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The stability of sucrose monolaurate: rate of formation of lauric acid

R. A. ANDERSON AND A. E. POLACK*

At concentrations below the critical micelle concentration, sucrose monolaurate hydrolyses to give lauric acid at first-order rates. At concentrations above the critical micelle concentration first-order kinetics are not obeyed. In systems buffered to pH values sufficiently high for the liberated lauric acid to ionize, the laurate ions appear to form mixed micelles with the ester and these carry negative charges. Coulombic repulsion of hydroxyl ions by these negative charges protects neighbouring ester molecules from attack and so reduces hydrolysis rates.

THE sucrose fatty acid esters are non-ionic surface-active agents which differ from many non-ionic water-soluble surfactants in that they do not possess polyoxyethylene chains. They appear to be useful pharmaceutical adjuncts, but little information is available about their stability.

Osipow, Snell & others (1956) heated a solution containing 0·5% sucrose stearate and 1% sodium tripolyphosphate (pH 9·5) at 60° and found 8·9% hydrolysis after 1 hr and 14·9% after 4 hr. Stability to acid was determined using 0·1% ester in 0·1N hydrochloric acid. After 2 hr at 37°, stearic acid equivalent to 2·5% of the ester had been formed, and 30 min at 100° showed 6·9% hydrolysis. Kakemi, Arita & others (1962) have examined the decomposition of sucrose monostearate in some ethanol–water media.

Sucrose monolaurate is the most water-soluble of the common sugar esters and has been chosen for further study.

Experimental

MATERIALS

Sucrose monolaurate, "purified", 27-2037 (Colonial Sugars Co.) was further purified by the method of Mima & Kitamori (1964). The product was checked by thin-layer chromatography for absence of free sucrose, sucrose dilaurate and higher esters, and was shown by complete hydrolysis and quantitative gas chromatography to be free of residual solvents and esters of other fatty acids.

Lauric, myristic and palmitic acids were "specially pure" laboratory reagent grade, B.D.H.

The following buffer solutions were used to prepare the sucrose monolaurate solutions: hydrochloric acid–potassium chloride (pH 2·10; 2·51); chloroacetic acid–sodium hydroxide (pH 3·00); acetic acid–sodium acetate (pH 4·06; 5·08); potassium dihydrogen phosphate–dipotassium hydrogen phosphate (pH 5·80; 6·80; 7·20); boric acid–sodium hydroxide (pH 9·30). With the exception of one of the phosphate buffers (specifically noted in Fig. 6), the ionic strength of all buffer solutions

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was 0·2. The pH measurements were made at 25° using a "Radiometer" 23 pH meter.

METHODS

Degradation of ester. Solutions of sucrose monolaurate were made in the selected buffer and accurately measured volumes were pipetted into thin-glass ampoules. In a few cases palmitic acid was included by adding an appropriate volume of an ethanolic solution of the acid just before adjustment to volume. A fine suspension was formed and the suspension was measured into ampoules as before.

The ampouled solutions were heated at 100°, 71°, 46° or 25° ± 0·1°. At selected intervals, ampoules were withdrawn and cooled in an ice bath.

Estimation of free lauric acid. The contents of an ampoule of sucrose laurate solution was quantitatively transferred to a separating funnel along with diethyl ether and an accurately measured volume of a standard ethanolic myristic acid solution. The pH of the aqueous phase was lowered (if necessary) to about 5 by addition of 0·1N hydrochloric acid and the funnel shaken. The aqueous layer was removed and the ethereal layer washed twice with ether-saturated water. The procedure was shown to completely extract the free fatty acids and reject sucrose monolaurate (Polack, 1967).

The fatty acids in the ethereal solution were converted to the methyl esters by diazomethane (Schlenk & Gellerman, 1960), and the ethereal solution was analyzed by gas chromatography using a "Panchromatograph" (Pye) fitted with a Speedomax G recorder. A 5 ft column of 10% Silicone Elastomer 30 on Chromosorb W (HDMS) 60–80 mesh was used under isothermal conditions at 175°. Using nitrogen as the carrier gas at a flow rate of 80 ml/min, the retention times for lauric, myristic and palmitic acids (as methyl esters) are 2, 4 and 8 min respectively.

Peak heights were measured and the ratio of the concentrations of lauric acid to myristic acid was calculated from the relationship shown in Fig. 1. At least four chromatograms were run for each ethereal solution and the results averaged.

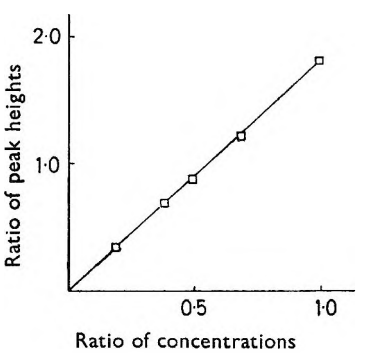

FIG. 1. The relation between the ratio of peak heights (lauric acid : myristic acid) and the ratio of concentrations.

Results and discussion

The degradation of sucrose monolaurate could possibly proceed in two ways. The molecule might split at the bond joining the dextrose and fructose units giving a hexose laurate, or hydrolysis might take place at the ester link liberating lauric acid.

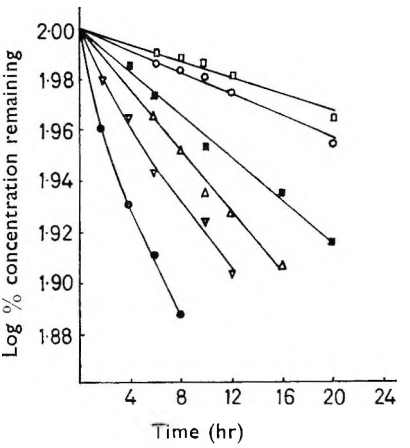

FIG. 2. Hydrolysis of sucrose monolaurate at 100° at various pH values. ● pH 2·10 Initial concentration 0·0096 M. ▽ pH 2·51 Initial concentration 0·0097 M. △ pH 3·00 Initial concentration 0·0099 M. □ pH 4·06 Initial concentration 0·100 M. ○ pH 5·08 Initial concentration 0·0102 M. ■ pH 5·80 Initial concentration 0·0099 M.

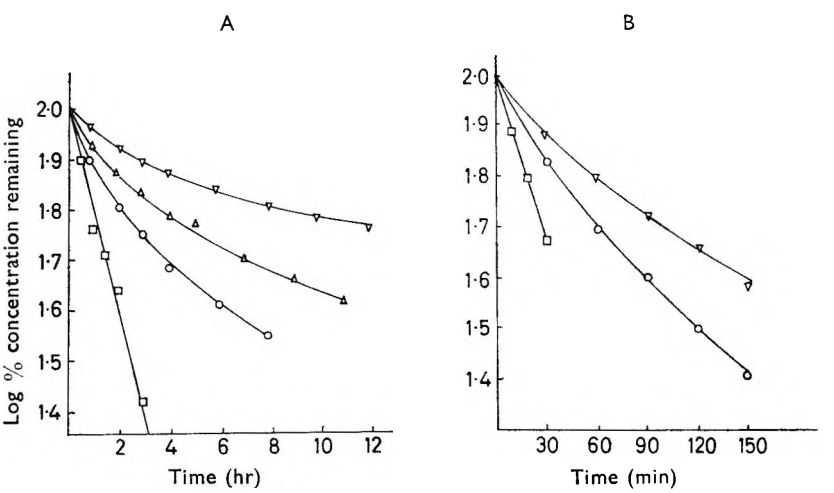

FIG. 3. A. Hydrolysis of sucrose monolaurate at 100° at pH 7·20 at various initial concentrations. ▽ Initial concentration 0·0200 M. △ Initial concentration 0·0100 M. ○ Initial concentration 0·0040 M. □ Initial concentration 0·0004 M.
B. Hydrolysis of sucrose monolaurate at 71° at pH 9·30 at various initial concentrations. ▽ Initial concentration 0·0200 M. ○ Initial concentration 0·0100 M. □ Initial concentration 0·0002 M.

Using thin-layer chromatography a faint spot at an Rf value similar to that of sucrose dilaurate was detected from some acid solutions (pH <4) but not from neutral or alkaline solutions, and this seems to indicate that hydrolysis of the sucrose part of the molecule is unimportant in neutral and alkaline solutions.

The concentrations of sucrose laurate remaining undegraded, and which are seen in Figs 2 to 6, are calculated on the assumption that the only quantitatively important degradation is hydrolysis to give free lauric acid. Fig. 2 shows the rates of hydrolysis of about 0·01M solutions at 100° at various pH values (2·10 to 5·80) and indicates a maximum stability at pH 4 to 5.

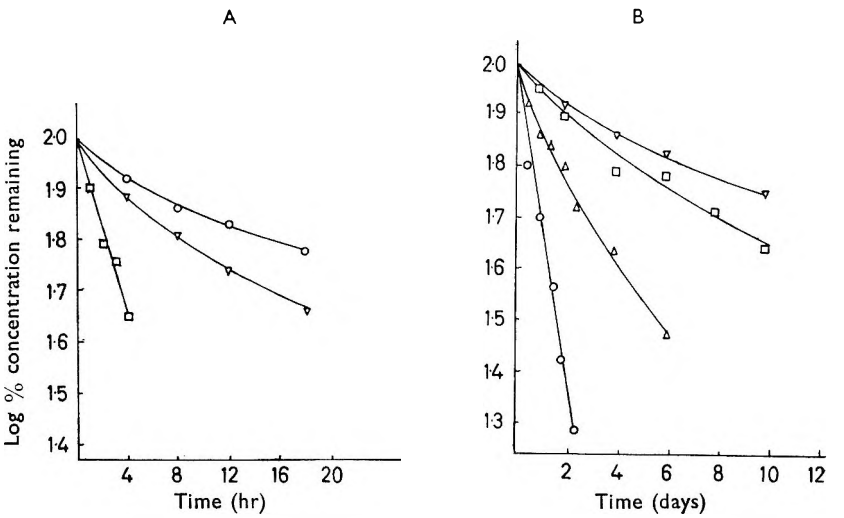

FIG. 4. A. Hydrolysis of sucrose monolaurate at 46° at pH 9·30 at various initial concentrations. ○ Initial concentration 0·0200 M. ▽ Initial concentration 0·0100 M. □ Initial concentration 0·0002 M.
B. Hydrolysis of sucrose monolaurate at 25° at pH 9·30 at various initial concentrations. ▽ Initial concentration 0·0200 M. □ Initial concentration 0·0100 M. △ Initial concentration 0·0010 M. ○ Initial concentration 0·00025 M.

Figs 3 and 4 show the effect of variation of initial concentration of sucrose laurate on hydrolysis rates. Above the critical micelle concentration (CMC), sucrose monolaurate is present both as individual molecules and associated as micelles, and differences would be expected for the rate constants for hydrolysis of free and micellar forms. The CMC for sucrose monolaurate was reported by Osipow, Snell & Hickson (1957) to be $3{\cdot}4 \times 10^{-4}$M at 27°. Using the same technique (surface tension measurements), Polack (1967) found a CMC of $2{\cdot}4 \times 10^{-4}$M at 25°, this value being essentially unaffected by the presence of 10^{-3}M lauric acid at pH 7·2.

At concentrations below the CMC the hydrolysis proceeds at first-order rates; at concentrations above the CMC the rate constant decreases with increase in initial concentration of ester but does not remain constant during the degradation. If, as seems reasonable, the rate constant for

hydrolysis of molecules in the micellar state is lower than that of unassociated molecules, plots of the logarithm of the percentage of ester remaining against time would be expected to show increased (negative) slope as the degradation proceeds; in the extreme case that micellar material does not undergo hydrolysis, the overall reaction would proceed at zero-order rates and plots of concentration against time would be linear. Clearly, some other factor is involved.

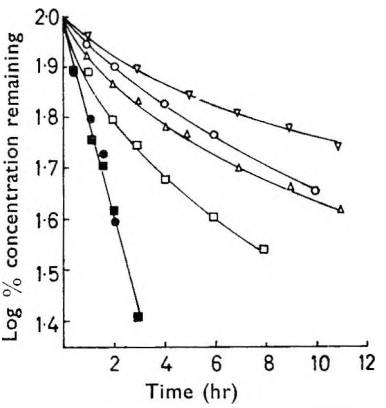

FIG. 5. Effect of palmitic acid on the hydrolysis of sucrose monolaurate at 100° at pH 7·20. □ Initial concentration 0·0040 M sucrose monolaurate. ○ Initial concentration 0·0033 M in presence of 0·0056M palmitic acid. △ Initial concentration 0·0100M sucrose monolaurate. ▽ Initial concentration 0·0102M in presence of 0·0140M palmitic acid. ■ Initial concentration 0·0004M sucrose monolaurate. ● Initial concentration 0·0004M in presence of 0·0006M palmitic acid.

Fig. 5 shows that the inclusion of palmitic acid slows the rate of hydrolysis when concentrations are appreciably above the CMC of the ester but not when the concentration of ester is in the region of the CMC. It seems likely that in systems containing micelles of sucrose laurate, free lauric acid or laurate ions will be taken up to form mixed micelles. The pKa value of lauric acid is about 5-6 (Rosano, Breindel & others, 1966), so that at pH 7·2 (and higher) most of the fatty acid will be in the form of laurate anions and the mixed micelles will carry a negative charge, the density of the charge increasing as the degradation proceeds. Coulombic repulsion of hydroxyl ions by these negative charges will serve to protect neighbouring ester molecules from attack. Similar effects have been reported for other systems. Riegelman (1960) has suggested that the anionic head groups of sodium lauryl sulphate provide a barrier to the approach of hydroxyl ions and protect solubilized benzocaine from hydrolysis; low (but not high) concentrations of cetyltrimethylammonium bromide increase the hydrolysis rate, probably by attracting hydroxyl ions to the benzocaine environment. Nogami, Awazu & others (1960) and Nogami & Awazu (1962) found that the hydrolysis of methantheline bromide in the presence of sodium lauryl sulphate was markedly dependent on pH; the base catalysis was suppressed by the surfactant whereas the

acid catalysis was promoted. Swarbrick (1965) has reported that this effect is probably due to the attraction and repulsion between the electrical charge on the micelle and the hydronium ion and hydroxyl ion respectively.

The effect of increasing ionic strength is shown in Fig. 6. The increased hydrolysis rate at the higher ionic strength reflects the reduced coulombic repulsion. The change of concentration of phosphate ions appears to have no effect, provided the ionic strength is held constant.

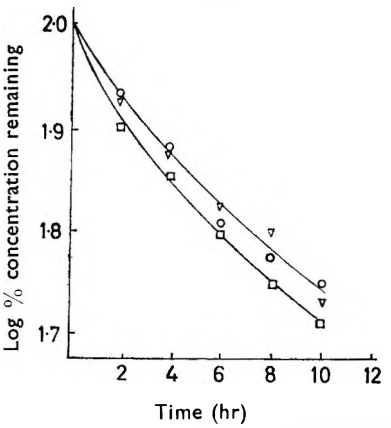

FIG. 6. Effect of buffer strength and ionic strength on hydrolysis of sucrose monolaurate at 100° at pH 6·80 in various phosphate buffer solutions. ▽ Initial concentration 0·0100M. Buffer strength 0·0917M. Ionic strength 0·2. ○ Initial concentration 0·0099M. Buffer strength 0·1834M. Ionic strength 0·2. □ Initial concentration 0·0102M. Buffer strength 0·0917M. Ionic strength 0·4.

