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Solubilization of griseofulvin by nonionic surfactants

P. H. ELWORTHY AND F. J. LIPSCOMB

Four nonionic surfactants, $Me[CH_2]_{15}\cdot[OCH_2\cdot CH_2]_xOH$ where x = 10, 22, 45 and 60, and polyoxyethylene glycols 200, 400 and 1000 increased the solubility of griseofulvin over its value in water. Cetomacrogol micelles solubilize two molecules of griseofulvin per micelle. The site of solubilization seems likely to be the polyoxyethylene part of the micelles. The partial molal heats and entropies of solution decrease as surfactant or polyoxyethylene glycol concentration is increased.

SURFACTANTS may play an important role in the dissolution step of an insoluble drug, such as griseofulvin, before absorption (Bates, Gibaldi & Kanig, 1966). Bates, Lin & Gibaldi (1967) have shown that lysolecithin is capable of solubilizing large amounts of griseofulvin. To effect an increase in absorption of griseofulvin by the body by artificial means, solubilized systems, or admixtures of detergent and drug, might be effective. Some nonionic detergents have been examined, as in general these are the least toxic of detergents.

Experimental

The materials used were commercial detergents based on hexadecanol with varying numbers of ethylene oxide units (abbr. to HE_x) e.g. $Me[CH_2]_{15}[OCH_2\cdot CH_2]_xOH$, where x = 10, 22, 45 and 60 (Glovers Chemicals). The material with x = 22 is Cetomacrogol B.P. The detergent P40 (Shell) or Triton X100, $Me_3C\cdot CH_2\cdot CMe_2\cdot C_6H_4[OCH_2\cdot CH_2]_{10}\cdot OH(OE_{10})$ was used, and for comparative purposes, polyoxyethylene glycols (PG) 200, 400 and 1000. Deionization of these compounds did not affect the solubility results. The ethylene oxide content of the detergents was checked using the method of Siggia, Stark & others (1958). Apart from the nominal HE_{45} and HE_{60}, which contained 38 and 50 ethylene oxide units respectively, the detergents had the composition stated above.

Solubilities were determined by the method of Elworthy & Lipscomb (1968), and measurements were made at different temperatures to evaluate the partial molal heats ($\Delta\bar{H}$) and entropies ($\Delta\bar{S}$) of solution. The concentrations of the saturated solutions were determined by suitably diluting the solution to give a final concentration of 50% v/v ethanol; calibration curves were prepared in the same solvent, arranged to contain the same amount of detergent as present after dilution of the saturated solution.

The maximum wavelength of absorption was determined in the various "solvents" used giving the following results for λ_{max} (in mμ): water 296, HE_x detergents and polyoxyethylene glycols 295, OE_{10} 294, benzene 291, heptane 287.

Viscosities of detergent solutions relative to water were measured at 25° in an Ostwald capillary viscometer.

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Results and discussion

The results of the solubility determinations are given in Table 1, expressed as moles griseofulvin dissolved in 1000 g solution i.e., molality, counting the detergent or polyoxyethylene glycol as part of the solvent.

With the detergent HE_{10}, cloudy solutions were formed at concentrations below 4%, and erratic results were obtained. In view of this experimental difficulty, the two results given in Table 1 for this detergent are considered approximate. Fig. 1 shows that solubilization in the detergent solutions increases linearly with detergent concentration. When the detergents are compared on a percentage basis (i.e., Table 1) the compounds containing the shortest polyoxyethylene chains appear to solubilize the largest amount of griseofulvin. However, when equimolar concentrations are compared (Fig. 1) the detergents containing the longest polyoxyethylene chains have greatest solubilizing power, solubilization decreasing with decreasing polyoxyethylene chain length.

TABLE 1. SOLUBILITY OF GRISEOFULVIN IN SOLUTIONS OF NONIONIC SURFACTANTS AND POLYOXYETHYLENE GLYCOLS

Concentration % w/v	Solubility (molality × 10^5) at 15°	25°	35°	45°
Cetomacrogol				
0·007	$2{\cdot}0_4$	$2{\cdot}8_6$	$3{\cdot}9_5$	$6{\cdot}3_3$
0·997	16·9	17·9	21·9	26·4
5·500	$92{\cdot}_6$	$97{\cdot}_6$	107·1	$116{\cdot}_8$
15·01	$245{\cdot}_8$	$257{\cdot}_6$	$285{\cdot}_8$	$344{\cdot}_6$
OE_{10}				
0·30	7·2	$8{\cdot}3_4$	11·3	
1·005	$20{\cdot}_6$	$24{\cdot}_4$	$29{\cdot}_3$	
5·019	$97{\cdot}_9$	$107{\cdot}_1$	$132{\cdot}_0$	
10·02	$196{\cdot}_3$	$210{\cdot}_3$	$262{\cdot}_2$	
19·74	363	413	$509{\cdot}_4$	655
HE_{10}				
7·45		128		
11·88		203		
HE_{28}				
0·997		13·0		
5·003		$56{\cdot}_6$		
15·011		$169{\cdot}_4$		
HE_{50}				
0·999		12·5		
5·007		$50{\cdot}_5$		
15·012		$155{\cdot}_3$		
PG200				
18·35	10·0	10·3	19·4	33·8
19·71	—	12·0	18·6	33·0
45·90	$42{\cdot}_7$	$61{\cdot}_3$	$103{\cdot}_6$	$193{\cdot}_3$
75·21	293	398	567	831
112·8	1737	2028	2531	3021
PG400				
11·15		5·7		
21·35		12·0		
33·69	25·6	30·3	73·5	110·5
63·87	228	248	409	782
86·18	923	1104	1516	2445
112·9	2880	3206	3852	4386
PG1000				
8·40		4·8		
14·80		7·1		
24·25		15·7		
36·64		35·6		
69·98		414		
82·79		1170		

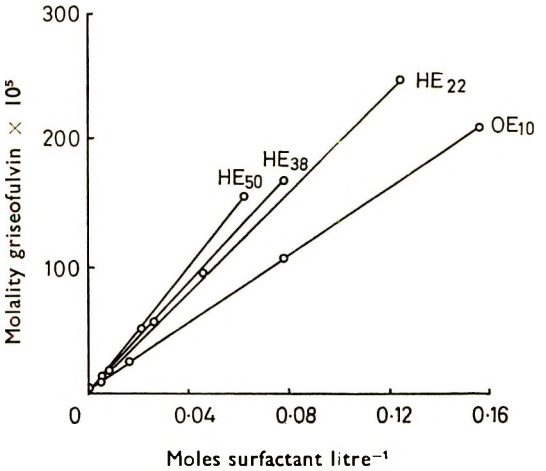

FIG. 1. Solubilization of griseofulvin by various nonionic surfactants.

But the detergents solubilize far more griseofulvin than polyoxyethylene glycol mixtures of equal concentration.

The ratios of mole detergent/mole griseofulvin solubilized calculated at 10% w/v detergent concentrations are:

Detergent	OE_{10}	HE_{10}	HE_{22}	HE_{38}	HE_{50}
Molar ratio	74	(85)	49	46	40

As the critical micelle concentrations (CMC) of these detergents are very low, e.g., 0·007% for cetomacrogol (Elworthy, 1960a), the solubility of griseofulvin in water has been subtracted from the total solubility in the detergent solution, in calculating the ratio. Use of the solubility at the CMC would have been strictly correct, but the error introduced by using the solubility in water is small, as the CMC values are so low, and the detergent concentration high.

It can be seen that the larger the polyoxyethylene chain, the greater is the amount of solubilization per mole of detergent. Because of the experimental difficulties, the molar ratio for HE_{10} is approximate.

The micelle of the OE_{10} compound has been shown to contain 140 detergent monomers (Kushner & Hubbard, 1954), while that of cetomacrogol contains 83 monomers (Elworthy, 1960b). For these detergents, roughly two griseofulvin molecules are solubilized per micelle. Micelle sizes for the HE_{38} and HE_{50} compounds are not known.

The solubilization of griseofulvin by detergent and polyoxyethylene glycol solutions increased with temperature; Fig. 2 gives the solubilities at different temperatures in cetomacrogol solution.

The solubility of griseofulvin is increased by polyoxyethylene glycols. Fig. 3 shows the relation between log molality of griseofulvin, and % w/w of polyoxyethylene glycol. Between 20 and 70% w/w, the graph is linear, and there appears to be very little difference between the

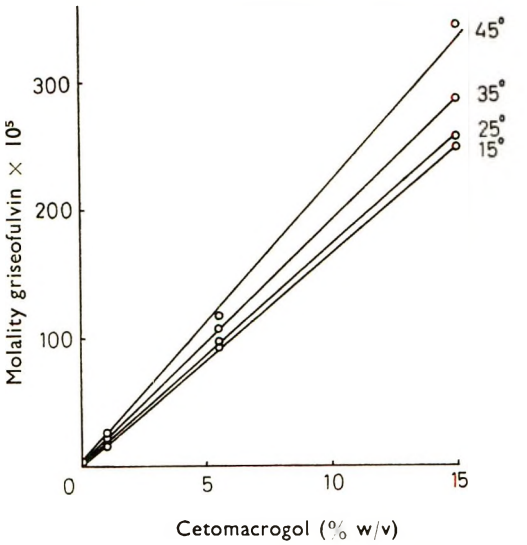

FIG. 2. Effect of temperature on the solubilization of griseofulvin in cetomacrogol solutions.

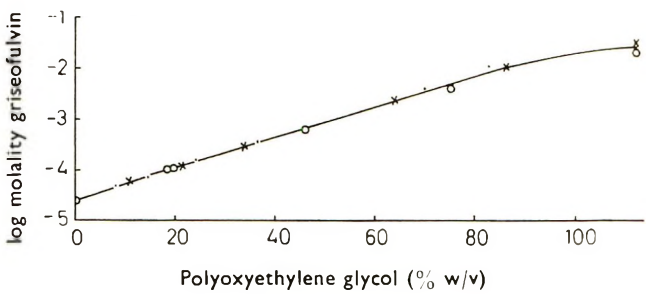

FIG. 3. Solubility of griseofulvin (log molality) in various polyoxyethylene glycol-water mixtures. ○ = PG200. × = PG400. ● = PG1000.

behaviour of griseofulvin in PG200, 400, or 1000. That the polyoxyethylene glycols increase the solubility of griseofulvin in water, and the length of the polyoxyethylene chain also affects solubility, may indicate that solubilization takes place in the polyoxyethylene-water layer of the micelles.

It has been shown (Elworthy, 1960b), that cetomacrogol forms spherical micelles, which are hydrated with 1·96 g water/g detergent. Similar conclusions on sphericity have been reached by Kushner & Hubbard (1954) for the OE_{10} compound, and from their viscosity results a hydration of 1·26 g water/g detergent can be calculated from

$$[\eta] = 2{\cdot}5\ (\bar{v} + w\ v_1^{\circ})$$

where $[\eta]$ is the intrinsic viscosity, obtained by plotting the specific

viscosity divided by the concentration against concentration, where

$$[\eta] = \left(\frac{\eta_{sp}}{c}\right) c = 0$$

$\bar{v}$ is the specific volume of detergent, w is the hydration, and v_1° is the specific volume of water. Elworthy & Macfarlane (1963) have shown that micelles formed by monohexadecyl polyoxyethylene ethers were spherical when the numbers of ethylene oxide units present in the chain exceeded nine. It seems a reasonable assumption that the micelles of HE_{38} and HE_{50} are also spherical. Hence the measured viscosities are plotted in Fig. 4 giving intercept of 8·7 and 9·4 respectively, and the corresponding hydrations are 2·6 and 2·9 g/water/g detergent respectively.

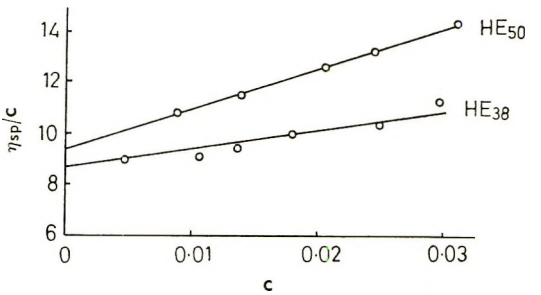

FIG. 4. Graph of $\frac{\eta_{sp}}{c}$ against concentration (g ml^{-1}) for HE_{38} and HE_{50}.

An attempt was made to calculate the solubility of griseofulvin in the micelles from the data on its solubility in polyoxyethylene glycols. In 1 g of cetomacrogol, which is associated with 1·96 g water, there is present 0·814 g glycol; thus we can consider the polyoxyethylene glycol-water part of the micelle as a 29·3% w/w solution. The solubility of griseofulvin in a solution of this concentration is 26×10^{-5} molal (Fig. 3) and 1 g of glycol is thus associated with $0{\cdot}09 \times 10^{-5}$ mole of griseofulvin. 1 g of cetomacrogol therefore has $0{\cdot}07 \times 10^{-5}$ mole griseofulvin theoretically solubilized in its glycol-water complex. From the solubility data, 1 g of cetomacrogol in a 10% w/v solution solubilized $1{\cdot}7 \times 10^{-5}$ mole of griseofulvin. Therefore about 25 times more griseofulvin is actually solubilized than can be accounted for on the basis of the calculation. The same picture emerges for the other detergents. For OE_{10} the practical value at 10% w/v detergent concentration is $2{\cdot}1 \times 10^{-5}$ mole/g detergent ($0{\cdot}07 \times 10^{-5}$), for HE_{38} $1{\cdot}1 \times 10^{-5}$ mole/g detergent ($0{\cdot}07 \times 10^{-5}$), for HE_{50} $1{\cdot}0 \times 10^{-5}$ mole/g detergent ($0{\cdot}07 \times 10^{-5}$). The theoretical figures are in brackets.

For the cetyl chain-containing compounds, calculations of the solubility of griseofulvin in the hydrocarbon core, based on the solubility in pure heptane, give values 10^3–10^4 times too small. For OE_{10}, solubility data in an alkyl-aryl chain hydrocarbon resembling the hydrophobic

moiety of this detergent are not available, but even using the solubility data in benzene, a figure of 50% of the observed solubility is obtained; it must be borne in mind that the hydrocarbon chain of the OE_{10} compound contains only about 40% of the phenyl group, so that the solubility of griseofulvin in it would be expected to be less than that in benzene, thus giving an even lower calculated solubility.

The above calculations have been based on the assumptions: (1) That the size and hydration of the micelles are unaltered when the solubilizate is present. (2) That the solubility in bulk polyoxyethylene glycol-water mixtures, and in bulk hydrocarbon liquids can be used to calculate the solubility in the micelles. (3) That the polyoxyethylene-water layer is homogeneous from the surface of the hydrocarbon core to the micelle surface.

As there is only a small amount of solubilization, the first assumption is probably reasonable. The assumption on using bulk solubility in hydrocarbons for the purposes of calculation is perhaps not important, at least for the aliphatic hydrocarbon chain detergents, as the calculated solubilities are 10^3–10^4 times too low. The third assumption, and part of the second relating to polyoxyethylene glycol-water mixtures require further examination. Schick (1963), has shown that in nonionic detergent micelles, the polyoxyethylene chain is arranged in an expanding spiral, i.e., a cone shape, with the narrower end of the cone at the surface of the hydrocarbon core. Macfarlane's work (1963) gave a similar idea, and molecular models showed that while there was plenty of room for hydrating water in the outer parts of the micelles, there was virtually no space for it close to the surface of the hydrocarbon core, due to crowding of the polyoxyethylene chain.

This region, adjacent to the hydrocarbon region, seems the most likely site for the griseofulvin within the micelles, as it is largely purely polyoxyethylene (rather than polyoxyethylene-water) in nature, and the solubility of griseofulvin in pure polyoxyethylene glycols is quite large. (Unfortunately, no calculation of theoretical solubility can be made, as the volume of the purely polyoxyethylene glycol region is unknown.) Also, the λ_{max} values are quite close in the detergents and polyoxyethylene glycol solution (probably reflecting a somewhat similar environment), and different from those in heptane and benzene.

The thermodynamics of solubilization also indicate a similarity between the behaviour of griseofulvin in polyoxyethylene glycol-water mixtures, and in the detergents, and a distinct difference from the behaviour in water and hydrocarbons. The probable site of solubilization seems to be in the polyoxyethylene-water layer of the micelles, with the possibility of it being close to the hydrocarbon-polyoxyethylene boundary.

THERMODYNAMICS OF SOLUBILIZATION

Partial molal heats and entropies calculated as before (Elworthy & Lipscomb, 1968) are listed in Table 2. The "solvent" here is the detergent solution, so the thermodynamic properties relate to the change from the crystalline state to a very large amount of saturated solution.

TABLE 2. THERMODYNAMIC PROPERTIES FOR SOLUTION OF GRISEOFULVIN IN NONIONIC DETERGENT AND POLYOXYETHYLENE GLYCOL SOLUTIONS* (% w/v)

		Temperature		
		20°	30°	40°
Water	$\Delta\bar{H}$	5·1	6·5	10·0
	$\Delta\bar{S}$	17	21	32
Cetomacrogol 1%	$\Delta\bar{H}$	1·2	2·5	3·5
	$\Delta\bar{S}$	4·1	8·3	11
Cetomacrogol 5%	$\Delta\bar{H}$	1·2	1·7	2·1
	$\Delta\bar{S}$	4·1	5·6	6·7
Cetomacrogol 10%	$\Delta\bar{H}$	1·2	1·5	1·7
	$\Delta\bar{S}$	4·1	5·0	5·4
Cetomacrogol 15%	$\Delta\bar{H}$	1·2	1·5	1·6
	$\Delta\bar{S}$	4·1	5·0	5·1
PG200 34%	$\Delta\bar{H}$	6·5	9·5	10·7
	$\Delta\bar{S}$	22	31	34
PG200 54%	$\Delta\bar{H}$	6·0	8·4	9·3
	$\Delta\bar{S}$	21	27	30
PG200 75%	$\Delta\bar{H}$	5·3	7·6	8·5
	$\Delta\bar{S}$	18	25	27
PG200 112·8%	$\Delta\bar{H}$	3·1	3·6	3·8
	$\Delta\bar{S}$	11	12	12
OE_{10} 1%	$\Delta\bar{H}$	2·2	4·2	—
	$\Delta\bar{S}$	7·5	14	—
OE_{10} 5%	$\Delta\bar{H}$	1·4	3·2	—
	$\Delta\bar{S}$	4·8	11	—
OE_{10} 10%	$\Delta\bar{H}$	1·4	3·6	—
	$\Delta\bar{S}$	4·8	12	—
OE_{10} 15%	$\Delta\bar{H}$	1·3	3·7	—
	$\Delta\bar{S}$	4·4	12	—
PG400 34%	$\Delta\bar{H}$	6·4	11·3	14·5
	$\Delta\bar{S}$	22	37	46
PG400 54%	$\Delta\bar{H}$	4·6	9·2	12·6
	$\Delta\bar{S}$	16	30	40
PG400 75%	$\Delta\bar{H}$	3·0	6·8	10·8
	$\Delta\bar{S}$	10·3	22	35
PG400 112·9%	$\Delta\bar{H}$	2·3	2·8	3·1
	$\Delta\bar{S}$	7·9	9·3	9·9

$\Delta\bar{H}$ in kcal $mole^{-1}$, $\Delta\bar{S}$ in cal $mole^{-1}deg^{-1}$.

* Solubilities used for calculation of the thermodynamic properties were obtained from smoothed curves of solubility against concentration for surfactants, and log solubility against concentration for polyoxyethylene glycols.

As either detergent or polyoxyethylene glycol concentration is increased the thermodynamic properties decrease, e.g., $\Delta\bar{H}$ and $\Delta\bar{S}$ become less positive. In 15% cetomacrogol and pure PG400, at 20°, $\Delta\bar{H}$ is 1·2 and 2·3 kcal $mole^{-1}$, and $\Delta\bar{S}$ is 4·1 and 7·9 cal $mole^{-1}$ deg^{-1} respectively. These figures are less than half the value obtained in pure water. Inspection of Table 2 reveals some correspondence of the thermodynamic values obtained in detergent solutions and in concentrated polyoxyethylene glycols, which indicates the possibility that griseofulvin is held in a polyoxyethylene glycol environment when solubilized in the micelle. These figures cannot be expected to correspond exactly because of the difference in the types of solutions being compared. Above 10% cetomacrogol and OE_{10} concentrations, $\Delta\bar{H}$ lies in the regions 1–2 kcal $mole^{-1}$

and $\Delta\bar{S}$ 4–5 cal mole^{-1} deg^{-1}, which corresponds moderately well with figures obtained at high polyoxyethylene glycol concentrations, $\Delta\bar{H}$, 2–3 kcal mole^{-1}, $\Delta\bar{S}$, 8–11 cal/mole^{-1} deg^{-1} (values at 20°). In the less concentrated polyoxyethylene glycol solutions the thermodynamic properties are fairly close to those obtained in water.

Considering concentrated detergent solutions and pure polyoxyethylene glycols, the lower entropies obtained suggest that the griseofulvin is in a more ordered state than when dissolved in pure water, possibly due to its association with the glycol or detergent. $\Delta\bar{H}$ is also lower than in water, and as the heat required to break up the crystal will be the same for dissolution in water as in a detergent or polyoxyethylene glycol solution, either the heat of dilution differs from that in water, or an interaction between griseofulvin and its polyoxyethylene "environment" accounts for the different heat changes. $\Delta\bar{H}$ is also less temperature-dependent in concentrated detergent solutions and in pure polyoxyethylene glycols than in water. This again may reflect the "environment", as water structuring breaks down with temperature increase (see Elworthy & Lipscomb, 1968), while the polyoxyethylene "environment" of micelles will be maintained.

