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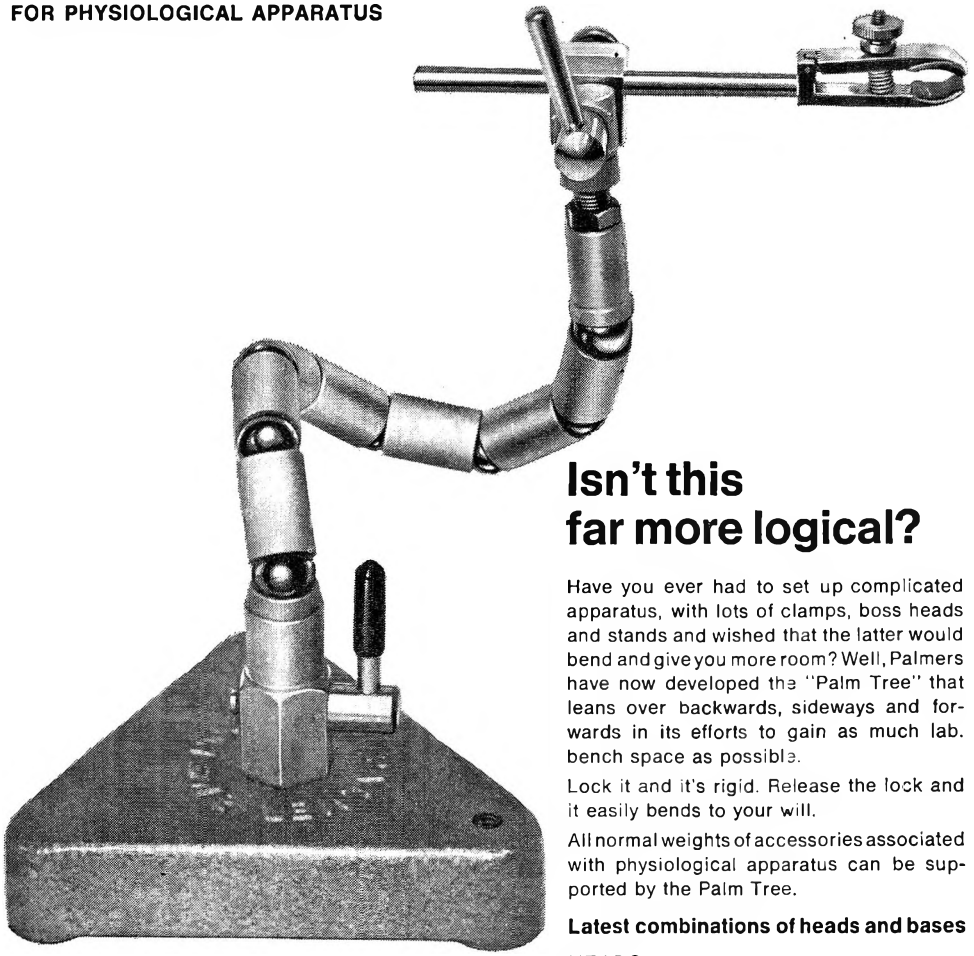

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Dissolution rates of sparingly soluble tablets

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The automated dialysis method offers an opportunity for the accurate evaluation of the dissolution rates of sparingly soluble dosage forms. By the analysis of kinetic models, the dissolution rates for disintegrating and non-disintegrating dosage forms may be calculated. The theory is used to examine the effect of additives and compression force on the dissolution rates of sulphathiazole tablets. At the fairly low compression forces used (640–1430 kg) the dissolution rate gradually increases due to penetration of the tablet by the dissolution medium. Polyethylene oxide causes an initial rapid increase in dissolution rate, but the formation of a mucilaginous film results in a constant rate. Using starch, dissolution rate increases rapidly as a result of tablet disintegration. This disintegration is shown to make available less than half the surface area of the original powder. The dissolution rate constant for sulphathiazole under the experimental conditions is $2{\cdot}75 \times 10^{-4}$ cm min^{-1}.

In a previous communication (Barzilay & Hersey, 1968) an automated dialysis method of measuring dissolution profiles of tablets was described. In common with other dialysis methods (Ferrari & Khoury, 1967; Marlowe & Shangraw, 1967; Krogerus, Kristoffersson & Kehela, 1967) dissolution rate is not measured directly. The purpose of this paper is to extend the utility of our method to the measurement of dissociation rates of sparingly soluble medicaments.

Dissolution rate may be expressed theoretically by the Noyes & Whitney (1897) relation

$$\frac{dW}{dt} = KS\,(C_S - C_M) \quad \ldots \quad (1)$$

where dW/dt is the rate of dissolution, K is the dissolution rate constant, S is the surface area, C_S is the solubility of the drug in the dissolution medium and C_M the concentration of drug in the medium at time t. Providing $C_S \gg C_M$, the above relation simplifies (Parrott, Wurster & Higuchi, 1955; Nelson, 1957) to

$$\frac{dW}{dt} = KSC_S \quad \ldots \quad (2)$$

Equation (2) is normally used as the basis for dissolution rate determination in which the dissolution process is allowed to proceed in a large volume of solvent. With sparingly soluble drugs, the solubility C_S is low, and build up of concentration in the dissolution medium will have a retarding effect on the dissolution rate (eqn 1). Thus many of the established *in vitro* dissolution rate methods are unable to accurately evaluate the dissolution rate of sparingly soluble drugs (Hersey, 1968).

The absorption of dissolved substances *in vivo* from the gastrointestinal tract is generally quite rapid and consequently in those instances where sparingly soluble

drugs are considered, absorption will depend upon the rate at which dissolution occurs, i.e. the absorption process is dissolution rate-limited. It is among low solubility medicaments that dissolution rate *in vitro* must be evaluated with the greatest care.

A drug dissolving in the gut contents is immediately available for absorption. Hence the concentration of the dissolved drug is kept at a minimum, that is, a natural sink condition exists. Gibaldi & Feldman (1967) have described an *in vitro* dissolution rate method using a sink condition, in which dissolved drug is partitioned into an organic phase. The use of an adsorbent material may also achieve the sink condition (Wurster & Polli, 1961), but may affect the viscosity and considerably retard the dissolution process (Wurster & Polli, 1964).

Dialysis provides a useful alternative to the sink conditions described. The automated dialysis method may, therefore, provide a powerful tool for the evaluation of *in vitro* dissolution rates of sparingly soluble drugs.

EXPERIMENTAL AND RESULTS

The apparatus used and method have previously been described (Barzilay & Hersey, 1968). In most cases the results given there are also used in the present discussion. Further results were obtained for 500 mg sulphathiazole tablets containing 5 and 10% of starch as a disintegrating agent (Fig. 1A) and for 250 mg of sulphathiazole powder (Fig. 1B) over a period of 4 h. For the powder, only half the quantity used in the

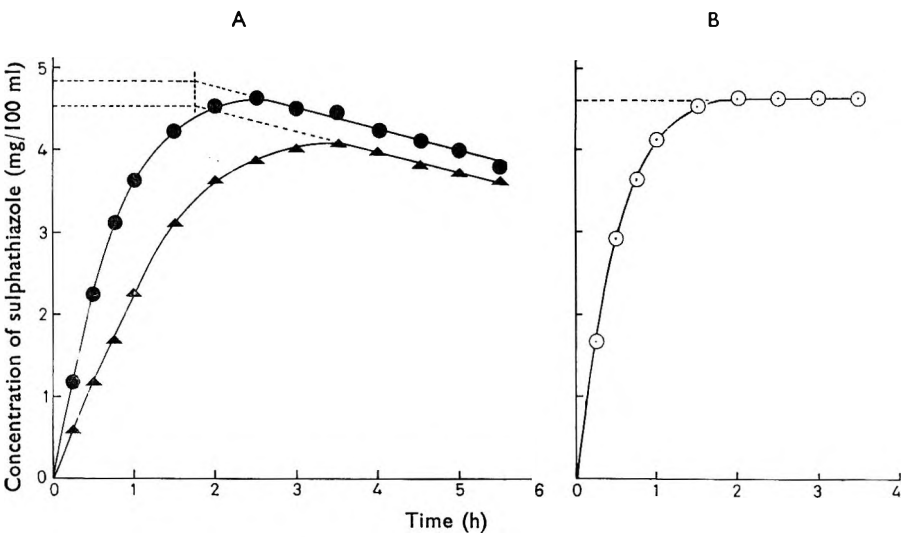

FIG. 1A. Dissolution of sulphathiazole tablets containing maize starch. ● 10% Starch ▲ 5% Starch. B. Dissolution of sulphathiazole powder.

tablets could be examined because of the sensitivity of the assay procedure. The time for disintegration, as measured by standard apparatus (B.P.), for the sulphathiazole tablets containing starch was within 25 s.

The surface area of the sulphathiazole was determined using the Fisher Sub-Sieve Sizer. The value of the surface mean diameter determined at a porosity between 0·45 and 0·60 was 19·4 μm equivalent to a specific surface area of 2000 $cm^2\ g^{-1}$ assuming spherical particles.

The kinetics of the dialysis were examined by placing solutions of 5 and 10 mg of sulphathiazole in the dialysis cell and the concentration measured as previously at suitable time intervals. The results are given in Fig. 2, which shows the dialysis to

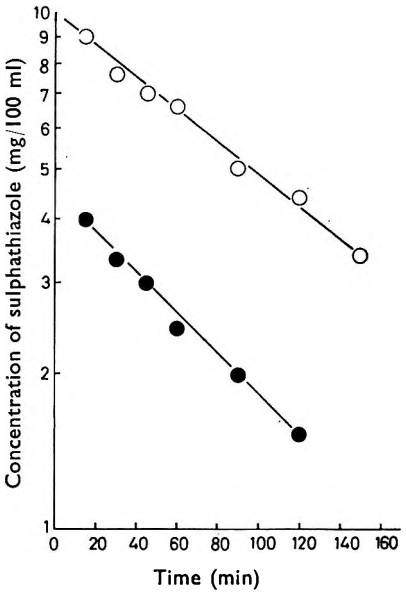

FIG. 2. Dialysis of sulphathiazole through cell membrane. ○ Initial concentration 10 mg/100 ml. ● Initial concentration 5 mg/100 ml.

be of first-order kinetics in the system investigated. The mean value of the dialysis constant (K_2) was calculated to be $3{\cdot}55 \times 10^{-3}$ min^{-1}.

DISCUSSION

In order to evaluate the dissolution rate, it is necessary to examine a kinetic model of the system. For non-disintegrating tablets a suitable kinetic model for the dissolution in the automated dialysis apparatus is given in Fig. 3.

The rate of dissolution of drug from the non-disintegrating tablet is given by the Noyes-Whitney equation (1). When the drug is dissolved it is available for dialysis, hence a sink condition is in operation and the condition $C_S \gg B$ (the concentration in the surrounding medium) may be assumed. Thus equation (1) reduces to

$$\frac{dW}{dt} = K_1 \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

providing there is negligible change in surface area due to dissolution (see eqn 2).

The rate of dialysis was shown to obey first order kinetics (Fig. 2) due to a second sink condition, whereby solute dialysing is continuously removed and replaced with fresh vehicle. Thus

$$V_B \frac{dB}{dt} = K_1 - V_B K_2 B \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where V_B is the volume of solution of concentration B, surrounding the dissolving tablet.

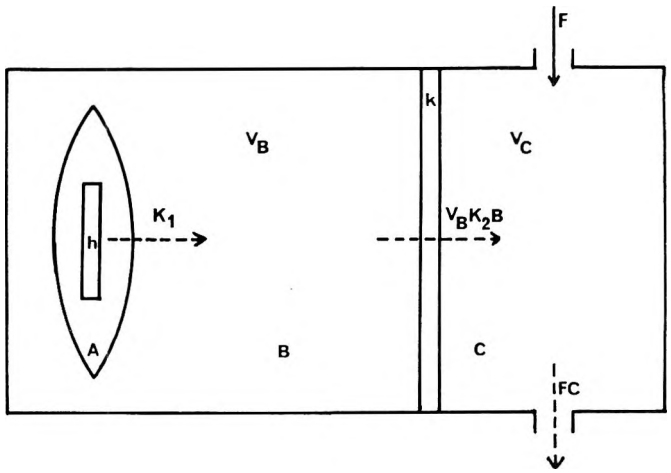

FIG. 3. Proposed kinetic model for the dissolution of non-disintegrating tablets. Drug dissolves from tablet h into the stationary region of saturated solution, A. Drug, K_1, diffuses into the surrounding medium, volume V_B concentration B. Drug, V_BK_2B, dialyses through membrane k, into compartment, volume V_C, concentration C. Diluent is pumped into this compartment at rate F and drug out at rate FC.

The violent agitation occurring on the measuring side of the dialysis membrane ensures a uniform measured solute concentration C at time t. Since F is the flow rate of solution through the measuring cell and also of replacement vehicle into the dialysis cell, the volume V_C of solution outside the dialysis membrane remains constant. Then

$$V_C \cdot \frac{dC}{dt} = V_BK_2B - FC \quad .. \quad .. \quad .. \quad (5)$$

Integration of equation (4) and using the limit $t = 0$, $B = 0$ gives

$$V_BK_2B = K_1(1 - e^{-K_2t}) \quad .. \quad .. \quad .. \quad .. \quad (6)$$

Substitution of equation (6) in equation (5) and using the limit $t = 0$, $C = 0$ gives

$$\frac{C}{K_1} = \frac{K_2V_C(e^{-Ft/V_C} - 1) + F(1 - e^{-K_2t})}{F(F - K_2V_C)} \quad .. \quad .. \quad .. \quad (7)$$

Since all the constants are known, K_1 can be evaluated by substitution of measured values of concentration C at times t, in equation (7). Results of this analysis are shown in Fig. 4. In all cases K_1 is shown to increase with time. For tablets of sulphathiazole compressed at different pressures (Fig. 4A) the increase of K_1 is linear. Comparison of equations (2) and (3),

$$K_1 = KSC_S \quad .. \quad .. \quad .. \quad .. \quad (8)$$

suggests that this is due to a gradual increase in the functional surface area. This may be explained by penetration of the surface of the tablet by the dissolution medium increasing the area available for dissolution. The tablets used in this study were made at pressures between 573 and 1266 kg cm^{-2}, well below those normally used for intrinsic dissolution rate measurements as, for example, by the rotating disc method (Levy & Sahli, 1962). At these low pressures, penetration might be expected to cause a large increase in the surface available for dissolution, although the tablets did not disintegrate during the test.

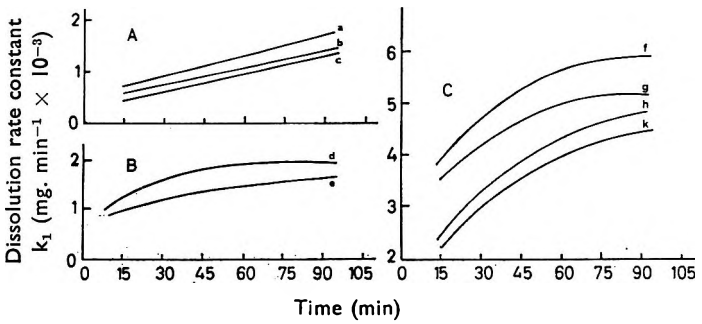

FIG. 4. Effect of time on the dissolution rate constant of 500 mg sulphathiazole tablets. A, compressed at different pressures: applied pressure a, 500 kg cm^{-2}; b, 770 kg cm^{-2}; c, 1090 kg cm^{-2}. B, containing polyethylene oxide: d, 5% Polyox; e, 1% Polyox. Mean applied pressure 790 kg cm^{-2}. C, containing maize starch: f, 10% starch; g, 7·5%; h, 5%; k, 2·5%. Mean applied pressure 660 kg cm^{-2}.

The results using polyethylene oxide (Fig. 4B), especially at the 5% concentration in 500 mg sulphathiazole tablets, indicate an initial rapid increase in the functional surface area, in agreement with the observed splitting of the tablet. This is followed by a much less rapid increase, probably due to the formation of a mucilaginous film around the tablet similar to that observed by Huber, Dale & Christenson (1966). Higher concentrations of polyethylene oxide gave identical results to the 5% concentration. The results for the rapidly disintegrating sulphathiazole tablets containing starch (Fig. 4C) are included for comparison to show the effect of the increasing surface area of sulphathiazole available for dissolution.

If the value of K_1 could have been established as a constant over the period of the investigation, an alternative solution to equation (7) is available. If it is assumed that the dissolution of the sulphathiazole does not effectively alter the surface area of the non-disintegrating dosage form, then at some finite time depending on the flow rate through the dialysis cell, the rate of dialysis and the recipient volume for dialysis

$$C = K_1/F \quad .. \quad .. \quad .. \quad .. \quad .. \quad (9)$$

Since, rearranging equation (7)

$$C = \frac{K_1}{F} + \text{constant}_1\, e^{-Ft/V_C} + \text{constant}_2\, e^{-K_2 t} \quad .. \quad .. \quad (10)$$

then as $t \to \infty$, $C = K_1/F$.

When examining disintegrating dosage forms it is necessary to know the relation between surface area and time for complete evaluation of the kinetic model. The shape of the graph for disintegrating tablets (Fig. 4C) suggests this relation should be exponential in form approaching a limiting value asymptotically. A general relation for this type of curve is given by

$$S = S_o\,(1 - e^{-Qt}) \quad .. \quad .. \quad .. \quad .. \quad (11)$$

where S_o is the surface area at complete disintegration and, for total disintegration, will be equal to the surface area of the original component drug particles assuming no breakdown on compaction, and Q is a rate constant of disintegration. Using equation (11) in the kinetic model (Fig. 3) then

$$\frac{dW}{dt} = KC_S S_o(1 - e^{-Qt}) \quad .. \quad .. \quad .. \quad (12)$$

and $$V_B \cdot \frac{dB}{dt} = KC_S S_o(1 - e^{-Qt}) - K_2 V_B B \quad .. \quad .. \quad .. \quad (13)$$

while $$V_C \cdot \frac{dC}{dt} = K_2 V_B B - FC \quad .. \quad .. \quad .. \quad (5)$$

as before.

Integration of equation (13) using the limits $t = 0$, $B = 0$ gives

$$K_2 V_B B = \frac{KC_S S_o}{K_2 - Q} [K_2(1 - e^{-Qt}) - Q(1 - e^{-K_2 t})] \quad .. \quad .. \quad (14)$$

Substitution of equation (14) in equation (5), integrating and using the limits $t = 0$, $C = 0$ gives an equation similar in form to equation (10), thus

$$C = \text{constant}_1 + \text{constant}_2 e^{-tF/V} + \text{constant}_3 e^{-Qt} + \text{constant}_4 e^{-K_2 t} \quad .. \quad (15)$$

where

$$\text{constant}_1 = \frac{KC_S S_o}{F} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (16)$$

$$\text{constant}_2 = \frac{-KK_2 C_S S_o Q}{F(F/V_C - Q)\,(F/V_C - K_2)} \quad .. \quad .. \quad (17)$$

$$\text{constant}_3 = \frac{-KK_2 C_S S_o}{V_C(K_2 - Q)\,(F/V_C - Q)} \quad .. \quad .. \quad (18)$$

$$\text{constant}_4 = \frac{-KC_S S_o Q}{V_C(K_2 - Q)\,(F/V_C - K_2)} \quad .. \quad .. \quad (19)$$

Since both Q and K are unknown, even assuming complete disintegration to the the original particle size, S_o, solution of equation (15) cannot be accomplished by simple substitution. Indeed, since this equation is similar to equation (10), a similar technique offers the most practical solution. As t approaches some finite value, depending on the values of K_2, Q and F, then C will approach its asymptotic value, i.e.

$$C = \frac{KC_S S_o}{F} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (20)$$

Examination of Fig. 1B shows that the lag time, T, for attainment of this asymptotic value for the sulphathiazole powder is 105 min due to dialysis and pumping rates chosen (Barzilay & Hersey, 1968). Substitution of the measured concentration 4·6 mg/100 ml at time T in equation (20) using the values for solubility, C_S, 97·7 mg/100 ml and flow rate F of 2·90 ml min^{-1} and specific surface area, S_o, 2000 $cm^2\ g^{-1}$ (i.e. surface area for 250 mg powder = 500 cm^2) gives the dissolution rate constant

$$K = 2{\cdot}75 \times 10^{-4} \text{ cm min}^{-1}$$

for sulphathiazole under the experimental conditions.

Examination of Fig. 1A shows a lag time, T of 2·5 h for sulphathiazole tablets containing 10% starch and 3·5 h for those containing 5% starch. This difference in lag time between the powder T = 1·75 h and the formulated products (Table 1) may be taken as a measure of the disintegration time under the prevailing agitation conditions in this test. Whereas the pharmacopoeia disintegration test could not distinguish between the two formulated tablets, under the extremely mild 'peristaltic' action in the dialysis cell a long disintegration time is evident.

Using the value of K calculated for the powder, a measure of the degree of disintegration can be obtained from equation (20). Thus in Table 1 it can be seen that

Table 1. *Disintegration of sulphathiazole tablets formulated with starch*

% Starch (maize)	Lag time T	Disintegration time T–T powder	% S_0 at T	% S_0 at T powder
5%	3·5 h	1·75 h	42·2	46·5
10%	2·5 h	0·75 h	47·6	49·7

the calculated surface area available for dissolution subsequent to disintegration is below 50% of that available from a consideration of the original powder compressed. The pressure of 660 kg cm^{-2} used to compact these tablets has evidently caused much bonding, which has not been disrupted by the disintegration mechanism of the starch.

At times in excess of the lag time, a decrease is observed in the measured concentration for the formulated tablets (Fig. 1A) in contrast to the powder (Fig. 1B). Such a decrease may be explained by the reduction in surface area, which accompanies the dissolution process. The discrepancy between the formulated products and the original powder suggests that the compression and disintegration of the formulated tablets has caused a complete change in the particle size distribution. The large reduction in surface area suggests an increased particle size, but the decrease in measured concentration is indicative of a smaller particle size. Such small particles probably formed during compaction rapidly dissolve and cause the significant drop in measured concentration. An alternative reason may be that the starch forms a mucilage surrounding some of the particles and only a few of the particles are therefore available for dissolution. As dissolution proceeds from these few particles the surface area is significantly reduced. In either event the degree of disintegration should be corrected for the amount of dissolution that has occurred in the difference between the lag times for the tablets and powder (T — T powder). Even with this correction, however, the calculated surface area remains below 50% of that based on the original powder.

Acknowledgements

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Stabilization of oil-in-water emulsions by non-ionic detergents: properties of synthetic detergents at anisole- and chlorobenzene-water surfaces

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As part of an investigation into the mechanisms of stabilization of emulsions, the interfacial activity of three synthetic non-ionic detergents, $CH_2{\cdot}[CH_2]_{15}{\cdot}[O{\cdot}CH_2{\cdot}CH_2{\cdot}]_xOH$, with x = 3, 6 and 9, at the anisole-water and chlorobenzene-water interface has been determined by measurement of interfacial tension. Surface pressure (π)-area (A) relations have been derived. The films are more expanded than at the air-water interface and an equation of the form $(\pi-\pi_k)A = xkT$ fits the results. It is suggested tentatively that π_k, which is in most cases positive, and which increases with polyoxyethylene chain length, arises from the interaction between the ethylene oxide chains as they approach closely. The apparent critical micelle concentrations determined from the interfacial tension-concentration curves are higher than the true critical micelle concentrations obtained at the air-water interface.

Although a knowledge of the behaviour of detergents at oil-water interfaces is of importance in any investigation of their mode of action as stabilizers of emulsions, few investigations of this nature have been made with synthetic non-ionic detergents. Becher's recent review (1967) on emulsification with non-ionic detergents mentions the only comprehensive study (Crook, Fordyce & Trebbi, 1963) with synthetic polyoxyethylene octylphenyl ethers at the water-iso-octane interface. Wrigley, Smith & Stirton (1957) and Becher (1963) have studied the interfacial tension lowering properties of a number of commercial non-ionic detergents at various oil interfaces.