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Concentric cylinder creep investigation of pharmaceutical semi-solids

B. WARBURTON AND B. W. BARRY*

The rheological examination of pharmaceutical preparations has not often taken into account the contributions of viscoelasticity. Many semi-solids have been treated as liquids and observed in continuous shear. The present paper describes a concentric cylinder creep apparatus designed to enable viscoelastic parameters to be measured. A resumé of current theory is given and some experimental data analysed.

IT has become obvious in recent years that pharmaceutical preparations such as creams and pastes are complex rheological bodies; e.g. they exhibit viscoelasticity (linear and non-linear). Until recently these viscoelastic properties have been ignored, and continuous shear experiments have been made to measure such properties as hysteresis loop area and shear stress at a spur point in the shear stress-shear rate diagram. These experiments are used to examine the complex phenomenon of breakdown and classical parameters such as viscosity and elasticity are not measured directly. It is valuable both theoretically and practically to consider a system in the rheological ground state, that is without the method of testing significantly altering the structure, and to express the results of such tests in combinations of simple physical entities.

Many of the preparations used in pharmacy are multiphase where the phase boundaries are stabilized with complex films built from moderately low molecular weight components, e.g. Aqueous Cream B.P. Although historically the application of the theory of linear viscoelasticity has been most noticeable in the field of synthetic high polymers (Ferry, 1958, 1961a; Leaderman, 1958; Turner Alfrey & Gurnee, 1956) there is no fundamental reason why the theory should not be applied to the pharmaceutical systems mentioned above, and probably to many others.

The purpose of this paper is to present details of an apparatus which is designed to measure the necessary rheological parameters that are required to describe rheologically complex materials used in pharmacy. A resumé of current theory associated with linear viscoelastic behaviour is also given for the case of the creep test, that is deformation of the sample under constant stress.

Theory

LINEARITY OF RHEOLOGICAL BEHAVIOUR

Viscoelastic considerations can only readily be applied to a sample which has a linear strain response to an applied stress and vice versa. For any particular material there is a definite range of strain over which linearity holds.

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* Initial work on this apparatus formed part of a thesis accepted for the degree of Ph.D. in the University of London. Present address: School of Pharmacy, Portsmouth College of Technology, Portsmouth, Hants.

The requirements for linearity have been dealt with by Boltzmann (1876) and Van Wazer, Lyons & others (1963a). These are implicit in the principle of superposition which has the same mathematical form both in mechanical systems and electrical networks (Goldman, 1966). In mechanical terms, the principle of superposition states that if the system under investigation can be represented by a network of rheological models each of which has constant parameters, and if several stresses are applied either at the same or different times, then the total strain is equal to the sum of the strains produced by the stresses applied separately.

SPECTRA OF TIMES

A fundamental rheological parameter of a simple viscoelastic solid is the retardation time. This may be defined as the ratio of viscosity to shear elasticity in the sample. A complex viscoelastic solid would have to be represented by more than one retardation time.

An experimental set of data of total shear strain $\gamma(t)$ at constant applied stress ($=\sigma_1$) such that

$$J(t) = \frac{\gamma(t)}{\sigma_1}$$

may be analysed in two ways, either to give a continuous spectrum of retardation times or a line spectrum of retardation times (Ferry, 1961b). The choice of analysis really depends on the number and spacings of retardation times in the system under investigation. Resolution of individual retardation times is made difficult or impossible if the number is greater than ten and the smallest spacing between any two adjacent retardation times is less than five times the value of the smaller retardation time. In such a case the continuous spectrum method is most tractable, but for fewer retardation times a more accurate representation would be the use of a line spectrum.

The number of retardation times in an experimental system will depend on the degree of parity between the various ratios of bond energy to bond modulus for the various entities present (Lethersich, 1949). If the degree of parity is good (e.g. in simple fluids such as water and ethanol) the number of retardation times will be small. However if the above ratios extend over a wide range, the number of retardation times may be immense (e.g. aqueous solutions of high molecular weight cellulose ethers).

The range of these ratios will in turn depend on several factors: (*a*) The size of the particles or molecules involved. (*b*) Their shape (large or small aspect ratio). (*c*) The number of molecules or particles taking part.

In general the range of ratios will be small for low molecular weight species and for particles or molecules which are nearly spherical or ellipsoidal in shape.

The authors suspect that many pharmaceutical emulsions, creams, suspensions and pastes have component parameters which would yield a fairly simple line spectrum analysis. It should be noted that the most complicated cases are those involving high molecular weight thread like molecules, made up of components of widely differing molecular weights.

Here, factors (*a*), (*b*) and (*c*) mentioned above give rise to a complicated spectrum of retardation times. A continuous spectrum of retardation times is commonly calculated for these systems (Ferry, 1961b).

Sometimes, where the spectrum of retardation times extends over several factors of ten of time, it is possible that only part of the spectrum may be determined due to the time scale of the experiment. The present apparatus does not give useful data for determination of times less than 30 sec.

DERIVATION OF SHEAR BEHAVIOUR IN TERMS OF SERIES VOIGT MODEL ELEMENTS UNDER CONSTANT STRESS

The apparent time dependent shear compliance J(t)* of a linear viscoelastic solid or semi-solid may be represented by the algebraic sum of a set of hypothetical series compliances which may or may not have a direct physical significance in the system of interest (Karas & Warburton, 1961).

Thus
$$J(t) = \sum_{i=0}^{n} J_i(t) \qquad (1)$$

It is also demonstrable that these systems can also be represented by a set of parallel time dependent moduli such that

$$G(t) = \sum_{r=0}^{n} G_r(t) \qquad (2)$$

However, data obtained under constant stress conditions (creep) are more readily analysed using equation (1).

In the essentially solid system each individual contributory compliance $J_i(t)$ is a function of time and in the most elementary case may be represented by a Voigt model (Van Wazer & others, 1963b). The Voigt model consists of an elastic element and a viscous element coupled in parallel. In shear, the appropriate moduli involved are the shear modulus G_i and the shear or Newtonian viscosity η_i. If σ_i is the total constant shear stress

$$\sigma_i = \gamma_i G_i + \frac{d\gamma_i}{dt}\eta_i \qquad (3)$$

by using Hooke's and Newton's laws. Re-arranging equation (3)

$$\frac{d\gamma_i}{\sigma_i - \gamma_i G_i} = \frac{1}{\eta_i}\,dt \qquad (4)$$

If the retardation time for the 'i'th element is defined as

$$\tau_i = \frac{\eta_i}{G_i} \text{ or } \eta_i J_i$$

equation (4) becomes

$$\frac{d\gamma_i}{\frac{\sigma_i}{G_i} - \gamma_i} = \frac{dt}{\tau_i} \qquad (5)$$

* All symbols are defined in Appendix 1, p. 268.

On integration without limits

$$-\ln\left\{\frac{\sigma_1}{G_1}-\gamma_1\right\}=\frac{t}{\tau_1}+k_1 \quad .. \quad .. \quad .. \quad (6)$$

At the start of the experiment $t = 0$, $\gamma_1 = 0$; thus:

$$k_1=-\ln\{\sigma_1 J_1\};\ \ln\left\{\frac{\sigma_1 J_1-\gamma_1}{\sigma_1 J_1}\right\}=-\frac{t}{\tau_1} \quad .. \quad (7)$$

$$\gamma_1=\sigma_1 J_1\left\{1-e^{-t/\tau_1}\right\} \quad .. \quad .. \quad .. \quad (8)$$

$$J_1(t)=J_1\left\{1-e^{-t/\tau_1}\right\} \quad .. \quad .. \quad .. \quad (9)$$

Compliances connected in series are additive so:

$$J(t)=\sum_{i=0}^{n} J_i\left\{1-e^{-t/\tau_1}\right\} \quad .. \quad .. \quad (10)$$

It is usual to assume also, for a real material, that the spectrum of values of τ_1 can include at the limits $\tau_1 = 0$ and ∞.

In the first case ($\tau_1 = 0$) the Voigt model reduces simply to an elasticity of compliance J_0 and in the second case ($\tau_1 = \infty$) the Voigt model reduces simply to a viscosity of compliance t/η_0. In this way the treatment can be extended to cover viscoelastic liquids:

$$\eta_0\frac{d\gamma_\eta}{dt}=\sigma_1 \quad .. \quad .. \quad .. \quad .. \quad (11)$$

Integrating:

$$\gamma_\eta=\frac{\sigma_1 t}{\eta_0}+k_2;\ t=0,\ \gamma_\eta=0,\ k_2=0$$

Hence:

$$J_{\eta_0}(t)=\frac{t}{\eta_0} \quad .. \quad .. \quad .. \quad (12)$$

For a line spectrum

$$J(t)=\sum_{i=0}^{n} J_i\left\{1-e^{-t/\tau_i}\right\}+\frac{t}{\eta_0};\ (\tau_0=0) \quad .. \quad (13)$$

In equation (13) the residual instantaneous shear compliance J_0 is regarded as being contained in the summation term. Mathematically it is not useful to include $J_{\eta_0}(t)$ in the summation term.

LINE SPECTRUM ANALYSIS OF CREEP RESULTS

Rheological creep data may be subjected to a stepwise analysis to yield discrete pairs of values of J_1, τ_1 for n models. It is usual to start the analysis of the creep data, starting with data collected at long times in the experiment. The analysis is conveniently divided into a number of steps.

Step 1. It must first be decided whether $J_{\eta_0}(t)$ is zero, finite or infinite: normally some intermediate state $0 < J_{\eta_0} < \infty$ is observed for a real material. Whichever of the three conditions apply, a plot of J(t) against time will produce one of the three curves shown in Fig. 1. Equilibrium or steady state is reached when a straight line portion of the plot is attained. This condition determines the suitable duration of the experiment.

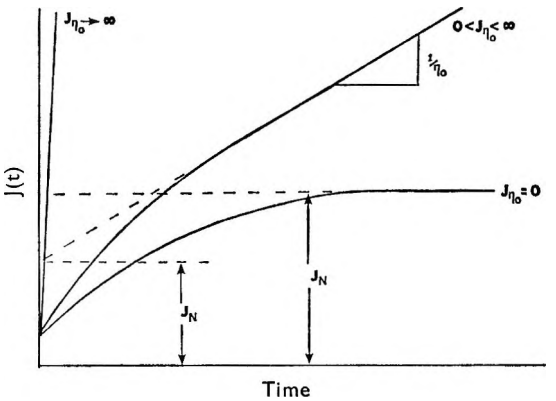

FIG. 1. Theoretical creep curves. For symbols see Appendix 1, p. 268.

It may be assumed for the purpose of the analysis that when the linear portion of the plot is reached all the component Voigt models present in the representation are fully extended:

$$J_i(t = \infty) = J_i \qquad 1 \leqslant i \leqslant n$$

From equation (13) the slope of the linear region of the graph is $1/\eta_0$ and so η_0 is obtained. Since the contribution to the creep compliance of $J_{\eta_0}(t)$ is now known, $(J(t) - J_{\eta_0}(t)$; 13a) is calculated. The contribution to the total creep compliance of the models can be calculated in turn starting with the one of largest retardation time τ_n.

Step 2. For the general Voigt model, equation (9) may be rewritten to give:

$$\frac{J_i - J_i(t)}{J_i} = e^{-t/\tau_1} = R_i(t) \qquad .. \quad .. \quad (14)$$

$$J_i(t) = \{1 - R_i(t)\} J_i \qquad .. \quad .. \quad .. \quad (15)$$

$$\text{Let} \sum_{i=0}^{n} J_i = J_N \quad .. \quad .. \quad .. \quad .. \quad .. \quad (16)$$

$$J(t) = \sum_{i=0}^{n} \{1 - R_i(t)\} J_i + J_{\eta_0}(t) = J_N - \sum_{i=0}^{n} R_i(t) J_i + J_{\eta_0}(t) \qquad (17)$$

After a certain time from loading all models except the nth may be considered to be essentially fully extended (for absolute maximum extension, infinite time would be required) so:

For $t \gg \tau_n$, $\sum_{i=0}^{n-1} R_i(t)J_i = 0$

$$J(t) = J_N - R_n(t)J_n + J_{\eta_0}(t) \qquad .. \quad .. \quad (18)$$

For the experimental data as calculated at 13a the following parameter is evaluated

$$Z_n = \frac{J_N - J(t) + J_{\eta_0}(t)}{J_N} \qquad .. \quad .. \quad (19)$$

The quantities of interest, but not directly available, however, are J_n, τ_n and $R_n(t)$. (17), (18) and (19) give:

$$R_n(t) = \frac{Z_n J_N}{J_n} \qquad .. \quad .. \quad .. \quad (20)$$

From (14) and (20)

$$\frac{-t}{\tau_n} = \ln Z_n + \ln \frac{J_N}{J_n} \qquad .. \quad .. \quad (21)$$

Thus a plot of $\ln Z_n$ against t gives a straight line of slope $-1/\tau_n$ for $t \gg \tau_n$. See example (Fig. 4). For $t \leqslant \tau_n$, the data will not lie on the extrapolated line. The latter makes an intercept of $-\ln (J_N/J_n)$ on the $\ln Z_n$ axis.

Since J_N is known J_n can be calculated.

Step 3. The values of $J_n(t)$ are now evaluated from the determined values of J_n and τ_n for all values of t and subtracted from the corresponding values of $J(t) - J_{\eta_0}(t)$.

The residuals now represent

$$\sum_{i=0}^{n-1} J_i \left\{1 - e^{-t/\tau_i}\right\}$$

It is now possible to return to the beginning of Step 2 and treat the processed data once more.

Equation (19) is now replaced by

$$Z_{n-1} = \frac{J_{N-1} - J'(t)}{J_{N-1}} \qquad .. \quad .. \quad .. \quad (22)$$

where $J_{N-1} = J_N - J_n$

$$J'(t) = J(t) - J_n(t) - J_{\eta_0}(t)$$

but the process is unaltered in principle. Successive repetitions of

Steps 2 and 3 yield J_{n-1}, τ_{n-1}, J_{n-2}, τ_{n-2} etc. until the data is exhausted. The approximation is made that the remaining points at short times (see dotted line, Fig. 5) are due to one further Voigt unit. Any remaining compliance is approximated to a residual compliance referred to as J_0. However, a high frequency oscillation experiment might allow this J_0 to be resolved into further pairs of J_1, τ_1 values.

TRUE VALUE OF J_N

The accuracy of the whole analysis depends on the best fit value of J_N. This is the equilibrium or steady state value because t approaches infinity and is not readily accessible experimentally.

The best fit value of J_N in practice has to be guessed in a series of approximations before the first model can be evaluated.

Differential approach. Another method of estimating J_N is from the slope of the J(t) versus t plot in the curved region of Fig. 1 before the final linear region.

In this region

$$J(t) - J\eta_0(t) = J_{N-1} + J_n\left\{1 - e^{-t/\tau_n}\right\} \quad .. \quad .. \quad (25)$$

where

$$J_N \simeq J_{N-1} + J_n \quad .. \quad .. \quad .. \quad (26)$$

On differentiation:

$$\frac{d\{J(t) - J_{\eta_0}(t)\}}{dt} = \frac{1}{\tau_n} \cdot J_n.e^{-t/\tau_n} \quad .. \quad .. \quad (27)$$

from which J_n and τ_n are readily accessible.

