucosamine hydrochloride and sodium hexametaphosphate, have been added to oral dosage forms to improve the absorption of drugs, but Wagner (1961) emphasizes that the attribution of beneficial results to them is not always unequivocal. With sorbitol in paracetamol tablets, it has been suggested that the improved absorption is due to the sorbitol acting on the metabolism of paracetamol and as a dispersing agent (Gwilt, Robertson & others, 1963). The combination of paracetamol and sorbitol provides a readily assayable system with which to examine the role of the adjuvant.

I have examined the effect of sorbitol on the aqueous solubility and partitioning of paracetamol and have studied the dissolution rate of paracetamol from commercial tablets containing paracetamol with or without sorbitol with the aim of evaluating the effect of the adjuvant on the availability of the drug.

EXPERIMENTAL

Materials. *p*-Acetamidophenol (B.D.H. Laboratory Reagent). n-Octanol (B.D.H. Laboratory Reagent). Sorbitol (Kerfoot Biochemical Reagent).

Proprietary tablets A and B containing paracetamol; A with, B without sorbitol, and tablet C, paracetamol tablets B.P. 0·155M buffer solutions, pH 2·0 were made from A.R. potassium chloride and hydrochloric acid, those of pH 7·4 from A.R. potassium dihydrogen phosphate and sodium hydroxide. pH was measured with a Pye Dynacap pH meter.

Solubility of paracetamol in sorbitol solutions. Water (10 ml) containing 0, 0·5, 1·0, 2·0, 4·0 and 8·0% sorbitol was added to excess (250 mg) paracetamol in 50 ml Quickfit flasks which were shaken (24 hr) at 25°. Aliquots were filtered through 13 mm 0·45 μ pore diameter Millipore membrane filters, diluted, and the concentration of paracetamol in solution measured at 243 mμ. Paracetamol in water or 0·1N HCl had λ_{max} 243 mμ and within the concentration range 0–16 μg/ml the solutions obeyed Beer's Law. The regression line equation was used to determine the concentration present.

Partition coefficients. n-Octanol (20 ml) was added to separate weighed quantities (6–20 mg) of paracetamol in Quickfit flasks and 20 ml of one of the following: water, water containing sorbitol 1/5th of the weight of paracetamol, buffer solutions pH 2·0 and pH 7·4 was added to successive duplicate flasks. The flasks were shaken at 25° for 24 hr. The absorption of the diluted aqueous phases was measured at 243 mμ and the apparent partition coefficients (Reese, Irwin & others, 1964) calculated.

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Transfer in three-phase model. A model similar to that described by Perrin (1967) was used at 25°. Buffer (500 ml; pH 2·0) containing paracetamol (100 mg) with and without sorbitol (20 mg) was placed in compartment A and buffer (500 ml; pH 7·4) in compartment C. n-Octanol (350 ml) was layered onto the aqueous phases. All aqueous phases were pre-saturated with n-octanol and the octanol with the pH 7·4 buffer. 1·6 ml samples of the aqueous phases were removed at intervals, suitably diluted with 0·1N HCl and assayed at 243 mμ. The volumes were kept constant by replacement with 1·6 ml of fresh buffer solutions.

Tests made on tablets included: (*a*) Assay for paracetamol (B.P. 1963) (*b*) Disintegration at 37° (B.P. 1963) (*c*) Dissolution as follows: 500 ml 0·1N HCl in a 600 ml beaker was equilibrated in a water bath at 37°. A stainless steel stirrer with 4 blades of 3·5 cm diameter was rotated at 113 rev/min at a depth of about 8·2 cm. Two tablets were placed in a tube, as used for the B.P. disintegration test, and this was moved vertically as for the B.P. test. 2 ml samples of the 0·1N HCl solution were removed at intervals by means of Luer syringes and the solutions filtered through Millipore HA membrane filters in Swinnex-13 holders. The extinction of the diluted filtrates was measured at 243 mμ. The total amount of drug in solution at each sampling time was calculated after taking into consideration the increase in volume due to disintegration and dissolution (about 1·3 ml), the decreasing volume of the dissolution medium after each sample was removed, and the amount of drug removed in each sample.

RESULTS AND DISCUSSION

The solubility of paracetamol in solutions containing 0–8% sorbitol was 1·43% (standard deviation 0·025). The mean partition coefficients were as follows: n-octanol–water, 2·03; n-octanol–water containing sorbitol (1/5th of the quantity of paracetamol), 2·02; n-octanol–buffer (pH 2·0), 2·00; and n-octanol–buffer (pH 7·4), 2·06. At equilibrium in the three-phase model the value for n-octanol–buffer solutions was 2·01. Equilibrium distribution of paracetamol was attained slowly (about 50 hr) in this system (Fig. 1). The results both with and without sorbitol were in close agreement and an analysis of variance showed that there was no significant difference ($P = 0{\cdot}05$). Distribution curves comparable with those in Fig. 1 have been obtained with barbitone (Doluisio & Swintosky, 1965) and salicylic acid (Khalil & Martin, 1967). The mean apparent rate constants for the transfer from A during the first 7 hr were calculated from the first-order equation:

$$k = \frac{2{\cdot}303}{t} \log \frac{C_o - C_\alpha}{C_t - C_\alpha}$$

where C_c and C_t are the concentrations at zero time and after time t and C_α the concentration at equilibrium. The value of k hr^{-1} for paracetamol in the absence of sorbitol was 0·259 and in the presence of sorbitol, 0·257,

The mean disintegration times for tablets A, B and C were 4·3, 2·8 and 7·75 min whereas the $t_{0{\cdot}9}$ (time for solution of 900 mg of drug) read from

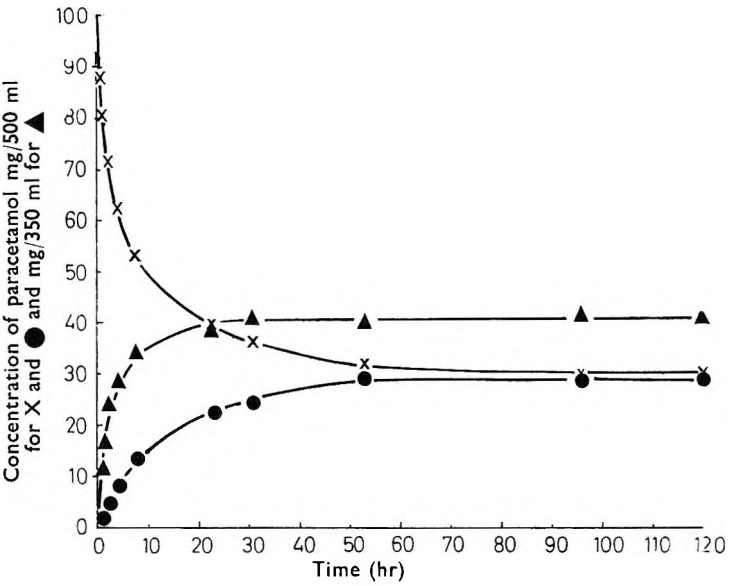

FIG. 1. Transfer of paracetamol from pH 2·0 buffer (×) through n-octanol (▲) to pH 7·4 buffer (●) at 25°.

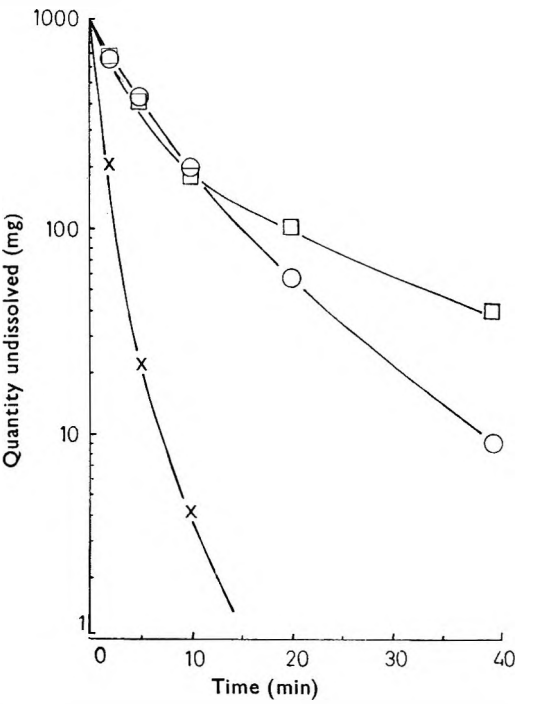

FIG. 2. Log paracetamol undissolved with time from commercial tablets (2) of paracetamol + sorbitol (×), paracetamol (○) and paracetamol tablets B.P. (□). Dissolution medium: 500 ml 0·1N HCl at 37°. (The points are the means of three replicates).

the dissolution rate curves (Fig. 2) were 3, 14·25 and 19 min, using the mean assay figures of 493·2, 505·0 and 502·2 mg paracetamol per tablet respectively. An analysis of variance showed that there was no significant difference between replicate dissolution tests ($P = 0{\cdot}05$). In agreement with the results of Levy & Hayes (1960) and Brudney, Stewart & Eustace (1964), the disintegration times did not correlate with the dissolution times. Varying degrees of correlation have, however, been claimed (Middleton, Davies & Morrison, 1964; Schroeter, Tingstad, & others, 1962; see also Morrison & Campbell, 1965; Wood, 1967). The characteristics of the material passing through the sieve during disintegration differed according to the source of the tablet. Tablets A and C appeared as a dispersible powder with more fine granules from the latter tablets, whereas tablet B disintegrated into aggregates smaller than 10-mesh sieve size which sedimented rapidly.