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A note on the stability of emulsions at high dilution

E. SHOTTON AND S. S. DAVIS*

The behaviour of liquid paraffin–potassium laurate emulsions has been investigated at high dilution using the Coulter Counter, microelectrophoresis, and centrifugation. The results show that the state of the oil–water interface is dependent on emulsifier concentration below and above the critical micelle concentration.

AN investigation has recently been made to examine the effect of emulsifier concentration on the rheology and physical properties of emulsions of liquid paraffin stabilized by potassium laurate (Shotton & Davis, 1967, 1968a). Further experiments are now described in which the emulsions have been subjected to high dilution. The behaviour of the diluted droplets was found to depend on the emulsifier concentration in the original emulsion.

Experimental and results

Materials and formulation details have been given elsewhere (Shotton & Davis, 1968a). In addition to the emulsions described therein, emulsions containing 0, and 0·25% of potassium laurate as emulsifier at volume fractions of 0·11 and 0·43 were also prepared and the more stable fraction that remained after 6 hr creaming was used in the experiments. The effect of diluting the emulsions was examined by the three methods below.

(1) *The Coulter Counter.* Emulsions, of volume fraction 0·11 at potassium laurate concentrations of 0, 0·25, 0·5, 1·0, 5 and 10% w/w were diluted $1:5 \times 10^5$ by a two-step dilution using normal saline and examined using a Coulter Counter model A (industrial) with a 30 μ orifice tube (Shotton & Davis, 1968b). The change in the total number of particles greater than 0·9 μ with time was then followed (Fig. 1). Those systems originally containing soap concentrations above the critical micelle concentration (CMC) in the region of 0·5% (Cockbain & McRoberts, 1953) show a small drop in cumulative count in 45 min whilst for concentrations below the CMC the fall in count is great. At all soap concentrations the fall in count depended on the soap concentration in the original emulsion. Counting the particles at 7 μ demonstrated a slight gain in particle number. Microscopic examination of the diluted emulsion showed the absence of aggregates and it is concluded that the count loss at $> 0{\cdot}9\ \mu$ level is due to coalescence, rather than to the aggregation phenomenon described by Groves (1966) for emulsions stabilized by a soap-amphiphile complex. The particles had a mean volume diameter in the region of 4 μ which from Stoke's Law gives a creaming distance of about 0·25 cm in 45 min. Any effects due to creaming were minimized by stirring the counting dilution; the same arbitrary stirring speed was used throughout so that the effect of stirring on coalescence was kept constant. The slight increase in

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* This work formed part of a thesis accepted for the degree of Ph.D. in the University of London.

particle count (Fig. 1) for 10% soap may be accounted for by coalescence of particles below the sizing limits of the apparatus.

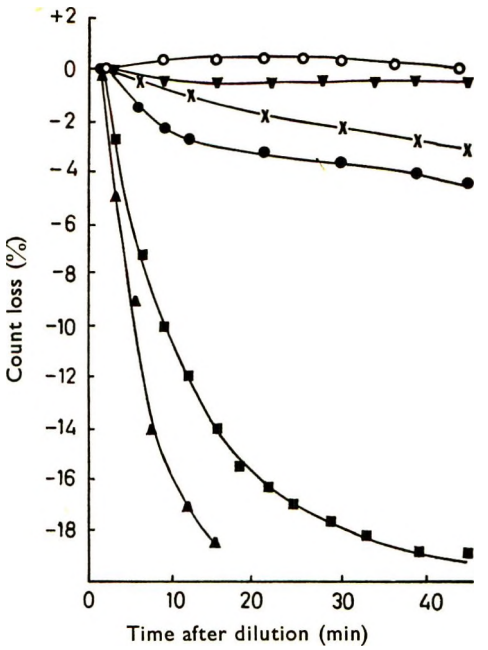

FIG. 1. The loss of count with time on the Coulter Counter for particles greater than 0·9 μ and its dependence on the soap concentration in the original emulsion. Concentration of soap % w/w. ▲ 0·0; ■ 0·25; ● 0·5; × 1·0; ▼ 5·0; ○ 10·0.

It is concluded that coalescence and hence the strength of the interfacial film depends on the concentration of emulsifier in the original emulsion.

(2) *Microelectrophoresis.* Emulsions, of volume fraction 0·11, were diluted $1:1 \times 10^4$ with distilled water and examined in a cylindrical microelectrophoresis cell similar to that described by Bangham, Heard & others (1958). Mobility measurements were made at different depths in the cell and the mobility at the stationary level (zero electro-osmotic flow) determined. The charge on the particles (zeta potential) depended on the emulsifier concentration in the original emulsion (Fig. 2). The quantity of surfactant that reached the final dilution, although small, may have had some effect. Measurements were therefore repeated using different dilutions, i.e. different quantitites of surfactant in the final dilution, but the mobilities were unaffected.

(3) *Washing of emulsions.* The method used was that of repeated centrifugation and removal of the aqueous phase (Shotton & Wibberley, 1960). A convenient volume of emulsion was diluted to twice its volume with water and centrifuged in an M.S.E. refrigerated centrifuge at 6000 rev/min for 30 min. The separated aqueous phase was carefully removed and replaced by a similar volume of water and the emulsion remixed. The process was repeated three times. The volume of separated oil was then

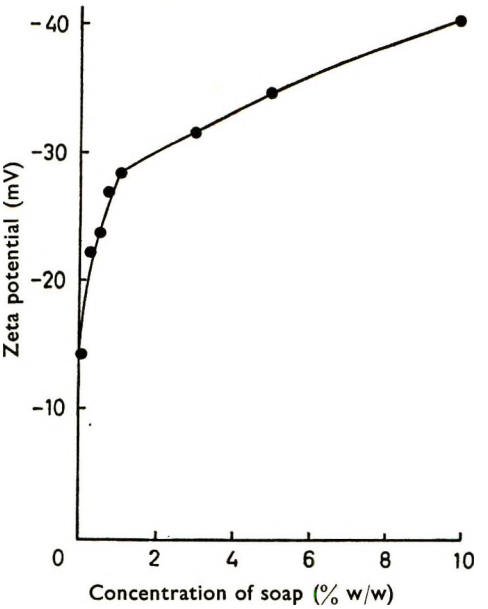

FIG. 2. The change in zeta potential of liquid paraffin–potassium laurate emulsions dispersed in distilled water with the soap concentration in the original emulsion.

measured and was found to depend on the soap concentration in the original emulsion (Fig 3). The amount of oil separating with decrease in emulsifier concentration above the CMC was much less than that separating below it.

Finally, the original emulsions were sized using the Coulter Counter and stored for 6 months when they were sized again. Table 1 shows that the emulsion coalesced to a small extent when the emulsifier concentration was above the CMC. Below the CMC the emulsion was unstable.

TABLE 1. THE EFFECT OF STORAGE ON THE PARTICLE SIZE DISTRIBUTION AND SPECIFIC SURFACE AREA OF POTASSIUM LAURATE/LIQUID PARAFFIN EMULSIONS. (LOG NORMAL DISTRIBUTION) Volume fraction = 0·43.

Soap concentration % w/w	Mean volume diameter μ	Standard deviation σ	Specific surface area $cm^2 g^{-1}$
	4 days		
10·0	2·09	2·07	$2·02 \times 10^4$
5·0	2·62	2·16	$1·62 \times 10^4$
2·5	3·41	2·00	$1·28 \times 10^4$
1·0	4·26	2·27	$8·83 \times 10^3$
0·75	4·17	2·23	$8·89 \times 10^3$
0·50	5·31	2·18	$7·16 \times 10^3$
0·25	$\simeq 10$	$\simeq 2·2$	$\simeq 3·6 \times 10^3$
	6 months		
10·0	2·30	2·20	$1·61 \times 10^4$
5·0	2·76	2·26	$1·37 \times 10^4$
2·5	3·23	2·20	$1·19 \times 10^4$
1·0	4·26	2·21	$9·27 \times 10^3$
0·75	4·24	2·11	$8·75 \times 10^3$
0·50	5·67	2·19	$6·67 \times 10^3$
0·25	∞	—	—

Discussion

The results from the Coulter Counter and centrifugation show that the emulsion droplets retain some or all of their original interfacial film on dilution. The strength of the film (resistance to coalescence) depends on the potassium laurate concentration in the original emulsion for concentrations above and below the CMC. In both cases a discontinuity is found at the CMC. The decrease in stability with concentration below the CMC is greater than that above it. The microelectrophoresis results show that the charge on the droplet, and hence the amount of surfactant that remains adsorbed on dilution, depends on the emulsifier concentration in the undiluted emulsion. In this case a discontinuity was found at 1·0% soap.

King (1941) was one of the first to suggest that the emulsifier concentration was a quantitative source of interfacial film strength, and Osipow, Birsan & Snell (1957) derived an equation relating the logarithm of emulsion stability to the root of the ratio of the emulsifier concentration to the CMC.

Vold & Groot (1962) and Rehfeld (1962) found that the stability of sodium dodecyl sulphate emulsions to ultracentrifugation (without dilution) increased with increasing soap concentration up to the CMC. The latter author further found that the amount of oil separating was linearly related to the log of the soap concentration with a discontinuity at the CMC. A similar relation has been found in the present work (Fig. 3).

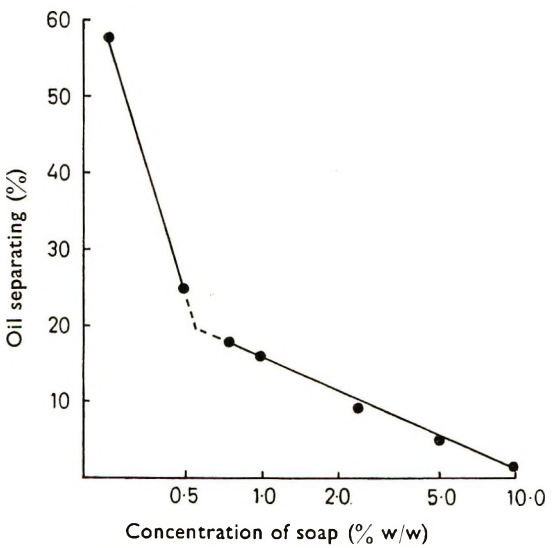

FIG. 3. The effect of the soap concentration in the original emulsion on the stability to dilution and centrifugation of potassium laurate–liquid paraffin emulsions.

However, both Vold & Groot and Rehfeld stated that the stability of the emulsions above the CMC was independent of emulsifier concentration through it is interesting to note that in a later paper, Vold & Groot (1964), when commenting on their earlier publication, have changed "independent" to "only slightly affected".

In comparison, Cockbain & McRoberts (1953) found that the stability to coalescence of soap stabilized droplets was at a maximum at concentrations above the CMC. These concentrations were close to those where aggregation of the emulsions occurred due to polymolecular adsorption of soap molecules (Cockbain, 1952). Aggregation of potassium laurate-liquid paraffin emulsions is known to commence in the region of 1·0% soap (Cockbain, 1952; Shotton & Davis, 1967, 1968a). We found that a discontinuity in the zeta potential concentration plot (Fig. 2) also occurred in this region.

Both Rehfeld (1962) and Vold & Groot (1962) concluded that the packing of surfactant molecules at the oil-water interface reached a maximum at the CMC. The results from the present work show that the state of the interface is markedly dependent on emulsifier concentration below the CMC and that emulsions containing less than 0·5% potassium laurate break down completely on storage. The state of the interface above the CMC is influenced by soap concentration but to a lesser extent. This may be due either to closer packing of the molecules and/or the adsorption of polymolecular layers although it is doubtful that such layers would be retained on dilution.

Closer packing may result from the reduction of charge on adjoining molecules due to an increased counter ion concentration (Powell & Alexander, 1952). Vold & Groot (1964) have found that the adsorption of soap at the o/w interface increases directly with the log of the total concentration of counter ion.

The nature of both the emulsifier and disperse phase may well be important. The chemical nature of the disperse phase is known to have an influence on the stability of an emulsion (Davis, 1967) and Rehfeld (1967) has found that layers of sodium dodecyl sulphate adsorbed at the unsaturated hydrocarbon interface were much more expanded than at the saturated hydrocarbon interface. Further work may show whether the retention of surfactant at the interface is restricted to systems where the disperse phase and lipophilic groups of the surfactant are similar structurally, as is the case in the present work.

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The solubility of sulphadiazine in water–dimethylformamide mixtures

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Precise measurements of the solubility of sulphadiazine in water, dimethylformamide (DMF), and a range of mixtures of these solvents have been made at 20°, 30°, and 40°. The solubility of sulphadiazine in the mixtures increases with their DMF content. The partial molar heats and entropies of solution decrease with increasing DMF concentration.

THE influence of particle size on the efficiency of absorption of certain drugs has been described in many publications. The uptake of sulphadiazine has been shown to be dependent on its particle size (Rheinhold, Phillips & Flippen, 1945). This drug was therefore considered suitable for investigations on methods capable of producing drugs in fine particle form.

The production of small sized crystals of various drugs, by a continuous process involving pumping a solution of the drug into a second miscible liquid in which the drug is insoluble, is being investigated. To study the fundamentals of this process of precipitation it was necessary to determine precise solubilities of the drug. These are reported here for the system sulphadiazine–water–dimethylformamide (DMF).

Experimental

MATERIALS

Sulphadiazine (B.P. quality) was twice recrystallized from an ethanol–DMF mixture (3 : 1 by volume) and dried over phosphorus pentoxide. M.p. 255° (Roblin, Williams & others, 1940, give 255–6°). Assay by the Pharmacopoeial method gave 100·0% purity calculated with reference to the material dried at 105°. Dimethylformamide (May & Baker Ltd.) was distilled under reduced pressure and gave $n_{25}^{D} = 1{\cdot}4283$ ($n_{25}^{D} = 1{\cdot}4294$ Dawson, Golben & others, 1952 and $n_{25}^{D} = 1{\cdot}4269$ Ruhoff & Reid, 1937).

SOLUBILITY DETERMINATIONS

Solvent mixtures were prepared by weight. Solutions were presaturated by shaking with powdered sulphadiazine for 24 hr, and transferred to the solubility apparatus, which was of the percolation type, and a modification of that used by Davies & Griffiths (1953). The percolator was thermostated at the required temperature $\pm 0{\cdot}05°$. The solution was recycled in the apparatus until saturated (in general 7–14 days). Use of porosity 5/3 sintered glass filters gave the same results as porosity 4 filters.

The concentration of sulphadiazine in solution was determined by one of three methods: (a) concentration greater than 1·5%, by evaporation of solvent and drying to constant weight; (b) concentration 0·02 to 1·5%, samples were diluted to give a 70% DMF solvent mixture and assayed

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spectrophotometrically measuring the extinction at 270 mμ; (c) concentration less than 0·02%, samples were diluted with water and assayed as in (b). In all spectrophotometric assays suitable calibration lines were prepared. Each solubility determination was duplicated and each solution assayed in duplicate.

Zimmerman (1952) emphasizes the need to verify the stability of the system during the equilibration period. There is evidence that DMF is stable under the experimental conditions used (Lang, 1960). There are no published quantitative studies on the stability of sulphadiazine solutions. Each solubility apparatus was painted black to protect the solutions from light. No change was detected in the ultraviolet absorption curve of sulphadiazine in water and 50% DMF water solutions when the solutions were heated at 40° for 14 days. The pH of all solutions lay in the range 5 to 6 and any difference in solubility over the pH range was within experimental error.

Density. The density of solutions was determined in a 10 ml pycnometer.

Results and discussion

Fig. 1 shows that the solubility of sulphadiazine increases in a non-linear manner as the DMF concentration is increased. The solubility behaviour

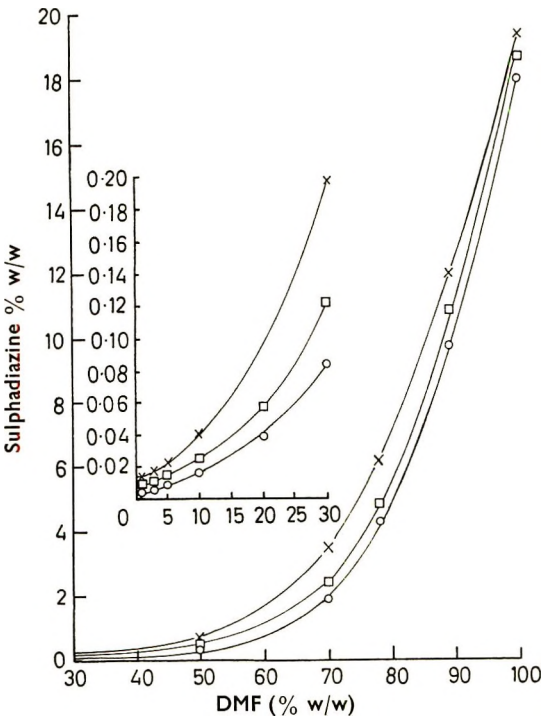

FIG. 1. Solubilities of sulphadiazine in water–DMF mixtures. Insert: solubilities at low DMF concentrations. ○——○ 20°, □——□ 30°, ×——× 40°.

TABLE 1. THE SOLUBILITY OF SULPHADIAZINE IN WATER–DMF MIXTURES

% w/w DMF in solvents	Sulphadiazine % w/w saturated solution			Mole fraction, N^S, sulphadiazine in solution		
	20°	30°	40°	20°	30°	40°
0·0	0·00454	0·00760	0·0129	$3·27 \times 10^{-6}$	$5·47 \times 10^{-6}$	$9·29 \times 10^{-6}$
0·5	0·00490	0·00828	0·0138	$3·54 \times 10^{-6}$	$5·92 \times 10^{-6}$	$9·97 \times 10^{-6}$
1·0	0·00520	0·00881	0·0147	$3·78 \times 10^{-6}$	$6·39 \times 10^{-6}$	$1·07 \times 10^{-5}$
2·0	0·00598	0·00987	0·0166	$4·37 \times 10^{-6}$	$7·21 \times 10^{-6}$	$1·21 \times 10^{-5}$
3·0	0·00679	0·0111	0·0187	$5·00 \times 10^{-6}$	$8·18 \times 10^{-6}$	$1·38 \times 10^{-5}$
5·0	0·00861	0·0141	0·0233	$6·44 \times 10^{-6}$	$1·06 \times 10^{-5}$	$1·74 \times 10^{-5}$
10·0	0·0170	0·0252	0·0410	$1·33 \times 10^{-5}$	$1·97 \times 10^{-5}$	$3·19 \times 10^{-5}$
20·0	0·0395	0·0579	0·0968	$3·35 \times 10^{-5}$	$4·87 \times 10^{-5}$	$8·21 \times 10^{-5}$
30·0	0·0850	0·123	0·188	$7·91 \times 10^{-5}$	$1·14 \times 10^{-4}$	$1·75 \times 10^{-4}$
50·0	0·352	0·502	0·758	$4·08 \times 10^{-4}$	$5·84 \times 10^{-4}$	$8·81 \times 10^{-4}$
70·0	1·90	2·40	3·50	$2·94 \times 10^{-3}$	$3·73 \times 10^{-3}$	$5·50 \times 10^{-3}$
78·0	4·28	4·85	6·20	$7·75 \times 10^{-3}$	$8·84 \times 10^{-3}$	$1·14 \times 10^{-2}$
89·0	9·80	10·9	12·0	$2·32 \times 10^{-2}$	$2·60 \times 10^{-2}$	$2·90 \times 10^{-2}$
100·0	18·0	18·7	19·4	$6·02 \times 10^{-2}$	$6·29 \times 10^{-2}$	$6·57 \times 10^{-2}$

appears to be complex. Liquid water is known to be highly structured due to the formation of intermolecular hydrogen bonds (Robinson & Stokes, 1959). There is also a substantial interaction between water and DMF which must affect the structuring of the former considerably. Evidence for the formation of hydrates of the form $HCONMe_2(H_2O)_n$ where n = 2 to 4 is available (Blankenship & Clampitt, 1950; Geller, 1961). Either solvent component may interact with the sulphadiazine. The ability of a number of amides to increase the solubility of a third substance in water is well documented by Higuchi & Connors (1965), and these authors endeavour to relate solute-solvent-cosolvent interactions on a molecular basis to the total solubility.

Here we test the validity of equations for calculating solubilities, n^S, from those in the pure solvents, n_1^S and n_2^S. The first method of calculation uses a simple mole fraction equation

$$n^S = n_1^S N_1 + n_2^S N_2 \qquad (1)$$

where N_1 and N_2 are the mole fractions of the two solvents.

A molar volume mixture rule in which

$$n^S = n_1^S V_1^* + n_2^S V_2^*$$

can be used, where $V_1^* = \dfrac{N_1 V_1}{N_1 V_1 + N_2 V_2}$ and $V_2^* = \dfrac{N_2 V_2}{N_1 V_1 + N_2 V_2}$ (2)

V_1 and V_2 being the molar volumes of the two components. Substitution of molar volumes by partial molar volumes in equation (2) gives a third method of calculation (Fleming, 1954). The densities used in the calculation of partial molar volumes are given in Table 2.

