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The phase diagram of cetomacrogol 1000–water–benzaldehyde in the presence of gallate antioxidants

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The phase equilibria of the system cetomacrogol 1000–water–benzaldehyde have been examined in the presence of a homologous series of gallates. Differential scanning calorimetry indicates that a bonded complex is formed in solution between the cetomacrogol and the gallate. The surface-active agent has, therefore, been considered as being the total amount of cetomacrogol and gallate in the system and different ratios have been used to prepare the phase diagrams. As the proportion of gallate increases, the liquid crystal phases become smaller and eventually disappear. It is suggested that this is because the bulky cetomacrogol 1000-gallate complex is unable to form the highly orientated liquid crystal micelles.

Equilibria in systems containing surface-active agents are often complex and may best be described by ternary diagrams in which water and the surface-active agent are two of the components and the solubilizate the third (Mulley, 1964). There is a general similarity in the pattern and nature of the phases found, but the concentration and temperature at which analogous regions occur vary with the chemical nature of the solubilizate and surface-active agent. Information about non-ionic surface-active agents is sparse but it has been suggested (Hyde, Langbridge & Lawrence, 1954) that the same regions will be present as in anionic and cationic surface-active agents. In most cases, however, the chain lengths of the surface-active agents studied have been low because of difficulties in preparation and determining the phases present. The effect of a fourth component on the system has received little attention. The fourth component has either been at a fixed concentration for the determination of the whole solubility diagram or present in a fixed ratio to one of the other components.

The present work describes the phase diagrams of a cetomacrogol 1000–water–benzaldehyde system in the presence of a series of gallate antioxidants. This was of interest as a precursor to a study of gallates as antioxidants for solubilized systems. Because of their phenolic nature, these materials are likely to form bonded complexes with the non-ionic surface-active agent and as a consequence the surface-active agent has been considered to be a cetomacrogol 1000–gallate mixture throughout this text.

MATERIALS AND METHODS

Cetomacrogol 1000. The commercial product Texofor AIP (Glovers Chemicals Ltd.) was used. M.p. 45–46°. Acetyl value 41·1. C:H:O ratio 59·3:10·1:30·3.

Surface-active agent was prepared by melting a mixture of cetomacrogol 1000 with different proportions of gallate esters. Ratios are expressed as w/w.

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Benzaldehyde was obtained from British Drug Houses Ltd. Refractive index 1·545; weight per ml 1·045 at 20°. Assay 99·5%.

Gallates. Propyl gallate (British Drug Houses Ltd.) had m.p. of 147°. Ethyl, octyl and dodecyl gallates (NIPA Laboratories) had m.p. 150–151°, 92–93° and 96–98° respectively, after crystallization.

Water was triple distilled from an all glass still.

Preparation of phase diagrams. A mixture of surface-active agent with either water or benzaldehyde was prepared, depending on the position in the diagram being examined. To the cetomacrogol: gallate mixtures, in ungreased glass stoppered flasks, the third component was gradually added and allowed to equilibrate at the appropriate temperature ±0·1°. Equilibrium was rapidly established and was unchanged for up to three days at the chosen temperature. The phase changes, if any, were noted between each addition of the third component. As far as possible, initial cetomacrogol: gallate mixtures were chosen so that on the addition of the third component the phase boundary was crossed as near as possible at 90°. Phase changes involving liquid crystal were observed with plane polarized light. The liquid crystal phases were all birefringent and, depending on their position within the phase diagram, varied from rather fluid jelly like liquids to solid gels. Where the position of the boundaries could not be determined more accurately than within 1% they are shown as dotted lines.

Differential scanning calorimetry. Cetomacrogol 1000 and propyl gallate were warmed together to give homogeneous mixtures. An accurately weighed quantity was placed in the standard sample pans of the differential scanning calorimeter (Perkin-Elmer DSC-1), the covering hood flushed with liquid nitrogen and a scan made between 240–450° K. With a uniform rate of scan the height of the peak was found to give a similar proportionality to the area under the curve.

RESULTS

Figs 1 and 2 show the effect on the solubility phase diagram of gradually increasing the proportion of propyl gallate in the cetomacrogol: gallate mixture which has been considered as one component for the purposes of constructing the solubility diagram.

Fig. 1 shows the basic solubility diagram for cetomacrogol 1000–water–benzaldehyde. A large region of unstable emulsions of the two conjugate liquids (L_1 and L_2) exists over the lower part of the curve. Within this region compositions towards the left-hand portion are the more stable.

In the lower left-hand corner an isotropic liquid phase, L_1, exists containing hydrophilic spherical micelles. The reverse type of micellar system, L_2, in which benzaldehyde is the continuous phase and the water the solubilizate, occurs at high concentrations of benzaldehyde in the lower right side of the diagram. These two isotropic phases merge above the two phase region and have been designated, L, as it was impossible to determine the proportionate composition of the two micellar types.

Anisotropic liquid crystal phases, LC, exist both in a central position and associated with high concentrations of surface-active agent on the left-hand side of the diagram. Surrounding the central liquid crystal is a heterogeneous mixture of LC + isotropic phase. This is a relatively narrow band where the liquid crystal faces onto the single isotropic liquid phases, but is more extensive on its lower boundary where it is

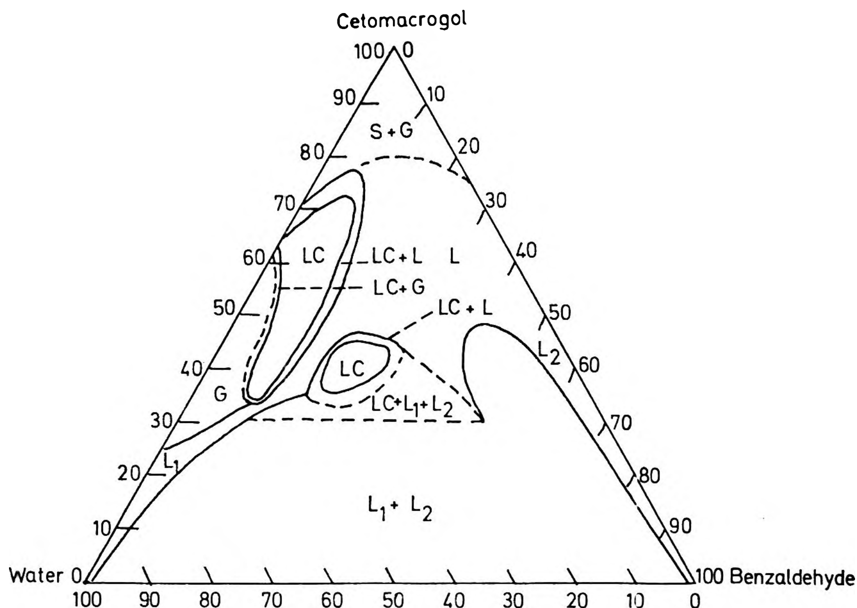

FIG. 1. Phase equilibria in the system cetomacrogol–benzaldehyde–water at 25°. L_1 and L_2 isotropic spherical micelles, LC liquid crystal, G isotropic gel phase, S solid.

associated with the two phase $L_1 + L_2$ systems. The boundary of this mixed region is difficult to determine exactly, because it is temperature sensitive; it has been shown by a dotted line in the diagrams.

The second and larger liquid crystal phase occurs as a band between 35 and 72·5% cetomacrogol. Most of this region is associated with 6 to 10% benzaldehyde, but it tapers towards the binary cetomacrogol–water axis and liquid crystal exists on this axis between 64 and 65·5% cetomacrogol. This second liquid crystal phase is surrounded, as usual, by a region of isotropic liquid + liquid crystal particularly narrow on its lower side. Bordering this lower edge, the isotropic liquid phase, L_1, becomes very viscous and gel like, but shows none of the properties associated with liquid crystal. The gel-like isotropic phase reappears at the apex of the diagram, but in this region it is associated with the separation of solid cetomacrogol.

Fig. 2 shows the effect of gradually increasing proportions of propyl gallate. It is apparent that as the proportion of gallate in the surface-active agent increases, the number of phases which co-exist decreases, thus simplifying the solubilization diagram.

The size of the large $L_1 + L_2$ region shows little variation up to a ratio of cetomacrogol:propyl gallate of 6:1 (Fig. 1). At cetomacrogol:propyl gallate ratios below this, the main $L_1 + L_2$ boundary begins to move to higher surface-active agent concentrations and by the ratio of 2:1 has risen so far as to smooth out the curve of the $L_1 + L_2$ area.

Until a cetomacrogol:propyl gallate ratio of 9:1 is reached, the width of the L_1 phase increases at the expense of the $L_1 + L_2$ region, whilst the onset of formation of the isotropic gel phase recedes gradually to higher surface-active agent concentrations. At a ratio of 4:1 this latter pseudo phase exists only as a narrow strip and disappears when the proportion of propyl gallate is increased to give a ratio of 2:1 cetomacrogol:gallate.

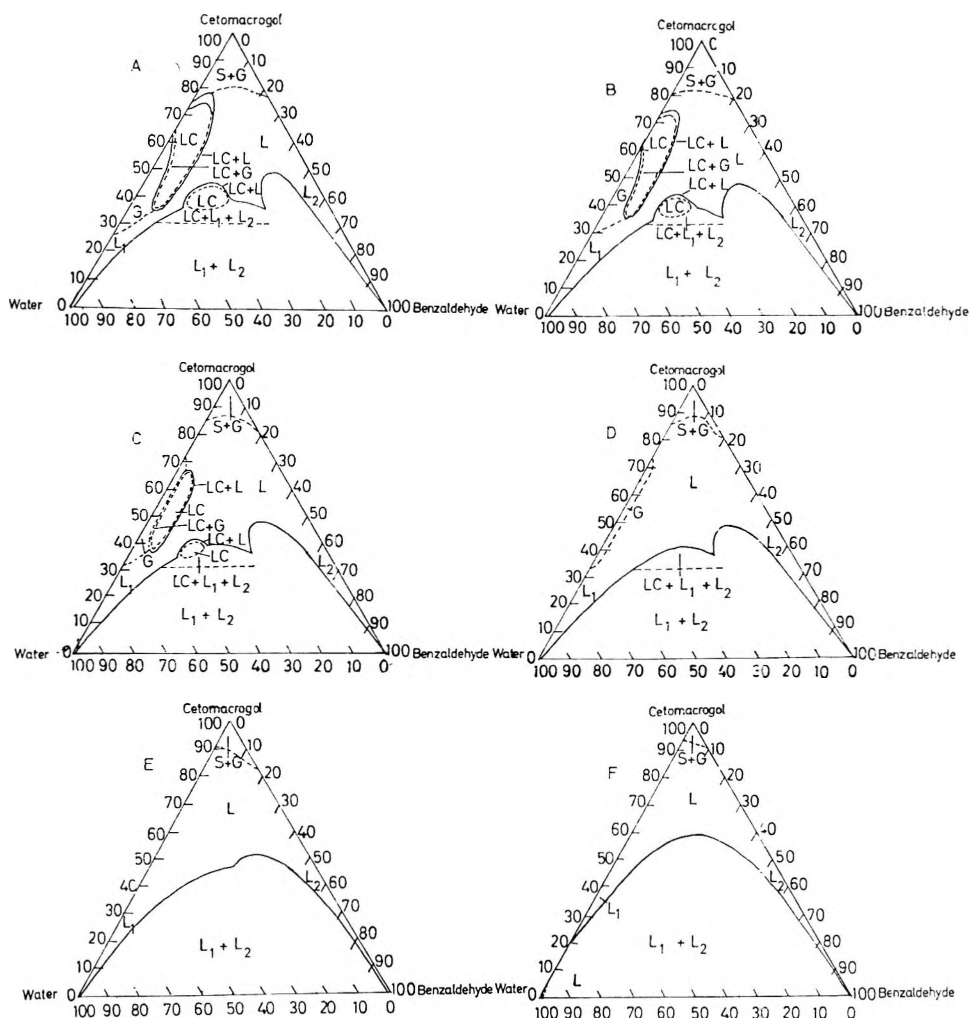

FIG. 2. Phase equilibria in systems containing cetomacrogol–propyl gallate–benzaldehyde–water at 25°. Ratio w/w cetomacrogol : propyl gallate; A 29 : 1, B 19 : 1, C 9 : 1, D 6 : 1, E 4 : 1, F 2 : 1.

The L_1 phase increases with increasing proportion of propyl gallate in the surface-active complex to a maximum and then when the relative proportion of gallate increases above the cetomacrogol : gallate ratio of 6 : 1, the width of the isotropic L_1 phase decreases swiftly until at a ratio of 2 : 1 no benzaldehyde solubilization occurs until the concentration of surface-active agent exceeds 23%. Corresponding changes in the width of the L_2 region are small, although a decrease in the amount of this phase present occurs at a 2:1 ratio of cetomacrogol : gallate.

The most dramatic change that occurs relative to the proportion of propyl gallate present is the disappearance of the liquid crystal phases. The boundary between pure liquid crystal and the surrounding heterogeneous phases becomes difficult to determine accurately and at a ratio of 6 : 1 cetomacrogol : propyl gallate, a phase containing only liquid crystal is absent. All traces of liquid crystal disappears when the ratio was 4 : 1.

The liquid crystal associated with the binary axis increases in extent until a ratio of cetomacrogol to gallate of 29:1: thereafter it quickly decreases to disappear from the binary axis when the ratio is 9:1.

The isotropic gel present also initially increases as the proportion of propyl gallate increases. This appears to occur at the expense of the liquid crystal, but once again the gel phase is absent at higher propyl gallate concentrations. A small quantity of isotropic gel phase remains throughout all these changes associated with the separation of solid cetomacrogol 1000, but this recedes to the region of higher concentrations of surface-active mixture as the proportion of propyl gallate increases.

Temperatures up to 45° do not eliminate liquid crystal from the system if the ratio cetomacrogol:propyl galate exceeds 29:1, but with higher proportions of propyl gallate the temperatures at which liquid crystal finally disappear were: 42° 29:1, 36° 19:1, 32° 9:1, 27° 6:1. In general, increasing temperature diminishes the liquid crystal regions and extends the L_1 region. The increase in the L_1 region is at the expense of the $L_1 + L_2$ phase and the isotropic gel phase associated with the surface-active agent-water axis.

The pattern of phases present, in position and extent is similar when gallates other than propyl gallate are used with cetomacrogol as the surface-active mixture.

A closer study of the boundaries of the L_1 and L_2 phases with the heterogeneous $L_1 + L_2$ phase shows that the gallates at low concentrations increase the solubility of benzaldehyde in the L_1 phase and of water in L_2. An exception is that the presence of dodecyl gallate does not increase the size of the L_2 phase. At higher ratios of cetomacrogol:gallate the proportion of solubilizate within the micelle decreases except with L_2 systems with cetomacrogol:ethyl gallate as surface-active complex. The solubility of benzaldehyde in the cetomacrogol:gallate mixtures is in the reverse order of the gallate molecular weights (Table 1).

Differential scanning calorimetry of cetomacrogol and propyl gallate gave peaks at 324–325° and 427° K respectively. Mixtures of the components in the same proportions as in the solubilization diagrams altered both the position and the height of the cetomacrogol peak. Calculated on the basis of total sample present, the height decreased as the proportion of propyl gallate increased (Fig. 3). If a molecular

Table 1. *The effect of ethyl, octyl and dodecyl gallates on the solubility of benzaldehyde in L_1 systems at 25°*

Gallate	Surface active mixture % w/w	Maximum solubility % w/w of benzaldehyde in L_1 type system with the given cetomacrogol 1000:gallate ratio					Maximum solubility % w/w of water in L_2 type system with the given cetomacrogol 1000: gallate w/w ratio				
		4:1	6:1	9:1	19:1	29:1	4:1	6:1	9:1	19:1	29:1
Ethyl gallate	5	1·6	2·0	1·9	2·0	1·5	2·0	2·0	2·0	2·0	1·8
	10	2·7	3·5	3·2	3·4	3·1	2·5	2·5	2·5	2·3	2·1
	15	4·0	5·1	5·0	5·0	4·6	2·9	2·8	2·7	2·6	2·3
	20	5·8	7·2	7·1	7·0	6·3	3·4	3·2	3·1	3·0	2·7
	25	7·9	10·0	9·9	9·4	8·6	4·0	3·8	3·7	3·5	3·0
	30	10·6	13·4	12·8	12·5	12·0	4·7	4·6	4·5	4·0	3·8
Octyl gallate	5	1·2	1·6	1·7	1·8	1·5	1·9	1·7	1·9	1·8	1·8
	10	2·1	2·7	2·9	3·1	2·9	2·0	2·0	2·2	2·1	2·0
	15	3·0	4·0	4·2	4·6	4·2	2·4	2·4	2·8	2·5	2·2
	20	4·0	5·2	5·8	6·1	6·1	2·8	2·8	3·3	2·8	2·7
	25	5·2	6·8	7·5	8·5	8·0	3·1	3·3	4·0	3·1	3·2
	30	7·0	8·2	9·5	10·5	10·0	4·0	4·3	4·4	4·0	4·0
Dodecyl gallate	5	1·4	1·6	1·7	2·2	1·6	1·5	1·9	1·9	1·8	2·0
	10	2·1	2·7	2·9	3·4	2·8	1·8	2·0	2·2	2·2	2·3
	15	2·9	3·6	4·2	5·0	4·2	2·0	2·5	2·4	2·4	2·8

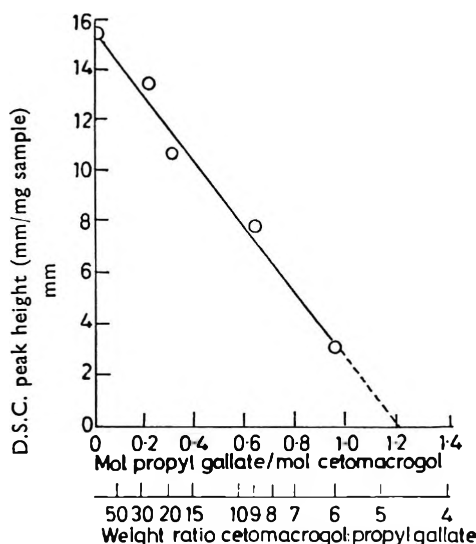

FIG. 3. The effect of propyl gallate on the height of the differential scanning calorimetry peak at 324–325° K.

weight of 1200 is assumed for cetomacrogol then the DSC peak reaches zero at a ratio of cetomacrogol propyl gallate of 1·2:1. In the presence of cetomacrogol, the propyl gallate peak ceased to exist at all concentrations.

DISCUSSION

The addition of the gallate antioxidants to cetomacrogol–benzaldehyde–water systems may be regarded as the simple introduction of a fourth component into the system or the combination of cetomacrogol and gallate can be considered as a surface-active complex possibly arising from the occurrence in solution of a bonded complex between the –OH grouping of the gallate and the polyoxyethylene group of the surfactant. Evidence for this is obtained from the DSC results where the endothermic peak is due to the unbonded cetomacrogol. At ratios in excess of 1·2:1M, the cetomacrogol peak is absent and all cetomacrogol will have gallate associated with it. This may be present as a solid solution of the two components or as a complex. The shape and relative position of the phases in the solubilization diagram also support the suggestion that the cetomacrogol gallate mixture should be regarded as forming the surface-active agent. The gradually increasing amounts of gallate present produce a higher molecular weight molecule which behaves in much the same way as when solubility diagrams are prepared for a homologous series of surface-active agents. If the mixture of surfactant and gallate was not acting as a complex in solution then the relative position of the phases present would have been displaced.

In a liquid crystal phase the surface-active agent molecules are highly orientated. The decrease, and then disappearance, of the liquid crystal regions when the proportion of gallate present was increased suggests that the orderly arrangement of the liquid crystal was disturbed. This could be because the association of the gallate with the cetomacrogol produces a geometric arrangement of the molecules which cannot close-pack in the manner characteristic of the liquid crystal state.

Winsor's (1954) theories of micelle formation suggest that the surface-active agent functions as a cosolvent between the water and the water-immiscible compound, depending almost exclusively on the strength and balance of the interactions between the components of the system. The presence of gallates in the surface-active complex will decrease the hydrophilic character and, as the proportion of gallate is raised, the increasing lypophilic nature of the complex will allow the formation of L_1 type micelles at a higher water and lower benzaldehyde concentration.

When the molar ratio cetomacrogol: gallate exceeds 1 : 1 (approximately 6: 1 w/w) the liquid crystal is absent and only spherical micelles are formed.

With phases containing spherical micelles the increased bulk of the cetomacrogol gallate complex tends to reduce the amount of benzaldehyde solubilized although small amounts of gallate, by increasing the pallisade space between adjacent molecules of the micelle, at first allow a slight but significant increase in benzaldehyde solubilization. There are two possible reasons for this phenomenon; either the increased molecular bulk and increased lypophilic nature prevents the formation of micelles until higher surface-active concentrations are reached, or the competition for space within the pallisade layer of the micelles favours the gallate which can complex with the cetomacrogol at the expense of the benzaldehyde which cannot. Probably both factors play a part in the overall solubility picture. Table 1 shows that the maximum additive concentration of benzaldehyde in the L_1 phase occurs at a cetomacrogol: gallate ratio of 6: 1 for ethyl gallate, 9: 1 for propyl gallate and 19: 1 for octyl and dodecyl gallates. This appears to indicate that with increasing size of the gallate molecule less can be accommodated within the pallisade before competition with the benzaldehyde becomes a limiting factor in the solubilization of the latter.

With micelles of the L_2 type, a similar situation should occur when water is the solubilizate and benzaldehyde is the continuous phase. However, the distribution of the higher molecular weight gallates between benzaldehyde and cetomacrogol appears to be overwhelmingly in favour of the former. Because of this the size of the micelle remains approximately constant with respect to gallate concentration and hence there is little change in the amount of water solubilized. Any maximum additive concentration noted represents only a small increase over the amount solubilized in the absence of gallates. With ethyl gallate no maximum additive concentration was found.

Acknowledgements

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The effect of temperature and of cetrimide on the rate of loss of refractility of spores of *Bacillus megaterium*

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The rate of germination of spores of *Bacillus megaterium* at 30° is not significantly different from the rate at 37° but the onset of germination is delayed; outgrowth is normal. At 45° germination of some spores occurs but the rate is much slower than at 37°, and there is no lag; emergence occurs from only a proportion of the germinated spores and after 3 or 4 vegetative cells have been produced, replication ceases. A single regression equation can represent the germination rate of the spores at 37° in the presence of from 0·0005 to 0·02% w/v of cetrimide and in its absence. In 0·0005% w/v of cetrimide, germ cells emerge from some of the germinated spores but many of them become swollen and disintegrate. Concentrations of 0·00125% w/v or more progressively inhibit swelling and completely inhibit emergence.

Campbell (1957) has described some of the criteria of germination of bacterial spores. These criteria include loss of heat resistance and of refractility, acquisition of stainability and decrease of optical density of spore suspensions. In addition, loss of calcium ion and of dipicolinic acid, and loss of resistance to toxic chemicals have been investigated as criteria of germination.

Campbell regarded loss of heat resistance as a basic criterion of germination and the subsequent stages of swelling of the germinated spore, emergence of the germ cell, and its elongation and division, he termed outgrowth.

Powell (1957) argued the convenience of using loss of refractility as a criterion of germination, since an examination by phase contrast microscopy is easy to perform and there is, he stated, a close association between the phase contrast appearance and the heat resistance of a spore. Levinson & Hyatt (1966) made a comprehensive investigation of the sequence in which the different germination events occur. They found loss of resistance to heat and to mercuric chloride were the first germination events to occur and these were followed by loss of dipicolinic acid, the acquisition of stainability, loss of turbidity of a suspension, and phase darkening of the spores. Levinson & Hyatt stressed the effect on the sequence of the germination events, of species differences, the sporulation and germination media used, the temperature of incubation during germination, the method of stabilizing samples taken for quantitative examination, the definition of the end point for the particular germination event and the technique of measurement of the degree of germinative change.

Gould (1964) has described the effect of a number of preservatives on growth from spores of six *Bacillus* spp. and the stage of development inhibited by each substance.

Gibbs (1964) and Thorley & Wolf (1961) have described the effect of change of incubation temperature on the germination of anaerobic and aerobic spores, and have shown that temperature optima for germination exist for the organisms they investigated.

In the work reported in this paper, loss of refractility has been employed as the criterion of germination and the rate of germination of spores of *B. megaterium* ATCC 8245 at 30°, 37° and 45° has been examined. The effect of cetrimide on the rate of germination at 37° of the spores and on the morphology and viability of germinated spores has been investigated.

MATERIALS AND METHODS

Bacillus megaterium ATCC 8245 *spore suspension.* This was prepared using potato agar as described by Chiori (1964). The washed suspension was stored at 4°. Only an occasional phase dark spore was observed when the suspension was examined by phase contrast microscopy. The total count, estimated by means of a 0·1 mm depth haemocytometer chamber was about $2{\cdot}5 \times 10^{10}$/ml.

MRVP broth (Difco Bacto) and *double strength MRVP broth* were autoclaved in 15 ml volumes at 115° for 15 min, *MRVP agar* was prepared by the inclusion of 1·5% w/v of Ionagar No. 2 (Oxoid) in MRVP broth and *Plain agar* was a 1·5% w/v solution of Ionagar No. 2 clarified and sterilized by filtration whilst molten, through a millipore filter (a.p.d. 0·22 μm). *Phenol agar* was prepared by including 0·5% w/v of phenol B.P. in plain agar; cetrimide B.P. and mercuric chloride (Analar) were used for inhibition experiments and for the stabilization of culture samples respectively.

A Patholux research microscope (Vickers Instruments, Metron Works, Purley Way, Croydon, Surrey) was used for phase contrast examinations of spore samples.

Preparation of agar slides. A layer of molten plain agar or phenol agar, about 0·8 mm thick was poured between two sheets of plate glass. When the agar had set, the upper glass sheet was removed and the layer of agar dried for 10 min at 37°. The peripheral regions of the layer were discarded and the remainder was cut into strips about 1·5 cm × 5·5 cm, each of which was transferred to a microscope slide (0·6 to 0·8 mm thick) on which were etched three sequentially numbered circles.

Sampling and stabilization of samples. To estimate the proportion of germinated spores in samples from an incubating suspension of spores in nutrient medium, it is necessary to ensure that germinative processes are stopped immediately after removal of the sample. Three methods were compared during the course of the work (*a*) a loopful of incubating suspension was transferred to a numbered site on a plain agar slide maintained at 4°, and refrigeration continued until microscopic examination (see below) could be made, (*b*) a loopful of incubating suspension was transferred to a phenol-agar slide at room temperature, (*c*) 0·1 ml of incubating suspension was transferred to an ignition tube containing 0·1 ml of 4 mM $HgCl_2$; after mixing, a loopful was transferred to a plain agar slide at room temperature.

When dry (approximately 20 s), each agar slide was covered with a thin cover slip. All slides were stored in large Petri dishes containing moist cotton wool to prevent drying out.