Intrinsically, this is the more accurate method, although it depends on experimental data of a high order of precision for the evaluation of

$$\frac{d\{J(t) - J_{\eta_0}(t)\}}{dt}.$$

An alternative method of the treatment of line spectrum analysis of creep results has been given by Inokuchi (1955), Sharma & Sherman (1966) and Sherman (1966).

Experimental

GENERAL ARRANGEMENT

Since many pharmaceutical preparations are of a liquid or soft gel-like consistency, it is very often difficult to obtain sufficient coupling through a sample to make rheological measurements in cone and plate or parallel plate geometry. If the diameter of the cone and plate is increased to obtain more torque, eventually the edge gap becomes so wide that the material easily flows out of the gap. Alternatively, if the cone angle is decreased the angular movement for any particular strain is reduced, leading to measuring difficulties. There is also the attendant and serious problem

of evaporation of water from emulsions and creams of the oil-in-water type.

The apparatus described is a concentric cylinder creep device built as a modification to the Weissenberg Rheogoniometer (Sangamo Controls Ltd.*). This is shown in Fig. 2a. The concentric cylinder geometry is particularly effective in overcoming the problems of lack of sample coupling and evaporation of water. There is a large area available for providing torque but only a small area from which water may evaporate. The concentric cylinders are made of stainless steel. The outer cylinder is machined with an annular trough to hold water and is located by means of two stainless steel pins which fit into a Perspex disk adapter. This adapter is screwed into the top cap flange of the fixed vertical stub shaft of the rheogoniometer (which is available from the manufacturers for rotational work not involving the measurement of normal force).

The Perspex disk reduces heat loss from the outer cylinder and sample into the large heat sink of the base of the rheogoniometer. The inner cylinder is hollow to reduce weight and end correction and a shallow angled nylon cone can be fitted to close the end if required. For greater accuracy, the end correction may either be eliminated (Couette, 1890, Hatschek, 1913, Mooney & Ewart, 1934, Mooney, 1946, Turner Alfrey & Rodewald, 1949, Moore & Davies, 1956) or calculated (Dinsdale & Moore, 1962, Lillie, 1930, Lindsley & Fischer, 1947). The inner cylinder is attached to an air bearing rotor by a short rod which also supports a Perspex shield. The latter, being cylindrical and of a diameter equal to the mean diameter of the trough described above, can be adjusted just to dip into the water when the inner cylinder is lowered into the outer cylinder. A vapour seal is thus provided, reducing evaporation from the sample.

In using the apparatus, the drag on the shield of the water in the trough will appear as an error in the results. This is usually negligible, but may be relatively more important with samples of low viscosity, in which cases the shield may be adjusted to a position just clear of the water surface. This arrangement still functioned efficiently as a humidity chamber.

The top of the air-bearing rotor carries the 10 cm arm to the transducer (Bolton Paul Meter Type C51) and above this is fitted a fixed grooved wheel of diameter 4·00 cm about which the torque may be applied.

APPLICATION OF TORQUE

The necessity of reproducing an accurately defined torque of suitable value in the horizontal plane in this type of apparatus confronts the experimenter with a particularly difficult problem. The most convenient source of force of constant magnitude which is independent of distance of application is the action of the earth's gravitational field on a fixed mass. However, this acts in a vertical plane. The line of action of an applied force may be turned through a right angle by means of a string

* Sangamo Controls Ltd., North Bersted, Bognor Regis, Sussex, England.

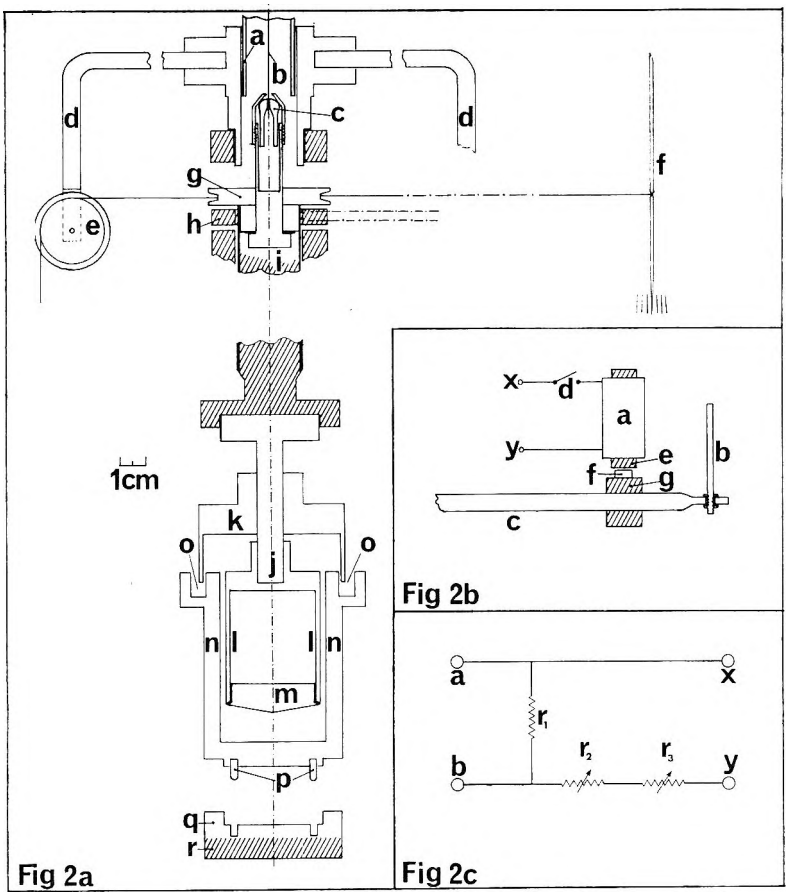

FIG. 2. a. Section of creep apparatus. Key: a. Lower part of 66 cm long support cylinder. b. 0·015 cm diam. beryllium-copper wire. c. Chuck, gripping the beryllium-copper wire. d. Stainless steel arms supporting the pulleys e. e. Pulley wheels (only the left-hand one shown) for loads greater than 5 g. f. Chemical balance pointer arm, showing the notched point. g. 4 cm diameter grooved wheel for torque application. h. Collet holding transducer arm (transducer arm is shown dotted, although not in the same plane as the drawn section). i. Upper part of air-bearing rotor. j. Rod, supporting the inner cylinder. k. Perspex shield. l. Inner cylinder. m. Shallow-angled nylon cone, closing inner cylinder. n. Outer cylinder. o. Annular trough in top of outer cylinder. q. Perspex disk, supporting outer cylinder. r. Steel base disk, existing part of rheogoniometer. Hatched areas of the section represent existing components of rheogoniometer. Water jacket not shown.
b. Quick release mechanism. Key: a. Electromagnet. b. Transducer core. c. Transducer arm. d. Switch for immediate application of stress to sample. e. Core of electromagnet. f. Brass stud. g. Armature. Power supply, 12 V., 50 cycles, is applied at x-y.
c. Recorder attenuating network. R_1 is a high stability fixed resistor of 1·8 ohms, R_2 and R_3 are wire wound resistances of maximum values 250 and 25 ohms respectively. Terminals a and b are connected to the Kent Recorder and x and y to the Boulton Paul meter.

passing over a pulley. For high torques, two opposed pulleys have been used with half the applied load in each pan, but even with the best types of pulley, at low applied forces, static friction losses in the bearings may account for variable force losses of up to 5% of that applied.

The laboratory balance has been used as a device which enables the required transformation of force to be accomplished without measurable losses at low torques. The applied torque to the inner cylinder of the creep apparatus is produced by the horizontal tension in a string between the balance pointer arm and the 4 cm grooved wheel described above. The string naturally falls into a catenary even when under tension but only the horizontal component is effective in providing a horizontal torque on the inner cylinder and this is equal in magnitude to the horizontal component at the pointer. The string is attached to the balance pointer at the notched point, f, Fig. 2a. The distance between the notched point of the pointer arm and the main balance knife-edge is made exactly half the outer knife-edge interdistance. In this way a one-to-one ratio is obtained in the conversion of force through a right angle. Hence if a mass of M g is placed in the left hand balance pan a torque of 2Mg dyne cm is applied to the inner cylinder of the apparatus when the balance is raised. The one-to-one ratio of forces is only strictly maintained when the pointer arm of the balance is on the centre zero. This condition is achieved either by making a small adjustment in the distance between the balance and the rheogoniometer or by the addition of small masses to the centre of the string.

QUICK RELEASE MECHANISM

It is essential in creep experiments to apply the constant stress during a very short period of time (theoretically instantaneously) and then maintain it constant during the experiment. It was found using the present equipment that it was not possible to achieve reproducible initial loading by simply raising the balance arm.

The electromagnetic release mechanism shown in Fig. 2b was devised to this end. A small soft iron armature is clamped to the mid point of the 10 cm transducer arm and is normally held stationary by an electromagnet which is energized by 50 cycle AC. The armature is held away from the soft iron core of the electromagnet by the small brass stud to ensure that a residual magnetic circuit cannot remain when the current is switched off.

An experiment is normally started by first energizing the electromagnet then raising the balance arm and finally cutting the current to the electromagnet. The stress is applied to the sample at the instant of de-energizing the electromagnet.

SUSPENSION ARRANGEMENTS FOR THE INNER CYLINDER

Attached to the 4 cm diameter grooved wheel at the top of the inner cylinder is a chuck which grips a beryllium-copper wire, the other end of which is soldered into an adjustable boss situated at the upper end of a 66 cm long support cylinder. This cylinder, at the lower end, fits into the

top of the rheogoniometer head and is clamped in the same manner as a standard torsion bar. The air bearing, which is practically frictionless and which maintains the axis of the inner cylinder against side thrusts produced by the applied torque, has been used by Oldroyd, Strawbridge & Toms (1951). The beryllium-copper wire has a diameter of 0·015 cm and was annealed for 3 hr at 320°. The torque produced by this wire during a typical creep experiment (when the applied torque was $9{\cdot}6 \times 10^3$ dyne cm) was so low that the variation in applied torque during the experiment due to the wire was not more than $7{\cdot}3 \times 10^{-4}$ %. Although the wire has to support the whole of the inner cylinder assembly, a load of 650 g, it was found that a 1 kg load was still within the elastic limit for the wire. This condition is essential so that the inner cylinder should not ground at the bottom during a run and also that the core of the stress-transducer should not become misaligned. The boss at the top of the supporting tube is adjustable in position so that the relative height of the inner cylinder assembly can be adjusted independently of the rheogoniometer lead-screw.

THERMOSTAT

A constant temperature water bath passes water through a jacket (fitted with a thermometer, 0·1° divisions) which surrounds the outer cylinder. So that the apparatus could in future also be used as a normal coaxial cylinder viscometer, it was manufactured so that a gap was left between the inner surface of the constant temperature jacket and the outer surface of the cylinder, i.e. the outer cylinder could be rotated by the rheogoniometer motors if required. However for the present work a brass tube, lubricated with silicone grease, was inserted to fill the gap; this decreases the time required for temperature equilibration to take place.

RECORDING ARRANGEMENTS

The output of the Boulton Paul Meter Type C51 is connected to a single point Kent recorder by means of an attenuating network, shown in Fig. 2c.

R_2 (coarse) and R_3 (fine) are adjusted to give full scale deflection on the Kent recorder with the Boulton Paul meter switched to CAL.

METHOD FOR CREEP TESTING

The general procedure is as follows. A sample is placed into the outer cylinder and the inner cylinder is slowly lowered until its top is level with the top of the outer cylinder. The excess material is removed and the vapour shield placed in position to minimize evaporation; the apparatus is left overnight for the temperature to equilibrate and for any stresses in the sample to relax.

Immediately before a run is made, most of the water in the outer cylinder trough is removed with a hypodermic syringe. A constant stress is then suddenly applied to the sample as described in a previous section.

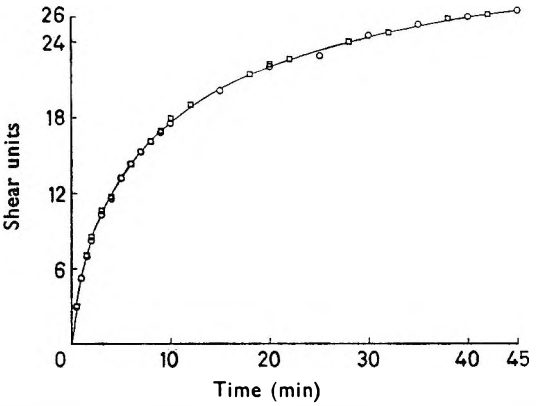

FIG. 3. Experimental and theoretical creep shear data as functions of time. □ — Experimental points ○ — Theoretical points.

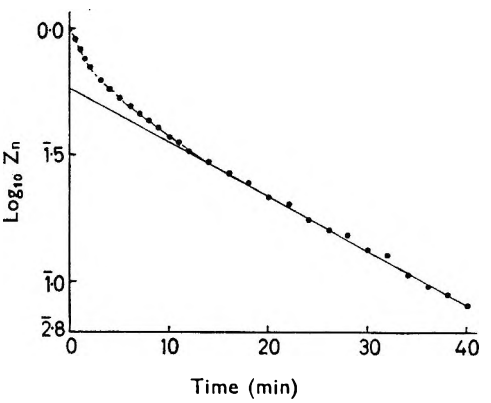

FIG. 4. Plot of $\log_{10} Z_n$ against time.

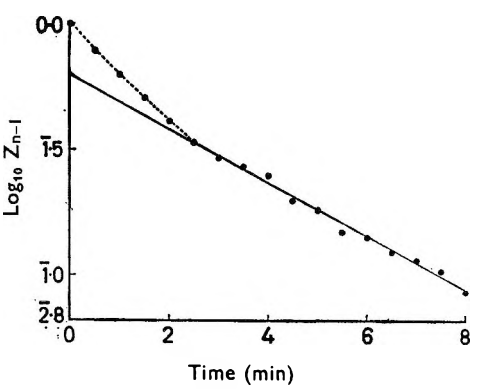

FIG. 5. Plot of $\log_{10} Z_{n-1}$ against time.

EXAMPLE OF TREATMENT OF RESULTS

The following set of data (Barry, 1967) is analysed according to the above theoretical treatment.

A plot of the experimental shear data versus time in minutes is given in Fig. 3. The shear units plotted are 0·1 mV units of the 12 mV Kent Recorder used. A deflection 0·1 mV is equivalent to a sample shear of $17{\cdot}98 \times 10^{-4}$. It will be noted that the initial instantaneous deflection due to the unshunted shear compliance J_0 and the continuous steady shear rate due to the unshunted viscosity η_0 have already been subtracted. The shear stress used in the experiment is 79·8 dynes cm^{-2}. The theoretical points shown in Fig. 3 are calculated from the derived components of the total viscoelastic model given in Table 1. The agreement is good.

Fig. 4 shows the second stage of the analysis where $\log_{10} Z_n$ is plotted against time. The use of logarithms to the base ten is more convenient, in practice, than the use of natural logarithms and the correct value of τ can be calculated from the slope of the best straight line using the necessary conversion factor for logarithms to different bases. Fig. 5 shows a further stage where $\log_{10} Z_{n-1}$ is plotted against time.

The values of the components of the total viscoelastic model are given in Table 1.