In Fig. 2 the calculated solubilities from the equations given above are shown. There is a large divergence between the experimental results and the calculated solubilities indicating that the basis of calculation of the solubility in water–DMF mixtures from values for the pure separate solvents is not correct. The addition of DMF does not increase the solubility of the sulphadiazine as much as might be expected from the solubility in pure

TABLE 2. THE DENSITY OF WATER–DMF MIXTURES

% w/w DMF	Density		
	d_4^{20}	d_4^{30}	d_4^{40}
0·000	0·9982	0·9957	0·9922
9·746	0·9978	0·9944	0·9902
19·591	0·9985	0·9941	0·9889
29·321	0·9996	0·9940	0·9879
49·978	0·9999	0·9922	0·9841
70·045	0·9911	0·9821	0·9728
78·929	0·9823	0·9731	0·9636
82·876	0·9773	0·9681	0·9586
89·322	0·9677	0·9583	0·9487
95·582	0·9570	0·9477	0·9381
100·000	0·9490	0·9395	0·9299

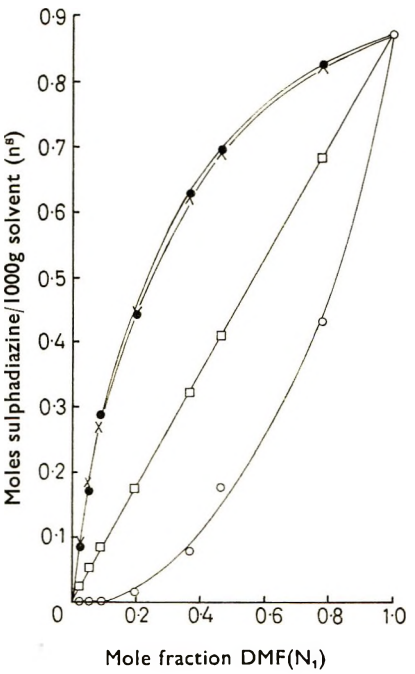

FIG. 2. Calculated and experimental solubility curves for sulphadiazine in water–DMF mixtures at 20°. ○——○ experimental. □——□ calculated from equation 1. ×——× equation 2. ●——● equation 2 using partial molar volumes. See text.

DMF. This may well be due to the interaction of water and DMF. The complex (or complexes) formed between the two solvents has a poorer solvent power for sulphadiazine than the ideal case (mole fraction line in Fig. 2). It seems possible that a complicated series of equilibria between water and DMF are in operation, as well as possible associations between the complexed solvents and the solute.

THERMODYNAMICS OF SOLUBILITY

The Clausius–Clapeyron equation was used to calculate partial molar heats ($\Delta\bar{H}$) of solutions, and the partial molar entropies obtained from

$\Delta\bar{S} = \Delta\bar{H}/T$, since $\Delta\bar{G} = 0$ at the saturated, equilibrium condition. Activity coefficients have been neglected in these calculations, although error arising from this assumption may become significant in solutions containing a high proportion of DMF, where the solubility is substantial. The results are summarized in Table 3.

TABLE 3. THE PARTIAL MOLAR HEATS AND ENTROPIES OF SOLUTION, $\Delta\bar{H}$ AND $\Delta\bar{S}$, FOR SULPHADIAZINE IN WATER–DMF MIXTURES

% w/w DMF in solvent	$\Delta\bar{H}$ k cal/mole		$\Delta\bar{S}$ cal/mole degree	
	25°	35°	25°	35°
0·0	9·1	9·9	30·5	32·4
0·5	9·3	9·5	31·5	31·0
1·0	9·2	9·7	31·0	31·4
2·0	8·9	9·7	29·9	31·6
3·0	8·7	9·8	29·3	31·8
5·0	8·7	9·5	29·2	30·8
10·0	7·1	9·1	23·7	29·7
20·0	6·6	9·8	22·3	31·8
30·0	6·4	8·1	21·6	26·2
50·0	6·4	7·7	21·6	25·2
70·0	4·2	7·3	14·1	23·8
78·0	2·3	4·8	7·7	15·6
89·0	2·0	2·1	6·7	6·7
100·0	0·75	0·82	2·5	2·7

The principal trends in the thermodynamic properties are the decrease of both $\Delta\bar{H}$ and $\Delta\bar{S}$ as the DMF content is increased and the slightly larger values obtained at 35° compared with 25°. The heat of solution will be made up of three main parts: the heat necessary to break the bonds in the crystal lattice ΔH^{f}, the heat of dilution ΔH^{d} and any heat changes arising from solvent–solute interactions. Since the heat change involved in disrupting the crystal lattice will be independent of DMF concentration, the decrease in $\Delta\bar{H}$ seems likely to be due to changes in heat of dilution or heat of interaction with DMF concentration.

Water structuring around sulphadiazine molecules will probably be replaced by hydrogen bonding between DMF and sulphadiazine as the concentration of the latter solvent is increased from zero. The concurrent formation of DMF–water complexes may lead to interaction of the complex with the sulphadiazine. It is impossible to decide, on the basis of the present data, whether heat of dilution effects, or those due to heat of interaction, or both, are responsible for the decrease of $\Delta\bar{H}$ with increase of DMF concentration.

The increase of $\Delta\bar{H}$ with temperature may represent reduced solute–solvent and DMF–water interaction at the higher temperature.

The entropy changes ($\Delta\bar{S}$), as expected, imply a far greater disorder of sulphadiazine molecules in solution than in the crystal. Also, the more concentrated solutions, with respect to sulphadiazine, present at high DMF concentrations, represent a state closer to that in the crystal than the very dilute solutions present at high concentrations of water. Consequently $\Delta\bar{S}$ for the high concentrations of water is high, while for solutions rich in DMF it is low.

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A note on the evaluation of results from creep tests using a computer

S. S. DAVIS AND B. WARBURTON

The accuracy of line spectrum creep analysis depends ultimately on the accuracy with which the steady state compliance of the system with the longest retardation time can be derived. The presented treatment shows how the maximum theoretical value of the creep compliance may be calculated and describes an iterative method for obtaining the correct value using the least sum of squares.

THE rheological examination of pharmaceutical semisolids by creep testing has been reported by Barry & Shotton (1967) and Warburton & Barry (1968). The method is simple and the results may be expressed in terms of fundamental rheological parameters. However, although the analysis of creep curves can be made graphically the procedure is lengthy and not suited to routine investigations. To overcome this, an automatic data logging system and analysis has been devised.

The apparatus used is that of Warburton & Barry (1968). The signal from a transducer bridge, which is a record of the change of strain with time, is attenuated and passed to both a Kent Chart recorder and a digital voltmeter with associated print out. The printer is triggered every 30 sec so that a record of the creep curve is available in graphical and digital forms the latter being punched onto tape for analysis by computer.

The computer analysis is dealt with in two parts.

(1) CREEP CURVE ANALYSIS

Most of the computer analysis is a straight mathematical interpretation of the graphical analysis of creep curves (Warburton & Barry, 1968). However, there are two steps requiring special consideration (the notation used is that of Warburton & Barry, 1968).

Step 1. *The original creep curve.* The essential part of this analysis is to find where the curve becomes linear. The approximate region for the onset of linearity can be deduced from the graph and the experiment allowed to proceed so that the linear region is greater than 25% of the total curve. The computer makes a linear regression on the last 20% of the points and the equation for the straight line is derived. Starting with the longest time, the strain at each time interval is generated from the regression equation and compared with the actual strain. The point for the onset of linearity is obtained when the difference between the two exceeds the maximum difference due to experimental error. The value of the intercept of the regression line on the strain axis leads to an *approximate* value for J(N), the total creep compliance at equilibrium.

Step 2. *Voigt model analysis.* To derive the parameters of the Voigt models that describe a viscoelastic system, a plot is made of the function $\ln (J(N) - J(t))/J(N)$ [abbreviated to ln (Zn)] against time where J(t) is

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the total creep compliance at time t. As in step 1 the point where a curve becomes a straight line must be found so that the best straight line can be drawn through the points obtained at longer times. The first method of approach was to assume that the curve was linear over the last 20% of the points and then adopt a procedure similar to that in step 1. However the values of ln (Zn) obtained at longer times are dependent on the accuracy of the data and the value chosen for J(N) so that in many cases the derived regression line is nowhere near the best straight line that can be drawn when the whole of the linear region is considered. The points that lie closest to a straight line are obtained immediately after the point where linearity commences. This region must be found before the regression line is calculated and this can be achieved by the method of second derivatives. Starting with time zero the gradient of the straight line through points 0–4 is calculated, this is repeated with points 1–5 and so on until the whole curve has been followed. The 1st derivatives so obtained are then subjected to the same process to give the second derivative values. For a straight line the second derivative values should be zero. In practice, for the linear region, their values fluctuate from small negative to small positive, so by setting limits the extent of the linear region can be determined. The regression line is then calculated, and by comparing the actual value of ln (Zn) with the calculated values, the point for the onset of linearity, found by the derivative method, can be checked. The analysis then follows directly that of Warburton & Barry (1968). Successive Voigt units may be analysed in a similar manner.

(2) THE CHOICE OF THE VALUE OF J(N)

The accuracy of the whole analysis depends on the best fit value of J(N). This is the equilibrium or steady state value as t approaches infinity and is not readily accessible. With computer facilities the best fit value for J(N) is obtained using an iterative procedure. The maximum theoretical value for J(N) can be found by considering the creep compliances at two times t_1 and t_2 where $t_2 = 2t_1$ and where the creep behaviour is due to the Voigt unit of longest retardation time only. Then for time t_1

$$e^{-t_1/\tau} = \frac{J(N) - J(t_1)}{J(N)} \quad .. \quad .. \quad .. \quad (1)$$

and for time t_2

$$e^{-t_2/\tau} = \frac{J(N) - J(t_2)}{J(N)} \quad .. \quad .. \quad .. \quad (2)$$

where τ is the longest retardation time. Dividing (2) by (1)

$$\frac{J(N) - J(t_2)}{J(N) - J(t_1)} = e^{-t_1/\tau} \quad .. \quad .. \quad .. \quad (3)$$

But

$$e^{-t_1/\tau} = \frac{J(N) - J(t_1)}{J(n)} \quad .. \quad .. \quad .. \quad (4)$$

(Warburton & Barry, 1968)

where J(n) is the compliance due to the Voigt unit of longest retardation time

$$\therefore J(n) = \frac{[J(N) - J(t_1)]^2}{J(N) - J(t_2)} \quad .. \quad .. \quad .. \quad (5)$$

$J(n) = xJ(N)$ where x is the antilog of the intercept of the $J(N) - J(t)/J(N)$ versus t plot on the ordinate

$$\therefore J(N)^2 - xJ(N)^2 - 2J(N)J(t_1) + xJ(N)J(t_2) + J(t_1)^2 = 0 \quad .. \quad (6)$$

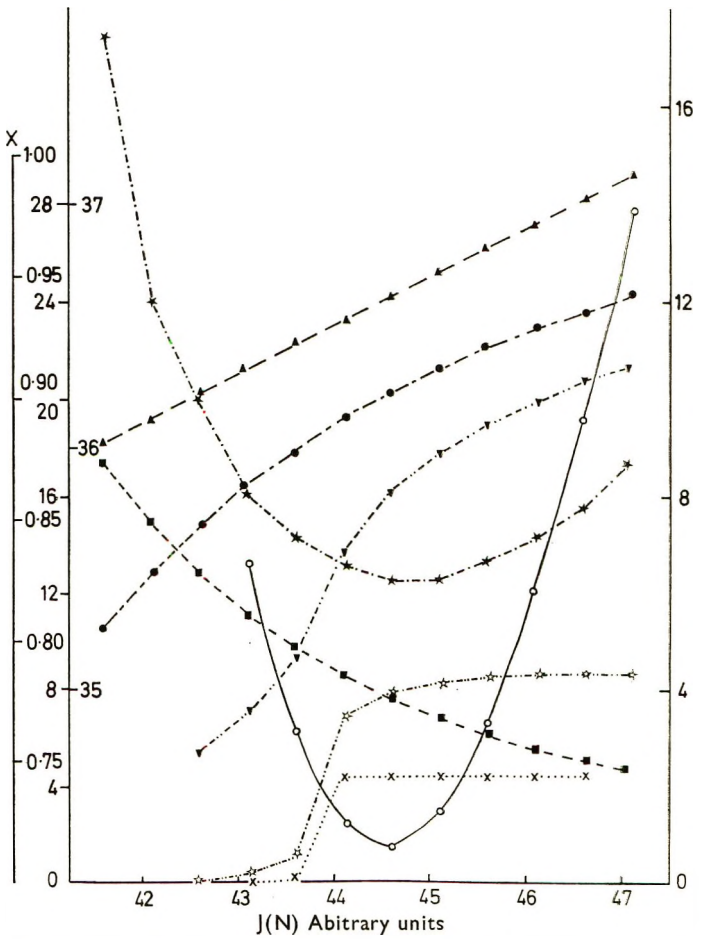

FIG. 1. The effect of the value of J(N) on the viscoelastic parameters of a 3 Voigt Unit System (Emulsifying Ointment B.P.). For notation see Warburton & Barry (1968). —○—, (S); —■—, X; —▲—, τ_2; —▼—, τ_1; —X—, τ_3; —★—, J(n); —●—, J(N) - J(n); —☆—, $J(n_3)$. Ordinates—left hand axes: the 1st represents X; the 2nd represents τ_1, τ_3 (min × 10), which are short and shortest retardation times, and also (S) = sum of squares; the third represents Jn. The right hand axis represents $J(n_3)$, J(N)—J(n), these together with Jn are arbitrary units, one unit being equivalent to a compliance of $1{\cdot}15 \times 10^{-7}$ cm^2 dyne^{-1}. The right hand axis also represents τ_2 (min), which is the longest retardation time.

As the equation is a quadratic, J(N) has a unique and maximum value when

$$'B^2 - 4AC = 0'$$

$$\text{i.e. } x^2J(t_2)^2 - 4J(t_1)xJ(t_2) + 4xJ(t_1)^2 = 0 \quad .. \quad .. \quad .. \quad .. \quad (7)$$

i.e. when $x = 0$

$$\text{or } x = \frac{4[J(t_1)\,J(t_2) - J(t_1)^2]}{J(t_2)^2} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (8)$$

$$J(N)_{MAX} = '-B/2A'$$

$$\text{i.e.} \qquad J(N)_{MAX} = \frac{2J(t_1) - J(t_2)x}{2(1 - x)} \quad .. \quad .. \quad .. \quad .. \quad (9)$$

$J(N)_{MAX}$ can therefore be determined from the derived values of J(t). To find the best fit value of J(N) the approximate value obtained from the linear regression in step 1 is reduced by 10% and then increased progressively to $J(N)_{MAX}$. At each value of J(N) the full creep curve analysis is made and the creep equation for the system is derived. The compliance at each given time interval is then calculated and compared with the experimental values (Barry & Shotton, 1967). The sum of the squares of the differences between the two is calculated (S) and the best fit value of J(N) is obtained where the sum of squares is a minimum.

The effect of the value of J(N) on the derived viscoelastic parameters for a 3 Voigt unit system is shown in Fig. 1. S has a well defined minimum value which can be easily found by this iterative process. It can be seen that the value of J(N) has a profound influence on all the viscoelastic parameters. The best fit value of J(N) is greater than the value found from Step 1 of the creep curve analysis but is less than the maximum value obtained from equation (9).

It is concluded that the use of computer methods will greatly facilitate creep curve analysis and that by using iterative methods accurate values are obtained for viscoelastic parameters.

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A high content (—)-deguelin concentrate from a commercial derris resin by steady-state distribution

E. BRIERLEY AND H. J. SMITH

The isolation of a high content (−)-deguelin concentrate from a commercial derris resin using steady-state distribution is described. The separation was followed by noting the change in ratio of extinctions at two wavelengths.

OF the six rotenoids occurring in *Derris* sp. only optically active deguelin is not known in a pure or crystalline form, although the crystalline (±) form has been obtained by treating a crude optically active deguelin concentrate with alkali (Haller & La Forge, 1934). An early claim to have isolated optically active crystalline deguelin from the resin (Takei, Miyajima & Ono, 1933) was later attributed to isolation of (±)-deguelin containing a small amount of rotenone responsible for the optical activity (Haller & La Forge, 1934).

We describe the isolation from a commercial derris resin of a high content (−)-deguelin concentrate which, although not crystalline was homogeneous and superior in deguelin content to that of Haller & La Forge (1934).

Experimental

The proton nmr spectra were determined at 60 megacycles on a Varian A-60 spectrometer with tetramethylsilane as internal standard. The light petroleum used had b.p. 60–80°.

STEADY-STATE DISTRIBUTION OF A COMMERCIAL DERRIS RESIN

A commercial derris resin (1·25 g), from which the rotenone had been removed as its carbon tetrachloride solvate, was dissolved in benzene (25 ml) and an aliquot (0·5 ml) of this solution was placed in cell (O) of a Quickfit and Quartz Steady-State Distribution apparatus. Distribution was made using benzene–light petroleum/acetic acid–water (6 : 14, 13 : 7) mixtures as the two phases by alternate top (T; upper phase) and bottom (B; lower phase) transfers. Aliquots (0·5 ml) of the resin solution were injected into cell (0) after each transfer and the distribution continued until 25 fractions of each phase had been collected from the ends of the train. Aliquots (0·05–0·5 ml) from the upper and lower phases were separately evaporated with a stream of nitrogen and the residues dissolved in spectroscopic ethanol (95%, 10 ml). The extinctions of the ethanolic solutions were determined at 237 and 272 mμ using a Uvispek H700 spectrophotometer.

The resin used had $E(1\%, 1\text{ cm})$ 529 and 356 at λ_{max} 237 mμ and λ_{max} 272 mμ respectively. The amount of material (mg) in each fraction from the distribution was calculated at each wavelength from the equation, $100 \times a \times b/c$, where a = dilution factor for aliquot taken, b = extinction of the aliquot in 10 ml of spectroscopic ethanol and c = $E(1\%, 1\text{ cm})$

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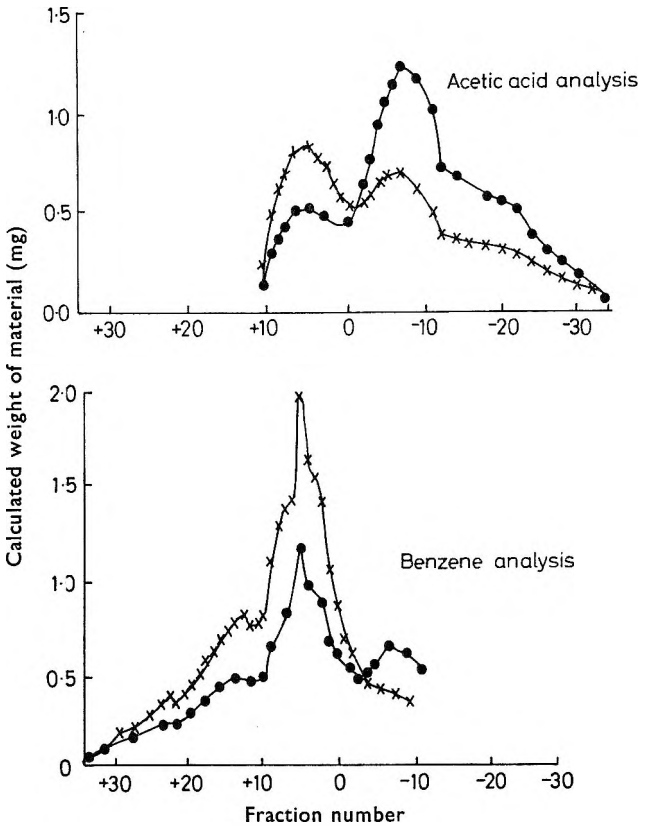

FIG. 1. Steady-state distribution of derris resin using benzene–light petroleum/acetic acid–water solvent mixture (●—based on D at 237 mμ, ×—based on D at 272 mμ).

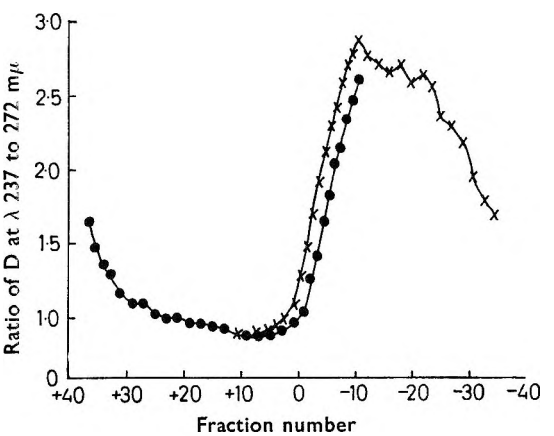

FIG. 2. Variation in ratio of D at λ 237 to 272 mμ with fraction number. (×—acetic acid analysis; ●—benzene analysis).

for the resin. The results of the analysis are shown in Fig. 1. The true fraction weight for absorbable material lies between the weights calculated at the two wavelengths assuming that absorbable materials have similar molecular weights. A graph of the ratio of extinctions at λ 237 and 272 mμ for the fractions shows a separation into high and low ratio fractions (Fig. 2).

Using this method, resin (11·1 g) was distributed between the same solvent mixture but with 16 cycles of the sequence TBBB. The fractions which had D 237/272 ratio < 1 were bulked and separated. The lower phase was diluted with water, extracted with ether and the organic phase neutralized ($NaHCO_3$), washed, dried (Na_2SO_4) and evaporated. The upper phase was neutralized, washed, dried and evaporated. The pooled residues (3·36 g) were dissolved in the minimum amount of benzene and chromatographed on a column of neutral alumina (Grade III) using ether–light petroleum (1 : 1) solvent mixture. The eluate was collected in five fractions (25 ml). Fractions (2) and (3), which contained the main bulk of the material, deposited crude dehydrorotenone crystals (44 mg) on standing. After removal of the crystals, the mother liquor was evaporated to give a white residue (2·5 g) which when dissolved in the minimum amount of carbon tetrachloride deposited impure rotenone-carbon tetrachloride solvate (0·56 g). The mother liquors were evaporated to give a non-crystallizable residue (1·55 g), m.p. 70° (glass) which gave positive reactions in the Goodhue (1936), Durham, and Rogers-Calamari (1936) tests but no colour with ferric chloride. Chromatography of the residue (0·32 g) on alumina with chloroform–light petroleum (1 : 1) solvent mixture with collection of the main fraction followed by removal of solvent left a crisp brown deguelin concentrate (0·3 g). The concentrate had a Goodhue value of 68·2%, $[\alpha]_{20}^{D} - 110{\cdot}5°$ (*c* 1%; benzene), λ_{max} [log *E*(1%, 1 cm)], 237 (2·667), 250 (2·651), 271 (2·735), 295 (2·367) and 315 (i) (2·197) mμ, ν_{max} ($CHCl_3$), 1667 (C=O), 1592, 1573, 1368, 1337, 1110, 1089, 909 and 893 cm^{-1}, and the proton nmr spectrum showed signals at τ 6·18, 6·21 (CH_3O-), 3·17, 3·52 (ring A, aromatic protons), 8·54, 8·61 (6′-$(CH_3)_2$-), doublets at 2·22 (J = 9 cycles/sec, 11 H), and 3·51 (J = 9 cycles/sec, 10 H) and doublets at 3·30 (J = 9·5 cycles/sec, 4′H) and 4·42 ppm (J = 9·5 cycles/sec. 5′H).