Differential counts of about 1000 spores in a partially germinated suspension were made by each of the three methods; there was no significant difference between the % of phase bright spores stabilized by each of the three methods. There was no increase in the % of phase dark spores after 8 h storage, nor did phase dark spores outgrow. The phenol-agar preparations were the most convenient, and phenol-agar was used for most of the stabilizations.

Estimation of rate of loss of refractility. To 10 ml of double-strength MRVP broth warmed to 37° ± 0·5° in a water bath were added 5·0 ml of sterile water or

of a solution of cetrimide, and 5·0 ml of a spore suspension containing about 10^8 mature spores/ml, which had been previously heat activated at 80° for 10 min and cooled. The mixture was then incubated with shaking at 37°, samples being withdrawn by sterile platinum loop at zero, and at 2 or 3 min intervals, during the subsequent 1 h, and then at 30 min intervals for 4 h. A final sample was withdrawn after 24 h incubation.

Duplicate slide preparations were made at each sampling time. All prepared slides were stored as previously described.

Differential counts of spore samples. Within 2 h of sampling, the slides were examined by phase contrast microscopy using a ×100 "Fluorite" phase contrast objective and ×10 eye-pieces. Photomicrographs were taken of many suitable fields of each sample, so as to have records of from 500 to 700 spores for each. In general, more photomicrographs were taken of the samples in which 40 to 60% of the spores had become dark. The negatives were projected onto white paper, differential counts were made and the % of phase dark spores at each sampling time calculated. Spores which were completely dark or in which the outer phase dark ring of the dormant spore had thickened, were scored as phase dark.

RESULTS

Rate of loss of refractility at 37°. From the estimates of % phase dark spores at each time interval, regressions of % phase dark spores on time were calculated using data representing from about 25 to about 75% of dark spores; six experiments were made.

A variance ratio test showed that the six regression equations could be fitted with a common line having the equation:

$$y = 2{\cdot}78x - 28{\cdot}25$$

observed variance ratio $F_{55,10} = 1{\cdot}49$

tabulated variance ratio

(Fisher & Yates, 1963) $F_{55,10} = 2{\cdot}63\ (P' = 0{\cdot}05)$

The regression equation constant (−28·25) which determines the position of the regression line can be given expression by the term GT50, i.e. the time required for 50% of the spores to lose refractility. The variance of y, (Vy), the variance of the regression coefficient b, (Vb), the GT50 and its limits of error for the common regression are shown in Table 1.

The plot of rate of loss of refractility is sigmoidal, arising from the heterogeneity of the spore population with respect to microlag (Vary & Halvorson, 1965). (Micro-

Table 1. *Regression equations for the rate of loss of refractility of* B. megaterium *spores incubated at different temperatures in MRVP broth*

Temperature	Regression equation ($y = bx + c$)	Vy	Vb	t-test of difference of b from b at 37°	GT50 and its limits of error at $P = 0{\cdot}95$ (min)	t-test of difference of c from c at 37°
30°	$y = 2{\cdot}68x - 49{\cdot}2$	5·6	0·017	0·74 (n = 75)	37·0 ± 2·1	6·8 (n = 75)
37°	$y = 2{\cdot}78x - 28{\cdot}25$	3·95	0·0012	—	28·15 ± 1·4	—
45°	$y = 1{\cdot}24x + 1{\cdot}0$	2·86	0·005	19·3 (n = 74)	39·2 ± 3·3	7·7 (n = 74)

n = degrees of freedom.
For n = 74 and 75, $t = 1{\cdot}99$ at $P' = 0{\cdot}05$ (Fisher & Yates, 1963).

lag can be interpreted as the time, from the commencement of incubation, required for any particular spore to commence phase darkening.) The progressively slower rate of phase darkening in the spore population after about 75% had lost refractility which we observed was therefore to be expected, and the small proportion (up to 3%) of spores which failed to become phase dark even after several hours incubation may be regarded as superdormant (Gould, Jones & Wrighton, 1958).

Effect of temperature change. Estimations of the rate of loss of refractility at 30° and at 45° were made and the data are shown in Table 1.

Rate of loss of refractility in the presence of cetrimide. This was investigated by replacing the 5 ml of sterile water in the normal germination procedure, by 5 ml of a sterile solution of cetrimide. The cetrimide concentration in the medium in each of the seven experiments made was (% w/v), 0·0005, 0·00125, 0·0025, 0·005, 0·02, 0·1 and 0·25 respectively.

A regression equation was calculated for each experiment and the data are shown in Table 2. The 13 equations (6 for normal germination and 7 for cetrimide germination) could be fitted with a common regression coefficient:

observed variance ratio $F_{12,105} = 1{\cdot}58$
tabulated variance ratio $F_{12,105} = 1{\cdot}89$ ($P' = 0{\cdot}05$)

Table 2. *Regression equations for rate of loss of refractility of* B. megaterium *spores incubated at 37° in MRVP broth containing cetrimide*

Cetrimide % w/w	Regression equation ($y = bx + c$)	Vy	Vb	GT50 and its limits of error at $P = 0{\cdot}95$ (min)
0·0005	$y = 2{\cdot}91x - 28{\cdot}5$	9·9	0·024	27·0 ± 2·70
0·00125	$y = 2{\cdot}90x - 33{\cdot}8$	8·7	0·023	28·9 ± 2·47
0·0025	$y = 2{\cdot}94x - 35{\cdot}6$	13·7	0·032	29·1 ± 3·40
0·005	$y = 3{\cdot}14x - 39{\cdot}6$	13·9	0·043	28·5 ± 2·96
0·02	$y = 3{\cdot}08x - 38{\cdot}6$	5·1	0·021	28·8 ± 1·73
0·1	$y = 2{\cdot}78x - 20{\cdot}3$	12·7	0·034	25·3 ± 3·22
0·25	$y = 2{\cdot}91x - 15{\cdot}9$	11·6	0·045	22·6 ± 3·01
Nil (Common regression for 6 experiments)	$y = 2{\cdot}78x - 28{\cdot}25$	3·95	0·001	28·15 ± 1·40

The variance of the regression constants was too great for a common equation to be fitted:

observed variance ratio (with respect to constants) $F_{12,105} = 23{\cdot}3$

After omission of the regressions for 0·25% and 0·1% w/v cetrimide germination, it was found that the remaining 5 cetrimide and 6 normal regressions could be represented by the single equation $y = 2{\cdot}84x - 29{\cdot}6$

observed variance ratio $F_{91,20} = 1{\cdot}67$
tabulated variance ratio $F_{91,20} = 1{\cdot}69$ ($P' = 0{\cdot}05$)

Outgrowth of vegetative cells. Outgrowth is regarded as embracing pre-emergent swelling of the germinated spore, emergence and elongation of the new cell and cell division (Hitchins, Gould & Hurst, 1963). Within 30 min incubation at 37°, about half of the *B. megaterium* spores had become phase dark and many of the dark spores had begun to swell (Fig. 1A); the degree of swelling corresponded to

germination swelling as reported by Hitchins & others (1963). After 1 h virtually all the spores were phase dark and most of them exhibited considerable one-sided swelling (Fig. 1B) corresponding to the pre-emergent swelling described by Hitchins & others (1963). Within 3 h, almost all dark spores had produced a germ cell and about half of the germ cells had divided (Fig. 1C).

At 45° most of the emerged germ cells were slightly swollen and after 24 h incubation, most had replicated but had produced only 3 or 4 vegetative cells, many of which were distorted or had disintegrated (Fig. 1D). At 30° each of the stages of outgrowth occurred later than at 37° but the outgrown cells appeared normal.

No emergence of germ cells occured in MRVP broth containing 0·00125% w/v or more of cetrimide, and little or no swelling of the spores occurred. Even in

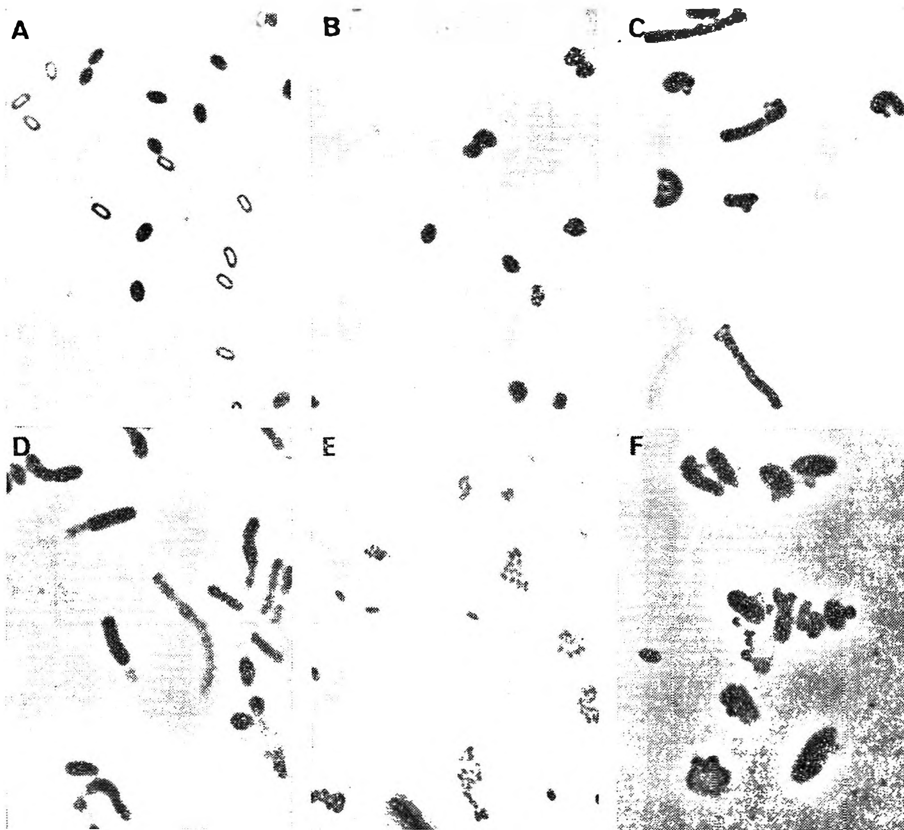

FIG. 1. A. Spores incubated in MRVP broth at 37° for 28 min, showing phase bright and phase dark spores exhibiting varying degrees of enlargement (× 1750).

B. Spores incubated in MRVP broth at 37° for 50 min, showing pre-emergent swelling (× 1750.)

C. Spores incubated in MRVP broth at 37° for 170 min, showing emerged germ cells and vegetative replication (× 1750).

D. Spores incubated in MRVP broth at 45° for 24 h, showing distorted germ cells and vegetative cells, and disintegrated cells (× 1750).

E. Spores incubated at 37° for 24 h in MRVP broth containing 0·00125% w/v of cetrimide, showing clumping and only partial enlargement (× 1100).

F. Spores incubated at 37° for 4 h in MRVP broth containing 0·0005% w/v of cetrimide, showing disintegration of germ cells (× 1750).

0·00125% w/v of cetrimide, no pre-emergent swelling was seen (Fig. 1E). At the higher cetrimide concentrations (0·1% w/v and 0·25% w/v) the microgermination time, i.e. the period commencing at the inception of phase darkening of each spore and ending when the spore is completely phase dark (Vary & Halvorson, 1965), was prolonged, being 4–5 min compared with about 1 min in the absence of cetrimide. A proportion of spores never became completely phase dark in these concentrations of cetrimide.

In 0·0005% w/v of cetrimide, germination swelling and pre-emergent swelling appeared to be normal and to occur at a normal rate. Some germ cells emerged and replicated in an apparently normal manner, whilst others became swollen and then disintegrated, often before they had completely emerged from the germinated spore (Fig. 1F).

DISCUSSION

Loss of refractility by a spore has been used as a criterion of germination to determine the percentage of spores that have germinated after a measured period of incubation. From the data, regressions representing the rate of germination of the spore population have been calculated. A comparison of the regression equation for rate of germination at 37° (regarded as standard), with those for germination at 30 and 45°, shows that at 45° a proportion of the spores germinated soon after incubation commenced and that the rate of germination, 1·24 spores/min, was significantly lower than at 37° (2·78 spores/min) ($t = 19·3$). The GT50 value (39·2 min) was significantly greater than at 37° (28·15 min). Emergence occurred at 45° from only a proportion of the germinated spores; many of the germ cells were swollen and replication ceased after only 3 or 4 cells had been produced.

At 30°, over the range 25 to 75% of germinated spores, the rate of germination was not significantly lower than at 37° ($t = 0·74$). The GT50 value was significantly greater than at 37° ($t = 6·8$), due to lag in the onset of germination; the theoretical lag (estimated by solving the regression equation for x at $y = 0$) was 18·3 min, compared with 10·2 min at 37°. Outgrowth was apparently normal, although delayed.

Inclusion of from 0·0005% w/v to 0·02% w/v of cetrimide in the medium did not produce a significant change in the rate of germination of spores at 37°, or in the GT50 values; a single regression equation can represent the rate of germination in MRVP broth at 37° in either the absence or the presence of cetrimide over this range of concentration.

In the presence of 0·25% w/v of cetrimide, germination commenced earlier (theoretical lag 5·5 min) than in all other cetrimide experiments (mean lag 11·0 min) but the rate of germination was normal; the theoretical lag in the case of 0·1% w/v cetrimide (7·3 min) was also less than normal. In these two experiments, the effect of the prolonged microgermination time was to produce only very gradual phase darkening of each individual spore so that a decision to count a spore as phase bright or phase dark was very subjective.

Cetrimide, 0·0005% w/v, did not inhibit swelling of incubated spores but inhibited the emergence of germ cells from many of the spores. Most of those cells which did emerge were sensitive to this low level of cetrimide and became swollen and then burst. Concentrations of 0·00125% w/v or more, progressively inhibited swelling, completely inhibited emergence, and induced clumping of the spores (Fig. 1E).

These results show that the germinative processes of *B. megaterium* spores are not sensitive to concentrations of cetrimide of up to 0·02% w/v. The outgrowth processes

of the germinated spore on the other hand, are sensitive to 0·00125% w/v of cetrimide or more, and indeed work which we are at present carrying out, suggests that the germinated spore is in fact killed by low concentrations of cetrimide. Levinson & Hyatt (1966) have reported the sensitivity of germinated but not of ungerminated spores of *B. megaterium* to 2 mM $HgCl_2$ and Vinter (1970), has reviewed the inhibition both of germination and of outgrowth. Our results using 0·0005% w/v of cetrimide indicate that emerged germ cells are even more sensitive than are germinated spores.

In short, mature spores of *B. megaterium*, germinated spores and vegetative cells are progessively more sensitive to cetrimide.

Parker, Barnes & Bradley (1966), using a Coulter counter to detect spore swelling, reported swelling of an order that must be interpreted as emergence or as pre-emergent swelling in *B. subtilis* NCTC 3610 spores incubated in broth containing 0·02% w/v of cetrimide. Parker (1969) reported finding up to 5% non-proliferating, swollen and distorted forms when he repeated the work. We detected some swelling, but not outgrowth of *B. subtilis* spores supplied by Parker when they were incubated in similar conditions and examined by the method we have described; the spores behaved similarly to our *B. megaterium* spores, i.e. semi-abortive emergence occurred only when the cetrimide concentration was reduced to 0·001% and 0 0005%. It may be that the swollen cells which Parker detected were already present in his suspension. At any rate, Parker reported no extensive emergence of germ cells and, indeed, only 27% increase in volume of his spores during germination in 0·02% w/v of cetrimide, and in these respects, his results are similar to ours. The use of thin strips of plain agar or agar containing phenol to mount either stabilized or unstabilized spore samples, we found preferable to other methods of mounting samples, because it preserved the phase properties of the spores throughout each experiment and also prevented Brownian movement of the spores; in addition it was not necessary to re-hydrate the spores for phase microscopy, as is necessary when spore samples are allowed to dry on slides or coverslips.

Acknowledgement

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The buccal absorption of some barbiturates

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The buccal absorptions of five barbiturates have been determined over the pH range 3 to 9. The absorptions increased as the pH decreased until pH 5·5 when they remained constant. No correlation between the absorptions and chloroform–0·1N HCl partition coefficients was apparent, indicating that the adsorptive power of the buffer-buccal membrane interface may represent more exactly the real affinity of the membrane for barbiturates than do partition coefficients with chloroform.

The 5 min cumulative buccal absorption test of Beckett & Triggs (1967) has been used to examine the buccal absorptions of alkyl substituted carboxylic acids (Beckett & Moffat, 1968, 1969a), *N*-alkyl amines (Beckett & Moffat, 1969b) and imipramine and its metabolites (Bickel & Weder, 1969). These results have also been related to aqueous-organic phase partition coefficients (Beckett & Moffat, 1969b; Bickel & Weder, 1969). This test is now used to examine the absorptions of some barbiturates.

MATERIALS AND METHODS

Aqueous solutions of the sodium salts of the barbiturates were used with the method of Beckett & Moffat (1968).

For analysis, the method of Beckett & Moffat (1968) was used, omitting the treatment with diazomethane. The gas-liquid chromatographic conditions used were: $\frac{1}{4}$ inch o.d. glass tube packed with Chromosorb G (acid washed, DMCS treated, 80–100 mesh) coated with 0·75% Neopentylglycol sebacate; nitrogen pressure 13 lb/inch^2, hydrogen pressure 18 lb/inch^2 and air pressure 30 lb/inch^2; injection block temperature approximately 50° above the oven temperature. The oven temperature and internal standard used for each barbiturate are summarized in Table 1.

RESULTS AND DISCUSSION

Analysis

The use of gas-liquid chromatography allowed multicomponent mixtures of barbiturates to be separated and analysed individually. Although the column had an efficiency equivalent to only 210 plates, nearly symmetrical peaks were obtained (e.g., Fig. 1). All calibration graphs were linear over the range 0·1 to 1·0 mg barbiturate in buffer solution or buffer solution containing saliva; the calibrations were identical for both solutions. Standard deviations, obtained from twelve replicate assays, were 0·88, 1·08 and 1·68% for barbitone, probarbitone and methylphenobarbitone respectively.

Buccal absorption

Absorptions increased as the pH decreased and the concentration of unionized barbiturate increased (e.g., Fig. 2) indicating that the unionized, and not the ionized,

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Table 1. *Gas-liquid chromatographic conditions for the analysis of some barbiturates on a 0·75% Neopentylglycol sebacate column*

Barbiturate	Retention time (min)	Oven temperature (°C)	Internal standard	Retention time (min)
Barbitone	6·0	190	Diallylbarbituric acid	10·0
Probarbitone	7·6	190	,,	10·0
Methylphenobarbitone	18·1	190	,,	10·0
Thiopentone	5·8	205	Hexobarbitone	3·7
Phenobarbitone ..	18·6	205	,,	3·7

species is absorbed. This is in agreement with Katz (1954), who found the free barbiturates to be more effectively absorbed by the oral mucosa than their ionized sodium salts. The pK_a values of the barbiturates are between 7·4 and 8·0 (Table 2), so at pH 5·5 all were at least 99% unionized which explains the relatively constant absorptions obtained at pH values below 5·5.

Since the absorptions above pH 5·5 were small, and therefore difficult to measure accurately, the mean absorptions below pH 5·5 were calculated (Table 2). Thiopentone was absorbed to the greatest extent, followed by methylphenobarbitone. These results are similar to the findings of Kakemi, Arita & others (1967a,b) who showed that the gastric and intestinal absorptions of barbituric acid derivatives were in the order oxy-<*N*-methyl-<thio-. These differences are also reflected in the chloroform–0·1N HCl partition coefficients (Table 2), although no correlation between buccal absorption and partition coefficients is apparent.

The n-heptane–0·1N HCl partition coefficients of thiopentone, barbitone and benzoic acid are 3·3, 0·002 and 0·19 respectively (assuming no dimerization in either phase; Hogben, Tocco & others, 1959). Thus, since the buccal absorption of benzoic acid at pH 3·0 is 70% (Beckett & Moffat, 1968), a much larger absorption for thio-

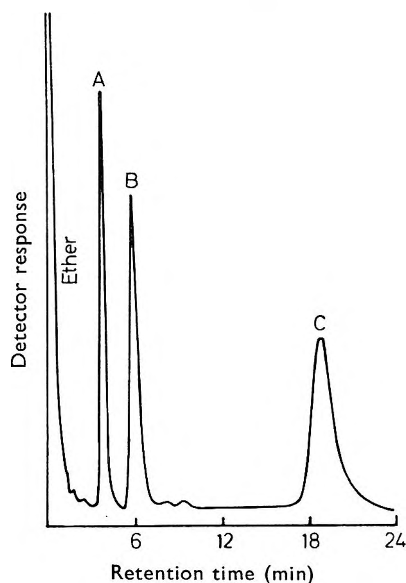

FIG. 1. Chromatogram of some barbiturates, extracted from saliva-buffer solution, on a 0·75% Neopentylglycol sebacate column at 205°: A, hexobarbitone; B, thiopentone; C, phenobarbitone.

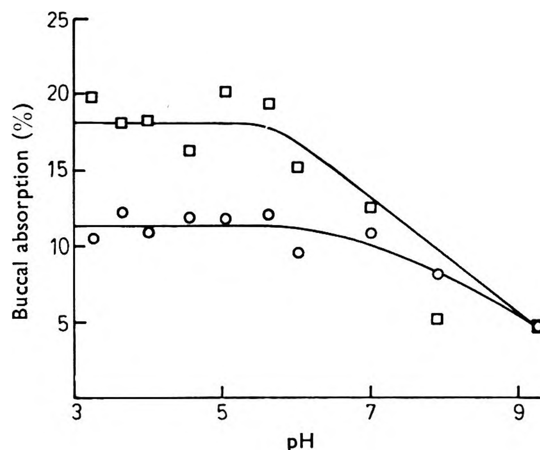

FIG. 2. Buccal absorption of some barbiturates (Subject I): □, thiopentone; ○, phenobarbitone.

Table 2. *Buccal absorption data (Subject I), chloroform*-0·1N *hydrochloric acid partition coefficients and* pK_a *values for some barbiturates*

Barbiturate	Mean maximum absorption (%)	Partition coefficient (37°)*	pK_a (25°)
Phenobarbitone	11·4	4·44	7·41†
Probarbitone	11·6	1·60	8·01†
Barbitone	16·3	0·72	7·91†
Methylphenobarbitone ..	16·4	95·5	7·7‡
Thiopentone	18·5	321	7·6§

* Kakemi & others (1967c) (assuming no association of molecules in either phase).
† Krahl (1940).
‡ Butler (1955).
§ Schanker & others (1957).

pentone than that actually obtained would be expected. Conversely, a very small absorption, compared to that of thiopentone, would be expected for barbitone. These comparatively small absorptions for the barbiturates are similar to those obtained using other tissues, e.g., Schanker, Shore & others (1957) showed that thiopentone, barbitone and benzoic acid were passively absorbed at pH 1 into the rat gastric mucosa in the same time, to the extent of 46, 4 and 55% respectively.

Thus, unlike the buccal absorption of the amines and carboxylic acids studied previously (Beckett & Moffat, 1968, 1969a,b, 1970), the relation between lipid solubility and absorption into the buccal mucosa is not clear for barbiturates. From the above results, the buccal mucosa is clearly selectively permeable to the unionized form. However, it is likely that this step is preceded by interaction or complex formation between the drug molecule and the protein of the mucosa, and, unlike the situation which obtains with the carboxylic acids and amines already studied, the release of the barbiturates into the lipid is the rate controlling step in the buccal absorption of barbiturates.

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The effects of pentobarbitone sodium on the carbon dioxide response and production, and oxygen consumption of the rabbit

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The effect of intravenous doses of pentobarbitone sodium on the ventilatory response to CO_2, CO_2 production and O_2 consumption were investigated in the rabbit. Pentobarbitone sodium depressed the ventilatory response to CO_2, but the time course of this effect showed a biphasic character whereas depression of unchallenged minute volume did not. O_2 consumption and CO_2 production were reduced by pentobarbitone, but although the degree and duration of the depression increased with increasing dose, the values returned to control values before depression of respiratory minute volume and response to CO_2. The possibility that these results may explain the lack of progressive depression of respiratory minute volume with increasing dose of barbiturate, previously reported by the authors, is discussed.

It has been demonstrated, in the rabbit, that increasing doses of pentobarbitone, amylobarbitone and barbitone sodium do not cause progressive depression of respiratory minute volume (Hunter, Pleuvry & Rees, 1968). There is a plateau in the dose response curves so that, for example, between 7·5 and 30 mg/kg pentobarbitone there is no significant increase in the depression of minute volume.

The present study describes further investigation of this phenomenon, by means of a detailed examination of the effects of pentobarbitone sodium on the rabbits respiratory response to carbon dioxide (CO_2). Since such measurements can be modified by changes in CO_2 production and O_2 consumption, these two parameters were also measured.

METHODS

Groups of five Flemish rabbits, 2 to 4 kg, were used. The three parameters measured in this study were examined independently.

Responses to inhaled gases. CO_2 gas mixtures were fed into the Gaddum respirometer circuit (Gaddum, 1941) avoiding positive pressure. The mixtures were 4·5, 7·2, and 14·3% CO_2 in oxygen. Respiratory minute volume was recorded until no further displacement of respiration was seen over a 30 s period (usually 2–3 min exposure).

After stable control values had been obtained, pentobarbitone sodium was administered into the lateral ear vein of the rabbit. Recordings of minute volume with the rabbit breathing air and then challenged with CO_2 were taken at 10 min intervals, the first being 5 min after injection, and continuing until responses had regained control values.

The effects of inhalation of 100% O_2 on the respiratory effects of 30 mg/kg of pentobarbitone sodium were also investigated.

CO_2 production. Minute volume was measured in the usual way using the Gaddum respirometer. After the rabbit had been breathing through the apparatus for about half a minute, the outlet valve was connected to an evacuated rubber bag and a 90 s sample of expired air collected. The gas filled bag was attached to the inlet of a CO_2 analyser (Hartman & Braun A.4 URAS 4) and the % CO_2 read from the scale. CO_2 production in ml/min kg^{-1} was calculated from the minute volume, CO_2 percentage in the expired air and the weight of the rabbit.

O_2 consumption. A sample of room air was pumped into an O_2 analyser (Servomex Type O A 101 Mk.2.) to obtain the percentage of O_2 in the inspired air. The expired air, collected as above, was then passed through the apparatus and the two readings, when subtracted from each other, gave the O_2 consumption expressed as a percentage of the total gas expired. Thus O_2 consumption in ml/min kg^{-1} can be calculated from the minute volume and weight of the rabbit.

To obtain stable control readings, it was necessary to accustom the animals to the procedure and to maintain a relatively constant sound level, e.g. a radio playing light music, during experiments.

After consistent control readings had been obtained for both O_2 consumption and CO_2 production, pentobarbitone was injected intravenously. Measurements were taken at 5 min intervals for the first 30 min and then at 10 min intervals until control values were regained. One group of five animals was injected with 1 ml of sterile saline and CO_2 production changes measured.

Percentage changes were generally used to express results in this study as these showed less variability than absolute values, however, some raw data have been included so that comparisons can be made with the findings of other workers.