The lowering of interfacial energy by adsorption of surface-active species is important in the process of emulsification, but its role in maintaining stability once the emulsion has been formed is not clear. In a previous paper (Elworthy & Florence, 1967), the stability of emulsions of anisole and chlorobenzene in the presence of the commercial non-ionic detergent, cetomacrogol 1000, was discussed. It was concluded that the main source of stability was the steric or hydrational repulsive force which arose from the interaction of the long polyoxyethylene chains. It appeared that the zeta potential of the oil globules play a secondary role in stabilization, as an increase of cetomacrogol concentration above the critical micelle concentration (CMC) decreased the zeta potential significantly yet resulted in only a small reduction in stability.

In the present paper this work is extended to include the effects of synthetic detergents with the structure $C_{16}H_{33}[O{\cdot}CH_2{\cdot}CH_2{\cdot}]_xOH$ with x = 3, 6 and 9, on the interfacial tension at anisole-water and chlorobenzene-water interfaces, in order to determine the effect of polyoxyethylene chain length.

EXPERIMENTAL

Apparatus and Methods

Interfacial tensions were measured using a pendant drop apparatus based on the design of Andreas, Hauser & Tucker (1938) as previously described by Elworthy & Florence (1967). At 20° ± 0·01° a value for the surface tension of water of 72·68 dynes cm^{-1} was obtained (72·79 dynes cm^{-1}) [literature values in brackets] for hexane-water interfacial tension, 51·03 dynes cm^{-1} (51·1 dynes cm^{-1}), and for carbon tetrachloride-water 44·85 dynes cm^{-1} (45·05 dynes cm^{-1}). Measurements were made at intervals to determine ageing in the systems. Chlorobenzene and anisole were fractionally distilled, and water was distilled from potassium permanganate. The detergents were synthesized using methods described previously (Elworthy & Macfarlane, 1962a, b; 1963) and were chromatographed on alumina and recrystallized before use. The Williamson ether method was used to prepare $Me[CH_2]_{15}[O{\cdot}CH_2{\cdot}CH_2]_3{\cdot}OH$. Redistilled hexadecyl bromide (Eastman Kodak), 1 mole, was condensed with monosodium trioxyethylene glycol, prepared by reaction of sodium (1 mole) with redistilled trioxyethylene glycol (BDH) (4 mole). The detergent was separated from the reaction mixture, from which the sodium bromide had been removed, with a light petroleum–ether mixture, the residue after evaporation fractionally distilled, redistilled and then purified as described above. All compounds had the same melting points as described (Elworthy & Macfarlane, 1962a, b; 1963) and assays for ethylene oxide content gave the theoretical amount (±0·2%).

RESULTS AND DISCUSSION

The equilibrium interfacial tensions of the detergents, $C_{16}H_{33}{\cdot}[O{\cdot}CH_2{\cdot}CH_2{\cdot}]_xOH$ with x = 3, 6 and 9 (abbreviated to $C_{16}n_3$, $C_{16}n_6$, $C_{16}n_9$) were determined as a function of concentration at 20° ± 0·01°. Some results are shown in Fig. 1.

Interfacial tension

For trioxyethylene and the hexaoxyethylene ethers, the detergents were dissolved in the oil phase and the interfacial tension measured against pure water. The nonaoxyethylene compound was also studied in this way but, in addition, the detergent was placed in the aqueous phase and equilibrated with pure oil. Table 1 summarizes the results, showing the apparent CMC's of the detergents and the interfacial tension at this concentration and areas/molecule in the immediate pre-CMC region, calculated on the assumption that the Gibbs' adsorption equation holds for the present systems. (Table 1). Gibbs' adsorption equation was used in its simple form,

$$\Gamma_2 = -\frac{1}{RT}\frac{d\gamma}{d\ln c}$$

where $\Gamma_2 AN = 10^{16}$, N being Avogadro's number and A the area/molecule (Å²), neglecting solute activities. This was considered to be admissible because of the low concentrations of surfactant involved in the pre-CMC region. Errors occur in the estimation of areas/molecule using the equation in this form only when the mole fraction of solute, N_2, is large (greater than 0·01) (Hutchinson, 1949). N_2 of the surfactants in the regions in which the areas/molecule were calculated was in all cases less than 4×10^{-4}. At the air-water interface, only when the concentration of similar non-ionic surfactants approached 30% ($N_2 \sim 10^{-2}$) did appreciable errors occur in areas/molecule when the simple form of Gibbs' equation was used (Florence, 1965).

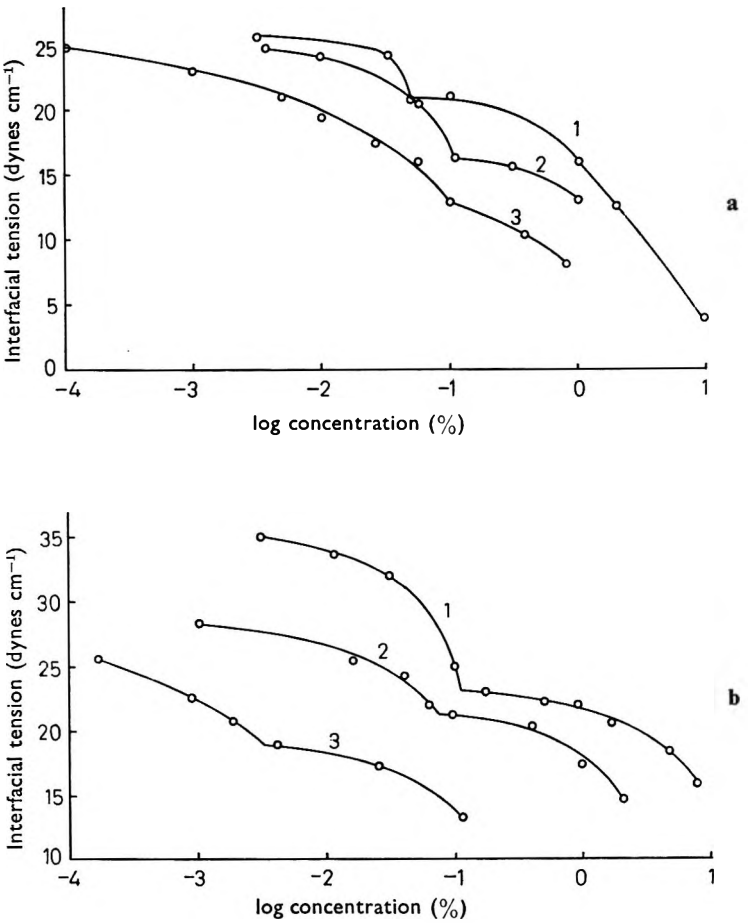

FIG. 1a. Anisole-water system. Effect of (1) $C_{16}n_3$, (2) $C_{16}n_6$, and (3) $C_{16}n_9$ on the interfacial tension. b. Chlorobenzene-water system. Effect of (1) $C_{16}n_3$, (2) $C_{16}n_6$, and (3) $C_{16}n_9$ on the interfacial tension.

Table 1. *Apparent critical micellar concentrations (mole litre^{-1}), areas per molecule and interfacial tensions at the* CMC

Oil phase	Compound	CMC mole litre^{-1}	Interfacial tension at CMC dynes cm^{-1}	Area/molecule Å^2	Area/molecule (Å^2) at air/water interface
Anisole	$C_{16}n_3$	$1{\cdot}74 \times 10^{-2}$	20·0	45	31†
	$C_{16}n_6$	$6{\cdot}92 \times 10^{-3}$	16·2	56	33*
	$C_{16}n_9$	$1{\cdot}59 \times 10^{-3}$	12·8	76	53
	$C_{16}n_{25}$‡	$0{\cdot}23 \times 10^{-3}$	5·2	154	120
Chlorobenzene	$C_{16}n_3$	$3{\cdot}17 \times 10^{-3}$	23·1	48	31†
	$C_{16}n_6$	$1{\cdot}55 \times 10^{-3}$	21·3	80	33*
	$C_{16}n_9$	$1{\cdot}05 \times 10^{-3}$	14·3	100	53
	$C_{16}n_{25}$‡	$0{\cdot}06 \times 10^{-3}$	7·5	184	120

† From surface pressure data using $C_{16}n_3$ spread on water

* Areas/molecule at air-water interface from Elworthy & Macfarlane (1962a) and Elworthy (1960).

‡ Cetomacrogol 1000, data from Elworthy & Florence (1967).

Greenwald, Kice & others (1961) have shown that the distribution coefficients of surfactants between oil and water show no concentration dependence below the CMC.

The areas/molecule at the oil-water interfaces so calculated are greater than those at the air-water interface; and the areas at the chlorobenzene-water are greater than at the anisole-water interface.

Interfacial tensions at the CMC fall with increasing ethylene oxide chain length, characteristic of non-ionic detergents at aromatic hydrocarbon interfaces (Becher, 1963). At aliphatic hydrocarbon-water interfaces (e.g. iso-octane) Crook & others (1963) found that the interfacial tension increased with increasing polyoxyethylene chain length, for a series of nonylphenyl polyoxyethylene ethers.

The work of adhesion of anisole to water is 81·6 dynes cm^{-1} compared with 67·9 dynes cm^{-1} for chlorobenzene suggesting that the methoxy group of the anisole is strongly oriented to the water (Adamson, 1960). This may account for the lower interfacial tensions in the anisole systems. There is also likely to be a difference in the structure of the interfacial films formed by long and short polyoxyethylene chain detergents. Becher, (1963) has suggested that some of the ethylene oxide units of short polyoxyethylene chain detergents reside in the oil phase, but as the chain length is increased the surfactant molecules become drawn more into the aqueous phase.

In contrast to the situation in some aqueous solutions the CMC in the oil-water systems studied here decreases as the polyoxyethylene chain of the detergent is lengthened. The CMC's are also many times higher in the oil-water system than the CMC's of the detergents in aqueous solution in the absence of oil. The same trends are observed in the results of Crook & others (1963) who suggest that the higher CMC's result from the preferential distribution of the detergent to the non-aqueous phase. Thus a large amount of detergent has to be present in the system before sufficient is present in the aqueous phase to form micelles. Obviously this has practical consquences as the CMC of oil-soluble surfactants determined from measurements at the air-water interface thus bear little relation to the minimum concentration which can be used to prepare the emulsions. The distribution explanation seems to be correct as the discrepancy between the true and apparent CMC's decreases as the chain length increases, in accord with the decreasing partition coefficient, K_w^o, as the series is ascended. All the systems studied show a further decrease of interfacial tension above the apparent CMC, which may be due to a further change in the structure of the interfacial film.

Ageing effects

Ageing at the oil-water interface is not pronounced with these short-chain detergents, but some results are presented in Fig. 2, for surfactants dissolved in chlorobenzene compared with those disolved in pure water. Concentrated systems came to equilibrium more rapidly than dilute ones (compare B and C, D and E, in Fig. 2).

Equation of state

π (surface pressure) *vs* A (area/molecule) plots were constructed from interfacial tension data, and in Fig. 3, these results are presented as plots of πA *vs* A. It can be seen that none of the lines obey the simple relation.

$$\pi A = kT$$

but that over large portions of the lines an equation of the type

$$\pi A = mA + xkT$$

is obeyed. Writing $m = \pi_R$ we obtain

$$(\pi - \pi_R)A = xkT \quad .. \quad .. \quad .. \quad (1)$$

where π_R may be considered to be a measure of the interaction between the adsorbed molecules. The values of π_R and xkT are recorded in Table 2.

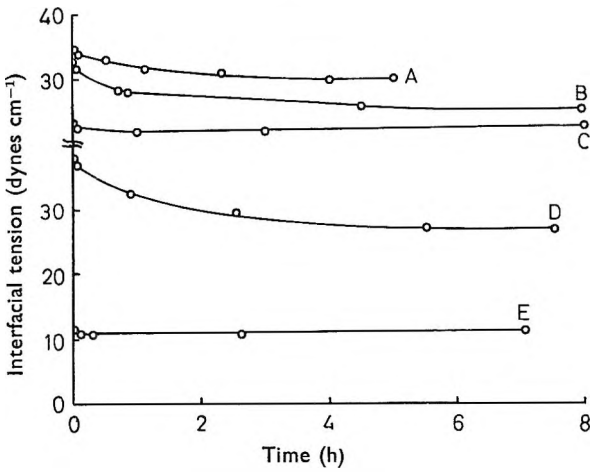

FIG. 2. Ageing effects ot the chlorobenzene-water interface.
(A) 0·00029% $C_{16}n_3$ (B) 0·00018% $C_{16}n_6$
(C) 0·19% $C_{16}n_6$ (D) 0·0001% $C_{16}n_9$
(E) 1·018% $C_{16}n_9$

Table 2. *Values of* π_R *and* xkT *from* πA vs A *plots at the oil-water interface*

Oil	Detergent	π_R dynes cm^{-1}	xkT	x
Anisole	$C_{16}n_3$	−0·6	200	0·5
	$C_{16}n_6$	+1·0	400	1·0
	$C_{16}n_9$	2·9	760	1·9
	$C_{16}n_{25}$	8·9	1340	3·4
Chlorobenzene	$C_{16}n_3$	2·8	398	1·0
	$C_{16}n_6$	10·8	400	1·0
	$C_{16}n_9$	12·5	800	2·0

NOTE: The change in sign of π_R suggests that it is made up of two components such that

$$\pi_R = \pi_{HC} + \pi_{PEG}$$

where π_{HC} is the interaction between the adsorbed hydrocarbon chains and π_{PEG} is the force between the polyoxyethylene chains. At the air/water interface π_{HC}, which will be negative, will predominate and π_R will thus be negative as indeed was found: π_R for $C_{16}n_3$ at the air-water interface = −14·7 dynes cm^{-1}.

Two factors are usually considered to cause the product πA to deviate from kT; molecular attraction between the adsorbed surfactant (causing it to decrease below kT) and the finite size of the molecules (causing it to increase above kT). At the non-polar oil-water interface lateral intermolecular attractions are so slight that an equation

$$\pi(A - Ao) = kT \quad .. \quad .. \quad .. \quad .. \quad (2)$$

has been found to hold for some surfactants, e.g. an equimolecular film of $C_{18}H_{37}\overset{+}{N}Me_3$ and $C_{22}H_{45}SO_4^-$ gives results obeying the equation $\pi(A - 20) = kT$ (Davies & Rideal, 1963). For equation (2) to hold, (*a*) there must be no electrical repulsive forces operating, (*b*) there must be no cohesive forces between the hydrocarbon chains, and, (*c*) there must be no repulsive forces of other origin. It is likely that conditions

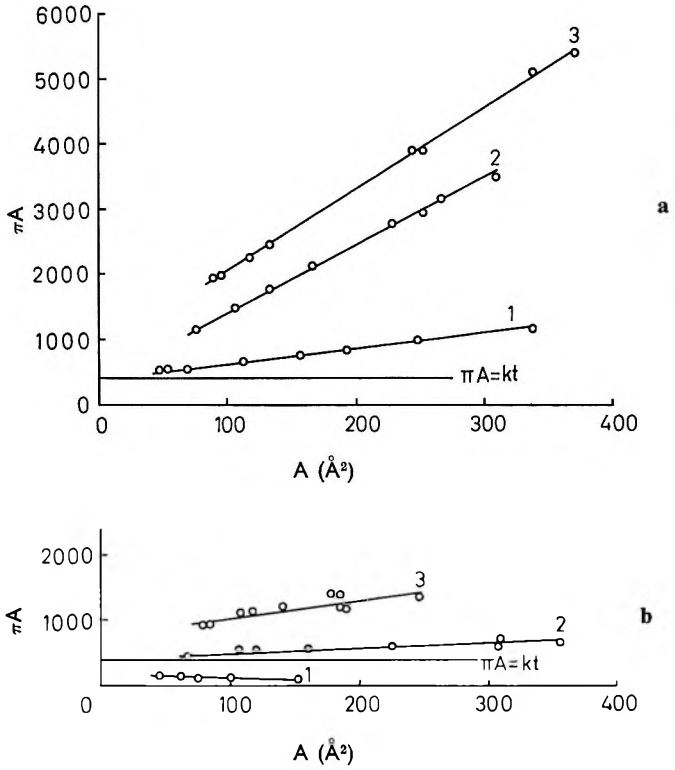

FIG. 3a. πA-A plots for (1) $C_{16}n_3$, (2) $C_{16}n_6$, and (3) $C_{16}n_9$ at the anisole-water interface. b. πA-A plots for (1) $C_{16}n_3$, (2) $C_{16}n_6$ and (3) $C_{16}n_9$ at the chlorobenzene-water interface.

(*a*) and (*b*) hold for non-ionic films, the latter because the polyoxyethylene chains are the determinants of closest approach. For the compounds studied here equation (2) does not hold, indicating that some repulsive force is in operation. This repulsion seems likely to arise from steric interactions of the ethylene oxide chains.

The fact that π_R increases with ethylene oxide chain length would imply that this interaction plays some part in the determination of the character of the πA *vs* A plot, as a positive value of π_R indicates the operation of repulsive forces. The small negative value obtained for $C_{16}n_3$ in anisole may be partially due to experimental error, and partially to the possibility that only a small part of the polyoxyethylene chain of this molecule resides in the aqueous phase.

The increase of π_R with chain length is probably due to an entropic effect arising from the penetration of adjacent polyoxyethylene chains into one another's available space. From the theory of Mackor (1957), this will give an increased free energy in the system. Such effects are also important in the interpretation of stability data on emulsions, and preliminary work has shown that the stability increases with increasing polyoxyethylene chain length.

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Solubilization and rheology of the system ascorbic acid-water-polysorbate 80: temperature effects

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Changes induced by temperature in the relative position and extent of the isotropic and liquid crystal phases present in the ascorbic acid–water–polysorbate 80 system have been recorded. Increases in temperature reduce the size of the liquid crystal phase. This phase is pseudoplastic, but a small central region also shows thixotropic hysteresis loops. On recycling, most systems show Newtonian behaviour. In the isotropic regions, an increase in temperature results in a fall in log of viscosity proportionate to $1/T_{abs}$, but in the liquid crystal region an increase in temperature may cause a similar fall or, because of a change in the relative position of the system within the liquid crystal region, an increase.

In previous papers the authors have reported on the solubility and rheology, at 25°, of ascorbic acid–water systems containing polysorbate 20 and polysorbate 80 (Nixon & Chawla, 1965, 1967). In the systems containing polysorbate 20, no anisotropic liquid crystal phase existed and the viscosity was Newtonian throughout all the single phase systems. In similar systems using polysorbate 80 as the solubilizer a far more complex picture was found. A large band of viscous anisotropic liquid crystal phase occurred. The rheological behaviour within this region was pseudoplastic when only small amounts of liquid crystal were present, but revealed complex thixotropic properties when liquid crystal was the only phase present. The present work reports on the changes produced by temperature on the solubility and rheological properties of ascorbic acid–water–polysorbate 80 systems.

EXPERIMENTAL

Ascorbic acid, polysorbate 80 and *water* complied with the specifications given previously (Nixon & Chawla, 1967).

Determination of viscosity. The Ferranti-Shirley cone and plate viscometer was used as described by Nixon & Chawla (1965). Stress-strain diagrams showing the rheological properties of the systems were prepared.

Determination of solubility. Solubility was taken as the average between an under and over saturated solution containing a difference of 10 mg ascorbic acid per 5 ml solution. In the viscous liquid crystal regions the dispersion was first warmed to 60° to speed equilibration. The end point was determined after storage at the desired temperature for 24 h.

RESULTS AND DISCUSSION

The solubility of ascorbic acid in polysorbate 80–water systems showed a negligible change at temperatures between 25° and 40° (Fig. 1). In no case could ascorbic acid

be dissolved in concentrations of polysorbate 80 above 97% w/w. The highest concentration of dissolved ascorbic acid was found in the absence of polysorbate 80, but even here the increase in solubility was only from 20·75% w/w at 25° to 22·1% w/w at 40°. As the concentration of polysorbate 80 increased, the solubility of ascorbic acid in the system fell.

The principal effect of temperature was on the type and position of the micellar phases present. At 25°, in the absence of ascorbic acid, birefringence commenced at 41% w/w polysorbate 80 and continued up to a concentration of 64% w/w. Pure liquid crystal appeared to be present from about 44% w/w polysorbate 80. The upper limit of the liquid crystal phase was imprecise and difficult to determine. At higher temperatures the width of this liquid crystal band was very much smaller as shown by Fig. 1 and Table 1.

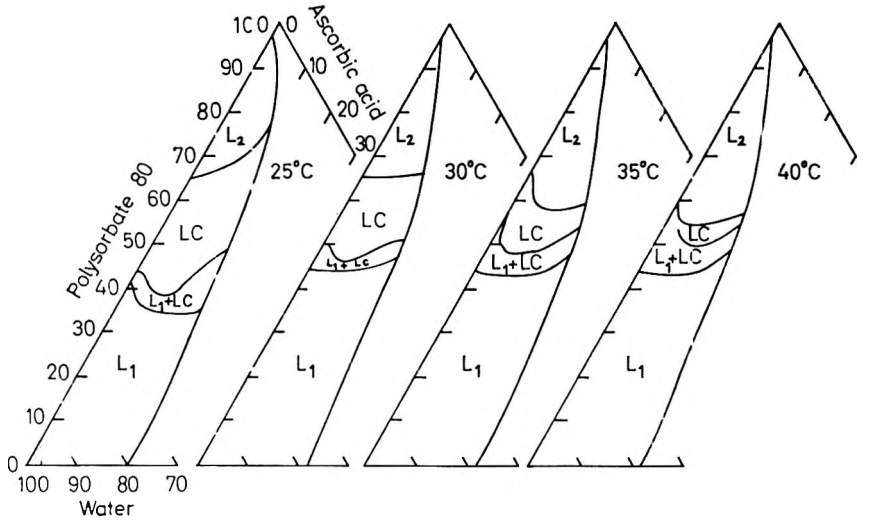

FIG. 1. Solubility of ascorbic acid in polysorbate 80–water systems: effect of temperature on phases present. L_1 and L_2, isotropic phases: LC, liquid crystal.

Table 1. *Boundary of liquid crystal regions (% w/w polysorbate 80)*

Temperature °C	Absence of ascorbic acid			5% w/w ascorbic acid			10% w/w ascorbic acid			Solubility limit of ascorbic acid		
	a	b	c	a	b	c	a	b	c	a	b	c
25	41	44	64	36	39	67	34·5	40	—	34	49	76
30	44	50	66	44	46	65	44	48	66	47	51	66·5
35	44	57	66	43	48	59	43	48·5	58	48	54	59
40	44	—	59	43	—	54	43	50	55	48	55	56

(a) Lower limit of liquid crystal + L_1; (b) lower limit of liquid crystal only; (c) upper limit of liquid crystal + L_2.

In the presence of ascorbic acid an increase in temperature between 30° and 40° made little difference in the polysorbate concentration at which liquid crystal first appeared, but between 25° and 30° an appreciable increase in the concentration was necessary to produce liquid crystal. At the higher temperatures the concentration of polysorbate 80 producing liquid crystal showed only a slight dependence on ascorbic

acid concentration until the acid solubility limit was approached, then the polysorbate concentration required rose slightly.

Greater changes, than in the lower boundary, occurred in the concentration of polysorbate 80 required to maintain liquid crystal. At 25° this concentration rose gradually as the solubility limit was approached. There was little effect between 25° and 35° on the limiting concentration of polysorbate required to maintain liquid crystal, but at 40° it fell. With ascorbic acid present the necessary concentration of polysorbate 80 fell with increasing temperature, but it was virtually independent of the ascorbic acid concentration at any individual temperature, except that it rose slightly as the solubility limit was approached.

It is a thermodynamic necessity that between the liquid crystal region and the single isotropic phase there should be a region where the two micellar types coexist. In the present system it was impossible to distinguish this at the upper limit of liquid crystal's existence, possibly because the band was too narrow to distinguish by the technique of solubility determination. At the lower limit for liquid crystal formation, the co-existent phase, liquid crystal plus isotropic liquid, was readily distinguishable as an opaque dispersion contrasting with the clear yellow of both the isotropic liquid and liquid crystal phases on their own.

This band of liquid crystal plus isotropic liquid tends to a minimum width between 5 and 10% w/w ascorbic acid. At 40° at low acid concentrations the band extends the full width of the liquid crystal region.