The results indicate that sorbitol does not form an absorbable complex with paracetamol. The improved absorption, claimed by Gwilt & others (1963), of paracetamol from tablets containing paracetamol and sorbitol (tablet A) may result from their higher dissolution rate. This rate is a function of formulation and of compression force (Levy & Gumtow, 1963; Ganderton, Hadgraft, & others, 1967; Marlowe & Shangraw, 1967; Polderman & Braakman, 1968) and these factors need to be standardized, leaving the presence or absence of sorbitol as the sole variable, before any improved *in vitro-in vivo* effects can be attributed to it.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 232S–238S Received May 1, 1968

Dissolution rate measurement by an automated dialysis method

R. B. BARZILAY AND J. A. HERSEY

An automated dialysis method for measuring the dissolution profiles of unit dose forms is described. The suitability of the method for the evaluation of these profiles has been demonstrated by an examination of tablets of sulphathiazole prepared under different conditions of pressure and excipient content. The addition of the water soluble resin, polyethylene oxide, has been shown to cause a small increase in the dissolution rates of the tablets.

DISSOLUTION rate tests for tablets and capsules have been developed in an attempt to obtain a more realistic *in vitro* determination of the availability of the drug *in vivo*. The beaker method of Levy & Hayes (1960), although widely used, has the disadvantage of a continually diminishing volume of dissolution fluid and, sometimes, random movement of the tablet under test. Beaker methods also involve a filtration in the sampling technique and where such methods have been automated (Schroeter & Wagner, 1962; Niebergall & Goyan, 1963; Michaels, Greely & others, 1965) continual passage of solution through a filter covered with drug particles may present an erroneous picture of the dissolution process. A constant volume of dissolution medium may be used in a circulatory system, provided dilution is not necessary for analysis of the dissolving drug. The sampling flow rate must also be considered in the continuous method because it may constitute an additional agitation process. Much evidence is available to show that agitation rates affect dissolution profiles. Dialysis techniques have been adopted to overcome the problem of obtaining a representative sample of the dissolution fluid without substantially affecting the fluid volume (Patel & Kostenbauder, 1958; Patel & Foss, 1964). More recently Marlowe & Shangraw (1967) have used a dialysis cell method to examine the release of sodium salicylate from tablet matrices. The disadvantages of their method have been summarized as follows: "The dissolution procedure is not as simple as would be desirable. The manual removal of samples and their assay are time consuming and interruption of rotation of the cell is necessary while samples are withdrawn. Even though the tablet remains immersed in the liquid and removal procedure standardized, there is a chance for a slight distortion in dissolution profile. Obviously an automatic sampler and analyser would be advantageous".

Ferrari & Khoury (1967) developed an automatic technique for use with the Technicon Autoanalyser and attempted to overcome the problem of fine particles passing from the dissolution flask into the dialyser unit by placing a fine plastic screen over the tip of the sampling pipette and introducing air segmentation to prevent fine particles from settling.

Krogerus, Kristoffersson & Kehela (1967) developed a method for dissolution studies involving dialysis, similar to the method which has

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been developed in this laboratory. Unlike the method of Krogerus & others however, the method described here maintains constant volumes and is fully automatic.

The object of this work was to develop and evaluate a suitable standard technique which could be used to examine dissolution as well as simultaneous disintegration and dissolution. Tablets were prepared at different pressures with and without the addition of starch or polyethylene oxide. The latter substance was chosen for its ability to reduce the onset of turbulence (Giles & Pettit, 1967) and hence its possible use to delay the absorption of drugs.

Experimental

Tablets of sulphathiazole were prepared on an instrumented Lehman tabletting machine. Flat surfaced, 12 mm diameter punches were used and the applied pressure determined as previously described (Shotton & Ganderton, 1960). The die was cleaned and lightly dusted with magnesium stearate before tablet preparation. 500 mg sulphathiazole powder of mean particle size 19·4 μ (Fisher Sub-Sieve Sizer) with the added excipient where indicated was handfilled into the die. Tablets of sulphathiazole alone were prepared at three different pressures. Tablets containing various concentrations of polyethylene oxide (Polyox WSR 301) or maize starch were prepared at 792 kg cm^{-2} or 660 kg cm^{-2} respectively.

Tablet disintegration test equipment readily obtainable in pharmaceutical laboratories was considered to be adaptable for work on dissolution. The only alteration was the replacement of the tablet holder with a cylindrical cell, the walls of which were formed from a dialysis membrane. The membrane was in the form of a tube, Visking Tubing, 32 mm inflated diameter. The lower end of the dialyser cell was formed by inserting a cylindrical Perspex block 13 mm high inside the dialysis tube to provide a tight fit and then clamping this inside a tough, flexible, polythene cap. The upper end of the tubing was held by a thin Perspex ring 12 mm high which could be inserted into the tube and held by a rubber band. This ring contained two holes for suspending the dialyser from the motor unit of the apparatus. The effective area available for dialysis was that supplied by the walls of a cylinder 75 mm high and 32 mm in diameter i.e. 75 cm^2.

For use, 150 ml of distilled water was placed in the outer vessel from which samples were continuously taken at a rate of 2·9 ml/min and replaced with distilled water at an identical rate. The tablet under test was placed inside the dialyser and at the beginning of the experiment 50 ml of distilled water previously equilibrated at 37° was poured into the dialyser and the motor was started.

The dialyser was raised and lowered 30 times a minute. At its highest position it was completely withdrawn from the water and at its lowest position the top of the dialyser remained clear of the water. The temperature of the system was maintained at 37° $\pm$ 1°.

For the purpose of comparison, the method of Ferrari & Khoury (1967)

was adapted for use with the unmodified disintegration apparatus. In place of the dissolution cell an Autoanalyser Dialyser was included in the circuit (Fig. 1B).

METHOD OF ANALYSIS

The method of Werner (1939) was adapted for continuous automatic sampling and assay using the Autoanalyser. This is shown diagrammatically in Fig. 1A. Colour intensity was examined at 460 mμ after passing the reaction stream through double mixing coils for 9 min.

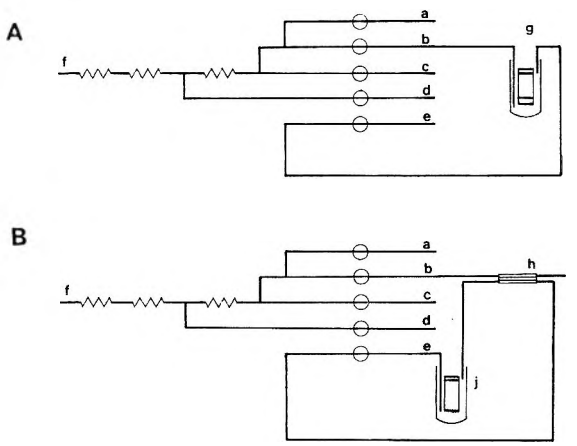

FIG. 1. Diagrammatic representation of the analysis systems. a. Pump tube 0·6 ml/min, air. b. Pump tube 2·9 ml/min, sample. c. Pump tube 2·0 ml/min, distilled water. d. Pump tube 0·1 ml/min, Ehrlich's reagent. e. Pump tube 2·9 ml/min, distilled water. f. to colourimeter, wavelength 460 mμ and recorder. g. Dialysis cell. h. Autoanalyser dialyser. j. Disintegration tube.