CONVERSION OF DEGUELIN CONCENTRATE TO (±)-DEGUELIN WITH ALKALI

The method of Haller & La Forge (1934) in our hands gave appreciable quantities of tephrosin and was therefore modified. The concentrate (1·5 g; 44% Goodhue value) in methanol (75 ml) under an atmosphere of hydrogen was mixed with sodium hydroxide solution (0·6 ml, 2N). After 15 min the solution was neutralized with mineral acid and allowed to stand overnight. On cropping the solution, crystals (413 mg) were deposited which gave a single spot (Rf 0·6) on thin film chromatography on alumina, which fluoresced yellow with ultraviolet light and stained yellow when sprayed with solutions of either iodine or 2,4-dinitrophenylhydrazine. Recrystallization (180 mg) from methanol gave (±)-deguelin (120 mg), m.p. 170–171° [lit. cites 165° (Cahn, Phipers & Boam, 1938) and 171°

(Clark, 1931)] which had a Goodhue value of 83·6% (Goodhue & Haller, 1939, cite 81%). (Found: C, 70·0; H, 5·7. Calc. for $C_{23}H_{22}O_6$: C, 70·0; H, 5·6%).

OPTICAL ROTATION—RACEMIZATION STUDIES ON ROTENONE AND A DEGUELIN CONCENTRATE

The optical rotation of a solution of a deguelin concentrate (1%) in "Analar" benzene (10 ml) was measured using a 1 dm cell at 20°. A solution (0·75 ml) of methanolic potassium hydroxide (0·34 N) was then added and the optical rotation noted at intervals. The $[\alpha]_{20}^{D}$ declined from an initial reading of −78° to zero within 10 min. Under similar conditions, solutions of rotenone (1, 1·5, 2·0%) which gave initial $[\alpha]_{20}^{D}$ values of −216°, −208° and −217° respectively declined to a final value of −33°, −44° and −49° respectively.

HIGH RATIO FRACTION

Fractions from the steady-state distribution of the crude resin with a ratio > 2 were bulked, the upper and low phases separated and processed as previously described. The resinous material (1·11 g) from the lower phase was chromatographed on alumina using benzene-chloroform (1 : 1) solvent mixture. The green ultraviolet fluorescent eluate was collected in two fractions, which were then evaporated to give residues (1) 0·57 g, (2) 0·30 g. Residue (1) alone gave a positive Durham reaction and had a Goodhue value of 43%. This residue (200 mg) was dissolved in methanol and kept at 0°. An oily mass was deposited which on standing at room temperature (20°) gave crystals (40 mg) of (−)-elliptone, which had m.p. and mixed m.p. 170–171·5° and 173° respectively (with an authentic sample of (−)-elliptone m.p. 174·5–175°), λ_{max} (log ϵ) 238·7 (4·575) and 274·7 mμ (3·949) (−)-elliptone had λ_{max} 238·7 (4·616) and 274·7 mμ (3·959).

Discussion

The main constituent of the low ratio fraction was recognized as deguelin by its proton nmr spectrum which was similar to that described for (±)-deguelin (Crombie & Lown, 1962) and is readily distinguishable from other known non-phenolic rotenoids by the signals at τ 3·3 and 4·42 ppm (doublets, J = 9·5 cycles/sec) corresponding to the two ring E olefinic protons. The infrared and ultraviolet absorption spectra were in accord with this interpretation which was confirmed by the isolation of (±)-deguelin from the concentrate after treatment with alkali.

The homogeneity of the deguelin concentrate was investigated by thin-layer chromatography and optical rotation-racemization studies. Thin layer chromatography on alumina gave a single spot (Rf 0·6) showing that tephrosin (Rf 0·3), known to be formed by oxidation of deguelin on the column (Brierley, 1966; Rangaswani & Sastry, 1956), was absent. In the optical rotation-racemization studies, rotenone had a final average specific rotation value of −42°, corresponding to the formation of mutarotenone

by racemization of the C-6a, C-12a centres whereas the deguelin concentrate had a final rotation of zero. It is concluded that the optical activity of the concentrate is due to (—)-deguelin and that rotenone is absent within the limits of sensitivity of the method used.

The (—)-deguelin concentrate isolated in this work although it could not be obtained crystalline, was apparently homogeneous as judged by the techniques employed and contained at least 85% deguelin as shown by colorimetric assay. In these respects it is superior to the concentrate previously described by Haller & La Forge (1934) which contained about 50% deguelin together with an appreciable quantity of rotenone.

The ultraviolet absorption spectrum for the high ratio fraction obtained in this work suggested a similarity to several recently isolated flavanoids from other *Leguminosae* sp. (nepseudin, Crombie & Whiting, 1962; neotenone, Crombie & Whiting, 1963) and differed considerably from that quoted for (—)-elliptone by Meijer (1941). However, the presence of (—)-elliptone in the fraction was indicated by the proton nmr spectrum and confirmed by isolation of crystalline (—)-elliptone.

The method described here for location of the high and low ratio fractions in the counter-current train is rapid for locating the constituents of a mixture, and is particularly advantageous where incomplete separation has occurred. The usual tedious gravimetric procedure is avoided and furthermore the constituents can be located in instances where a weight analysis shows little separation (cf. Figs 1 and 2). However, for its successful application the constituents must have different ratios for their ultraviolet absorption extinction coefficients at two pre-selected wavelengths. The method described here could prove useful in detecting and following the isolation of impurities in drugs where steady state distribution or column chromatography is being used.

Acknowledgements. We wish to thank Messrs. Cooper, McDougall and Robertson Ltd., for a gift of commercial derris extract and Dr. Th. M. Meijer for a sample of (—)-elliptone.

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Quantitative method for determining aminofurantoin†

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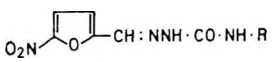
A quantitative colorimetric procedure for the estimation of aminofurantoin is described. The red colour of the Schiff base produced by the reaction of dimethylaminobenzaldehyde with aminofurantoin, is stable after a period of 40 min for a further 20 min and can be measured at 582 mμ. Maximal colour is developed at room temperature (20°) under acid conditions (pH 2·0). This technique was found suitable for the determination of aminofurantoin in tissue homogenates. The formation of aminofurantoin as a probable metabolite of nitrofurantoin was investigated but its production was not confirmed.

IN view of the extensive use of nitrofurantoin (5-nitro-2-furfurylideneamino hydantoin) as a urinary antiseptic, it is surprising that the metabolic fate within mammalian tissue is still uncertain. Paul, Ellis & others (1960) discussed the probable metabolites produced by the physiological degradation of substituted semicarbazone derivatives of 5-nitro-2-furfurylidene (I).



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The compounds appear to be biochemically degraded by hydrolysis of the side chain to give nitrofuraldehyde followed by oxidation to nitrofuroic acid (Paul, Austin & others, 1949; Paul & others, 1960) and by the reduction of the nitro-group followed by ring cleavage. Evidence suggests that the intermediate hydroxylamine is formed but the aminofurans could not be isolated.

Studies on the degradation of nitrofurantoin by bacterial systems have shown that a single stage breakdown occurs involving the simultaneous reduction of the nitro-group and the cleavage of the furan ring (Beckett & Robinson, 1956). However, Ebetino, Caroll & Geur (1962) found that the catalytic reduction of nitrofurantoin produced 1-(5-aminofurfurylidene-amino)hydantoin, which was quite stable and showed *no ring cleavage*. Although aminofurantoin is a postulated intermediate in the metabolic degradation of nitrofurantoin, there is still no direct evidence for its formation in mammalian tissues.

The difficulties inherent in isolating unchanged nitrofurantoin and its metabolic products from urine or tissue homogenate have made it tedious to separate and characterize the metabolites. Therefore a simple colorimetric procedure for the quantitative estimation of aminofurantoin in tissue homogenates has been developed in an attempt to facilitate the characterization of this compound as a possible metabolite; the need for preliminary tedious chromatographic separation is thereby avoided.

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† Aminofurantoin is the accepted trivial name for 1-(5-amino-2-furfurylidine-amino)hydantoin.

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The method developed by Bratton & Marshall (1939) for the quantitative estimation of aromatic amines using naphthylethylenediamine was found to be unsuitable for the determination of aminofurantoin. Therefore, the method of Venkataraman, Venkataraman & Lewis, (1948) has been investigated as a possible alternative.

The red colour produced by the interaction of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde 1% in ethanol) with aminofurantoin is sensitive, unaffected by the presence of nitrofurantoin and is sufficiently stable for accurate measurements to be made.

METHODS

Ehrlich's reagent (1% *p*-dimethylaminobenzaldehyde (BDH) in ethanol) (100 ml) containing concentrated hydrochloric acid (1 ml) was used to develop the colour which was measured on a Unicam SP800 at 582 mμ. Aminofurantoin (50 mg) was dissolved in *NN*-dimethylformamide (1 ml) and this was diluted with water to give a stock solution containing 100 μg/ml.

Dilutions of the stock solution of aminofurantoin were prepared to give concentrations between 2 and 15 μg/ml. The aminofurantoin solution was adjusted to pH 1·8 by the addition of Clark & Lubbs buffer (3·5 ml to 1 ml of the aminofurantoin solution); Ehrlich's reagent (0·5 ml) was added to the acidified aminofurantoin (4·5 ml) and the mixture was allowed to stand at room temperature (20°) before reading the absorption at 582 mμ.

The procedure was modified to study the effect of time (colour determined after 10 min intervals), temperature (20–48°) and pH 1·0–2·0. In each case a solution containing 20 μg/ml of aminofurantoin was used. The results are presented in Figs 1 and 2.

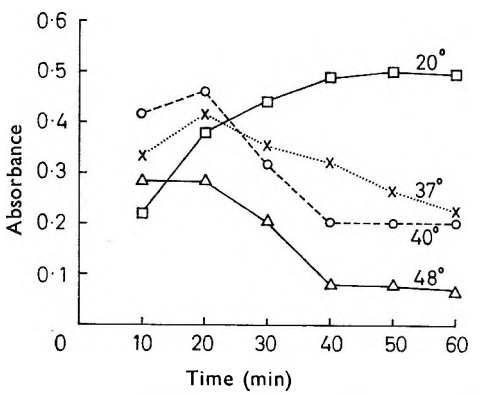

FIG. 1. Effect of temperature and time on the colour development (582 mμ) resulting from the interaction of aminofurantoin with Ehrlich's reagent.

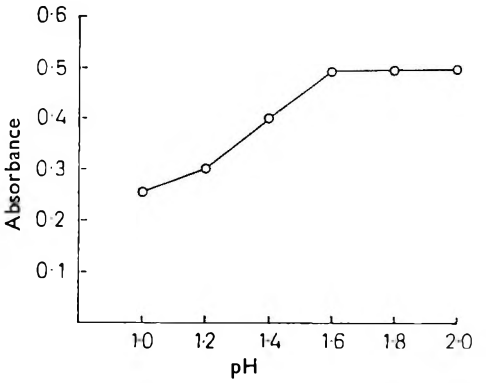

FIG. 2. Effect of pH on the colour development resulting from the interaction of aminofurantoin with Ehrlich's reagent.

Recovery of aminofurantoin from tissue homogenates. Aminofurantoin solutions (1·5 ml) were added to freshly homogenized rat liver (5 g in 10 ml of phosphate buffer, pH 7·4) (2 ml) to give final concentrations of 4–15 μg/ml. Trichloroacetic acid 10% in water (10 ml) was added to the homogenate preparation and the precipitated protein removed by centrifugation. Dilute ammonium hydroxide solution was added to correct the pH of the supernatant to pH 1·8. The above process was repeated replacing the homogenate with water. The volumes of the final solutions were adjusted to 15 ml, Ehrlich's reagent (0·5 ml) was added to 4·5 ml aliquots and the colour determined at 582 mμ. The percentage recovery of aminofurantoin at different concentrations is presented in Table 1.

TABLE 1. RECOVERY OF AMINOFURANTOIN FROM BIOLOGICAL FLUIDS

Concentration of added aminofurantoin		Concentration of "recovered" aminofurantoin μmole/ml			% Recovery		
μg/ml	μmole/ml	Water	Homo-genate	Urine	Water	Homo-genate	Urine
4	0·019	0·019	0·019	0·019	100%	100%	100%
7·5	0·036	0·036	0·034	0·034	100%	97%	97%
10	0·048	0·048	0·047	0·046	100%	97%	95%
15	0·072	0·072	0·068	0·064	100%	98%	95%

Incubation of nitrofurantoin with tissue homogenates. Nitrofurantoin and *p*-nitrobenzoic acid solutions (1 ml) were added to separate samples of a guinea-pig liver homogenate as described for aminofurantoin above, to give concentrations of 1 μmole/ml. The homogenates were fortified by the addition of a co-factor solution (2 ml) containing NADPH (1·1 μmole), glucose-6-phosphate (19 μmole), riboflavine (2·5 μmole) and nicotinamide (1·5 μmole) in phosphate buffer (pH 7·4).

The preparations were incubated at 37° for 1 hr under nitrogen, the reactions stopped with trichloroacetic acid and the colour developed as described above. *p*-Aminobenzoic acid (1·25 μmole) was produced in

the reaction mixture containing nitrobenzoic acid as substrate whereas no aminofurantoin could be detected in that preparation containing nitrofurantoin.

Incubation of aminofurantoin with tissue homogenates. Aminofurantoin (1 μmole) was added to freshly homogenized rat liver as described above. The homogenate was incubated at 37°, samples were removed at 10 min intervals, and the aminofurantoin content determined (see Fig. 3).

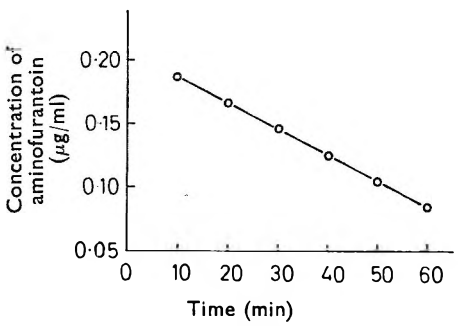

FIG. 3. Decrease in aminofurantoin concentration on incubation with rat liver homogenate.

Attempted recovery of aminofurantoin from urine. Furadantin (200 mg) was given orally to two men whose urinary pH was maintained at 5·0 by ammonium chloride (1 g orally every 3 hr). A 4 hr urine sample was collected and analysed for aminofurantoin content as described. No aminofurantoin was detected although nitrofurantoin was shown to be present by a spectroscopic examination of a urine extract.

Results

The colour development was found to be: (i) time dependent, reaching a maximum at 20° after a period of 40–60 min (see Fig. 1), (ii) temperature dependent, the optimal temperature being 20° (see Fig. 1), (iii) pH dependent, requiring an acidic pH optimal at 1·8 (see Fig. 2).

The absorption by the Schiff base formed during the reaction was shown to obey the Beer Lambert law below concentrations of 15 μg/ml, and was sensitive enough to detect concentrations as low as 2 μg/ml.

A mixture of Ehrlich reagent and rat liver homogenate under the conditions described produced no measurable absorption at 582 mμ, and excellent recoveries were obtained of aminofurantoin added to an homogenate (see Table 1).

However, upon incubating aminofurantoin with the homogenate a significant loss was observed as shown in Fig. 3.

Discussion

Ehrlich's reagent forms a Schiff base with aminofurantoin having an absorption maximum at 582 mμ which is stable under defined conditions of temperature and pH, and whose intensity is quantitatively related to

low concentrations of the aminofurantoin. The colour development with aromatic amines reported by Vankataraman & others (1948) was maximal at a pH of 2·0–2·2. However, using aminofurantoin a pH of 1·8 was required for maximum colour development.

Good recoveries were obtained from rat liver homogenate; quantities of 2 μg/ml being detected by the method. The technique described appears to offer a reasonably sensitive and convenient method for the detection of aminofurantoin in the presence of biological material.

Although the system used was shown to reduce *p*-nitrobenzoic acid to *p*-aminobenzoic acid it was not possible to demonstrate the formation of aminofurantoin as a reduction product from nitrofurantoin. However, it was shown that aminofurantoin is broken down at an appreciable rate under the conditions used, and therefore if it is formed by the action of nitroreductase on nitrofurantoin it is probably immediately converted by some mechanism to another reaction product. Therefore it was not suprising that aminofuranotin was not detected in the urine of persons receiving nitrofurantoin, even though the urinary pH favoured maximum excretion of any basic material.

Acknowledgements. Aminofurantoin was kindly supplied by Norwich Pharmacal Company, New York.

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Simulation methods in drug screening

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Simulation is used to give a quantitative explanation of the phenomenon frequently observed in routine drug screening, that activities found in the initial tests are not confirmed on retest. Simulation is also used to assess the efficiency of a screening test procedure and to compare the efficiencies of different procedures.

SCREENING tests must be designed to make efficient use of the animals in a test, and to apply the appropriate amount of effort to test each compound so as to maximize the probability of finding a suitable drug. Much literature exists on the design of screening tests, and an extensive bibliography is given in Federer (1963).

A frequently occurring phenomenon is that activity found in an initial test is not confirmed on retest, an observation sometimes referred to as the "first experiment disease". This arises in the following way.

Compounds classified as positive in the primary screening test are usually retested experimentally to obtain more reliable estimates of their activities. But on retest the activities are generally appreciably lower than those observed initially. As a result the test or compound is often suspected of unreliability. Yet it can be readily shown statistically that on average a lower activity can be expected as a natural consequence of the usual testing error, and is not evidence of abnormality in the test. The reasons for this are that testing error alone can give rise to an apparent activity which may be greater than the actual activity, and when selecting compounds with highest apparent activities there is a tendency to select results which are high because of normal testing error. This can be pronounced when the proportion of positives is small.

There is no difficulty in demonstrating this phenomenon mathematically and to measure its magnitude. Another and convincing method of doing this is by simulation. This method does not require any specialized knowledge of mathematics and can be used by any experimenter preferably with some knowledge of computer programming, because simulations are most easily carried out on a computer.

PROCEDURE OF SIMULATION

First the compounds to be tested must be simulated. The compounds are assumed to be selected randomly from a large pool of possible compounds which could be tested. It is necessary to assign a distribution of activities to the pool of compounds. Much information is available on biological activities of compounds in general and there may be enough information available on the particular activity being investigated to allow at least a good guess of the form of the distribution. There must be some expectation of activity, otherwise the screen would not be

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undertaken. Since the aim is to demonstrate an effect, a rough indication of the distribution is all that is needed.

In an actual screen which was simulated and the results of which are given later in this paper, the distribution of activities given in Table 1 was assumed. According to this distribution of activities 85% of the compounds are inactive and the remainder have activities ranging from 7·5 to 30% in the proportions shown.

TABLE 1. ASSUMED DISTRIBUTION OF ACTIVITIES IN THE POOL OF COMPOUNDS AVAILABLE FOR TEST

% Activity	0	7·5	15	22·5	30
Proportion of compounds ..	0·85	0·08	0·04	0·02	0·01
Cumulative proportions ..	0·85	0·93	0·97	0·99	1·00

The procedure of selecting a compound randomly from the distribution is as follows. A random number between 0 and 1 is chosen from a table of random numbers (Tippett, 1959). The cumulative distribution is used to identify this number with a compound. If the number is less than or equal to 0·85, then it refers to a compound of zero activity; if the number is more than 0·85 and less than or equal to 0·93, it refers to a compound of activity 7·5% and so on. The complete Table is as shown in Table 2.

TABLE 2. CORRESPONDENCE BETWEEN RANDOM NUMBERS AND ACTIVITIES

Random number x	Activity of compound %
$0 < x \leqslant 0{\cdot}85$	0
$0{\cdot}85 < x \leqslant 0{\cdot}93$	7·5
$0{\cdot}93 < x \leqslant 0{\cdot}97$	15
$0{\cdot}97 < x \leqslant 0{\cdot}99$	22·5
$x > 0{\cdot}99$	30

The next step is to simulate the result of a test on the compound. The only information needed about the test is the distribution of the testing errors. If required, a numerical distribution of the errors can be drawn up in much the same way as that for the activities of the compounds, and an error selected randomly from the distribution again using a similar procedure. The distribution of errors will contain negative as well as positive errors. Usually it can be assumed that the testing error distribution is normal and then the standard error is all that needs to be known. Tables exist for random sampling from a normal distribution (Wold, 1948); these tables are based on a standard error of unity, and the selected random number must therefore be multiplied by the standard error of the test. The result represents the error to be added to the activity of the compound selected to give the simulated test results.

The above procedure has to be repeated many times and is best done by computer. Facilities exist in all modern computers to select a random number and also a random Normal deviate.

The steps in the full simulation procedure can now be set out.

(1) Draw up a distribution of expected activities for the compounds (see Table 1).

(2) Select compounds successively at random from this distribution and note the results. This simulates the compounds which are presented for test. Repeat a sufficient number of times, e.g. 300 or more times.

(3) Simulate a test result by adding a random error, based on knowledge of the test, to the known activity of the compound selected. Repeat for successive compounds and note the results.

(4) Identify those compounds which on first test are considered positive, e.g., all compounds giving a test result greater than a given amount decided beforehand.

(5) Simulate a retest on these compounds by adding a random error to the known activities of the compounds.