RESULTS

Effect of inhaled CO_2 in pentobarbitone-treated animals

The effect of CO_2 inhalation in control rabbits has been fully described by Pleuvry & Rees (1969). Minute volume and tidal volume increase with increasing concentrations of CO_2, but respiratory rate is unchanged or falls slightly.

The time course of the effect of 30 mg/kg of pentobarbitone on resting minute volume and CO_2 challenged minute volume is shown in Fig. 1. The results are expressed as % change from pre-injection values. Although the increase in respiratory minute volume depends upon the concentration of CO_2 inspired, the % change from control CO_2 challenged minute volume in the presence of pentobarbitone is similar for all three concentrations of CO_2 and thus the values obtained for Fig. 1 are the bulked results for all three concentrations of CO_2.

The time course of the changes induced in the CO_2 challenged minute volume by pentobarbitone sodium is significantly different from the changes in unchallenged minute volume.

Fig. 2 shows the results expressed as % change in respiratory minute volume induced by CO_2 compared with the immediately preceding unchallenged minute volume. In the absence of drug this is a constant measure for a given concentration of CO_2, but in the presence of various doses of pentobarbitone the patterns shown in Fig. 2 are obtained. Although the results illustrated were obtained with 4·5% CO_2, similar patterns were obtained with 7·2 and 14·3% CO_2, except that the actual % changes were higher with greater concentrations of CO_2.

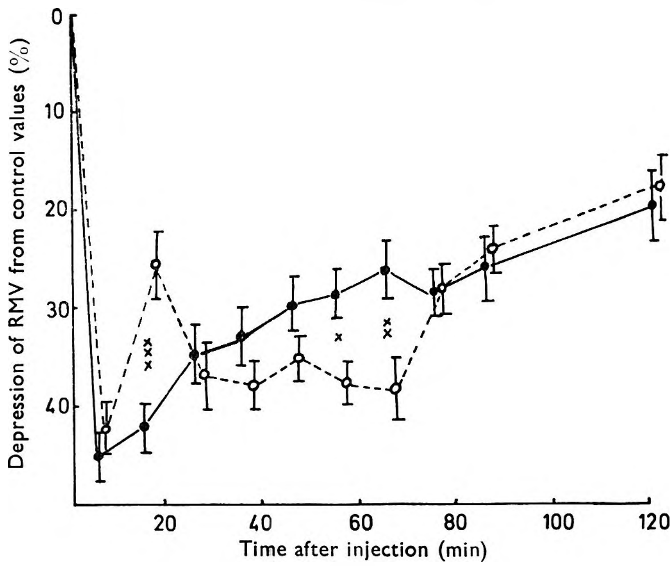

FIG. 1. The effect of 30 mg/kg pentobarbitone sodium on resting and CO_2 challenged minute volume in the rabbit. The closed circles represent the % change in unchallenged minute volume from pre-injection controls and the open circles are the % change of CO_2 challenged minute volume from pre-injection controls. Although the control values of CO_2 challenged minute volume increase with increasing concentration of CO_2, the % change from these values in the presence of pentobarbitone are similar and thus bulked results from all 3 concentrations of CO_2 are shown in the figure.

The means and standard errors of both groups were calculated on the basis of 15 experiments and significant differences between them are indicated by crosses; 1 cross P 0·02, 2 crosses P 0·01, 3 crosses P 0·001.

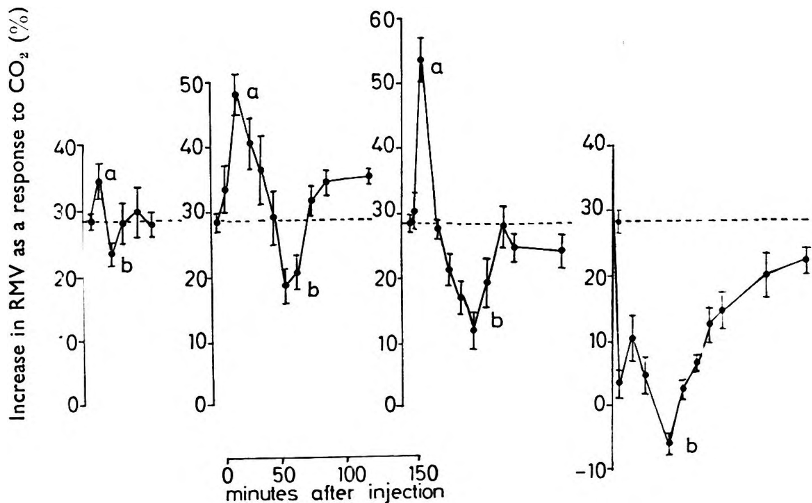

FIG. 2. The effect of various doses of pentobarbitone sodium on the minute volume response of the rabbit to 4·5% CO_2, measured as the percentage increase in minute volume caused by CO_2 inhalation over the immediately preceding unchallenged minute volume. All means and standard errors were calculated from five experiments and the first point on each graph indicates the mean preinjection response to CO_2.

The lettering a and b indicate the highest and lowest response to CO_2 measured after the various doses of pentobarbitone. The doses used were (from left to right) 7·5, 15, 30, 45 mg/kg.

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Table 1. *The effect of pentobarbitone sodium on the O_2 consumption and CO_2 production of the rabbit.*

Time after injection (min)	Percentage change from control values (mean ± standard errors) CO_2 production	O_2 consumption
1. *Saline controls*		
5	−0·2% (±1·5)	−0·9% (±2·5)
10	−3·8% (±2·1)	−0·2% (±2·8)
15	−2·2% (±2·6)	−1·1% (±3·5)
20	−4·8% (±2·6)	−2·7% (±2·9)
25	−2·3% (±2·3)	−1·0% (±2·3)
35	−1·8% (±2·6)	−1·2% (±4·2)
45	−3·0% (±2·1)	+3·1% (±4·1)
55	−3·2% (±2·9)	+2·4% (±8·6)
65	−0·6% (±0·4)	+8·8% (±5·6)
2. 3·75 *mg/kg pentobarbitone sodium*		
5	−11·8% (±2·2)	−13·7% (±1·5)
10	+2·9% (±2·2)	−0·7% (±3·1)
15	+1·7% (±1·7)	+2·7% (±6·4)
20	−1·4% (±3·5)	+6·2% (±2·5)
25	+1·0% (±3·5)	+1·2% (±3·2)
3. 7·5 *mg/kg pentobarbitone sodium*		
5	−14·9% (±3·3)	−18·7% (±2·4)
10	−12·6% (±3·6)	−13·3% (±2·5)
15	+1·6% (±3·0)	+3·8% (±4·5)
20	+4·1% (±3·0)	+1·9% (±2·1)
25	+2·4% (±4·8)	−2·9% (±2·2)
35	+12·1% (±7·0)	−3·9% (±3·8)
45	+3·3% (±3·2)	−3·0% (±2·8)
55	+4·7% (±5·2)	+0·9% (±2·0)
4. 15 *mg/kg pentobarbitone sodium*		
5	−29·3% (±2·5)	−21·7% (±2·5)
10	−23·2% (±4·9)	−20·2% (±3·2)
15	−28·5% (±5·2)	−13·5% (±3·7)*
20	−22·2% (±5·1)	−9·0% (±3·9)*
25	−11·1% (±6·6)	−4·1% (±5·5)
35	−13·1% (±6·9)	−6·1% (±5·5)
45	−6·5% (±2·6)	−3·4% (±6·4)
55	+2·2% (±2·6)	+3·5% (±9·0)
65	+0·9% (±5·6)	+7·8% (±5·0)
75	−1·4% (±6·5)	+8·7% (±5·7)
85	−0·8% (±5·2)	+8·0% (±6·4)
120	+0·5% (±3·2)	+11·9% (±7·2)
5. 30 *mg/kg pentobarbitone sodium*		
5	−34·8% (±3·2)	−30·5% (±0·3)
10	−32·5% (±3·8)	−22·2% (±1·7)*
15	−34·7% (±3·8)	−18·0% (±2·1)*
20	−28·9% (±4·2)	−15·2% (±5·3)*
25	−24·7% (±3·9)	−11·5% (±1·8)*
35	−24·9% (±1·7)	−8·6% (±1·5)*
45	−9·0% (±4·4)	−9·0% (±2·4)
55	−3·7% (±6·6)	−6·5% (±5·2)
65	−8·4% (±7·8)	−8·9% (±2·8)
75	−1·6% (±7·3)	−7·6% (±3·5)
85	−1·9% (±6·8)	−4·9% (±4·5)
120	−4·5% (±2·7)	−4·7% (±2·2)

Time after injection (min)	Percentage change from control values (mean ± standard errors) CO₂ production	O₂ consumption
6. 45 *mg/kg pentobarbitone sodium*		
5	−44·6% (±2·6)	−44·0% (±12·4)
10	−36·2% (±2·1)	−40·3% (±9·8)
15	−31·9% (±6·4)	−40·5% (±3·8)
20	−32·2% (±5·8)	−34·0% (±4·4)
25	−31·4% (±2·9)	−33·2% (±9·7)
35	−30·5% (±4·9)	−38·1% (±5·2)
45	−29·5% (±5·3)	−38·8% (±4·4)
55	−22·3% (±9·7)	−33·9% (±6·8)
65	−19·3% (±10·4)	−32·5% (±5·5)
75	−11·6% (±11·8)	−33·7% (±5·1)
85	+2·2% (±11·8)	−29·7% (±6·9)
120	−12·5% (+9·4)	−29·8% (±7·8)

Note: Except for 45 mg/kg of pentobarbitone sodium, all means and standard errors were calculated on the basis of 5 experiments. With 45 mg/kg, the CO_2 production was calculated from 5 experiments, but the O_2 consumption was calculated from 3 experiments.

* Significant differences ($P < 0·05$) between the % change in O_2 consumption and CO_2 production.

The points a and b on Fig. 2 represent the highest and lowest % increase in minute volume obtained in the presence of pentobarbitone. Fig. 3 is obtained when the points a and b are plotted for each concentration of CO_2 in the inspired air. The % increase in minute volume for each concentration of CO_2 in the absence of drug is included for comparison. Fig. 3 illustrates that the highest % change increases and the lowest percentage change decreases in value with increasing dose of pentobarbitone.

Although the actual value of the highest % increase in minute volume increases with dose, the duration of its occurrence above control levels decreases until there is no initial increase in minute volume with the LD 50 dose of 45 mg/kg of pentobarbitone sodium.

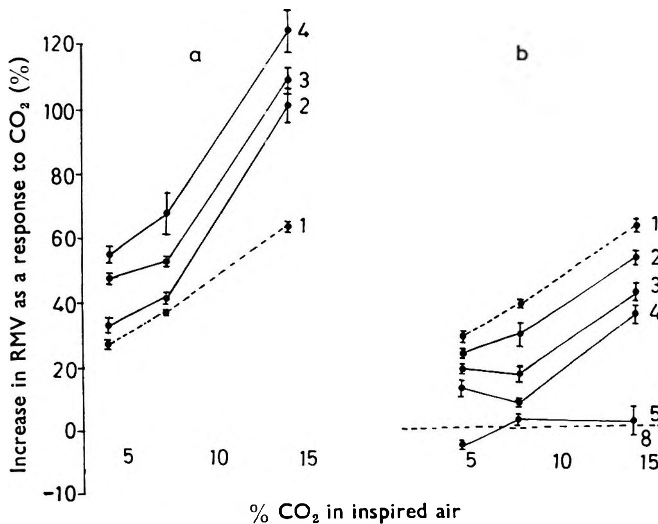

Fig. 3. The effect of increasing concentrations of CO_2 on the highest and lowest responses to CO_2 obtained after injection of pentobarbitone sodium. The highest responses to CO_2 obtained with 4·5% CO_2 are labelled a on Fig. 2. and the lowest are labelled b. 1. Dotted line. Control values in the absence of drug. 2, 7·5 mg/kg; 3, 15 mg/kg; 4, 30 mg/kg; 5, 45 mg/kg of pentobarbitone sodium.

Inhalation of 100% O_2 had no effect on control minute volume or on minute volume depressed by the administration of 30 mg/kg of pentobarbitone sodium.

The effect of pentobarbitone on CO_2 production and O_2 consumption

All doses of pentobarbitone sodium examined (3·75 to 45 mg/kg) depressed both CO_2 production and O_2 consumption of the whole rabbit. The maximum depression increases with increasing dose. Table 1 shows the results obtained.

Table 2. *Selected raw data from which Figs* 1, 2 *and* 3 *and Table* 1 *were constructed*

A. CO_2 challenge		Respiratory minute volume ml/min ± s.d.		
	Pentobarbitone	resting	CO_2 challenged	
4·5% CO_2	Control	829±189	1051±218	(23)
	5 min after 7·5 mg/kg	554±174	771±244	(5)
	25 min ,,	734±250	920±275	(5)
	15 min after 15 mg/kg	420±92	715±81	(5)
	55 mins ,,	573±48	678±47	(5)
	15 min after 30 mg/kg	489±59	752±97	(5)
	55 mins ,,	622±96	694±74	(5)
	15 mins after 45 mg/kg	373±11·5	4[illegible]1±11	(3)
	55 mins ,,	447±11	457±5·7	(3)
7·2% CO_2	Control	769±148	1045±163	(25)
	5 min after 7·5 mg/kg	589±39	796±103	(5)
	25 min ,,	572±99	844±146	(5)
	15 min after 15 mg/kg	609±104	945±202	(5)
	55 min ,,	672±136	806±247	(5)
	15 min after 30 mg/kg	469±65	779±164	(5)
	55 min ,,	529±103	602±161	(5)
	15 min after 45 mg/kg	398±131	462±147	(5)
	55 min ,,	506±220	526±260	(5)
14·3% CO_2	Control	870±106	1380±127	(23)
	5 min after 7·5 mg/kg	619±38	1242±46	(5)
	25 min ,,	726±102	1121±146	(5)
	15 mins after 15 mg/kg	636±105	1242±205	(5)
	55 min ,,	725±139	1042±246	(5)
	15 min after 30 mg/kg	563±65	1169±182	(5)
	55 min ,,	651±103	870±74	(5)
	15 min after 45 mg/kg	343±85	426±139	(3)
	55 min ,,	392±56	400±78	(3)
B. O_2 consumption of the rabbit		ml/min/kg ± s.d.		
	Control	6·6 (±0·9)		(25)
	Minimum value after			
	3·75 mg/kg	5·4 (±0·7)		(5)
	7·5 mg/kg	5·4 (±0·5)		(5)
	15 mg/kg	4·7 (±0·4)		(5)
	30 mg/kg	4·3 (±0·3)		(5)
	45 mg/kg	4·0 (±1·7)		(3)
C. CO_2 production of the rabbit		ml/min/kg ± s.d.		
	Control	5·5 (±0·7)		(25)
	Minimum value after			
	3·75 mg/kg	4·6 (±0·6)		(5)
	7·5 mg/kg	4·5 (±1·0)		(5)
	15 mg/kg	3·8 (±0·4)		(5)
	30 mg/kg	3·5 (±1·1)		(5)
	45 mg/kg	2·9 (±0·4)		(5)

Note: the numbers in brackets at the end of each line indicate the number of animals in the group from which the means and standard deviations (s.d.) were calculated.

There is no significant difference between the maximum % depression of CO_2 production and O_2 consumption and the duration of the depression, which also increases with increasing dose of pentobarbitone, is approximately the same. However, there is a significant difference between depression of O_2 consumption and depression of CO_2 production, 15 to 20 min after 15 mg/kg pentobarbitone and 10 to 35 min after 30 mg/kg, CO_2 production is depressed more than O_2 consumption indicating a fall in the respiratory quotient. A similar observation has been reported by Ament, Suskin & Rahn (1949) for thiopentone.

Some of the raw data, from which the preceding figures and tables were constructed are shown in Table 2. The standard deviations of the means are included to show the wide scatter values between rabbits, particularly with minute volume measurements.

DISCUSSION

The respiratory effects of pentobarbitone sodium described are in marked contrast to the respiratory effects of morphine. Depression of respiratory minute volume increases progressively with increasing dose of morphine (Hunter, Pleuvry & Rees, 1968); the time course of the action of morphine on CO_2 challenged minute volume shows no biphasic character (Pleuvry & Rees, 1969) and unpublished work of the authors' indicates that although morphine does initially lower CO_2 production, its effects are very transient and control values are regained 10 min after injection.

We have demonstrated that pentobarbitone can cause a reduction in minute volume by two mechanisms.

(*a*) Depression of metabolism. Less CO_2 is produced per minute and thus a lower alveolar ventilation is required to maintain the P_{CO_2} of the blood at normal levels.

(*b*) Depression of the response of the respiratory reticular formation to CO_2 (Fig. 1). Changes in CO_2 response will mean that less increase in ventilation will result from rises in P_{CO_2} of the blood. Since ventilation is normally driven by P_{CO_2}, ventilation will fall and P_{CO_2} rise.

It is not possible to point to the exact mechanism by which a drug may depress CO_2 response as this may occur at a variety of sites described fully by Lambertsen(1964). Some of these sites are unlikely to apply to the barbiturates as it has been shown that the barbiturates only raise the threshold for stimulation of skeletal muscle in very high doses (Thesleff, 1956); there is no evidence that the barbiturates possess any local anaesthetic activity in central depressant doses and Dripps & Dumke (1943) have shown that they have little effect on peripheral chemoreceptors. In view of this last point it might be suggested that, as minute volume and respiratory rate are depressed by the barbiturates, the ensuing hypoxic stimulation of the aortic and carotid body chemoreceptors might tend to flatten the depression of minute volume/log dose graph and contribute to the plateau. However, it has been demonstrated that 100% O_2 does not affect the minute volume depressed by 30 mg/kg of pentobarbitone indicating that hypoxic drive is absent.

The principal alternative sites of action of pentobarbitone on CO_2 response are the chemosensitive areas of the respiratory reticular formation or generalized depression of the neurons within the respiratory reticular formation.

It has been shown that the barbiturates depress the reticular formation as a whole (French, Verzeono & Magoun, 1953) and both Harris & Borison (1954) and Robson, Houseley & Solis-Quiroga (1963) suggested that depression of conduction through the

respiratory reticular formation was important in the respiratory depressant effects of the barbiturates. Thus although depression of central chemoreceptors cannot be ruled out, it appears that barbiturates cause a more generalized depression.

Both depression of metabolism and depression of CO_2 response increase progressively with dose of pentobarbitone. However, depression of minute volume does not increase progressively with dose, there being a plateau in the log dose response curve where increasing the dose of pentobarbitone causes no further depression of minute volume.

It is possible that this anomaly, together with the curious pattern of changes in minute volume challenged with CO_2 after pentobarbitone, is related to the interactions of the two mechanisms by which pentobarbitone causes a fall in minute volume, i.e. depression of metabolism and general depression of the respiratory reticular formation.

Jennett (1968) demonstrated, in man, that, in the presence of metabolic depression, the alveolar ventilation "metabolic hyperbole" when breathing CO_2 moved to the left. Thus there might be a similar result in terms of alveolar ventilation for a given inspired CO_2 concentration when metabolism alone is depressed and when respiration is depressed. It was pointed out that it was impossible to distinguish the two unless alveolar or arterial P_{CO_2} were measured during the CO_2 response readings. In the rabbit this is very difficult in practice.

However, the depression in CO_2 response obtained after CO_2 production and O_2 consumption have returned to normal is probably indicative of true respiratory depression.

Before this, the CO_2 response obtained will be reflecting the effects of both depression of metabolism and depression of the respiratory reticular formation. Table 1 shows that the maximum effect of pentobarbitone on CO_2 production and O_2 consumption occurs almost immediately after injection and then returns rapidly to normal values, the actual duration of the return to normal being dependent upon the dose of pentobarbitone. During this time there is a reduction in the sensitivity of the respiratory reticular formation to CO_2. Bearing in mind Jennett's findings described above, the possible effects of these two factors on CO_2 challenged minute volume will be considered.

Depression of metabolism and depression of the respiratory reticular formation will cause a fall in challenged minute volume, but as the metabolism returns to normal the challenged minute volume will tend to rise. However this will be partially overcome by the depression of CO_2 sensitivity, the greater the depression of CO_2 sensitivity the shorter the duration of the rise. A mechanism such as described above may account for the shape of the time course of depression of challenged minute volume shown in Fig. 1.

It was suggested earlier that the respiratory depressant activity of pentobarbitone sodium was more likely to be due to direct depression of the respiratory reticular formation as a whole rather than a specific effect on receptor systems. In view of this it seems reasonable to suppose that the actions of all factors centrally modifying respiration will be depressed including changes in metabolism. The graph constructed by Jennett (1968) showed that even in respiratory depression of undefined aetiology the alveolar ventilation "metabolic hyperboles obtained breathing air at different metabolic rates tend to come closer together as respiratory depression increases".

The maximum depression of resting minute volume obtained with pentobarbitone,

in the rabbit, occurs during the first 15 to 20 min after injection when depression of metabolism and presumably its effects on minue volume are at a maximum. As the dose of pentobarbitone increases, the depression of the respiratory reticular formation increases faster than the depression of metabolism. Thus the actual effect of metabolism depression on minute volume will tend to be reduced as the effects of depression of the respiratory reticular formation on minute volume increase. It is possible that this interaction could cause a flattening of the log dose/depression of minute volume curve obtained with pentobarbitone sodium in the rabbit.

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Adenyl cyclase activity of rabbit aorta

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Adenyl cyclase activity in homogenates of thoracic and abdominal aorta from young and old rabbits was neither stimulated nor inhibited by isoprenaline, noradrenaline, 5-hydroxytryptamine, or histamine but was markedly increased by sodium fluoride. Sodium fluoride-stimulated adenyl cyclase activity was significantly higher in the abdominal than in the thoracic aorta. Neither the basal nor sodium fluoride-stimulated adenyl cyclase activity of thoracic aorta changed with increasing age of the aorta. On the other hand, the abdominal aorta showed an increase in sodium fluoride-stimulated activity but no change in basal activity with increasing age. After the ATP pool of intact thoracic aorta was prelabelled with [^{14}C]-adenine, isoprenaline did not enhance the formation of labelled cyclic AMP in intact aortic strips. Based on these data no correlation could be made between adenyl cyclase activity and the β-receptor activity of this tissue.

Fleisch, Maling & Brodie (1970) showed that rabbit thoracic but not abdominal aorta could be relaxed by isoprenaline, a β-receptor agonist. In addition this activity of isoprenaline on thoracic aorta decreased with increasing age of the aorta. Since Robison, Butcher & Sutherland (1967) have postulated that adenyl cyclase (AC) is part of the β-adrenergic receptor system in many tissues, we examined AC activity of thoracic and abdominal aorta of young and old rabbits to determine if there was a relation between AC activity and the reported responses to isoprenaline in aortic smooth muscle. The data obtained did not permit a correlation to be made.

MATERIALS AND METHODS

Materials were obtained from the following sources: ATP-^{3}H(G) (15·7 Ci/mmol), New England Nuclear Corp.; [8–^{14}C]adenine (50 mCi/mmol), Schwarz Bioresearch; theophylline, Z. D. Gilman Inc.; (−)-isoprenaline (+)-bitartrate and (−)-noradrenaline-bitartrate, Winthrop laboratories; 5-hydroxytryptamine creatinine sulphate (5-HT), Aldrich Chemical Co.; histamine dihydrochloride, Mann Research Laboratories; dibenamine HCl, gift of Smith, Kline and French; phentolamine methanesulphonate, gift of Ciba; and propranolol HCl, gift of Ayerst Laboratories.

Male and female New Zealand rabbits (1·6–3·3 kg) of known age were used. The animals were killed by administration of 20 to 30 ml of air into the marginal ear vein. Spirally cut thoracic and abdominal aortic strips were prepared by the method of Furchgott & Bhadrakom (1953). The tissues were kept moist with Krebs-bicarbonate solution during the preparation. The aortas were then homogenized in an all-glass

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homogenizer for about 1 min with 10 volumes of a buffer containing 80 mM Tris-HCl pH 7·4, 6·6 mM $MgSO_4$ and 2 mM theophylline.

For each experiment aortic tissues of either one old or two young rabbits were used. Incubations were performed at 30° for 10 min in a total volume of 0·6 ml containing homogenate equivalent to 40 mg of tissue, 2 mM ATP-^{3}H (specific activity 100–200 μCi/μmol), and either 10^{-3}M drugs or 10^{-2}M sodium fluoride. The AC activity was measured by the method of Krishna, Weiss & Brodie (1968). The protein content of homogenates was determined by the method of Lowry, Roseborough & others (1951).

To measure adenyl cyclase activity of intact tissue, a modification of the method of Shimizu, Daly & Creveling (1969) was used. Aortic strips were pre-incubated with [^{14}C] adenine for 45 min in Krebs-bicarbonate solution at 37°. The tissues were then washed and incubated for 1, 2 and 10 min with isoprenaline in the presence of phentolamine (3 μg/ml). The cyclic AMP formed was measured by the method of Krishna & others (1968).

RESULTS AND DISCUSSION

Table 1 shows the AC activity of thoracic and abdominal aortas from young (8 weeks old) male and female rabbits. Since there were no sex differences in the AC activities, data from both sexes were pooled. Although the basal AC activity appeared to be higher in abdominal aorta, the difference was not statistically significant. At concentrations of 10^{-3}M, isoprenaline, 5-HT and histamine did not change the AC activity in thoracic or abdominal aorta. NaF (10^{-2}M), however, significantly increased the AC activity in both thoracic and abdominal aorta. After NaF stimulation the AC activity of abdominal aorta was significantly higher than that of thoracic aorta (Table 1).

Table 2 shows the AC activity of thoracic and abdominal aortas from 2- to 3-year-old rabbits. As with young animals, there was no stimulation by isoprenaline, 5-HT, or histamine, whereas NaF significantly increased the AC activity of both thoracic and abdominal tissue. In contrast to the results obtained in tissue from young animals, however, the basal as well as the NaF-stimulated AC activity was significantly higher in abdominal than in thoracic aorta.

Table 1. *Adenyl cyclase activity in homogenates of aortic tissue from 8-week-old rabbits*

	Thoracic aorta		Abdominal aorta	
Drugs	No. Exp.	pmol cAMP/mg protein/min	No. Exp.	pmol cAMP/mg protein/min
No drug	10	1·79 ± 0·29	10	2·90 ± 0·61
		$P < 0.01$		$P < 0.01$
10^{-2}M NaF	10	3·91 ± 0·53	10	6·27 ± 0·54
10^{-3}M Isoprenaline	6	1·83 ± 0·52	5	3·31 ± 0·87
10^{-3}M 5-HT	3	1·20 ± 0·31	3	1·72 ± 0·82
10^{-3}M Histamine	3	1·31 ± 0·34	3	2·19 ± 0·84
mg protein/g tissue	10	124 ± 7	10	97 ± 4

Values are means ± s.e. of the number of experiments indicated. Significant differences between groups were established by paired comparison (Student's *t*-test).
cAMP = cyclic AMP.