The rheological properties of the system under consideration may conveniently be divided into two sections. In the isotropic liquid phases the viscosity was Newtonian, whilst in those regions where liquid crystal was present, far more complex non-Newtonian rheological properties were shown.

Andrade (1930), Sheppard (1930) and Sheppard & Houck (1930) have shown that to a first approximation Newtonian fluids obey the temperature dependent relation $\eta = Ae^{-\epsilon/kt}$ where ϵ is the potential energy of adjacent molecules and k is the Boltzman constant. From this expression it is possible to derive the following expression $\log \eta = \log A + B/T \log e$ where B is ϵ/k and A is a constant. A plot of $\log \eta$ against $1/T$ will be a straight line. Typical data for both isotropic liquid phases are shown in Fig. 2 where it is apparent that in these regions of the solubility diagram a normal viscosity–temperature relation holds.

Systems containing liquid crystal exhibited non-Newtonian rheological properties. At the first appearance of liquid crystal, pseudoplastic flow occurred, but when the liquid crystal only phase was reached, thixotropic hysteresis loops occurred. These hysteresis loops were frequently accompanied by a "spur" value as reported previously (Nixon & Chawla, 1967) and also observed by others (Barry & Shotton, 1967).

At temperatures below 30° a small region in the liquid crystal phase gave anomalous rheological flow curves. In these systems a spur value occurred above which pseudo-plastic flow was noted until a rate of shear of 850 s^{-1} was reached. Above this value a slight dilatent flow curve was found. After the shear rate was allowed to decay, the curve showed a slight hysteresis loop to the left at all shear rates, the width of this loop being small over the dilatent portion of the curve. At temperatures above 30° this anomalous behaviour at high shear rates disappeared along with the "spur" on the curves. A thixotropic hysteresis loop was found in only one system studied (53% polysorbate 80–8% ascorbic acid–39% water) at temperatures above 30°. At the

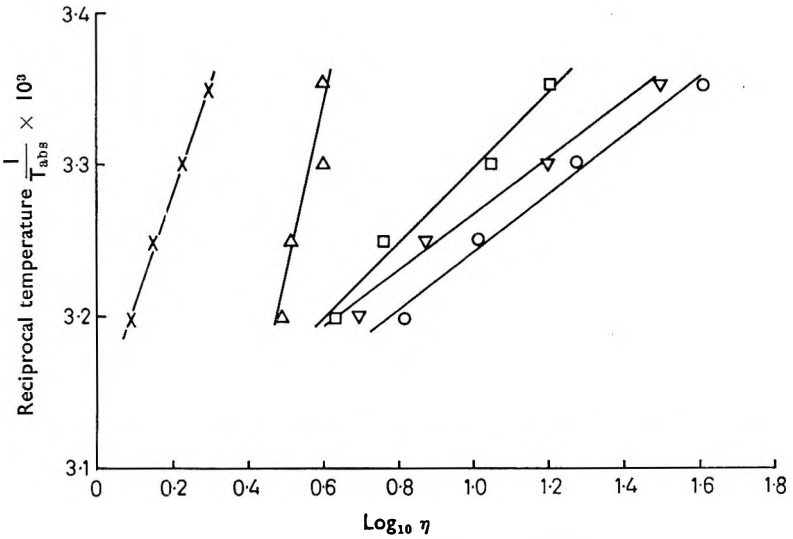

FIG. 2. Effect of temperature on the viscosity of isotropic dispersions of ascorbic acid–water–polysorbate 80.

% w/w Composition:	Ascorbic acid	Polysorbate 80	Water
×	0	9·36	90·64
△	19·17	11·14	69·69
□	4·86	80·76	14·38
▽	0	70·99	29·01
○	19·19	71·09	9·72

Abscissa in centipoises for ×, △: poises for □, ▽, ○.

higher temperatures the flow behaviour was pseudoplastic throughout the liquid crystal region.

Recycling of the systems resulted in the destruction of this hysteresis loop in all cases and the substitution of pseudoplastic flow, which on continuous recycling (more than 5 cycles) approached Newtonian behaviour. Where the original liquid crystal system had exhibited pseudoplasticity, recycling produced Newtonian viscosity. This occurred usually after two recycles at temperatures above 35° but occasionally required as many as 5 recycles at lower temperatures. On allowing the system to stand for up to 1 h, the original rheological behaviour tended to be re-established, particularly when this had been pseudoplastic. The thixotropic hysteresis loops did not, after this time, return to their full width.

As suggested by Barry & Shotton (1967) it would appear that systems such as those described do not exhibit true thixotropy, in that the area occupied by the hysteresis loop does not return to its full value even after prolonged standing. The findings suggest that the structural breakdown occurring at higher rates of shear does not completely reform on standing. The dilatent behaviour exhibited by certain systems at high rates of shear suggests that the structural breakdown has proceeded to the extent of "squeezing" water out of the liquid crystal gel and thus producing shear thickening. That this water is not taken back into the liquid crystal system in the same way is shown by the slight hysteresis loop produced on reducing the shear rate.

The spur values which have been noted on certain liquid crystal systems are a fairly frequent occurrence in rheograms (De Butts, Hudy & Elliott, 1957; Martin, Banker & Chun, 1964; Boylan, 1966; Barry & Shotton, 1967). They have been explained as

indicating the presence of three-dimensional gel structures. In the present system this would be formed by chains of liquid crystal micelles. As suggested by Barry & Shotton (1967), the resistence of these threads would have to be broken down before deformation could occur. Because of this breakdown of structure the spur does not show on subsequent recycling.

With these non-Newtonian systems it is not possible to give more than an "apparent" viscosity (Table 2) at a definite rate of shear (850 s^{-1}). Plots of apparent viscosity

Table 2. *Changes in the flow characteristics with temperature for representative systems*

System % w/w					
Ascorbic acid	Polysorbate 80	Temperature °C	Original flow properties	Flow after 5 recycles	Viscosity or apparent viscosity (at shear rate 850 s^{-1}) after recycling poises
		25	Newtonian	Newtonian	0·0396
19·17	11·14	30	Newtonian	Newtonian	0·0396
		35	Newtonian	Newtonian	0·0321
		40	Newtonian	Newtonian	0·0309
		25	Thixotropic	Pseudoplastic	14
15·0	51·5	27	Pseudoplastic	Newtonian	15·09
		35	Pseudoplastic	Newtonian	16·97
		40	Pseudoplastic	Newtonian	17·92
		25	Thixotropic	Pseudoplastic	13·41
		27 (a)	Thixotropic	Pseudoplastic	13·44
		27 (b)	Thixotropic	Pseudoplastic	13·23
10·39	45·86	30	Pseudoplastic	Newtonian	11·06
		35	Pseudoplastic	Newtonian	7·54
		40	Pseudoplastic	Newtonian	6·13
		25	Slightly thixotropic	Pseudoplastic	11·73
		30	Pseudoplastic	Newtonian	10·37
0·0	45·57	34	Pseudoplastic	Newtonian	10·83
		35	Pseudoplastic	Newtonian	9·90
		38	Pseudoplastic	Newtonian	9·28
		40	Pseudoplastic	Newtonian	7·07
		25	Thixotropic	—	—
		27	Thixotropic	Pseudoplastic	24·47
0·0	60·93	30	Thixotropic	Pseudoplastic	32·06
		31	Thixotropic	Pseudoplastic	23·22
		35	Thixotropic	Newtonian	13·68
		40	Newtonian	Newtonian	8·96
		25	Newtonian	Newtonian	35·20
8·0	81·0	30	Newtonian	Newtonian	17·91
		35	Newtonian	Newtonian	9·67
		40	Newtonian	Newtonian	6·60
		25	Newtonian	Newtonian	16·20
4·86	80·76	30	Newtonian	Newtonian	10·37
		35	Newtonian	Newtonian	5·66
		40	Newtonian	Newtonian	4·24

against temperature after 5 recycles were treated in a similar manner to the isotropic liquid phase and specimen curves are shown in Fig. 3. Not only is the apparent viscosity of these systems affected by the non-Newtonian flow, but also by differences in relative position within a solubilized phase brought about by temperature changes. Thus it is possible for a system exhibiting thixotropic properties at low temperatures, to pass through a pseudoplastic phase into Newtonian viscosity at high temperature,

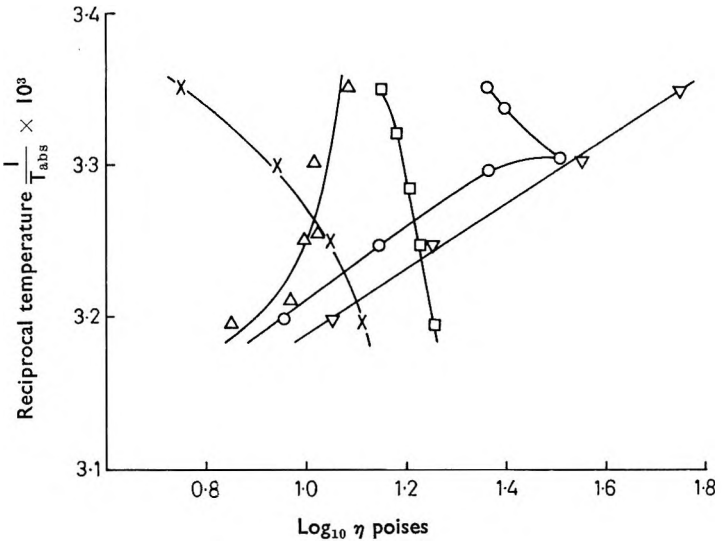

FIG. 3. Effect of temperature on the apparent viscosity after recycling of anisotropic dispersions of ascorbic acid–water–polysorbate 80. Shear rate at measurement 850 s^{-1}. Number of recycles 5.

% w/w Composition:	Ascorbic acid	Polysorbate 80	Water
×	15·5	48·5	36·0
△	0	45·6	54·4
□	15·0	51·5	33·5
▽	11·5	68·0	20·5
○	0	60.9	39·1

corresponding to a change from a system containing liquid crystal micelles to one containing small spherical micelles. Certain of these systems, again depending on the change in their relative position within the liquid crystal region, show higher apparent viscosities (measured at a shear rate of 850 s^{-1}) with increase in temperature. When the system becomes isotropic the Newtonian viscosity now exhibited falls in a similar manner to the other isotropic systems.

It is therefore apparent that examination of the rheological behaviour of solubilized systems, requires that not only temperature effects on the individual system be considered, but also the effect of changes in the relative position of the phases present within the solubilization diagram.

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A comparison of the effects on blood glucose and ketone-body levels, and of the toxicities, of pent-4-enoic acid and four simple fatty acids

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Some effects of the hypoglycaemic compound pent-4-enoic acid in rats and mice, are described. Pent-2-enoic acid, pentanoic acid, cyclopropanecarboxylic acid and cyclobutanecarboxylic acid, which were shown to be non-hypoglycaemic, were used as controls. Pent-4-enoic acid and cyclopropanecarboxylic acid caused ketosis in rats, with a lowering of the blood β-hydroxybutyrate/acetocetate (βHB/AcAc) ratio; some ketosis was caused by the other fatty acids but the βHB/AcAc ratio was not changed. Pent-4-enoic acid caused an increase in free fatty acid concentration in rat plasma. The acute toxicities of these compounds in mice were determined. The mechanism of the hypoglycaemic action of pent-4-enoic acid is discussed in relation to that of hypoglycin.

Pent-4-enoic acid has been reported briefly to be hypoglycaemic (Anderson, Johnson & others, 1958; McKerns, Bird & others, 1960; Senior & Sherratt, 1966). It is the simplest member of a series of hypoglycaemic fatty acids related to hypoglycin (L-α-amino-β-methylenecyclopropanepropionic acid) (Anderson & others, 1958), the toxic principle of the unripe ackee fruit, *Blighia sapida* (Hassall, Reyle & Feng, 1954). The structural requirement for hypoglycaemic activity is a vinyl group separated from a carboxyl group by two carbon atoms.

In this paper some effects of pent-4-enoic acid in animals are compared with those of four non-hypoglycaemic fatty acids which were used as controls. Pent-2-enoic acid and pentanoic acid were used because of their structural relationship to pent-4-enoic acid; and cyclopropanecarboxylic and cyclobutanecarboxylic acid were used because of their relationship to the hypoglycaemic compounds, methylenecyclopropaneacetic acid and 3-methylenecyclobutanecarboxylic acid (Anderson & others, 1958). These four control fatty acids were also used in a biochemical investigation of the mechanism of action of pent-4-enoic acid in order to establish which *in vitro* effects are correlated with its hypoglycaemic activity (Senior & Sherratt, 1968a, b; Senior, Robson & Sherratt, 1968). The effects of the fatty acids on blood ketone bodies were studied since both hypoglycin and cyclopropanecarboxylic acid were known to cause a strong ketosis in rats (Williamson & Wilson, 1965).

EXPERIMENTAL

Materials

Butyric acid, pentanoic acid, hexanoic acid, crotonic acid and pent-4-enoic acid (Fluka A. G. Buchs, Switzerland), acrylic acid, pent-2-enoic acid, hex-3-enoic acid,

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hept-2-enoic acid, sorbic acid, DL-carnitine, riboflavin and riboflavin phosphate (Koch-Light Laboratories Ltd., Colnbrook, Bucks.), cyclopropanecarboxylic acid and cyclobutanecarboxylic acid (Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.). Glucose oxidase kits for glucose estimation, NAD^+, NADH and crystalline D-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) 140 units/mg, were obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. Fatty acids were injected as aqueous solutions of their sodium salts, pH 7·4; the doses quoted refer to the free acids.

Animals

All animals used were albino males, the rats were Wistar strain.

Methods

Effects of pent-4-enoic acid and related fatty acids on blood glucose levels. Rats (150–200 g), mice (22–26 g) and rabbits (1·5–3·0 kg) were starved for 24 h to deplete their glycogen reserves and food was withheld during the experiments. Fatty acids or 0·153 M NaCl were given to rats intraperitoneally and to mice and rabbits subcutaneously. Blood was taken from the tails of rats or the ear veins of rabbits at intervals and collected in cold heparinized tubes. Mice were killed at intervals after injection and blood was collected from the heart. Blood was deproteinized with 0·6 N $HClO_4$ and glucose was determined using glucose oxidase (EC 1.1.3.4) with test-kits supplied by Boehringer. Samples taken at the nadir of hypoglycaemia gave very low optical density readings, so the reaction mixture was fortified with additional potassium phosphate to give a final concentration of 0·4 M, pH 7·0. Larger samples of the acid supernatant could then be assayed.

RESULTS

Effects of pent-4-enoic acid and related fatty acids on blood glucose levels

Rats. Only pent-4-enoic acid caused marked hypoglycaemia (Table 1), the time course of which is illustrated in Fig. 1. There was some individual variation in the

Table 1. *Blood glucose levels in rats after administration of pent-4-enoic acid and related fatty acids.* Blood glucose levels were determined in each animal at 1 h intervals for at least 7 h as described in the Methods section. Zero time blood glucose concentration ± s.d. were (mM) 3·33 ± 0·75 (41).

Fatty acid	Dose (mg/kg)	No. of animals	Range of blood glucose levels (mM) observed during experiments	Mean values ± s.d. or range of greatest percentage changes in, blood glucose levels	P where applicable
Pent-4-enoic acid	100	6	1·45–3·65	—23·2 ± 8·0	>0·001
	150*	6	1·74–4·00	—35·7 ± 8·6	>0·001
	200*	6	1·28–4·67	—41·9 ± 21·7	>0·01 <0·001
	250*	3	0·02–3·05	—79 ± 34·5	>0·01
Pent-2-enoic acid	50–250	3	2·50–3·50	0– —20	—
n-Pentanoic acid	50–250	3	3·22–3·88	0–+20	—
Cyclopropanecarboxylic acid	50–250	6	3·77–4·02	0– —18	Not significant
Cyclobutanecarboxylic acid	50–280	3	3·44–4·72	+23–+61	—
Control (given 0·153 M NaCl)	—	5	2·77–4·56	0–+24	—

* One death.

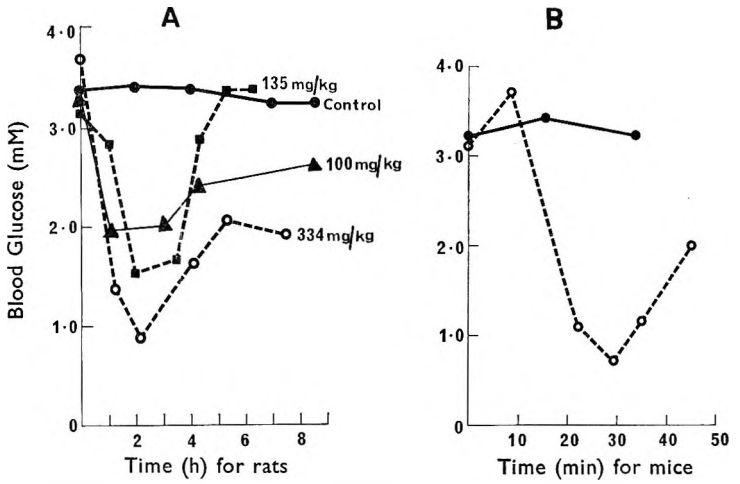

FIG. 1A. Time course of hypoglycaemia in individual rats caused by intraperitoneal administration of pent-4-enoic acid; control (●), pent-4-enoic acid 100 mg/kg (▲), pent-4-enoic acid 135 mg/kg (■), pent-4-enoic acid 334 mg/kg (○).

B. Hypolgycaemia in mice caused by subcutaneous administration of pent-4-enoic acid (1·0 g/kg); control (●), pent-4-enoic acid (○). Each point represents the blood glucose level in one animal.

hypoglycaemic effect, and pent-4-enoic acid (M = 100) was approximately half to a third as effective on a weight basis in producing maximum hypoglycaemia as hypoglycin (M = 141) (Feng & Patrick, 1958). Pent-2-enoic acid in large doses (250 mg/kg) caused a slight fall (20%) in blood glucose. All fatty acids caused depression and irregular respiration but pent-4-enoic acid was markedly most depressant. Similar depressant effects are caused by injection of saturated short-chain fatty acids (Samson & Dahl, 1955; Samson, Dahl & Dahl, 1956). Rats that recovered from hypoglycaemia appeared normal after 24 h and also after six months.

Mice. Pent-4-enoic acid caused pronounced hypoglycaemia in mice (Fig. 2). All treated animals became severely depressed after 15 min. Breathing was difficult and was deep and irregular, with epistaxis, exophthalmos and loss of righting reflex.

Rabbits. There were no consistent or significant effects on blood glucose levels for up to 28 h after injections of pent-4-enoic acid (30–425 mg/kg) in 14 rabbits. The lethal dose was variable (200–425 mg/kg) but the blood glucose did not fall even at the time of death, and in one animal it rose sharply to twice the normal level.

Effect of pent-4-enoic acid and related fatty acids on blood ketone body levels

Pent-4-enoic acid and cyclopropanecarboxylic acid increased the total ketone body content of blood and reduced the β-hydroxybutyrate (βHB)/acetoacetate (AcAc) ratio in starved rats (Table 2). Pent-2-enoic and pentanoic acid increased the total ketone body content without changing the βHB/AcAc ratio significantly (Table 2).

The control levels of AcAc and βHB were similar to those found in starved rats by Berry, Williamson & Wilson (1965) and the effects of cyclopropanecarboxylic acid confirm the results of Stewart (1962) and of Williamson & Wilson (1965).

Effects of pent-4-enoic acid on plasma free fatty acid levels

There was an increase in the plasma free fatty acid level in fed rats to double the control value 40 min after injecting pent-4-enoic acid (175 mg/kg) (Table 3). The

Table 2. *Effects of pent-4-enoic acid and related fatty acids on blood ketone body levels.* Rats (200–250 g) were starved for 24 h before use to increase the total blood ketone body content up to 15 times normal. Starvation also stabilizes the β-hydroxybutyrate (βHB)/acetoacetate (AcAc) ratio at a higher value (Berry & others, 1965); this enables more accurate assay of these compounds. Blood was collected from the tail and 0·40 ml was added to 1·80 ml of 0·6 N $HClO_4$ to precipitate protein, and βHB and AcAc were assayed in the supernatant by the enzymic method of Williamson, Mellanby & Krebs (1962). The fatty acids were given intraperitoneally 20 min after the first blood sample was taken. The results are expressed as μmole of AcAc plus βHB/ml blood. Data are given for one experiment with each compound, with lower doses similar though smaller changes were obtained.

Control	Time	—20 min	1 h 10 min	3 h 5 min	4 h 10 min	6 h 30 min
0·4 ml	Total ketones	2·46	2·61	2·79	2·26	2·84
0·135 M NaCl	βHB/AcAc ratio	2·84	2·73	2·72	3·18	3·11
Pent-4-enoic acid	Time	—20 min	1 h 5 min	3 h	4 h 5 min	6 h 25 min
200 mg/kg	Total ketones	2·71	4·82	5·16	4·60	2·86
	βHB/AcAc ratio	2·82	1·00	0·91	0·92	2·32
Pent-2-enoic acid	Time	—20 min	1 h 15 min	3 h 15 min	4 h 15 min	6 h 15 min
150 mg/kg	Total ketones	2·56	3·16	3·11	2·72	2·90
	βHB/AcAc ratio	2·88	2·95	3·38	4·22	3·14
Pentanoic acid	Time	—20 min	65 min	3 h 5 min	4 h 6 min	6 h 35 min
300 mg/kg	Total ketones	2·56	4·20	4·15	4·00	3·20
	βHB/AcAc ratio	2·88	2·50	2·20	1·50	1·91
Cyclopropane-carboxylic	Time	—20 min	1 h	3 h	4 h	6 h
acid	Total ketones	2·59	4·40	5·60	5·81	3·10
150 mg/kg	βHB/AcAc ratio	3·05	1·44	1·17	1·00	1·82
Cyclobutane-carboxylic	Time	—20 min	55 min	2 h 55 min	3 h 55 min	6 h 55 min
acid,	Total ketones	2·56	3·16	3·55	3·66	2·70
150 mg/kg	βHB/AcAc ratio	2·88	2·68	2·74	2·86	2·37

Table 3. *Effect of pent-4-enoic acid on plasma free fatty acid levels.* The method of Duncombe (1963, 1964) was followed using rats (240–260 g) allowed a normal diet up to 4 h before the experiment. Pent-4-enoic acid or 0·135 M NaCl was given intraperitoneally at zero time. Animals were stunned at intervals by a blow on the head and 3 ml of blood was taken by cardiac puncture. The blood was transferred to ice-cold heparinized tubes, centrifuged at 3500 rev/min for 10 min and 0·5 ml of plasma used for analysis. Each result represents the free fatty acid levels in a single rat.

Injection	Time after injection (h min)	Free fatty acid level (mμmole/ml plasma)
0·14 M NaCl (0·50 ml)	0·00	256
	0·33	273
	1·05	224
	2·01	262
	2·34	240
Pent-4-enoic acid 175 mg/kg	0·25	262
	0·40	510
	1·00	510
	1·30	516
	1·50	430
	2·35	346
	3·00	280
	3·30	416
	4·00	334

control values were slightly lower than those given in the literature (De Renzo, McKerns & others, 1958; Hales & Kennedy, 1964).

Toxicities of pent-4-enoic acid and related fatty acids

The LD50 for pent-4-enoic acid in starved mice (315 mg/kg, Table 4) was similar to the value for starved rats (about 250 mg/kg) estimated from the data in Table 1. Most deaths occurred within the first hour. Cyclopropanecarboxylic acid (which is not hypoglycaemic in mice, Stewart, 1962) was more toxic than pent-4-enoic acid, but all the other fatty acids tested, including some not used elsewhere in this work, were less toxic (Table 4). The toxicity of pent-4-enoic acid was increased by starvation, the LD50 being even less after 22 h than after 18 h (Table 4). In contrast the toxicity of cyclopropanecarboxylic acid was unchanged after 22 h of starvation.