(6) Compare the two series of tests on the "positives".

Example. Four hundred compounds were screened in a routine biological test and 40 of them were considered positive. These 40 were retested. The average degree of activity in the initial tests showed a reduction of 11% after retest. The standard error of the test was known to be 8%.

RESULTS OF THE SIMULATION

The assumed distribution of activities was that given in Table 1. The value of 8% was taken for the standard error of the test.

The results of the first simulation are given in Table 3. Of the 400 compounds tested only those that passed the first screen, i.e., which gave simulated test results of 12% or more, are included, and those test results are given in the second column. The first column represents the known activities of the compounds as selected randomly from the distribution of expected activities. The third column gives the results of the retest, and the fourth, the averages of the first and the second tests.

The simulation was repeated 20 times giving the means of Table 4.

These tables show that the grand mean of the retested activities is only about one-half of the corresponding mean of the initial test on the "active" compounds, and indicate that the apparent drop in activity on retest in the routine screening test noted previously, is normal and to be expected. It is in no way an indication either of loss in activities of compounds or of aberrations in the biological test.

EFFICIENCY OF SCREENING PROCEDURE

Further useful results can be obtained from the above simulations. For example they supply a direct and simple way of assessing the *efficiency* of the screening procedure. A brief indication of how this can be done will now be given. Screening divides the compounds tested into two lots, those that are considered to be of interest and those

TABLE 3. RESULTS OF FIRST SIMULATION

% Activity (1)	Activity on first "test" ⩾ 12% (2)	Repeat test (3)	Average test (4)
0·00	19·57	1·96	10·77
0·00	15·27	−15·05	0·11
0·00	20·38	10·25	15·32
30·00	28·88	29·93	29·41
0·00	14·18	− 4·84	4·67
7·50	18·03	7·34	12·69
0·00	15·76	− 4·68	5·54
30·00	33·17	49·45	41·31
0·00	17·12	5·91	11·52
15·00	12·60	21·88	17·24
22·50	21·99	23·04	22·52
0·00	22·66	1·88	12·27
0·00	12·82	− 6·62	3·10
0·00	12·56	4·16	8·36
15·00	21·06	21·96	21·51
22·50	34·42	18·59	26·51
0·00	24·61	− 4·98	9·82
22·50	23·88	24·86	24·37
0·00	12·69	− 3·57	4·56
30·00	36·72	29·48	33·10
0·00	18·39	−17·13	0·63
22·50	28·07	18·53	23·30
30·00	16·09	44·25	30·17
0·00	14·91	− 3·16	5·88
7·50	24·65	− 5·49	9·58
0·00	19·79	− 8·80	5·49
15·00	13·31	11·77	12·54
0·00	14·35	− 2·53	5·91
0·00	16·17	18·60	17·39
15·00	18·93	9·38	14·16
0·00	13·51	− 7·25	3·13
30·00	23·39	24·34	23·87
0·00	13·66	− 8·94	2·36
15·00	12·31	18·92	15·61
15·00	21·10	11·12	16·11
0·00	15·32	12·73	14·02
22·50	30·90	20·35	25·62
30·00	27·46	43·01	35·23
15·00	32·87	17·41	25·14
15·00	22·07	23·50	22·78
7·50	13·17	6·74	9·96
0·00	17·45	− 3·70	6·88
22·50	29·27	30·00	29·64
0·00	23·74	10·20	16·97
Mean 10·40	20·44	10·79	15·61

TABLE 4. MEAN RESULTS FROM EACH OF TWENTY SIMULATION RUNS (each conducted on a group of 400 compounds)

No. of runs (1)	Mean % activity per run (2)	Mean activity on first test per run (3)	Mean repeat tests per run (4)	Mean of (3) and (4) (5)
1	10·40	20·44	10·79	15·61
2	11·03	20·96	11·82	16·39
3	10·37	19·30	10·50	14·90
4	9·91	18·57	9·31	13·94
5	9·96	19·39	10·85	15·12
6	8·61	18·44	7·23	12·84
7	9·48	18·34	10·85	14·59
8	9·84	18·25	10·34	14·30
9	10·42	17·90	9·06	13·48
10	7·90	17·23	9·77	13·50
11	8·14	16·20	7·15	11·67
12	9·68	18·40	8·62	13·51
13	8·11	19·43	7·87	13·65
14	7·62	18·74	6·81	12·78
15	9·48	17·66	9·43	13·55
16	9·88	18·80	9·30	14·05
17	11·48	19·25	10·42	14·83
18	8·90	17·36	6·48	11·92
19	12·09	21·00	12·64	16·82
20	13·06	20·08	13·44	16·76
Grand Mean	9·818	18·787	9·634	14·211

considered not to be of interest. The screening is based on a test which is subject to error, and, therefore, some misclassifications will arise. For the first screen the procedure was to regard compounds giving results $\geqslant 12\%$ activity as of interest and all others of no interest. A total of 8,000 compounds were "tested" in the above simulations, and of these 1,106 were considered active and 6,894 inactive.

By noting the actual activities of the randomly selected compounds in the two groups—those accepted and those rejected—the distributions of the activities of the compounds in the two groups can be derived. These are given in Table 5.

TABLE 5. DISTRIBUTIONS OF ACTIVITY

	% Activity of compounds					Total
	0	7·5	15·0	22·5	30·0	
Compounds which passed the test ..	474	165	199	187	81	1106
Compounds which failed the test ..	6332	429	115	17	1	6894
Total	6806	594	314	204	82	8000
Probability of accepting compound ..	0·070	0·278	0·634	0·917	0·988	
Probability of rejecting compound ..	0·930	0·722	0·366	0·083	0·012	

This Table shows that only 1 out of 82 (i.e. 1·2%) of the compounds with activity of 30% and 17 out of 204 (i.e. 8·3%) of the compounds with activity of 22·5% fail to be detected. The chance of missing active compounds of interest is, therefore, quite small. Similarly, the chance of passing compounds is negligible for those of zero activity and fairly small for compounds with activity of 7·5%. This scheme is suitable in the situation where compounds of 7·5% activity are of no interest, compounds of 15% activity of marginal interest and compounds of activities 22·5% or larger of definite interest.

A greater degree of discrimination arises after the second test. If on retest only those compounds with apparent activities $\geqslant 15\%$ are selected, the probabilities in Table 6 are obtained.

TABLE 6. DISTRIBUTIONS OF ACTIVITIES FOR OTHER ACCEPTANCE CRITERIA

	% Activity of compounds				
	0	7·5	15·0	22·5	30
Number accepted on second test	12	18	93	147	81
Probability of accepting	0·002	0·030	0·296	0·721	0·988
Probability of rejecting	0·988	0·970	0·704	0·279	0·012
(Numbers accepted on alternative procedure) (see text)	(2)	(11)	(86)	(147)	(76)

This second screen has removed most of the inactive compounds at a slight sacrifice of active ones.

Another possible criterion of selection is to calculate the means of the first and second test and to accept compounds with means $\geqslant 18\%$. This is a more stringent criterion which rejects far more of the inactive compounds at some further slight sacrifice of the active ones, as seen from the results in the last row of Table 6.

An initial inspection of the test performance in this way is often sufficient to select a suitable procedure. It is, sometimes, desirable to attempt a more quantitative comparison of the schemes. One way of doing this is to place a cost on passing compounds of low activities and a value on some active compounds which have been detected by the test. A cost might also be placed on those active compounds that have not been detected. These costs may be largely subjective and therefore the sensitivity of this procedure of selecting a suitable scheme to the assumption made will need to be known. This can be done by investigating a range of different costing schemes. A range of different initial distributions of activities might also need to be tried.

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Some pharmacological effects of 1,2,3,4-tetrahydro-2-naphthylamine (β-tetra) and its secondary *N*-alkyl derivatives

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Some effects of 1,2,3,4-tetrahydro-2-naphthylamine (β-tetra, β-tetrahydronaphthylamine, 2-aminotetralin) and four of its secondary *N*-alkyl derivatives on blood pressure in the cat and in the pithed rat are described. Pressor responses were obtained with β-tetra in both preparations. The introduction of an *N*-methyl group to form 1,2,3,4-tetrahydro-2-naphthylmethylamine led to a decrease in pressor potency in both species. The substitution of higher alkyl groups yielded compounds which were depressor in the cat and had markedly reduced pressor potency in the pithed rat. Increases in substituent chain length resulted in slight decreases in adrenaline-blocking potency and slight decreases in the tendency of the compounds to depress breathing. Experiments with β-tetra in reserpine-treated, adrenalectomized and hexamethonium-treated animals led to the conclusion that the pressor effect of 1,2,3,4-tetrahydro-2-naphthylamine is mediated by an action at the sympathetic neuron resulting in the release of noradrenaline.

THE compound β-tetra was first prepared by Bamberger & Müller (1888) and its pharmacological properties have since been extensively studied. Bovet and colleagues (Bovet, Bovet-Nitti & others, 1951; Bovet, Bovet-Nitti & Longo, 1952; Bovet, Sollero & Marotta, 1952; Bovet & Virno, 1952; Bovet, 1959) synthesized and examined some pharmacological actions of the *N*-methyl, *N*-dimethyl, *N*-ethyl and *N*-diethyl derivatives of β-tetra. They reported that these drugs had sympathomimetic actions and also possessed anti-adrenaline activity. The effects of 1,2,3,4-tetrahydro-2-naphthylamine (β-tetra, β-tetrahydronaphthylamine, 2-aminotetralin) and of some secondary *N*-alkyl derivatives on the blood pressure, respiration and pressor responses to adrenaline in the cat are now described. Pressor potencies of the compounds in the pithed rat preparation (Shipley & Tilden, 1947) have also been examined, so too has the mechanism of the pressor action of β-tetra. The compounds used were prepared by Craig, Moore & Ritchie (1959).

Experimental

METHODS

Cats of either sex (1·5–4 kg) were anaesthetized with intravenous chloralose (80 mg/kg) after induction with ether. Tracheal cannulation was such that respiratory movement could be recorded by the method of Gaddum (1941), or artificial respiration could be applied if needed. Blood pressure was measured via a cannula in a carotid artery by a mercury manometer. Drugs were administered into a cannulated vein.

In some experiments with β-tetra, cats were made spinal by the method of Burn (1952) and maintained by artificial respiration. Some cats were

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treated with 3 to 4 mg reserpine in 20% ascorbic acid, injected intraperitoneally, on each of 2 days and used on the 3rd day.

The experimental procedure for cats was as follows: adrenaline (5–40 μg) was injected intravenously at regular intervals, usually of 7–12 min. When the mean resting blood pressure was steady and responses to adrenaline were constant, β-tetra or one of its derivatives was injected intravenously. Progressively increasing doses were given at intervals of at least 20 min during which the regular injections of adrenaline were continued.

Blood pressure in pithed rats prepared as described by Shipley & Tilden (1947) was recorded with a Statham P23Db pressure transducer and ink-writing recorder (Pennefather & Rand, 1960). Rats received 1 mg atropine/100 g subcutaneously 20 min before use. In some experiments heart rate was also recorded.

The compounds used were: 1,2,3,4-tetrahydro-2-naphthylamine hydrochloride (β-tetra) and the *N*-methyl-(methyl β-tetra), *N*-ethyl-(ethyl β-tetra), *N*-n-propyl-(propyl β-tetra), and *N*-n-butyl-(butyl β-tetra) 1,2,3,4-tetrahydro-2-naphthylamine hydrochlorides.

Results

CATS

Each compound was given in a dose of 0·5 mg/kg to at least 4 cats. The primary amine, β-tetra, caused large pressor effects (45–80 mm Hg). Methyl β-tetra had decreased pressor potency (30–45 mm Hg). The effects of both drugs lasted 10–20 min. Tachyphylaxis occurred when subsequent equal or larger doses of either drug were given. Ethyl and propyl β-tetra had pronounced depressor effects (−20 to −45 and −40 to −55 mm Hg respectively) which often lasted for more than 1 hr. Subsequent injections of ethyl-β-tetra often produced pressor effects. Fig. 1 shows a record of blood pressure from a cat given propyl β-tetra. Butyl β-tetra was a less potent hypotensive agent (−10 to −25 mm Hg) than propyl β-tetra and its effect was relatively short-lived.

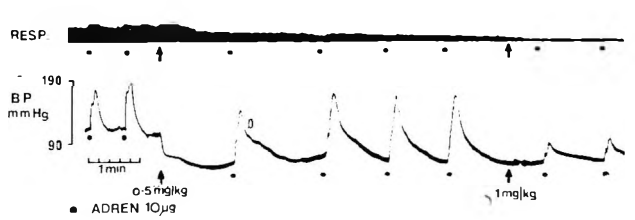

FIG. 1. Record of blood pressure (lower trace) and breathing (upper trace) in a cat anaesthetized with chloralose. Pressor responses at spots were produced by injection of adrenaline 10 μg. Propyl β-tetra (↑) lowered the blood pressure and depressed breathing. Subsequent response to injections of adrenaline were prolonged. A second dose of propyl β-tetra, 1 mg/kg, given while the blood pressure remained low, further depressed breathing: responses to adrenaline were reduced.

Anti-adrenaline activities of the compounds were compared by determining the dose (A) of each that caused 100% abolition of the pressor response to adrenaline. The respiratory depressant effects of the compounds were compared by determining for each the dose (R) that caused cessation of breathing. These doses (in μmole/kg) were: β-tetra A 10 (5–11), R 15 (5–22); methyl β-tetra A 10 (10), R 13 (10–20); ethyl β-tetra A 23 (7–48), R 26 (2–49); propyl β-tetra A 35 (22–44), R 26 (9–44); butyl β-tetra A 20 (8–21), R 20 (8–41). Thus potency in blocking pressor responses to adrenaline was similar with β-tetra and its methyl derivative but then showed a fall to the propyl derivative, rising again with the butyl compound. In some experiments with β-tetra and methyl β-tetra, adrenaline 'reversal' was observed after giving a number of doses.

The primary amine and its methyl derivative caused most depression of breathing with successive reductions from ethyl to propyl. Butyl-β-tetra showed an increased effect. Although β-tetra and methyl β-tetra had the most depressant activity, they caused least respiratory depression in doses which were effective in blocking the pressor effect of adrenaline. Thus, their R/A ratios were 1·5 and 1·3, whereas, those for the ethyl and propyl derivatives were 0·8 and 0·7 respectively.

RATS

Compared on a per kilogram basis the compounds were approximately ten times more potent as pressor agents in the pithed rat than in the cat and tachyphylaxis and cross-tachyphylaxis were not observed. All compounds were pressor, the relative potencies being: β-tetra 85–105; methyl β-tetra 40–65; ethyl β-tetra 1·5–2·5; propyl β-tetra 1·2–3; butyl β-tetra 1·2–2·3. In other experiments, the potency of β-tetra was compared with that of (±)-amphetamine and that of tyramine. Amphetamine and β-tetra were approximately equipotent and both were about 65% as potent as tyramine. In Fig. 2, a record of blood pressure in a

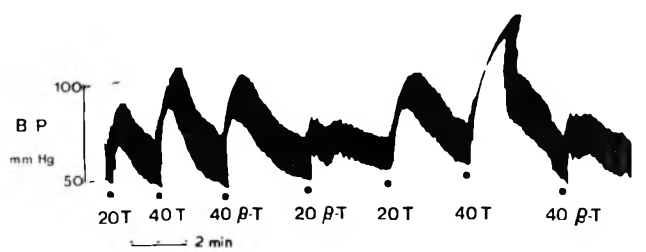

FIG. 2. Record of blood pressure of pithed rat. β-Tetra (40 μg) produced a pressor response intermediate in height between responses to tyramine (T) 20 and 40 μg. Subsequent responses to tyramine were larger than before β-tetra was given, whereas the response to a second injection of β-tetra was smaller.

pithed rat is shown. Injections of tyramine and of β-tetra were alternated at fairly short intervals. The responses to tyramine became larger with each successive injection while those to β-tetra became smaller. If injections of tyramine were discontinued there was no significant reduction

in the size of responses to repeated injections of β-tetra. The relative absence of tachyphylaxis to the actions of the compounds, and of any noticeable cross-tachyphylaxis between them suggests that the pithed rat preparation is suitable for the comparison of pressor activities of drugs of this type.

ACTION OF β-TETRA IN RESERPINE-TREATED ANIMALS

Cats. The pressor action in spinal cats of β-tetra (2–4 mg), first reported by Barger & Dale (1910), was confirmed in two experiments. Activity was much reduced in three reserpine-treated spinal cats; 15 min after infusion of noradrenaline (50 μg/kg/min) responses to injections of tyramine and of β-tetra (1–2 mg/kg) were still less than in untreated animals. At that time the blood pressure was low and steady.

Rats. β-Tetra had less pressor effect in reserpine-treated pithed rats than in untreated rats. Table 1 shows the results of experiments in which β-tetra (80 μg) was injected before and after infusions of noradrenaline. After the infusion of noradrenaline the pressor response to β-tetra was larger than before. Fig. 3 shows a record of blood pressure of a reserpine-treated pithed rat and illustrates the increase in response 15 min after a noradrenaline infusion.

ACTIONS OF β-TETRA AFTER ADRENALECTOMY AND GANGLION BLOCKADE IN RATS

Three rats were adrenalectomized two weeks before use and were then pithed. No reduction in the pressor activity of β-tetra relative to that of

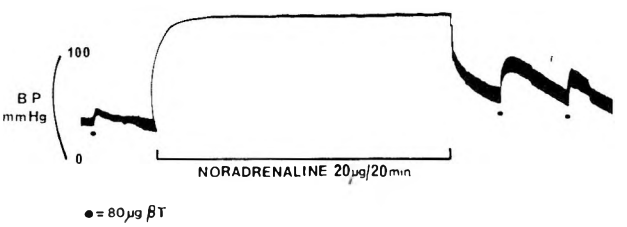

FIG. 3. Record of blood pressure from pithed rat which had received reserpine (2 doses of 2·5 mg/kg on each of two days before use, given intraperitoneally). β-Tetra (80 μg) had little pressor action. Noradrenaline (20 μg) infused during 20 min, caused a prolonged pressor response. Subsequent pressor responses to β-tetra were larger than before the infusion.

TABLE 1. THE PRESSOR RESPONSES TO β-TETRA IN RESERPINE-TREATED PITHED RATS MEASURED BEFORE AND AFTER INFUSION OF NORADRENALINE

Dose of β-tetra μg	Amount of noradrenaline infused μg	Pressor response before infusion mm Hg	Pressor response after infusion mm Hg	Ratio of pressor response after infusion to pressor response beforehand
80	20	10	45	4·5:1
80	10	12	29	2·2:1
80	10	10	15	1·5:1
80	20	8	27	3·4:1
60	20	7	15	2·1:1
60	—	17	—	—

Infusions of noradrenaline were given over 10 or 20 min period at the rate of 1 μg/min/rat.

tyramine was seen. In a fourth rat, acute adrenalectomy did not modify blood pressure responses to β-tetra and tyramine.

In other experiments, the action of hexamethonium on the blood pressure response to β-tetra was examined. There was only slight inhibition of the response to β-tetra, but complete block of the response to nicotine used at an initially equipressor dose.

Discussion

The potent pressor action of β-tetra in anaesthetized and spinal cats has been confirmed; this effect also occurs in the pithed rat. Substitution of one amine hydrogen atom by alkyl groups of increasing chain length alters activity. Methyl-β-tetra has less pressor activity in both cats and rats. Larger alkyl groups are depressor in the anaesthetized cat and much less potent than β-tetra as pressor agents in the pithed rat. Adrenaline-blocking potency and potency in producing depression of respiration were progressively reduced by the introduction of methyl, ethyl and propyl groups, but were again increased slightly when the substituent chain length was increased to four carbon atoms (butyl-β-tetra).

The tachyphylaxis to the pressor actions of β-tetra observed with cats has previously been noted by Barger & Dale (1910) and by Cloetta & Waser (1913) in dogs. Cross-tachyphylaxis between the β-tetra derivatives was also reported by the latter authors. Ethyl β-tetra, as found previously, had much less pressor action than either of the lower homologues. Respiratory depression following injection of β-tetra, methyl-β-tetra or ethyl-β-tetra as observed by Cloetta & Waser was also confirmed. Bovet & others (1952), on the other hand, did not mention the effects of the drug upon respiration.

Adrenaline blocking potency did not increase with increasing *N*-alkyl chain length. In contrast, Bovet & others (1952), who used dogs, reported that ethyl-β-tetra was more potent in blocking the pressor action of adrenaline than either the unsubstituted or the methyl derivative. In the present experiments with cats, the duration of adrenaline blockade produced by ethyl-β-tetra was longer lasting than the blockade produced by the compounds of lower molecular weight, but its adrenaline blocking potency was slightly less.

Reserpine treatment, but neither adrenalectomy nor effective ganglion-blocking doses of hexamethonium, reduced the pressor effect of β-tetra. These findings suggest that direct release of noradrenaline from sympathetic nerve endings may be a significant component of the drug's pressor action. Such an effect could explain the findings of Bacq (1936) that β-tetra failed to cause contraction of the denervated or cocaine-treated nictitating membrane in the cat, although there was contraction of the normal innervated membrane.

The failure of noradrenaline infusions to restore pressor responses to β-tetra in resperpine-treated cats is reminiscent of the findings of Burn & Rand (1958) with amphetamine in reserpinized cats. They were unable to obtain restoration of the pressor response to amphetamine if an

injection of amphetamine had been given before the infusion. Rand (personal communication) has pointed out that this is true for other amines which, like amphetamine and β-tetra, often produce tachyphylaxis. In the pithed rat, tachyphylaxis to the pressor action of β-tetra was not seen, and pressor responses to β-tetra were readily restored by noradrenaline infusions in reserpine-treated preparations. It may be that an anti-sympathomimetic acition of β-tetra, manifest in the present experiments as an antagonism towards the pressor action of adrenaline, might at least partly account for the tachyphylaxis occurring in normal cats, as well as for the failure of noradrenaline to "restore" the pressor action of β-tetra in reserpine-treated cats.