Table 2. *Adenyl cyclase activity in homogenates of aortic tissues from 2- to 3-year-old rabbits*

	Thoracic aorta		Abdominal aorta	
Drugs	No. Exp.	pmol cAMP/mg protein/min	No. Exp.	pmol cAMP/mg protein/min
No drug	4	1·51 ± 0·19	4	3·51 ± 0·35
		P < 0·01		P < 0·01
10^{-2}M NaF	4	4·68 ± 0·28	4	9·48 ± 0·96
10^{-3}M Isoprenaline	3	1·54 ± 0·22	3	3·95 ± 0·53
10^{-3}M 5-HT	4	1·25 ± 0·16	4	3·30 ± 0·37
10^{-3}M Histamine	4	1·40 ± 0·20	4	4·06 ± 0·58
mg protein/g tissue	4	116 ± 7	4	90 ± 18

Values are means ± s.e. of the number of experiments indicated. Significant differences between groups were established by paired comparison (Student's *t*-test).

A comparison of Tables 1 and 2 shows no marked differences in basal AC activities between young and old rabbits. After NaF stimulation, however, aortas from older animals showed a higher enzymatic activity in abdominal (young, 6·27 ± 0·54; old, 9·48 ± 0·96), but not in thoracic aorta (young, 3·91 ± 0·53; old, 4·68 ± 0·28). These age differences could not be explained by a change in the tissue protein content used as basis for the calculation of AC activity, since the protein content did not change with age. There was, however, a lower protein content in abdominal than in thoracic aorta (Tables 1 and 2).

Turtle & Kipnis (1967) and Abe, Robison & others (1969) showed that α-receptors are capable of mediating a fall in the level of cyclic AMP. Since isoprenaline can act as an agonist at α-receptors, experiments were made in which thoracic aortas from young rabbits were preincubated with an α-receptor blocking agent (1 μg/ml of either dibenamine or phentolamine) for 15 min before homogenization. No change in either basal AC activity or the activity in the presence of isoprenaline was observed. In another experiment, 10^{-3}M noradrenaline was used as an α-receptor agonist. Again, there was no change in the AC activity.

It also seemed possible that homogenization stimulated AC activity in some unknown way such that β-receptor activation could not manifest itself or that homogenization altered the mechanism responsible for β-receptor activation of AC. To overcome these possible objections, thoracic and abdominal aortas from young animals were treated with 10^{-3}M isoprenaline for 1 min after 3 μg/ml phentolamine. The aortas were then homogenized in the presence of 10^{-3}M isoprenaline and AC activity was measured in the presence of 10^{-3}M isoprenaline as described above. Neither isoprenaline nor phentolamine changed the basal AC activity (Table 3). Thus, under these conditions AC activity cannot be changed by β-receptor activation even when the tissue is intact. To preclude the possibility that adenyl cyclase was maximally stimulated by a substance released during homogenization, aortas were preincubated with 10^{-6}M propranolol, a β-receptor blocking agent, for 15 min, then homogenized in 10^{-6}M propranolol and the AC assayed. Propranolol did not influence the basal activity, indicating that homogenization plays no role in the stimulation of AC through a β-receptor mechanism (Table 3). In both sets of experiments, 10^{-2}M NaF added after homogenization, markedly increased AC activity (Table 3).

Table 3. *Adenyl cyclase activity in homogenates of rabbit aorta during adrenergic blockade**

Drugs added before homogenization	Drugs added during assay	Thoracic aorta	Abdominal aorta
		pmol cAMP/mg protein/min	
None	None	1·63	3·00
3 μg/ml Phentolamine	3 μg/ml Phentolamine	1·54	2·53
3 μg/ml Phentolamine	NaF	4·18	8·35
3 μg/ml Phentolamine and			
10^{-3}M Isoprenaline	10^{-3}M Isoprenaline	1·60	2·68
10^{-6}M Propranolol	10^{-6}M Propranolol	1·62	3·21

* Pool of three rabbit aortas. Each value is the result of duplicate determinations.

Isoprenaline is known to enhance activity more effectively in intact heart (Laraia & Reddy, 1969), fat cells (Kuo & Renzo, 1969) and brain slices (Shimizu & others, 1969) than in homogenates. For this reason, adenyl cyclase activity was measured in intact aortas prelabelled with [^{14}C]adenine for 45 min at which time, 85 to 90% of the total radioactivity in the tissue can be found in the ATP fraction (Krishna & others, 1968). In the presence of 5×10^{-4}M theophylline and 3 μg/ml of phentolamine isoprenaline 10^{-3} and 10^{-6}M failed to induce significant conversion of ATP into cyclic AMP at 1,2 and 10 min (pool of 6 thoracic aortas).

The present study shows that the AC activity in aortic tissue cannot be stimulated by various biogenic amines but is increased by 10^{-2}M NaF after homogenization. It also indicates that, although there are no age differences in the endogeneous AC activity of either the thoracic or abdominal aortic strips, the NaF-stimulated activity of the abdominal aorta increased with age. Thus, the difference in responsiveness to a β-adrenergic receptor agonist between thoracic and abdominal aorta and the decrease of this responsiveness with increasing age found by Fleisch & others (1970) could not be related to changes in AC activities of these tissues. It is still possible, however, that these negative results are due to the heterogeneity of aortic tissue and to the fact that β-receptors represent a small part of the whole tissue.

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The binding of indomethacin, salicylate and phenobarbitone to human whole blood *in vitro*

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The binding of indomethacin, salicylate and phenobarbitone to human whole blood, plasma and red cells has been determined by equilibrium dialysis. The red cells bound appreciable proportions of salicylate and phenobarbitone but not indomethacin. It is concluded that prediction of the extent of the drug binding to the circulating blood should be made from the results obtained with whole blood, red cells and plasma.

After entering the blood a drug may circulate in a bound form. The bound drug can be regarded as a storage depot since only the unbound fraction is able to penetrate the cells and initiate pharmacological actions. In addition, when a drug binds to the circulating proteins it may displace other small molecules, such as amino-acids and fatty acids (McArthur & Dawkins, 1969; Dawkins, McArthur & Smith, 1970), from their binding sites on the proteins. A common practice is to determine the binding of drugs to either separated plasma or to purified albumin fractions and to use the results to predict the circulating levels of bound and unbound drug. Albumin has been shown to be responsible for most plasma protein-drug interactions although some of the other proteins may also be concerned. The possible influence of the erythrocytes and other cellular components on drug binding in blood has been largely neglected. The present investigation is concerned with comparing the binding characteristics of indomethacin, phenobarbitone and salicylate in human whole blood and plasma *in vitro*.

MATERIALS AND METHODS

Materials

Samples of blood (200 ml) were obtained by venepuncture from healthy normal males and collected into plastic bags, 1 ml of heparin containing 1 mg sodium heparin (100 IU) being used as an anticoagulant. Approximately 150 ml of each sample of whole blood was centrifuged at 3000 *g* for 15 min and the separated plasma divided into two equal portions. One of these (undiluted plasma) was used as such and to the other (diluted plasma) was added sufficient 0·9% (w/v) NaCl solution to adjust its volume to that of the corresponding volume of whole blood from which it had been prepared. A similar procedure was used with the red cells separated by the centrifugation except that these were suspended and centrifuged in six quantities, each of 100 ml of 0·9% (w/v) NaCl solution, before the final volume was adjusted to that of the initial sample of whole blood (diluted red cells). No attempt was made to separate either the leucocytes or blood platelets and the red cell fraction includes both these components. Visking dialysis tubing (8/32 inch inflated diameter)

was obtained from the Scientific Centre, London, and powdered indomethacin was a gift from Merck, Sharp & Dohme Ltd., Hoddesdon, Herts. All chemicals were of analytical grade except for sodium phenobarbitone and sodium salicylate, which were of British Pharmacopoeial grade, and distilled water was used throughout.

Measurement of drug binding

Aliquots (2 ml) of either whole blood, plasma, diluted plasma or diluted red cells were placed inside sacs of Visking tubing which had been soaked in two changes of water for 20 min before use. They were dialysed against 3 ml of 0·9% (w/v) NaCl solution containing sufficient of the drugs to give initial concentrations as follows: phenobarbitone, 0–3 mM, indomethacin, 0–1 mM and salicylate 0–10 mM, in vessels shaken at 100 rev/min for 28 h in a water bath at 8°. The indomethacin was suspended in distilled water and sufficient N NaOH added to yield a 5 mM solution of the drug. Sufficient 0·9% (w/v) saline was added to give the range of indomethacin concentrations required and in each case the final solution was adjusted to pH 7·2 by the addition of 0·1N HCl. Preliminary experiments were made to ensure that no loss of drug occurred during dialysis due to adsorption to the tubing and each combination of drug and blood fraction was separately investigated to establish that dialysis was complete and equilibration attained within 28 h. Indomethacin and salicylate were estimated in samples taken from outside the sacs with an Aminco Bowman Spectrophotofluorimeter using activating wavelengths of 290 and 294 nm and detecting wavelengths of 385 and 413 nm respectively. The phenobarbitone concentration was determined by adjusting to pH 10·0 with 0·1M borate buffer and measuring the absorption at 240 nm using a Unicam SP800 spectrophotometer. The concentrations of the drugs outside the sacs at the end of dialysis are the unbound concentrations. At the end of the dialysis the concentrations of the drugs inside the sacs containing the blood preparations were calculated by subtracting the amounts of drug outside the sac when dialysis was complete from the total amounts of drug, i.e. inside and outside the sacs, found in the corresponding experiments when only saline was inside the sac. The concentrations of bound drugs inside the sacs at the end of dialysis were obtained by difference.

RESULTS

The binding curves of indomethacin to whole blood, plasma, diluted plasma and diluted red cells are shown in Fig. 1. In this and the subsequent figures the concentrations of unbound drug have been plotted against the concentrations of bound drug. The results with indomethacin show that the drug is not bound by the red cells but that at a total concentration of 0·2 mM approximately 75% is bound by the plasma. The binding curve for whole blood is almost identical to that of the plasma to which has been added a volume of saline equivalent to that of the erythrocytes (diluted plasma). Thus the contribution of the red cells to the binding of indomethacin is negligible.

The binding curves for salicylate (Fig. 2) and phenobarbitone (Fig. 3) differ from those of indomethacin in that appreciable proportions of both drugs are bound by the red cells and the plasma. Measurements of the binding to separated plasma *in vitro* would suggest that the free, i.e. unbound concentrations of the drugs are smaller fractions of the total concentrations that would be present *in vivo*.

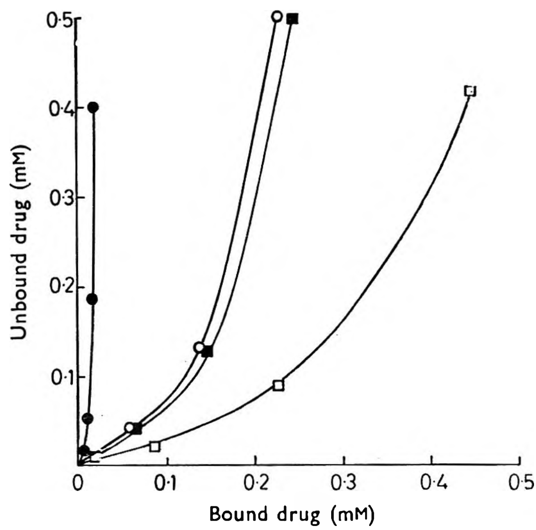

FIG. 1. Binding curve of indomethacin to blood fractions *in vitro*. ● Diluted red cells. ○ Whole blood. ■ Diluted plasma. □ Plasma.

DISCUSSION

The study of the binding of drugs in the circulation has conventionally been restricted to their interactions with plasma proteins. One reason is the obvious convenience of experimental techniques using either separated plasma or purified protein fractions. The availability of highly purified albumin preparations has provided an impetus to the detailed investigation of the binding sites and nature of the forces which cause drugs to interact with plasma albumin. Other plasma proteins can and do participate in drug binding but the possible influence of the cellular constituents in the blood have received scant attention (Goldstein, 1949; Meyer & Guttman, 1968)

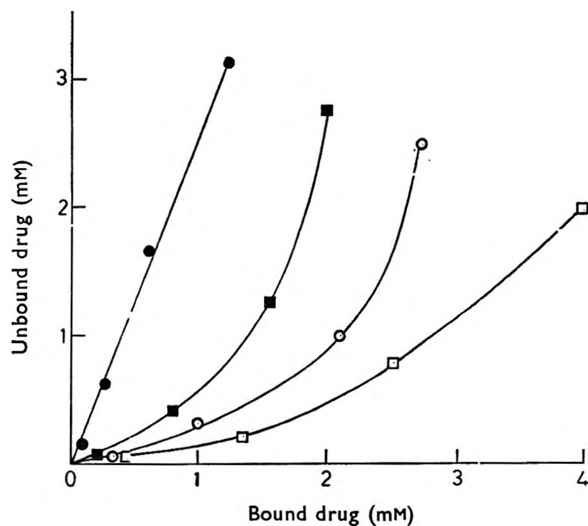

FIG. 2. Binding curve of salicylate to blood fractions *in vitro*. ● Diluted red cells. ○ Whole blood. ■ Diluted plasma. □ Plasma.

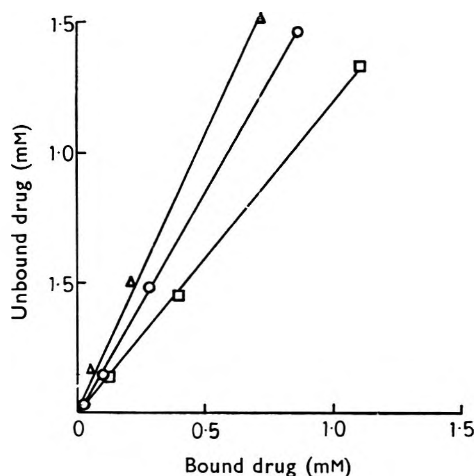

FIG. 3. Binding curve of phenobarbitone to blood fractions *in vitro*. △ Diluted red cells and diluted plasma. ○ Whole blood. □ Plasma.

The present results show that for three widely used drugs, indomethacin, salicylate and phenobarbitone, the determination of their binding to separated human plasma would yield misleading information about the ratio of bound to unbound drug in the circulation. It is the fraction of drug in the unbound form which is available, at any one time interval, to enter the cells and initiate pharmacological and toxic actions. The determination of this fraction is of obvious importance in relating *in vitro* effects to *in vivo* actions, in the assessment of the efficiency of a drug in different formulations and after various rates of administration, and in the investigation of untoward effects. Thus an individual may be at a higher risk after the ingestion of a particular dose of a drug because of a reduced capacity to bind the drug in the circulation.

The present results show that human red cells do not bind indomethacin. It would be possible to predict values for unbound indomethacin in the circulation from a plasma binding curve for the drug prepared *in vitro* if appropriate corrections were made for the volume occupied by the red cells, i.e. diluted plasma binding curve. This situation does not apply to salicylate and phenobarbitone since both these drugs bind to the red cells. It is necessary to measure their binding curves to whole blood rather than to either separated plasma or purified albumin fractions. The present results with salicylate do not agree with either those of Coburn (1943) who stated that the drug was excluded from human red cells or of Lester, Lolli & Greenberg (1946) and Smith, Gleason & others (1946) who concluded that the red cell was freely permeable to salicylate but did not bind the drug.

The concentrations of indomethacin and salicylate used in the *in vitro* experiments were equivalent to those observed in man either during therapy or in acute intoxication (Hucker, Zacchei & others, 1966; Smith, 1966). The plasma concentrations of phenobarbitone after medicinal doses is about 0·2 mM and concentrations up to 0·5 mM have been reported in acute poisoning (Broughton, Higgins & O'Brien, 1956). The range of concentrations of the drug used in the present work was higher because of the relative insensitivity of the analytical method at very low concentrations.

It is concluded that the determination of the extent of binding of a drug to either separated plasma or to purified protein fractions provides inadequate data about the binding of drugs in the circulation. The interaction of the drug with red cells should also be measured. If a drug binds to red cells, then the diluted plasma binding curves yield lower figures for bound drug than would be obtained with whole blood *in vitro* and expected to occur in the circulating blood. It is possible that a drug may not bind to plasma proteins but bind to the red cells. The determination of its binding to plasma or to albumin preparations would yield results suggesting that it would leave the circulation at a rapid rate whereas its complex with the red cells might enable it to persist for a considerable period. Studies of drug binding *in vitro* should therefore be made with whole blood in addition to any measurements performed on plasma or separated plasma protein fractions.

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Inhibition of acetylcholinesterase by dibenamine and dibenzyline

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Dibenamine and dibenzyline are irreversible inhibitors of acetylcholinesterase (AChE). Kinetic studies show that at pH 9·5 a fast reaction occurs between a group on the enzyme with pK_a 9·1 and the ethyleniminium ion derived from the inhibitor. Either the ϵ-amino-group of a lysine residue is alkylated or else a lysine residue catalyses the alkylation of a non-ionisable group (e.g. hydroxyl). At pH 6·5 there is a slow reaction between a carboxyl anion on the enzyme and the ethyleniminium ion. Studies of the alkylation reactions in the presence of the reversible competitive inhibitor of the enzyme, tetramethylammonium ion, show that alkylation occurs at some distance from the anionic site and probably on the borders of the active site.

The hypothesis concerning the identity of the enzyme acetylcholinesterase (AChE) and the cholinergic (acetylcholine) receptor of mammalian tissues was first proposed by Roepke (1937). Since then an accumulation of conflicting data has cast considerable doubt on its validity (Koelle, 1963; Webb, 1965; Podleski, 1967; Karlin, 1967). We have examined this problem by a comparative study of the reaction between certain alkylating agents and both the enzyme and receptor. This communication is concerned with our findings on the nature of the reactions of dibenamine and dibenzyline with the enzyme.

MATERIALS, METHODS AND RESULTS

Materials

Dibenamine hydrochloride and dibenzyline hydrochloride were recrystallized from isobutanol and had m.p. 187–8° and 136·5–137° respectively. The AChE used was a lyophilized preparation from bovine erythrocytes (Koch—Light Laboratories, 20 000 Schachter units per vial) and solutions were prepared in partially hydrolysed gelatin (2%) containing sodium chloride (0·2M) and stored at 4°. The concentrations of enzyme expressed here as mg refer to the lyophilized preparation. Acetylcholine bromide (BDH, biochemical grade) solutions (4×10^{-2}M) were prepared in sodium chloride (0·2M) and stored at 4°. Fresh solutions of enzyme and substrate were prepared every 3–4 days.

Dissociation constants of dibenamine and dibenzyline

The pK_a values of dibenamine and dibenzyline were determined by Shapiro's method (Shapiro, Isaacs & others, 1962) on solutions of the hydrochloride salts (1×10^{-4}M) in methanol–water mixture (1 : 1) at 25° by titration with alkali using a pH-stat (Radiometer, Copenhagen). The apparent pK_a values were 5·5 and 5·1 respectively.

Inhibition of AChE by dibenamine and dibenzyline

Standard procedure. The enzyme solution (1 ml, 3·6 mg) was added to a solution of the inhibitor (19 ml) in sodium chloride (0·2M). The mixture was mechanically stirred and adjusted to the required pH by the automatic addition of sodium hydroxide solution (20 mM) from a pH-Stat (Radiometer, Copenhagen) or by the dropwise addition of dilute hydrochloric acid to the mixture. The reaction mixture was then incubated in a thermostat bath at $25 \pm 0{\cdot}01°$. It proved extremely difficult even under an atmosphere of nitrogen to maintain the reaction mixture at pH values $> 7{\cdot}5$ due to absorption of atmospheric carbon dioxide each time a sample was withdrawn. A satisfactory method of sampling was devised which used the pressure of nitrogen gas to blow a sample of the mixture from the vessel when required. By this means the pH of the mixture during each experiment was maintained $\not> 0{\cdot}3$ pH unit below the commencing value. The pH values quoted are the means of the initial and final values observed.

Acetylcholine solution (1 ml) was added to a solution of sodium chloride (17 ml, 0·2M) in a jacketed vessel through which water from the thermostat bath circulated. The vessel was connected to the pH-stat and supplied with a continuous flow of dry, carbon dioxide-free nitrogen, the end of the nitrogen tube being placed just above the surface of the solution. Samples (2 ml) of the inhibition reaction mixture were withdrawn at suitable intervals of time and added to the assay vessel. The rate of substrate hydrolysis at pH 7·4 was given by the slope of the trace on the recorder over a period of 5 min. The rate of acid production in the absence of enzyme was negligible.

Irreversible inhibition

A solution of dibenamine hydrochloride ($7{\cdot}5 \times 10^{-5}$M) was incubated with the enzyme at pH 9·5 following the standard procedure. The enzyme activity remaining decreased with time and was eventually zero. The reaction with dibenzyline hydrochloride (1×10^{-4}M) followed a similar course.

Attempted regeneration of inhibited enzyme

Solutions of inhibited enzyme (20 ml) prepared by the standard procedure from either dibenamine or dibenzyline at both pH 5·0 and pH 9·5 were mixed with sodium thiosulphate (1 ml, 1·334M) to remove the alkylating species (*vide infra*). After readjustment of the pH to the initial values the solutions were stored at 25° for 18 h. Control solutions of the enzyme were subjected to the same treatment. There was no change in the enzyme activity of either the control solutions or inhibited enzyme solutions over the 18 h period. Consequently, the inhibition of AChE by dibenamine and dibenzyline is regarded as irreversible for this period of time.

Kinetics of the inhibition reaction

In the general case of a bimolecular reaction between two compounds (A) and (B) the rate of reaction is given by

$$\frac{dx}{dt} = k_2 (a - x)(b - x) \quad \ldots \quad (1)$$

where k_2 is the second-order rate constant, (a) and (b) are the initial concentrations of

(A) and (B) respectively and the concentration of products is (x) at time (t). Integration and rearrangement of equation (1) gives,

$$k_2 = \frac{2{\cdot}303}{t(a-b)} \log \frac{b(a-x)}{a(b-x)}.$$

In the situation where a $\gg$ b, this simplifies to,

$$k_2 = \frac{2{\cdot}303}{ta} \log \frac{b}{(b-x)} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

and gives,

$$t = \frac{2{\cdot}303}{k_2a} \log b - \frac{2{\cdot}303}{k_2a} \log (b - x),$$

so that a plot of t *vs* log (b − x) for the reaction is linear and the second-order rate constant can be calculated from the slope of the line. However, it is more usual to determine the second-order rate constant from the half life, $t_{0{\cdot}5}$, for the reaction when plotted as a first-order reaction. The first order rate equation on integration gives

$$k_1 = \frac{2{\cdot}303}{t} \log \frac{b}{(b-x)} \quad .. \quad .. \quad .. \quad .. \quad (3)$$

so that for a plot of t *vs* log (b − x),

$$t_{0{\cdot}5} = \frac{2{\cdot}303}{k_1} \log 2,$$

where k_1 is the first order rate constant. In the case of a pseudo unimolecular reaction, k_1 is an apparent first order rate constant and from equations (2) and (3), $k_1 = k_2a$. The second-order rate constant is then given by the slope of the line for a plot of k_1 *vs* concentration of (a) in a series of experiments where the concentrations of (b) are constant.

Dibenamine hydrochloride over the concentration range 0·05 − 2·5 × 10^{-5}M was incubated with AChE at pH 9·5 following the standard procedure. A similar study was made at pH 6·5 over the concentration range 3·75–6·25 × 10^{-5}M.

At each incubation pH, duplicate control experiments were conducted in which the inhibitor was replaced by sodium chloride (0·2M). There was no change in the enzyme activity of any of the control solutions which showed that AChE is stable at each pH studied during the time of the inhibition studies.

A graph of log (rate of acetylcholine hydrolysis) *vs t* for each inhibition reaction was linear showing that the reaction followed first order kinetics.

The lines of best fit were determined by regression analysis and the apparent first-order rate constants, k_1, calculated. The mean values for k_1 were a linear function of the inhibitor concentration at each pH studied (Fig. 1A) as expected for a pseudo-unimolecular reaction and the lines passed through the origin indicating that the inhibitor does not form a complex with the enzyme before formation of a covalent bond.* The second-order rate constants, k_2, were calculated from the slopes of the graphs and had the values 37·2 and 1093 M^{-1} min^{-1} at pH 6·5 and 9·5 respectively.

Similar results were obtained at pH 6·5 and 9·5 with dibenzyline hydrochloride over the concentration ranges 6·0–10·0 × 10^{-5}M and 2·0–6·0 × 10^{-5}M respectively (Fig. 1B).

* In a preliminary communication (Beddoe & Smith, 1967), complexing was erroneously reported for the dibenamine reaction, attributable to working in excess of the solubility of the base.

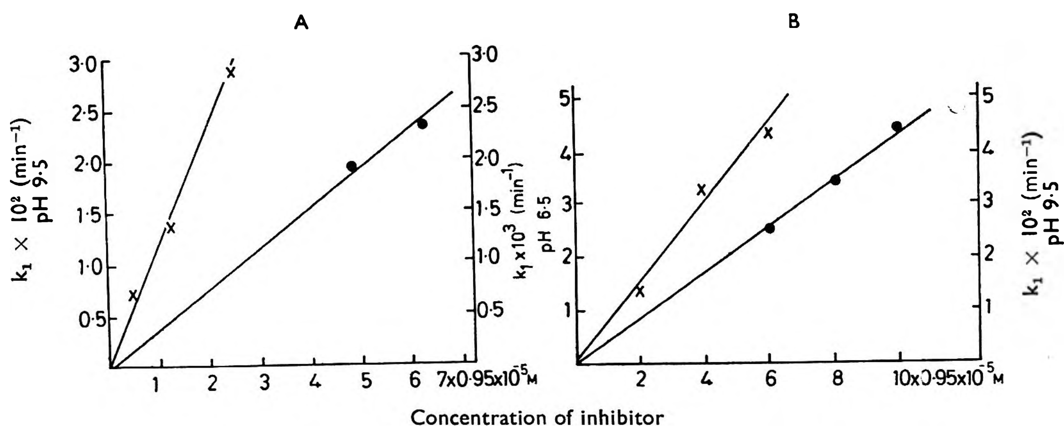


Fig. 1. Inhibition of AChE by dibenamine (A) and dibenzyline (B) at pH 9·5 (x) and pH 6·5 (●) as a function of inhibitor concentration.

The values obtained for the second order rate constants, k_2, at pH 6·5 and 9·5 were 43·5 and 735·4 M^{-1} min^{-1} respectively.

In kinetic studies of the reactions of inhibitors with enzymes the rate constants can vary as much as ± 10% between duplicates (Kézdy, Thomson & Bender, 1967). In this work the coefficient of variance (%) for six determinations at pH 6·5 was 6·1 and for five determinations at pH 9·5 was 8·9%. The coefficient of variance (%) for ten determinations in the enzyme assay alone was 1·18%.

Variation of the concentration of enzyme with constant inhibitor concentration showed that the second-order rate constants for the inhibition reaction were independent of enzyme concentration as expected for a pseudo-unimolecular reaction (Table 1).