Table 4. *Toxicities of pent-4-enoic acid and related fatty acids in mice.* The LD50 values were estimated by the method of Weil (1952), using 6 animals per dose level, the 95% confidence limits are given by the figures in brackets. Mice (20–25 g) were allowed, unless otherwise stated, a normal diet before use. The ambient temperature was maintained at 21 ± 1° and water allowed freely. Fatty acid solutions were given intraperitoneally or subcutaneously (the toxicity was not influenced by the route of administration). Mice were pre-treated with riboflavin phosphate (12·5 mg/kg) and DL-carnitine (800 mg/kg) intraperitoneally 1 h before use where indicated. Deaths were scored after 24 h.

Fatty acid	LD50 (g/kg)
Pent-4-enoic acid	0·891 (0·665–1·19)
Pent-4-enoic acid	1·00 (0·733–1·14)
Pent-4-enoic acid*	0·871 (0·671–1·13)
Pent-4-enoic acid, pre-treated with riboflavin phosphate and DL-carnitine	0·794 (0·509–1·01)
Pent-4-enoic acid*, animals starved 18 h	0·575 (0·445–0·744)
Pent-4-enoic acid, animals starved 22 h	0·315 (0·237–0·437)
Pent-4-enoic acid, animals pre-treated with riboflavin phosphate and DL-carnitine, starved 22 h	0·315 (0·237–0·437)
Cyclopropanecarboxylic acid	0·172 (0·127–0·232)
Cyclopropanecarboxylic acid	0·223 (0·161–0·310)
Cyclopropanecarboxylic acid, animals pre-treated with riboflavin phosphate and DL-carnitine	0·159 (0·126–0·200)
Cyclopropanecarboxylic acid, animals starved for 22 h	0·230 (0·139–0·378)
Cyclopropanecarboxylic acid, animals starved for 22 h and pre-treated with riboflavin phosphate and DL-carnitine	0·223 (0·161–0·310)
Acrylic acid	1·59 (1·26–2·00)
Crotonic acid	3·59 (2·62–4·50)
Pent-2-enoic acid	1·58 (1·26–2·00)
Sorbic acid	2·82 (2·45–3·24)
Hex-3-enoic acid	1·84 (1·29–2·20)
Hept-2-enoic acid	1·60 (1·05–2·41)
Butyric acid	3·18 (2·51–4·05)
Pentanoic acid	3·59 (2·62–4·50)
Hexanoic acid	3·18 (2·51–4·05)
Cyclobutanecarboxylic acid	1·27 (1·00–1·61)

*Estimations using 10 animals per dose level.

These figures may be compared with those for hypoglycin. Entman & Bressler (1967) gave doses of 500–750 mg/kg to mice starved for 18 h and did not report any deaths. The LD50 in fed rats was about 100 mg/kg and fasting halved this value (Feng & Patrick, 1958; Hassall & Reyle, 1955a, b). Hypoglycin therefore appears to be more toxic than pent-4-enoic acids in rats but not in mice. Hypoglycin is very toxic in rabbits (toxic dose 10–20 mg/kg; Chen, Anderson & others, 1957).

Holt & Holt (1959) reported that feeding riboflavin phosphate to rats and mice antagonized the toxicity and hypoglycaemic effects of hypoglycin. Entman & Bressler (1967) found that L-carnitine (12 mg/mouse; 360–600/mg/kg) given intravenously with hypoglycin also antagonized the hypoglycaemic effects. Attempts were therefore made to modify the toxicity of pent-4-enoic acid by intraperitoneal administration of these two unrelated compounds (though it is appreciated that there may be no simple relation between toxicity and hypoglycaemia). Pre-treatment with riboflavin phosphate (12·5 mg/kg) and DL-carnitine (800 mg/kg) had no significant effect on the toxicity of pent-4-enoic acid in fed or in starved mice (Table 4). They also had no effect when given at the same time as pent-4-enoic acid; or when given in divided doses one h before and at the same time as pent-4-enoic acid. Neither riboflavin phosphate or DL-carnitine given separately modified toxicity. Free riboflavin (12·5 mg/kg) also did not change the toxicity. In control experiments riboflavin phosphate plus DL-carnitine had no apparent effect when given to mice. These compounds had no effect on the toxicity of cyclopropanecarboxylic acid in fed or in starved mice (Table 4).

DISCUSSION

The maximum hypoglycaemic effect of pent-4-enoic acid is seen about 2 h after administration in the rat and after 30 min in the mouse. With hypoglycin the maximum effect in the rat is after 4–6 h (Feng & Patrick, 1958) and in the mouse after about 90 min (Entman & Bressler, 1967). This is consistent with the view that pent-4-enoic acid is active without further metabolism, but that hypoglycin must first be converted *in vivo* into methylenecyclopropaneacetic acid (Holt, 1966). None of the four structurally related fatty acids used here was hypoglycaemic in rats although cyclopropanecarboxylic acid causes hypoglycaemia in the guinea-pig and monkey by a mechanism which depends on circulating insulin (Stewart, 1962). No explanation can be offered for the lack of effect of pent-4-enoic acid on blood glucose levels in the rabbit, though hypoglycin is also not hypoglycaemic in some species (Chen & others, 1957).

Evidence that pent-4-enoic acid and hypoglycin do not lower blood sugar levels by influencing insulin secretion or activity may be summarized as follows. Blood glucose does not fall until liver glycogen is depleted (Patrick, 1954). Insulin reduces serum free fatty acid levels, whilst pent-4-enoic acid and hypoglycin increase them (McKerns & others, 1960). Infusion of ketone-bodies causes hypoglycaemia in dogs, most probably by stimulating insulin secretion (Mebane & Madison, 1964) although ketone-bodies also reduce glucose utilization since βHB and AcAc are preferred fuels in most tissues (Randle, Newsholme & Garland, 1964). Large amounts of AcAc given parenterally to rats produced small falls in blood glucose, though blood levels of AcAc reached during starvation were not hypoglycaemic (Tidwell & Axelrod, 1948). Both cyclopropanecarboxylic acid and pentanoic acid caused an increase in total ketone bodies similar to those caused by pent-4-enoic acid or by hypoglycin (Williamson & Wilson, 1965), yet neither was hypoglycaemic. It is therefore unlikely that ketosis contributes to the hypoglycaemic effects of pent-4-enoic acid or hypoglycin (cf. Chen & others, 1957). Lowered glucagon secretion may conceivably cause hypoglycaemia but Chen & others (1957), Leppla & Holt (1956) and Feng (1957) found no evidence for impaired glucagon secretion after hypoglycin treatment. Hypoglycin did not alter secretion of adrenal glucocorticoids (McKerns & others, 1960).

Both methylenecyclopropaneacetic acid (Holt, Holt & Böhm, 1966) and pent-4-enoic acid (Senior & Sherratt, 1967; Senior & others, 1968) strongly inhibit the

oxidation of long-chain fatty acids. This could account for raised levels of serum free fatty acids found in hypoglycin-treated rats (De Renzo & others, 1958) and in pent-4-enoic acid-treated rats.

Pent-2-enoic acid and pentanoic acid caused an increase in the total blood ketone body levels in mice without altering the βHB/AcAc ratio. Pent-4-enoic acid, and also hypoglycin and cyclopropanecarboxylic acid (Stewart, 1962; Williamson & Wilson, 1965), increased the total ketone body levels and reduced the βHB/AcAc ratio. A reduction of the blood βHB/AcAc ratio reflects reduction in the NADH/NAD^+ ratio within mitochondria (Berry & others, 1965). This cannot be related simply to inhibition of fatty acid oxidation as suggested by Williamson & Wilson (1965) since cyclopropanecarboxylic acid does not inhibit this process (Senior & others, 1968). The increase in total blood ketone bodies is probably due to greater impairment of their peripheral utilization than of their formation by the liver following administration of these three fatty acids (Williamson & Wilson, 1965; Senior & others, 1968).

Glucose utilization in hypoglycin-treated animals is either reduced or unchanged while palmitate or stearate utilization is strongly inhibited (McKerns & others, 1960; Holt & others, 1966). Holt & others (1966) suggested that gluconeogenesis may be inhibited following hypoglycin administration. We have shown that pent-4-enoic acid, but none of the four control fatty acids, strongly inhibits gluconeogenesis *in vitro* (Senior & Sherratt, 1968b). The most probable explanation of the hypoglycaemic effects of pent-4-enoic acid and of hypoglycin, therefore, is that when long-chain fatty acid and ketone body oxidation are blocked the only available major fuel is glucose. Glygocen reserves become exhausted and since glucose cannot be replaced by gluconeogenesis hypoglycaemia ensues (Senior, 1967). The greater toxicity of pent-4-enoic acid and of hypoglycin (Feng & Patrick, 1958) but not of cyclopropanecarboxylic acid, in starved than in fed animals agrees with this interpretation.

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The preferred conformation of noradrenaline and a consideration of the α-adrenergic receptor

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The preferred conformation of noradrenaline has been calculated using extended Hückel molecular orbital theory. The conformation was found to be identical to the previously calculated conformation of (−)-ephedrine in respect to the relation of the quaternary and hydroxyl groups and the phenyl ring. These findings reinforce the previous hypothesis of the nature of the α-adrenergic receptor and also support the view that these molecules function at the receptor in their preferred conformations.

In a previous study, the preferred conformation of ephedrine and pseudoephedrine was calculated using molecular orbital theory (Kier, 1968d). The results indicated that ephedrine and pseudoephedrine had preferred conformations represented by Fig. 1a and b, respectively. The presentations of the four isomers to an assumed relatively planar receptor led to a hypothesis of a receptor pattern that was consistent with the ranking of α-adrenergic potency of the four compounds (see Fig. 2).

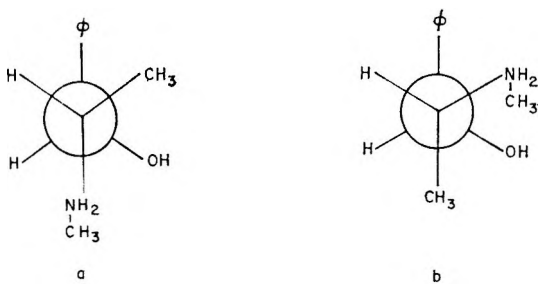


FIG. 1. Calculated preferred conformations of ephedrine (a) and ψ-ephedrine (b).

In view of the results of this work on ephedrine isomers, it now seems appropriate to pursue the same kind of approach with noradrenaline, the most potent of the α-adrenergic agonists, to determine whether the calculated preferred conformation of this molecule is consistent with the postulated hypothetical α-adrenergic receptor model.

A few comments on the molecular orbital theory and the use of the preferred conformation to describe the receptor are in order. Molecular orbital calculations are made on conservative molecules, that is, molecules that do not interact with an environment. The relation between the calculated geometry and the geometry in a crystal or in solution is unknown. However, demonstration of consistency among calculated, crystal, and solution data in our laboratory (Kier, 1967a, b; 1968a, b, c, d)

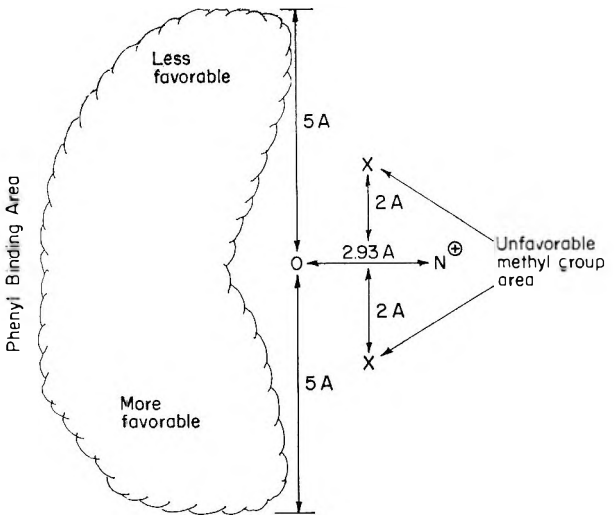


FIG. 2. Postulated α-adrenergic receptor features based on ephedrine isomer studies (Kier, 1968d), and noradrenaline studies (present work).

as well as by others (Giordano, Hamann & others, 1967; Jordan & Pullman, 1968) is an encouraging sign that a relation perhaps exists.

My calculations have revealed energy minima for a particular conformation, from which I have derived hypotheses concerning the corresponding biological receptors. Although it could be argued that a drug molecule may not interact to form a drug-receptor complex in its preferred conformation, it is assumed that interaction occurs in the preferred form. A further premise that has been made is that a calculated rotational barrier of sufficient magnitude will not be overcome by interaction with solvent or another molecule, provided the approach of the drug to the receptor does not permit covalent bond formation. This is certainly so in the highly reversible agonists I have examined to date. Even if the drug in its preferred conformation is not complexed with the receptor, some specific conformation related to the preferred one must engage the receptor or the high degree of structural specificity found for many agonists would not be experienced. The potent muscarinic agents acetylcholine, muscarine and muscarone were found (Kier, 1967b) to present three comparable heteroatoms in a similar relation in their calculated preferred conformations. This consistency suggested that, in this instance, the molecules function at their common muscarinic receptor in their preferred conformations. In a study of histamine (Kier, 1968b), it was found that histamine in its H_1 preferred conformation presented two nitrogen atoms separated by a distance comparable to the internitrogen distance in a potent antagonist molecule. In a third study, on 5-hydroxytryptamine, the internitrogen distance in the calculated preferred conformation corresponded to the internitrogen distance in the potent antagonist lysergic acid diethylamide (Kier, 1968c).

These three examples offer a reasonable justification for a working hypothesis that many drug molecules, engaging their receptors in noncovalent complexes, do so in their preferred conformations. As will be seen, the work on the ephedrine isomers and the present study on noradrenaline are a fourth example supporting this hypothesis.

EXPERIMENTAL

The parameters for the extended Huckel-theory calculations were those previously used (Kier, 1967a). The bond lengths were adopted from X-ray data (Carlstrom & Bergin, 1967) that were known, or were assumed to be, of standard length (Pople & Gordon, 1967). The protonated form of the molecule was considered. The phenolic hydroxyl groups were held stationary, *trans* to each other in relation to the ring plane. The C–C bond of the side-chain was rotated by 60° increments through a full cycle. The phenyl-C bond was considered every 90°.

RESULTS

The total energy versus angle of rotation of the side-chain C–C bond (Fig. 3) reveals a definite minimum at 180°. At this angle, the amino-group is *trans* to the phenyl ring. The distance separating the oxygen and nitrogen atoms is 2·86Å.

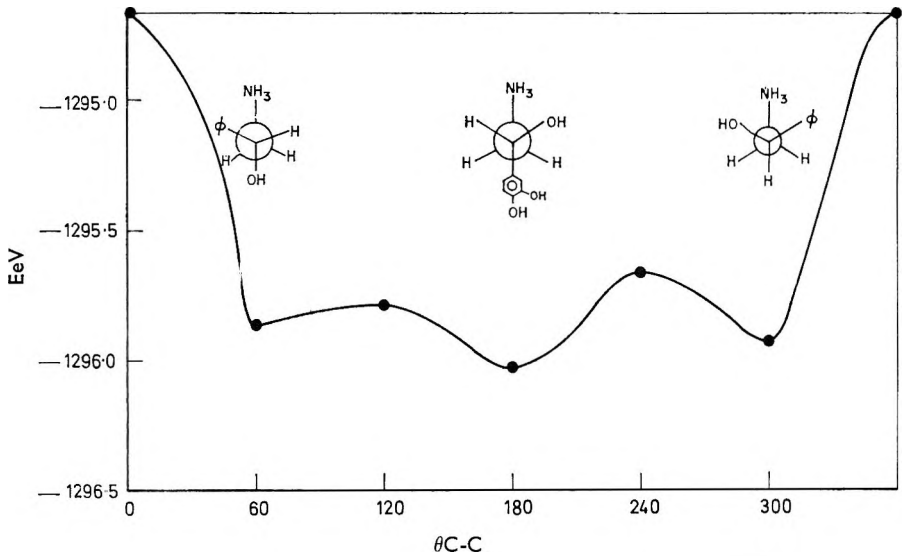
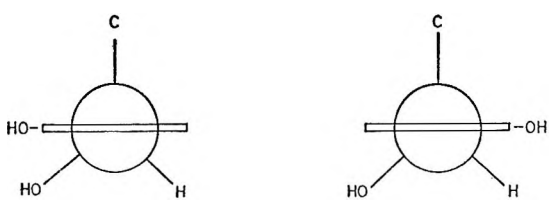


FIG. 3. Energy versus rotation angle of C–C bond in side chain of noradrenaline.



Ia Ib

Rotation of the phenyl group revealed identical energy minima for the 90° and 270° rotamers, depicted by Ia and Ib respectively. Thus, the *meta*-hydroxyl group does not discriminate in either of the two preferred conformations.

The calculated conformation and the conformation derived from X-ray analysis of crystalline (—)-noradrenaline hydrochloride (Carlstrom & Bergin, 1967) are identical.

DISCUSSION

The calculated preferred conformation of noradrenaline places the nitrogen, oxygen, and phenyl ring of the molecule in the same relation as was found in the calculations of the preferred conformation of ephedrine (Kier, 1968d). The oxygen-to-nitrogen atomic distance in noradrenaline (2·86 Å) is very close to the 2·93 Å interatomic distance calculated for ephedrine. The modest difference is due to the slightly different bond lengths used in calculations.

If a relatively planar receptor surface is assumed, the noradrenaline molecule, in its preferred conformation, could present to this receptor the oxygen, nitrogen, and phenyl ring in an identical manner as does (–)-ephedrine. The *meta* hydroxyl group could be positioned at either Ia or Ib with equal preference, according to these calculations. These findings support my hypothesis of the nature of the α-adrenergic receptor (Fig. 2). The greater potency of noradrenaline over (–)-ephedrine must be due to the presence of at least one phenolic hydroxyl group. That both noradrenaline and (–)-ephedrine are α-adrenergic agonists, and that both present key features in an identical manner in their calculated preferred conformation, and also that both calculations agree with physical data, lends validity to the calculations. It also supports the view that these two molecules engage their receptor in their preferred conformations.

Acknowledgement

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Adrenergic receptors in the guinea-pig trachea*

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The effects of adrenaline, noradrenaline and isoprenaline have been investigated on the guinea-pig isolated trachea in the presence of propranolol hydrochloride 10^{-5} and 10^{-6} g/ml. Adrenaline and noradrenaline were both shown to produce contractions of the tissue. The relative order of potency of the catecholamines (adrenaline > noradrenaline > isoprenaline), together with the antagonism exhibited by phenoxybenzamine, suggests that the contractions were due to α-adrenergic receptor involvement.

According to the classification of Ahlquist (1948, 1966), bronchodilatation by sympathomimetic amines is mediated via β-adrenergic receptors. There appears to be some controversy concerning the concomitant existence of excitatory α-adrenergic receptors in the respiratory tract. Castro de la Mata, Penna & Aviado (1962), demonstrated the existence of α-adrenergic receptors which were considered responsible for bronchoconstriction in the anaesthetized dog. The possible existence of similar receptors in cat isolated tracheal muscle has also been discussed by Türker & Kiran (1965). However, Foster (1966) could find no evidence for the presence of α-adrenergic receptors in the isolated trachea of the guinea-pig.

Propranolol has been shown to inhibit the action of β-agonist drugs on the isolated guinea-pig trachea (Foster, 1966). However, in the course of experiments made in the presence of propranolol, adrenaline was sometimes observed to produce contractions of the tissue. This observation suggested α-adrenergic receptor involvement, and further experiments were made to examine this effect in more detail. The ability of noradrenaline and isoprenaline to produce this response has also been investigated.

EXPERIMENTAL

Method

Guinea-pigs of either sex, weighing 400–700 g, were killed by dislocation of the neck. The trachea was removed, cut into a chain containing the equivalent of eleven rings, and suspended in a 25 ml organ bath containing Krebs-Henseleit solution maintained at 37°. The Krebs-Henseleit solution contained the following (per litre): NaCl, 6·9; KCl, 0·35; $CaCl_2$ 0·28; $MgSO_4 \cdot 7H_2O$, 0·11; KH_2PO_4, 0·14; D(+) glucose, 2·0; $NaHCO_3$, 2·1 g. The fluid reservoir and bath were gassed with a mixture of 5% carbon dioxide in oxygen. The tissue was allowed to stabilize to these conditions for 1 h. During this period the tissue was washed with pre-warmed and pre-gassed Krebs-Henseleit solution at 20-min intervals. Tissue responses (isotonic) were recorded on a Brush Mark 250 recorder via a semi-conductor strain gauge transducer. The tension on the tissue was 300 mg.

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Initial experiments consisted of the recording of responses to adrenaline, noradrenaline and isoprenaline before and during exposure of the tissue to propranolol hydrochloride 10^{-6} g/ml added 30 min previously. In later experiments the initial relaxing responses to the three catecholamines were omitted and propranolol hydrochloride 10^{-5} g/ml was in contact with the tissue throughout the duration of the experiment.

Catecholamine responses were recorded for 4 min after which the tissue was washed at 10 min intervals until it returned either to its original, or a steady, level. This usually resulted in an interval of 20–30 min between doses. L-(+)-Ascorbic acid 40 μg/ml and sodium edetate 40 μg/ml were included in the catecholamine solutions.

A maximum contraction and relaxation was produced by acetylcholine and aminophylline, respectively, at the commencement and termination of each experiment with propranolol hydrochloride 10^{-5} g/ml. The effects of between five and nine different concentrations of a catecholamine were studied in each experiment. The sequential method, in which the tissue is washed to recovery between each dose, was used to record concentration-effect. The difference between concentrations was usually two-fold and the responses ranged from minimal to supra-maximal. Those concentrations producing responses were repeated at least once during each experiment. Responses have been expressed as % of maximum. Regression lines were fitted by the method of least squares.

Catecholamine concentrations refer to final bath concentration of the base in g/ml. Other drug concentrations have been expressed as final bath concentration of the salt in g/ml.

Drugs

Drugs used were acetylcholine chloride (Sigma), aminophylline B.P., (±)-isoprenaline hydrochloride U.S.P., (—)-noradrenaline bitartrate monohydrate (Levophed, Winthrop), (—)-adrenaline tartrate (Parke Davis & Co.), propranolol hydrochloride (Inderal, I.C.I.), atropine sulphate, diphenhydramine hydrochloride (Benadryl, Parke Davis & Co.), phenoxybenzamine hydrochloride (Dibenyline, S.K.F.) and dibenamine hydrochloride.

RESULTS

Effect of propranolol 10^{-6} g/ml

Isoprenaline, adrenaline and noradrenaline produced relaxations of the norma trachea. The concentration ranges producing minimal to maximal relaxations to these three catecholamines were as follows: isoprenaline: $5 \times 10^{-10} - 5 \times 10^{-9}$ g/ml; adrenaline: $4 \times 10^{-9} - 3 \times 10^{-8}$ g/ml; noradrenaline: $4 \times 10^{-8} - 2 \times 10^{-7}$ g/ml.

A parallel shift to the right of the isoprenaline, adrenaline and noradrenaline log concentration: response (relaxation) lines occurred in the presence of propranolol 10^{-6}. However, contractions of the tissue were observed in response to concentrations of adrenaline and noradrenaline below those required to produce relaxations as a result of antagonism of the propranolol β-adrenergic receptor blockade. A similar effect was not observed with isoprenaline.

The size of the contractions was insufficient to permit estimations of the relative potency of adrenaline and noradrenaline to be made. In view of this, it was decided to increase the concentration of propranolol ten-fold in an attempt to enhance the

contraction size, and also to increase the concentration range within which these catecholamine contractions could be studied.

Effect of propranolol 10^{-5} *g/ml*

The use of propranolol at this concentration qualitatively increased both the contraction size and concentration range within which contractions to adrenaline and noradrenaline occurred.