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Metabolic effects induced by the interaction of reserpine with desipramine

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The administration of desipramine to reserpinized rats induces an increase of body temperature without any change in blood glucose and liver or muscle glycogen. Instead there is an increase in the utilization of blood glucose after a glucose load and an increase of blood lactic acid. Plasma and brown adipose tissue free fatty acids are also increased. These findings are discussed in relation to the event linking the central stimulation induced by desipramine with the peripheral metabolic changes associated with the increase of body temperature.

THE interaction between imipramine-like drugs and reserpine (Garattini, 1958; Costa, Garattini & Valzelli, 1960; Sulser, Watts & Brodie, 1960) has been the subject of extensive investigations in an attempt to elucidate the mechanism of action of tricyclic antidepressant agents. In particular this laboratory has examined the increase in body temperature induced by desipramine and similar drugs in rats made hypothermic by previously administered reserpine (Garattini & Jori, 1967).

The increase in body temperature is probably of central origin because it can be reproduced by injecting desipramine into the brain (Bernardi, Jori & others, 1966) or even into selected thalamic and hypothalamic areas (Rewerski & Jori, 1968). This effect is abolished by pithing the animals and is decreased relative to the level of spinal transection (Bernardi, Paglialunga & Jori, 1968). The interaction between desipramine and reserpine is not due to inadequate reserpinization (Manara & Garattini, 1967) but is probably mediated by the adrenergic system (Sulser, Bickel & Brodie, 1964; Sulser & Sorowo, 1965). It is well known that tricyclic antidepressant agents inhibit the uptake of noradrenaline peripherally (Hertting, Axelrod & Whitby, 1961; Iversen, 1965; Callingham, 1967) and in the central nervous system (Glowinski & Axelrod, 1964).

Inhibition of noradrenaline uptake leads to an increased availability of the adrenergic mediator at the adrenergic sites (Sulser, Owens & Dingell, 1967), particularly in reserpinized animals where the stores of catecholamines are depleted, and may be responsible for the potentiation of catecholamines. Consistent with this hypothesis is the finding that the hyperthermic effect of desipramine in reserpinized animals is blocked by inhibitors of noradrenaline synthesis (Jori, Carrara & Garattini, 1966a) and by adrenergic blocking agents (Jori, Paglialunga & Garattini, 1966b).

The mechanisms which presumably link the effect of desipramine in the central nervous system to the peripheral metabolic events responsible for the increase in body temperature have now been investigated.

Experimental

MATERIALS AND METHODS

Female Sprague-Dawley rats, 150 ± 10 g, were injected intravenously with reserpine (5 mg/kg) 16 hr before the experiments. After which the

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animals were kept at 20° with a relative humidity of 56%. At the beginning of the experiments body temperatures were 28–30°. Blood samples for the glucose tolerance test were obtained from the ophthalmic plexus according to Riley (1960). The rate of glucose disappearance was calculated by the analytical method of Amatuzio, Stutzman & others (1953).

Glucose and lactic acid were measured by the enzymatic methods of Hugget & Nixon (1957) and Scholz, Schmitz & others (1959) respectively. Glycogen was measured by the method of Kemp & Kits von Heijningen (1954) and free fatty acids (FFA) in plasma and in brown adipose tissue were determined according to Dole (1956) with minor modifications.

Results

EFFECT OF DESIPRAMINE ON GLUCOSE AND GLYCOGEN CONCENTRATION

The blood glucose level of fasted reserpinized rats (5 mg/kg i.v.) was not significantly modified by desipramine (7·5 mg/kg i.p.) over 5 hr. However, after a glucose load, desipramine increased the rate of glucose removal from the blood stream, under the same experimental conditions. This is particularly significant considering that reserpine or desipramine, given alone, reduced the rate of glucose disappearance. The effect of desipramine in reserpinized rats was rapid, persisted for at least 2 hr and did not appear to depend upon the body temperature (Table 1). Chlorpromazine, reduced the glucose disappearance in normal and reserpinized rats (Table 1). Liver or muscle glycogen was not affected by desipramine.

TABLE 1. EFFECT OF DESIPRAMINE ON THE RATE OF BLOOD GLUCOSE DISAPPEARANCE IN NORMAL AND RESERPINIZED RATS

No. of rats	Treatment	Time between treatment and glucose administration (min)	Glucose utilization (rate of removal %/min ±s.e.)	Body temperature change °C ± s.e.
15	Saline	—	7·6 ± 0·4	—
15	Desipramine	15	3·8 ± 0·4*	—
5	Chlorpromazine	60	3·0 ± 0·6*	−1·0 ± 0·2
27	Reserpine + saline	—	4·3 ± 0·4	—
29	Reserpine + desipramine	15	7·5 ± 1**	+0·5 ± 0·06
5	Reserpine + desipramine	45	9·7 ± 2**	+2·2 ± 0·5**
5	Reserpine + desipramine	90	9·1 ± 1·5**	+4·5 ± 0·8**
5	Reserpine + desipramine	120	8·2 ± 0·8**	+6·2 ± 0·5**
5	Reserpine + chlorpromazine	60	3·9 ± 0·6	−0·2 ± 0·1

Desipramine (7·5 mg/kg i.p.), chlorpromazine (5 mg/kg i.p.) or saline were given 16 hr after reserpine (5 mg/kg i.v.). Glucose was injected i.v. at a dose of 1 g/kg.
Body temperature was measured before glucose load.
Rate of removal represents the rate of fall of glucose level per min as % of the increase above the fasting level.
$P < 0.01$ in respect to controls (*) or reserpinized rats (**).

EFFECT OF DESIPRAMINE ON THE LEVEL OF LACTIC ACID IN BLOOD

The effect of desipramine, and chlorpromazine, on blood lactic acid levels of normal and reserpinized rats treated with saline or glucose, is summarized in Table 2. Blood levels of lactic acid were much lower in reserpinized animals than in fasted control rats, but were increased in both

TABLE 2. EFFECT OF DESIPRAMINE ON BLOOD LACTIC ACID CONCENTRATION OF NORMAL AND RESERPINIZED RATS

No. of rats	Treatment	Time between treatment and saline or glucose (min)	Blood lactic acid mg % ± s.e.
11	Saline	—	20·7 ± 1·1
14	Desipramine + saline	15	10·2 ± 0·8*
5	Chlorpromazine + saline	15	12·2 ± 1·8*
19	Reserpine + saline		6·6 ± 0·4
15	Reserpine + desipramine + saline	15	10·8 ± 0·8*
5	Reserpine + chlorpromazine + saline	15	7·3 ± 0·5
7	Saline + glucose	15	25·8 ± 1·7
6	Desipramine + glucose	15	23·3 ± 3·8
13	Reserpine + glucose		8·7 ± 0·3
9	Reserpine + desipramine + glucose	15	14·9 ± 0·5**
10	Reserpine + desipramine + glucose	45	14·7 ± 1·2*
5	Reserpine + desipramine + glucose	90	14·1 ± 0·8**
10	Reserpine + desipramine + glucose	120	11·2 ± 0·4*

Reserpine (5 mg/kg i.v.) was given 16 hr before desipramine (7·5 mg/kg i.p.) or chlorpromazine (5 mg/kg i.p.).
Glucose (1 g/kg i.v.) was given 20 min before lactic acid determinations.
Experiments were made in 15 hr fasted rats, at 20°.
* = $P < 0.05$; ** = $P < 0.01$ relative to controls of each group.

groups after glucose administration. Desipramine enhanced the level of blood lactic acid in reserpinized rats treated with saline or glucose. In normal rats, desipramine decreased the blood lactic acid of saline-treated groups but had no effect on glucose-loaded groups. The effect of chlorpromazine was similar to desipramine in that it lowered blood lactic acid levels in normal rats but it was without effect in reserpinized animals.

EFFECT OF DESIPRAMINE ON FFA

Desipramine increased the level of plasma FFA by about 30% in reserpinized rats for at least 1 hr after its administration. Since the intrascapular brown adipose tissue is important for maintenance of body temperature (Donhoffer, Sardy & Szegvàri, 1964) the level of FFA in this tissue in reserpinized rats treated with desipramine or saline was investigated. It is evident from Table 3 that desipramine, although ineffective in normal rats

TABLE 3. EFFECT OF DESIPRAMINE ON THE BROWN ADIPOSE TISSUE FFA OF RESERPINIZED RATS

No. of rats	Treatment	Time between desipramine, chlorpromazine or saline and determination (min)	Brown adipose tissue FFA (μ-equiv./g) ± s.e.	Temperature difference °C ± s.e.	
				Subcutaneous	Rectal
9	Reserpine + saline	20	5 ± 0·4	—	—
8	Reserpine + saline	180	5·8 ± 0·4	−1·1 ± 0·2	−0·5 ± 0·2
9	Reserpine + desipramine	10	6·8 ± 0·7	+0·3 ± 0·1	−0·9 ± 0·1
9	Reserpine + desipramine	20	7·9 ± 0·5*	+0·7 ± 0·2	+1·1 ± 0·1
9	Reserpine + desipramine	40	7·7 ± 0·6*	+2·1 ± 0·5	+2·3 ± 0·5
9	Reserpine + desipramine	180	7·8 ± 0·8*	+4·3 ± 0·6	+4·7 ± 0·4
5	Reserpine + chlorpromazine	40	5·8 ± 0·25	+0·5 ± 0·4	+1·0 ± 0·4
5	Saline	40	9·2 ± 0·3	—	−0·3 ± 0·2
5	Desipramine	40	9·8 ± 0·6	—	−1·2 ± 0·2
5	Chlorpromazine	40	11·5 ± 0·7*	—	−2·4 ± 0·3

Subcutaneous temperature was measured over the intrascapular brown adipose tissue.
Reserpine (5 mg/kg i.v.) was given 16 hr before desipramine (7·5 mg/kg i.p.), chlorpromazine (5 mg/kg i.p or saline. Experiments were made at 20°C.
* $P < 0.01$ versus saline groups.

increased FFA in the brown adipose tissue or reserpinized animals. On the other hand, chlorpromazine increased the FFA in brown adipose tissue, in normal, but not in reserpinized rats.

Discussion

The results indicate that when desipramine is given to hypothermic reserpinized rats, metabolic changes occur that are associated with the increase in body temperature. Although no changes could be obtained in the level of blood glucose or of liver or muscle glycogen, it was observed that desipramine increased the utilization of glucose. Evidence for this effect was obtained by measuring the rate of removal of glucose in blood after a glucose load and by determining the levels of blood lactic acid. The increase of rate of glucose removal and the rise of blood lactic acid levels induced by desipramine in reserpinized animals are particularly significant considering that desipramine has opposite effects on the same parameters in normal rats. Chlorpromazine did not increase body temperature in reserpinized rats nor did it enhance glucose utilization or raise blood lactic acid levels. Desipramine also increased the level of FFA in plasma as well as in the intrascapular brown adipose tissue.

These biochemical manifestations of desipramine could be interpreted as causing an increase in energy metabolism with a resultant increase in body temperature thus allowing reserpinized animals to recover from the hypothermic condition.

Lipolysis in brown adipose tissue appears to be important in restoring body temperature after hibernation (Joel, 1965), and in normal rats for the maintenance of body temperature in emergency conditions (Donhoffer & others, 1964; Imai, Horwitz & Smith, 1968). The hydrolysis of triglycerides would serve the function of producing heat *in situ* (Joel, 1965) where are located large vessels important also for the brain circulation. It is difficult, however, to establish to what extent the observed biochemical changes precede or follow the increase of body temperature induced by desipramine in reserpinized animals. It is, however, significant that at 15–20 min after desipramine administration, the increases in glucose removal, blood lactic acid levels and plasma and brown adipose tissue FFA are accompanied by only a modest rise of blood temperature as measured in the subcutaneous tissue or in the rectal cavity.

Assuming that these biochemical changes are an important event in the interaction between desipramine and reserpine it remains to be established which hormonal or neurohormonal pattern links the central stimulation to the observed peripheral effects.

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Observations on the efficacy of oral hyoscine *N*-butyl bromide

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Hyoscine *N*-butyl bromide (Buscopan) antagonized the spasmogenic action of acetylcholine on guinea-pig isolated ileum, having a pA_2 of 7·8. The drug also had intestinal antispasmodic activity in the anaesthetized cat after either parenteral or enteral administration. With the enterally administered drug larger doses were required, but the effects were longer lasting. Enterally administered in conscious dogs the drug sometimes produced a small increase in heart rate.

HYOSCINE *N*-butyl bromide is an anticholinergic drug used for relieving spasm of the gastrointestinal. biliary and urinary tracts. Its efficacy after injection has been thoroughly established in animal experiments (Wick, 1951), in clinical investigations (Heinkel, 1951; Laurence, 1966) and in experiments with normal human subjects (Brownlee, Wilson & Birmingham, 1965; Herxheimer & Haefeli, 1966). However, some doubts have been raised as to the efficacy of the orally administered drug in experiments with human subjects (Herxheimer & Haefeli, 1966), although there are numerous clinical reports that the drug is effective orally in the control of various gastrointestinal disorders. There has been little published information from animal experiments on the effects of the drug administered via the gastrointestinal tract, however, Wick (1967) produced evidence that after intraduodenal administration of the drug to rats the LD50 was less than that after subcutaneous injection.

Our observations provide evidence for the absorption of hyoscine *N*-butyl bromide from the gastrointestinal tract in amounts sufficient to exert pharmacological effects.

Experimental

GUINEA-PIG ISOLATED INTESTINE

Segments of guinea-pig ileum were suspended in a 25 ml bath containing Tyrode solution maintained at 34° and bubbled with 5% carbon dioxide in oxygen. Contractions were recorded by means of an isotonic lever writing on a smoked drum. pA_2 values were estimated in a manner similar to that described by Schild (1947).

ANAESTHETIZED CATS

Cats weighing 1·5–3·0 kg were anaesthetized with intraperitoneal injections of chloralose (80 mg/kg) and pentobarbitone sodium (6 mg/kg). The trachea was intubated and the animals were ventilated from a respiration pump. Blood pressure was measured from a femoral artery by means of a Statham pressure transducer (P23AA) and recorded on a Beckman Offner Dynograph. The electrocardiogram was recorded from

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leads corresponding to human limb lead II. The femoral vein was cannulated for intravenous administration of drugs. A midline incision in the abdomen was made and the duodenum cannulated for intraduodenal administration of drugs. Movements of a segment of ileum were measured by anchoring one end of the segment to a fixed rod and attaching the other to a strain gauge transducer recording on a Beckman Offner Dynograph. Resting tension was about 5 g. The skin was closed around the transducer, care being taken not to interfere with the movements of the ileum.

The right vagus nerve was cleared, separated from the vago-sympathetic trunk and tied centrally. The peripheral end was stimulated with 1 msec, 8 to 10 V pulses at a frequency of 20/sec for 30 sec.

Contractions of the nictitating membrane were elicited by stimulation of the left preganglionic cervical sympathetic nerve with 1 msec, 3 V pulses at a frequency of 10/sec for 10 sec at 2 min intervals, and recorded by means of a strain gauge transducer.

CONSCIOUS DOGS

The electrocardiogram was recorded from leads corresponding to human limb lead II. Drugs were administered by mouth or by intravenous injection into one of the leg veins.

DRUGS

The drugs used were: acetylcholine hydrochloride, atropine sulphate, histamine acid phosphate, hyoscine *N*-butyl bromide (Buscopan). All doses of drugs are quoted in terms of these salts.

Results

GUINEA-PIG ISOLATED INTESTINE

The pA_2 values for hyoscine *N*-butyl bromide against acetylcholine and histamine were 7·8 and 4·6 respectively, from which it can be seen that the drug is a relatively specific acetylcholine antagonist. It was less potent than atropine, for which the corresponding pA_2 values were 8·5 and 5·2.

ANAESTHETIZED CATS

Intravenous injections of 0·3 to 3 mg/kg of hyoscine *N*-butyl bromide in 8 cats produced an immediate fall in blood pressure, decrease in gut motility and reduction in the size of contractions of the nictitating membrane elicited by preganglionic sympathetic nerve stimulation. The heart rate did not change significantly during the depressor response. The cardiac responses to vagal nerve stimulation, as measured from the ECG or blood pressure records, were abolished and the depressor responses to injections of acetylcholine were diminished by doses of hyoscine *N*-butyl bromide greater than 1 mg/kg. Intestinal responses to vagal stimulation were diminished and intestinal responses to acetylcholine

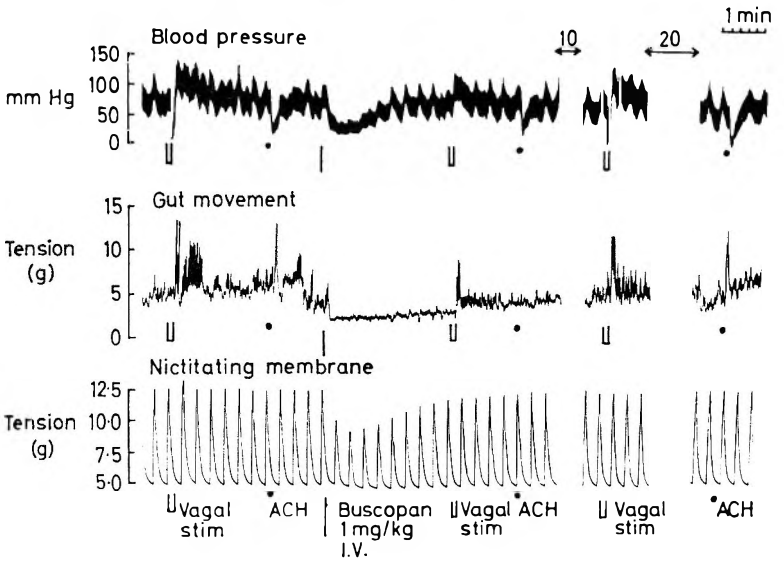

FIG. 1. Effects of intravenous hyoscine *N*-butylbromide in an anaesthetized cat. Records of blood pressure, gut movement and contractions of the nictitating membrane elicited by preganglionic sympathetic nerve stimulation. The drug (1 mg/kg) caused a fall in blood pressure, depression of gut movements and reduction in contractions of the nictitating membrane. The cardiovascular effects of vagal stimulation (∪) were abolished by the drug for a period of 40 min. The intestinal responses to vagal stimulation were reduced and recovered in 40 min. The depressor response to acetylcholine (• ACH) (2 μg) was slightly reduced by the drug and the intestinal response was abolished but recovered within 60 min.

were abolished immediately after the drug. Cardiovascular and intestinal responses to vagal stimulation and acetylcholine, recovered from the effects of the drug given intravenously within 1 hr (Fig. 1). After a 1 mg/kg dose, there was an immediate fall in blood pressure, decrease in gut motility and reduction in contractions of the nictitating membrane with a gradual return to control levels. The depressor response to vagal stimulation was blocked but returned within 40 min. The depressor response to acetylcholine was diminished but returned within 60 min. The intestinal response to vagal stimulation was reduced and that to acetylcholine was abolished for periods of 40 and 60 min respectively.

Intraduodenal administration of 5 to 10 mg/kg of hyoscine *N*-butyl bromide in ten cats was without effect on the blood pressure, heart rate or contractions of the nictitating membrane, but decreased gut motility and affected the cardiovascular and intestinal responses to vagal stimulation and acetylcholine. The depressor response to vagal stimulation was either reduced or abolished and the depressor response to injected acetylcholine was reduced but never abolished. Intestinal responses to vagal stimulation and acetylcholine were both abolished after intraduodenal administration of the drug and these effects lasted for longer than 2 hr. Fig. 2 illustrates the effect of intraduodenal administration of 10 mg/kg of the drug. The decrease in gut motility was evident within 15 min. The

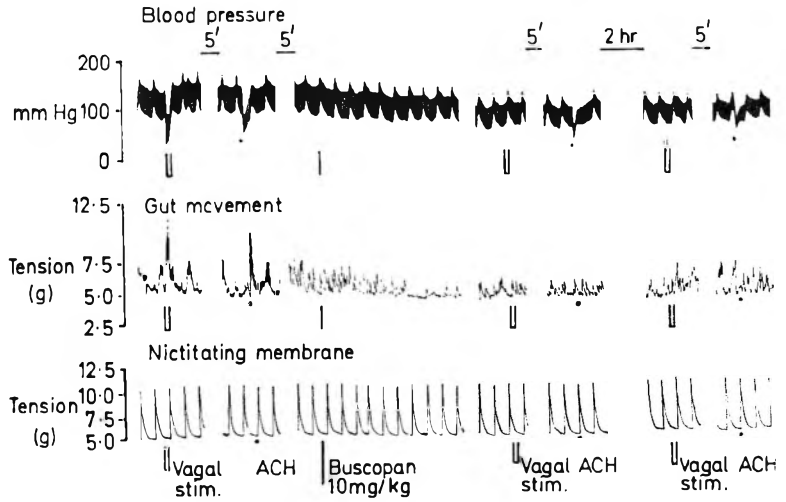

FIG. 2. Effects of intraduodenal hyoscine *N*-butylbromide in an anaesthetized cat. Records as in Fig. 1. The drug (10 mg/kg) decreased gut movements but did not affect blood pressure or contractions of the nictitating membrane. The cardiovascular, and intestinal effects of vagal stimulation (∪) were abolished within 20 min of the administration of the drug and had not returned in 2 hr. The depressor response to acetylcholine (• ACH) (2 μg) was reduced and the intestinal response to acetylcholine was abolished within 30 min of the administration of the drug and these effects lasted for longer than 2 hr.

depressor response to injected acetylcholine was reduced. Intestinal responses to vagal stimulation and acetylcholine were both abolished. These effects lasted for more than 2 hr.