The method was chosen after a comparison with the method of Bratton & Marshall (1939) as modified by Wrightman & Holl (1965). The standard deviations of ten results for three sulphathiazole concentrations (1, 5, 10 mg/100 ml) are: 0·0011, 0·0041, 0·0111 for these concentrations by Werner's method and 0·0035, 0·0122 and 0·0334 by Bratton & Marshall's method.

The accuracy and simplicity of Werner's method of analysis has already been pointed out by Andrews & Strauss (1941) when comparing these two methods. In addition, the absorbance of solutions of sulphathiazole over a range of 1 to 20 mg/100 ml was measured for both methods of analysis. Regression lines were plotted and the average variation of absorbance values about the regression lines found were: for Werner's method, 0·0004 (2 exp.) and for Bratton & Marshall's method, 0·0010 (2 exp.). At lower concentrations, the response in Bratton & Marshall's method was non-linear. The passage of small nitrogen bubbles through the flow cell may also constitute an additional source of variation.

Results

The dissolution profile of tablets containing 500 mg of sulphathiazole, compressed at different pressures is shown in Fig. 2. The position of the tablet on the Perspex block at the bottom of the dialyser remained constant in relation to the dialyser itself throughout the experiment. As the tablet remained intact, dissolution could only have occurred from the top surface and from the edge of the tablet. The results indicate a decreasing dissolution rate with increasing applied pressure over the range of pressures used.

In spite of the reduced area available for dialysis much higher dissolution profiles were obtained using the unmodified disintegration apparatus in

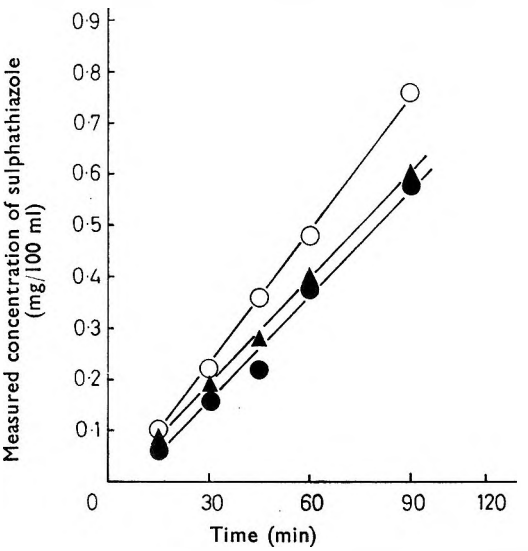

FIG. 2. Dissolution profiles of sulphathiazole tablets prepared at different applied pressures. ○ Applied pressure 500 kg cm^{-2}. ▲ Applied pressure 770 kg cm^{-2}. ● Applied pressure 1090 kg cm^{-2}.

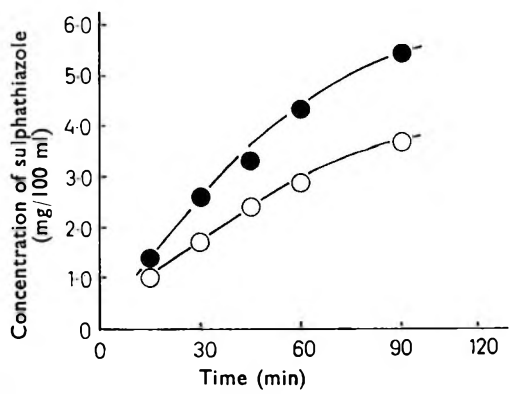

FIG. 3. Dissolution profiles of sulphathiazole tablets prepared at different pressures using the Autoanalyser dialyser system. ● Applied pressure 730 kg cm^{-2}. ○ Applied pressure 1140 kg cm^{-2}.

conjunction with the Autoanalyser dialyser (Fig. 3). This may be explained by the higher agitation intensity experienced by the tablets in the disintegration apparatus and also by the fact that dissolution of drug may occur from the whole of the tablet surface. Once again the tablets did not disintegrate during the period of investigation.

The effects of polyethylene oxide (Polyox WSR 301) and of maize starch on the dissolution profiles of the sulphathiazole tablets (Figs 4 and 5) show that an increasing concentration of either excipient increases the

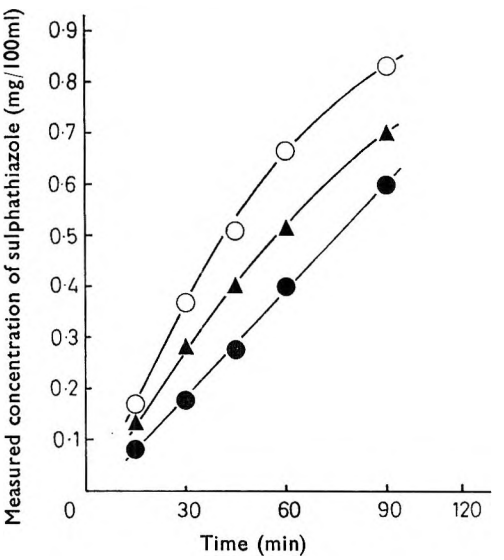

FIG. 4. Effect of polyethylene oxide on the dissolution profile of sulphathiazole tablets. ○ 5% Polyox. ▲ 1% Polyox. ● Sulphathiazole tablets.

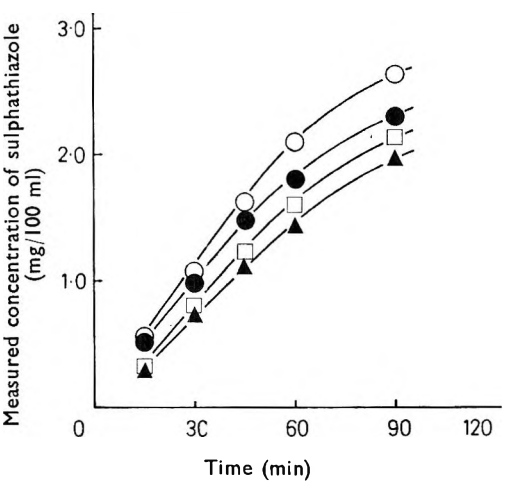

FIG. 5. Effect of starch (maize) on the dissolution profile of sulphathiazole tablets. ○ 10% maize starch. ● 7·5%, □ 5·0%. ▲ 2·5%.

dissolution rate. At low concentrations of polyethylene oxide, the tablets split at right angles to the axis whereas at higher concentrations the tablet became swollen and many fissures were observed. Tablets containing starch disintegrated and increase in the concentration produced a more rapid disintegration. In all cases the results quoted are the mean of two determinations.

Discussion

When adapted for dissolution tests, the British Pharmacopoeia disintegration apparatus has proved satisfactory, mainly because it allows a tablet to dissolve into a constant volume of liquid.

The problem of selecting and standardizing a dissolution test system, with respect to the agitation intensity necessary to provide representative sampling of the solution, has proved to be formidable. Efficient sampling of homogeneous solutions is a prime consideration in these tests, but also, it has been held by Levy (1963) that a certain degree of agitation could represent the conditions prevailing in the stomach. The main object of *in vitro* tests is the attempt to evaluate *in vivo* effects and hence any possible simulation of the latter should be earnestly considered.

Since we used a modified disintegration test apparatus, it was convenient to provide agitation similar to that provided in the official test. The vertical movement of the dialyser at a rate of 30 cycles/min produced suitable turbulence in the recipient fluid and smooth continuous dissolution profiles were obtained. Also, the oscillation of the dialyser produced alternate extensions and relaxations of the membrane when the dialyser was withdrawn from or immersed in the solution.

The dissolution profiles for the tablets made at different pressures indicates a decreased dissolution rate with increasing applied pressure over the range studied. Since the tablets remained intact the area available for dissolution is reduced on increasing the applied pressure by reduction in the tablet porosity. The method of determining dissolution profiles described appears to differentiate adequately between tablets prepared at different pressures. Using the Autoanalyser dialyser, much higher concentrations, indicating higher dissolution rates, were observed, due to the increased agitation of the tablet in the disintegration assembly.

It would be expected that the use of polyethylene oxide to reduce the onset of turbulence would result in an increase in the thickness of the stationary film adjacent to the tablet with an associated decrease in rate of dissolution. In fact, at the concentrations used, the swelling and slightly more hydrophilic nature of the polyethylene oxide appeared to override any retardation effect on dissolution rate and an increased rate was observed. Starch was also used to determine the ability of the apparatus to evaluate differences in dissolution rate of tablets of various disintegration properties. The dissolution rate increased with increasing starch concentration, due to the increased area available for dissolution.