TABLE 1. VALUES OF VISCOELASTIC PARAMETERS

Voigt unit i value	τ (sec)	J($cm^2 dyne^{-1}$)	$\eta = \tau \times \frac{1}{J}$ (poise)
(J) 0	—	$4{\cdot}22 \times 10^{-6}$	—
1	$1{\cdot}23 \times 10^3$	$9{\cdot}35 \times 10^{-6}$	$1{\cdot}32 \times 10^7$
2	$2{\cdot}45 \times 10^3$	$4{\cdot}15 \times 10^{-6}$	$5{\cdot}91 \times 10^6$
3	$5{\cdot}88 \times 10$	$2{\cdot}47 \times 10^{-6}$	$2{\cdot}38 \times 10^6$
(η) 0	—	—	$2{\cdot}38 \times 10^7$

The creep shear compliance J(t) was calculated as follows:

Using the well-known expression for concentric cylinders (Dinsdale & Moore, 1962)

$$G(t) = \frac{T}{4\pi h}\left(\frac{1}{R_1^2} - \frac{1}{R_2^2}\right)(\theta(t))^{-1}$$

it follows that:

$$J(t) = \frac{4\pi h}{T}\left(\frac{1}{R_1^2} - \frac{1}{R_2^2}\right)^{-1}\theta(t)$$

Where the important dimensions of the cylinders and gap are: R_1 = radius of inner cylinder = 1·84 cm; R_2 = radius of outer cylinder = 2·10 cm; h = height of inner cylinder = 5·0 cm; T = applied torque (dyne cm); θ = angular deflection of inner cylinder (rads.); G(t) = 1/J(t).

Acknowledgements. The authors wish to thank Dr. K. Ridgway for suggesting the use of the chemical balance for the application of low torques, and Mr. J. Deer for help with the design and construction of the apparatus.

APPENDIX 1

List of symbols used:

σ_i = shear stress applied to the 'i'th Voigt model

γ_i = shear strain of the 'i'th Voigt model

$\frac{d\gamma_i}{dt}$ = rate of shear strain of the 'i'th Voigt model

$\gamma(t)$ = total shear strain at time t

G_i = shear modulus of the 'i'th Voigt model

η_i = shear viscosity of the 'i'th Voigt model

$J_i = {}^1/G_i$ = shear compliance of the elastic part of the 'i'th Voigt model

$J_i(t)$ = the apparent shear compliance at time t of the 'i'th Voigt model

J_o = unshunted or residual shear compliance

η_o = unshunted or residual shear viscosity

t = time in sec

J_N = total creep compliance at equilibrium

J(t) = total creep compliance at time t

$J\eta_o(t)$ = equivalent compliance of η_o at time t

τ_i = retardation time of the 'i'th Voigt model

$R_i(t)$ = compliance normalizing factor for the 'i'th Voigt model = e^{-t/τ_i}

Z_n = compliance parameter defined by equation (19)

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Use of the analogue computer to examine the quantitative relation between urinary pH and kidney reabsorption of drugs partially ionized at physiological pH

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A method, involving the use of an analogue computer, is described for determining a quantitative relation between measured urinary pH and the kidney tubular reabsorption of certain drugs under normal conditions of fluctuating urinary pH. The method is based on the use of drug excretion versus time profiles under normal conditions and of absorption, metabolism and excretion rate constants, determined under conditions of controlled urinary pH, e.g. constant acidic pH (<5) in the case of basic drugs. In support of the theoretical treatment, experimental results, using amphetamine as a model drug, are presented. The implications of the method are discussed with respect to the evaluation of drug formulations under normal urinary pH conditions.

THE dependence of the urinary excretion of acidic and basic drugs on urinary pH is now well established (Milne, Scribner & Crawford, 1958; Weiner & Mudge, 1964; and others), and although the effect may be explained by the change in pH reversing the direction of an active transport system (Baer, Paulson & others, 1956), it is more generally considered that passive or non-ionic diffusion is responsible (Orloff & Berliner, 1956; Torretti, Weiner & Mudge, 1962; Weiner & Mudge, 1964). Furthermore, evidence indicates that diffusion of drugs across the lipid membranes of the kidney tubules is predominantly in a reabsorptive direction, from the urine back into the blood (Torretti & others, 1962; Ullrich, Kramer & Boylan, 1961).

This hypothesis has recently been used to explain excretion versus time profiles of amphetamine (Beckett & Rowland, 1965a) and other related amines such as the "ephedrines" (Beckett & Wilkinson, 1965) under normal conditions in which the pH of urine fluctuates. When urinary pH was maintained at a constant acidic level (pH about 5), variations in the excretion rates of the amines, in response to normal rhythmic diurnal changes in urinary pH, were abolished, thus allowing an evaluation of the kinetics of drug absorption and elimination from the smooth excretion versus time profiles so obtained.

Maintenance of a constant acidic urinary pH is a valuable procedure for examining biological availability of basic drugs from various formulations in volunteers (Beckett & Tucker, 1966, 1968), but it is impractical and probably undesirable under clinical conditions. Thus, only general indications of how a drug, sensitive to urinary pH, will be eliminated under the conditions of its use, can be deduced from studies in which urinary pH is closely controlled at acidic values (or alkaline values for

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acidic drugs). Furthermore, although it is important to know how the drug in a particular formulation is being released from the preparation and absorbed from the alimentary tract, it is equally important to know how different formulations will affect the amount of drug in the body when the urinary pH is fluctuating.

The present paper attempts to show how a numerical relation between drug reabsorption and urinary pH can be determined, with the aid of an analogue computer. Such a relation is a necessary prerequisite for the evaluation of drug formulations by studying urinary excretion of drugs under normal conditions. In support of the theoretical treatment, experimental results, using amphetamine as a model drug, are also presented.

Theoretical

When the urinary pH is maintained at a constant acidic level, the absorption, metabolism and excretion of drugs such as amphetamine (see Beckett & Tucker, 1968) and the "ephedrines" (Wilkinson, 1966), administered in aqueous solution of "free" forms, can essentially be described by simple compartmental models of the type shown in Fig. 1, where k values represent apparent first-order rate constants.

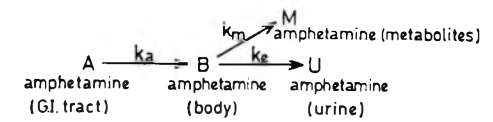

FIG. 1. Pharmacokinetic model for the absorption, metabolism and excretion of amphetamine in man at constant acidic urinary pH.

In addition to the assumptions implicit in the use of these models (see Beckett & Tucker, 1968), the following assumptions are made for the purposes of the present theoretical treatment: (i) The value of the excretion rate constant, ke, determined when urinary pH is controlled, is a direct measure of the rate at which drug is presented to the kidney for excretion, and reabsorption is negligible under such conditions. This is reasonable if essentially smooth exponential curves can be drawn through experimental rate of excretion versus time data when urinary pH is controlled at an acidic value (about 4·7 for amphetamine), and minor variations in pH at such levels have little effect on excretion rate. (ii) Intra-subject variation in the values of the rate constants determined under controlled acidic urine conditions is small. (iii) The values of these rate constants are the same under conditions of controlled and uncontrolled urinary pH. (iv) Of the factors determining the extent of tubular reabsorption, i.e. the amount of drug in the glomerular filtrate plus that actively excreted, the permeability of the tubules at the reabsorption site(s) to unionized and ionized drug species, respectively, the pH of the peritubular fluid, and the pH of tubular fluid, only the latter is significantly altered by the induction and maintenance of an acidic urine.

Even if the first assumption is not completely valid, but providing that

the second assumption is verified experimentally and any changes making the final assumptions invalid are progressive and consistent, it should still be possible to establish a relation between measured urinary pH and the urinary excretion of appropriate drugs. In the following treatment, excretion figures obtained under closely controlled conditions are merely used as a reference to calculate perturbing effects on urinary excretion due to normal changes in urinary pH.

Equations 1 and 2, derived from the model shown in Fig. 1, describe the rate of change of body level of drug and of its excretion, respectively, under acidic urine conditions.

$$\frac{dB}{dt} = ka.A - km.B - ke.B \quad .. \quad .. \quad .. \quad 1$$

$$\frac{dU}{dt} = ke.B \quad .. \quad .. \quad .. \quad .. \quad .. \quad 2$$

Thus, the term ke.B represents the rate of presentation of drug to the kidney at any time. At relatively alkaline pH, some of the drug which has passed into the kidney tubule is reabsorbed back into the body compartment, thus effectively acting as an additional dose of the drug. For drug initially appearing in the tubules, the value of the constant ke is assumed to remain the same as under controlled acidic urine conditions, but the amount of drug in the body will differ for any particular time depending on the amount which has previously been reabsorbed. This new body level of the drug, which depends on the amount of reabsorbed drug, is designated B* where:

$$B^* = B + R \quad .. \quad .. \quad .. \quad .. \quad 3$$

(R = amount of drug which has been reabsorbed).

Reabsorbed drug, as well as drug already in the body, will be susceptible to metabolism in the body and filtration in the kidney and thus equation 1 must be re-written (see eqn 4) by substituting B* for B.

$$\frac{dB^*}{dt} = ka.A - km.B^* - ke.B^* \quad .. \quad .. \quad 4$$

When integrated, equation 4 gives the amount of drug present in the body at any time (B*) if the amount of drug previously reabsorbed in in the kidney tubules (R) can be determined. The value of R, at any time during a drug excretion study in which urinary pH is not controlled, may be calculated using the analogue computer. Using the appropriate values of ka, km and ke for a particular subject, a continuous calculation of U (see eqn 2) as a function of time can be made on the computer. At the same time, the actual rate of excretion of drug when the subject's urinary pH is fluctuating, (dU*/dt), can be programmed on the computer as a function of time using a variable diode function generator. By integrating the simulated function dU*/dt, the value of U* (the amount of drug excreted when urinary pH is fluctuating), can be continuously calculated on the same time scale as that used to calculate U. The value of R can be determined up to any time by continuously subtracting

U* from U. Knowing the value of R at any time, equation 3 can then be solved on the computer by continuously adding R into a new body compartment, multiplying by the constants km and ke, and integrating. The results, B*, will be calculated as a function of time, taking into account the metabolism of reabsorbed drug. The analogue computer program required to carry out these calculations is represented diagrammatically in Fig. 2.

The term ke.B* in equation 4 represents, at any time, the rate of presentation of the drug to the kidney for excretion when urinary pH is fluctuating. The difference between rate of presentation of drug to the kidney and rate of appearance of drug in the urine is a measure of the rate of reabsorption of drug from the kidney tubules. Therefore, by subtracting the actual rate of excretion (dU*/dt) from the value of ke.B* at the same time, the rate of reabsorption (dR/dt) at that time can be calculated. Since dU*/dt and ke.B* are produced continuously on the computer, with the same time scale, their difference (dR/dt) can be plotted as a continuous function (see Fig. 2). Percentage reabsorption can be calculated by taking the ratio of dR/dt to ke.B* at any time, and likewise the ratio of dU*/dt to ke.B* gives the percentage excretion at any time.

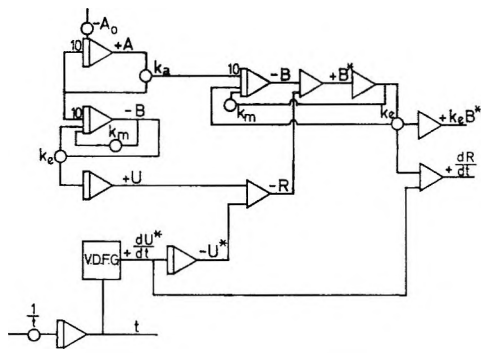

FIG. 2. Analogue computer program† for the absorption, metabolism and excretion of amphetamine in man at constant acid urinary pH.

When urine is collected at known time intervals and the pH accurately measured, the percentage drug reabsorption and percentage excretion derived as described above can be plotted against the average pH for each particular time interval.

† An alternative treatment of the problem is possible using a program based on the following equations:

$$\frac{dA}{dt} = -ka.A$$

$$\frac{dB}{dt} = ka.A - km.B - ke.B + \frac{dR}{dt}$$

$$\frac{dU}{dt} = ke.B - \frac{dR}{dt}$$

However, the introduction of the two different body level terms, B and B*, in the present treatment, was chosen to emphasize the fact that results are obtained by examination of two separate sets of experimental data, i.e. controlled and uncontrolled data.

The computer program given in the next paper of the series, for the prediction of urinary excretion under uncontrolled conditions (Beckett, Boyes & Tucker, 1968) is essentially derived from the above equations.

Experimental

Apparatus. An analogue computer (Electronic Associates Limited TR-20R), a digital computer (Elliot Automation 803B), a Perkin Elmer F11 gas chromatograph and a Pye Dynacap pH meter with a screened glass/saturated calomel electrode system.

CALCULATION OF REABSORPTION

Rate constants previously obtained for subjects given (+)-amphetamine under acidic urine conditions were used (Beckett & Tucker, 1968).

One subject was given two separate 15 mg doses and another subject was given one 15 mg dose of (+)-amphetamine sulphate in aqueous solution, on separate occasions, and the urinary pH was not controlled. Urine samples were collected at $\frac{1}{2}$ hrly intervals for 16 hr. The pH of each sample was determined immediately after collection and after cooling to room temperature; the amphetamine content was measured by the gas chromatographic assay (Beckett & Rowland, 1965b). Further uncontrolled urine pH amphetamine excretion figures were also used to calculate reabsorption values (Rowland, 1965).

The analogue computer was programmed as shown in Fig. 2 and the experimentally determined rate of excretion during each trial, corrected for "lag time", was simulated using a variable diode function generator. The values of ka, km and ke, previously determined for each subject,

TABLE 1. URINARY EXCRETION OF AMPHETAMINE AFTER ORAL ADMINISTRATION OF 15 MG (+)-AMPHETAMINE SULPHATE TO SUBJECT 1 (UNCONTROLLED URINE pH)

Sample time (hr)	Mean time (hr)	Urine pH	Urine Vol (ml)	Total excretion (μg base)	μg base/min	% dose/hr	$\left(=\frac{dU^*}{dt}/ke.B^* \times 100\right)$ % excretion
0	0	5·56	—	—	—	—	—
0·5	0·25	7·10	66·0	35·7	1·19	0·67	8·5
1·0	0·75	6·66	53·0	73·8	2·46	1·39	17·3
1·5	1·25	6·32	61·0	26·3	4·21	2·38	22·9
2·0	1·75	5·42	114·0	165·0	5·50	3·11	32·4
2·5	2·25	5·50	238·0	197·4	6·58	3·72	40·1
3·0	2·75	5·19	110·5	229·8	7·66	4·33	75·5
3·5	3·25	5·48	176·0	215·4	7·18	4·06	43·0
4·0	3·75	5·60	161·0	186·3	6·21	3·51	36·0
4·5	4·25	5·76	126·0	155·1	5·17	2·92	32·9
5·0	4·75	6·00	110·0	121·5	4·05	2·29	26·9
5·5	5·25	6·17	59·0	101·4	3·38	1·91	24·1
6·0	5·75	6·28	63·0	78·6	2·62	1·48	17·4
6·5	6·25	6·68	53·5	63·6	2·12	1·20	14·7
7·0	6·75	6·59	41·0	39·9	1·33	0·75	11·5
7·5	7·25	6·35	41·0	52·5	1·75	0·99	18·1
8·0	7·75	5·96	48·0	75·3	2·51	1·42	29·7
8·5	8·25	5·95	60·5	111·9	3·73	2·11	36·5
9·0	8·75	5·34	120·0	132·6	4·42	2·50	46·1
9·5	9·25	5·43	140·0	138·6	4·62	2·61	48·2
10·0	9·75	5·30	140·0	139·5	4·65	2·63	53·0
10·5	10·25	5·29	63·5	138·0	4·60	2·60	55·1
11·0	10·75	5·26	42·0	134·4	4·48	2·53	56·4
11·5	11·25	5·19	40·0	130·5	4·35	2·46	58·1
12·0	11·75	5·49	28·5	106·8	3·56	2·01	55·3
12·5	12·25	5·40	31·0	97·8	3·26	1·84	51·4
13·0	12·75	5·70	41·5	84·9	2·83	1·60	48·5
13·5	13·25	5·58	26·0	74·4	2·48	1·40	42·4
14·0	13·75	5·90	25·0	50·4	1·68	0·95	30·5
14·5	14·25	6·15	18·0	34·5	1·15	0·65	23·3
15·0	14·75	8·00	21·0	11·7	0·39	0·22	0·076
15·5	15·25	7·85	24·0	6·3	0·21	0·12	3·7

were set using the appropriate potentiometers. The values of ke.B*, dR/dt and dU*/dt were plotted against time. The percentage reabsorption and excretion was then calculated for the times at which the urinary pH was known.