A typical record of a section of an experiment made with adrenaline in the presence of propranolol 10^{-5} g/ml is shown in Fig. 1. The responses shown are the result of

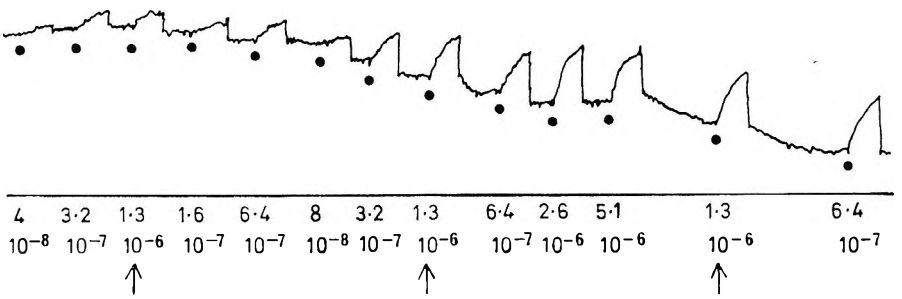

FIG. 1. Guinea-pig isolated trachea suspended in Krebs-Henseleit solution at 37° containing propranolol hydrochloride 10^{-5} g/ml. Responses shown are due to adrenaline at bath concentrations (g/ml) indicated. Adrenaline contact time: 4 min. Interval between doses: 20–30 min. Line above the responses was drawn between the two maximum acetylcholine contractions; line below was drawn between two maximum aminophylline relaxations. Note the influence of loss of inherent tone on the three responses to adrenaline $1{\cdot}3 \times 10^{-6}$ g/ml indicated by arrows (↑).

various concentrations of adrenaline indicated in the figure. The line above the responses was drawn between the two maximum acetylcholine contractions; the line below was drawn between the two points of maximum relaxation to aminophylline. This procedure was carried out in each experiment. The distance between these two lines was essentially the same at both the beginning and end of all experiments indicating that the tissues were still able to produce their full range of response as exhibited by aminophylline and acetylcholine. With these two lines, it is possible to assess the inherent tone of the tissue at any stage of an experiment. The catecholamine contractions may also be expressed in terms of the maximum acetylcholine response of the tissue. A gradual loss of tone occurred during all experiments. This effect, illustrated in Fig. 1, usually occurred within 2–4 h of commencement of an experiment. The rate of loss of tone between preparations was variable. In some preparations a sudden, and relatively rapid, loss occurred over a 2 h period, whilst in others the effect was much slower and a period of 6 h was required before a steady level was reached. At this point, further relaxation could be produced by aminophylline in all tissues.

The tone of the tissue was found to influence the size of the response to a standard dose of adrenaline. This is illustrated also in Fig. 1 where the arrows indicate the responses produced by a standard dose ($1{\cdot}3 \times 10^{-6}$ g/ml) of adrenaline at various times during the same experiment. A large increase in the size of the response was observed as inherent tone was lost; however, when the tone of the tissue had reached a

steady level, the responses to the same concentration of adrenaline were constant. Hence, the increase in contraction amplitude to a standard dose of adrenaline is dependent upon the loss of tone of the tissue. Very similar results were obtained in the experiments made with noradrenaline.

The ability of isoprenaline to produce contractions in the presence of propranolol 10^{-5} g/ml was also investigated. Contractions were not observed at concentrations below those antagonizing the β-adrenergic receptor blockade.

The dose-response relation for adrenaline and noradrenaline is shown in Fig. 2. Six experiments were made with each catecholamine. The regression lines were

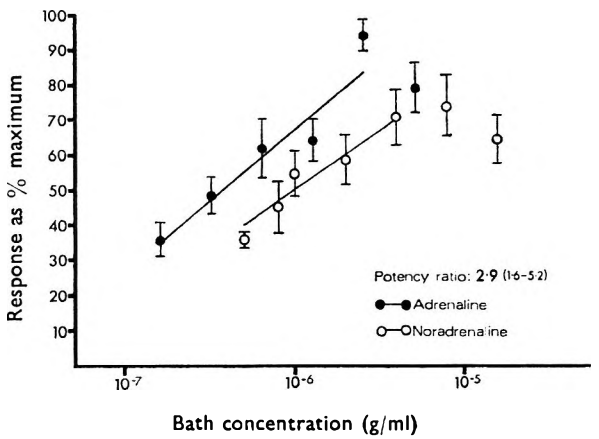

FIG. 2. Mean dose-response lines for adrenaline (●) and noradrenaline (○) contractions of guinea-pig isolated trachea in the presence of propranolol hydrochloride 10^{-5} g/ml. Each line represents the mean results from six experiments. Vertical bars represent standard errors of the means.

calculated from the pooled results of each series of experiments. The large standard errors of the means of the points plotted are due to the variation in responses within the same experiment, as shown in Fig. 1, and also to the differences in sensitivity between preparations. Statistical analysis of the results established that both regressions were linear over the concentration range indicated. No significant departure from parallelism was found and the lines did not coincide. Adrenaline was 2·9 (95% limits, 1·6–5·2) times more potent than noradrenaline. The responses to adrenaline and noradrenaline diminished at concentrations greater than those producing peak effects. If higher concentrations than those shown in Fig. 2 were used, then a relaxation occurred due to antagonism of the propranolol β-adrenergic receptor blockade.

An estimate of the size of the catecholamine contractions in relation to the maximum acetylcholine response was obtained by expressing the largest catecholamine response in each experiment as a percentage of the acetylcholine maximum. The mean response from the six adrenaline experiments was 38·4 (s.e. $\pm$ 2·1)%. The corresponding figure from the noradrenaline series of experiments was 32·0 (s.e. $\pm$ 3·5)%. There was no significant difference (Student's test) between the two means.

Effect of α-adrenergic blocking agents on adrenaline contractions

The effect of phenoxybenzamine and dibenamine was investigated on adrenaline responses at various points on the adrenaline dose response curve. At concentrations

ranging from 10^{-6} to 10^{-5} g/ml, both phenoxybenzamine and dibenamine exerted partial to almost complete antagonism of the responses elicted by adrenaline in the presence of propranolol 10^{-5} g/ml. The effect of phenoxybenzamine 8×10^{-6} g/ml on the contractions due to adrenaline 2×10^{-6} g/ml is illustrated in Fig. 3. Potentiation of

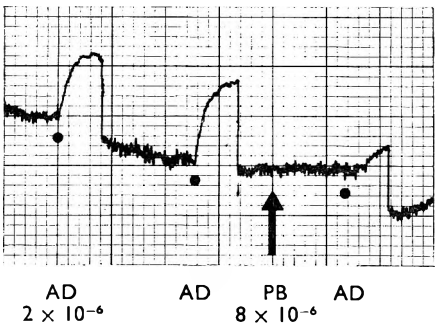

FIG. 3. Isolated guinea-pig trachea suspended in Krebs-Henseleit solution at 37° containing propranolol hydrochloride 10^{-5} g/ml. Antagonism of adrenaline (AD) contractions by phenoxybenzamine (PB). Concentrations are in g/ml. Adrenaline contact time: 4 min.

the adrenaline contractile responses by either phenoxybenzamine or dibenamine was never observed.

Effect of other blocking agents on adrenaline contractions

The contractions produced by adrenaline 2×10^{-6} g/ml in the presence of propranolol 10^{-5} g/ml, were unaffected by either atropine (5×10^{-7} g/ml) or diphenhydramine (10^{-6} g/ml). These concentrations of antagonists were shown to be sufficient to block the effects of acetylcholine and histamine respectively.

DISCUSSION

The guinea-pig isolated trachea has been shown to contract in response to adrenaline and noradrenaline, when propranolol is present in sufficient concentrations to block the β-adrenergic effects of these catecholamines. The size of the responses was shown to be related to inherent tone of the tissue. The largest responses elicited by either adrenaline or noradrenaline occurred when the tissue had minimal tone. Adrenaline was more potent than noradrenaline; however, isoprenaline exhibited no activity under the same conditions. This finding substantiates the observation by Widdicombe (1963) concerning the lack of reports of airway constriction with isoprenaline. The contractions elicited by adrenaline in the presence of propranolol were antagonized by the α-adrenergic blocking agents, phenoxybenzamine and dibenamine, but were unaffected by atropine or diphenhydramine. Thus, the antagonism exhibited would seem to be unrelated to the reported anticholinergic and antihistamine activities of phenoxybenzamine and dibenamine (Goodman & Gilman, 1965).

The order of potency of the three catecholamines in the experiments described coincides with the order of α-adrenergic activity found by Ahlquist (1966) in a wide range of biological actions. This, together with the antagonism exhibited by phenoxybenzamine and dibenamine, satisfies the adrenergic receptor characterization criteria of Ahlquist, and leads to the conclusion that the catecholamine contractions of the guinea-pig isolated trachea are mediated by α-adrenergic receptors.

The results reported here are in agreement with the findings of Takagi, Osada & others (1967), but at variance with those of Foster (1966). Foster never observed contractions to noradrenaline in the presence of propranolol in the concentrations as high as 2×10^{-5} g/ml and concluded that the adrenergic receptors of the guinea-pig trachea were β-receptors. The potentiation of the catecholamine relaxing responses by α-adrenergic blocking agents was explained as being solely due to inhibition of catecholamine uptake (Foster, 1966). Blockade of the α-adrenergic receptors may contribute to this phenomenon.

The previously reported existence of α-adrenergic receptors in dog bronchioles (Castro de la Mata & others, 1962) and now in the guinea-pig trachea, raises the possibility of the existence of similar receptors in human bronchial tissue. This would seem especially pertinent in view of the similar drug sensitivities of guinea-pig tracheal and human bronchial preparations (Hawkins & Schild, 1951), and the similar behaviour of guinea-pig bronchial and human asthmatic bronchial tissues (Herxheimer, 1967). If such receptors were shown to exist, the question should then be raised as to the role of these receptors in the regulation of bronchial tone in both the normal and asthmatic states.

Acknowledgements

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Relative activity of prostaglandins E_1, A_1, E_2 and A_2 on lipolysis, platelet aggregation, smooth muscle and the cardiovascular system

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The relative activities of four prostaglandins (PGE_1, PGA_1, PGE_2 and PGA_2) were determined in several biological tests. They were compared as intestinal muscle stimulants on rabbit duodenum and guinea-pig ileum, as inhibitors of adrenaline-induced lipolysis in rat isolated epididymal fat, inhibitors of platelet aggregation in rabbit plasma, as vasodepressor agents in anaesthetized rats and dogs, and on both blood pressure and cardiac output in unanaesthetized dogs. Formation of PGAs by dehydration and introduction of one additional double bond virtually abolished activity in all of these systems except the cardiovascular system. PGE_2 was more active than PGE_1 on isolated rabbit duodenum and as an antilipolytic agent, but less active in the other systems. Only PGE_1 had high potency as an inhibitor of platelet aggregation.

Prostaglandins are a group of acidic lipids, widely distributed in mammalian tissues, which are highly active in many diverse biological tests. Prostaglandin E_1 (PGE_1) is the most thoroughly studied member of the group, and shows outstanding activity as a smooth muscle stimulant, a nasal vasoconstrictor, a vasodepressor agent, and as an inhibitor of gastric secretion, lipolysis and platelet aggregation. Although published comparisons of natural prostaglandins have, in general, revealed only quantitative differences, preliminary studies on a series of synthetic prostaglandin analogues and derivatives of natural prostaglandins indicate that there are also some qualitative differences (Pike, Kupiecki & Weeks, 1967). The pharmacology of the prostaglandins has recently been extensively reviewed (Pickles, 1967; Bergström, Carlson & Weeks, 1968).

The PGA compounds are analogues of the corresponding PGE compounds wherein an additional double bond is introduced at C 10:11 by dehydration (Fig. 1).

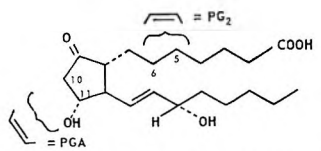

FIG. 1. Structure of prostaglandin E_1 (PGE_1). PG_2s have an additional *cis* double bond at C 5:6. The PGAs are dehydrated derivatives with a double bond at C 10:11 (for nomenclature see Nugteren, van Dorp & others, 1966; Hamberg & Samuelsson, 1967).

The PG_1 and PG_2 compounds differ in that the latter have an additional *cis* double bond at C 5:6. Because of the provocative pharmacological activities of the prostaglandins and possible qualitative differences between some of them, we undertook

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Table 1. *Relative activity of four prostaglandins as smooth muscle stimulants, inhibitors of lipolysis and vasodepressor agents*

Prostaglandin	*In vitro* concns (ng/ml) or *in vivo* doses (μg/kg i.v.)	No. replicate comparisons*	Average response†	Relative activity ($PGE_1 = 1{\cdot}0$)	95% confidence limits	λ‡
			Rabbit isolated duodenum			
E_1	18		24·1			
	56		34·3			
A_2	1000		21·6	0·010	0·0060–0·016	
	3200		27·3			
		7				0·26
E_2	5·6		20·6	3·1	1·9–5·0	
	18		37·7			
A_2	56		13·0	0·084	0·040–0·14	
	180		21·6			
			Guinea-pig isolated ileum			
E_1	1·8		24·6			
	5·6		48·8			
A_2	320		16·6	0·0020	0·0012–0·0028	
	1000		27·1			
		9				0·22
E_2	1·8		25·3	0·85	0·60–1·19	
	5·6		43·4			
A_2	320		31·2	0·0057	0·0040–0·0080	
	1000		42·6			
			Rat epididymal fat			
E_1	10		72·1			
	100		46·0			
A_1	1000		82·5	0·0037	0·0022–0·0060	
	3200		72·5			
	10000		54·6			
		3				0·18
E_2	10		55·8	2·9	1·7–5·2	
	100		38·8			
A_2	1000		63·6	0·029	0·018–0·049	
	3200		45·3			
	10000		33·4			
			Rat blood pressure			
E_1	1·8		20·0			
	5·6		31·0			
A_1	1·8		13·3	0·30	0·12–0·53	
	5·6		17·7			
		6				0·28
E_2	1·8		2·0	0·13	0·04–0·27	
	5·6		15·7			
A_2	1·8		12·0	0·33	0·14–0·58	
	5·6		20·7			
			Dog blood pressure			
E_1	0·10		14·0			
	0·32		45·3			
A_1	0·018		7·7	2·7	1·5–4·1	
	0·056		24·0			
		6				0·22
E_2	0·18		22·7	0·71	0·46–1·13	
	0·56		45·2			
A_2	0·018		8·0	2·5	1·4–3·9	
	0·056		21·7			

a quantitative comparison in several tests systems of PGE_1, E_2, A_1 and A_2. *In vitro*, they were compared as smooth muscle stimulants (rabbit duodenum and guinea-pig ileum), as antilipolytic agents against adrenaline-stimulated lipolysis in rat epididymal fat and as inhibitors of platelet aggregation in platelet-rich rabbit plasma. *In vivo*, they were compared as depressor agents in anaesthetized rats and dogs, and in unanaesthetized dogs for their effect on both blood pressure and cardiac output.

EXPERIMENTAL

PGE_1 and PGE_2 were prepared by biosynthesis using sheep vesicular glands and the appropriate precursor fatty acid (Daniels & Pike, 1968). PGA_1 and PGA_2 were prepared from the respective PGE compound by heating with glacial acetic acid (Pike & others, 1967).

Methods.† Isotonic contractions of longitudinal segments of rabbit duodenum and guinea-pig ileum were recorded kymographically. Two concentrations of each prostaglandin were selected, separated by 0·5 units log concentration, such that the lower concentration gave small but distinct responses. Relative potencies were calculated from the movement of the writing point (mm).

Antilipolytic activity was evaluated using pooled, chopped epididymal fat of rats, incubated for 1 h with 0·1 μg/ml of adrenaline (Walk, Schultz & Weeks, 1968). Lipolysis was measured by glycerol production. Relative potencies were calculated from glycerol production, but are expressed in Table 1 as % of glycerol formed in presence of adrenaline alone (average μ30·6 mole/g h^{-1}).

Inhibition of platelet aggregation was evaluated using ADP-induced aggregation in platelet-rich rabbit plasma by the revolving-loop method of Silver (1965). The times required for the appearance in succession of visible aggregation, "snowstorm phenomenon" and platelet-head formation were recorded. The prostaglandin concentrations tested were increased progressively (100 μg/ml maximum) until the time for appearance of either visible aggregation or the snowstorm phenomenon differed significantly ($P < 0{\cdot}05$) from control values. Since relative potencies were estimated comparing single threshold concentrations, further statistical evaluation was not possible. However, differences between prostaglandins were so great that more detailed evaluation seemed unnecessary.

The vasodepressor activity in anaesthetized dogs and rats was evaluated using pentobarbitone anaesthetized, pentolinium tartrate pre-treated, vagotomized animals. Femoral (dogs) or carotid (rats) arterial pressures were recorded on a polygraph. Prostaglandins were injected into a femoral vein, using the same experimental design as for isolated intestine experiments. Calculations were based upon mm Hg fall in pressure.

† Details of the several experimental methods used as well as a complete tabulation of experimental results and their statistical interpretations are available on specific request with authors' reprints or order NAPS Document 00185 from ASIS National Auxiliary Publications Service, c/o CCM Information Sciences, Inc., 22 West 34th Street, New York, New York 10001; remitting $1.00 for microfiche or $3.00 for photo copies.

Footnotes to table

* Indicates number of muscles, dogs or rats used. In fat tissue, number of replicate vessels each concentration.

† Isolated muscles; mm pen deflection; fat tissue, glycerol production as % of adrenaline stimulated control; rat and dog blood pressure, mm Hg fall.

‡ Standard deviation/slope, an estimate of the precision of the assay.

Vasodepressor activity was also evaluated in four trained unanaesthetized dogs. Blood pressure was measured from a femoral artery by needle puncture. No more than two doses (one compound) could be tested at a session, since dogs would not remain quiet longer than 15 to 20 min. Every dog received two doses of each prostaglandin separated by 0·5 log interval. A four-point assay calculation could not be used because the doses were not the same in all dogs. Individual dogs varied in sensitivity to the prostaglandins. Furthermore, doses which caused over 30 mm Hg fall in pressure often disturbed the dogs, and their moving or struggling masked effects of the prostaglandin. Consequently, low doses sometimes elicited only equivocal responses. The rank order of potency was therefore estimated graphically by plotting mm Hg fall against log dose for each dog, giving greatest weight to doses which produced approximately equivalent effects.

Effects on cardiac output and blood pressure were evaluated in four trained, unanaesthetized dogs with chronically implanted electromagnetic flow-probes on the ascending arch of the aorta and chronic indwelling cannulas in the abdominal aorta. Only a single dose of each prostaglandin was given to each dog. From the dose and magnitude of the response, the rank order of potency was estimated.

RESULTS

The assay results and relative activities of PGA_1, PGE_1 and PGA_2 relative to PGE_1 as smooth muscle stimulants, inhibitors of lipolysis and vasodepressor agents are summarized in Table 1. All four prostaglandins were qualitatively alike as vasodepressor agents, but the PGAs were very weak as smooth muscle stimulants or inhibitors of lipolysis. Relative activities and other statistical interpretations were derived by treating the data as a parallel-line assay with a randomized block design (Finney, 1964). In all cases there was a clear relation between the effect and the concentration or dose, and the values for λ, ranging between 0·18 and 0·28, are reasonably good for these types of assays.

Table 2 summarizes the effectiveness of the prostaglandins as inhibitors of platelet aggregation. Relative potencies are calculated from the minimally effective concentrations. Only PGE_1 was a potent inhibitor of platelet aggregation.

Table 2. *Relative activity of four prostaglandins as inhibitors of ADP-induced aggregation of rabbit platelets*

Prostaglandin	Concentration μg/ml	Time(s) ± s.e. until appearance of: Visible aggregation	"Snowstorm phenomenon"	Platelet head formation	Relative activity (PGE_1 = 1·0)
Control ..		18·3 ± 1·4	25·3 ± 2·3	32·8 ± 3·5	
PGE_1 ..	0·05	27·7 ± 2·8	>40*	—†	
PGA_1 ..	90	18·0 ± 0·7	>57*	—†	0·0006
PGE_2 ..	10	21·2 ± 1·7	42·8 ± 9·1*	>59*	0·005
PGA_2 ..	100	14·0 ± 1·8	22·2 ± 4·0	25·2 ± 2·3	inactive (<0·0005)

* Difference from control significant at $P < 0.05$.

† Change did not occur.

Figures are based upon the average of replicate values in plasma from 6 rabbits for PGE_1 and PGE_2, 5 rabbits for PGA_2 and 4 rabbits for PGA_1.

As vasodepressor agents on unanaesthetized dogs, the activity of the prostaglandins ranked $PGA_1 = PGA_2$ slightly $> PGE_1 > PGE_2$. In all four dogs PGE_2 was clearly the least active, and in two dogs there was virtually no difference between the PGAs and PGE_1. One of the dogs was uniformly more sensitive to the prostaglandins. In this dog the doses used for PGE_1, PGA_1 and PGA_2 ranged between 0·10 and 0·56 μg/kg, for the other dogs between 0·56 and 5·6 μg/kg. PGE_2 was also more active in this dog than in the other dogs. Unfortunately, there were no comparative data available to determine whether the greater sensitivity in this dog was specifically for prostaglandins or for all vasodepressor agents.

Blood pressure and cardiac output measurements were made after 3·2 μg/kg i.v. of PGE_1, PGA_1 and PGA_2 and after 5·6 μg/kg i.v. of PGE_2. There was considerable individual variation in responses, but for all prostaglandins the peripheral resistance decreased since blood pressure fell and cardiac output increased. The rank order was the same as for dogs in which only blood pressure was measured.

DISCUSSION

The influence of structural changes on biological activities of these prostaglandins may be considered from two aspects: (1) the dehydration and introduction of a double bond at C 10:11 (PGE to PGA); and (2) the presence of an additional *cis* double bond at C 5:6 (PG_1 to PG_2).

Dehydration to PGAs causes the most striking changes in activity. There is virtual loss of contractile activity on intestinal smooth muscle, as well as on inhibition of lipolysis and platelet aggregation. On the other hand vasodepressor activities of the PGAs are relatively unaffected, being only about one-third less than PGE_1 in the rat and nearly three-fold greater than PGE_1 in the dog. The preliminary observations previously reported for PGA_1 are confirmed (see Kloeze, 1967; and Table 1, Bergström & others, 1968).

These results are in agreement with the lack of antilipolytic activity of PGA_1 *in vivo* in the dog (Steinberg & Pittman, 1966). Likewise, the greater *in vivo* vasodepressor activity of PGA_1 seen here agrees with its greater *in vitro* relaxing action on dog isolated arterioles (Strong & Bohr, 1967).

The influence of the additional double bond in the PG_2s is not always the same. PGE_2 was more active than PGE_1 on rabbit duodenum but not on guinea-pig ileum. This observation is in agreement with more limited comparisons previously reported (see Table 1; Bergström & others, 1968). As an antilipolytic agent, PGE_2 was significantly more active than PGE_1, but as a vasodepressor agent, less active. The intestinal muscle stimulant and antilipolytic activities of PGA_2, while very weak relative to PGE_1, were several fold greater than PGA_1. This increase was true even in the guinea-pig ileum, which showed little difference between PGE_1 and PGE_2.

Of the four prostaglandins, only PGE_1 was a potent inhibitor of platelet aggregation. Hampton, Harrison & others (1967) reported that several unrelated vasodilators also inhibited platelet aggregation. Since the PGAs are virtually inactive as inhibitors of platelet aggregation and yet at least equal to PGE_1 as vasodilators, no parallelism between vasodilatation and inhibition of platelet aggregation appears to exist.

The comparison of these four prostaglandins in several systems shows that there is no consistent relation between activities and chemical structure.

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Relation of brain sensitivity and hepatic metabolism of hexobarbitone to dose-response relations in infant and young rats*

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The dose-response relation to hexobarbitone of infant (5 day) and young (44 day) male rats was examined, and the relative contribution of hepatic metabolism (measured *in vitro*) and changes in brain sensitivity to the overall response were evaluated. The infant rat shows a parallel shift to the left in its dose-response curve with the relative potency of hexobarbitone almost 5 times greater than for the 44 day old animal. The slope of the curves show marked changes at the first lethal dose level of drug. This and other evidence suggest that death may not be merely an extension of the mechanism causing hypnosis. Infant rats exhibited a shorter increment in sleep time for increasing doses of hexobarbitone than is predictable from their low rate of *in vitro* metabolism. Although this is also true for the young rats, the two values are in much closer agreement than for the infant animals. Brain concentrations of hexobarbitone, measured upon regaining of the righting reflex, were lower in the infant than in the young rat. This suggests the central nervous system of the infant rat has an increased sensitivity to the drug.