The automated dialysis system is not restricted in use to the Autoanalyzer measuring system. It could, to advantage, be used with any

suitable pumping system and coupled with a recording spectrophotometer using a flow cuvette. In this way a wider range of materials could be examined.

Acknowledgements. We wish to thank the Science Research Council for a grant which permitted the acquisition of the Technicon Autoanalyser and for the financial support of one of us (R.B.B.) which enabled this work to be undertaken. Our thanks are also due to Technicon Instruments Ltd. for the loan of an Autoanalyser Dialyser and to Union Carbide Ltd. for supplying samples of polyethylene oxide.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 239S–247S Received May 1, 1968

The influence of alkyl substitution in acids on their performance in the buccal absorption test

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A method to determine the passage of acids into the buccal membrane of man is presented. The absorption is shown to be the passive entry into a lipid phase. There is an increase in absorption of the acids due to alterations in partition characteristics with increasing chain length of n-fatty acids from butyric to dodecanoic. Methyl substitution of benzoic acid increases the absorption with mono- and di-substituents, but decreases the absorption with the tri- and tetra-substituents by lowering the pK_a. Predictions of the relative absorption and excretion in man of these acids is included.

KNOWLEDGE of the factors affecting the passive transfer of drugs across biological membranes has enabled predictions to be made about the extent that drugs will be absorbed, distributed and excreted in man (Beckett & Triggs, 1967). The buccal absorption test of these authors is now used to indicate the relative importance, in buccal absorption, of pK_a and alkyl chain length in a closely related series of acids.

Experimental

Buffer solutions in the range pH 3·00–9·09 (at 37°) were prepared using McIlvane's citric acid-phosphate buffer for pH values between 3·00–8·00 (Documenta Geigy, 1962a) and borax (0·05M) for pH 9·09. All pH values were measured at room temperature with a Pye Dynacap pH meter.

Solutions of the acids for buccal absorption tests (2 mg acid/ml) and internal standard solutions for chromatography (0·2 mg acid/ml) were prepared by dissolving a suitable quantity of the acid in a slight excess of sodium hydroxide solution.

BUCCAL ABSORPTION MEASUREMENTS

Men aged 20 to 30 were used.

General method. The method of Beckett & Triggs (1967) was used, with the following refinements. A measuring cylinder was used to measure the volume of the expelled buffer solution after the froth had subsided (or a nominal 0·5 ml was given for the froth) and the pH was also measured. The expelled solutions were combined, the internal standard solution (5 ml) added, the volume adjusted to approximately 200 ml and an aliquot (5 ml) used for analysis. The waiting time between successive tests was 30 min.

Drug mixtures. Mixtures containing 1 mg of two to six different acids were investigated using the general procedure.

Analytical technique. The 5 ml aliquot of the expelled solution was placed in a glass-stoppered centrifuge tube together with 6N hydrochloric acid (0·5 ml). The solution was then extracted with 3 × 2·5 ml freshly distilled Analar diethyl ether using a mechanical tilt-shaker, centrifuged,

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and the ether extracts transferred to a 15 ml Quickfit test tube with a finely tapered base (see Beckett, 1966). The extract was then concentrated to about 50 μl on a water bath at 40°. After cooling in ice, a few drops of an ice-cold ethereal solution of diazomethane was added and the tube was shaken. After 1 min 1–2 μl was injected into the gas chromatograph.

A Perkin-Elmer F11 gas chromatograph, with a flame-ionization detector, was used with the following conditions: a 2 m, $\frac{1}{4}$ inch o.d. glass tube packed with Chromosorb G (acid washed, DMCS treated, 80–100 mesh) coated with 2·5% SE-30; nitrogen pressure 20 lb/inch^2, hydrogen pressure 24 lb/inch^2, and air pressure 30 lb/inch^2; injection block temperature approximately 50° above the oven temperature. The oven temperature and internal standard used for each acid are summarized in Table 1.

TABLE 1. GAS-LIQUID CHROMATOGRAPHY CONDITIONS FOR THE ANALYSIS OF SOME ACIDS

Methyl ester of acid	Retention time (min)	Oven temp (° C)	Methyl ester of internal standard	Retention time (min)
n-Butyric	2·6	40	Hexanoic	18·0
n-Valeric	7·0	40	Hexanoic	18·0
n-Hexanoic	3·7	65	Benzoic	13·0
n-Heptanoic	5·6	75	Benzoic	8·7
n-Octanoic	4·3	100	*p*-Toluic	7·6
n-Nonanoic	10·2	95	*p*-Toluic	8·6
n-Decanoic	6·6	125	*p*-Chlorobenzoic	3·9
n-Undecanoic	12·6	125	*p*-Chlorobenzoic	3·9
n-Dodecanoic	23·5	125	*p*-Chlorobenzoic	3·9
Benzoic	4·0	95	*p*-Toluic	8·6
o-Toluic	6·6	100	*p*-Chlorobenzoic	9·6
m-Toluic	7·3	100	*p*-Chlorobenzoic	9·6
p-Toluic	7·6	100	*p*-Chlorobenzoic	9·6
2,4-Dimethylbenzoic	11·8	100	*p*-Toluic	7·6
2,4,6-Trimethylbenzoic	8·2	115	*p*-Toluic	3·9
2,3,5,6-Tetramethylbenzoic	19·4	115	*p*-Toluic	3·9
Phenylacetic	6·1	100	*p*-Chlorobenzoic	9·6
Ibufenac	20·3	125	*p*-Chlorobenzoic	3·9
p-Chlorobenzoic	9·6	100	*p*-Toluic	7·6

The amount of acid remaining in the mouthwash was calculated from the peak height ratio of the acid/internal standard and a calibration curve. The calibration curve was constructed by plotting the peak height ratio against amount of acid, using results obtained from a series of McIlvane buffer solutions containing from 0·1–1·0 mg of acid. All absorption curves were plotted as percentage of acid absorbed against the mean pH of the buffer solution before and after the test. The pH of borax buffer at 37° was calculated from the room temperature measurement and standard correction tables.

Results and discussion

ANALYSIS

The use of gas-liquid chromatography allowed acids in mixtures to be separated (as their methyl esters) and analysed individually. The peaks were almost symmetrical (Fig. 1) and the column had an efficiency equivalent to over 2,500 theoretical plates. One interfering peak was observed

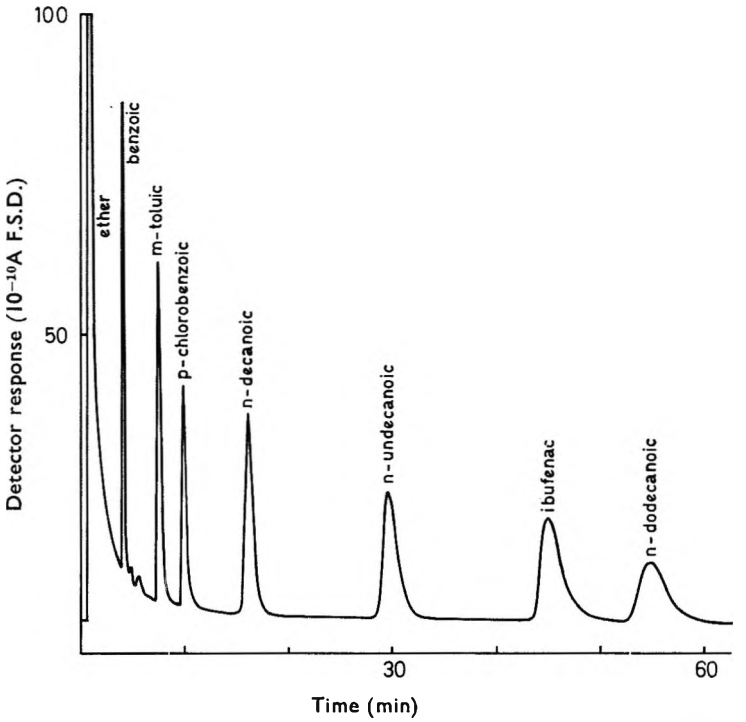

FIG. 1. Chromatogram of some acids (as their methyl esters) on the 2·5% SE-30 column at 100°.

due to methyl citrate (retention time 18·5 min at 125°): this became larger with buffers of low pH.

All calibration graphs were linear over the range 0·1 to 1·0 mg acid in buffer solutions or buffer solutions containing saliva, and the curves were identical for both solutions. Since n-valeric and n-butyric acids were volatile, the error of the analysis of these two acids was high, necessitating duplicate analyses. With all the other acids studied, the standard deviation, obtained from 12 replicate assays, was not greater than 2·5%.