Results

Fig. 3 shows ke.B*, dR/dt and dU*/dt (for one subject) as calculated by the computer and plotted against time; Table 1 summarizes the data from which the curves were plotted. Similar results were obtained in all the trials. In Fig. 4 the logarithm of percentage excretion has been

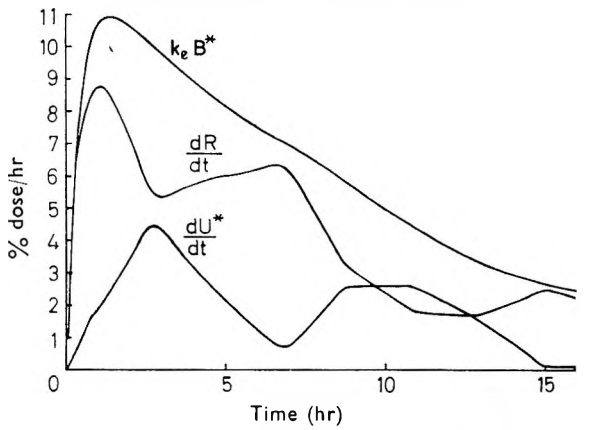

FIG. 3. Computer calculations of rate of presentation of amphetamine to the kidney (ke. B*); rate of kidney reabsorption of amphetamine $\left(\frac{dR}{dt}\right)$; and rate of urinary excretion of amphetamine as functions of time under controlled urinary pH conditions (Subject 1).

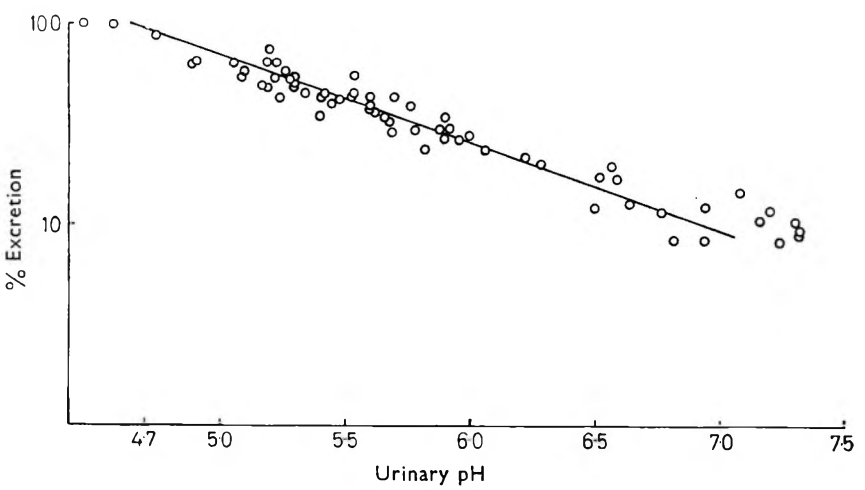

FIG. 4. Relation between log percentage urinary excretion of amphetamine and measured urinary pH (data from 2 trials in 2 subjects).

plotted against the corresponding urinary pH values and using a digital computer a straight line represented by equation 5 fitted by the method of least squares.

$$\log \% \text{ excretion} = -0{\cdot}4383 \text{ pH} + 4{\cdot}0384 \quad .. \quad .. \quad 5$$

The calculated correlation coefficient was −0·955.

Discussion

The method used to calculate the relation between kidney tubule drug reabsorption and measured urinary pH takes into account the higher body levels which result from reabsorption of the drug and the increased amount of metabolism which occurs compared with conditions under which reabsorption is assumed to be negligible. The high correlation coefficient obtained for the line resulting from a plot of log percentage excretion of amphetamine against pH (Fig. 4) supports the theory on which the calculations are based. Equation 5 gives a logarithmic relation between excretion (or reabsorption) of the drug and the measured urinary pH. Thus, there is a direct relation between the concentration of unionized drug in the kidney tubules and the extent of reabsorption. The present result apparently substantiates the theory that reabsorption is a passive process dependent on the concentration of unionized drug in the kidney tubules.

The rate of urine output varied between 0·5 and 5 ml/min in the present experiments. However, this does not explain the scatter of the points about the line in Fig. 4 since no direct relation between distance from the line and urine flow rate was apparent. In addition to departures from the assumptions listed in the theoretical section, possibly such factors as differences in measured urine pH and the actual pH at the site(s) of reabsorption, rate of change of urinary pH, and minor variations in kidney function, contribute to the relatively minor scatter of points.

The urinary excretion of most basic or acidic drugs will be influenced by urinary hydrogen ion concentration. If, using a basic drug, a smooth curve of rate of excretion against time can be obtained by rendering the urine acidic (about pH 5) (or alkaline for an acidic drug), the present method will reveal a relation between drug reabsorption (or excretion) and the pH of urine. For those drugs which are relatively slowly metabolized in man and are excreted in high percentage in the urine as unchanged drug, an estimate of the duration of action, under conditions of fluctuating urinary pH, should then be possible. Having established that there is little inter- or intra-subject variation in the rate constants describing absorption, metabolism and excretion of a drug, it should be possible to predict the excretion pattern for the drug in subjects whose urinary pH is not controlled, solely from a knowledge of the collection times and pH of urine samples. With amphetamine, inter- and intra-subject variation in the values of rate constants for absorption (ka) and excretion (ke) are relatively small (at least in healthy male subjects), although the inter-subject variation in the value of km, the metabolic constant, is somewhat larger (see Beckett & Tucker, 1968). However,

since the metabolism of amphetamine in man is a relatively slow process, differences in the value of km are very much less important in controlling the fate of the drug than major changes in the excretion rate of the drug, caused by fluctuations in urinary pH under normal conditions.

Once the necessary initial results have been obtained in volunteers under controlled conditions, it should, at least in theory, be possible to predict the performance of drug formulations solely on the basis of urine data from patients.

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Use of the analogue computer to predict the distribution and excretion of drugs under conditions of fluctuating urinary pH

A. H. BECKETT, R. N. BOYES* AND G. T. TUCKER†

An analogue computer program, incorporating a previously established relation between urinary pH and amphetamine excretion, has been used to predict the quantitative excretion of the drug under normal conditions of fluctuating urinary pH. Under the same conditions, a comparison has been made of computer predicted body levels of amphetamine after administration of the drug in a single solution dose, three divided solution doses, and in various prolonged-release formulations. Advantages of sustained-release preparations are indicated and specifications for a theoretically ideal sustained-release formulation are suggested.

IN a previous paper, Beckett, Boyes & Tucker (1968) described a method for determining a quantitative relation between urinary pH and kidney tubular reabsorption of drugs. A mathematical relation between percentage excretion of amphetamine and urinary pH was established, and it was considered that this relation could be used to predict the excretion of the drug under normal conditions of fluctuating urinary pH.

The ability to predict excretion of a drug will allow an assessment of its behaviour in the general population to be made with increased confidence and precision. Also, the advantages and disadvantages of various pharmaceutical formulations, like "prolonged release" products, with regard to useful availability of drugs, could be better considered. The present paper describes an attempt to make such predictions from the mathematical relation previously established.

Theoretical

The proposed relation between excretion of amphetamine and urinary pH is:

$$\log \% E = -0{\cdot}4383\ pH + 4{\cdot}0384 \quad .. \quad .. \quad 1$$

where % E = percentage amphetamine excreted.

The differential equation describing the body level of amphetamine when the urinary pH is maintained at a constant acidic level ($4{\cdot}7 \pm 0{\cdot}2$) is:

$$\frac{dB}{dt} = ka.A - km.B - ke.B \quad .. \quad .. \quad .. \quad 2$$

(Beckett & others, 1968).

When the urinary pH is fluctuating, the body level of the drug B is designated B*, where:

$$B^* = B + R \quad .. \quad .. \quad .. \quad .. \quad 3$$

(R = amount of drug which has been reabsorbed from the kidney tubules).

On substituting B* for B in equation 2 the term ke.B* represents the rate of presentation of drug to the kidney for excretion at any time.

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Taking equation 1 into account, and multiplying this term (ke.B*) by a factor equivalent to the expected % excretion of the drug, for the pH at a particular time, the result will be the predicted rate of excretion of the drug at that time, i.e.

$$\frac{dB^*}{dt} = ka.A - km.B^* - ke.B^* \times \frac{\%\,E}{100} \quad .. \quad .. \quad 4$$

$$\frac{dU^*}{dt} = ke.B^* \times \frac{\%\,E}{100} \quad .. \quad .. \quad .. \quad 5$$

where

$\frac{dU^*}{dt}$ = predicted rate of excretion; % E is calculated from equation 1.

The analogue computer program, shown in Fig. 1, combines equations 1, 4 and 5 to give an output proportional to predicted rate of excretion as a function of time.

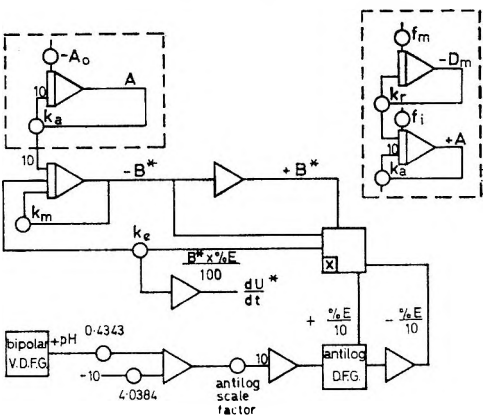

FIG. 1. Analogue computer program for the prediction of amphetamine excretion and body levels, from urinary pH data, after oral administration of the drug in solution and in a sustained-release form. (Inset—program for sustained-release.)

Experimental

Apparatus. An analogue computer (Electronic Associates Limited TR-20R) with an X-Y recorder (Advance Electronics Limited), a Perkin Elmer F11 gas chromatograph and a Pye Dynacap pH meter with a screened glass/calomel electrode system.

METHODS

Two male volunteers were given 15 mg oral doses of D(+)-amphetamine sulphate in aqueous solution and urine samples were collected at 30 min intervals for 16 hr; urinary pH was uncontrolled and the pH of each urine sample was accurately measured. The pH measurements for a particular subject, corrected for "lag-time" (Beckett & Tucker, 1968), were programmed on the analogue computer as a function of time. The previously determined values of ka, km and ke for the subject were set on the computer and a plot of $\frac{dU^*}{dt}$ against time was made.

The amphetamine contents of the urine samples were determined by

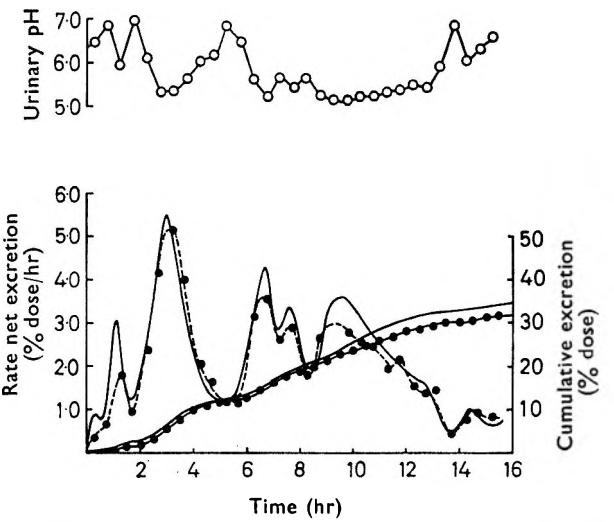

FIG. 2. A comparison of computer predicted and actual rates of excretion and cumulative excretion of amphetamine after oral administration of 15 mg (+)-amphetamine sulphate in solution (subject 1). Continuous lines: computer predictions. - -●- - Experimental data. —○— Urinary pH.

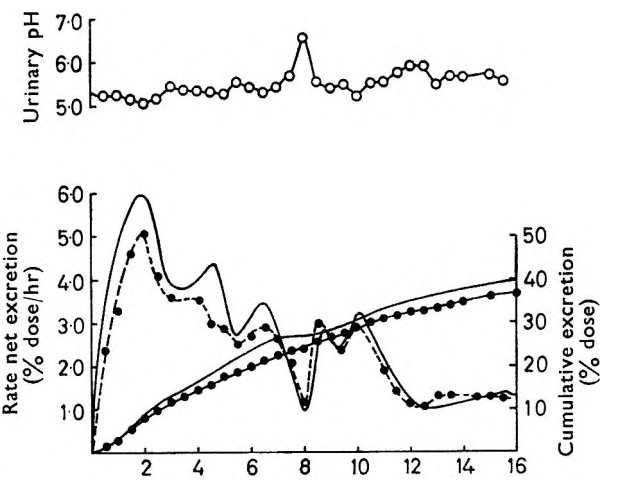

FIG. 3. A comparison of computer predicted and actual rates of excretion n cumulative excretion of amphetamine after oral administration of 15 mg (+)-amphetamine sulphate in solution (subject 2). Continuous lines: computer predictions. - -●- - Experimental data. —○— Urinary pH.

gas chromatography (Beckett & Rowland, 1965). When the analyses were completed, a plot of actual rate of excretion of amphetamine against time was constructed and compared with the computer calculated prediction. Predicted and actual cumulative excretion curves were also compared for each subject.

Results and discussion

Comparisons of predicted and actual excretion rate and cumulative excretion curves for amphetamine in the two subjects are very close (Figs 2 and 3). Even when the urinary pH fluctuated markedly (see Fig. 2) the prediction is in excellent agreement with the actual data. The greatest differences are apparent in the first few hours after drug administration, when absorption is still occurring and distribution is being established. The cumulative excretion curves indicate that over a period of 16 hr the difference between actual and predicted amount of drug excreted is only 2·5 and 3% of the dose respectively in the two subjects.

The close correlation of the predicted and actual excretion curves is acceptable evidence that the relation between excretion or kidney tubular reabsorption and urinary pH is valid (eqn 1).

The calculations necessary to predict the rate of excretion of the drug at any time involve a prediction of the body level of the drug at the same time. If the predicted rates of excretion are reasonably close to the actual rates, it may be assumed that the predicted body levels will also be close to the actual body levels. Since it is possible to plot the predicted body levels as a function of time using the analogue computer, detailed studies of the effect of urinary pH on the body levels of drugs can be made.