The low activity of microsomal liver enzymes of newborn guinea-pigs, mice and rabbits was shown by Jondorf, Maikel & Brodie (1958) and Fouts & Adamson (1959). Kato, Vassanelli & others (1964) surveyed the response to, and metabolism of, several drugs including pentobarbitone, in female rats 1–250 days old. They found that hepatic drug-metabolizing activity increased with increasing age to a maximum at 30 days and then slowly declined. This was opposite to the duration of drug response. Catz & Yaffe (1967) observed that the plasma level of hexobarbitone was the same in 2, 3 or 4 week old mice upon awakening even though the duration of hypnosis differed. In contrast, brain pentobarbitone levels were lower in young than in adult rats at time of death (Bianchine & Ferguson, 1967) which they interpreted as indicative of greater sensitivity of young rats to pentobarbitone. However, toxicity and hypnosis may be unrelated properties (Catz & Yaffe, 1967).

EXPERIMENTAL

Dose-response curves for hexobarbitone hypnosis. On each of 3 trials, 5 and 44 day old, fed, male, Sprague-Dawley rats were divided into four groups of 5 animals. For the 5 day old rats, littermates were evenly distributed between the groups; for the 44 day old rats there was even matching of weights in all groups. Hexobarbitone sodium (Winthrop Laboratories, New York, N.Y.) was injected intraperitoneally in the volume of 5 ml/kg in a dose range of 15–480 mg/kg. Since infant rats lose heat rapidly, they were kept at 35°; the older rats were kept at 22°. Sleeping time was

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recorded as the time interval between losing and regaining the righting reflex. Animals not showing loss of righting reflex 5 min after injection were decapitated and the brain removed and immediately frozen on dry ice. The brains of rats that died from the hexobarbitone were also frozen at the time of death. All other rats were decapitated at the time of regaining of the righting reflex and then brains were frozen. All brains were kept frozen until analysed one or two days later.

Metabolism. *In vitro* metabolic activity was measured using the 9000 *g* supernatant from a 20 min centrifugation (5°) of liver homogenate (1 g liver + 2 ml 1·15% KCl). Incubation was for 30 min at 37° under 5% carbon dioxide in oxygen. The final incubation mixture (molar concentrations), in the order added, consisted of: 2·3 ml pH 7·4 phosphate buffer (0·1); 1·0 ml of a mixture of glucose-6-phosphate (0·02), nicotinamide (0·1) and $MgSO_4$ (0·05); 0·2 ml NADP (0·0025); 0·5 ml hexobarbitone sodium (0·006) and 1·0 ml 9000 *g* supernatant. The amount of unchanged hexobarbitone at the end of the incubation was assayed by the method of Cooper & Brodie (1955). Ten livers, each assayed in duplicate, were used for 44 day old rats; 6 pairs of livers, each assayed in duplicate, were used for 5 day old rats.

Brain concentration of hexobarbitone. Brains from all animals used in the 3 trials were assayed for their hexobarbitone content. Each brain was analysed individually for the 44 day old rats; 2–3 brains were needed for each measurement at time of regaining righting reflex for the 5 day old animals. The brain levels at death were high enough to allow single brains to be analysed in all groups. Brains were weighed, homogenized with 0·001 N NaOH (2 ml/g brain), and 3 ml of the homogenate was immediately added to 4·0 ml pH 5·5 citrate buffer (0·5 M). Hexobarbitone was then extracted into heptane containing 1·5% isoamyl alcohol by the same method used for the liver homogenates (Cooper & Brodie, 1955).

RESULTS

Dose-response relation of hexobarbitone. The 5 day old rat showed major differences from the 44 day old rat in its dose-response relation to hexobarbitone

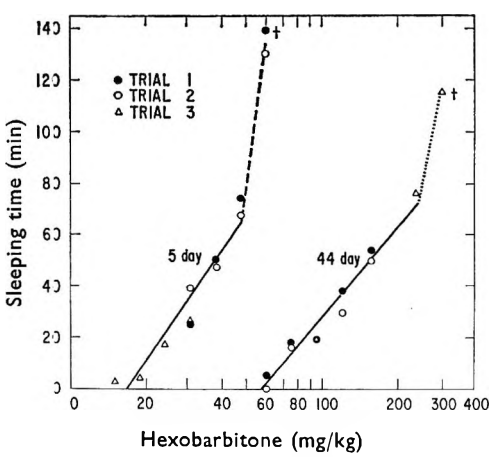

FIG. 1. Dose-response relation for hexobarbitone in 5 and 44 day old rats. Fed, male Sprague-Dawley rats were injected i.p. with 5 ml/kg hexobarbitone sodium in the doses indicated. Sleeping time was measured as the interval between loss and regaining of the righting reflex. Slopes of 140 ± 7·5 (s.e.) and 120 ± 3·7 were obtained for the 5 and 44 day old rats by regression line calculation in the hypnotic range. † Indicates some deaths occurred at that dose. Time till death is not included.

(Fig. 1). Linear regression lines (fitted by least squares; Burn, Finney & Goodwin, 1952) show that the response curves of the two groups are parallel up to the minimal dose causing death, the relative potency of hexobarbitone is about 5 times greater for infant than for young rats, and a distinct change in slope occurs for both age groups at the minimum lethal dose level. Although among 5 day old rats one rat from each litter was in each dose level, there was no rank correlation between littermates for duration of the hypnotic response and no correlation of duration of hypnosis with body weight. The 44 day old rats do not show the latter correlation, either.

Table 1. *Sleeping time*[a] *and brain concentrations*[b] *of hexobarbitone in 5 and 44 day old rats*

		5 day		44 day	
Dose (mg/kg)	Trial	Sleep time (min ± s.e., n)	Brain concn (μg/g ± s.e., n.)	Sleep time (min ± s.e., n)	Brain concn (μg/g ± s.e., n)
15·0	3	3·2 ± 0·6 (5)	17·6 ± — (1)		
18·9	3	4·4 ± 3·0 (5)	14·9 ± 0·9 (2)		
23·8	3	17·4 ± 3·3 (5)	14·9 ± 2·1 (2)		
30·0	1	25·0 ± 4·0 (4)	19·8 ± 1·4 (4)		
	2	38·8 ± 6·8 (5)			
	3	26·0 ± 1·1 (4)	20·1 ± 1·4 (2)		
37·8	1	50·0 ± 15·2 (5)	21·6 ± 1·7 (4)		
	2	47·0 ± 10·6 (5)			
47·6	1	74·2 ± 13·5 (5)	22·0 ± 1·5 (4)		
	2	67·0 ± 9·8 (5)			
60·0	1	†138·8 ± 14·6 (4)	21·7 ± 1·8 (4)	0 ± 0 (5)	20·6 ± 0·5 (2)
	2	130·4 ± 8·9 (5)		5·4 ± 3·0 (5)	
75·6	1	†227·0 ± 57·0 (2)	25·7 ± 2·6 (2)	17·7 ± 6·7 (3)	28·6 ± 1·8 (7)
	2	†287·3 ± 23·0 (3)		16·4 ± 5·4 (5)	
95·2	1			19·0 ± 1·0 (3)	29·9 ± 0·9 (8)
	2			*32·0 ± 5·9 (5)	
	3			19·2 ± 2·1 (5)	32·9 ± 1·2 (5)
120·0	1			38·0 ± 5·9 (4)	28·8 ± 1·0 (8)
	2			29·0 ± 2·9 (4)	
155·9	1			53·5 ± 4·5 (4)	31·2 ± 1·6 (10)
	2			49·8 ± 3·7 (5)	
240·0	3			75·8 ± 10·1 (5)	31·4 ± 0 (2)
302·4	3			†115·8 ± 23·5 (4)	40·3 ± 3·2 (4)
480·0	3			†55·0 ± — (1)	31·5 ± — (1)

[a] Time between loss and regaining of righting reflex after i.p. administration of hexobarbitone sodium in dose indicated; 5·0 ml/kg injected into fed, male Sprague-Dawley rats.

[b] Brain concentration at time of awakening (regaining of righting reflex). 2–3 brains pooled for analysis of 5 day old; individual brains analysed in adults. Brains from rats not falling asleep analysed separately 5 min after injection; rats dying analysed at time of death.

* Group inadvertently fasted overnight, omitted from Fig. 1.

† Each experiment had 5 rats/group. Where numbers after sleeping times differ from these, either animals died or did not lose their righting reflex by 5 min after the injection.

Brain concentration. Table 1 provides figures for the sleep times and the respective brain concentrations upon regaining of the righting reflex. Six dose levels in the hypnotic range as well as two dose levels at which deaths occurred are included. At doses defining the middle two-thirds of the hypnotic range (23·8–37·8 and 95–156 mg/kg for 5 and 44 day old, respectively), brain concentrations within an age group do not differ with dose. There is a significant difference in the brain concentration of

hexobarbitone between the two age groups; the 5 day old rat awakens at significantly lower brain levels than the 44 day old rat (Table 2). Also in this Table are additional measurements of brain concentrations obtained from the few rats exhibiting only ataxia, and from those rats dying from the injected dose. The infant rat seems to exhibit ataxia and death at lower brain levels than does the 44 day old rat.

Table 2. *Brain concentration of hexobarbitone at various stages of response in 5 and 44 day old rats*

Stage[a]	Brain concentration μg/g ± s.e. (n)	
	5 day	44 day
Ataxic	14·2 ± (1)	20·8 ± 2·34 (4)
Awakening from hypnosis	19·8 ± 0·81 (18)	**29·8 ± 0·80 (42)
Dead	77·9 ± 4·21 (6)	**256·5 ± 13·23 (5)

[a] Fed male Sprague-Dawley rats (mean body weights 11·4–14·8 and 180·8–192·6 g) injected i.p. with varying doses of hexobarbitone sodium in volume of 5 ml/kg. Animals decapitated and brain concentrations of unchanged hexobarbitone estimated: (1), 5 min after injection in animals ataxic but not showing loss of righting reflex; (2), upon awakening (regaining righting reflex), or (3) at time of death. Single brains used for analysis in 44 day old; 2–3 brains pooled for analysis in 5 day old.

** Significantly different from the corresponding concentration in the 5 day old at $P < 0{\cdot}01$.

Hepatic metabolism, in vitro. Similarly enriched preparations of 9000 *g* liver supernatants from 5 and 44 day old rats were compared for their ability to metabolize hexobarbitone *in vitro*. The respective metabolic rates (μg/g liver in 30 min ± s.e.) were for 5 day rats 98 ± 27 (6 assays), 39 ± 12 (6 assays), while the 44 day old rats showed rate of metabolism 10–25 times greater: 922 ± 46 (10 assays) and 900 ± 123 (2 assays, each of the pooled livers of 3 rats).

Approximation of LD50. From doses over the range 60–480 mg/kg the limited data yielded approximations of LD50 values which were 75 and 375 mg/kg for the 5 and 44 day old rats, respectively. The curves appear to be parallel on a semi-log plot, but too few values are available to test for parallelism.

DISCUSSION

No single factor was exclusively responsible for the five-fold increase in potency of hexobarbitone in the 5 versus the 44 day old rats. A slower hepatic metabolism and an increased brain sensitivity exist, but do not account quantitatively for the entire prolongation of sleep time. During maturation, glial cells increase in proportion to neurons and an altered brain sensitivity on this basis is considered later.

To evaluate the role of hepatic metabolism, the *in vitro* results were taken as indicative of relative capacities under the conditions used. A 50 min increment in sleep time, from 10 to 60 min (Fig. 1) is produced by a dose increase of 24 or 120 mg/kg in the 5 or 44 day old rat, respectively. Using the *in vitro* rates of metabolism for the two groups, and the liver weights (3·0 and 4·4 g/100 g for 5 and 44 day old), a time can be calculated of 350 and 90 min to metabolize the doses causing an increased sleep time of only 50 min in the 5 and 44 day old rats, respectively. The discrepancy between the observed and calculated sleep times is obviously much greater in the 5 day old animals. A discrepancy of similar magnitude to that calculated for the 44 day old rats has been noted between *in vivo* and *in vitro* results for pentobarbitone (Kato & Takanaka, 1967).

Although Catz & Yaffe (1967) found similar plasma levels in 2, 3 and 4 week old mice at the time of their awakening from hexobarbitone, we find that the 5 day old rat awakens at a lower brain level than the 44 day old (20 *vs* 30 μg/g). Although it is possible that the lower brain concentration in the infant is due to more difficulty in determining the end point because of their slower emergence from hypnosis, we do not believe this to be so since the standard errors for both age groups are similar. We find that the water content of the infant brain is higher (89 *vs* 78%) and comprises a greater percentage of the body mass (4·4 *vs* 0·9%).

During maturation, the proportion of glial cells to neurons increases from 1 : 1 to 2 : 1 between 5 and 44 days (Brizzee, Vogt & Kharetchko, 1964). If central nervous system sensitivity is determined by the concentration at the neuron and if there is an equal distribution of drug between glial cell and neuron, then the relative brain concentrations found for the 44 and 5 day old rats (30 and 20 μg/g) would represent equivalent neuron concentrations of hexobarbitone.

The sharp change in slope at the minimal lethal dose (Fig. 1) and the differing ratios between lethal and hypnotic brain concentrations for 4 and 44 day old rats (4 : 1 and 8 : 1) suggest that the toxic effect may not be merely an extension of the hypnotic effect. Catz & Yaffe (1967) have made such a suggestion on the basis of relative differences in hypnosis and toxicity between strains of mice. The enhanced toxicity of the 5 day old seems at variance with the known resistance of infants of many species to withstand anoxia (and hence the respiratory depression caused by the barbiturates) better than the older animal (Fazekas, Alexander & Himwich, 1941). This protective effect was evident in the delayed time to death of the 5 day old (13–83 min) compared with the 44 day old (3–10 min) rather than in the brain concentration at death (78 *vs* 256 μg/g).

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The effects of osmotic pressure on procaine-induced vacuolation in cell culture

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The effect of increased tonicity on procaine-induced vacuolation in the H.Ep.2 cell has been investigated. An increase in tonicity equivalent to about 1·3% NaCl was found to reduce the formation of the drug-induced vacuoles and also to reduce established vacuolation. There appeared to be no difference in the effectiveness of the osmotic adjusting substances used (sodium chloride, sodium sulphate, glucose, fructose and sucrose) when employed in osmotically equivalent amounts.

A wide variety of weakly basic substances induce cytoplasmic vacuolation in cells in culture (Lettré & Albrecht, 1941, 1943, 1951; Pomerat & Emerson, 1945; Buchsbaum & Kuntz, 1954; Belkin, Hardy & others, 1962). Yang, Strasser & Pomerat (1965) examined the effects of procaine and other substances on Hela cells and chick embryo fibroblasts and discussed possible mechanisms for the vacuolation observed. These workers found that increasing the osmotic pressure of the growth medium by the addition of sucrose did not reduce the rate or extent of the vacuolation.

This paper describes the effects of increased tonicity on procaine-induced vacuolation in human epithelial cells in continuous culture and provides evidence that increased tonicity can influence the course of procaine-induced cell vacuolation.

EXPERIMENTAL

The human heteroploid epithelial-like cell line H.Ep.2 derived from a carcinoma of the larynx by Fjelde (1955) was used in all experiments. The growth medium in which the cells were maintained throughout all experiments consisted of Medium 199 described by Morgan, Morton & Parker (1950), modified by Salk, Youngner & Ward (1954) and supplemented with 20% bovine serum. Sodium benzylpenicillin and streptomycin sulphate were added to a final concentration of 500 units/ml and 150 μg/ml respectively. All media and the H.Ep.2 cell line were supplied by Commonwealth Serum Laboratories, Melbourne. Media were adjusted to the required pH with sodium bicarbonate solution and were sterilized by filtration (Millipore 0·47μm) before use. All experiments were made in 30 ml polystyrene culture flasks (Falcon Plastics) or silicone-stoppered Pyrex T-flasks each containing 5 ml of medium. Each flask was seeded with approximately 750,000 cells and incubated at 37° for 24 h before use.

Experiments were conducted by removing the growth medium from 24 h cultures and replacing it with fresh growth medium alone (controls) or with fresh growth medium plus procaine hydrochloride (3·7mM) and incubating at 37°. The effects of the following osmotic adjustments were observed on both control and procaine-treated cells. In one series, additional solid NaCl was added to the medium to increase the concentration by 0·2, 0·3, 0·4, 0·5, 0·6, 0·7, 0·8, 0·9 or 1% NaCl

(10 flasks were used at each of these concentrations). In other series, osmotic pressure was increased by the addition of anhydrous dextrose to the extent of 1·7, 2·3, 2·8 or 4·7%, fructose 2·3%, sucrose 4·1% or $Na_2SO_4 \cdot 10H_2O$ 1·8% (10 flasks at each concentration). All substances added to the growth medium were added as solids and were dissolved by thorough agitation before the medium was made to volume. The medium was then warmed to 37° and added to the culture flasks.

The progress of vacuolation was followed by phase contrast microscopy (300X). Photographs were taken every hour for the first 24 h, then less frequently, at which times the extent of vacuolation in several random fields of each culture flask was compared with that of cultures treated with procaine alone. Experiments were continued for up to 10 days with the adjustment of pH and replacement of procaine, osmotic adjusting substance and growth medium being made each day. pH measurements were taken before and after media were changed to determine the pH range over which experiments were made. The pH was maintained between 7 and 8 in all experiments; in this range, Yang & others (1959) found the effects of procaine were not pH dependent. Measurements were made with a combination-electrode pH meter immediately after flasks were opened; this minimized the loss of dissolved CO_2 from solutions.

RESULTS

Effects of procaine on cells

In preliminary experiments, the effects of procaine hydrochloride in concentrations ranging from 1–10mM on vacuolation were observed, to determine the concentration producing the most marked vacuolation without producing undue toxic effects. The optimal concentration of procaine was between 3 and 4mM, and as Yang & others (1965) used 3·7mM procaine for Hela cells and chick embryo fibroblasts, it was decided to use this same concentration in all subsequent experiments.

The course of procaine-induced vacuolation in H.Ep.2 cells appeared identical to that described by Yang & others (1965) for Hela cells and chick fibroblasts. The vacuoles started to appear in the perinuclear area and gradually grew in size and number until large vacuoles packed the cells (Fig. 1a). Vacuolation was maximal

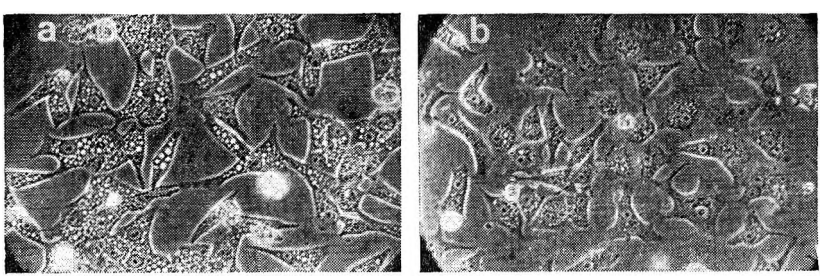

FIG. 1. a. H.Ep.2 cells that have become fully vacuolated after about 24 h exposure to 3·7mM procaine. b. The reduction in vacuolation seen after 30 h exposure to 3·7mM procaine and an additional 0·4% NaCl (bringing the total osmotic pressure of the medium to the equivalent of 1·3% NaCl). Note that occasional cells are vacuole-free.

after about 24 h exposure to procaine (3·7mM) and the cells remained heavily vacuolated for the duration of the experiments. Vacuoles of this nature were never seen in control cultures.

Effects of increased osmotic pressure on vacuolation

Procaine-induced vacuolation was markedly reduced both in the extent and size of vacuoles by the addition of 0·3–0·5% NaCl (bringing the total osmotic pressure to the equivalent of 1·2–1·4% NaCl, since the original growth medium was approximately isotonic). The difference between treatment with procaine alone, and procaine with added NaCl was obvious after about 24 h, and was marked after about 30 h. Cells treated with procaine plus NaCl appeared more epithelial in shape and occasional vacuole-free cells were seen in each field. All cells had fewer vacuoles than those exposed to procaine alone. Maximal vacuolation was never attained in the presence of these strengths of NaCl (Fig. 1b). The addition of 0·2% NaCl exerted little effect on the process while the addition of 0·6% or more NaCl produced toxic effects. This toxicity was also noted in control cultures containing an additional 0·6–1·0% NaCl and no procaine.

The addition of 0·3–0·5% NaCl appeared optimal for the modification of the procaine-induced effects. The addition of 1·7, 2·3 and 2·8% dextrose, providing total osmotic pressures equivalent to 1·2–1·4% NaCl (since 0·9% NaCl is iso-osmotic with 5·1% dextrose), also reduced the extent of vacuolation in a similar manner to equivalent strengths of NaCl. Dextrose 4·7% caused cell death within a few hours. The addition of fructose (2·3%), sucrose (4·1%) or $Na_2SO_4 \cdot 10H_2O$ (1·8%), providing total osmotic pressures in the medium equivalent to 1·3% NaCl (since 5·1% fructose, 9·3% sucrose or 4·0% $Na_2SO_4 \cdot 10H_2O$ is iso-osmotic with 0·9% NaCl), also reduced the extent of procaine-induced vacuolation in a similar manner to osmotically equivalent strengths of NaCl.

When the osmotic adjustments listed above were made to cultures that had become fully vacuolated after 24 h exposure to procaine, it was noted after 1–2 days that vacuolation was markedly less than in cultures treated with procaine alone.

While the vacuolation with procaine was very much reduced in media adjusted to an osmotic pressure equivalent to approx. 1·3% NaCl, cells kept under these conditions were less uniform in shape and had a much more granular cytoplasm than untreated controls. These effects became more pronounced as the strength of the osmotic adjusting substance was increased beyond the equivalent of 1·3% NaCl; there was also a concomitant increase in the proportion of dead cells.

DISCUSSION

Yang & others (1965) found that procaine 3·7mM produced pronounced but reversible vacuolation in Hela cells and chick embryo fibroblasts. We found this also to be true for the H.Ep.2 cell line. Lower strengths of procaine did not produce maximal vacuolation in H.Ep.2 cells and higher strengths proved toxic to the cells within a few hours.

Yang & others (1965) added 10% sucrose to their growth medium to test whether an increased osmotic pressure could reduce drug-induced vacuolation. These workers found no difference in the extent or rate of vacuolation during the 16–18 h the cells were observed. Our study has confirmed that increased osmotic pressure produced no detectable difference during this period, and it was only after about 24 h exposure to procaine and the osmotic adjusting substances that the effect of increased osmotic pressure became obvious. This difference was maintained for the remainder of the experiments (up to 10 days). An increase in osmotic pressure equivalent to

approx. 1·3% NaCl reduced the formation of vacuoles and was also effective in reducing established vacuolation. There appeared to be no difference between the effects of NaCl, glucose, fructose, sucrose or Na_2SO_4 when used in osmotically equivalent amounts. A high concentration of dextrose (4·7%) was added to the growth medium in one series because there are reports (Setnikar & Temelcou, 1959; Vaille & Souchard, 1965) that about 10·5% dextrose (instead of the usual 5·1%) is isotonic with human erythrocytes. Since the addition of 4·7% dextrose destroyed the cells within a few hours, it is unlikely that 10·5% dextrose is isotonic with human epithelial cells. It is interesting that a final tonicity equivalent to approx. 1·3% NaCl appeared optimal for reducing procaine-induced vacuolation since Setnikar & Temelcou (1959) found that about 1·3% NaCl was necessary to render procaine solutions isotonic to rabbit erythrocytes (rabbit red cells are normally isotonic with 0·93% NaCl). These workers suggested that procaine increased the permeability of the erythrocyte to NaCl. They also noted that low concentrations of dextrose (0·6 iso-osmolar) not only abolished the increase in permeability produced by procaine but rendered the cell membrane partially impermeable to procaine itself. Hönig, Malm & Persson (1964) found that dextrose appeared to counteract the haemolytic effects of lignocaine hydrochloride on rabbit erythrocytes below a certain concentration of the local anaesthetic. Under the conditions of our experiments, we were not able to detect a specific protective effect of dextrose on procaine-induced cell vacuolation.