Influence of thiosulphate ion on the inhibition reaction

AChE was added to a solution of dibenamine hydrochloride (7·5 × 10^{-5}M) or dibenzyline hydrochloride (1 × 10^{-4}M) in a medium containing sodium thiosulphate [6·67 × 10^{-2}M; ionic strength (I) = 0·2] and the pH was adjusted to 10. The mixture was left at room temperature for 10 min and then the standard inhibition procedure followed. Control experiments were conducted in a similar manner in a medium containing sodium chloride (0·2M). Other experiments were made in a

Table 1. *Influence of AChE concentration on the second-order rate constant for the inhibition reaction*

	Enzyme concentration in inhibition mixture (mg/ml)	Incubation pH	Mean k_2 ($M^{-1}min^{-1}$)
Dibenzyline			
(1 × 10^{-4}M)	0·18	6·5	48·9
		9·5	435·8
	0·36	6·5	46·9
		9·5	391·4
Dibenamine			
(7·5 × 10^{-5}M)	0·18	6·5	24·4
		9·5	532·1
	0·36	6·5	22·1
		9·5	534·4

medium containing sodium thiosulphate ($9{\cdot}4 \times 10^{-5}$M) in addition to sodium chloride. The results show that thiosulphate ion prevents the reaction of dibenamine and dibenzyline with AChE.

Influence of ionic strength on the rate of the inhibition reaction

Dibenamine hydrochloride ($7{\cdot}5 \times 10^{-5}$M) and dibenzyline hydrochloride (1×10^{-4}M) were separately incubated with AChE at pH 6·5 and pH 9·5 following the standard procedure but in media of different ionic strengths over the range, $I = 0\text{–}20 \times 10^{-2}$.

A three to four fold increase in the value of the observed second-order rate constant, k, was noted for both inhibitors at pH 6·5 when the ionic strength was decreased from 0·2 to zero (Fig. 2). There was a slight increase (about 8%) in the rate constant when chloride ions were replaced by sulphate ions but this was not regarded as having a special significance. The second-order rate constant at pH 9·5 was independent of

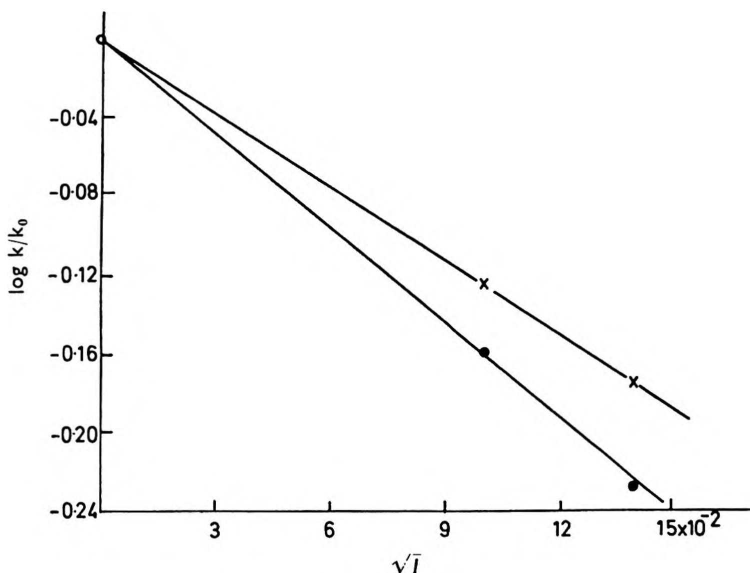


FIG. 2. Influence of ionic strength on the rate of inhibition of AChE by dibenamine (o) and dibenzyline (x) at pH 6·5.

the ionic strength of the medium over the range $I = 0{\cdot}05 - 19{\cdot}05 \times 10^{-2}$ for dibenamine but for dibenzyline the reaction was slowed to half rate at the high ionic strength.

It can be shown (Scatchard, 1932) that for two interacting species,

$$\log k/k_0 = 1{\cdot}018\, Z_A\, Z_B \sqrt{I} \qquad (4)$$

where k_0 is the second-order rate constant when $I = 0$, and Z_A and Z_B are the charges on the two interacting species. A plot of log k/k_0 *vs* $\sqrt{I}$ for the results at low pH gave a linear curve with slope $-1{\cdot}63$ for dibenamine and $-1{\cdot}24$ for dibenzyline over the low ionic strength range, $I = 0 - 1{\cdot}95 \times 10^{-2}$.

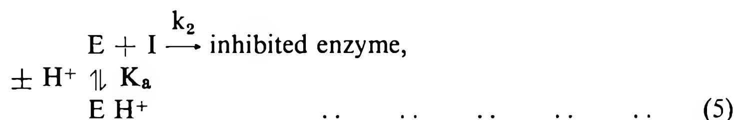
Effect of pH on the inhibition reaction

Solutions of dibenamine hydrochloride ($7 \cdot 5 \times 10^{-5}$M) and dibenzyline hydrochloride (1×10^{-4}M) were separately incubated with the enzyme over the pH range 4·7–10·6 following the standard procedure.

Duplicate control experiments in which the inhibitor was replaced by sodium chloride (0·2M) were made at pH 4·7 and 10·6 for the same intervals of time as the corresponding inhibition experiments and showed no loss of enzyme activity at the end of these periods.

The observed second-order rate constants, k, for the reactions were pH-dependent and plots of k *vs* pH were sigmoid-shaped.

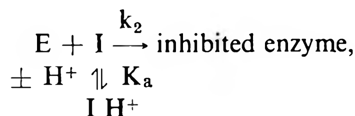
The relation between the hydrogen ion concentration and the observed second-order rate constant k, for the system



where a single ionizable group is on the enzyme, is given (Dixon & Webb, 1964) by

$$\frac{1}{k} = \frac{1}{k_2} + \frac{1}{k_2 K_a} [H^+] \qquad (6)$$

where k_2 is the second-order rate constant for the reaction with the active form of the enzyme. Equation (6) must also apply in the system,



where the ionizable group is on the inhibitor.

Graphs of $1/k$ *vs* $[H^+]$ for the inhibition reactions with dibenamine and dibenzyline over the pH range 6–10 gave non-linear curves (Fig. 3A and B).

Ionization of a single group would have shown a linear relation so that two ionizable groups must be involved in the inhibition reaction over this pH range. Each graph was divisible into two separate linear sections over the pH ranges 7·8–9·5 ad 6·0–7·3 for dibenamine and 8·5–9·9 and 5·5–6·5 for dibenzyline. The lines of best fit were calculated for each linear section and the pK_a value of the group ionizing over each pH range was calculated from the gradient ($1/k_2K_a$) and intercept ($1/k_2$). The pK_a values for the ionizable groups involved in the inhibition reaction between dibenamine and AChE were 5·8 and 9·05 and the corresponding values for dibenzyline were 5·4 and 9·22. Using these observed values, theoretical ionization curves were constructed and fitted to the experimental points for the reactions (Fig. 4A and B). The theoretical curves fitted the data well except for the points at very high pH. These deviations are readily explicable since small differences in the $t_{0 \cdot 5}$ values are much more significant when the reaction is fast as at high pH. Accordingly, these points were not used in calculating the pK_a values.

Influence of tetramethylammonium ion (TMA) on the inhibition reaction

Dibenamine hydrochloride ($0 \cdot 75 \times 10^{-5}$M) and dibenzyline hydrochloride (1×10^{-4}M) were separately incubated with the enzyme at pH 9·5 in media containing

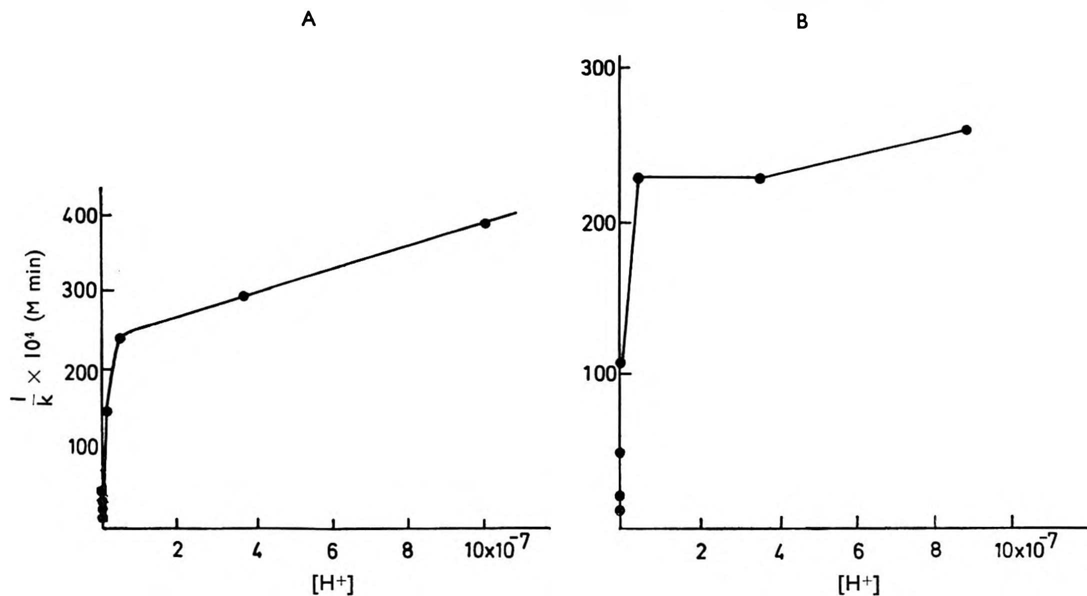
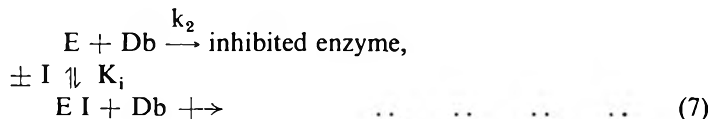


FIG. 3. Inhibition of AChE by dibenamine (A) and dibenzyline (B) as a function of hydrogen ion concentration.

tetramethylammonium ion (0–0·2M) and sodium chloride such that $I_{total} = 0{\cdot}2$ and the standard procedure followed.

In the system



$$\begin{array}{l} E + Db \xrightarrow{k_2} \text{inhibited enzyme,} \\ \pm I \;\; \rightleftharpoons \;\; K_i \\ E\,I + Db \not\rightarrow \end{array} \qquad (7)$$

where Db = dibenamine or dibenzyline and I = TMA, then, provided the (EI) complex is unreactive it can be shown that

$$\frac{1}{k} = \frac{1}{k_2} + \frac{1}{k_2 K_1} \cdot [I], \qquad (8)$$

where k_2 is the second-order rate constant for the reaction with the active form of the enzyme and k is the observed second-order rate constant in the presence of inhibitor.

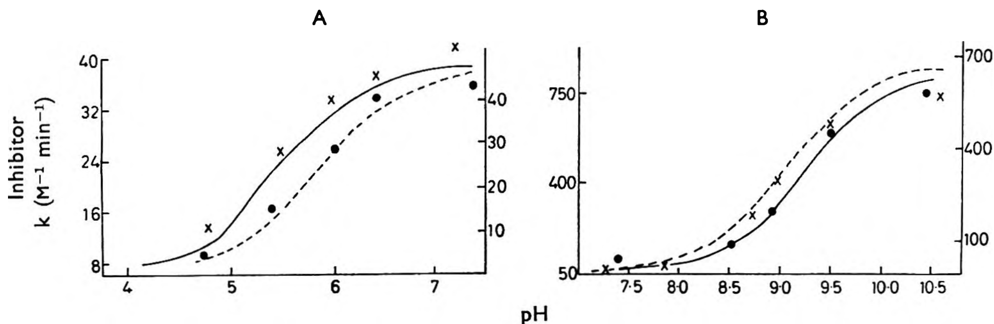
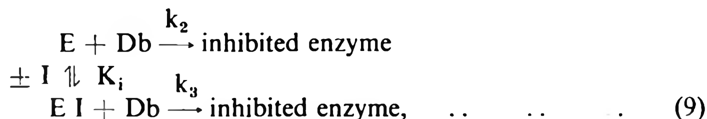


FIG. 4. Inhibition of AChE by dibenamine (left hand ordinate) (● in A, × in B) and dibenzyline (right hand ordinate) as a function of pH. The curve is that calculated for the dissociation of a group with A pK$_a$ 5·8 (- - - -) and 5·4 (———) respectively; B pK$_a$ 9·05 (- - - -) and 9·22 (———) respectively.

This equation resembles equation (6) for the effect of hydrogen ions on the second-order rate constant.

The relation between $\frac{1}{k}$ and TMA concentration was non-linear (Fig. 5A and B) and consequently it was inferred that TMA did not completely prevent access of the irreversible inhibitor to a reactive group at the active site of the enzyme. An acceptable explanation is that in the system



$$\begin{array}{l} E + Db \xrightarrow{k_2} \text{inhibited enzyme} \\ \pm I \upharpoonleft\downharpoonright K_i \\ E\,I + Db \xrightarrow{k_3} \text{inhibited enzyme,} \end{array} \qquad (9)$$

the (EI) complex is capable of reacting with dibenamine or dibenzyline at a reduced rate (i.e. $k_2 > k_3$). The relation between the rate constants k_2 and k_3 and the observed second-order rate constant, k, is then given by the expression

$$\frac{1}{k} = \frac{1}{\left(\frac{k_2}{(1 + \frac{[I]}{K_I})}\right) + \left(\frac{k_3}{(1 + \frac{K_I}{[I]})}\right)} \qquad (10)$$

The theoretical curve calculated using equation (10) approximately fitted the experimental values for dibenamine inhibition, where $K_I = 5 \times 10^{-3}$M, $\frac{1}{k_3} = 90 \times 10^{-4}$M min and $\frac{1}{k_2} = 16{\cdot}4 \times 10^{-4}$M min, and for dibenzyline inhibition, where $K_I = 10 \times 10^{-3}$M, $\frac{1}{k_3} = 85 \times 10^{-4}$M min and $\frac{1}{k_2} = 27{\cdot}8 \times 10^{-4}$M min.

DISCUSSION

The reaction between dibenamine and dibenzyline with AChE leads to progressive loss of enzyme activity until eventually the enzyme is completely inactivated. After

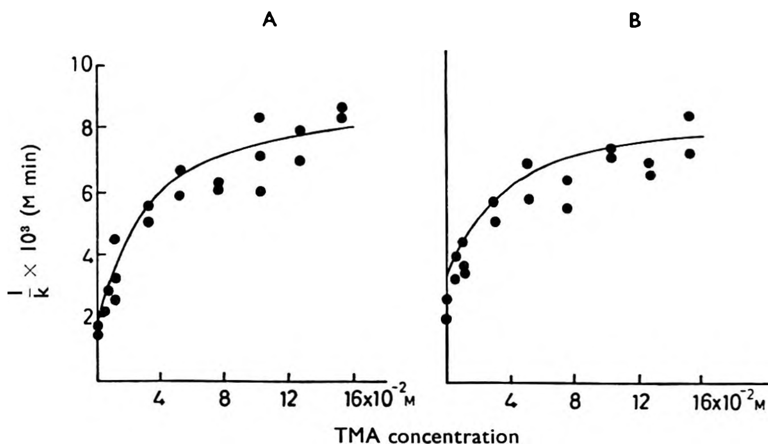
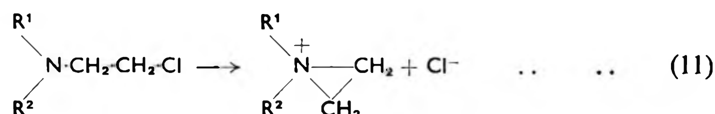


Fig. 5. Inhibition of AChE by dibenamine (A) and dibenzyline (B) at pH 9·5 in the presence of TMA. k is the observed second-order rate constant for the inhibition.

removal of the inhibiting species, enzyme activity cannot be regenerated by prolonged exposure of the inhibited enzyme to either acidic or alkaline conditions. This establishes that these compounds are irreversible inhibitors of the enzyme, and that the inhibitor residue is firmly attached to the enzyme by a very stable covalent bond.

The kinetics for the inhibiton reaction are first-order and are in accord with the occurrence of a biomolecular reaction between the enzyme and the inhibitor where the concentration of inhibitor is in excess. Work with other irreversible inhibitors of AChE such as carbamates and organophosphates (Reiner & Aldridge, 1967) has shown that in a few cases the second-order rate constant for the reaction is dependent on the inhibitor concentration. This has been interpreted in terms of initial complex formation between the inhibitor and enzyme preceding the formation of a covalent bond between the enzyme and inhibitor (Smith & Williams, 1965; Baker, 1964; Singer, 1967). In this work, complex formation between the inhibitor and the enzyme was not observed. However, this may be a result of the low inhibitor concentration used (due to the low aqueous solubility of the bases) which would tend to prevent such an observation (see Ryan, Ginsburg & Kitz, 1969).

Nitrogen mustards (Barlett, Ross & Swain, 1947; Hanby, Hartley & others, 1947) and certain monofunctional β-halogenoethylamines (1) (Graham 1962) are known to cyclize to the corresponding ethyleniminium ions (2) in neutral aqueous solutions, i.e.



and several of these ions have been shown to be the pharmacologically active species present in solutions of the parent compounds (Chapman & James, 1954; Graham, 1957; Allen & Chapman, 1960). Dibenamine is considered to exert its adrenergic blocking action through its ethyleniminium ion (Nickerson & Gump, 1949) since this action is prevented by the presence of thiosulphate (Nickerson & Goodman, 1948) which is known to react rapidly with ethyleniminium ions but only slowly with alkyl halides (Golumbic, Fruton & Bergmann, 1946).

The reactions of both dibenamine and dibenzyline with the enzyme are prevented by high thiosulphate concentration in the pH region where both inhibitors exist in the base form. The action of the thiosulphate may be attributable to removal of the active intermediate (presumably ethyleniminium ion) formed from the base.

The reaction between the inhibitors and the enzyme is pseudounimolecular and follows first order kinetics at pH 6·5 and 9·5 where the inhibitors are present mainly in the base form. The observation that the active alkylating species is the intermediary ethyleniminium ion requires accommodating within this kinetic scheme. This can be achieved if the concentration of the ion is rapidly built up to a constant level which is maintained thoughout the life of the experiments. That this situation exists is supported by the linear nature of the first order plots for the reactions over the concentration ranges studied together with evidence from previous studies where it was found that the level of ethyleniminium ion in aqueous-organic solvent solutions of dibenamine and dibenzyline is low (Chapman & Tomsett, 1961) and reasonably constant over a period of several hours (Harvey & Nickerson, 1953).

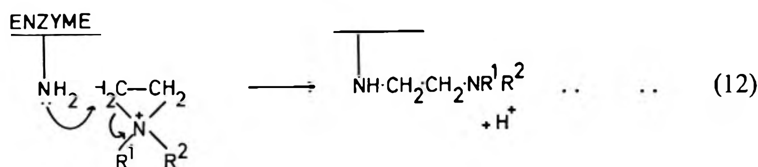
The pH-inhibition rate constant (k_2) profiles for the reactions between both dibenamine and dibenzyline with the enzyme over the pH range 4–10 were sigmoid. Close

examination showed that two ionization steps were involved over this range, one attributable to a group with a low pK_a value and the other to a group having a high value.

The group with a high pK_a has a value of 9·05 and 9·22 for the inhibition reactions using dibenamine and dibenzyline respectively. We have attributed the similarity in both systems to the ionization of an identical group. This group must be on the enzyme surface since dibenamine (pK_a 5·5) and dibenzyline (pK_a 5·1) exist in the base form above pH 7·5 and remain unchanged as the pH is increased.

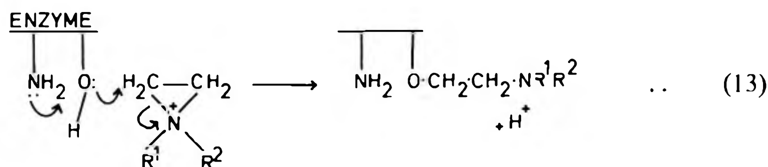
The effect of ionic strength on reaction rates has been well documented for organic reactions and successfully applied to enzyme-substrate interactions although misleading results have sometimes been obtained (Kézdy, Clement & Bender, 1964; Lumry, Smith & Glantz, 1951). The absence of a significant ionic strength effect on the second-order rate constants, k_2, for the inhibition reactions with both inhibitors at pH 9·5 suggests that an uncharged group on the enzyme is alkylated by the ethyleniminium ion since interaction between two charged ions would have exhibited a considerable effect.

We consider that the neutral group on the enzyme involved in the reaction is the ϵ-amino group of lysine (free base form) which exists in proteins with a pK_a value within the range 9·4–10·6 (Edsall, 1965). This group can either be directly alkylated by the ethyleniminium ion (eqn 12) or else act as a general base and catalyse the



.. .. (12)

alkylation of an adjacent unionized group such as a hydroxyl group (eqn 13). There is no evidence to suggest that the conformation of the enzyme changes over the pH range 7–10 [cf. α-chymotrypsin (Bender, Gibian & Whelan, 1966)]. This eliminates the possibility that a conformational change, dependent on a group with pK_a 9·1, leads to the unmasking of a neutral group which enters into the alkylation reaction.



.. (13)

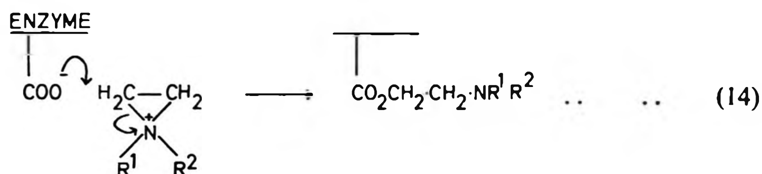
The group with a low pK_a value involved in the inhibition reaction has values of 5·8 and 5·4 for the reaction with dibenamine ($pK_{a_{app.}}$ 5·5) and dibenzyline ($pK_{a_{app.}}$ 5·1) respectively. These values compare favourably with those for the inhibitors. It is to be expected that the second-order rate constants for the reaction would increase in the observed manner with increase in pH since the concentration of the alkylating species is dependent on the proportion of the inhibitor present in the base form.

The effect of ionic strength on the second order rate constant for the reactions at

pH 6·5 was considerable and contrasted with the absence of such an effect at high pH. The noted values for the slopes of the graphs of log k/k_0 *vs* $\sqrt{I}$ for the reactions with dibenamine (— 1·63) and dibenzyline (— 1·24) are considered high values for a reaction with a slope of — 1. This is the expected value for a reaction occurring between oppositely charged monovalent ions.

We consider that all these data are in accord with alkylation by the ethyleniminium ion of a negatively charged group on the enzyme. This group does not change its state of ionization throughout the low pH range studied and consequently does not influence the pH-rate profile for the reaction. This group is most likely a carboxyl group which in proteins is almost completely ionized over the range considered (α-carboxyl, pK_a 3·0–3·2; aspartyl, 3·0–4·7; glutamyl ca 4·4; Edsall, 1965) and, furthermore, is known to react with dibenamine and dibenzyline (Harvey & Nickerson, 1954).

The reaction between the carboxylate ion on the enzyme and the ethyleniminium ion leads to the formation of an ester (eqn 14) which is stable under acidic and basic conditions for prolonged periods. This observation is in accord with the known resistance to hydrolysis of esters derived from these inhibitors (Graham & Al Katib, 1966).



.. .. (14)

The pH-activity profile for the reaction between AChE and its substrates is bell-shaped and is attributable to general base catalysis and general acid catalysis by imidazole (histidine) and phenolic hydroxyl (tyrosine) respectively in both the acylation and de-acylation steps (Wilson & Bergmann, 1950a; Bergmann, Segal & others, 1956). The reactions between the enzyme and organophosphorus compounds, e.g. TEPP (Wilson & Bergmann, 1950b) and carbamates, e.g. neostigmine (Reiner & Aldridge, 1967) also have bell-shaped pH-activity profiles. Consequently, it is generally accepted that the reactions of AChE with its substrates (i.e. acylation), organophosphorus compounds and carbamates are analogous processes (Wilson, Harrison & Ginsburg, 1961; Reiner & Simeon-Rudolf, 1966; Winteringham & Fowler, 1966). By contrast, the reactions of both dibenamine and dibenzyline with the enzyme have pH-activity profiles which are sigmoid-shaped and have a different pH-dependence.

Reaction between the alkylating agents and AChE resulted in total loss of activity. This observation is partial evidence that alkylation occurs at, or very near, the active site.

The question arises as to whether the point of attachment of the alkylating agents to the enzyme is to serine at the active site, (Schaffer, May & Summerson, 1953; Jansz, Berends & Oosterbaan, 1959), as for the acylation, phosphorylation and carbamoylation reactions, or elsewhere. The alkylation reaction occurring at low pH is with a carboxylate ion which rules out this possibility and the proposal that alkylation at high pH occurs on lysine similarly eliminates the involvement of serine.

However, the alternative interpretation of the data for the reaction at high pH, where lysine acts as a general base catalyst for the alkylation of an unionized group, could involve the hydroxyl group of serine. The sigmoid-shaped pH-activity profile for the alkylation reaction does not preclude this possibility since the charged nitrogen atom of the alkylating ethyleniminium ion, by acting as a "built-in" electron deficient centre, removes the necessity of a general acid catalyst for the reaction. It seems unlikely for the catalysis, however, that the ϵ-amino group of a lysine residue at the active site is preferred to the adjacent and correctly aligned imidazole nucleus of the histidine residue.

Studies were conducted with a reversible competitive inhibitor, the tetramethylammonium ion (TMA) (Wilson, 1952), to obtain further information on the point of attachment of the inhibitors to the enzyme. TMA is known to bind to AChE at the anionic site and alkylation of a group at the active site should be impeded by this inhibitor in a predictable manner.

TMA slowed the rate of inhibition of the enzyme by both dibenamine and dibenzyline, as would be expected if the reaction were to occur with an amino acid in the vicinity of the active site. However, the quantitative effect of TMA on the reaction differed from that expected for an inhibitor which binds to the active site and completely prevents the alkylation reaction from occurring by either steric or electrostatic repulsive interactions. The results obtained were in agreement with the view that alkylation occurs at some distance from the anionic site and on the borders of the active site so that the bound TMA ion can retard, but not prevent, the alkylation reaction. The equations developed for this situation approximately fit the observed experimental data.

Acknowledgements

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A new approach to the study of serum concentrations of orally administered cephalexin

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Studies of cephalexin in man showed that the conventionally constructed average serum level curves could give misleading results, with the average peak serum level from the curve being lower than the peak titres of the individual curves contributing to it It was also difficult to determine whether there were significant differences between such curves obtained from different preparations. Smooth curves were constructed from the observed data by computer methods; they were then arranged by the computer to have their peaks coincident in time and then averaged to give a curve much more similar in shape to its components. After the computer had fitted the smooth curve for each individual, it was possible to calculate a number of different parameters for each volunteer. Analysis of variance could then be done on these parameters, and thus significant differences between the results from tests on different preparations could readily be determined.