Fig. 4 (curve 2) shows the predicted body levels of amphetamine corresponding to the rate of excretion curve shown in Fig. 2 (a similar curve was obtained with subject 2); the body levels of the drug after 16 hr corresponds to 40% of the total administered dose. This represents about 6 mg of amphetamine, calculated as the sulphate, in the body, and it is significant that the volunteer experienced considerable difficulty in going to sleep after the 16 hr period. Curve 3 of Fig. 4 indicates that if the dose had been given to the subject in the form of three 5 mg solution doses at 4-hrly intervals, 50% (7·5 mg) of the total dose would be present in the body 16 hr after the administration of the first dose.

By programming typical urinary pH against time figures, it is possible to study the effect of administration of a formulated product on the body levels of a drug. The body level against time curves illustrated in Fig. 5 were obtained after programming two different pH against time patterns, and "administering" the dose to the computer, in solution and in a typical prolonged-release form [20% (f_l) initial "free" dose; 80% (f_m) "maintenance" dose (D_m): first-order release rate constant $kr = 0{\cdot}300\ hr^{-1}$, see insert on Fig. 1 for modification of computer program for prolonged-release dosage forms]. Curves 1 and 3 in Fig. 5 were obtained using pH/time pattern A and curves 2 and 4 using pattern B. Comparison of curve 1 with curve 2, and curve 3 with curve 4 indicates that there is a greater difference between body levels, with the two urinary pH/time patterns, when the drug is given in solution compared with drug given in the prolonged-release form; the latter makes the dose available to the body over a longer period of time, and this tends to compensate for changes in body levels of drug resulting from large changes in urinary pH.

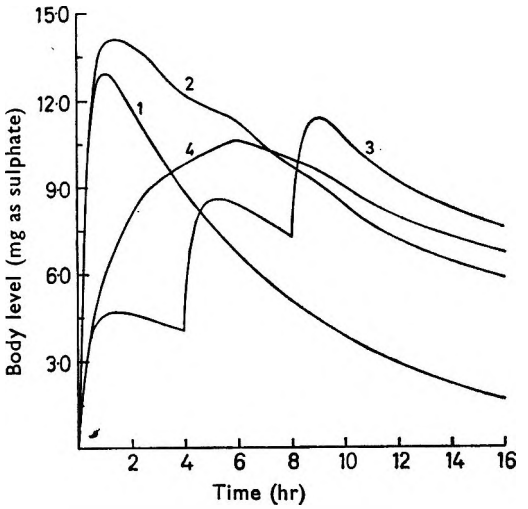

FIG. 4. Computer predicted body level (subject 1) of amphetamine (as sulphate) after oral administration of 15 mg doses in various dosage forms.

1. Single solution dose; acidic urinary pH control. 2. Single solution dose; fluctuating urinary pH as in Fig. 2. C. Three divided solution doses at 4 hrly intervals; fluctuating urinary pH as in Fig. 2. 4. Prolonged release form (20% free dose; 80% maintenance dose; kr = 0·300 hr^{-1}); fluctuating urinary pH as in Fig. 2.

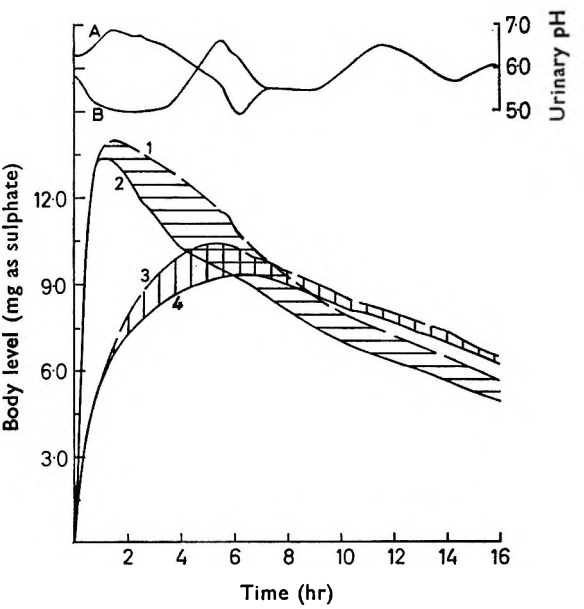

FIG. 5. The effect of urinary pH on computer predicted body levels of amphetamine after oral administration of the drug in solution and a prolonged-release form.

1 and 2. After the solution form with urinary pH/time patterns A and B respectively. 3 and 4. After the prolonged-release form with urinary pH/time patterns A and B respectively.

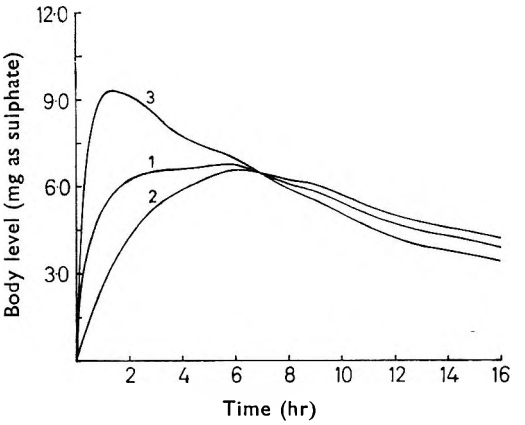

FIG. 6. A comparison of computer predicted body levels of amphetamine after oral administration of 10 mg (+)-amphetamine sulphate to subject 1 (urinary pH/time pattern as in Fig. 2). 1. 'Ideal' sustained-release form (40% free dose; 60% maintenance dose; kr = 0·300 hr^{-1}). 2. As in curve 1, but total dose in maintenance form. 3. Total dose in free (solution) form.

More effective and economical formulations could thus be designed by taking into account the urinary pH dependent excretion of drugs. Curve 4 in Fig. 4 shows the predicted body levels pattern of amphetamine if the subject had been given 15 mg (+)-amphetamine sulphate in the prolonged-release preparation previously described while curve 3, Fig. 4, shows the body level using a 3 × 5 mg regimen. Assuming a body level of about 6 mg unchanged amphetamine produces an optimum therapeutic effect, reduction of the total dose to 10 mg and alteration of the proportions of "free" and "maintenance" dose to 40 and 60% respectively, will give a much better sustained release formulation (see curve 1, Fig. 6); a similar curve was obtained with the pH figures for subject 2.

Thus correctly designed sustained-release preparations can eliminate not only the peaks and troughs resulting from the use of divided doses, but also reduce differences in the body levels of the drug obtained when the dose is administered during a period in which the urine is relatively acidic and when it is relatively alkaline. Furthermore, such formulations would allow the maintenance of constant therapeutic drug levels with smaller total dosage requirements, compared with the use of divided dose regimens. Convenience to the patient is thus not the only justification for the use of sustained-release formulations.

Acknowledgements. One of us (G.T.T.) thanks the Pharmaceutical Society for a research scholarship and the Science Research Council for a research studentship.

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Cocaine-sensitive uptake of sympathomimetic amines in nerve tissue

S. B. ROSS, A. L. RENYI AND B. BRUNFELTER

The uptake of [^{3}H]octopamine, [^{3}H]norephedrine and [^{3}H]phenylethanolamine in slices of cerebral cortex and heart of the mouse was investigated. Cocaine inhibited the uptake of octopamine but had no effect on that of the other two amines, whose uptake seems to be a pure physico-chemical partition between the tissue and the solution. Together with previous results, these indicate that the cocaine-sensitive uptake is linked with the hydroxyl groups in the benzene nucleus and particularly with that in the *meta* position. The results bear out the view that the potentiating effect of cocaine on the direct effect of sympathomimetic amines is due to inhibition of the uptake of the amines in sympathetic nerves, but they contradict the assumption that the antagonizing effect of cocaine on the indirect action of the amines is due to inhibition of the amine uptake.

IN previous experiments it was found that the uptake of tritiated sympathomimetic amines in brain slices which had been incubated with the amines for a short time was greatly reduced by cocaine and desipramine but not by reserpine (Ross & Renyi, 1966a, b, c). This indicates that under these conditions the uptake of the amines via the neuron membranes of adrenergic nerves was the most important factor governing the amine accumulation. The rate of uptake was found to be dependent on the chemical structure of the amines. For instance noradrenaline and dopamine were taken up with similar velocities while tyramine showed a slower uptake. (−)-Amphetamine was not actively taken up at all.

To obtain more information the investigation has been extended with three amines, namely [^{3}H]octopamine, [^{3}H]norephedrine and [^{3}H]phenylethanolamine.

Experimental

MATERIALS AND METHODS

[^{3}H]Octopamine {(±)-*p*-[7-^{3}H]hydroxyphenylethanolamine; specific activity 2·5 c/mmole} was obtained from New England Nuclear Corp., USA. [^{3}H]Phenylethanolamine {(±)-[α-^{3}H]phenyl-β-aminoethanol; specific activity 110 mc/mmole} and [^{3}H]norephedrine {(±)-[α-^{3}H]-hydroxy-β-aminopropylbenzene; specific activity 100 mc/mmole} were synthesized by reducing α-aminoacetophenone and α-aminopropiophenone with tritiated sodium borohydride (The Radiochemical Centre, Amersham, England; specific activity 750 mc/mmole). The purity of the tritiated amines was checked by paper chromatography (n-butanol–acetic acid–water, 4 : 1 : 5).

The uptake of the amines in brain slices was determined as described previously (Ross & Renyi, 1966a). Unless otherwise specified the incubation solution contained 100 mg of brain slices (cerebral cortex of

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mouse) 0·2 nmole of the tritiated amine and the inhibitor to be tested in 2 ml of Krebs-Hensleit buffer containing 0·1% glucose. The incubation was made in an atmosphere of 93·5% oxygen and 6·5% carbon dioxide, at 37°. The tritiated amine was added to the solution after 5 min pre-incubation of the slices with or without the inhibitor.

The amount of the tritiated amine in the slices was measured as the total radioactivity in an ethanol extract of the homogenized slices. It was expressed in nmole/g of tissue. No attempt was made to separate metabolites formed in the tissue from the amine.

Results

Although the 3 amines were all accumulated in the slices above the external concentration, only the uptake of [^{3}H]octopamine was reduced by cocaine (Figs 1–3, Table 1). Pre-treatment of the animals with pheniprazine had scarcely any effect on the amount of [^{3}H]octopamine accumulated (Fig. 1), whereas reserpine effected a marked reduction with prolonged incubation (Table 2). These results indicate that the amine taken up is rapidly bound in noradrenaline storage granules.

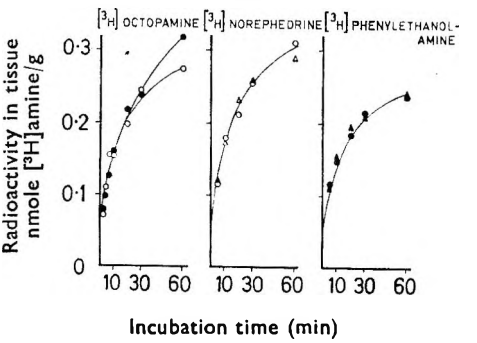


FIG. 1. Accumulation of [^{3}H]octopamine, [^{3}H]norephedrine and [^{3}H]phenylethanolamine (0·1 nmole/ml) in slices of mouse cerebral cortex (100 mg). —○— control slices. —△— slices with cocaine, 3×10^{-5}M. Filled symbols indicate pre-treatment with pheniprazine, 10 mg/kg i.p., 24 hr before the experiment.

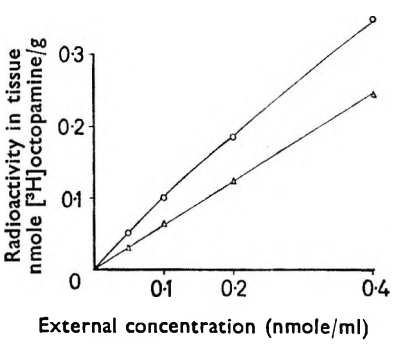


FIG. 2. Uptake of [^{3}H]octopamine in cortex slices at different external concentrations. Incubation time, 5 min. —○— control slices. —△— slices with cocaine, 3×10^{-5}M.

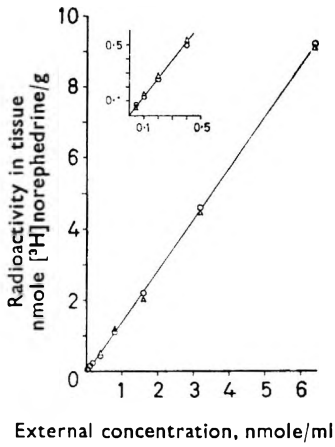


FIG. 3. Uptake of [^{3}H]norephedrine in cortex slices at different external concentrations. For conditions see legend to Fig. 2.

TABLE 1. INHIBITION OF THE UPTAKE OF [^{3}H]OCTOPAMINE IN SLICES OF CEREBRAL CORTEX OF THE MOUSE

	Conc. M	n	Uptake nmole/g in 5 min	-Δ Uptake nmole/g in 5 min
Control	—	6	0·119 ± 0·008	—
Cocaine	3×10^{-5}	3	0·075 ± 0·003*	0·044
	6×10^{-6}	3	0·082 ± 0·001*	0·037
	$1{\cdot}5 \times 10^{-6}$	3	0·089 ± 0·001*	0·030
	3×10^{-6}	3	0·100 ± 0·005	0·019
Desipramine	3×10^{-7}	3	0·078 ± 0·003*	0·041
	3×10^{-8}	3	0·089 ± 0·003*	0·030
	3×10^{-9}	3	0·094 ± 0·005	0·025

* $P < 0{\cdot}01$.

The slices were pre-incubated with the inhibitor for 5 min and with [^{3}H]octopamine (0·1 nmole/ml) for a further 5 min.

If the uptake of [^{3}H]octopamine in the first 5 min incubation is taken as the initial rate (Fig. 4), the approximate values of K_M and V_{max} for the cocaine-sensitive part of the uptake were 7×10^{-7}M and 0·06 nmole/g min. The value of K_M is about the same as that previously found for the uptake of noradrenaline, dopamine and tyramine. The value of V_{max} was close to that obtained for tyramine but lower than values for noradrenaline and dopamine (Ross & Renyi, 1966a).

TABLE 2. INHIBITION OF THE ACCUMULATION OF [^{3}H]OCTOPAMINE IN CEREBRAL CORTEX SLICES OF THE MOUSE BY RESERPINE

Incubation time (min)	Control	Reserpine (5 mg/kg)
5	0·111 ± 0·004	0·100 ± 0·004
30	0·310 ± 0·005	0·193 ± 0·014*

* $P < 0{\cdot}001$.

Reserpine was injected 18 hr before the experiment. The values are means ± s.e. for 4 animals.

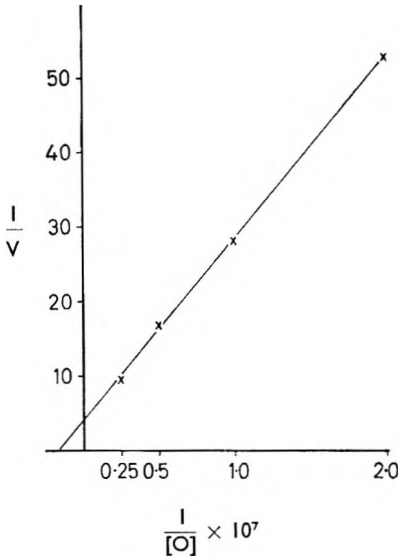


FIG. 4. Double reciprocal plot of the cocaine sensitive uptake of [^{3}H]octopamine in slices of mouse cerebral cortex. The plot is based on the experiments shown in Fig. 2. V = rate of uptake expressed in nmole/g and 5 min. The concentration of [^{3}H]octopamine [O] expressed in mole/litre.