It is known that the extent of drug-induced cytoplasmic vacuolation is related to the concentration of vacuolating agent and that the vacuolation is fully reversible even after 24 h exposure to such substances. Vacuolated cells appear to exhibit normal motility and have frequently been observed to undergo mitosis. It has also been established that pH has a marked effect on vacuolation, presumably because of its influence on the ionization state of the vacuolating agent and thus on its availability to the cell (Yang & others, 1965). The present study indicates that osmotic effects can also influence procaine-induced vacuolation but sufficient information is not yet available for the significance of this finding to be fully interpreted.

Acknowledgements

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LETTERS TO THE EDITOR

Setaria cervi, a test organism for screening antifilarial agents

The methods currently available for the screening of antifilarial agents are time-consuming and inconvenient, since an intermediate orthopod vector is required to transmit the infective larvae to the experimental host. Even then there is a long incubation period before microfilaria appear in the blood; 50 days for *Litomosoides carinii* (Hewitt, Wallace & others, 1947) and 8 months for *Dirofilaria immitis* (Webber & Hawking, 1955).

In the present method, two male and two female adult *Setaria cervi*, a common filarial parasite of cattle, were implanted intraperitonially into rats within 2 h of collection from the slaughter house. Microfilaria appeared in the rat blood in the second week after implantation and continued to be present up to six weeks after which the adult worms were found dead at autopsy.

Rats which showed the presence of microfilaria in peripheral circulation were given diethylcarbamazine orally in doses of 2·5, 5 and 10 mg/100 g. Blood was examined daily by spreading a thick film on the slide, dehaemoglobinizing, and then staining with Leishman stain. Complete disappearance of the microfilaria during 3 consecutive days was accepted as a positive antifilarial response.

Table 1. *The antifilarial activity of diethyl carbamazine against groups of rats infected with* Setaria cervi

Daily oral dose of diethylcarbamazine mg/100 g	Rats surviving	No. of rats cleared of microfilaria	Response %
Control	10/10	0/10	–
10	9/10	9/9	100
5	10/10	7/10	70
2·5	10/10	2/10	20

As shown in Table 1 diethylcarbamazine was found to be completely effective in a dose of 10 mg/100 g and the response diminished with the reduction of the dose. When the drug was discontinued microfilaria reappeared in the blood.

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Protection against semicarbazide-induced convulsions in mice at a hypobaric pressure

Exposure to hyperbaric oxygen causes marked reduction of brain γ-aminobutyric acid (Wood, Watson & Ducker, 1967) and also convulsions in mice (Faiman & Hable, 1966). Although effects opposite to those of hyperbaric oxygen have been reported on brain γ-aminobutyric acid in mice after exposure to decompression hypoxia (Wood, Watson & Ducker, 1968) the relation of this environment to convulsions has not been investigated. We have found decompression hypoxia to antagonize the convulsant properties of semicarbazide.

Male, albino mice, random bred of the Swiss Webster strain (Charles River Farms), weighing 25–35 g, were injected intraperitoneally with a freshly prepared aqueous solution of semicarbazide (200 mg/kg). They were placed in pairs, in Plexiglass desiccators and either exposed to a hypobaric environment equivalent to an altitude of 19,000 ft (364 mm Hg; pO_2 = 76 mm Hg) for 5 h, the pressure being reduced at a rate of 50 mm Hg min^{-1}, or kept at room atmosphere (760 mm Hg; pO_2 = 159 mm Hg). Room air was used. Plexiglass desiccators had a height of 14 inches and inside diameter of 10 inches, they were connected in series to a vacuum pump (Baumel, Robinson & Blatt, 1967). Clonic or tonic convulsions were recorded in individual mice by two trained observers. The experiments were made at room temperature of 21–23°.

The incidence of convulsions and the course of their onset were altered markedly in the mice exposed to the hypobaric environment (Fig. 1).

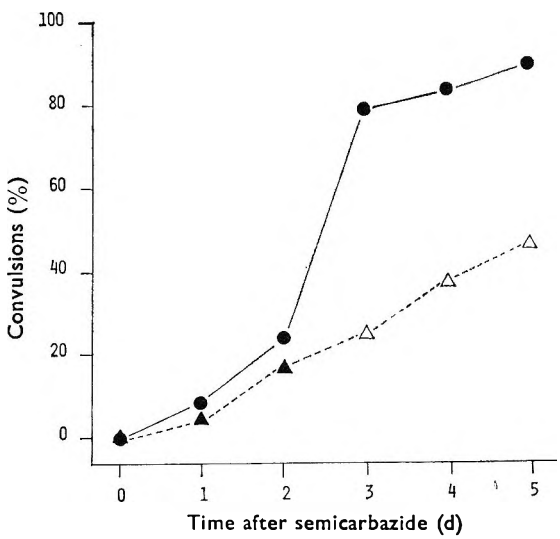

FIG. 1. Effect of exposure to hypobaric environment on convulsions due to semicarbazide. Each point represents % convulsed out of 24 mice. Differences between two groups indicated by open symbols are statistically significant ($P < 0.005$). Circles = 760 mm Hg. Triangles = 364 mm Hg.

Wood & others (1967) proposed that impairment of oxidative metabolism while breathing hypoxic air (Gurdjian, Webster & Stone, 1949) led to decreased use of γ-aminobutyric acid in the shunt pathway of the tricarboxylic acid cycle. Since convulsions produced by semicarbazide are associated with decreased levels of

γ-aminobutyric acid (Killam & Bain, 1957), the depleting action of semicarbazide on the level of the acid is likely to be compensated for by a decreased use of the acid during altitude exposure, resulting in an anticonvulsant effect.

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On the locus of the airway constricting effect of histamine in the dog

Although the ability of histamine to constrict the airway smooth musculature of a variety of animal species (including man) has been recognized for a number of years, little evidence has appeared in the literature concerning the precise location within the tracheobronchial tree at which this effect of histamine manifests itself. We now report the results of experiments which shed light on the problem.

The experiments were made on five mongrel dogs (6 to 10 kg) anaesthetized with a solution containing 100 mg of allobarbitone, 400 mg of urethane and 400 mg of monoethylurea in each ml (0·6 ml/kg i.p.). The animals were intubated with a cuffed Murphy endotracheal tube and their interpleural spaces were cannulated. Airflow was measured with a Fleisch pneumotachograph in conjunction with a differential strain gauge (Sanborn 270). A volume signal was obtained by electrical integration of the flow signal. Transpulmonary pressure was measured with a differential pressure transducer (Sanborn 267B), one side being connected to the interpleural cannula and the other side to an opening in the endotracheal tube. Individual parameters of airflow, volume and transpulmonary pressure were recorded simultaneously on a Sanborn multi-channel recorder and from these tracings values for total lung resistance were obtained by the method of Amdur & Mead (1958).

Constriction of airway smooth muscle was induced by administering five inhalations of a 2% solution of histamine base from a DeVilbiss No. 42 nebulizer. Arterial blood samples were collected anaerobically via a polyethylene cannula inserted into a femoral artery. The oxygen tension (pO_2) of the arterial blood was determined with a Radiometer oxygen microelectrode. The pH of each blood sample was measured as drawn at 38° with a Radiometer ultra-micro capillary electrode unit and again after the blood was equilibrated with two different known CO_2 mixtures. The nomogram of Siggaard Andersen & Engel (1960) was then used to determine the carbon dioxide tension (pCO_2) of each sample.

The results of a typical experiment are presented in Table 1. Intrapulmonic administration of the histamine solution evoked a rapid increase in respiratory minute volume and total lung resistance. The increased minute volume was the

consequence of a highly elevated respiratory rate and not the result of an increase in tidal volume which, in most instances, was much diminished. As a rule, tachypnoeic breathing began after an initial period of apnoea lasting 10–30 s, reached a maximum within 2 or 3 min, then gradually subsided. The precise cause of the respiratory stimulation was not investigated. Certainly, the high arterial pCO_2 and low pO_2, produced as a result of the histamine inhalations, must have contributed to the observed increase in respiratory frequency. However other factors, such as reflex stimulation of the respiratory centre through reflexes originating in the carotid sinuses and homologous regions sensitive to the hypotensive effect of histamine, may also have played a significant role. The important finding here is that a nearly twofold increase in respiratory minute volume was virtually ineffective in restoring the arterial blood gas values to normal. As shown in Table 1, the histamine induced hypercapnic hypoxia began within 30 s after the last breath of histamine was administered and persisted for the duration of the measurement period. It is apparent then, that the observed increase in respiratory minute volume merely reflected enhanced movement of dead space air and was not associated with augmented alveolar ventilation.

Table 1. *The effect of histamine inhalation on the respiratory minute volume, pulmonary resistance and blood gases of an anaesthetized, spontaneously breathing dog*

	Minute volume (litres/min)	Pulmonary resistance (cm H_2O/litres s^{-1})	pO_2 (mm Hg)	pCO_2 (mm Hg)
Control	3·39	8·3	97	42
30 s	4·69	16·7	70	50
1 min	4·75	21·8	62	52
2 min	5·96	19·6	62	52
3 min	6·04	18·2	67	54
5 min	4·35	14·4	72	51

These findings suggest that when histamine is administered intrapulmonically it exerts a strong constricting action on the peripheral airways (i.e., the terminal bronchioles or alveolar ducts) thereby diminishing the total number of lung units being ventilated. This effect manifests itself as an increase in total lung resistance and a decrease in alveolar ventilation. It should be pointed out that these results do not preclude a possible secondary effect of histamine on the proximal airways, either directly or reflexly via pulmonary vagal efferents. Additional studies will be required to determine the extent to which the increase in pulmonary resistance was due to an effect on the upper air passages.

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Jejunal ulcers produced by indomethacin

While studying the anti-inflammatory effect of indomethacin in rats we frequently observed acute perforating jejunal ulcers leading to generalized peritonitis. The following experiments were made to verify this accidental observation.

Female Sprague-Dawley rats (Holtzman Farms, Madison, Wisconsin, U.S.A.) weighing about 100 g and kept on Purina Laboratory Chow and tap water *ad libitum* received single doses of indomethacin (1, 2, 4 and 8 mg) into the jugular vein. Depending on the dose, 20–90% of the rats died within two to four days. The survivors were killed at different intervals after indomethacin treatment.

After macroscopic inspection specimens of jejuna were fixed in ethanol-formol and embedded in paraffin for staining with haematoxylin-phloxine and with the PAS technique.

On opening the abdominal cavity, generalized peritonitis was observed. A serofibrinous exudate filled the abdomen and many adhesions developed between the intestinal loops. Twenty-four h after treatment numerous circular or elongated ulcers 2 to 4 mm in size became evident in the jejunum. Some of these ulcers were superficial, affecting only the mucosa, others penetrated into the muscularis and destroyed the entire intestinal wall. The most severe lesions were observed between the second and the fourth days. Doses as low as 1 mg indomethacin caused ulceration while the administration of 8 mg led to extensive lesions and accelerated death. No ulcers were found in the stomach, ileum or colon.

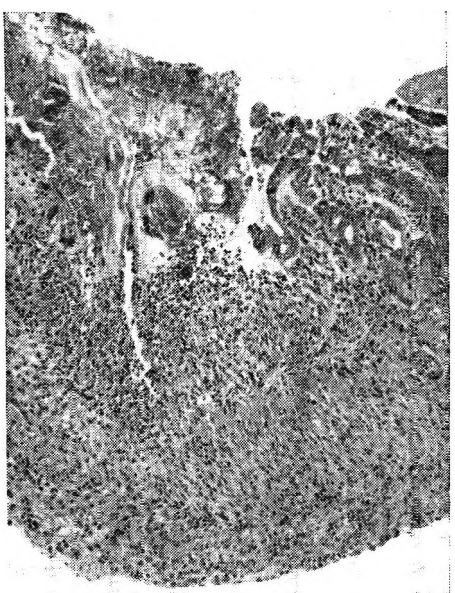

FIG. 1. Acute ulcer in the jejunum four days after the intravenous injection of 4 mg indomethacin. (PAS 75X).

Histologic examination confirmed the macroscopic findings. Marked inflammation was observed in the vicinity of the ulcers extending into the mesentery. The small vessels were dilated and filled with erythrocytes but there were no thrombi or vascular occlusions. Numerous polymorphonuclear leukocytes and fibrin strands could be seen on the serosa and in the mesenteric fat.

Patients treated with indomethacin have developed gastrointestinal symptoms such as nausea, vomiting, dyspepsia, peptic ulcer and gastrointestinal haemorrhage, particularly when large doses were given (Lövgren & Allander, 1964; Ballabio, 1965; Rothermich, 1966). Anderson (1965) found that indomethacin can cause gastric erosion and haemorrhage in starved guinea-pigs. In dogs, Nicoloff (1968) observed that the administration of indomethacin induced antral and gastric ulceration with melena and perforation, as well as jejunal ulcers.

Our investigations show that in rats the jejunum is most susceptible to the ulcerogenic effect of indomethacin. The pathogenesis of jejunal ulcers still needs elucidation and it is felt that this easily reproducible experimental model may facilitate the study of factors influencing jejunal ulcers in rats.

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Effect of subcutaneously administered degraded carrageenan on the production of histamine-induced gastric and duodenal ulceration

We recently reported the pronounced and prolonged inhibitory effect of parenterally administered degraded carrageenan on the acid gastric secretory response of the guinea-pig to histamine (Eagleton, Watt & Marcus, 1968). We have now made a comparison of the protection afforded by parenteral carrageenan against histamine-induced gastric ulceration and histamine-induced duodenal ulceration in the same species.

For the selective production of gastric and duodenal ulcers, histamine acid phosphate was given to fasted adult male albino guinea-pigs, 550–650 g, and the lesions evaluated (Eagleton & Watt, 1965, 1967). Freshly prepared degraded carrageenan (5% aqueous solution) derived from the red seaweed *Eucheuma spinosum* was given as a single subcutaneous injection, 400 mg/kg, to all test animals 12 h before administration of histamine; control animals received no carrageenan. For the production of gastric ulcers, the animals were injected intraperitoneally with 5 mg of histamine (doses refer to the salt) per kg and killed 3 h later, i.e. 15 h after receiving carrageenan. For the production of duodenal ulcers, 8 injections of 0·25 mg histamine/kg were given intramuscularly at $\frac{1}{2}$ h intervals; the animals were killed 4 h after the first injection of histamine, i.e. 16 h after receiving carrageenan.

We also investigated the effect of degraded carrageenan in doses ranging from 195 to 550 mg/kg on the incidence and severity of histamine-induced duodenal ulceration, the carrageenan being injected 12 h before histamine. The volume and total acid concentration (titration with phenolphthalein as indicator) of the gastric juices removed at autopsy were measured.

Table 1. *Effect of 12 h pretreatment with subcutaneously administered degraded carrageenan on histamine-induced gastric ulceration*

Group	Incidence of gastric ulceration	No. of animals with gastric lesions of severity†			
		±	+	++	+++
Control (7)*	100%	0	0	4	3
	P > 0·30				
Test (15)	87%	1	4	8	0

* No. of animals.
† Arbitrary scale. ± = Tiny areas of epithelial loss just visible to the naked eye. +++ = Numerous extensive areas of epithelial loss involving at least 2 of the three divisions of the stomach.

Table 2. *Effect of 12 h pretreatment with subcutaneously administered degraded carrageenan on histamine-induced duodenal ulceration*

Group	Duodenal ulceration			Mean length of duodenum involved (cm)
	Incidence	No. with	No. without	
Control (14)*	100%	14	0	8·5 ± 1·7
	P < 0·01			P < 0·01
Test (20)	55%	11	9	0·3 ± 0·6

* No. of animals.

Table 1 shows the effect of degraded carrageenan on the production of gastric ulceration. There was slight reduction in the incidence of ulceration, 2 animals being completely protected. Of the 13 animals with ulceration, 5 presented lesions of low severity.

The effect of degraded carrageenan on the production of duodenal ulceration is shown in Table 2. Both the incidence of ulceration and the severity of damage were greatly reduced.

In the dose-response study, at 7 dose levels below 400 mg/kg the incidence of duodenal ulceration was 56% and the average length of duodenum involved was 0·6 cm. At 6 dose levels at and above 400 mg/kg, the incidence of ulceration was 17% and the average length of duodenum involved was 0·2 cm. In all the animals in the dose-response study, the gastric juice volumes at autopsy were 1 ml or less and the total acid concentrations ranged from 43 to 79 m-equiv/litre, values which are below fasting for the guinea-pig. In control animals, juice volumes ranged from 9 to 12 ml and total acid concentrations from 105 to 112 m-equiv/litre.

From these results it is evident that subcutaneously administered degraded carrageenan offers marked protection against histamine-induced duodenal ulceration. This is in contrast to antihistamines which show no protective action against such lesions (Watt & Eagleton, 1966). On the other hand, the degree of protection afforded by antihistamines against histamine-induced gastric ulceration (60% protection) is much greater than that afforded by carrageenan in the above study (13% protection) (Watt & Eagleton, 1964).

In animals given parenteral degraded carrageenan, repeated tests have failed to demonstrate the presence of toluidine blue metachromasia either in the fasting or

histamine-stimulated gastric secretions. The absence of detectable amounts of degraded carrageenan in the gastric or duodenal contents indicates that the protection against histamine-induced duodenal ulceration afforded by parenteral carrageenan is not attributable to a local antipeptic action. On the other hand, in contrast to antihistamines which neither suppress histamine-stimulated acid secretion nor reduce the severity of histamine-induced duodenal ulceration, parenterally administered carrageenan both suppresses acid secretion and has a marked protective effect against histamine-induced duodenal ulceration. It is reasonable to suppose, therefore, that this inhibition of acid gastric secretion by systemic carrageenan is a major factor in preventing histamine-induced duodenal damage.

The sulphated polysaccharides are in general classified as antipeptic agents and their role in preventing experimental gastroduodenal ulceration as well as in the therapy of peptic ulceration in man is largely attributed to this property (Houck, Bhayana & Lee, 1960; Sun, 1967). From our own observations we consider that the systemic anti-secretory action of sulphated polysaccharides, in particular degraded carrageenan, is an important aspect of their pharmacological activity that should not be ignored.

In several species, viz. rabbit, rat and guinea-pig, we have observed that when degraded carrageenan—and indeed even a crude aqueous extract of the seaweeds *Chondrus crispus* or *Eucheuma spinosum*—is given orally, both the urine and faeces show the presence of metachromatic staining material demonstrable by toluidine blue. Similarly staining material is also present in macrophages in colonic mucosa and mesenteric lymph nodes, in Kupfer's cells in the liver, as well as epithelial cells of the convoluted tubules and collecting ducts of the kidney. This is strong evidence that some of the degraded carrageenan is absorbed into the blood stream, as Anderson & Soman (1963) had already suspected from their studies in the guinea-pig. It may be, therefore, that apart from any local action in the stomach, degraded carrageenan when given orally is absorbed from the bowel and thereafter exerts a systemic anti-acid secretory and anti-ulcer effect, either by complexing with histamine or by stimulating the release into the blood stream of diamine oxidase as occurs in response to the sulphated polysaccharide heparin (Dahlbäck, Hansson & others, 1968). In retrospect, this could adequately explain the inhibition of histamine-stimulated gastric secretion observed in guinea-pigs fed oral carrageenan over a 2 week period (Anderson, Marcus & Watt, 1962). Similarly, such a systemic anti-acid secretory action could also account for the protection against histamine-induced duodenal ulceration which Houck & others (1960) observed in dogs fed carrageenan over a 30 day period.

Recently, it has been shown both in experimental animals and in man that the sulphated polysaccharide heparin given intravenously has an inhibitory action on acid gastric secretion (Thompson, Lerner & Musicant, 1966; Watt, Eagleton & Marcus, 1966). This inhibitory action in man affects basal secretion as well as stimulated secretion. Heparin's anticoagulant properties, however, would contra-indicate its therapeutic use in the management of patients with peptic ulceration. Such contraindication would not apply to degraded carrageenan (Anderson & Duncan, 1965). Nor is there any granulomatous reaction at the site of injection of degraded carrageenan as occurs with the undegraded preparation. The therapeutic possibilities of parenterally administered sulphated polysaccharides, whether synthetic or naturally occurring, would appear to warrant further exploration, particularly in relation to duodenal ulceration.

We thank Dr. G. B. Shirlaw of Laboratories Glaxo-Evans, Paris, for supplying degraded carrageenan.

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Parallel assay of prostaglandin-like activity in rat inflammatory exudate by means of cascade superfusion

Pharmacological activity was found in inflammatory exudates from rats treated with carrageenan, and was predominantly attributable to the presence of E-type prostaglandins. Some of this work has been briefly reported to the British Pharmacological Society (Willis 1968). Novel features of the experimental methods used are presented here in more detail.

Cascade superfusion for the detection and assay of prostaglandins has been used previously (Ferreira & Vane, 1968; Gilmore, Vane & Wyllie, 1968). However the modifications described below allow reduced flow rates and thus increased sensitivity for the repeated parallel assay of small amounts of prostaglandin-like activity.

Isolated tissues were suspended in chambers formed from non-wettable polypropylene and superfused in series with a stream of Tyrode solution delivered at constant rate by a roller pump (Watson Marlowe MHRE). Tyrode in the reservoir was gassed with air and contained atropine (10^{-6}M), mepyramine (10^{-6}M) and methysergide bimaleate (5×10^{-7}M). The entire cascade was enclosed in a Perspex-fronted box maintained at near 37° and saturated with water vapour from a humidifying device (Fig. 1). This arrangement permitted prolonged survival of up to four tissues with superfusion rates of only 2 to 4 ml/min. Standard prostaglandins (PGs) and extracts in Tyrode solution (0·8 ml or less) were injected into the inlet side of the silicone rubber roller pump tubing. Responses of the tissues were recorded on a kymograph by pendulum levers (Paton 1957) with lengthened writing arms (Schild 1947).

The principal isolated tissue used was the rat stomach strip as prepared by Vane (1957). It was found that a prolonged settling period of several hours resulted in a steady base-line and high sensitivity to prostaglandins (0·5 to 1 ng of PGE_2). This tissue was often used in conjunction with the chick rectum (Mann & West, 1950, Ferreira & Vane, 1967). It was found that under the conditions described, the Tyrode superfused rectum from chicks of 150–200 g responded in a selectively sensitive manner to E-type prostaglandins. This tissuewhile virtually equi-

sensitive to PGs E_1 and E_2 was at least 100 times less sensitive to $PGF_1\alpha$ and 20 times less sensitive to $PGF_2\alpha$.

Other tissues used have been the gerbil (*Meriones shawii*) colon, the guinea-pig proximal colon and rat colon (see review by Bergstrom, Carlson & Weeks, 1968).

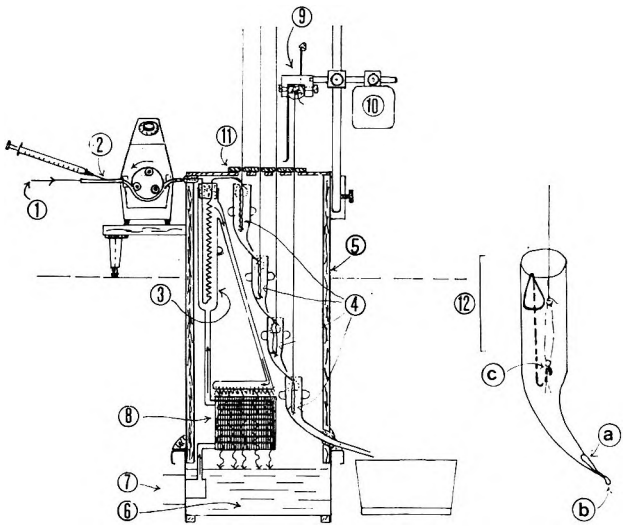

FIG. 1. The modified cascade apparatus. (1) Aerated atropinized Tyrode delivered at constant rate by a roller pump. (2) Extracts and standard prostaglandins injected through a small needle into the self-sealing inlet side of the silicone-rubber roller pump tubing. (3) Warming coil of fine polythene tubing. (4) Isolated tissues suspended in polypropylene chambers arranged in cascade. (5) Perspex-fronted cabinet constructed from marine plywood and sealed with polyurethane. (6) Water tank thermostatically maintained at 38°. (7) Pump. (8) Humidifying and air warming device. Warm water circulated through a series of finned tubes over which water returning from the warming coil trickles back to the tank. (9) Pendulum-type recording levers with lengthened frontal writing arms. (10) Vibrator. (11) Adjustable Perspex discs to seal top of the cabinet where strings pass to the recording levers. (12) Detail of isolated tissue chamber drawn from a polypropylene tube. (*a*) Open channel to provide free drainage of fluid to next tissue; (*b*) Rounded lip to avoid friction with thread; (*c*) Isolated tissue suspended from stainless steel hook clipped to side of the chamber.