Ethereal solutions of the methyl esters were stored on ice to prevent evaporation. The standard deviation of the method, for benzoic acid, when the ester solution is held at room temperature, is 8·7% and when stored at 0°, 2·1%.

BUCCAL ABSORPTION METHOD

The error in transferring the buffer solution from the beaker to the subject's mouth was 1·0% and has been neglected. Opalescent solutions were obtained when n-undecanoic and n-dodecanoic acids were placed in buffer solutions of low pH, but their absorptions were not affected.

The percentage buccal absorption of a water-soluble acid, phenylacetic acid, and a water-insoluble acid, ibufenac, at pH 4 and at various time intervals is shown in Fig. 2. The rate of absorption was rapid initially

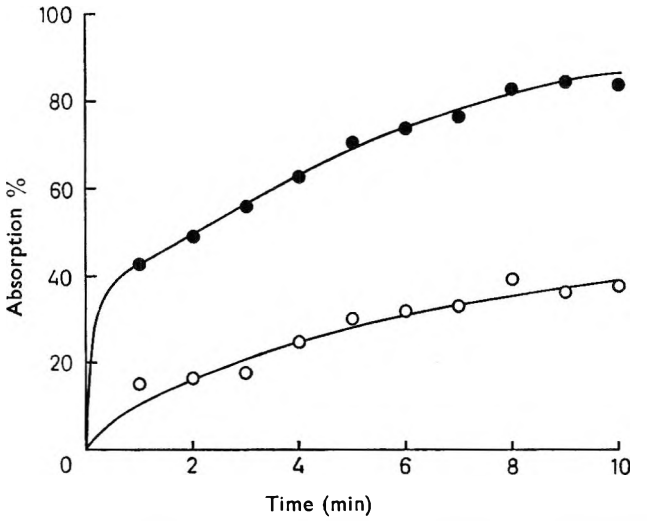

FIG. 2. The effect of solution contact time on the buccal absorption of ibufenac and phenylacetic acid. (Subject PGJ) ●, ibufenac; ○, phenylacetic acid.

but slowed later. A contact time of 5 min was chosen for subsequent experiments with the various acids.

The absorption of *m*-toluic acid at pH 4·0 was determined eight times on one subject, with 30 min wait between each test. The percentage absorption remained constant at about 50% throughout this test and 30 min was therefore allowed to elapse between all tests.

The normal output of saliva from man is 1–2 litres daily, i.e., approximately 3·5–7·0 ml in 5 min, with a mean pH of 6 (Documenta Geigy, 1962b). Thus any solution in the mouth will increase in volume by the amount of saliva and the pH will change towards 6. The buffer solutions must therefore be efficient over a wide pH range and must not interfere with the assay procedure or have a large pH change with temperature. Beckett & Triggs (1967) used Sörensen's phosphate and potassium hydrogen phthalate buffers. However, these are unsuitable for the present studies because the first is not a good enough buffer and the second interferes with the chromatography of the acids. Borax buffer (0·05M) had a large pH-temperature coefficient but standard tables are available for change of pH with temperature. The changes of pH of McIlvane's buffer between room temperature (20°) and 37° did not exceed 0·05 pH units at any pH and its ionic strength remained constant. McIlvane's and the borax buffer were therefore used in the general method.

A pH range of 9·09 to 3·00 was chosen for the general method because solutions of less than pH 3·0 were unpleasant to use and both the volume and pH changes in the mouth were large.

During the early stages of these experiments, many subjects swallowed during the test period and practice was needed before the swallowing reflex could be overcome. The head is best held forwards whilst effecting the test and a second task reduces the temptation to swallow.

The inter-subject variations, determined for ten subjects, in volume and pH changes of buffer solutions of pH values 9·09, 7·0, 5·0 and 3·0 during the 5 min buccal absorption test were mean (with range): 4·7 (1·5 to 10·5); 4·1 (0·0 to 8·5); 8·2 (3·0 to 14·0); 18·3 (8·0 to 31·0) ml and −0·08 (−0·02 to −0·12); −0·01 (0·01 to 0·18); 0·17 (0·07 to 0·44); 0·46 (0·16 to 0·71) respectively. Intrasubject variations, determined for a single subject on ten different occasions were 2·0 (1·0 to 3·0); 2·3 (1·0 to 4·5); 3·5 (2·5 to 5·0); 10·0 (7·0 to 13·0) ml respectively and −0·15 (−0·10 to −0·22); −0·04 (0·00 to −0·09); 0·12 (0·07 to 0·23); 0·24 (0·18 to 0·31) respectively. The absorption of 1 mg of *m*-toluic acid for the same ten subjects at pH 5·0 was 32·1 (22·6 to 40·6) % absorption and at pH 3·0, 61·1 (47·2 to 74·5) % absorption, compared with 49·0 (45·6 to 56·2) % absorption at pH 4·0 for a single subject. Since intra-subject variations in volume and pH changes of the buffer solutions and absorption of *m*-toluic acid were less than the inter-subject variations, a single subject was used for measurements of the buccal absorption of the series of acids below.

Since the concentration of unionized molecules was reduced by increased pH and volume of the test solution, the absorption-pH curves were drawn using the mean pH value before and after the buccal absorption test.

Fig. 3A shows the absorption of *p*-chlorobenzoic acid on three separate occasions using the same individual. Nearly all the experimental points

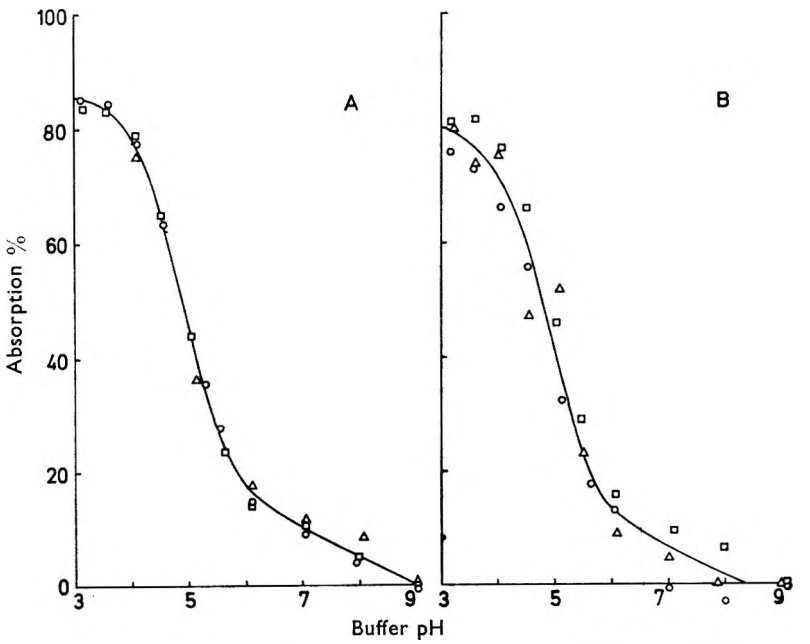

FIG. 3A. The buccal absorption of *p*-chlorobenzoic acid on three separate occasions. (Subject ACM) △, trial 1; ○, trial 2; □, trial 3.
B. The effect of acid concentration on the buccal absorption of *m*-toluic acid (Subject ACM). △, 0·01 mg/25 ml; □, 0·1 mg/25 ml; ○, 1·0 mg/25 ml.

lie on the drawn line showing the reproducibility of absorption of an acid using the buccal absorption test over the whole pH range studied.

The use of 0·01 or 0·1 or 1 mg of acid in the buccal absorption test does not affect its percentage absorption (Fig. 3B), although the analytical error increases with small quantities of acids. Therefore with acids that have not previously been given to man only 0·1 or 0·01 mg quantities of acids need be used, and a borax buffer mouthwash afterwards will return some of the acid to the mouth. Also the number of tests at low pH values should be reduced. For instance, with n-heptanoic acid, at pH 3·15, 91% was absorbed after 5 min, but the use of a borax buffer mouthwash afterwards returned 8%. Thus if 0·01 mg had been used—only 8·3 μg would have remained in the body.

The absorption of individual acids in mixtures containing up to six acids, was the same as the absorption of the acids determined singly. Thus the measured absorption is a true passive transfer into the lipid membrane of the mouth and no specialized transport system for these acids exists.