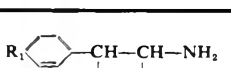
TABLE 3. ACCUMULATION OF [^{3}H]OCTOPAMINE, [^{3}H]NOREPHEDRINE AND [^{3}H]-PHENYLETHANOLAMINE IN HEART SLICES OF THE MOUSE

Amine	Accumulation of the amine nmole/g			
	n	Control	n	Cocaine 10 µg/ml
Octopamine	2	0·148 (0·152, 0·144)	2	0·074 (0·073, 0·076)
Norephedrine	4	0·244 ± 0·004	4	0·232 ± 0·004*
Phenylethanolamine ..	4	0·225 ± 0·012	4	0·205 ± 0·010*

* $P > 0·05$.

The heart slices were incubated with [^{3}H]amine (0·1 nmole/ml) for 15 min.

TABLE 4. RELATIONSHIP BETWEEN THE COCAINE SENSITIVE UPTAKE OF SYMPATHOMIMETIC AMINES AND THE EFFECT OF COCAINE ON THE DIRECT AND INDIRECT EFFECT OF THE AMINES



Amine	R_1	R_2	R_3	R_4	Uptake inhibited by cocaine	Direct action potentiated by cocaine*	Indirect action antagonized by cocaine*
Noradrenaline ..	OH	OH	OH	H	++	+	
Dopamine	OH	OH	H	H	++		
Metaraminol ..	H	OH	OH	Me	++	+	
Octopamine ..	OH	H	OH	H	+	−	+
Tyramine	OH	H	H	H	+		+
Norephedrine ..	H	H	OH	Me	−	−	+
Phenylethanolamine	H	H	OH	H	−	−	+
Amphetamine ..	H	H	H	Me	−		+

* According to Trendelenburg & others (1962).

Within a large concentration range the accumulation of [^{3}H]norephedrine in the slices was linear (Fig. 3); this indicates a pure physicochemical partition of the amine between the tissue and the solution.

The uptake of [^{3}H]octopamine, but not that of [^{3}H]norephedrine or [^{3}H]phenylethanolamines, was also inhibited in heart slices of the mouse by cocaine (Table 3).

Discussion

These experiments strengthen the earlier supposition of a relation between the chemical structure and the rate of uptake of sympathomimetic amines in brain tissue (Ross & Renyi, 1966a). As shown in Table 4 the cocaine-sensitive uptake seems to be linked with the hydroxyl groups in the benzene nucleus and in particular with that in the *meta* position. The hydroxyl group in the side-chain seems to be of secondary importance. Amines having no hydroxyl group in the benzene nucleus were not taken up by a cocaine-sensitive mechanism. Since similar results have been obtained for heart slices (Ross & Renyi, 1966b and this report) it seems probable that the relations obtained may be generally valid for the uptake of the amines in sympathetic nerves.

The similar pattern between the potentiating effect of cocaine on the direct component of the sympathomimetic effect of the amines described by Trendelenburg, Muskus & others (1962) and the cocaine-sensitive uptake of the amines bears out these authors' supposition that the potentiating action of cocaine is a result of inhibition of the uptake of the amine in sympathetic nerves. They found, however, that the direct action of octopamine (norsynephrine) on the nictitating membranes of the cat was not potentiated by cocaine. This observation may have its explanation in the rather weak direct action of this amine and its sensitivity to monoamine oxidase. Since the capacity of the uptake reaction is limited, it seems likely that the inactivation mechanism for those amines which had to be given in large doses is less dependent on the uptake reaction. It should be recalled that the action of the other more potent *p*-hydroxylated amines, or those that were metabolically more stable, studied by the above-mentioned authors, namely synephrine, *p*-hydroxyephedrine and *p*-hydroxyphenylpropanolamine, was significantly potentiated by cocaine.

Unlike the direct component of the sympathomimetic effect the indirect one is antagonized by cocaine (Trendelenburg, 1963). Among the amines listed in Table 4 the last 5 compounds exert an important indirect effect, and two of them—tyramine and amphetamine—act only indirectly (Trendelenburg, 1963). Our observations that three of these amines were not taken up by a cocaine-sensitive mechanism seems to contradict the supposition that the antagonizing effect of cocaine is due to inhibition of the uptake of these amines before they release noradrenaline (Trendelenburg, 1961). Ross & Renyi (1966a) proposed that the noradrenaline released by the indirectly-acting amines emanates from a small

extraneuronal store, possibly located in the carrier in the neuron membrane. However, the possibility also remains that the outward passage via the neuron membrane of noradrenaline released from an intraneuronal store is inhibited by cocaine.

Recent observations that in cat and rat the tachyphylaxis to the pressor effect of (+)-amphetamine, phenylethylamine, and mephentermine is only poorly or not at all crossed to tyramine and α-methyltyramine (Bhagat, 1965; Bhagat, Gordon & Kopin, 1965; Eble & Rudzik, 1965; Fawaz & Simaan, 1965; Day, 1967) indicate that the indirectly-acting amines may produce their effects by two distinct mechanisms (Day, 1967). It is interesting to note that the difference in chemical structure between these two groups of indirectly-acting amines is the same as that determining the uptake reaction discussed above.

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Subcellular distribution of some phenothiazines in blood platelets of rabbit

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Platelets incubated in plasma containing 5×10^{-5} and 3×10^{-4} M chlorpromazine, desmonomethylchlorpromazine or chlorpromazine sulphoxide were homogenized and fractioned by differential centrifugation. After fractionation most of the accumulated chlorpromazine and desmonomethylchlorpromazine were found in the particulate fractions, while most of the chlorpromazine sulphoxide was in the supernatant. The phenothiazines studied had a certain affinity for that fraction containing most of the platelet 5-hydroxytryptamine (5-HT). These phenothiazines released half of the platelet 5-HT without altering its intracellular distribution. The distribution of 5-HT was affected only by concentrations of chlorpromazine and desmonomethylchlorpromazine which released nearly all of the platelet 5-HT.

CHLORPROMAZINE and some other phenothiazines are known to cause 5-hydroxytryptamine (5-HT) liberation from blood platelets *in vitro* (Bartholini, Pletscher & Gey, 1961; Paasonen, 1964, 1965; Ahtee & Paasonen, 1965). Alterations in the chemical structure change the ability of these compounds to release 5-HT (Ahtee, 1966). The platelets accumulate many times more phenothiazines than are present in plasma, and there are certain relations between the ability of various compounds to liberate 5-HT from platelets and the uptake of phenothiazines by platelets (Ahtee & Paasonen, 1966). Most of the platelet 5-HT is bound in a certain granule fraction which can be separated by differential centrifugation (Solatunturi & Paasonen, 1966). Using similar fractionation the present experiments were undertaken to study how some of the phenothiazine derivatives are distributed in platelets.

Experimental

Male albino rabbits weighing 2·8–3·4 kg were bled under ether anaesthesia from the carotid artery through a polyethylene cannula. The platelet-rich plasma was obtained as described by Paasonen (1964) and 5 or 10 ml of it was incubated in air with gentle shaking at 37°. One ml of platelet-rich plasma contained $9{\cdot}5 \times 10^8 \pm$ s.d. $2{\cdot}6 \times 10^8$ platelets ($n = 19$). The experiments were made in polyethylene or polypropylene vessels, and polypropylene pipettes were used. Into each ml sample was added 0·1 ml of the drug solution or saline.

The drugs used were: chlorpromazine hydrochloride (May & Baker Ltd., Dagenham), desmonomethylchlorpromazine maleate and chlorpromazine sulphoxide (Rhône-Poulenc, Paris).

After incubation for 1 hr the platelets were separated by centrifugation at 4,000 *g* for 20 min at a temperature below 5°, and homogenized in 4 ml of 0·32M sucrose by ultrasound (Branson Sonifer S-75 with a $\frac{1}{8}$ inch micro tip, setting No. 3, 1 min). After homogenization 5 ml of 0·32M sucrose was added. The diluted homogenates were fractionated by differential

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centrifugation (Buckingham & Maynert, 1964; Solatunturi & Paasonen, 1966). From the particulate fractions of 2,500, 18,500 and 100,000 *g* sediments, and from the supernatant obtained, the phenothiazines were estimated spectrophotometrically (Salzman & Brodie, 1956) and 5-HT spectrophotofluorometrically (Bogdanski, Pletscher & others, 1956).

Results and discussion

DISTRIBUTION OF PHENOTHIAZINES

After incubation of the platelet-rich plasma with 5×10^{-5}M concentrations of phenothiazines, the platelets accumulated per ml of platelet-rich plasma $2{\cdot}0 \pm 0{\cdot}8$ μg (mean $\pm$ s.d., n = 4) of chlorpromazine, $3{\cdot}7 \pm 2{\cdot}6$ μg of desmonomethylchlorpromazine and $4{\cdot}1 \pm 0{\cdot}8$ μg of chlorpromazine sulphoxide. The corresponding accumulation in the platelets incubated with 3×10^{-4}M concentrations were $11{\cdot}4 \pm 3{\cdot}4$ μg, $13{\cdot}3 \pm 6{\cdot}2$ μg and $15{\cdot}5 \pm 4{\cdot}9$ μg, respectively. Fig. 1 represents the percentage distribution of these compounds in particulate fractions and

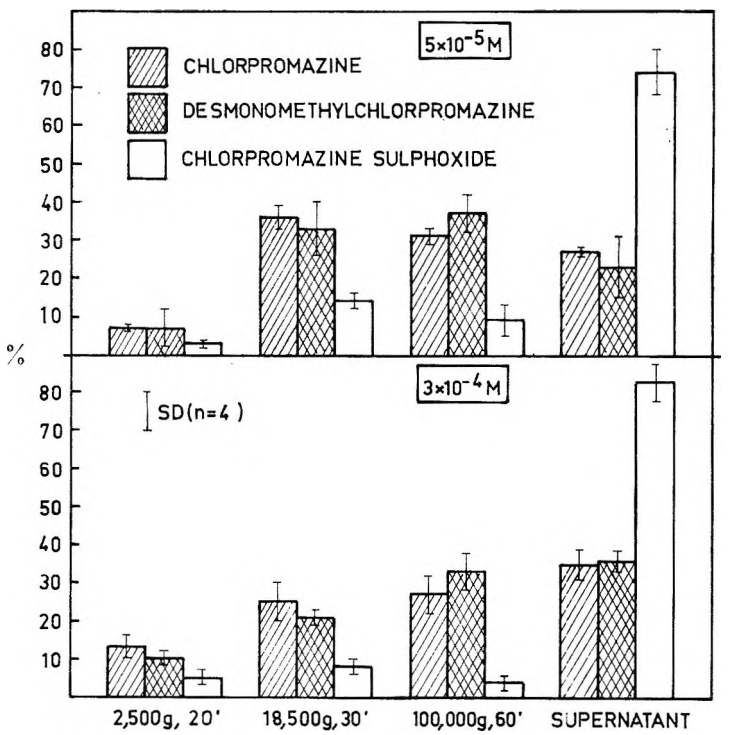

FIG. 1. Percentage distribution of phenothiazines in subcellular fractions of rabbit platelets incubated for 1 hr with 5×10^{-5} and 3×10^{-4}M concentrations of these compounds. Fractions were obtained by differential centrifugation of platelet homogenate. The three particulate fractions are indicated by the *g* values and the centrifugation times used to sediment them.

the supernatant after incubation of platelet-rich plasma with the two concentrations of phenothiazines mentioned above. After incubation with the higher concentrations the percentage of phenothiazines had increased more in the supernatant and in the 2,500 *g* sediment than in the other two fractions.

The distribution of chlorpromazine and its desmonomethyl derivative was similar in the supernatant and the particulate fractions, but they were present in much larger amounts in the particulate fractions. On the other hand, most of chlorpromazine sulphoxide was present in the supernatant fluid. These findings are related in the same way as is the behaviour of the three compounds on the central nervous system, chlorpromazine sulphoxide possessing much weaker central nervous system effects than chlorpromazine and desmonomethylchlorpromazine.

Among the particulate fractions, most of the phenothiazines were found in the 18,500 and 100,000 *g* sediments. The 100,000 *g* sediment contains microsomes and membranes but no 5-HT. On the other hand, the 18,500 *g* sediment contains two-thirds of the platelet 5-HT (Solatunturi & Paasonen, 1966). It is therefore interesting to note that this granule fraction also accumulated considerable portions of the phenothiazines. In this fraction 36% of the accumulated chlorpromazine, 33% of the desmonomethylchlorpromazine and 14% of the chlorpromazine sulphoxide were found after incubation with 5×10^{-5}M concentrations. After incubation with 3×10^{-4}M concentrations the corresponding percentages were about one-third lower.

To test the experimental procedure, the platelet-rich plasma was incubated without phenothiazine and the platelets from 5 ml of platelet-rich plasma were then homogenized in sucrose solution containing 60 μg of chlorpromazine. This is equal to the amount accumulated in the platelets from the 3×10^{-4}M incubation concentration. The distribution of chlorpromazine in subcellular fractions was similar to that presented above. Therefore the subcellular distribution of phenothiazines may be modified by the fractionation method used.

When the platelets incubated with 3×10^{-4}M chlorpromazine, desmonomethylchlorpromazine or chlorpromazine sulphoxide were washed twice with saline, their total phenothiazine contents decreased by 66, 51 and 90%, respectively. However, the percentages of these phenothiazines remaining in the platelets after washing were larger than initially in the 18,500 *g* sediment. In this fraction before washing there was 25% of the accumulated chlorpromazine, 21% of the desmonomethylchlorpromazine and 8% of the chlorpromazine sulphoxide. After two washings these relative phenothiazine amounts were increased to 30, 35 and 15%, respectively. In the other fractions, especially in the supernatant, the relative amounts of the phenothiazines correspondingly decreased. An exception was the 2,500 *g* sediment from chlorpromazine-treated platelets, in which the percentage of chlorpromazine also slightly increased, possibly because this fraction contains some poorly homogenized cells among the subcellular particles. The above experiments suggest that chlorpromazine and related phenothiazines have a certain affinity for the 18,500 *g* sediment.

EFFECT OF PHENOTHIAZINES ON THE DISTRIBUTION OF 5-HT

The effect of phenothiazines on the intracellular distribution of 5-HT in platelets was also studied. During 1 hr incubation of platelet-rich plasma, none of the phenothiazines released 5-HT from platelets in the concentration of 5×10^{-5}M. In 3×10^{-4}M concentration chlorpromazine released 37%, desmonomethylchlorpromazine 73% and chlorpromazine sulphoxide 24% of the platelet 5-HT. In a concentration of 10^{-3}M, chlorpromazine caused a release of 98%. This agrees with earlier data (Ahtee, 1966). In 5×10^{-5}M concentration none of the phenothiazines, and in 3×10^{-4}M concentration only chlorpromazine and chlorpromazine sulphoxide did not alter the percentage distribution of 5-HT in the above fractions. However, in concentrations which release nearly all of the platelet 5-HT, chlorpromazine and desmonomethylchlorpromazine caused changes also in the intracellular distribution of this amine. The 18,500 *g* sediment contained 60% of the platelet 5-HT in controls incubated without phenothiazines. Incubation for 1 hr with 10^{-3}M chlorpromazine lowered the proportion of 5-HT in this fraction to 35% and incubation with 3×10^{-4}M desmonomethylchlorpromazine lowered it to 48% of the total platelet 5-HT found after these treatments. Correspondingly the relative amount of 5-HT in the supernatant increased.