Cephalexin, a new orally administered cephalosporin antibiotic, is virtually completely absorbed from the upper small intestine, both in experimental animals (O'Callaghan Ryan & others, 1970) and in man (Muggleton, O'Callaghan & others, 1968) and is excreted unchanged in the urine, partly by glomerular filtration and partly by tubular secretion. Some problems posed by the studies of blood levels in men after administration of different doses and different types of preparation have been investigated in detail. The solutions adopted and the conclusions drawn are presented here.

MATERIALS AND METHODS

Cephalexin (7β-(D-α-aminophenylacetamido)-3-methylceph-3-em-4-carboxylic acid) is sparingly soluble in water (about 1·5% at neutral pH). It was given as a 5·25% suspension in 60% sucrose solution, or as two different solid preparations in capsules. All three preparations are commercially available. Of the two types of capsule used, one contained 500 mg cephalexin and virtually no excipient, the other contained 250 mg cephalexin and an approximately equal weight of corn starch. Both products were presented in the same type of size 0 capsule.

Assay of cephalexin

Cephalexin was assayed in serum by the large plate agar diffusion method against *Bacillus subtilis* (Muggleton & others, 1968). A spore suspension of *B. subtilis* ATCC 6633 was inoculated at 0·01% into agar made up as follows: 0·5% peptone, (Oxoid), 0·3% Lab Lemco, 1% sodium citrate and 1·5% agar (Oxoid) at pH 7.

Standard solutions

Standard solutions of cephalexin were prepared at 500 μg/ml in 0·2M phosphate buffer at pH 6 and stored at 4° for up to 4 days. Working standards at the required concentrations were freshly prepared each day by dilution into pooled human serum.

Volunteers

Young, healthy, men were given one dose of cephalexin in the morning about 1 h after a standard light breakfast (2 slices of buttered toast and preserve and 2 cups of tea or coffee), unless otherwise stated. Blood was taken by venepuncture at ½, 1, 1½, 2, 3, 4 and 6 h after the dose, although not all the men were bled at all the times. The serum was separated and its antibiotic content estimated as described above.

RESULTS

Average serum level curves

The magnitude and duration of the serum concentrations of cephalexin were obtained for each individual. The averaged results for each type of preparation and each dose are given in Table 1. Such values have been reported for cephalexin

Table 1. *Average serum concentrations as observed after oral doses of cephalexin*

Dose in mg	Type of preparation	Fed or Fasting	Serum concentration in μg/ml at h ½	1	1½	2	3	4	6
1000	Tight fill capsule	Fasting*	15·5	28·0	NT**	20·2	NT	4·7	<1·6
1000	,, ,,	Fed, 1 dose	1·25	11·6	NT	18·5	NT	9·4	1·8
1000	,, ,,	Fed, 5 doses	2·9	8·0	NT	22·5	NT	11·6	2·3
500	Tight fill capsule	Fasting	4·3	11·5	12·4	11·5	6·0	2·7	<1·9
500	,, ,,	Fed, 1 dose	4·2	13·2	NT	11·5	NT	3·9	<1·25
500	,, ,,	Fed, 5 doses	1·0	11·5	NT	14·4	NT	5·8	NT
500	Loose fill capsule	Fasting	3·0	11·5	12·9	12·5	7·1	2·8	NT
500	Suspension	Fasting	14·8	13·9	NT	8·6	NT	1·3	<1·3
300	Solution	Fasting	14·6	10·1	NT	3·1	2·0	<1·2	NT
250	Tight fill capsule	Fasting	<2·9	5·1	6·4	6·8	NT	1·3	<0·4

* Fasting volunteers had had a minimal breakfast consisting of two small slices of toast and two cups of tea or coffee one hour before the dose.
** NT = Not tested.

(Gower & Dash, 1969; Griffith & Black, 1968; Kind & Kestle & others, 1968; Perkins, Carlisle & Saslaw, 1968). There were wide variations in the time and magnitude of the peak titre similar to those shown by Clark & Turck (1968). When the results from the individuals in any one experiment were compared with the average serum levels the peak serum level of the average was lower than the peak serum level of every individual contributing to it (Table 2). When serum concentration was plotted against time, the curve from the average was much flatter and wider than the curves from the individual values (Fig. 1). It was concluded that no meaningful statistical comparison could be made between the types of preparation given, nor could the effect of the different doses be estimated.

Mathematical expression of the cephalexin serum level curve

Examination of the individual serum level curves obtained from 95 volunteers showed that in most cases the serum level curve could be expressed by the formula:—

$$y = A(e^{-p(t-t_0)} - e^{-q(t-t_0)})$$

Table 2. *Individual serum concentrations obtained after one dose of two loose fill low density* 250 *mg capsules and the average calculated from them*

Volunteer	Serum concentration in μg/ml at h					
	½	1	1½	2	3	4
1	0·7	17·1*	16·4	9·7	4·9	2·2
2	0·5	12·0	13·8	13·9*	5·2	2·2
3	9·4	18·1*	9·7	5·1	2·2	1·1
4	0·7	7·2	9·2	14·2*	11·7	7·7
5	<0·5	4·3	13·3	19·5*	6·0	3·2
6	2·9	6·5	11·0	15·7*	5·7	3·3
7	12·0	23·7*	13·2	4·1	2·3	1·3
8	<0·5	3·3	16·7	17·0*	4·3	2·1
Average	3·4	11·5	12·9*	12·4	5·3	2·9

* Peak titres.

in which y is the serum level at time t after administration, and the constant t_0 is the time taken for the dose administered to reach the site of absorption. The constants p and q are respectively related to the rate constant concerned with the speed of absorption and the rate constant concerned with the speed of excretion, there being no loss due to destructive metabolism. The constant A, given the values of p and q, and t_0 determines the area under the serum level curve. The same model was used by Mueller & Lieberman (1970) for salicylate blood levels.

The rationale behind this simple equation is that cephalexin, once it has reached the site of absorption, is absorbed so rapidly that it behaves as if it were an injectable

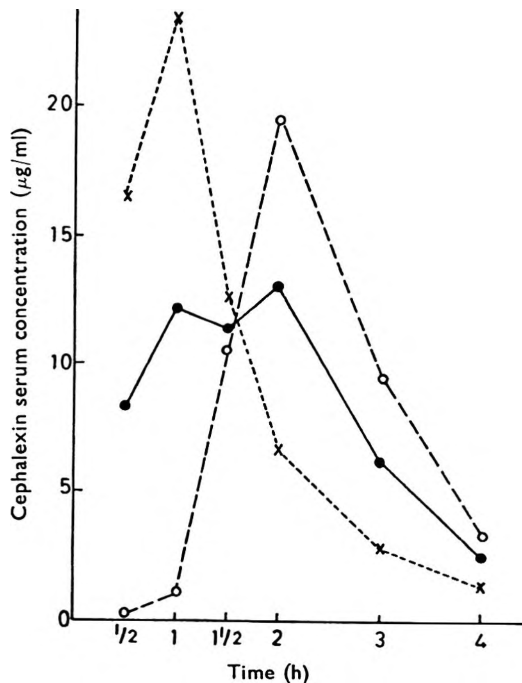

FIG. 1. Serum level curves from 2 volunteers after 1 oral dose of 500 mg cephalexin (observed values) and the conventional average curve constructed from them.

antibiotic. The chemically related injectable antibiotic cephaloridine is known to comply with an equation of this form but with $t_0 = 0$.

The constants A, p, q and t_0 are estimated from the observations by the method of least squares. However, the usual methods do not generally converge and it was necessary to use a descent method derived from that of Fletcher & Powell (1963). Even this method can lead to overflow in the computer unless special precautions are taken when p and q happen to be approximately equal.

A serum level curve, obtained from concentrations measured at arbitrary times, may miss the peak concentration actually achieved. When the formula was fitted to the data, however, the rate constant for absorption, the rate constant for excretion, the delay before any cephalexin was detected in the serum and the area under the curve, could all be calculated. Using these constants, fitted values were interpolated at close time intervals and, in this way, fitted curves were drawn. One of these, as drawn by the computer, is shown in Fig. 2; it has a sharp peak and illustrates that the highest observed serum concentration may not be the actual peak titre.

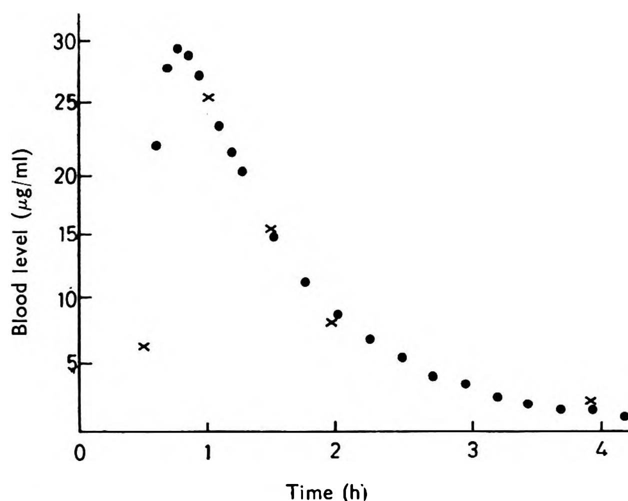

FIG. 2. An example of a computer fitted curve for the observed serum levels from one volunteer after 1 oral dose at 500 mg cephalexin. X Observed points. ● Computer plotted points.

With some volunteers or preparations, the serum concentration measured at one or more times was zero; if this meant that there were then less than 4 actual values measured, the calculation could not be applied. It was also not possible to calculate the constants if the earliest or the latest value obtained was the highest. The fitted curves were drawn for 95 of the 124 sets of serum levels actually measured, i.e., 77% overall. In the later experiments, the times chosen for bleeding were grouped more closely around the expected peak time, and over the last 30 volunteers the number where computer calculation was possible increased to 90%.

Construction of average serum level curves

The fitted curves for all the individual volunteers in any one experiment were, in a sense, moved along the time scale by the computer so that each peak occurred at the

origin of the time scale. Average curves were then computed and were found to be very much more like their individual component curves; they avoided the anomaly of having an average peak value markedly lower than all the values contributing to it.

Parameters calculated from the fitted curves

The calculation whereby the fitted curves were obtained also gave the following information for each individual:

1. Time of first blood level. 2. Time of peak serum level. 3 Time interval from first blood level to peak. 4. Time interval from peak to half peak concentration. 5. Ultimate half life, as determined from the rate of loss of cephalexin from the serum when absorption had ceased. 6. Peak serum level. 7. Area under the curve. 8. Length of time for which a nominated serum concentration would be exceeded. Calculations were made for 6·25, 8 and 12·5 μg/ml for all volunteers.

The average values for (2), (6) and (8) are given in Table 3, and the remainder in Table 4.

Table 3. *Average peak serum concentrations, time of peak concentration and time in excess of nominated serum levels after oral doses of cephalexin*

Dose in mg	Type of preparation	Fed or fasting	Peak level in μg/ml	Time of peak (min after dose)	No of min concentration exceeded (in μg/ml)		
					6·25	8	12·5
1000	Tight fill capsule	Fasting	40·8	54·4	189·6	169·8	132·5
1000	,, ,,	Fed, 1 dose	18·6	112·1	245·7	212·1	137·5
1000	,, ,,	Fed, 5 doses	22·5	113·5	245·2	217·1	158·6
500	Tight fill capsule	Fasting	19·5	75·7	126·0	104·8	53·8*
500	,, ,,	Fed, 1 dose	15·6	84·4	148·7	117·3	41·8*
500	,, ,,	Fed, 5 doses	16·7	62·8	174·9	140·4	72·7
500	Loose fill capsule	Fasting	17·3	85·5	122·1	101·3	50·1*
500	Suspension	Fasting	15·7	53·8	131·7	108·2	48·2
300	Solution	Fasting	18·4	26·0	71·0	58·0	29·2*
250	Tight fill capsule	Fasting	9·9	84·6	55·1*	32·7*	3·0*

* not all the volunteers reached the nominated level.

Table 4. *Average time of first serum level, serum half life and area under the curve after oral doses of cephalexin*

Dose in mg	Type of preparation	Fed or fasting	Time of first serum level (min)	Time from peak to half peak (min)	Ultimate half life (min)	Area under curve
1000	Tight fill capsule	Fasting	23·5	72·9	50·1	74·0
1000	,, ,,	Fed, 1 dose	32·5	133·8	56·0	65·6
1000	,, ,,	Fed, 5 doses	41·6	120·7	50·3	72·7
500	Tight fill capsule	Fasting	38·4	73·1	42·7	34·2
500	,, ,,	Fed, 1 dose	30·2	97·6	49·2	38·0
500	,, ,,	Fed, 5 doses	27·8	112·1	82·6	47·9
500	Loose fill capsule	Fasting	46·8	72·8	39·2	31·8
500	Suspension	Fasting	9·1	81·3	40·8	32·7
300	Solution	Fasting	9·9	41·4	33·2	19·8
250	Tight fill capsule	Fasting	50·8	65·5	37·5	16·5

Statistical analysis of the computer fitted serum level curves

The parameters calculated were examined for each preparation and any significant differences were noted. The comparisons made and the results obtained were as follows.

The relation between the dose given, the peak serum concentrations, the area under the curve and the time of the peak. Comparisons were made between volunteers who received single doses of 1000, 500 and 250 mg, presented virtually without excipient in hard gelatin capsules. After a single dose of 1000 mg, the average peak titre in 10 volunteers, as calculated by the computer, was 40·8 (±4·6) μg/ml at 54·4 min after dosing. This was almost exactly double the value for the 20 volunteers who received 500 mg; they had a peak titre of 19·5 (±1·9) μg/ml, at 75·7 min after dosing. Seven volunteers who had 250 mg gave an average of 9·9 (±0·8) μg/ml at 84·6 min after the dose. Thus, there was an almost exact arithmetic relation between the magnitude of the peak titre and the dose given, the titre doubling as the dose was doubled. The area under the curve also doubled as the dose doubled, doses of 1000, 500 and 250 mg giving respective areas of 74·0 (±4·3), 34·2 (±1·4) and 16·5 (±1·1), and the time taken to achieve peak titre diminished as the dose increased. The average serum levels following these doses are given in Fig. 3.

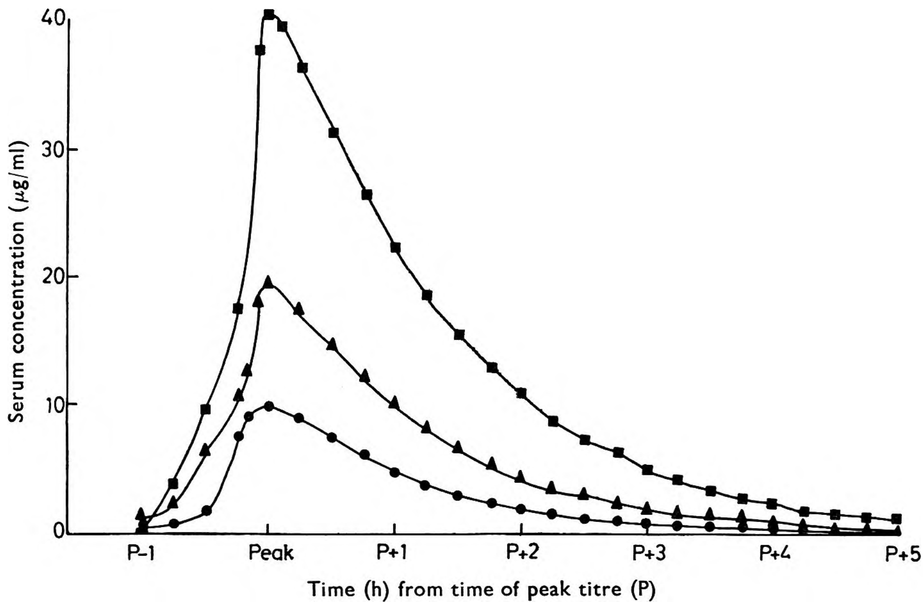

FIG. 3. Average serum levels of cephalexin as calculated by the computer after 1 oral dose of 250 mg (●—8 volunteers), 500 mg (▲—20 volunteers) and 1000 mg (■—10 volunteers). The origin of the time axis is peak time (P).

Comparison of serum concentrations following administration of cephalexin in capsules with and without excipient. The comparison was made between serum levels obtained after administration of one capsule containing 500 mg cephalexin with no excipient (20 volunteers) and those obtained after administration of two capsules, each containing 250 mg cephalexin loosely packed with approximately the same weight of corn starch (14 volunteers). The calculated average peak serum levels of 19·5

(±1·9) and 17·3 (±1·4) μg/ml respectively, occurring at 75·7 and 85·5 min after dosing, were not significantly different. The average serum level curves from the two preparations were virtually identical, none of the calculated parameters differing significantly from one preparation to the other.

The effect of giving the dose with a meal. Single doses of 1000 mg (2 × 500 mg capsules, no excipient) were given to volunteers immediately after they had eaten a large meal. This significantly reduced the peak titre and delayed the time at which it occurred. It also significantly increased the length of time that the titre exceeded 8 μg/ml and the length of time taken for the peak concentration to fall to half. When a dose of 500 mg was given after a large meal, the differences seen were less extreme than with the 1000 mg dose. The rate of fall from peak titre to half peak titre in the volunteers who had had the large meal was significantly slower but none of the other parameters was significantly affected.

Comparison of the serum levels obtained after one dose and after 5 doses. Doses of 1000 mg were given at 6-hourly intervals, the first and fifth being given immediately following a large meal. Between the 1st and the 5th doses the volunteers ate at normal meal times. The peak titre and the length of time the serum levels exceeded 12·5 μg/ml were significantly increased after the fifth dose, which suggested that at certain amount of accumulation was taking place, although not very rapidly.

When 5 doses of 500 mg were given in the same manner, there was no significant increase in any of the parameters after the fifth dose. This suggests that the initial dose of 500 mg was completely excreted in 6 h, whilst the initial dose of 1000 mg was not.

The effect on serum levels of giving the antibiotic in suspension or solution. After a dose of 500 mg given as a 5% suspension in a flavoured paediatric syrup, cephalexin appeared in the serum in 9 min, compared with the 38 min taken when the antibiotic was given as a capsule. The 29 min difference presumably represents the opening time of the capsule. A dose of 300 mg, given entirely in solution, gave cephalexin in the serum in 9·9 min and the peak time at 26 min after the dose was significantly earlier than the peak times after capsules had been taken.

CONCLUSIONS

Several conclusions can be drawn about factors which would have an influence on the size and duration of cephalexin serum concentrations. These are:

The average levels likely to be found after a given dose cannot be predicted with any accuracy from averages arrived at in the conventional way after trials in several persons. The variation in the time taken to absorb an oral dose is sufficiently large, from one person to another, both to make the conventional average misleading and to markedly reduce the apparent average peak level. More meaningful averages are found by calculating the serum level curves on a time scale with its origin at peak titre and then averaging the curve.

A heavy meal delays and also reduces the peak titre obtained after a single oral dose. It lengthens the time that an arbitrary level of 8 μg/ml is exceeded and also lengthens the period between peak and half peak levels. There could be an initial gradual increase in the peak titre after a series of doses of 1000 mg, but this was not found by us with doses of 500 mg.

The serum level curve can be affected by gross differences in the presentation. If,

for example, the cephalexin is partly or wholly in solution, it appears much more rapidly in the serum than when it is given as a solid in a capsule.

The presence of excipient makes no difference to the serum levels attained. After administration of cephalexin in capsules with no excipient or in capsules containing an equal amount of starch, the average serum level curves are indistinguishable.

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LETTERS TO THE EDITOR

Interaction and acute cross tolerance between ethanol and hexobarbitone in the rat

Sleeping times (the times without righting reflex) have been extensively used to evaluate the interaction between ethanol and barbiturates (see for instance Forney & Hughes, 1968; Gruber, 1955; and Wiberg, Coldwell & others, 1968). Some controversy exists about the mode of interaction (Forney & Hughes, 1968). From theoretical considerations Gruber (1955) concluded that the data so far suggested an additive effect.

After single administrations of ethanol (Mirsky, Piker & others, 1941) or barbiturate (Brodie, Mark & others, 1951) a phenomenon occurs which has been called acute tolerance. Acute tolerance may be measured as a difference between the concentrations of the drug at the appearance and subsequent disappearance of symptoms of CNS depression. It has also been measured as a positive correlation of the blood concentration of the drug at the disappearance of certain neurological signs of intoxication, and the magnitude of the administered dose (Brodie & others, 1951; Maynert & Klingman, 1960). The study of Maynert & Klingman (1960) included two barbiturates, ethanol, trichlorethanol and paraldehyde. Acute tolerance thus seems to be a common property of depressant drugs and it is possible that there is a cross-tolerance between different drugs. Such an acute cross-tolerance has been recorded in the present investigation between hexobarbitone and ethanol.

The threshold dose of hexobarbitone needed to obtain suppression for 1 s or more of the bursts in activity seen in the EEG after barbiturate was determined (Wahlström, 1966a). Briefly, hexobarbitone as the racemate was infused at a constant rate of 0·25 mg/kg s^{-1} into the tail vein of male rats (350 g). When the first EEG burst suppression with a duration of 1 s or more appeared in the record, the infusion was stopped and the ensuing sleeping times recorded (Wahlström, 1966b). Animals were kept in darkness between 8 a.m. and 8 p.m. and were well adapted to this rhythm before the experiments. All experiments were done between 9 a.m. and 3 p.m.

Ethanol was given intraperitoneally as a 20% (w/v) solution in isotonic saline. All blood samples were taken from a tail vein; in the threshold experiments the sample was obtained with a new needle and syringe after the infusion. Ethanol was measured by gas-chromatography (F and M model 402) (Curry, Walker & Simpson, 1966), with 1-pentanol as internal standard. Injection port and column temperature 85°; detector temperature, 105°; carrier gas, N_2, flow rate, 50 ml/min. The relation between peak heights were used to determine the ethanol concentration. Planimetry gave no higher precision.

The approximate times at which the threshold determinations needed to be made were estimated by measuring blood ethanol after intraperitoneal injection of 2 g/kg in a separate group of 5 animals (Fig. 1A).

In the rats in the main experiment (n = 18) four barbiturate threshold analyses were done before ethanol was given. The first one was discarded (Wahlström, 1966a) and a pre-ethanol mean of threshold dose and ensuing sleeping time was calculated on the remaining three. The mean and standard error for all participating rats were 59·7 ± 1·1 mg/kg and 18·9 ± 1·3 min respectively. The threshold measurements were then done for groups of 6 rats 1·25, 2·75 and 5·75 h after the injection of 2·0 g/kg ethanol. Each group was tested at one time only. Average blood concentrations of ethanol when the threshold measurements were made are in Fig. 1A.

The relation between ethanol concentration and hexobarbitone threshold dose is

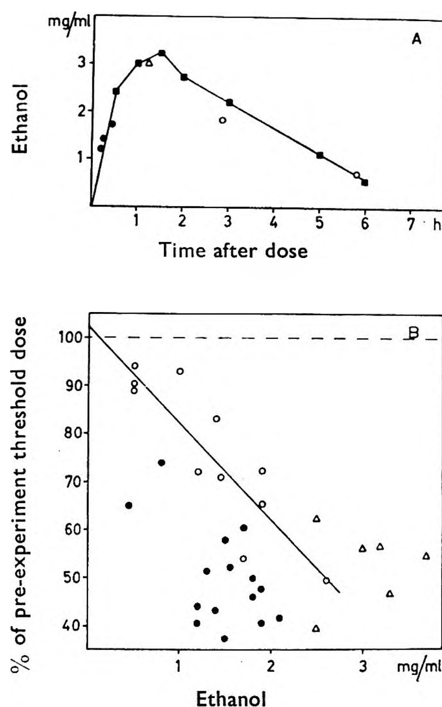

Fig. 1. A. Average ethanol concentration in blood after intraperitoneal injection of 2 g/kg. ■ Preliminary experiment in a separate group of rats (n = 5). The averages were obtained by graphical interpolation of the curves obtained from the individual rats. Samples were obtained approximately at the times used. △○● Average ethanol concentrations obtained after two threshold determinations in 3 groups of rats as described in text.
B. Relation between ethanol blood concentrations and per cent of pre-ethanol threshold dose of hexobarbitone needed to obtain a burst suppression of 1 s or more. The unbroken line indicates the regression line calculated on the data obtained on the falling ethanol concentration curve (○). The same animals were used on two occasions. A few threshold determinations could not be evaluated because of subcutaneous infusions. ● Ethanol increasing. ○ Ethanol decreasing. △ Ethanol around maximum.

given in Fig. 1B. It is evident that there was a linear relation (linear regression coefficient = $-20{\cdot}0 \pm 3{\cdot}5$ d.f. = 9) between blood ethanol concentration and decrease in barbiturate threshold dose when the threshold measurements were made on the decreasing part of the blood ethanol concentration curve (Fig. 1B). This line does not deviate significantly from the point 0 mg/ml; 100%. This is a strong indication that Gruber's (1955) conclusion of an additive effect is correct for hexobarbitone and ethanol on the decreasing blood ethanol curve, 1 mg/ml of ethanol corresponding roughly to a decrease of 20% (approx. 12 mg/kg) of hexobarbitone. The sleeping times before ethanol were not different from those after ethanol, the difference $\pm$s.e. being $-1{\cdot}3 \pm 3{\cdot}5$ min (n = 10). The decrease in threshold dose thus compensated for the additional effect of ethanol. A small decrease would be expected as the ethanol concentration decreased.

The threshold measurements made around the peak ethanol blood concentration (Fig. 1B) deviated slightly to the right compared with the regression line for the decreasing ethanol blood concentrations. More observations are needed to decide whether this deviation is significant. The sleeping time in this group of animals was $14{\cdot}5 \pm 2{\cdot}7$ min longer than the pre-ethanol time (n = 6) which might indicate that the threshold was measured before the peak ethanol concentration in the brain.

Before the subsequent experiments a new threshold determination was made without ethanol. 0·2–0·4 h before barbiturate infusion, saline was given intraperitoneally: barbiturate dose and the sleeping time were unchanged compared with the pre-experimental ones.

Threshold measurements on the rising part of the blood ethanol curve were then made on the same animals (3 groups of 6 rats in each) 0·20, 0·25 and 0·40 h after ethanol injection. The interval between the two ethanol experiments in the same animal was 2–3 weeks. The average ethanol concentrations are shown in Fig. 1A, and the threshold measurements in Fig. 1B. Larger decreases in the barbiturate threshold doses were encountered on this part of the ethanol curve than on the decreasing part. Because of the accumulation of data between 1 and 2 mg/ml of ethanol, no definite statement about linearity can be made. The sleeping times were increased by 18·0 ± 3·6 min (n = 13) over the pre-ethanol times. As the experiments were done on a rapidly rising part of the ethanol curve such an increase is to be expected.

Two kinds of systematic error will affect the information obtained from the ethanol determinations more markedly on the rapidly rising part of the blood ethanol curve than on the more slowly decreasing part. Since the ethanol concentration is rising, blood sample measurements taken after the threshold determinations (the time lag approximately 0·1 h) will give an over-estimate of the ethanol concentration in the blood at the time of the threshold determination. Also, blood concentration will be greater than CNS concentration.