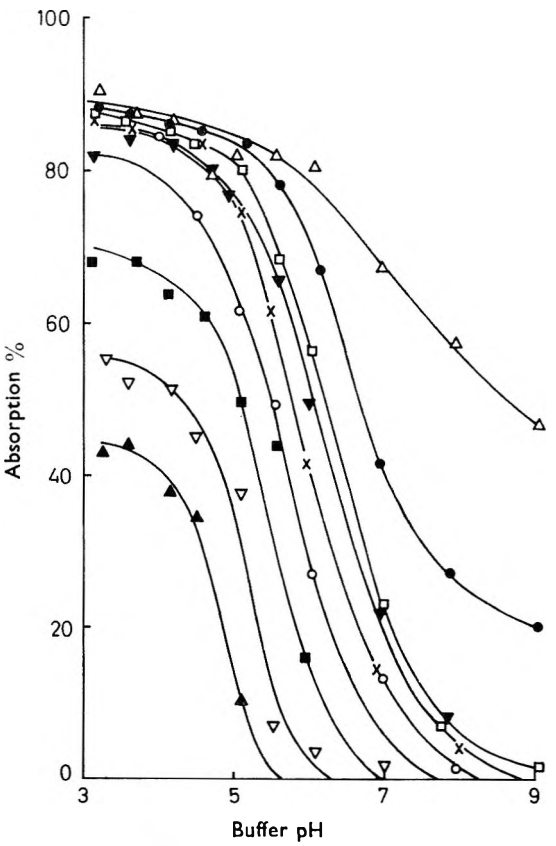

FIG. 4. The buccal absorption of straight chain fatty acids (Subject ACM) △, dodecanoic; ●, undecanoic; □, decanoic; ▼, nonanoic; ×, octanoic; ○, heptanoic; ■, hexanoic; ▽, valeric; ▲, butyric.

BUCCAL ABSORPTION MEASUREMENTS

Three groups of acids have been examined: (*a*) those with the same pK_a value and different lipid solubilities; (*b*) those with different pK_a values and the same lipid solubility; (*c*) those with different pK_a values and lipid solubilities.

(*a*) The absorption of all the long-chain fatty acids studied increased as the pH decreased and the concentration of unionized acid increased (Fig. 4). Since all the acids have approximately the same pK_a value (n-butyric acid, 4·82; n-octanoic acid, 4·85; at 25°, Fieser & Fieser, 1956) the different absorptions of the acids at each pH is due to the different partition characteristics of the unionized forms between the aqueous buffer solution and the cells constituting the epithelium of the buccal cavity. The absorption-pH curves for acids from butyric to decanoic show a gradation of increased absorption of the unionized form with chain length. n-Butyric acid has the lowest lipid-water partition coefficient and the smallest absorption at each pH value. Increasing the chain length by one methylene group greatly increases the rate of entry into the biological membrane. The addition of another one or two methylene groups increases this effect by approximately the same amount. The differences in absorption of n-dodecanoic and n-undecanoic acids from that of n-decanoic acid at pH 9·0 are due to the increased lipid solubility of the ionized forms of the C_{11} and C_{12} acids, so that they penetrate the membrane. On the other hand, surface-active properties would concentrate the molecules at the buffer-membrane interface and also organize some of the ions into micelles.*

(*b*) The curves for the buccal absorption of the three toluic acids are the same (Fig. 5A) displaced from each other by approximately the differences in their pK_a values. This would be expected since the partition characteristics of the unionized forms of the three acids are the same (Beckett & Moffat, unpublished observation).

(*c*) Fig. 5B shows the increased absorption of mono- and di-methyl substituted benzoic acids, over that of benzoic acid. An increase of pK_a by only 0·03 and 0·07 unit respectively (Wilson, Gore & others, 1967) would not account for such large differences between the curves (Fig. 5A). The increased absorption of these two acids is therefore primarily due to the expected increase in the lipid-water partition characteristics of the unionized forms. Absorption of the tri- and tetra-methyl substituted acids were less than that of benzoic acid. This anomolous behaviour cannot be explained simply by their pK_a differences from benzoic acid of $-0{\cdot}65$ and $-0{\cdot}72$ unit respectively (Wilson & others, 1967) unless the pH of the buffer solution is not that at the buffer-buccal membrane interface where absorption takes place. Were this true, changes of pH of the buffer solution would be greater than those at the interface and the above behaviour of the tri- and tetra-methyl benzoic acids is explained.

Beckett & Triggs (1967) showed that results from buccal absorption

* McBain & Hutchinson (1955) state the critical micelle concentration of potassium laurate to be 0 0234M, whilst in these studies the equivalent of a 0·000168M solution was used.

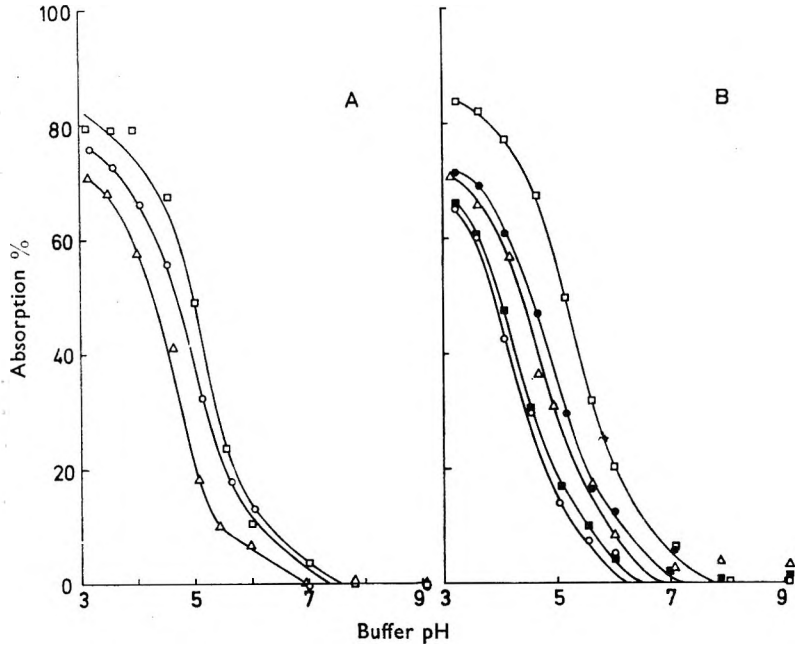

FIG. 5A. The buccal absorption of toluic acids (Subject ACM). □, *para* (pK_a 4·33); ○, *meta* (pK_a 4·24); △, *ortho* (pK_a 3·92).
B. The buccal absorption of methyl substituted benzoic acids (Subject ACM.) □, 2,4-dimethylbenzoic acid (pK_a 4·28); ●, *m*-toluic acid (pK_a 4·24); △, benzoic acid (pK_a 4·21); ■ 2,4,6-trimethylbenzoic acid (pK_a 3·56); ○, 2,3,5,6-tetramethylbenzoic acid (pK_a 3·49).

measurements may be used to predict the excretion of drugs by man. Thus, assuming that there is no binding of the acids nor active transport mechanisms and that the nature of all the cell membranes in the body is the same, predictions may be made of the fate of the long-chain acids in man.

They would be rapidly absorbed from both the stomach (pH 1) and the small intestine (pH 5), (Hogben, Tocco & others, 1959), and since the average pH of human urine is 6·3, all the acids would be reabsorbed from the glomerular filtrate. However, if the urine became alkaline, the excretion of the shorter-chain acids would increase until, at pH 8·0–8·5, both n-butyric and n-valeric acids would be excreted without reabsorption.

The differences in the passage into a biological membrane of acidic molecules differing by even a single methylene group, or having small differences in pK_a values, are clearly shown by the buccal absorption test. The test, whether for acidic or basic substances, is thus a powerful tool for distinguishing between the partition characteristics of members of a closely related group of compounds and has advantage over classical partition coefficient experiments where organic solvents are used which may bear little resemblance to biological membranes.

Acknowledgments. The authors wish to thank all those who participated in the trials. We also thank Boots Pure Drug Company Limited for ibufenac and Professor J. M. Wilson for 2,3,5,6-tetramethyl-benzoic acid.

One of us (A.C.M.) also thanks the Science Research Council for a research studentship.

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J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 248S–253S Received April 3, 1968

The effect of acetylsalicylic acid, phenylbutazone and indomethacin on the binding of 11-hydroxysteroids to plasma proteins in patients with rheumatoid arthritis

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Plasma concentrations of total and unbound 11-hydroxysteroids in patients with rheumatoid arthritis have been determined using a fluorimetric method before and after administration of acetylsalicylic acid, phenylbutazone and indomethacin. Unbound 11-hydroxysteroids were measured in plasma ultrafiltrates obtained using the Toribara apparatus. Enhancement and quenching of fluorescence by acetylsalicylic acid, phenylbutazone, and indomethacin, and heparin used as an anticoagulant in the blood samples, have been shown to be absent. The results show that acetylsalicylic acid, phenylbutazone and indomethacin given in recommended therapeutic doses for periods of one week have no significant effect on plasma protein-binding of 11-hydroxysteroids.