Similar effects on gut motility and cardiovascular and intestinal responses to vagal stimulation and acetylcholine were produced by intraduodenal and intravenous administration of the drug, but the effects of the enterally administered drug were much longer lasting and much higher doses were required by this route.

Evidence of sympathetic ganglionic blockade, that is a decrease in blood pressure and reduction in contractions of the nictitating membrane, was demonstrated only when the drug was given intravenously.

DOGS

Intravenous injections of atropine-like drugs produce tachycardia in conscious dogs by blocking vagal inhibitory tone to the heart. The six dogs used in these experiments had control heart rates of 70 to 120 beats/min, and their heart rates increased to more than 220 beats/min after intravenous injections of atropine methylnitrate.

Hyoscine *N*-butyl bromide was administered orally to these dogs in tablets, solution and mixed with food, and their heart rates were determined from ECG records taken before and at intervals after the drug. In one dog given 8 tablets (6·1 mg/kg), the heart rate was slightly increased

above the control level (to 140 beats/min) between 1½ and 3 hr after dosing. However, in another dog of the same weight given the same number of tablets (6·1 mg/kg) there was no increase in heart rate. Further, in two dogs given 4 tablets each, in one there was a slight but drawn-out increase in heart rate to 120 beats/min (with 2·6 mg/kg), but in the other there was no change (with 2·5 mg/kg). In two dogs given 2 tablets each (doses corresponding to 1·2 and 2·0 mg/kg) there were no changes in heart rate. An intravenous injection of 1·6 mg/kg of the drug produced an immediate increase in heart rate to 220 beats/min, but the effect declined rapidly, the heart rate returning to the control level in 45 min.

Discussion

Our findings indicate clearly that hyoscine *N*-butyl bromide can be absorbed from the gastrointestinal tract of cats and then exert its anticholinergic effects as evidenced by blockade of the effects of vagal stimulation on the heart rate, blood pressure and intestine, and blockade of intestinal responses to injected acetylcholine. The time course of the effects of the drug by the intravenous and enteral routes differed considerably. The effects by the parenteral route were short lasting, presumably because the drug was rapidly eliminated, either from metabolism or excretion. This confirms earlier observations. Thus, Brownlee & others (1965) and Herxheimer & Haefeli (1966) reported that in man the effects of the subcutaneously administered drug disappeared within an hour. However, when intraduodenally administered, the drug had a long duration of action. This was probably a result of its relatively slow absorption, which is to be expected since the drug is a quaternary nitrogen compound. The maximal effect apparently depends on the concentration of the drug in the circulation, and its effect enterally must depend, therefore, on the balance between the rates of absorption and elimination. It follows from these considerations that it is impractical to compare doses given by the two routes in terms of the magnitude of effects at any one time.

A further complication that arises in comparing the effects of different blood concentrations of hyoscine *N*-butyl bromide (arising from administration by the two different routes) is that it has two distinct pharmacological actions: it antagonizes the muscarinic effects of acetylcholine (the so-called anticholinergic action) and it antagonizes the nicotinic actions of acetylcholine and thereby blocks ganglionic transmission (Herman, Shaw & Rosenblum, 1958). The latter effect undoubtedly explains the reduction in contractions of the nictitating membrane and the depressor action of the drug intravenously, but these effects were not observed enterally which suggests that the blood concentration may not rise to a level sufficient for the drug to exert a significant ganglion blocking action.

Our experiments were not designed to investigate whether hyoscine *N*-butyl bromide is more effective on the gastrointestinal tract than on other tissues but they did not disprove that this might be so. In one experiment we observed a relaxation of a segment of intestine (which

had been made hypermotile by injections of neostigmine) with enteral doses of the drug that did not abolish the cardiac responses to vagal stimulation. If it is true that effectors other than the gut (e.g., the heart, salivary glands and ciliary muscle) are relatively insensitive to the drug, then indirect tests for the efficacy of orally administered drug involving responses of these effectors may not be valid, even though it is possible to produce fleeting effects on, say, the heart rate by giving the drug parenterally. The weak and inconstant effect after the drug orally on heart rate of dogs is in accord with the findings on heart rate in man (Herxheimer & Haefeli, 1966). Unfortunately, these authors base their conclusions on the lack of efficacy of the drug orally entirely on effects which are unrelated to the therapeutically desired actions on the gastro-intestinal tract, and which would have amounted to side-effects if they had occurred.

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The guinea-pig isolated vas deferens: a method for increasing sensitivity to drugs

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A preparation of the guinea-pig vas deferens was developed in which it was opened along its length. It was sufficiently sensitive to obtain reproducible dose-response curves to acetylcholine, noradrenaline, histamine, nicotine, 5-hydroxytryptamine and potassium chloride; it was more sensitive than the unopened vas deferens.

THE isolated hypogastric nerve–vas deferens preparation (Huković, 1961) has been widely used for physiological and pharmacological, investigations. The mixed pre- and post-ganglionic nature of the hypogastric nerve and the proximity of the ganglia to the wall of the organ (Sjöstrand, 1962; Ferry, 1963; Ohlin & Strömblad, 1963) led to the development of a method for transmural stimulation to obtain a preparation stimulated through its post-ganglionic nerves (Birmingham & Wilson, 1963). Even when stripped of its mesenteric investment this adrenergically innervated organ is comparatively insensitive to exogenously applied drugs, and has therefore been rarely employed except in conjunction with hypogastric nerve or transmural stimulation (Bentley & Sabine, 1963; Birmingham, 1966). We have been able to modify this preparation, making it sufficiently sensitive for the study of drugs in the absence of electrical stimulation.

Experimental

Vasa deferentia from albino guinea-pigs (250—350 g), killed by cervical dislocation and bled by section of the carotid ateries, were isolated without dissection of the hypogastric nerve; the organs were carefully freed of the mesenteric investment according to the method of Bentley & Sabine (1963). Next, one blade of a pair of fine scissors was inserted into the urethral end of the lumen and the organ cut open longitudinally. The inside surface of the opened vas was carefully scraped free of mucilagenous material. The preparation was placed in a 5 ml organ bath, containing modified Krebs Ringer bicarbonate buffer (Hukovic, 1961), prepared with distilled, demineralized water and aerated with 5% carbon dioxide in oxygen. The temperature was kept at $37 \pm 0{\cdot}5°$. Tension, 200–300 mg, was applied to the organ.

At the beginning of each experiment the preparation was permitted to equilibrate for 1 hr, during which period the bath fluid was exchanged three to four times by bottom drainage. Dose-response curves to acetylcholine, noradrenaline, histamine and potassium chloride were obtained by adding doses every 4 min and washing repeatedly between each application. Doses of 5-hydroxytryptamine (5-HT) and of nicotine were added at 20- and at 90-min intervals, respectively. The agonists were randomly alternated in successive experiments. Between each dose-response curve a washing and resting period of 10 to 20 min was interposed. The maximum contractile responses were recorded as grams of

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tension by a Grass force transducer (FT-03) using a Grass or Sanborn polygraph.

The compounds studied, expressed as salt concentrations per ml of bath fluid, are: Acetylcholine bromide (Eastman Organic Chemicals, Inc.), histamine dihydrochloride (Mann Research Laboratories), (−)-noradrenaline bitartrate (Sterling Winthrop Research Institute), nicotine hydrogen tartrate $2H_2O$ (British Drug Houses), 5-HT creatinine sulphate, H_2O (Mann Research Laboratories) and potassium chloride (reagent grade, Fisher Scientific Co., Inc.). Concentrated stock solutions were made in distilled, demineralized water except that of noradrenaline which was dissolved in 0·1 N HCl; they were frozen between use and stored no longer than 10 days. Appropriate serial dilutions were made with the Krebs buffer. Linear regression analysis of the results was obtained by the method of least squares (Snedecor, 1956), using an Olivetti Underwood Programmer 101, programmed by the manufacturer.

Results

The preparation contracted maximally to all agonists within 1 min except to 5-HT for which 2–3 min were required. Contractions to each agonist declined quickly, even before washing, except to nicotine which caused a contraction lasting up to 1 min, and to 5-HT which sometimes induced long lasting rhythmic waves of contractions. The results are summarized in Fig. 1.

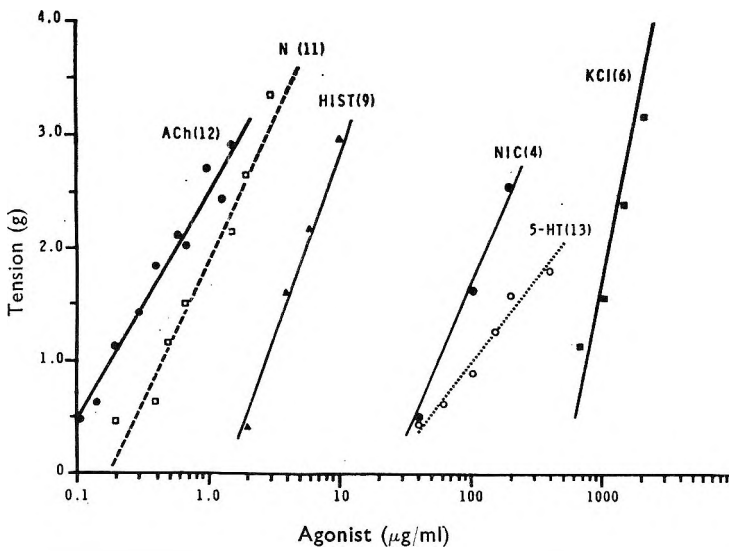

FIG. 1. Dose-response curves with regression lines, obtained by least squares calculations, to acetylcholine (ACh ● slope ± s.e. 2·00 ± 0·13), noradrenaline (N □ 2·50 ± 0·24), histamine (HIST ▲ 3·21 ± 0·26), nicotine (NIC ● 2·88 ± 0·08), 5-HT (5-HT ○ 1·5 ± 0·13) and KCl ■ 5·91 ± 0·46) are depicted. The figures in brackets indicate the number of organs studied for each point shown for each line. The dose-response regression line to nicotine was obtained, using single organs of animals weighing over 300 g.

As can be seen, the steepest slope was obtained with potassium chloride which was significantly greater than that obtained with histamine ($P<0.01$). The dose-response curve to noradrenaline had a slope similar to that to acetylcholine, and was significantly lower than that to histamine ($P<0.001$). Although the curve to 5-HT appears flatter (Fig. 1), the difference in slope when compared with noradrenaline was not significant ($P<0.1$).

The tissue was most sensitive to acetylcholine, which produced contractions at concentrations as low as 0·1 μg/ml. Noradrenaline, at a concentration of 0·2 μg/ml, consistently produced a contraction, whereas it was necessary to use 2μg/ml of histamine and as much as 40μg/ml of nicotine and 5-HT. Potassium chloride at its threshold concentration, 7,500 to 10,000 times that of acetylcholine, produced a very brisk response. If left in contact with the tissue for a longer period, potassium chloride often induced secondary contractions; therefore, it was washed out immediately after a response was observed. All dose-response curves were reproducible, except that not every vas responded equally well to 5-HT and to nicotine. The contractile responses to 5-HT were greater in organs of animals weighing less than 275 g, while nicotine produced greater contractions in organs taken from guinea-pigs weighing more than 300 g. The regression line to nicotine was obtained using organs only of animals weighing over 300 g.

When the vas was stripped but not opened and scraped, much higher concentrations of all agonists had to be applied, and these concentrations often induced spontaneous waves of contractions which persisted even after repeated washing. Under these conditions reproducible dose-response curves could not be obtained.

Discussion

The opened and scraped vas deferens of guinea-pigs can be used to obtain reliable dose-response curves to acetylcholine, noradrenaline, histamine, nicotine, 5-HT and potassium chloride. With the exception of 5-HT which sometimes induced rhythmic contractions, washing of the preparation after agonist administration produced a rapid return to the baseline. These findings are in contrast to those of Ohlin & Strömblad (1963) who showed that noradrenaline and acetylcholine induced spontaneous movements in vasa deferentia, even at 31°. Laporte, Jané & Valdecasas (1966) studied guinea-pig vasa deferentia at 30° and reported that 5-HT did not produce a contractile response. Furthermore, their preparation, which was not stripped, was considerably less sensitive than the stripped and opened organ. They reported an ED50 for acetylcholine of 2 μg/ml and for noradrenaline of 5 μg/ml, while our results showed the opened vas to be approximately 10 times more sensitive. The ED50 of our preparation was 0·3 μg/ml for acetylcholine and 0·6 μg/ml for noradrenaline. The opened vas deferens thus is a reliable and sensitive preparation which permits the study of effects of drugs on this smooth muscle.

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Cortisone acetate crystal forms

SIR,—Different crystalline forms of cortisone acetate have been distinguished by several workers (see Table 1). Unfortunately the various methods used to investigate them have often prevented correlation with previous work, so that a single form may have received several different designations. This is exemplified by the hydrated form recently described by Carless, Moustafa & Rapson (1968a, b) as form IV*, apparently on the basis of similarities between its infrared spectrum and that of their form IV. In fact, examination of this spectrum (Moustafa & Rapson, personal communication) showed it to be identical with that of a form described as form VI (Mesley, 1966), the X-ray diffraction pattern of which agrees with one listed in the X-ray Powder Data File as form γ (card 10-559) and with form V of Williams (1961) and form 5 of Majek (1951, 1954). The method of preparation and the rather sparse X-ray data given by Magerlein, Dale & Wachtel (1958) for their form A suggest that this again is the same form, although this is denied in the patent specification. Table 1 represents an attempt to correlate all those forms about which data have been published.

TABLE 1. CRYSTAL FORMS OF CORTISONE ACETATE PREVIOUSLY REPORTED

a	b	c	d	e	f	g	Solvent
I	III	III	α	2	II		—
II	II	II	β	1	I	B	—
III	I	I		3		C	—
IV	IV	IV	E				ethanol
IV*		VI	γ	5	V	A	water
V	V	V	F				methanol
		VII					tetrahydrofuran/water?
		VIII	δ				dimethylacetamide
					VI		acetone
					VII		dimethylformamide

Notes
a Carless, Moustafa & Rapson (1966, 1968a).
b Callow & Kennard (1961).
c Mesley & Johnson (1965); Mesley (1966).
d *X*-ray Powder Data File (methods of preparation of E & F transposed).
e Majek (1951, 1954).
f Williams (1961).
g Magerlein, Dale & Wachtel (1958).
Other solvated forms have been obtained from methyl ethyl ketone, n-propanol, isopropanol, glacial acetic acid, dioxan and diethyl ether (Kuhnert-Brandstätter & Grimm, 1968).

It is generally accepted that many of these forms contain solvent and are therefore not strictly polymorphic modifications. Recently, however, Kuhnert-Brandstätter & Grimm (1968) have prepared a large number of solvated forms and have disputed earlier results relating to some of them. In particular they claim that forms IV and V, stated by Callow & Kennard (1961) and by Carless & others (1966) to be hydrated, in fact contain ethanol and methanol respectively, and that form III of the latter workers (Callow's form I) is a hydrate. The assertions regarding forms IV and V appear to be correct: these forms have been prepared using anhydrous ethanol and methanol respectively, and the vapours evolved on heating have been examined by infrared spectroscopy, using a multi-reflection gas cell of one metre pathlength; in each instance the vapour was found to be the pure alcohol. The molecular weight and elemental composition quoted by Callow & Kennard (1961) would be consistent with one molecule of solvent per molecule of cortisone acetate in each form.

The statement by Kuhnert-Brandstätter & Grimm (1968) that form III is a hydrate, apparently based solely on the evolution of bubbles when the crystals were heated in silicone oil, cannot be supported. This form has been prepared

by recrystallization from 30% water in acetone, giving a product with an infrared spectrum identical with that of Carless & others (1966) and an X-ray pattern agreeing with that of Callow & Kennard (1961). Differential scanning calorimetry confirmed the findings of Carless & others in showing no transition consistent with evolution of water; a small, very broad, endothermic transition below 55°C was confirmed by infrared examination of the vapour as being due to removal of traces of acetone from the surface of the crystals (there was no evidence for the presence of the acetone solvated form in this sample).

Several other solvated forms have been prepared, and found to agree with previously published data. However the material obtained by recrystallization from, or evaporation of, a tetrahydrofuran solution is apparently different from that reported by Kuhnert-Brandstätter & Grimm (1968). These workers reported infrared absorptions due to OH and C=O groups at 3375, 1750, 1725 and 1708 cm^{-1}, whereas our material, previously designated form VII (Mesley, 1966) had absorptions at 3290, 1739, 1724 and 1701 cm^{-1}. In addition, when obtained by evaporation of tetrahydrofuran solution in a current of air, a peak at 3640cm^{-1} was present; this frequency is improbably high for an organic hydroxyl group in a crystalline solid, and is attributed to inclusion of water molecules in a clathrate type of structure. Whether this form also contains tetrahydrofuran has not been established with certainty.

It was noted during this work that some of the solvated forms were unstable after grinding with liquid paraffin. In particular the forms obtained from acetone, tetrahydrofuran and isopropanol solutions, when examined as Nujol mulls, showed spectral changes over a period of 30 min or less. This phenomenon has been observed occasionally with other polymorphic substances, but despite this the Nujol mull method is still considered to be preferable to the alkali halide disc method for examining polymorphic substances.

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The effect of probenecid on the elimination from CSF of intraventricularly injected 5-hydroxyindoleacetic acid in normal and hydrocephalic dogs

SIR,—In earlier investigations (Andersson & Roos, 1968 a, b) we examined the elimination of 5-hydroxyindoleacetic acid (5-HIAA) from cerebrospinal fluid (CSF) in normal and hydrocephalic dogs after intravenous injection of the precursor 5-hydroxytryptophan (5-HTP). The current investigation is a further attempt to get information about this problem using intraventricular injection of 5-HIAA with and without pretreatment with probenecid. Probenecid has been shown not only to reduce the renal excretion of 5-HIAA (Despopulos & Weissbach, 1957) but also to decrease the outflow of this acid from the brain and cerebrospinal fluid (Neff, Tozer & Brodie, 1964; Guldberg, Ashcroft & Crawford, 1966; Werdinius, 1967).

Twelve mongrel dogs of different ages were used. Hydrocephalus was induced in four dogs about two weeks before the actual investigation, using the kaolin method of Andersson (1968). The animals were kept under pentobarbitone anaesthesia during the experiments.

After puncture of a lateral ventricle, 5 μg 5-HIAA, and in one experiment 5 μg 5-hydroxytryptamine (5-HT), in 0·2 ml saline was injected. One ml of CSF was removed at regular intervals from hydrocephalic dogs by puncture of the contralateral ventricle and from normal animals by percutaneous puncture of the cisterna magna. In one hydrocephalic dog it was possible to obtain cisternal CSF at the end of the experiment. In separate experiments CSF was taken at regular intervals from the ventricle of a normal dog after an intraventricular injection of 5-HIAA and in another from the cistern after a cisternal injection. In one group (3 normal and 2 hydrocephalic dogs) the experiments were made 30 min after intravenous injection of probenecid (50 mg/kg body weight).

5-HIAA and 5-HT were determined according to Ashcroft & Sharman (1960), Roos (1963), Andén & Magnusson (1967) and Werdinius (1967).

The mean value of 5-HIAA in the ventricle of the normal dog is 0·19 $\pm$ 0·02 μg/ml (Andersson & Roos, 1968 b). Intraventricular injection of 5-HIAA to normal dogs caused a slight increase of the acid in the cisternal CSF. The highest concentration was observed in the samples taken 30 min after the injection (Fig. 1a). After pretreatment with probenecid there was a higher

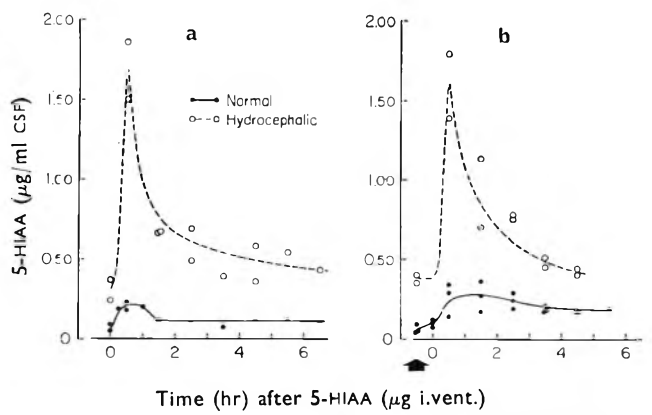

FIG. 1. CSF levels of 5-HIAA in normal (●—●) and hydrocephalic (○—○) dogs (a) after injection of 5-HIAA 5 μg, intraventricularly, (b) after probenecid, 50 mg/kg, i.v. (at arrow) given 30 min before the injection of 5-HIAA.

concentration of 5-HIAA 30 min after the injection and the return of the concentration to within normal levels did not occur over 5·5 hr (Fig. 1b).

After intraventricular injection of 5-HIAA to hydrocephalic dogs, the concentration of this acid much increased and the subsequent decrease was slow. The level of 5-HIAA 180 min after its administration was still about twice that before the injection (Fig. 1a). After pretreatment with probenecid, the 5-HIAA concentration at the various intervals was about the same as in animals which had not received probenecid (Fig. 1 b). From these data it appears that in the normal animal the elimination of 5-HIAA in CSF is reduced by probenecid; this is in agreement with earlier findings (Guldberg & others 1966; Bowers & Gerbode, 1968). In the hydrocephalic animals, however, where it has been suggested that the elimination of 5-HIAA is decreased (Andersson, 1968; Andersson & Roos, 1968 b), probenecid had no detectable influence upon the levels of this acid after intraventricular injection. If the decrease in elimination of 5-HIAA in CSF in hydrocephalus depends on some disturbance in the active transport mechanism, as suggested by Pappenheimer (1961), a mechanism sensitive to probenecid (Guldberg & others, 1966), our findings could be explained by the hypothesis that in hydrocephalus little if any of the mechanism remains for the probenecid to act upon.