Since both errors overestimate the critical concentration of ethanol on the increasing part of the ethanol concentration curve, and to a much smaller extent underestimate them on the decreasing part, the relevant values are probably even smaller than those recorded (Fig. 1B). The difference in hexobarbitone needed to obtain a suppression of EEG bursts of 1 s or more at similar ethanol concentrations on the increasing and decreasing part of the concentration curve is thus probably even larger than the one recorded (Fig. 1B). An acute cross tolerance thus seems to exist between hexobarbitone and ethanol.

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Regional variations in the distribution of noradrenaline along the rat vas deferens

The vas deferens is widely used as a model to study different aspects of adrenergic mechanisms due to the rich sympathetic innervation of its smooth muscle layers (Falck, 1962). The density of this adrenergic supply varies morphologically between the prostatic and the epididymal ends in several species. Sections of vas deferens processed histochemically for the demonstration of monoamines show the population of adrenergic nerves to be denser in the prostatic than in the epididymal end (Norberg, 1967; Owman & Sjöstrand, 1965; Sjöstrand, 1965; Norberg, Risley & Ungerstedt, 1967; Bell & McLean, 1970). These observations agree with ultrastructural studies which demonstrate variations in the amount of nerve fibres present in both ends of the vas deferens (Farrell, 1968). For example, in equivalent surfaces of thin sections through the muscle layers, 152 endings were identified in the prostatic end, 121 in the medial third and 52 in the epididymal end.

In the course of experiments in which slices of vas deferens of the rat were incubated *in vitro* (Zieher & Jaim-Etcheverry, unpublished), we noticed a great diversity in the noradrenaline content of slices in different incubation flasks. We now report the distribution of noradrenaline in thin transverse segments obtained from the entire length of the vas deferens.

Vasa deferentia were removed from Wistar rats of 250–300 g along with a portion of the prostate and the epididymis. For each experiment, groups of 4–5 vasa deferentia were pinned together at their *in situ* length (38–40 mm) on dental wax and sectioned with a special chopper consisting of 20 stainless steel razor blades whose cutting edges were maintained exactly 2 mm apart by interposing between them acrilic plates of the appropriate thickness. The entire vas deferens was sectioned in one operation so that 19–20 tissue segments of 2 mm thickness were obtained from the complete length of the organ. The segments corresponding to the various vasa deferentia cut by two

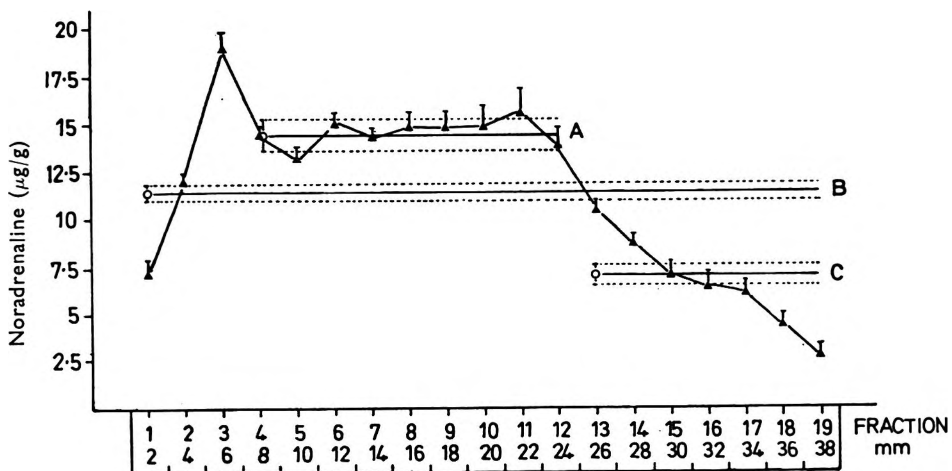

FIG. 1. Concentration of noradrenaline assayed in 2 mm segments obtained from the entire length of the vas deferens of the rat. Fractions are numbered starting from that corresponding to the prostatic end of the organ. Triangles represent mean values ±s.e. from results of 5 experiments. Mean values ±s.e. (dotted lines) corresponding to segments comprised between fractions 4–12 are represented in A, to segments 1–19 (entire length of the organ) in B and to segment between fractions 13–19 in C. Significance of differences between A and B, $P < 0.05$; between B–C and A–C, $P < 0.001$.

adjacent blades and found between them were pooled together, weighed and homogenized in 0·4 N perchloric acid for extraction of noradrenaline. Fractions were numbered 1 to 19 starting from that of the prostatic end. Tissue extracts were purified by column chromatography on Dowex 50W-X4 resin (column size 4·2 × 50 mm), eluted with HCl and measured fluorometrically (Häggendal, 1963). The fluorescence was read at 400–515 nm (excitation and emission wavelengths respectively) and the recovery of noradrenaline was 90%.

Three different zones may be identified along the vas deferens (Fig. 1). The prostatic end of the organ (except the 2 mm corresponding to its termination in the prostate) comprising the following 6–8 mm, has a high concentration of noradrenaline (18·9 μg/g in fraction 3). From there-on the concentration of noradrenaline remains stable at a constant level (between 13·2 and 15·7 μg/g) for the following 18 mm, and in fraction 12 initiates a progressive and sustained decline, reaching the lowest level at the epididymal end (6·5 μg/g in fraction 19). The mean value obtained for grouped data corresponding to all fractions is 11·5 ± 0·4 μg/g, while the mean value of the segment comprising fractions 4–12 is 14·5 ± 0·8 μg/g and that of fractions 13–19 is 7·0 ± 0·9 μg/g. The differences between the values of both segments are statistically significant and they also differ significantly from the mean value for the entire organ.

The marked differences observed in the regional distribution of noradrenaline in rat vas deferens confirm previous morphological experiments since the quantity of noradrenaline correlates with the density of adrenergic innervation in sympathetically innervated tissues. Moreover, they reflect the lack of homogeneity of the nervous supply within a given tissue. The preganglionic fibres of the hypogastric nerve penetrate the vas deferens for the initial 6–8 mm where postganglionic neurons lie in close vicinity to the muscle coat of the organ (Sjöstrand, 1962, 1965; Ohlin & Strömblad, 1963; Owman & Sjöstrand, 1965). The presence of this nervous plexus is probably responsible for the peak in noradrenaline concentration found at this level. It is in this zone where the adrenergic nerve fibres are first apparent during development of the innervation and from where they extend to the rest of the organ (Furness, McLean & Burnstock, 1970). This possibility of distinguishing biochemically between zones of the organ where the adrenergic cell bodies or their terminals are localized, might prove to be useful in the analysis of the effects of drugs which have differing effects on the concentration of noradrenaline in both portions of the adrenergic neuron.

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An interaction between hydrocortisone and hemicholiniums in mice

Toxicity of hemicholinium-3 (HC-3) has been attributed to failure of acetylcholine synthesis due to interference with the passage of choline to its intracellular sites of acetylation (MacIntosh, Birks & Sastry, 1958; Gardiner, 1961). The work of Schueler (1955) and Reitzel & Long (1959), who have reported that choline is the specific antagonist to HC-3 toxicity, supports this. Perfusion studies on the cat superior cervical ganglion have shown that the presence of choline in the perfusion fluid is essential for optimal synthesis and release of acetylcholine and that acetylcholine synthesis is inhibited by HC-3 (Birks & MacIntosh, 1961). Drugs which can influence the plasma levels of choline might therefore be expected to modify the toxicity of HC-3. Cortisone has been reported to lower the plasma choline of dogs by 60–80% within 30 min of injection (MacIntosh, 1963). We now report the effects of a water-soluble hydrocortisone derivative (hydrocortisone sodium succinate) on the toxicity in mice of HC-3 and its *p*-terphenyl analogue (TPHC-3) (Gardiner & Lee, 1969).

Albino mice of either sex weighing 16–24 g were used. The mice were pretreated with hydrocortisone (10 mg/kg) or 0·9% sodium chloride (saline). One h later the mice were injected with different doses of HC-3 or TPHC-3. 20 mice were used for each dose.

All drugs were made up in saline and administered by intraperitoneal injection. The volume of drug solutions injected was 0·1 ml per 10 g mouse. The number of mice that died at the end of 2 h were noted.

The mortality in mice increased with increasing doses of HC-3 and TPHC-3 (Table 1). TPHC-3 was the more toxic (Gardiner & Lee, 1969). Pretreatment with hydrocortisone (10 mg/kg) for 1 h reduced the mortality produced by all doses of HC-3 and TPHC-3.

It was anticipated that, if hydrocortisone produced a fall in plasma choline, as

Table 1. *Partial protection of mice against HC-3 and TPHC-3 by pretreatment with hydrocortisone* (10 mg/kg). Hydrocortisone or saline was administered 1 h before HC-3 or TPHC-3. The values given are % mice dead 2 h after injecting HC-3 or TPHC-3 (20 mice/group) and each value represents the mean ± s.e. of four experiments.

	HC-3			TPHC-3	
	Animals receiving:			Animals receiving:	
Dose (μg/kg)	hydrocortisone	saline	Dose (μg/kg)	hydrocortisone	saline
120	16 ± 2	29 ± 4	80	10 ± 5	19 ± 6
150	33 ± 3	53 ± 9	100	16 ± 5	45 ± 8
180	55 ± 3	70 ± 5	120	63 ± 3	76 ± 4
220	81 ± 3	90 ± 0			

it was reported to do in dogs (MacIntosh, 1963), it might enhance the toxicity of the hemicholiniums. However, it was found that hydrocortisone protected the mice to a small extent from the toxicity of the hemicholiniums.

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[³H]Dopa in [³H]tyrosine with high specific activity: a serious complication in the study of catecholamine metabolism

Decomposition by self-irradiation is a great problem for users of tritium-labelled compounds. Although some factors controlling the stability of labelled compounds have been elucidated, much is unknown and stored compounds may suddenly show a markedly accelerated rate of decomposition (cf. Bayly & Evans 1966, 1967).

In many cases traces of an impurity may be relatively harmless. If, however, it interacts with chemical or biochemical processes to be studied, the result may lead to serious misinterpretations It has been shown that one of the radiolysis products of tyrosine is dopa (Rowbottom 1955). The present report will demonstrate the effect of traces of [^{3}H]dopa in [^{3}H]tyrosine when studying the catecholamine metabolism using [^{3}H]tyrosine.

In the following experiment the [^{3}H]tyrosine* used was found by radiopaper chromatography (isopropanol-2N HCl, 65:35, v/v) to contain about 12% impurities, more than half of which could be identified with dopa.

^{3}[H]Tyrosine, 5 μg/kg weight, was given intravenously to male rats grouped in pairs. Fifteen min later the animals were killed. [^{3}H]Noradrenaline (^{3}H-NA) and [^{3}H]dopamine (^{3}H-DA) in the caudate nucleus, the spinal cord and in the heart were determined, after separation on alumina and Dowex 50 columns (for details see Persson & Waldeck 1968, Persson 1969). The results are presented in Table 1. This Table also shows results obtained from pure [^{3}H]tyrosine and [^{3}H]dopa in a previous investigation (Persson 1969) under experimental conditions similar to those described above. From these values the expected yield of ^{3}H-NA and ^{3}H-DA from [^{3}H]tyrosine containing 10%[^{3}H]dopa has been calculated. It appears that the values obtained in the present experiment in four out of six cases are rather close to these calculated values. ^{3}H-NA in the caudate nucleus and ^{3}H-DA in the spinal cord were considerably higher than would be expected. These irregularities may be caused by decomposition products other than [^{3}H]dopa.

* L-Tyrosine, ring-3,5-^{3}H in an aqueous solution containing 2% ethanol, specific activity 47 Ci/mmol, was obtained from The Radiochemical Centre, Amersham.

Table 1. *Effect of* [^{3}H]*dopa as a contaminant on the yield of* [^{3}H]*noradrenaline* (^{3}H-NA) *and* [^{3}H]*dopamine* (^{3}H-DA) *formed from* [^{3}H]*tyrosine* in vivo. Male rats, grouped in pairs, recieved an intravenous injection of 5μg/kg [^{3}H]tyrosine (containing about 7% of a decomposition product identified with [^{3}H]dopa). Fifteen min later the animals were killed. ^{3}H-NA and ^{3}H-DA in various tissues were determined. Shown are the means in pmol/g tissue ± s.e. of three groups. Also shown are data from a previous investigation (Persson 1969) using pure [^{3}H]dopa and [^{3}H]tyrosine respectively. From these data the expected result of a 10% contamintaion of [^{3}H]tyrosine with [^{3}H]dopa has been calculated.

	caudate nucleus		spinal cord		heart	
Treatment	^{3}H-NA	^{3}H-DA	^{3}H-NA	^{3}H-DA	^{3}H-NA	^{3}H-DA
[^{3}H]Tyrosine, 5 μg/kg, i.v. contaminated with [^{3}H]dopa	0·19 ±0·05	0·79 ±0·06	0·06 ±0·00	0·20 ±0·01	0·18 ±0·02	0·24 ±0·01
[^{3}H]Tyrosine, 4, 5 μg/kg, i.v. +[^{3}H]dopa 0·5 μg/kg, i.v. calculated from the data below	0·01	0·68	0·03	0·03	0·21	0·19
[^{3}H]Dopa 2·5 μg/kg, i.v.	0·04	0·80	0·11	0·12	0·98	0·92
[^{3}H]Tyrosine 5 μg/kg, i.v.	0·002	0·55	0·01	0·01	0·01	0·01

The heart appeared to be the organ most sensitive to the [^{3}H]dopa contamination of [^{3}H]tyrosine. From the figures shown in Table 1, even 1% of [^{3}H]dopa in the [^{3}H]tyrosine would give a yield of ^{3}H-NA three times the normal. This should be considered when using [^{3}H]tyrosine in the study of catecholamine metabolism.

Our experience shows that the radiochemical purity of labelled compounds has to be checked carefully. As mentioned above, decomposition may occur suddenly. Therefore the user should not rely on the analysis certificate of the manufacturers alone, but should also obtain his own data concerning the purity of the material, both on arrival and immediately before use (c.f. Hempel & Männl 1967). This is of particular importance when using [^{3}H]labelled compounds with high specific activity

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Uptake of [^{14}C]histamine by tissues of the guinea pig

Schayer (1952) reported that guinea-pigs, unlike cats and rabbits, were unable to take up and retain systemically administered histamine. Duncan & Waton (1970) queried the use by Schayer of only a single animal at each of two time intervals and speculated whether a different route of administration and a larger dose of histamine might have yielded results consistent with those for the cat and the rabbit.

We now report the uptake of histamine by guinea-pig tissue using larger numbers of animals and a higher dose of histamine than employed by Schayer (1952) and over a more extended period. Male guinea-pigs (250 g; Hartley strain) were injected with ring-[2-^{14}C]histamine (80 mg/kg; 2·5 μCi/kg) intravenously. At various time intervals the levels of the [^{14}C]histamine, [^{14}C]-1-methyl-4-(aminoethyl)imidazole(1,4-methyl histamine) and [^{14}C]imidazole acid metabolites in several tissues were measured (Snyder, Axelrod & Bauer, 1964).

Table 1. [^{14}C]*content of guinea-pig tissues* (nCi/g *of tissue*) *after intravenous injection of ring*-[2-^{14}C]*histamine* (2·5 μCi/kg)

	Time (h) after injection of [^{14}C]histamine									
	2 (n = 3)			4 (n = 3)			8 (n = 2)*		24 (n = 2)*	48 (n = 1)*
	Hi	1,4 Me Hi	Acids	Hi	1,4 Me Hi	Acids	1,4 Me Hi	Acids	Total ^{14}C	Total ^{14}C
Lung	0·01	0·05	0·41	0·02	0·03	0·36	0·02	0·23	0·13	0·11
Ileum	0	0·07	0·53	0	0·05	0·49	0·02	0·33	0·29	0·19
Kidney	0·08	0·07	1·41	0·09	0·06	4·47	0·05	3·94	0·47	0·25
Liver	0·02	0·06	2·48	0·01	0·06	2·39	0·04	1·48	0·32	0·16
RBC	0·15	—	—	0·06	—	—	—	—	0·03	0·01
Plasma	0·06	—	—	0·06	—	—	—	—	0	0

Hi, [^{14}C]histamine; 1,4 Me Hi, [^{14}C]-1,4 methyl histamine.
Acids, [^{14}C]acid metabolites of Hi.
Levels of ^{14}C in tissues, nCi/g; ^{14}C in plasma, nCi/ml; values represent the means.
* No [^{14}C]histamine detectable in the tissues at these times.

Initially (Table 1), there was an uptake of radioactivity in the tissues examined but very little of this was found to be histamine. In fact from 8 to 48 h after administration of histamine none of this labelled amine was detectable in the tissues. The [^{14}C]acid fraction comprised of metabolites like imidazole-4-acetic acid, 1-ribosyl-imidazole-4-acetic acid and 1-methyl-imidazole-4-acetic acid, was measured at intervals up to 8 h after injection of histamine and was found to represent most of the radioactivity in the tissues. The results are in close agreement with those of Schayer (1952) although he measured only [^{14}C]histamine and total radioactivity. Thus it is confirmed that the guinea-pig does not possess a significantly effective mechanism for the uptake of histamine by the tissues.

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A note on the chloroform-soluble alkaloids of *Fagara macrophylla*

A preliminary chromatographic screening of the stem and root barks of *Fagara macrophylla* (Oliv.) Engl. indicated the presence of at least six chloroform-soluble bases of which skimmianine, angoline, and angolinine were named and fagaramide was tentatively proposed (Calderwood & Fish, 1966). Further work has resulted in the isolation and characterization of fagaramide and skimmianine together with chelerythrine, nitidine, and trace amounts of a weakly basic acridone alkaloid which on silica gel (tlc, 3 solvent systems) corresponds to 1-hydroxy-2,3-dimethoxy-10-methyl-acridan-9-one. The latter compound has been found in *Fagara leprieurii* Engl. (Fonzes & Winternitz, 1968a).

The previously reported angoline is now known (Fonzes & Winternitz, 1968b) to be 9-methoxychelerythrine and occurs as an artifact following extraction. Our isolation of chelerythrine from *F. macrophylla* confirms the report of this alkaloid by Torto Sefcovic & others (1969). Likewise our report of nitidine confirms that of Torto & Mensah (1970) from the root bark but we have also proved the presence, in smaller concentrations, of the above-named bases (except the acridone alkaloid) in the stem bark. From tlc characteristics and ultraviolet spectrum, nitidine appears to be identical with the previously reported angolinine and from our present work it seems that it is the predominant alkaloid in this species giving yields of about 0·025 % from root bark and only 0·002 % from stem bark.

Fagaramide ($C_{14}H_{17}O_3N$), white crystals m.p. 119–120° (softening at 104°) (EtOH) (m.p., tlc, i.r.), u.v. $\lambda_{max.}^{EtOH}$ 219, 236, 282, 290, 323 nm (log ϵ 3·23, 3·18, 3·14, 3·15, 3·19): fagaramide hydrochloride m.p. 136° (EtOH).

Skimmianine ($C_{14}H_{13}O_4N$), white crystals m.p. 175–6° ($CHCl_3$–light petroleum 40–60°) (m.p., tlc, i.r.), u.v. $\lambda_{max.}^{EtOH}$ 249, 320, 332 nm (log ϵ 4·90, 3·90, 3·90).

Chelerythrine isolated as the chloride ($C_{21}H_{18}O_4N^+Cl^-$), yellow needles m.p. 198–9° (EtOH/Et_2O) (m.p., tlc, i.r.), u.v. $\lambda_{max.}^{EtOH}$ 227, 273, 281(sh), 320, 343 nm (log ϵ 4·26, 4·53, 4·46, 4·34, 4·16): Chelerythrine nitrate m.p. 235–7° (EtOH/2N HNO_3).

Nitidine isolated as the chloride and purified as the nitrate ($C_{21}H_{18}O_4N^+NO_3^-$) green prisms m.p. 275–7° (EtOH–2N HNO_3) (m.p., tlc, i.r.), u.v. $\lambda_{max.}^{EtOH}$ 231, 273, 281, 304(sh), 329 nm (log ϵ 4·37, 4·50, 4·49, 4·39, 4·36).

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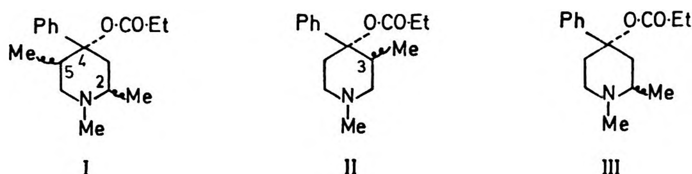
Analgesic potency and stereochemistry of trimeperidine and its isomers and analogues

Reports on the analgesic trimeperidine (γ-promedol) and its isomers that have appeared since 1956 (Prostakov & Mikheeva, 1962) have failed to provide either pharmacological detail or firm evidence of stereochemistry. A generous gift of the precursor 4-piperidone by Dr. N. S. Prostakov has enabled us to investigate these compounds and to apply modern physical techniques to solving their stereochemistry that were not available when the work was originally done. We isolated three 1,2,5-trimethyl-4-propionyloxy-4-phenylpiperidines (I) which corresponded



with the isomeric γ-, β- and α-forms of the Russian workers. The compounds were assayed for their analgesic properties in mice by the hot-plate test along with analogues lacking 2-methyl (α- and β-prodine, II) or 5-methyl substituents (III). We thank Dr. E. L. May of the National Institutes of Health for these data. Hot-plate ED50 values and stereochemical findings, given in Table 1, enable the following points to be made:

(1) The high activities of the promedol isomers and the fact that replacement of *N*-methyl by *N*-phenethyl in γ-promedol has a potency enhancing effect (Portoghese, 1965), provide good evidence of these esters having a morphine-like action at the analgesic receptor.

(2) The fact that the most active promedol isomer (β-) is equipotent with β-prodine (β-II) further demonstrates the superiority of *cis* 3-Me/4-Ph geometry over the *trans* arrangement in 4-phenylpiperidine analgesics (Casy, 1968; Casy, Chatten & Khullar, 1969).

Table 1. *Stereochemical findings and hot-plate ED50 values*

Compound	Configuration*	Hot-plate ED50 in mice mg/kg, subcutaneous inj.
γ-I	*cis* 2Me/4Ph, *trans* 5Me/4Ph	1·6
γ-I†		0·91
β-I	*cis* 2Me/4Ph/5Me	0·18
α-I	*trans* 2Me/4Ph, *cis* 5Me/4Ph	0·58
α-II	*trans* 3Me/4Ph	0·92
β-II	*cis* 3Me/4Ph	0·18
α-III	*trans* 2Me/4Ph	1·32
β-III	*cis* 2Me/4Ph	1·37
Pethidine	—	4·7

* The preferred conformation of γ-I is a chair with 4-phenyl equatorial; there is evidence that significant skew-boat populations (with 4-phenyl pseudo-equatorial) arise in the case of β- and α-I.
† *N*-Methyl replaced by *N*-phenethyl.

(3) The similar orders of activity of the isomeric analogues of the promedols that lack a 5-methyl substituent (α- and β-III) shows that the orientation of the 2-methyl group has little influence upon the activity of 4-phenylpiperidine analgesics.

In view of recent studies of brain levels of α- and β-II in mice (Abdel-Monem, Larson & others, 1970), it is probable that potency differences between isomeric promedols are due to differences in their affinities for the receptors rather than distribution and metabolism.

Details of evidence establishing the stereochemistry of the promedol isomers and their 2-methyl analogues will be given elsewhere.

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Lipid depletion of bacteria induced by biotin deficiency and its relation to resistance to antibacterial agents

Previous communications from this laboratory have shown that Gram-positive bacteria grown in the presence of glycerol increase in lipid content and that this is accompanied by an increase in resistance to penicillins (Hugo & Stretton, 1966a, b), and phenols (Hugo & Franklin, 1968). We now report that bacteria grown under conditions of biotin deficiency are depleted in their lipid content and this in turn is accompanied by a decrease in resistance to a variety of antibacterial agents.

The organisms used were *Staphylococcus aureus* (Oxford) NCTC 6571 which includes biotin amongst its growth requirements, and *Escherichia coli* T94A (strain 58–278 M), obtained from Professor W. W. Umbreit, which requires biotin and phenylalanine for growth. The biotin requirement of the *E. coli* can be alleviated by the presence of aspartate in the medium. The Oxford staphylococcus was grown in nutrient broth and the cells contained 68·4 μg/mg dry weight lipid in agreement with previous findings (Hugo & Stretton, 1966a, b; Hugo & Franklin, 1968). Growth in Difco biotin assay medium at half strength and supplemented with 2 μg/litre of biotin (optimum 10 μg/litre) produced cells in which the lipid content had fallen to 55·4 μg/mg dry weight, significantly less (19%) than the nutrient broth grown cells.

E. coli was grown in the synthetic medium of Gavin & Umbreit (1965), biotin deficient cells were obtained by growth in this medium from which biotin had been omitted and biotin adequate cells in the same medium containing 10 μg/ml of biotin. Biotin-adequate cells had a lipid content of 179 μg/mg dry weight and biotin deficient cells, 109 μg/mg dry weight, a decrease of 39%.

The biotin adequate and biotin deficient cells of both species were challenged with a series of antibacterial agents. Table 1 gives the minimum inhibitory concentrations.

Table 1. *Minimum inhibitory concentrations, μg/ml, for organisms grown in (a) a nutrient broth, (b) Difco biotin assay medium + 2 μg/litre biotin, (c) a synthetic medium, biotin adequate, (d) a synthetic medium, biotin-deficient*

Agent	*S. aureus* a	b	$\frac{b}{a}$	*E. coli* c	d	$\frac{d}{c}$
Phenol	2200	1550	0·70	1060	1000	0·95
4-Methylphenol	1390	1160	0·84	540	510	0·95
4-Ethylphenol	640	520	0·82	260	240	0·89
4-n-Propylphenol	390	220	0·79	155	145	0·94
$C_{12}H_{25}-N^{+}(Me)_3Br^{-}$	13·6	1·5	0·11	26·5	25·0	0·95
$C_{14}H_{29}-N^{+}(Me)_3Br^{-}$	7·5	1·2	0·16	14·5	12·0	0·83
$C_{16}H_{33}-N^{+}(Me)_3Br^{-}$	5·7	0·6	0·11	4·4	4·2	0·96
$C_{18}H_{37}-N^{+}(Me)_3Br^{-}$	10·5	1·2	0·11	4·0	3·6	0·90
Tetracycline	0·12	0·012	0·10	—	—	—
Oxytetracycline	0·12	0·016	0·13	—	—	—
Chlortetracycline	0·12	0·012	0·100	—	—	—
Benzylpenicillin	0·05	0·012	0·24	27·0	13·0	0·48
Chloramphenicol	—	—	—	10·0	9·0	0·90
Polymyxin B	—	—	—	0·53	0·50	0·95
Proflavine	—	—	—	31·0	27·0	0·88
Actinomycin D	25	1	0·04	>200	>200	—

A statistical analysis of the results with cetrimide (C_{18}) and phenol against the *E. coli* strains, using the method of paired comparisons, showed a significant difference, the former at the 5% level, the latter at the 2·5% level.