ACETYLSALICYLIC acid, phenylbutazone and indomethacin are well established as effective analgesic and anti-inflammatory drugs in the management of patients with rheumatoid arthritis, although the mechanism of their action is little understood. It has been suggested (Brodie, 1965) that in the rat they liberate corticosterone from its binding sites on the α-globulin carrier-protein, transcortin, and that their action is due to the then unbound corticosteroid, which is considered to be the physiologically active fraction of the total plasma corticosteroid (Booth, Dixon & others, 1961). Acetylsalicylic acid decreases the protein-binding of thyroxine (Christensen, 1959), and phenylbutazone is bound to plasma proteins to the extent of 98% at therapeutic plasma levels (Brodie & Hogben, 1957). We have investigated the effects of acetylsalicylic acid, phenylbutazone and indomethacin on the plasma levels of total and unbound 11-hydroxysteroids in patients with rheumatoid arthritis.

Experimental

METHODS

The investigation was made on twelve patients with rheumatoid arthritis. Five of these had been receiving high doses (4·5 g per day) of acetylsalicylic acid for several weeks before the experiments began, and continued to receive the same doses during the period in which the investigations were made. Blood was withdrawn at 09.00–09.30 hr on each of several days in the course of a week or 10 days, and an ultrafiltrate prepared from each sample. Total 11-hydroxysteroids in each sample were determined in duplicate by the standard procedure described below; unbound 11-hydroxysteroids were determined similarly in each sample of ultrafiltrate, and the protein-binding then calculated.

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The remaining seven patients had previously received a variety of drug treatments for rheumatoid arthritis. Each of these seven patients received a placebo consisting of calcium lactate (600 mg) in tablet form daily for one week, after which blood was withdrawn at 09.00–09.30 hr on the eighth day and examined as in the first series of patients. Throughout the following week the same seven patients were treated with acetylsalicylic acid [Solprin: 3 tablets (each containing 324 mg acetylsalicylic acid) five times a day] and protein-binding of 11-hydroxysteroids was determined in 09.00–09.30 hr blood samples taken on the fifteenth day. In four of of the seven patients aspirin was replaced in the third week by phenylbutazone (Butazolidin, 3 × 100 mg tablets daily) and in the fourth week by indomethacin (Indocid capsules, 2 × 25 mg daily). Protein-binding of 11-hydroxysteroids was determined at the end of each of the third and fourth weeks in 09.00–09.30 hr blood samples taken on the 22nd and 29th days respectively. Protein-binding was also determined on samples taken at 1800 hr from two of the four patients at the end of each regime.

The concentration of salicylates in plasma samples on which 11-hydroxysteroid samples were made was determined by the method of Trinder (1954) before and after treatment with acetylsalicylic acid.

COLLECTION OF BLOOD AND ULTRAFILTRATION OF PLASMA

Blood (60 ml) was withdrawn by venepuncture and immediately distributed in heparinized centrifuge tubes (10 ml). Erythrocytes were separated from the plasma by centrifuging for 10 min.

Protein-free ultrafiltrates of the plasma samples were obtained using the apparatus of Toribara (1953). Plasma was placed in Visking tubing (8/32 inch) in the special centrifuge tubes, and centrifuged at 2000 rev/min for 2 hr at 37°; about 5 ml of ultrafiltrate was obtained from each 20 ml of plasma. Pooled ultrafiltrates (one drop) were tested with salicylsulphonic acid 20% w/v in water (1 ml); only those ultrafiltrates, shown to be protein-free by this method, were used.

DETERMINATION OF 11-HYDROXYSTEROIDS

Plasma concentrations of total* and unbound 11-hydroxycorticosteroids were determined by the spectrofluorometric method of Mattingly (1962), observing his recommendations for the purification of dichloromethane, preparation of reagents and cleaning of glassware. The method is not specific for 11-hydroxycorticosteroids (hydrocortisone), but 90% of the plasma 11-hydroxysteroids is known to be hydrocortisone.

Up to four plasma samples, ultrafiltrates or other test solutions were assayed simultaneously. A reagent blank (de-ionized water, 2 ml) and a standard hydrocortisone solution (0·5 μg/ml; 2 ml) were carried through the procedure with each batch.

* For the purpose of this investigation total hydrocortisone means the total of unbound and protein-bound 11-hydroxysteroids. The term "protein-binding" will be used to mean the ratio of unbound hydrocortisone to the total of unbound and protein-bound hydrocortisone.

Hydrocortisone standards. Hydrocortisone (B.D.H.) (50 mg) was dissolved in absolute ethanol (50 ml) (absolute alcohol, James Burroughs Ltd). A stock solution (5 mg; 100 ml) was prepared by diluting this solution (5 ml to 100 ml) with de-ionized water. Further dilution of this solution as necessary gave a working standard of 50 μg/100 ml.

Apparatus. An Aminco-Bowman Spectrophotofluorimeter was used with 1 cm quartz cells, and slit program No. 3. The fluorimeter was coupled to an XY recorder, and excitation and emission spectra of all standard and test solutions were recorded. For excitation spectra the emission monochromator was set at 522 mμ and for emission spectra the excitation monochromator was set at 466 mμ.

TESTS FOR FLUORESCENCE OR QUENCHING FROM EXTRANEOUS SUBSTANCES

Heparin. De-ionized water (5 ml) was shaken in a heparinized centrifuge tube and an aliquot (2 ml) of the solution submitted to the standard procedure. Water alone was simultaneously submitted to the procedure. A standard hydrocortisone solution was similarly shaken in a heparinized tube and a sample (2 ml) of the resultant solution, and, also at the same time, the solution of hydrocortisone alone, was subjected to the standard procedure. Heparin neither gave rise to fluorescence when examined alone, nor did it quench the fluorescence of hydrocortisone (Fig. 1B).

Drugs. Acetylsalicylic acid (10 mg) and phenylbutazone (10 mg) were each added separately to the fluorescence reagent (5 ml), and the fluorescence measured under the standard conditions. Since the contents of an Indocid capsule fluoresced strongly in the reagent, a saturated solution of indomethacin (18 μg/ml) (2 ml) was likewise put through the standard procedure. The first two compounds gave rise to no measurable fluorescence, the third had the same fluorescence as the de-ionized water blank.

Solutions of acetylsalicylic acid (0·4 mg/ml; 1 ml), phenylbutazone (0·1 mg/ml; 1 ml) and indomethacin (18 μg/ml; 1 ml) were added to standard hydrocortisone solution (1 ml) and the mixture assayed by the standard procedure. The relative fluorescence intensity was the same as that produced by dilution of the hydrocortisone with deionized water.

Results and discussion

Wavelengths of the peaks in the excitation and emission spectra of both plasma and ultrafiltrate samples corresponded with those produced by the standard solution of hydrocortisone in each set of determinations (Fig. 1).

Plasma itself has been reported to produce non-specific fluorescence (Braunsberg & James, 1962; Daly & Spencer-Peet, 1964). Absolute values of hydrocortisone, however, were not required, since the object of the present investigation was to study the effect of the clinical treatment on the proportion of unbound to total 11-hydroxysteroids. Careful examination of excitation and emission curves for several of the treated plasma and ultrafiltrate samples revealed no abnormality as the curves were similar to those produced by authentic samples of hydrocortisone. It is thus reasonable to assume that hydrocortisone, as the major 11-hydroxysteroid, was being measured.