The first source of prostaglandin-like activity was the oedema fluid from rat feet inflamed by carrageenan (Winter, 1962). A 1% suspension of carrageenan (Gelazone S.T.1, Whiffen) in saline was injected (0·1 ml) into the subplantar surface of a hind foot through a 26 gauge needle. Between 1 and $5\frac{1}{2}$ h later, groups of 5 to 10 animals were killed and the injected feet excised at the ankle joint. The oedema fluid was then squeezed out using a hand vice with rubber jaws, pooled in graduated centrifuge tubes and immediately extracted.

It could be argued that some or all of the pharmacological activity present in the fluid from the feet was due to physical damage of the tissue, inherent in this method. To obviate this the "carrageenan air bleb technique" was developed from the granuloma pouch of Selye (1954). In this way large volumes of exudate are recoverable for extraction and the fluid in the bleb is relatively free from contact with physically damaged tissue. This technique also permits the study of changes in levels of the pharmacologically active principles with time, although one should not assume that events are necessarily parallel in the air bleb and in the carrageenan-inflamed foot.

Rats were anaesthetized with ether, the skin of the back shaved, swabbed with cetrimide 1% in ethanol, and 10 ml of air injected through a bacterial filter (Millipore) with a 23 gauge sterile needle. 5 ml of a 2% carrageenan suspension in sterile

saline, warmed to about 37°, was then injected into the air pouch which extended along the dorsal part of the thorax. In this case a 19 gauge needle was used. In some experiments the carrageenan suspension was autoclaved for 15 min at 121° before injection. Groups of animals were killed at intervals up to 24 h after carrageenan injection and the fluid in the bleb swiftly and cleanly withdrawn into plastic syringes using 19 gauge needles.

For prostaglandin extraction the individual samples of bleb fluid were transferred to graduated centrifuge tubes, acidified to pH 3 with hydrochloric acid and extracted twice with an equal volume of ethyl acetate. This was removed under partial vacuum at 60° using a rotary evaporator and the dried material reconstituted in 0·5 to 1 ml of Tyrode solution before assay. The dried extracts were stored at 3° for up to 4 days whilst awaiting assay.

Table 1.

Source of activity	Activity extracted into ethyl acetate at pH 3 assayed as ng/ml of PGE_2*			
	Rat stomach strip	Gerbil colon	Chick rectum	Guinea-pig prox. colon
Oedema fluid from foot	3·7	3·9	2·4	—
	5·3	6·1	3·6	—
	6·0	7·9	8·3	—
Contents of sub-cutaneous air pouch	7·7	5·8	5·0	—
	8·0	8·1	8·5	—
	17·5	17·8	25·0	—
	26·7	20·7	31·0	—
	31·3	28·7	27·0	—
	34·3	34·3	38·3	—
	53·7	—	—	74·4
	82·3	—	—	85·2
	100·0	—	—	74·0

* Figures given are in ascending order after varying time intervals.

Parallel assay of activity in extracts recovered during carrageenan inflammation are shown in Table 1. These results suggest that activity in the extracts is mainly attributable to the presence of E-type prostaglandins. This assumption has been supported in further studies using thin-layer chromatographic separation.

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A method for the measurement of therapeutic levels of (+)-amphetamine in human plasma

The absence of a specific and sensitive method for the measurement of therapeutic levels of unchanged and radioactively unlabelled (+)-amphetamine in human plasma has limited kinetic studies to investigations of urinary excretion (Beckett & Rowland, 1965a; Beckett, Tucker & Moffat, 1967). I present here an improved procedure based on the gas-chromatographic technique of Cartoni & Stefano (1963), subsequently developed by Beckett & Rowland (1965b), which has now enabled plasma levels to be estimated.

Procedure. Plasma (10 ml) was made alkaline by the addition of 1 ml N NaOH and extracted by gentle shaking with 10 ml and 5 ml aliquots of diethyl ether (distilled over Na wire to remove oxide contaminants) for 5 min in a 3-dimensional mechanical shaker (Desaga A.-G., Heidelberg). Vigorous shaking resulted in the formation of emulsions. After centrifuging at 4000 rev/min, the ether extracts were combined and stored in a glass-stoppered centrifuge tube (15 ml) at $-5°$ to freeze out dissolved aqueous phase containing plasma constituents which would interfere with the subsequent detection. Drying with anhydrous Na_2SO_4 caused appreciable losses due to adsorption. The organic phase was decanted and 1 ml of an ethereal solution of *NN*-dimethylaniline (0·04 μg/ml) added as the internal standard. Transfer of the bases into 0·5 ml of 0·1N H_2SO_4 (ether-washed) was accomplished by passing a steady stream of N_2 through the acid phase to disperse the layers and give accompanying solvent evaporation. After washing the acid phase with diethyl ether (4 ml), this was basified by the addition of 0·2 ml of N NaOH (ether-washed) and shaken for 1 min with diethyl ether (2 ml) on a Fisons "Whirlimixer". On separation in the centrifuge, the ether layer was transferred to a special tube for controlled evaporation to 50μl with a slow stream of N_2 (18°). This consists of a 15 ml centrifuge tube drawn out at the bottom into a sealed narrow bore (1 inch long) with an approximate capacity of 100 μl (Laboratory Glassware Manufacturers, Beckenham, Kent).

Mild conditions are essential for evaporation of the solvent. If evaporation to dryness occurs, 80% or more of the amphetamine and a large proportion of the internal standard are volatilized. The final extracts are stored at $-5°$ and removed immediately before analysis: this precipitates the aqueous layer and prevents evaporation of the organic phase.

Suitable peaks for (+)-amphetamine on the chromatogram recordings resulted when the columns were injected with 2–4 μl of the concentrate (Fig. 1). To calculate the concentration of (+)-amphetamine, a linear reference curve was constructed by plotting the ratio of the peak areas (height $\times$ width at half height) for (+)-amphetamine and *NN*-dimethylaniline over the concentration range 10–80 ng/ml of plasma.

Recoveries over this range were 75 $\pm$ 7%. Amphetamine is known to be bound to plasma proteins to the extent of approximately 15% (Axelrod, 1954). Comparison of the recovery of (+)-amphetamine obtained from plasma containing 10 ng/ml with that from an aqueous solution of the same concentration, indicated that both free and protein-bound forms of the drug were extracted *in toto* by the above procedure. This would infer that losses in recovery of (+)-amphetamine are in the main a consequence of its volatility.

A Series 104 model 24 chromatograph (W. G. Pye), equipped with dual flame ionization detectors, was used. The signal was recorded on a Honeywell $-0·1$ to $+0·1$ mV recorder. The column support was an 80–100 mesh acid-washed dimethyldichlorosilane-treated Chromosorb G, coated with 5% KOH and 15% Carbowax 6000. This was packed into silanized glass columns (1·5 m long $\times$ 4 mm internal diameter),

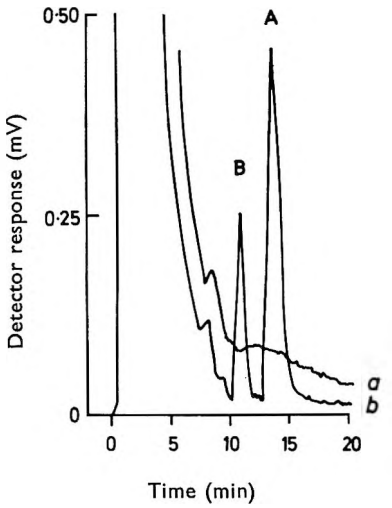

FIG. 1. The curve *a* represents an ether extract of plasma without (+)-amphetamine. Curve *b* represents an ether extract of plasma (3 μl injection) containing 40 ng of (−)-amphetamine (A) plus *NN*-dimethylaniline (B) as internal standard. An attenuation (×10) equivalent to a current of 10^{-11}A and a chart speed of 10 inches/h were used.

which were conditioned on the chromatograph for 24 h at 180° before use. The columns were maintained at 165° with the injection port at 215°. Inlet pressures for H_2, N_2 and O_2 were 20, 50 and 30 lb/inch^2 respectively. Under these conditions, flow rates for H_2, N_2 and O_2 were 27, 27 and 325 ml/min repsectively. Replacement of compressed air by O_2 (Jones & Green, 1966) gave an 80% improvement in sensitivity, but to prevent the detectors from burning out, the high flow rate used was found to be essential. On the recordings, (+)-amphetamine was located by means of its relative retention time in comparison with *NN*-dimethylaniline. (+)-Amphetamine was characterized by observing the shift in retention time on transforming the drug into its acetone-derivative (Brochmann-Hanssen & Svendson, 1962). Losses of (+)-amphetamine by adsorption onto the solid phase of the column were minimized by presaturation of the active adsorption sites before each set of determinations. This was achieved by injecting an ethereal solution of nicotine (1 μg/ml) repeatedly until a constant base line was maintained (nicotine retention time 31·4 min). The use of (+)-amphetamine for this purpose may lead to erroneous results due to its displacement from the column sites by water contaminating the final extract. Before the injection of each sample, sufficient time was allowed for nicotine to be completely eluted from the column, since this can interfere in the determination of amphetamine in blood taken from smokers. Human subjects given 10–15 mg of (+)-amphetamine sulphate had maximum blood levels of 40–50 ng/ml after 1½–2 h, falling to 2 ng/ml (the lowest limit of detection) after 8–10 h.

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Serosal transfer of glucose during peristalsis

We have recently described a method (Gwee & Yeoh, 1968) for comparing the serosal appearance of a substance under "quiescent" and "peristaltic" conditions in the same piece of intestine. A solution of the substance is perfused through the lumen of the intestine at a constant pressure and peristaltic contractions may be initiated by causing the intestine to distend slightly. We now report the serosal transfer of glucose.

The salt solution used in the preliminary isolation and mounting of intestinal segments and also to bathe the serosal surface of the intestine (serosal fluid), contained only a minimal quantity of glucose (0·01 g/litre); the electrolyte composition (g/litre) was as follows: NaCl 6·92, KCl 0·353, $CaCl_2$ 0·282, $MgSO_4$ 0·142, KH_2PO_4 0·161, $NaHCO_3$ 1·0. The fluid used to perfuse the lumen (mucosal fluid) had the same electrolyte composition but the glucose concentration was increased to 3·6 g/litre.

After an initial rest period of 15 min and thereafter at 30 min intervals, the serosal fluid was sampled to determine the glucose concentration by the method of Nelson (1944). An experiment consisted of four 30 min periods of alternating quiescent and peristaltic conditions. Mean values for the change in serosal glucose concentration for 30 min periods gave the following results.

In 15 experiments the rate at which glucose appeared in the serosal fluid was $56 \pm 5{\cdot}1$ (s.e.) μg/cm intestine h^{-1} with the intestine quiescent and $135 \pm 8{\cdot}4$ with the intestine undergoing peristaltic contractions ($P < 0{\cdot}001$).

These results are consistent with the view that the serosal glucose is mainly transferred from the lumen and is not endogenous, since the rate of appearance of glucose on the serosal aspect varied with its concentration in the mucosal fluid, and the rate of appearance declined only slightly over the 2 h period of the experiments. Also, experiments with [^{14}C] labelled glucose in guinea-pig small intestine showed similar "transfer" rates for glucose estimated chemically, and for glucose estimated by its radioactive content (Yeoh & Lee, 1968).

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BOOK REVIEWS

The British Pharmacopoeia 1968*

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The most obvious fact about the B.P. 1968 is that it is fatter than its precedessors, in spite of very strict economy in the white space, which has resulted in an overcrowding of the text on the page. The reason for this is to be seen on pp. xxvii–xxix where the list of 215 new monographs is offset by only about one quarter of its number of deletions. This is the inevitable result of the upsurge of "new" drugs in recent years and presents pharmacopoeia authorities with a difficult task of selection. The B.P. Commission is to be congratulated on having discharged this particular task with distinction, for the B.P. '68 may justly be claimed as the most complete, modern, and highly developed of all present pharmacopoeias.

A note of nostalgia appears on p. xiv where we are informed that the present edition will be the last to be published under the auspices of the General Medical Council which has been responsible for the work since its institution in 1858. Future editions will be the responsibility of whatever organisation is to be established under the auspices of the Medicines Commission provided for in the new Medicines Act of 1968. It is to be hoped that whatever the new system to be evolved, it will continue to recruit the goodwill and the high scientific endeavour of the many experts who have contributed to the successive editions of the B.P. throughout the last 110 years.

Unlike most of the other European pharmacopoeias published in recent years, the B.P. still contains many monographs on pharmaceutical preparations, including some antiquated ones such as sulphur ointment, coal tar solution, compound rhubarb tincture, camphorated tincture of opium, and turpentine liniment.

In the process of rationalization which must inevitably attend the institution of a new regime it may well be that a distinction could be made in future between standards for substances and standard formulae for preparations, with the possible removal of the latter into a separate compendium. This is not to cast doubt, however, on the importance of setting standards for the most widely used pharmaceutical forms, such as tablets, capsules, or injection solutions, of each of those substances which are the subject of monographs. The latter principle has been challenged in many countries, usually by industrial interests, who believe that the formulation of pharmaceutical preparations is now such a complicated and delicate matter that it cannot be adequately treated in the pharmacopoeia. They would prefer to leave the manufacturer free to develop his own formulation which would be divulged (as it must be) only to the authority granting permission for its release onto the market. This view, however, ignores the primary responsibility of the pharmacopoeia authority to protect the user by establishing and publishing objective norms which can be applied at any stage in the chain of distribution of the product.

* *THE BRITISH PHARMACOPOEIA* 1968. Pp. xxxi + 1423. Published for the General Medical Council by The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1. £8, post free.

An aspect of quality control of which awareness has increased rapidly in recent years is that of drug availability in the body. The pharmacopoeia has traditionally applied appropriate tests to delayed-action insulin preparations and has a control for the particle size of Griseofulvin but much remains to be done in devising suitable tests in connection with many other formulated products. So far very little has been done in this direction in any pharmacopoeia.

Of particular interest to the reviewer is the reference on page xiv to the relationship between the B.P. Commission and the European Pharmacopoeia Commission. The latter body, set up under the auspices of the Council of Europe (Partial Agreement), unites eight countries in the collaborative revision of pharmacopoeial standards. These will become the official standards in each of the participating countries and will replace the existing monographs in their respective national pharmacopoeias. The first volume of the European Pharmacopoeia is now in the press and its publication early in 1969 will cause some revision in the B.P. monographs concerned. The B.P. Commission, its experts and its staff have played and continue to play a considerable part in the elaboration of the Ph.Eur.

An inevitable development in the B.P. as in other pharmacopoeias is the use of physical and physico-chemical methods to replace classical chemical procedures in the identification or control of purity of drugs. Gas chromatography is introduced for the first time in this edition. Indeed many of the newer substances could not be adequately controlled by the older methods. In consequence it has to be recognized that pharmaceutical analysis is a speciality in its own right, and that it can be carried out satisfactorily only in appropriately equipped laboratories. This fact is not always palatable in some countries where law and tradition still presuppose that the practising pharmacist is directly responsible for the quality of the medicaments he supplies and that he should carry out the analytical procedures necessary to the discharge of that duty. It is clearly uneconomical to equip every pharmacy, or even a substantial proportion of them, with the apparatus and staff necessary for such work. However, the widespread introduction of techniques using thin-layer chromatography both for identification and for the detection of trace impurities should enable laboratories even of modest scope to give more attention to pharmaceutical analysis than has sometimes been the case in the past.

The use of techniques such as chromatography and light absorption has emphasized the need for reference substances of various kinds, such as samples of known purity and known impurities, authentic specimens, and even "pure" impurities. The work of the B.P. Commission in collaboration with the Pharmaceutical Society in making available such substances appears to have gone further than that of the WHO in this field. It is to be hoped that in due course some international agreement can be reached regarding these substances for they are obviously of great importance to all pharmacopoeia authorities.

The new edition continues to include brief statements about the actions and uses of drugs. The value of these statements is not obvious, nor is their presentation uniform. In some cases no statement is made (e.g. Eucalyptus oil) and in others the statement is not very precise, e.g. Eugenol is said to be a "local analgesic used in dentistry". The usual term in dentistry is "obtundent" which avoids confusion with local anaesthetic (? analgesic) solutions injected before extractions, etc. It would be better either to give fuller information, as is done in the B.P.C., or to omit it altogether. As it is not the function of the B.P. to become a handbook of therapeutics, perhaps the latter solution would be best.

This, however, is a small quibble to make concerning such an excellently edited work. The secretariat and the printers have admirably combined to maintain the traditional high quality of presentation. The typography is clear, though small,

and elderly users would do well to ensure good light and accurate visual correction if they are to avoid headaches when using it.

The British Pharmaceutical Codex 1968*

Reviewed by

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As a foreigner preparing a review on the latest edition of The British Pharmaceutical Codex, I was sufficiently disturbed, if not alarmed, by the future uncertainties referred to in a recent commentary in The Pharmaceutical Journal (Editorial, 1968), to feel that a discussion of comparable or parallel situations in my own country should preface the review.

United States law (the Federal Food, Drug, and Cosmetic Act, as amended) recognizes as "official compendia" the United States Pharmacopeia and the National Formulary. These two compendia are nonduplicative in content but are very similar in purpose and treatment of subject matter. Between them they serve to establish officially recognized standards of identity, purity, strength, and quality for virtually all therapeutically meritorious articles available for use in the U.S. Neither, however, provides more than the very briefest information on actions, uses, and doses of drugs, and nothing on side-effects, precautions, contra-indications, and so on. Moreover, there is currently no other such compilation in this country which enjoys legal recognition.

A growing desire appears to be developing on the part of various sectors and groups for the creation and issuance of a compendium of therapeutic information. Many plans have been offered by the Commissioner of the U.S. Food and Drug Administration and other interested parties. Several legislative proposals have been introduced into Congress to authorize and direct preparation of such a compendium by a government agency.

This incidental information is offered to demonstrate the need for authoritative information on actions, uses, and doses of drugs of the type presently provided in the United Kingdom by the B.P.C. It suggests that in the absence of such a compendium, the present practice of pharmacy and medicine would require that such a volume be prepared and made available for use by practitioners.

We must not overlook the fact, either, that such a compendium does not come into being simply by legislative decree. A comprehensive, scientifically sound, and medically accurate compilation requires considerable expertise and experience in a broad variety of areas. This must be combined with a special knowledge of the intricacies of committee organization, effective procedure, and productive direction which are comparable to the marshalling of a military force and its successful engagement on the field of battle.

* *THE BRITISH PHARMACEUTICAL CODEX* 1968. Pp. xxxvii + 1513. The Pharmaceutical Press, London. £7, post free.

In the reviews of the two most previous editions of the B.P.C. (Feldmann, 1960, 1963) emphasis was directed at the suitability of the Codex as a book of standards. We now read that the action of the British government in taking over the British Pharmacopoeia leaves the future of the Codex "a matter of speculation" (Editorial, 1968). Since the matter of standards has been apparently provided for in the new Medicines Bill, our principal attention is naturally drawn to the companion role played by the Codex in the area of providing useful therapeutic information. Historically the Codex has performed so important a function in this field that concern over its possible loss or disappearance is a natural consequence.

Close examination of the 1968 edition reveals that Editor S. C. Jolly, the Codex Revision Committee under the Chairmanship of H. G. Rolfe, and the numerous Subcommittees have admirably discharged the directive and responsibility given them by the Council of the Pharmaceutical Society of Great Britain. The excellence of the new edition is readily apparent to even the most casual reader.

There are five relatively objective methods of judging the quality of a new pharmacopoeia or formulary; these criteria are known to and utilized by those persons intimately involved with such revision and publication programmes. The five criteria are: (a) the perception shown in selection of new drugs for initial inclusion; (b) the wisdom demonstrated in weeding out and discarding drugs no longer worthy of recognition; (c) the technical ability of the scientific bodies in utilizing advanced scientific methods of drug testing; (d) the adoption of more sophisticated types of specifications which will provide greater assurance of drug quality; and (e) the general expertise demonstrated in the application of new specifications and the methods for their determination, as well as the accuracy and general reliability of the information in all areas, including the therapeutic commentary.

The new Codex measures up well when examined and assessed by any of these. There are almost 100 new drug substances including pharmaceutical adjuncts, plus about the same number of new dosage forms, including capsules, creams, elixirs, injections, lozenges, ointments, tablets, and tinctures. The effectiveness of the housecleaning is evident from the list of deletions which include buchu, gall, ipomoea, jalap, lard, linseed oil, mercury, and a few other nostrum-type articles which should have been placed among the discards long ago. Interestingly enough, however, some of the deletions are items of rather recent vintage, and, in addition to indicating the rapid advances of modern therapy, it will be interesting to see what reaction these deletions will generate on the part of those who use and rely upon the Codex.

Chromatography has been widely used with thin-layer and gas-liquid chromatographic methods in particular evidence. The utility of these procedures for small quantities, coupled with their selectivity, as well as their wide applicability to many classes of drugs, more than overcome the somewhat reduced precision associated with the techniques when compared to other quantitative procedures.

The greater emphasis on determination of potential impurities is representative of strides which have been made in applying the new techniques in an effort to provide better standards of purity and quality. While much has been done in this regard, it would be incorrect to imply that more could not yet be done. For example, the National Formulary (U.S.A.), currently in preparation, will introduce atomic absorption spectroscopy for the assay of certain metal-containing compounds, as well as X-ray diffraction for specifying the acceptable polymorphic forms of certain drugs known to exist in more than one form. [While the B.P.C. has introduced a novel infrared procedure for the examination of the crystal forms of chloramphenicol palmitate, recent reports in the research literature (Borka & Backe-Hansen, 1968) indicate that polymorphic transformations may take place not only in solution but even in the solid state under pressure when preparing a potassium bromide pressed

disk. At any rate, infrared spectroscopy appears to be less generally satisfactory for distinguishing polymorphs than X-ray diffraction.]

However, a much more significant difference exists in the standards of the Codex and those of the USP and NF. After a trial period in the 1965 editions of the NF and USP, tests for "content uniformity" are now being widely applied in the monographs for tablets, capsules, sterile solids with diluents, and certain other dosage forms, which will appear in the forthcoming editions of these compendia. These content uniformity tests are based upon the assay of a number of single dosage units and are in addition to the conventional assay of a composite sample—usually 20 tablets or capsules. The excellent review of pharmacopoeial standards and specifications prepared by the Canadian Food and Drug Directorate (French, Matsui & others, 1967) emphasized the desirability of such a test in light of the frequency of poor mixing and other factors which may result in lack of homogeniety in a particular batch or lot.

However, such shortcomings as may exist—such as the lack of content uniformity tests—are few in the new Codex, and it would be an injustice to imply otherwise. Indeed, one must search hard to find any real point of possible criticism.

Perhaps more than in any other respect, the care in preparing and compiling the B.P.C. 1968 is evident in the accuracy of its content. This attentiveness is particularly reassuring to the practitioner in connection with the actions and uses information. In this respect little can be added by way of additional praise to that stated in the early paragraphs of this review.

In conclusion, B.P.C. 1968—which is scheduled to become effective or "to come into force" on March 3, 1969—is not only a worthy addition to the series, but indeed represents a new high water mark from the standpoint of its overall excellence. In the expectation that many practitioners and others will find it to be an eminently useful text, it is respectfully suggested to the reader that he preserve his copy with due care; the uncertainties voiced concerning the future of the Codex make it appear that the 1968 edition may have to serve his purposes for many, many years to come!

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