With *E. coli* the minimum inhibitory concentration ratio is almost a constant irrespective of the drug used, although benzylpenicillin is an exception. We also note that despite the change in lipid content induced in *E. coli*, the increased susceptibility is generally less than that induced by EDTA treatment (Leive, 1965, 1968; Hamilton-Miller, 1966) which removes lipopolysaccharide and we were unable to induce susceptibility to actinomycin D (Leive, 1968).

With *S. aureus* an increase in sensitivity was found with all the antibacterial agents used, the largest occurring with actinomycin D. The ratios of the other antibacterial agents tested fell into two distinct groups, the phenols showing a smaller increase than the rest (Table 1).

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An improved field test for hallucinogens

Although the conditions of a forensic field test may be adjusted for optimum specificity (e.g. cannabis: de Faubert Maunder, 1969), the test is frequently a sorting procedure demonstrating the need for professional analysis or alternatively for avoiding the unnecessary detention of person or goods. A sorting test widely used by the police and drugs control officers relies on the blue fluorescence of substances such as lysergide (LSD) when illuminated with screened ultraviolet (365 nm) radiation. The test may be rendered more sensitive for psychotomimetic ingredients which have been dissolved – for instance – on a sugar cube, by leaching the object with water onto a filter paper and examining that in ultraviolet light (Government Chemist, 1969). However, many innocent substances will also give a blue fluorescence (e.g. caffeine, quinine and certain detergents) whereas a number of hallucinogens do not.

The van Urk (or Ehrlich) reagent 4-dimethylaminobenzaldehyde in strongly acid solution gives a suitable colour test with lysergic acid derivatives; it is used for erganes as a chromatographic spray (Clarke, 1967) and for photometric assay (B.P., 1968). Dechert (1968) described a field test based on this principle. Filter paper sheets are soaked in a solution of 4-dimethylaminobenzaldehyde (1g) in ethanol (B.P.) (10 ml) with 1 drop of acid-ferric chloride TS (U.S.P. XVII), dried, cut into 5 mm squares and stored in a dark place. A fragment of the suspect material is placed on one of these squares, the paper wetted with anhydrous methanol followed by a drop of sulphuric acid (1:1). A violet colour slowly develops with erganes. Alternative procedures employed in two commercial test kits require mixing two solutions, reagent and acid, with the suspect material on a porcelain tile (Narcodal Kit) or in a plastics tube (Narcotest).

All three tests lack sensitivity and may risk consuming the total sample. Admixed substances such as colouring matter often interfere. Reagents must be mixed at the time of the test and the extra apparatus must be washed or, with Narcotest, placed in a polythene bag and destroyed before the tube disintegrates within an hour.

Table 1. *Test response of various hallucinogens and related substances*

Immediate violet colour		*Lysergamide(§)
Tryptamine	†Dimethyltryptamine (DMT)	8β-Ergotamine
5-Methyltryptamine	†Diethyltryptamine (DET)	8α-Ergotamine
7-Methyltryptamine	*Psilocybin	Dihydroergotamine
‡α-Methyltryptamine	*Psilocin	8β-Ergocristine
5-Hydroxytryptamine	*Bufotenine	8α-Ergocristine
5-Methoxytryptamine	*5-Methoxydimethyltryptamine	Dihydroergocristine
‡*N*-Ethyltryptamine	*5-Benzyloxydimethyltryptamine	8β-Ergometrine
5-Hydroxy-*N*-methyltryptamine		8α-Ergometrine
		Methylergometrine
Slow violet colour	*No response*	
*8β-Lysergide (§)	*Mescaline	
‡1-Acetyl-lysergide	‡3,4-Methylenedioxyamphetamine (MDA)	
Methysergide	‡3-Methoxy-4,5-methylenedioxyamphetamine (MMDA)	
	†2,5-Dimethoxy-4-methylamphetamine (STP, DOM)	
5-Benzyloxygramine	‡Harmine	
5-Benzyloxytryptamine	‡Harmaline	
	‡Ibogaine	

Notes

*Indicates control under the Drugs (Prevention of Misuse) Act, 1964.

†Features in WHO proposals and specified in the 1970 Modification Order to the above act.

‡Other drugs reputedly psychotomimetic.

§When visualized on a TL plate, the response of the 8α-epimer is slightly faster than that of the 8β-epimer.

These disadvantages have been remedied. Although a solution of 5% 4-dimethyl-aminobenzaldehyde in hydrochloric acid: ethanol (1:1) turns from yellow to brown within a week, replacement of ethanol by methanol provides a solution which is stable for several months. The use of a porcelain tile or tube is avoided by placing a small amount of the suspect material on a filter paper and adding a drop of the reagent. Radial striations of colour develop from the centre of the spot. By chromatographic action the material responding to the reagent is carried away from the bulk of the sample, where dyestuffs and other materials interfere, and is concentrated into striations. It is possible to obtain a response with weak samples of lysergide which have failed to produce a fluorescence with an ultraviolet lamp. The Table summarizes the responses obtained using this technique for some known hallucinogens and structurally related substances.

Although similar colour reactions are observed with hallucinogens derived from lysergic acid and the tryptamines, as well as the natural ergot bases and dihydro-ergotamine, the test is convenient for non-scientific personnel and is much more restrictive than the observation of ultraviolet-induced fluorescence. When taken with adequate circumstantial evidence there is less likelihood of mistakingly seeking professional confirmation. The filter paper may be retained as a record of the test and can be signed and witnessed. A combination of the two techniques assists the examination of heterogeneous specimens.

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In vitro release of aspirin from various wax-coated formulations

Wax-coating of pharmaceuticals has been reported amongst techniques used for controlling drug releases. We have studied the *in vitro* release of aspirin from various wax-coated formulations in an attempt to explain the differences in release profiles. The waxy materials used were spermaceti, stearic acid, a hydrogenated grade of cottonseed oil and a blend of equal parts of these waxes. Two methods were adopted in the preparation of the formulations; the congealing method (Robinson, Moorestown & Svedres, 1957) and the aqueous dispersion method (Robinson & Becker, 1968). All formulations were prepared to contain 1 part of aspirin and 5 parts of the wax. The release experiments were made using the 23/30 mesh fraction.

The release rates were determined at 37° ± 0·1° in a rotating bottle apparatus similar to that of Souder & Ellenbogen (1958). Acid pepsin and alkaline pancreatin solutions of the B.P. 1963 were used as the dissolution media. After specified time intervals the contents were filtered and an aliquot was assayed for aspirin by spectrophotometric measurement of the salicylic acid (in 0·1N HCl at 305 nm) produced after preliminary hydrolysis with 0·1N NaOH. Blank experiments were made using equivalent amounts of the waxes. Fig. 1 shows the release patterns in both dissolution media. In acid pepsin the release rate followed the sequence: spermaceti > stearic

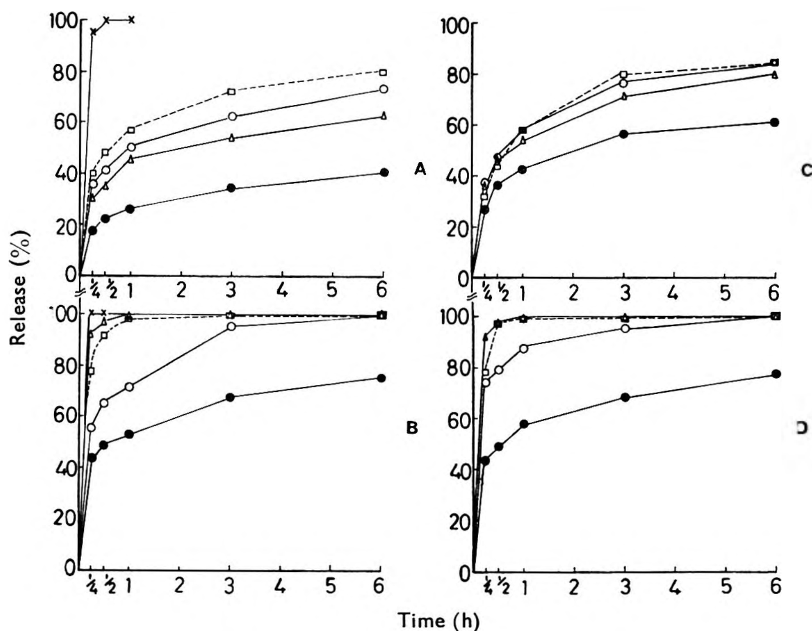

FIG. 1. *In vitro* release of aspirin from formulations prepared by the congealing method (A,B) and the dispersion method (C,D). A,C in acid pepsin solution (pH 1·2). B,D in alkaline pancreatin solution pH 8·3. ●—● hydrogenated cottonseed oil; △—△ stearic acid; ○—○ spermaceti; □—□ mixed waxes (equal parts). Ratio of aspirin to wax 1:5; ×—× plain aspirin crystals.

acid > hydrogenated cottonseed oil. In alkaline pancreatin solution the sequence was: stearic acid > spermaceti > hydrogenated cottonseed oil. In a given time the release in this medium was faster than in acid pepsin solution. The method of preparing the formulations affected the release in a particular medium. The dispersion method gave higher release rates for all the waxes particularly in acid pepsin solution (Fig. 1C). Products prepared using a blend of equal parts of the three waxes gave unexpectedly higher release rates in both dissolution media (Fig. 1). In this respect our results do not agree with those of John & Becker (1968). They found, for a blend of 1 : 1 combination of two waxes, a release profile intermediate between the two waxes.

It is difficult to attribute the difference in release through waxy matrices as solely due to the chemical composition of the waxes since other factors may also contribute. The relative hydrophilic nature of the waxes has been claimed to affect the release rate (Cusimano & Becker, 1968). We found a correlation between the release rate through a particular wax-aspirin mixture and the melting point of wax. In acid pepsin the release at 37° followed the sequence: spermaceti (m.p. 45–46°) > stearic acid (m.p. 58–60°) > hydrogenated cottonseed oil (m.p. 65–66°). Measurements of the relative softening of the congealed waxes were made at 37° using the penetrometer technique (Martin, 1962). The results (Table 1) showed that the relative softening followed the above sequence. In alkaline pancreatin solution it is probable that the dissolution rate of the waxes has a decisive effect. Experiments were made to compare the dissolution rate of the waxes (using the 25/30 mesh) at 37° in the alkaline pancreatin solution (pH 8·3). The results (Table 2) showed that the dissolution rate followed the sequence: stearic acid > spermaceti > hydrogenated cottonseed oil. This is similar to the sequence found in the release study in alkaline pancreatin solution. The release through the mixed waxes gave unexpectedly high release

Table 1. *Relative softening of the congealed waxes measured by the penetrometer at 37°.* (Penetrometer readings in 0·1 mm. after 5 s)

Wax	m.p. °C	Penetrometer reading*
Spermaceti	45–46	5·3
Stearic acid	58–60	2·4
Hydrogenated cottonseed oil ..	65–66	1·1
Mixed waxes†	48–50	12·7

* Average of 12 runs (±3%).
† A blend of equal parts of the three waxes.

Table 2. *Dissolution rates of the wax particles* (25/30 *mesh*) *in alkaline pancreatin solution* (pH 8·3) *at* 37° ± 0·1

Wax	% Dissolution* 1 h	3 h	6 h
Spermaceti	7·1	12·6	27·3
Stearic acid	48·3	57·6	62·6
Hydrogenated cottonseed oil ..	3·9	5·1	8·8

* Average of three experiments (±4%).

probably due to the appreciable softening, which occurred at 37° (Table 1) and the 'distortion' which may occur in the waxy matrix as a result of the dissolution of component(s) of the blend during the release experiment. This would be the case in alkaline pancreatin solution where a relatively high percentage of stearic acid dissolves (Table 2).

Determination of the salicylic acid content (using a modified B.P. method) in the plain aspirin powder used and in the freshly prepared formulations revealed an insignificant increase (within the B.P. limit for salicylic acid in aspirin powder).

We thank Prof. M. M. Abdel Khalek for his valuable suggestions.

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Aspirin polymorphism questioned

Aspirin, crystallized under various conditions, has recently been reported to display different melting points (Tawashi, 1968; Summers, Carless & Enever, 1970), heats of fusion (Tawashi, 1969), densities (Summers & others, 1970), and rates of dissolution (Mitchell & Saville, 1967, 1969; Tawashi, 1968). These differences were ascribed in every case to polymorphic behaviour. It seems, however, that this conclusion is not warranted by the submitted evidence, and that other explanations for the observed differences should be considered.

Particularly disturbing is the failure of the supposedly discrete structures, i.e., "polymorphs," to exhibit different x-ray powder diffraction properties. Even the one possible exception—described only as diffraction "differences" by Tawashi (1968)—may well have been due to preferred crystal orientation, inasmuch as two forms of extremely different shape, i.e., prisms and needles, were compared.

Mitchell & Saville (1968) proposed that x-ray diffraction methods failed to distinguish between two forms of aspirin because the forms were mixtures of polymorphs. In view of the fact that the powder diffraction data from commercial aspirin (presumably Form B) is reconcilable with the single crystal data (Smith, 1962), however, their proposal is questionable.

The findings of Summers & others (1970) that there are six polymorphs of aspirin and that these range in density from 1·29 to 1·50 g/ml *but have similar diffraction properties* are not consistent with the usual concepts of crystal structure and polymorphism.

It is unfortunate that the cited authors did not describe their crystals with regard to size and habit; variables that may conceivably have affected the various measurements. For example, differences in size and habit might:

(*a*) Affect the dissolution rate from a pressed disc through differences in capillarity or wetting;

(*b*) affect the determination of melting point and heat of fusion through differences in rates of sublimation and decomposition. Aspirin has a long history of giving trouble in melting point determinations (Hayman, Wagener & Holden, 1925);

(*c*) affect density measurements by variously interfering with the complete filling of cavities by the displacement fluid.

Another proposal consistent with the published observations would be that the crystals somehow differ with regard to imperfections, stresses or finer structural details, but these differences also would not justify the use of polymorphic designations.

Whatever the source of the apparent extra thermodynamic activity exhibited by some of the aspirin crystals, exposure to heat, ultrasound, solvent, etc. could cause them to anneal, grow or ripen; they would thus mimic polymorphic behaviour by reverting to a "more stable" form but would not undergo changes in their routinely determined x-ray diffraction properties.

In the interest of preserving the meaning of the term "polymorphism," it would be helpful if future publications on the subject of aspirin polymorphism were to clarify the questions raised in this letter. Furthermore, all articles claiming the existence of polymorphs should include explicit directions for their preparation and identification.

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The aggregation of chlorhexidine digluconate in aqueous solution from optical rotatory dispersion measurements

In our continuing investigations of the optical rotatory dispersion (ORD) and circular dichroism (CD) of optically active surfactants and the possible detection of micelle or aggregate formation by this technique (Bonkoski & Perrin, 1968, 1969; Mukerjee, Perrin & Witzke, 1970), we have for the first time investigated a system in which the optical activity is centred in the counterion rather than the core of the aggregate. The chlorhexidine digluconate solutions chosen for these studies were prepared from the recrystallized base (Ayerst Labs., Inc., Rouses Point, N.Y.) and the theoretical amount of 1,5-gluconolactone in de-ionized water. Heard & Ashworth (1968) have reported a CMC of $6{\cdot}6 \times 10^{-3}$M for chlorhexidine digluconate, but lower values could be extrapolated from their surface tension and conductance data. We have found, using a Beckman Model RC 16B2 conductivity bridge (Beckman Instruments, Cedar Grove, New Jersey), a CMC of approximately $4{\cdot}4 \times 10^{-3}$M at $25{\cdot}0 \pm 0{\cdot}01°$ (Fig. 1A). This value is in good agreement with the value obtained from optical rotatory dispersion measurements (Fig. 1B and 2). In the ORD investigations

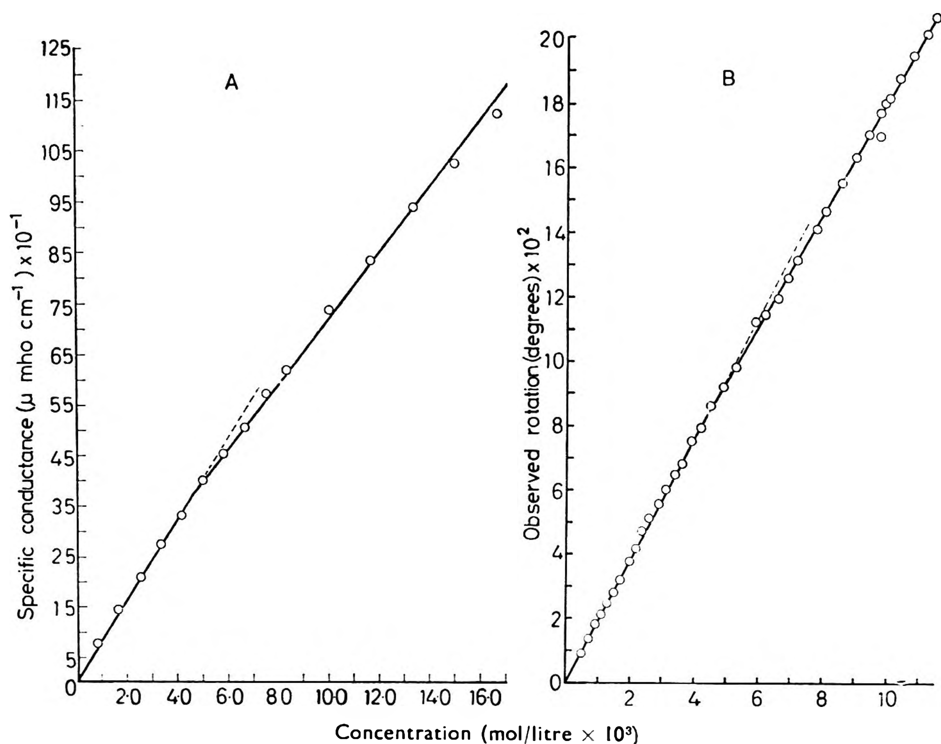

Fig. 1.A. Specific conductances of chlorhexidine digluconate in de-ionized water at 25°. B. Observed rotations at 317·5 nm for chlorhexidine digluconate solutions at 25°.

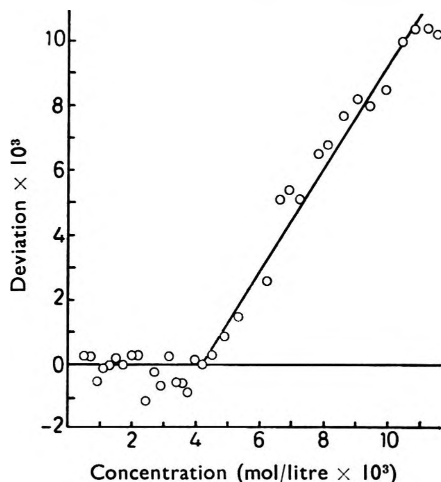

Fig. 2. Deviation plot of the data from Fig. 1B. Deviation being defined as observed rotation divided by (19·04 × concentration).

(Cary Model 60, Cary Instruments, Monrovia, California) at 25·0 ± 0·1° in 5 cm cells, the solutions were scanned from 400 down to 310 nm, where the absorption of the stronger solutions was too great for accurate quantitative analysis. Over this wavelength range the solutions gave apparently plain dispersion curves. Observed rotation for various concentrations of surfactant at 317·5 nm is shown in Fig. 1B, the real but small break at the CMC is emphasized by the deviation plot of Fig. 2. Analysis of the apparently plain curves by a single-term Drude equation (Drude, 1906), as was performed for the octyl glucoside (Mukerjee & others, 1970), proved unsuccessful; apparently more than one electronic transition is responsible for the gluconate curves. The change in rotation found after micelle formation is probably related to the change in ionization of the gluconate on aggregation; however, the dangers of mutorotation in extremes of pH make confirmation of this difficult. It should be noted that the rotations of the gluconate solutions had not changed 48 h after the experimental determinations.

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Tyrosine aminotransferase activity in a soluble extract of ergot fungus

The numerous ergoline alkaloids produced by the ergot fungus, *Claviceps purpurea* (Fries) Tulasne, and their several synthetic derivatives include a number of medicinally significant agents (Stoll, 1965). Medical, biological and chemical interests in these alkaloids have led to extensive use of saprophytic cultures of various strains of *C. purpurea* and *C. paspali* in many studies of the biosynthesis of the ergoline alkaloids (Stoll & Hofmann, 1965; Ramstad, 1968). Recently, Cavender & Anderson (1970) demonstrated the formation of clavine alkaloids from known precursors by a cell-free preparation of *C. purpurea*. However, few individual enzymes or enzyme reactions in claviceps have been established by direct experiments with cell-free fractions, and the relation between particular enzyme reactions and alkaloid formation remains to be elucidated.

Staba, Speaker & Schwarting (1961), using suspensions of washed mycelium of three different strains of claviceps for incubation with substrates, reported the association of alkaloid formation with aspartic: glutamic transaminase activity. We should like to report preliminary experiments with a soluble extract made from cultured mycelium of *Claviceps purpurea* which showed activity of tyrosine aminotransferase (tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5).

An *Agropyron* strain of *C. purpurea* which produced clavine alkaloids (cultured from sclerotium obtained from Dr. Matazo Abe of Tokyo University of Education, Japan) was grown in a synthetic medium (Abe, Yamano & others, 1952) in 500 ml Erlenmeyer flasks as still cultures at room temperature for 14–20 days. Seventy to 100 g (fresh wt) of mycelial tissue from 25–35 flasks of cultures (150 ml of growth medium per flask) were separated from the liquid by centrifugation, washed three times with water, and pressed dry between filter papers by hand, and weighed. This tissue was then frozen overnight, thawed at room temperature, and homogenized for 3 min at 4° in a pre-chilled Servall Omni-mixer with M/15 phosphate buffer, pH 7:3, containing 0·001 M ethylenediamine tetra-acetic acid tetrasodium salt (EDTA), using 5 ml of buffer for each g of mycelial tissue fresh wt. All subsequent steps from this point on until incubation with the substrates were carried out with pre-chilled containers at 0–4°. The homogenate was then centrifuged in a Servall RC-2 centrifuge at 1085 *g* for 30 min, and the residue discarded. The supernatant liquid was again centrifuged at 5090 *g* for 10 min and the residue again discarded. The 5090 *g* supernatant was re-centrifuged in a Beckman Spinco L-2 ultracentrifuge at 105 000 *g* for 30 min. The supernatant liquid was then dialysed against polyethylene glycol PEG 20M (Union Carbide) for 15–20 h at 4° to reduce the volume to about one-third. The resulting opaque liquid, with or without further dilution with buffer, constituted the soluble enzyme preparation which contained 2–4 mg of protein per ml (Miller, 1959).

The enzyme preparation was tested and assayed for tyrosine aminotransferase activity (Diamondstone, 1966) by determining the amount of *p*-hydroxyphenylpyruvate (pHPP) formed in the incubation mixture at 3, 6, 9, 12, 15, 20 and 30 min of incubation in a water bath at 30° with shaking at 90 strokes/min. The incubation mixture had a total volume of 4·5 ml, including 2 ml of the crude enzyme preparation, and containing (μmol) of L-tyrosine 11·25, of α-ketoglutaric acid (KG) 45, pyridoxal phosphate (PLP) 0·18, sodium diethyldithiocarbamate (DDC) 18, 2-mercaptoethanol 4·5, and M/15 phosphate buffer (pH 7·3) to make up to volume. The tyrosine was dissolved in 0·03N NaOH, DDC in water, and all the other components of the incubation mixture were each dissolved in an appropriate amount of the same buffer. The reagents were from commercial sources: L-tyrosine (Schwarz Bioresearch); KG (Nutritional Biochemical Corp.); PLP monohydrate (Mann Research Labs.); DDC sodium salt

(Fisher reagent); EDTA tetrasodium salt (British Drug Houses); 2-mercaptoethanol (Eastman); pHPP (Nutritional Biochemical Corp.).

For each incubation mixture, all the components excluding α-KG but including the enzyme preparation were incubated in the water bath shaker for 5 min before zero time when the reaction was started with the addition of α-KG. The reaction was stopped at each time interval by adding 0·3 ml of 10 N NaOH solution to the incubation mixture, with immediate shaking, and then followed by addition of 0·2 ml of the same buffer to make up to 5 ml volume. After standing at room temperature for 20 min, the mixture was centrifuged at 12 000 *g* for 10 min. This last centrifugation was found unnecessary by Diamondstone (1966) with mammalian preparations, but was essential with our

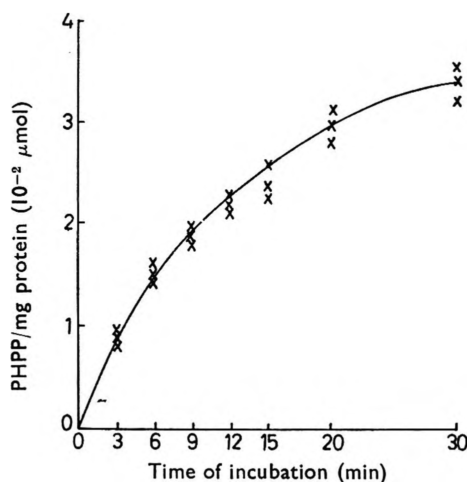

FIG. 1. Progress curve: Increase of *p*-hydroxyphenylpyruvate with time (per mg of protein of enzyme preparation). Each incubation mixture contained 2 ml of enzyme preparation, equivalent to 6·9 mg of protein, and other components as described in the text.

claviceps preparation to obtain a clear solution. Aliquots of the supernatant were then read for absorbance at 331 nm. This absorbance reading for each sample, minus the absorbance reading of a zero time control, was then converted to μmol of pHPP by referring to a standard curve made from assaying known concentrations of pHPP in the same buffer and carried through the same procedure in the presence of all the other components as the incubation mixtures but without the enzyme preparation. The zero time control sample contained the complete incubation system including the enzyme preparation, and at zero-time the 10 N NaOH solution was added to it immediately before adding the α-KG. After that, it was then carried through the same treatments of standing, centrifugation, and spectrophotometric reading as the other samples. Within the working range of concentrations of pHPP used in our experiments, the extinction coefficient was 20 530 M^{-1} (compared to 19 900 M^{-1} obtained by Diamondstone, 1966).

A progress curve, Fig. 1, shows the increase of reaction product (pHPP) with time of incubation. The specific activity (rate of pHPP formed per mg protein per unit time) determined from the slope of the initial portion of the progress curve was 0·16 μmol of pHPP formed/mg protein h^{-1}.

Vining (1970) recently reported evidence which showed that the influence of tryptophan on alkaloid formation in claviceps involved not merely the utilization of tryptophan as a precursor but had wider metabolic connections. Evidence for tryptophan: 2-oxoglutarate transamination has also been reported recently (Teuscher, 1970).

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