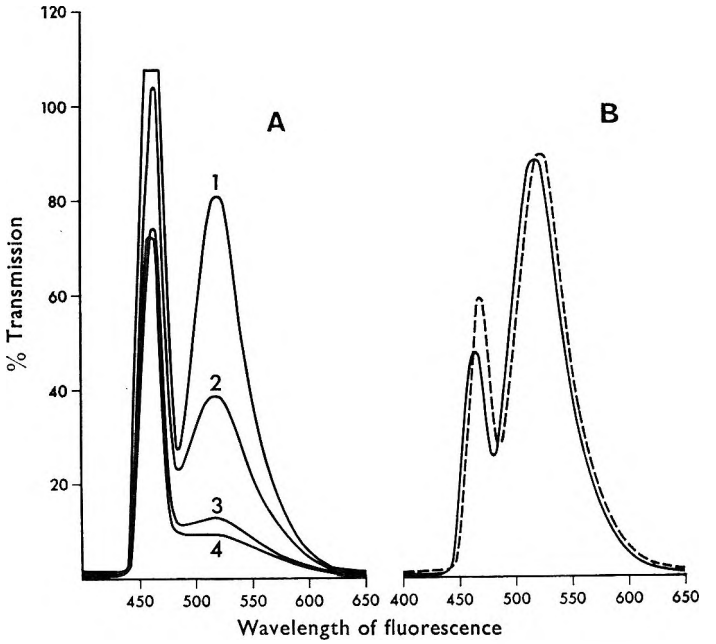

Fig. 1. Fluorescence spectra obtained with Aminco-Bowman Spectrofluorimeter of: A. 1, Standard cortisol, 2, Plasma total 11-hydroxysteroids, 3, Plasma unbound 11-hydroxysteroids. 4, Blank. B. ——— Standard cortisol. ------ Heparin in the presence of standard cortisol. The curves in B are strictly superimposable.

Plasma levels of 11-hydroxysteroids and protein-binding in patients with rheumatoid arthritis treated in the first clinical study with acetylsalicylic acid are given in Table 1. This shows that the protein-binding of corticosteroids in the plasma of rheumatoid arthritic patients treated with acetylsalicylic acid does not differ significantly from previously reported normal values. Using an isotopic technique, Plager, Schmidt & Staubitz (1964) have observed that the ratio of unbound hydrocortisone to the total hydrocortisone in normal adult plasma is 12.64 ± 3·20% (mean ± s.d.). We found that in 23 duplicate determinations upon the plasma of five rheumatoid arthritic patients, treated with aspirin, the protein-binding value was 11·20 ± 2·73% (mean ± s.d.).

TABLE 1. PLASMA LEVELS OF 11-HYDROXYSTEROIDS IN PATIENTS WITH RHEUMATOID ARTHRITIS TREATED WITH ACETYLSALICYLIC ACID

Patient No.	No. of duplicate determinations	Mean concentration of plasma 11-hydroxysteroids		Mean protein binding (%)
		Unbound μg %	Total μg %	
1	5	2·02	16·4	11·9
2	4	1·99	18·3	11·0
3	4	1·64	14·8	11·3
4	5	1·34	13·3	10·1
5	5	1·23	10·6	11·6

Mean (± s.d.) protein-binding (%) for all 23 determinations = 11·20 ± 2·73.

TABLE 2. PLASMA LEVELS OF TOTAL AND UNBOUND 11-HYDROSTEROIDS IN PATIENTS WITH RHEUMATOID ARTHRITIS TREATED IN EACH OF FOUR SUCCESSIVE WEEKS WITH A CALCIUM LACTATE PLACEBO, ACETYLSALICYLIC ACID, PHENYLBUTAZONE AND INDOMETHACIN RESPECTIVELY

Patient No.	Plasma 11-hydroxy-steroids*		Protein-binding (%)	Plasma 11-hydroxy-steroids*		Protein-binding (%)
	Unbound µg %	Total µg %		Unbound µg %	Total µg %	
	1st week placebo			2nd week acetylsalicylic acid		
6	3·15	21·1	14·9	2·21	15·2	14·5
7	1·40	12·0	11·7	1·16	12·0	9·7
8	1·33	8·3	16·0	1·38	9·2	15·0
9	1·37	11·7	11·7	0·93	9·5	9·8
10	1·82	19·5	9·3	2·07	18·0	11·5
11	1·21	15·1	8·0	1·78	15·1	11·8
12	2·47	19·0	13·0	0·96	10·8	8·9
	3rd week phenylbutazone			4th week indomethacin		
6	2·02	15·3	13·2	2·26	19·1	11·8
7	1·23	12·0	10·2	1·88	17·3	10·9
8	1·88	16·4	11·5	1·67	10·9	15·3
9	1·05	12·0	8·7	1·84	11·7	15·7

* Each result is the average of two determinations.

Mean (± s.d.) protein-binding (%): placebo 12·1 ± 2·85; acetylsalicylic acid 11·6 ± 2·38; phenylbutazone 10·9 ± 2·27; indomethacin 13·4 ± 2·43

Unbound and total hydrocortisone plasma levels (09.00 hr) and protein-binding in the seven patients treated successively with placebo, aspirin, phenylbutazone and indomethacin are presented in Table 2.

The results for these patients treated with a placebo and then with acetylsalicylic acid show no significant difference in protein-binding before and after treatment. The mean salicylate level in the plasma of patients before treatment with acetylsalicyclic acid was 1 mg % and that during therapy, 20 mg %, indicating that therapeutic levels had been achieved. The number of patients studied after treatment with phenylbutazone and indomethacin was too small for full statistical analysis, but the results suggest that no significant difference in protein-binding occurs as a result of treatment with these drugs.

The patients studied experienced definite relief of joint symptoms during treatment. The results therefore suggest that the anti-rheumatic action of these drugs is not associated with a significant degree of displacement of 11-hydroxysteroids from plasma proteins. This conclusion is in accord with the views of Theobold & Domenjoz (1956), of Winter, Risley & Nuss (1963) and of Winter, Risley & Silber (1967), based on experiments with acetylsalicylic acid, phenylbutazone and indomethacin in adrenalectomized rats. It has also been shown (Jansen & Schou, 1967) that neither phenylbutazone, sodium salicylate nor indomethacin influence binding of hydrocortisone to plasma proteins in the guinea-pig.

The level of total (bound and unbound) plasma unconjugated 11-hydroxysteroids is reported to be subject to diurnal variation (Bliss, Sandberg, Nelson & Eik-Nes, 1953). Diurnal variation was observed in the levels of both unbound and total plasma 11-hydroxysteroids of two

TABLE 3. PLASMA LEVELS OF TOTAL AND UNBOUND 11-HYDROXYSTEROIDS AT 09.00 AND 18.00 HR IN TWO PATIENTS WITH RHEUMATOID ARTHRITIS TREATED IN EACH OF FOUR SUCCESSIVE WEEKS WITH A CALCIUM LACTATE PLACEBO, ACETYLSALICYLIC ACID, PHENYLBUTAZONE AND INDOMETHACIN RESPECTIVELY

Treatment	09.00 hr			18.00 hr		
	Plasma 11-hydroxy-steroids*		Protein-binding (%)	Plasma 11-hydroxy-steroids*		Protein-binding (%)
	Unbound μg %	Total μg %		Unbound μg %	Total μg %	
Patent No. 6						
1st week placebo	3·15	21·1	14·9	1·55	14·0	11·1
2nd week acetylsalicylic acid	2·21	15·2	14·5	2·09	11·0	19·0
3rd week phenylbutazone	2·02	15·3	13·2	0·87	9·8	8·9
4th week indomethacin	2·26	19·1	11·8	1·82	18·3	10·0
Patient No. 7						
1st week placebo	1·40	12·0	11·7	0·40	6·0	6·7
2nd week acetylsalicylic acid	1·16	12·0	9·7	0·81	10·2	7·9
3rd week phenylbutazone	1·23	12·0	10·2	0·62	6·9	9·0
4th week indomethacin	1·88	17·3	10·9	0·75	10·7	7·0

* Each result is the average of two determinations.

patients with rheumatoid arthritis during treatment with the drugs (Table 3), but without apparent variation in the protein-binding.

Acknowledgements. This work, which was carried out during the tenure of a CIBA Clinical Research Fellowship (MKJ) and a Pharmaceutical Society Schclarship (AGD), was supported by grants from the Arthritis and Rheumatism Council of Great Britain and the Nuffield Foundation.

We are grateful to Dr. W. W. Buchanan for helpful criticism, to Miss Mary Gardner for plasma salicylate determinations, and also to Dr. T. B. Binns, Director of Medical Research, CIBA Laboratories, Horsham, England, for valuable discussions and encouragement.

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Printed by W Heffer & Sons Ltd Cambridge England

Journal of Pharmacy and Pharmacology

1968, Volume 20

With Supplement:

British Pharmaceutical Conference
One Hundred and Fifth Meeting
Birmingham

September 9 to 13, 1968

London
The Pharmaceutical Press
17 Bloomsbury Square, W.C.1

Journal of Pharmacy and Pharmacology
1968, Volume 20

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Journal of Pharmacy and Pharmacology

British Pharmaceutical Conference Supplement