The marked initial rise of the 5-HIAA after the intraventricular injection of the acid to hydrocephalic dogs together with the delayed decrease of the values, also supports the hypothesis of a decreased elimination of 5-HIAA in this condition. In one hydrocephalic dog it was possible to determine simultaneously 5-HIAA in the ventricle and in the cistern 4 hr after the injection of 5-HIAA. The finding that the concentrations were the same in both spaces again lends support to the hypothesis.

The samples were taken cisternally in the normal dogs and ventricularly in the hydrocephalic dogs. Accordingly control experiments were made (a) where 5-HIAA was injected intraventricularly to a normal dog and the concentration of the acid in the ventricular CSF followed at regular intervals and (b) where the acid was injected intracisternally and followed by determining the cisternal concentrations (Table 1). Only a moderate increase in 5-HIAA level could be detected in these two normal dogs.

TABLE 1. THE LEVEL OF 5-HIAA OVER 150 MIN IN THE CSF OF A DOG INJECTED WITH THE ACID INTRAVENTRICULARLY AND THE VENTRICULAR CSF LEVEL MEASURED, AND OF A DOG INJECTED INTRACISTERNALLY AND THE CISTERNAL CSF LEVEL MEASURED

	5-HIAA in CSF (μg/ml)			
	O min	30 min	90 min	150 min
Dog 1 (ventr.-ventr.)	0·25	0·29	0·36	0·17
Dog 2 (cistern.-cistern.)	0·05	0·21	0·12	0·11

No 5-HT could be detected in the CSF of the normal dog. Intraventricular injection of the amine to the animal caused a slow rise in the concentration of both 5-HT and 5-HIAA in the cisternal CSF. The highest levels of both amine and acid were found in the sample taken 30 min after the injection (0·03 μg/ml for 5-HT and 0·27 μg/ml for 5-HIAA). It is apparent from this that 5-HT, penetrating into the brain tissue surrounding the ventricles (Fuxe & Ungerstedt, 1967), where the enzymes necessary for the formation of 5-HIAA are found, is rapidly eliminated from CSF, and, in agreement with earlier suggestions, the elimination of 5-HIAA is also rapid (Guldberg & others, 1966; Andersson & Roos, 1968 a).

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The effect of promethazine on the antinociceptive actions of some narcotic analgesics

SIR,—Moore & Dundee (1961) showed that the clinical effectiveness of pethidine as an analgesic was reduced when given in association with promethazine. Siker, Wolfson, & others (1966) confirmed this finding in man by tests with an ear lobe algesiometer. Moore & Dundee also reported that promethazine had a hyperalgesic effect.

Dundee (1960) and Clutton-Brock (1960) had previously found that thiopentone and pentobarbitone would reduce the effectiveness of pethidine and were hyperalgesic. Neal (1965) was able to demonstrate, in mice, a reduction of the antinociceptive activity of morphine and pethidine with thiopentone and other barbiturates. He showed also that the barbiturates were hyperalgesic in mice.

We have tried to show an antagonism of the antinociceptive action of pethidine and other analgesics in mice using an electroshock method devised by Reinhard & DeBeer and described by Burn, Finney & Goodwin (1950).

SASTO strain female mice weighing between 15 and 20 g were first tested to ensure that they would vocalize in response to electroshocks applied at 1 sec intervals to their tails. Animals which did not respond to five or fewer shocks were rejected.

Groups of ten mice were given subcutaneous injections of either saline or a solution of promethazine hydrochloride (10 mg/kg) 15 min before the subcutaneous injection of the analgesic. Thirty min later the animals were again tested for a vocalizing response to the electroshocks. Failure to respond to five more shocks than were previously required to induce a response was our

TABLE 1. THE EFFECT OF PROMETHAZINE ON THE ANTINOCICEPTIVE ACTION OF ANALGESICS

Drug	Mean ED50 mg/kg s.c.	s.d.	No. of experiments	s.e.	Probability
Diamorphine HCl	1·9	0·3	7	0·1	< 0·001
,, ,, after promethazine	2·9	0·5	7	0·2	
Morphine sulphate	7·7	1·8	8	0·6	< 0·001
,, ,, after promethazine	12·1	2·5	8	0·9	
Codeine phosphate	73	12	7	4·5	< 0·001
,, ,, after promethazine	121	19	7	7·2	
Pethidine HCl	29	6	9	2	> 0·05
,, ,, after promethazine	32	7	9	3·5	

selected end point for antinociception for that animal. From the dose response curve, the dose of analgesic causing abolition of response in 50% of the animals (ED50) was measured Each determination of ED50 was repeated at least seven times.

Promethazine itself had no measurable effect on the nociceptive response at doses ranging from 2·5 to 40 mg/kg; it caused neither analgesia nor hyperalgesia. The mean number of shocks required to produce a vocalizing response in 80 mice treated with saline was 2·78 $\pm$ 0·4. The mean number required to produce a response in 80 mice treated with promethazine hydrochloride 10 mg/kg was 2·82 $\pm$ 0·5.

The results in Table 1 show that although promethazine antagonizes the antinociceptive actions of codeine, morphine and diamorphine in mice in this test, it does not show a significant effect on pethidine.

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A further application of the open-ended capillary method for measuring diffusion coefficients

SIR,—At the 1966 British Pharmaceutical Conference we described two methods for studying the tracer diffusion of sodium-22 in protein solutions and phospholipid sols, which were based on the open-ended capillary method of Anderson and Saddington and on the continuous monitoring method of Mills (Castleden & Fleming, 1966). These techniques suffered from the limitation that it was necessary to use an isotope with radiation sufficiently strong to penetrate the walls of the capillary and the metal surrounding the sodium iodide crystal of the scintillation counter. Preliminary experiments, in which the fluid surrounding the capillary has been analysed for radioactivity, have shown that this modification makes the above methods more versatile; for instance, it is possible to use tritium or carbon-14 labelled substances (or a mixture of both).

Using the Anderson and Saddington technique, we set the apparatus up as before, except that only one capillary was immersed in the inactive bulk solution. At various times, t, the activity of the bulk solution, C, was estimated by withdrawing 0·5 ml samples and assaying them for radioactivity using a liquid scintillation counter (Packard 3015). At the end of the experiment the contents of the capillary were added to the bulk solution, and after thorough mixing, its activity was determined and used to calculate the initial activity, C_0, at time $t = 0$. The relation between C, C_0, and C_t, which is the activity remaining in the capillary at time, t, is given by the expression: $C_t = C_0 - C$. Log C_t is linearly related to t, and the diffusion coefficient, D, was calculated as described previously. It was found that the accuracy was improved by weighing the samples into the counting vials, and this was done with the alanine experiment.

Table 1 shows values obtained for the diffusion coefficients of sodium-22 in sodium chloride solution, and of carbon-14 labelled alanine in alanine solution. From the results we conclude that a suitable method based on the above technique can be developed for studying the diffusion of compounds labelled with weak β-emitter isotopes.

TABLE 1. DIFFUSION COEFFICIENTS OF SODIUM-22 IN SODIUM CHLORIDE AND OF ALANINE IN ALANINE SOLUTIONS

Experiment	Compound	Concentration	$Dx10^5$ cm^2 sec^{-1}
1	NaCl	0·1M	$1{\cdot}27_0$
2	NaCl	0·1M	$1{\cdot}31_0$
Mills & Godbole (1958)	NaCl	0·1M	$1{\cdot}277_0$
3	Alanine	1%	$0{\cdot}476_5$
Longsworth (1952)*	DL-β-alanine	0·618%	0·45
" "	DL-α-alanine	0·624%	0·4317

* As there are no values for the self-diffusion coefficient of alanine in the literature, we quote the diffusion coefficient determined by Longsworth (1952) to indicate the order of magnitude to be expected.

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Tolerance experiments with barbitone-dependent rats

SIR,—Recently Ungar & Cohen (1966) found that morphine tolerance could be transferred to mice by the injection of a brain extract prepared from morphine-tolerant rats or dogs. It has also been reported that the respiration of brain slices removed from morphine-tolerant rats is refractory to the depressant effect of morphine (Takemori, 1961; 1962). Adopting experimental procedures similar to those of the above authors we have looked at barbiturate tolerance in experiments with barbitone-dependent and barbitone-withdrawn rats.

Female Wistar rats, about 50 g at the beginning of the experiment, were made dependent on barbitone by the administration of up to 400 mg/kg/day barbitone sodium in the drinking water over a five week period. Withdrawal was effected by replacing barbitone solution by drinking water. Control animals received drinking water throughout. Animals were killed after five weeks of barbitone treatment or 48 hr after withdrawal.

Groups of mice were injected intraperitoneally with a whole brain homogenate (0·5 ml/25 g) prepared from barbitone-dependent, withdrawn or control rats. Each ml of homogenate contained 400 mg of brain in normal saline. Either 3 or 24 hr after administration of the homogenate, the sleeping time after intraperitoneal injection of 100 mg/kg hexobarbitone sodium was measured. At the same time hexobarbitone metabolism *in vitro* was assayed on liver microsomal preparations from other groups of similarly treated mice. The method used involved the determination of the metabolite formed from ^{3}H-labelled hexobarbitone.

Respiratory rate was measured by conventional manometric techniques. Two cortical slices, one from each hemisphere, were placed in each flask and after an initial equilibration period of 15 min at 37° respiration was measured over 60 min. The effect of barbitone (5×10^{-3}M) was measured on the respiration of both unstimulated and stimulated (0·1M KCl) cortical slices taken from barbitone-dependent, withdrawn and control rats.

Although Ungar & Cohen (1966) found that pentobarbitone tolerance was not transferred, their method of producing tolerance was such that it is unlikely that the tolerance observed was due to a central mechanism but rather that the drug stimulated the activity of enzymes concerned with its metabolism. Our method to produce physical dependence on barbiturates was that first described by Crossland & Leonard (1963) who showed that a characteristic withdrawal syndrome developed on cessation of barbitone administration.

TABLE 1. HEXOBARBITONE SLEEPING TIME AND RATE OF HEXOBARBITONE OXIDATION *in vitro* BY LIVER MICROSOMES OF MICE TREATED WITH RAT BRAIN HOMOGENATE

	Pretreatment (hr)	Type of brain homogenate		
		Control	Barbitone-dependent	Withdrawn
Sleeping time (min)	3	61 ± 20(8)	56 ± 14(8)	49 ± 13(8)
Sleeping time (min)	24	66 ± 20(8)	46 ± 15(8)*	55 ± 16(8)
Hexobarbitone metabolism[1]	24	2·8 ± 0·9(4)	3·8 ± 0·9(4)	—

* $P < 0.05$.
[1] μmoles hexobarbitone oxidized/9,000 g supernate from 1 g liver/30 min.

TABLE 2. EFFECT OF BARBITONE (5×10^{-3}M) ON THE RESPIRATION OF BRAIN SLICES OF BARBITONE-DEPENDENT AND WITHDRAWN RATS

Incubation conditions	Oxygen uptake (μM/g/hr)		
	Control	Barbitone-dependent	Withdrawn
Unstimulated	49·0 ± 2	50·4 ± 9	50·0 ± 3
Unstimulated + barbitone	40·3 ± 7	41·3 ± 6	39·8 ± 5
Stimulated[1]	85·3 ± 4·5	90·2 ± 9	83·9 ± 6
Stimulated + barbitone	48·4 ± 7·5	51·6 ± 12	54·3 ± 12

[1] Incubations carried out in the presence of 0·1M KCl.
Figures denote mean ± standard deviation of six observations.

We find hexobarbitone sleeping time to be reduced in mice which 24 hr previously had been injected with brain homogenate prepared from barbitone dependent rats (Table 1). Since the injected brain extract might have affected the activity of hepatic drug-metabolizing enzymes the ability of microsomal preparations to oxidize hexobarbitone *in vitro* was measured in this group of mice. The fact that this activity was somewhat elevated suggests the possibility that the reduction in sleeping time was due to a stimulation of hexobarbitone oxidation and not to a reduced sensitivity of the brain to barbiturate. Support for this view comes from the observation that the barbitone sleeping time (250 mg/kg i.p.) was the same in this group of mice (210 ± 23(8) min) as in the control group (205 ± 18(8) min). It is possible that this enzyme induction may have been brought about by the barbitone present in the brain homogenate from barbitone-treated rats.

The respiratory rate of cortical slices removed from barbitone-dependent or withdrawn rats was the same, and was stimulated to the same extent by 0·1M KCl, as those taken from controls (Table 2). Barbitone depressed unstimulated respiration by about 20% and KCl stimulated respiration by about 40%, again there being no difference between the results obtained with slices from the 3 kinds of experimental animal. The greater sensitivity of stimulated respiration to barbiturates is well known (McIlwain, 1953, Ghosh & Quastel, 1954).

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Moisture permeation through polymer films

SIR,—Based on Fick's law of diffusion and Henry's law, the movement of a gaseous permeant through a film may be expressed as (Kumins, 1965):

$$\frac{W}{A\Delta p} = \frac{P}{t} \qquad (1)$$

where W is the weight of permeant passing through the film in g/hr, A is the area of the film in cm², Δp is the pressure gradient across the film in mm Hg, P is the permeability coefficient in g/hr/cm/mm Hg, and t is the film thickness in cm. In a previous paper concerned with the transmission of water vapour through polymer films (Banker, Gore & Swarbrick, 1966), the term $\frac{W}{A\Delta p}$ was referred to as R_{wvt}, the rate of water vapour transmission, with units of g/hr/cm²/mm Hg. Substitution of this term into equation 1 leads to:

$$P = R_{wvt}.t \qquad (2)$$

According to equation 2, the product of R_{wvt} and t will be constant if the film system is behaving in accordance with Fick's and Henry's laws. Graphically, a plot of R_{wvt} *vs* t will result in a hyperbola; when plotted on a log-log basis, a straight line with a slope of -1 will result. This ideal system is shown in Fig. 1A, 1B, line II. Moisture permeation data giving plots of log R_{wvt} *vs* log t with a slope of less than -1 (line I, Fig. 1A, 1B) indicates that the effect of increasing film thickness is not as predicted by Fick's and Henry's laws. In this case, moisture is permeating the film at a rate faster than would be expected. Conversely, data resulting in log-log slopes of greater than -1 (line III, Fig. 1A, 1B) are indicative of systems transmitting moisture at a rate below that predicted by equation 2 as the film thickness is increased.

If permeation is proceeding in accordance with equation 2, then P will be independent of t. Figure 1C shows the relation obtained when the data used in Fig. 1A and 1B is plotted in this manner. With the accelerated transmission of system I, P exhibits a positive deviation with increasing thickness. A negative deviation is shown by system III. Only in the case of system II, which is ideal, is P truly independent of t.

It is reasonable to suppose that, in these theoretical examples, the polymer films exemplified by system I have an affinity for moisture which is not taken account of by Fick's or Henry's laws. Likewise system III, where the data imply an active repulsion between water molecules and the film. In both cases, the closer the log-log slopes are to -1, the more ideal is the film.

In a previous investigation on the water vapour transmission properties of three polymer film systems (Banker & others, 1966), linear log-log relations typical of system I were observed. With hydroxypropyl cellulose films and mixed Methocel:Ethocel films, both relatively hydrophilic systems, the slopes ranged from $-0{\cdot}389$ to $-0{\cdot}468$. With the more lipophilic butyl methacrylate films the slopes ranged from $-0{\cdot}672$ to $-0{\cdot}701$. The present authors have found a similar log-log relation with cast films of cellulose acetate hydrogen phthalate (CAP, Eastman Organic Chemicals, No. 4642) plasticized with diethyl phthalate. The results are presented in Table 1. In all cases the slope values are less than -1 and are of the same magnitude as the other cellulosic films studied previously.

Taken as a whole, these results indicate that the greater the affinity of moisture for the polymer film system, the greater will the water transmission data deviate

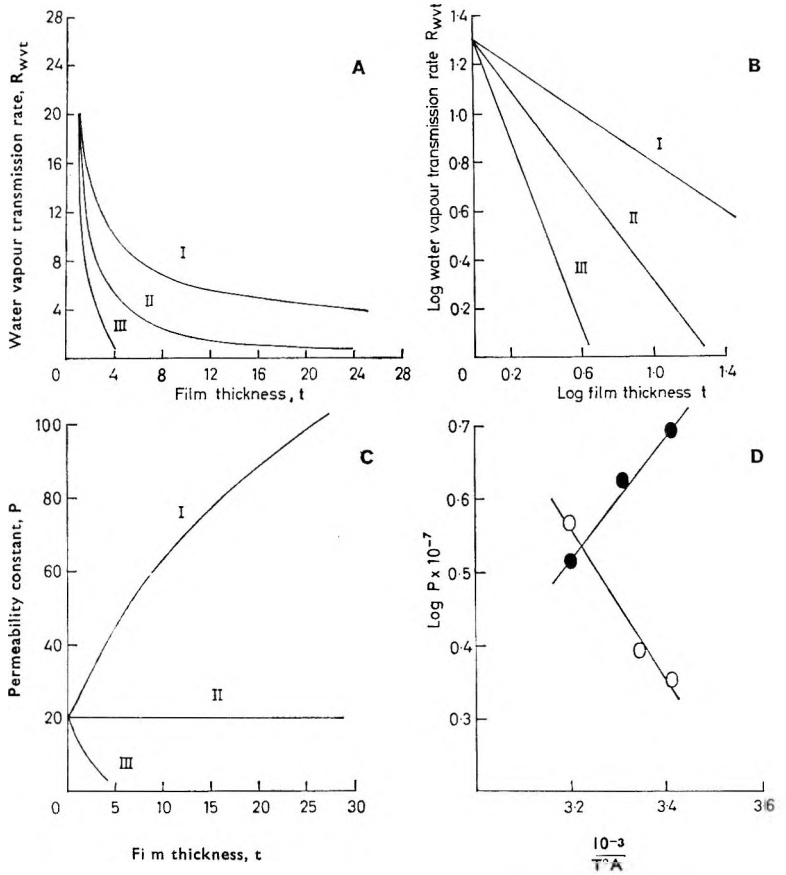

FIG. 1. Water vapour transmission through ideal and nonideal polymer films.

A. Effect of film thickness on rate of water vapour transmission. System I shows positive deviation (accelerated permeation) from equation 1; system II is ideal, conforming to equation 1; system III shows negative deviation (retarded permeation) from equation 1.

B. Data in A plotted on log-log basis. Slopes are −0·5, −1·0, and −2·0 for systems I, II, and III, respectively.

C. Effect of film thickness on permeability coefficient. Based on data used in A.

D. Variation of log permeability coefficient with temperature at a film thickness of 0·0064 cm and a vapour pressure difference of 17·5 mm Hg. Key: n-butyl methacrylate, ○; cellulose acetate hydrogen phthalate, ●. Film casting solution: n-butyl methacrylate 100·0 g; diethyl phthalate 2·5 g; methylene chloride and acetone equal parts to 1000 ml.

from ideality. This affinity of permeant for film will be a function of the hydrophilic nature of the film system, in particular the availability for hydrogen bonding. To test this latter hypothesis, we have also studied the effect of temperature on the permeability properties of CAP and *n*-butyl methacrylate [BMA, DuPont Elvacite (formerly Lucite) 2044] polymer films plasticized with diethyl phthalate. Typical results are shown in Fig. 1D. In the more ideal BMA films the permeability coefficient, increases with temperature in the expected normal manner. The slope of log *P vs* 1/T has a value of $-1{\cdot}08 \times 10^3$. In direct contrast, the permeability coefficient for CAP decreases

TABLE 1. STATISTICAL CONSTANTS FOR RELATION BETWEEN LOG WATER VAPOUR TRANSMISSION RATES AND LOG FILM THICKNESS OF CELLULOSE ACETATE HYDROGEN PHTHALATE FILMS*

Temperature °C	Vapour pressure mm Hg	Correlation coefficient	Slope of line	Ordinate intercept log
20	17·5	0·96	−0·388	−3·779
20	13·2	0·94	−0·396	−3·788
25	23·6	0·99	−0·430	−3·698
30	31·8	0·97	−0·479	−3·645
30	23·9	0·98	−0·466	−3·732
30	10·3	0·96	−0·505	−3·466
40	55·3	0·99	−0·361	−3·925
40	41·6	0·97	−0·315	−4·044
40	17·4	0·98	−0·399	−3·902

* Film casting solution: Cellulose acetate hydrogen phthalate .. 50·0 g
Acetone, to .. .. 400·0 ml
Diethyl phthalate .. .. .. 12·5 g
Methylene chloride, to .. .. .. 1000·0 ml

as the temperature is raised, the slope in Fig. 1D having a value of $+0{\cdot}75 \times 10^3$. This anomalous behaviour, also observed with other cellulosic films (Patel, Patel & Lemberger, 1964), would seem to be related to a breaking of hydrogen bonds between permeant and film, causing in effect dehydration of the film and a reduction in permeation. This view is supported by the effect of temperature on dehydration of ether linkages in nonionic surfactants (Greenwald & Brown, 1954; Schick, 1962) resulting in a cloud point, the anomolous aqueous solubility of methylcellulose, and the dehydration of starch (Knyaginichev, Chernyak & Lyapunova, 1966). With BMA films there is little opportunity for hydrogen bond formation, the log R_{wvt} *vs* log t slope is closer to -1 and hence the normal temperature effect on permeation is observed.

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Determination of methylimidazoleacetic acids in human urine by gas chromatography (Correction)

SIR,—In the addendum to a Letter to the Editor (*J. Pharm. Pharmac.*, 1968, **20**, 659–661) a typographical error has arisen which affects the gas chromatogram. Line 3 of the addendum should begin "0·05M acetate buffer, . . ." and not "0·5M acetate buffer, . . .".

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Letters to the Editor

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