The phenothiazines were able to release half of the platelet 5-HT without altering its intracellular distribution. Therefore, it is plausible that they exert their effect both on the cell membrane and on intracellular 5-HT-containing structures. The fractionation method used may have modified the subcellular distribution of phenothiazines. However, the potent 5-HT releasers, chlorpromazine and desmonomethylchlorpromazine, seem to possess a certain affinity for the 18,500 *g* sediment, which contains most of the platelet 5-HT.

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Effect of desipramine injected intracerebrally in normal or reserpinized rats

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Desipramine, 7·5–30 μg in 1 μl, injected under stereotaxic control into the brain of rats elicits a moderate hyperthermic response. This effect is more marked in animals made hypothermic by a previous treatment with reserpine. The response is not specific for any one of the six brain areas investigated (anterior part of the hypothalamus; nucleus rhomboideus thalami; third ventricle; posterior part of the hypothalamus; lateral part of the area amygdaloidea basalis; and lobus frontalis).

DESIPRAMINE given to animals made hypothermic by a previous administration of reserpine, induces a rapid and sustained increase of body temperature (Garattini & Jori, 1967). This effect requires the integrity of the central nervous system (Bernardi, Paglialunga & Jori, 1967). Since Bernardi, Jori & others (1966) showed that desipramine injected intracerebrally in reserpinized rats also elicits a hyperthermic reaction, the effect of desipramine injected by a stereotaxic technique into specific parts of the rat brain has been examined.

Experimental

Female Sprague Dawley rats of the average weight of 240 ± 10 g (brain weight 1·79 ± 1 g) were used. Under a light ether anaesthesia, microinjections of desipramine were made in different parts of the rat brain using a stereotaxic apparatus (C. H. Stoelting) with an adaptator to place a Hamilton 701 LT microsyringe with a cemented needle.

All operations were made at the same time in the morning. The average duration of an operation was 8 ± 1 min.

The microinjections were made into the nucleus rhomboideus thalami; anterior part of hypothalamus; posterior part of hypothalamus; third ventricle; lobus frontalis, and area amygdaloidea basalis pars lateralis.

The co-ordinates of these centres were calculated according to de Groot (1959) with the modification that the head of the rat was in the horizontal plane, and therefore the "O" point was in coincidence with the lambda point.

The volume of the microinjection was always 1 μl; pyrogen-free distilled water was used as a control. The pH of different concentrations of desipramine and water was the same (6·4 ± 0·05). After the operation, the animals were kept in individual Makrolon cages at 20° and a relative humidity of 60%. The body temperature was recorded with an automatic device (Jori & Paglialunga, 1966).

At the end of each experiment, the rats were decapitated and the brains were sectioned to check the site of the microinjection.

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Results

Table 1 summarizes the results obtained when desipramine was injected by stereotaxic technique into six different areas of the brains of normal rats. It is evident that a rise of body temperature, still present at 4–6 hr occurred after the injection of desipramine into the anterior part of the hypothalamus (AH), in the nucleus rhomboideus thalami (RH), in the third ventricle (V), and in the posterior part of the hypothalamus (PH).

TABLE 1. EFFECT OF DESIPRAMINE (DMI) ON BODY TEMPERATURE, AFTER MICRO-INJECTIONS IN DIFFERENT AREAS OF THE BRAIN OF NORMAL RATS

No. of rats	Area*	Treatment	Body temperature changes (°C ± s.e.) after hr			
			1	2	4	6
5	AH	Water	−0·1	+0·1 ± 0·1	−0·1 ± 0·1	−0·2
12	AH	DMI	+0·7	+0·8 ± 0·2	+1·0 ± 0·2	+0·8
5	RH	Water	−0·9	−0·4 ± 0·1	−0·6 ± 0·1	−0·6
5	RH	DMI	+0·3	+0·8 ± 0·1	+0·9 ± 0·1	+0·4
7	V	Water	−0·5	−0·2 ± 0·1	−0·2 ± 0·1	−0·3
4	V	DMI	+1·0	+1·3 ± 0·1	+1·3 ± 0·2	+0·8
4	PH	Water	−0·9	−0·4 ± 0·1	−0·5 ± 0·1	−0·4
5	PH	DMI	+0·3	+1·0 ± 0·2	+0·3 ± 0·1	0·0
4	LF	Water	−0·1	−0·1 ± 0·1	−0·1 ± 0·1	−0·1
5	LF	DMI	0·0	+0·2 ± 0·1	−0·2 ± 0·1	−0·2
4	ABL	Water	−0·6	−0·5 ± 0·1	−0·4 ± 0·1	−0·3
4	ABL	DMI	−0·2	−0·1 ± 0·1	+0·1 ± 0·1	0·0

See text.
DMI was given at the dose of 7·5 μg/brain in all the areas, except in V (30 μg/brain).

Desipramine (7·5 μg/brain) was without effect in the lobus frontalis (LF) and in the lateral part of the area amygdaloidea basalis (ABL).

Desipramine was also injected in three different doses in the anterior part of the hypothalamus (AH). Table 2 shows that there is no relation between the dose and the observed increase of body temperature.

TABLE 2. CHANGES IN BODY TEMPERATURE AFTER MICROINJECTIONS OF DESIPRAMINE (DMI) IN DIFFERENT DOSES IN THE ANTERIOR PART OF THE HYPOTHALAMUS OF RATS

No. of rats	Treatment	Dose μg/brain	Maximum increase in body temperature °C ± s.e. after microinjection	
			2 hr	4 hr
6	Water	—	no change	+0·2 ± 0·1
12	DMI	7·5	+0·8 ± 0·2	+1·0 ± 0·2
6	DMI	15·0	+1·1 ± 0·2	+1·2 ± 0·2
6	DMI	30·0	+1·1 ± 0·3	+1·1 ± 0·2

Other experiments were performed in rats reserpinized 16 hr before the injection of desipramine. Only rats showing a body temperature between 28° and 31° were used.

Fig. 1 summarizes the data obtained when desipramine (30 μg/brain) was injected into the six areas of the brains of reserpinized rats. In all cases desipramine elicited a rise of body temperature which was marked; controls injected with water showed a further hypothermic reaction. The

administration of desipramine at lower concentrations (7·5 μg/brain) appeared to be less effective in the three areas tested.

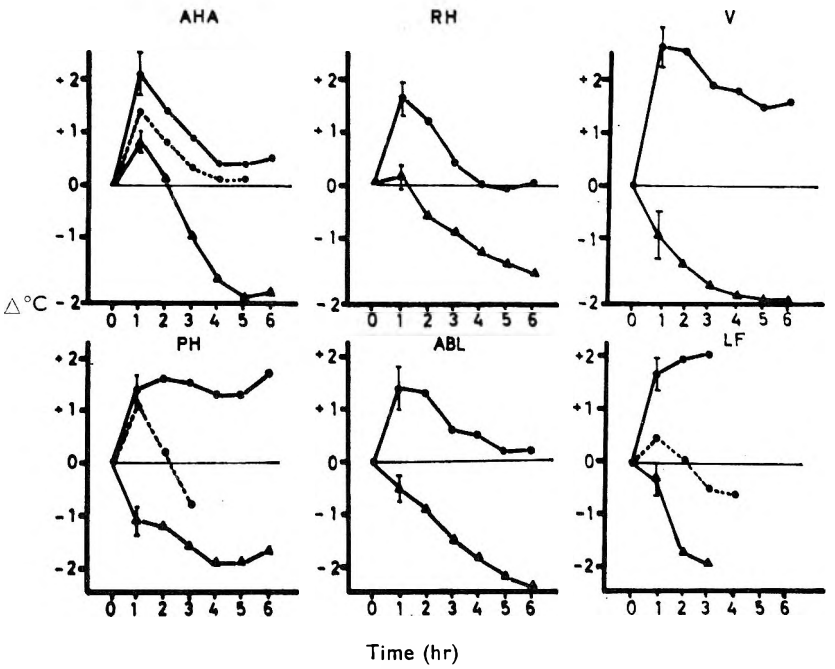

FIG. 1. Rats were injected with reserpine (5 mg/kg i.v.) 16 hr before the experiment and kept at a room temperature of 20°. Rats received a microinjection of distilled water (▲——▲) or desipramine 30 μg/brain (● —— ●) or 7·5 μg/brain (● - - - - ●). Each point represents the average of at least 5 animals. Vertical bars represent the standard errors. AHA (anterior part of hypothalamus); RH (nucleus rhomboideus thalami); V (third ventricle); PH (posterior part of hypothalamus); ABL (lateral part of area amygdaloidea basalis); LF (lobus frontalis).

To analyse the effect of desipramine it was also injected intravenously or into the nucleus rhomboideus thalami.

Table 3 shows that the effect of desipramine in reserpinized rats is more significant by intracerebral than by intravenous administration.

TABLE 3. EFFECT OF DESIPRAMINE (DMI) ON BODY TEMPERATURE OF RESERPINIZED RATS

No. of rats	Administration route	Treatment	Change of body temperature °C ± s.e.
10	Intravenously	Water	−0·23 ± 0·25
10	Intracerebrally (RH)	Water	−0·10 ± 0·19
10	Intravenously	DMI	+0·63 ± 0·15
10	Intracerebrally (RH)	DMI	+2·13 ± 0·34

Rats were injected with reserpine (5 mg/kg i.v.) 15 hr before the experiment and kept at room temperature of 20°. Body temperature was measured 1 hr after the treatment, at the time of the maximum increase observed. Desipramine (30 μg) and water were microinjected intracerebrally in the nucleus rhomboideus thalami (RH) and intravenously in the saphenous vein.

This factorial experiment was statistically analysed as 2 × 2 plan (treatment-route of administration). The interaction was significant ($P < 0·02$).

As the administration route does not modify the effect of the water injection it can be concluded that the effect of desipramine is significantly dependent on the route of administration.

Discussion

Desipramine induces a hyperthermic response when injected into the brain of normal rats by a stereotaxic technique. The effect is evident in the anterior and in the posterior part of the hypothalamus, in the thalamus (nucleus rhomboideus) and in the third ventricle, but not in the lobus frontalis or in the lateral part of the area amygdaloidea basalis. Desipramine injected intraperitoneally or intravenously is without effect on body temperature and only at high doses does it induce an hypothermic effect. When injected into the posterior part of the hypothalamus in a range of doses from 7·5 to 30 μg/brain, desipramine was hyperthermic without there being an evident relation between dose and effect.

The effect of desipramine was more marked in reserpinized animals. Better results were obtained when desipramine was injected into the posterior part of the hypothalamus and in the lobus frontalis.

Even in the thalamus nucleus rhomboideus, where the effect was less marked, the action of desipramine was significantly different from the effect elicited by the same dose of desipramine injected intravenously.

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Toxicity of cryoprotective agents at 30°

ARMAND M. KAROW, JR., OLIVER CARRIER, JR. AND BEN R. CLOWER

The toxicity of four cryoprotective agents (glycerol, dimethylsulfoxide, dextran, magnesium ion) as a function of time was assessed at 30° on isolated rabbit atria. Glycerol, 2·0 M, in Ringer solution killed all atria in less than 30 min. Atria tolerated 2·1 M dimethylsulphoxide in Ringer for 1 hr, 0·7 M glycerol in Ringer for 4 hr, 6% (w/v) dextran in saline for 6 hr and $6{\cdot}6 \times 10^{-2}$ M magnesium chloride in Ringer for 8 hr. After washing out the cryoprotective agents the tissue was examined histologically and for its response to isoprenaline, MJ-1999 [4,2-(isopropylamino-10-hydroxyethyl)methanesulphonilide, a β-blocking agent] and isoprenaline and ouabain. These tests were the more sensitive indicators of functional integrity.

PREVIOUSLY Karow & Webb (1965), Karow, Webb & Stapp (1965) have shown the relation of concentration, time and temperature during hypothermia and freezing with known cryoprotectants (glycerol, dimethylsulfoxide, dextran and magnesium ions). The toxicity of these agents at concentrations normally used for cryoprotection as a function of time under normal laboratory conditions is now reported.

Experimental

Rabbits weighing approximately 2·0 kg were stunned by a blow on the head, quickly bled and their hearts removed. The hearts were immediately placed in oxygenated (95% O_2, 5% CO_2) Ringer solution (composition: mM NaCl 153·9, KCl 5·4, $CaCl_3$ 2·4, $NaHCO_3$ 16·8, dextrose 11, distilled water to 1 litre) and the left atria removed. The atria were carefully trimmed of peripheral tissue, tied to a Plexiglass holder containing two platinum electrodes and immersed in oxygenated Ringer solution maintained at 30° in an isolated muscle bath. One g tension was placed on each atrium and they were stimulated continuously throughout the entire experiment with a square wave of 5 msec duration (120 Hz, 5V). Contractions were measured with a (Grass) strain gauge and recorded on a (Sanborn) polygraph.

After allowing the muscle to equilibrate in the tissue bath for 1 hr, the Ringer solution was drained from the bath and a solution of one of the test agents was substituted. The agents tested were 0·7 M and 2·0 M glycerol, 2·1 M dimethylsulphoxide and $6{\cdot}6 \times 10^{-2}$ M $MgCl_2{\cdot}6H_2O$ made up in Ringer solution. Also tested was commercially prepared 6% (w/v) dextran (average *M* 70,000) in saline. The atria were incubated with a given agent for 0·5, 1, 2, 3, 6, 8, 10, 18, 20 hr) and then washed three times in Ringer solution. After incubating again in the Ringer solution for 1 hr following the washout of the test compounds, the responses of the tissue to three drugs were observed. The three drugs were: isoprenaline (10^{-6} M), MJ-1999 [4,2-(isopropylamino-10-hydroxyethyl)methane sulphonilide; a beta blocking agent] (10^{-3} M) and ouabain (10^{-5} M).

The procedure used was a follows. Atria were subjected to the isoprenaline for 20 min, after which they were washed three times. They were

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kept in drug-free Ringer solution for 20 min and then subjected to the MJ-1999 for 20 min. After this, isoprenaline was added to the bath for a further 20 min period and they were again washed three times and incubated for 20 min in Ringer solution. Finally, they were subjected to ouabain for 20 min. The atria were then removed and placed in formalin to await histological study. In tabulating the results, the criterion used for physiological normality was resumption of rhythmic contractions in response to the electrical stimulus during the 1 hr washout period following the administration of the cryoprotectant. Pharmacological normality was assumed when the inotropic response to isoprenaline was at least a 50% increase in amplitude of contraction over the control amplitude for those particular atria, a reduction in this response to less than 5% after treatment with MJ-1999, and a positive inotropic response to ouabain of at least 50%.

HISTOLOGICAL METHODS

These have been described in detail previously (Clower & Williams, 1966) and follow the techniques as presented by Lillie (1965). Complete cross sections were made through the atria in a frontal or longitudinal plane, or both.

Organs were fixed in 10% aqueous formaldehyde (U.S.P.) or in Lovdowsky's (formaldehyde, water, ethanol and acetic acid) solution. The latter was used in most instances at $-6°$ to $-12°$ to attain better fixation of glycogen. The periodic acid-Schiff's reagent method was used to show fibrosis, glycogen and recent myocardial necrosis. Von Kossa and alizarin techniques were used to demonstrate calcium, if present. These tissues were also stained with haematoxylin and eosin.

Results

The results are summarized in Table 1 and Fig. 1. The most toxic agent at 30° was 2·0 M glycerol and the least toxic was the magnesium ion. The pharmacological test was more sensitive in defining tissue functional integrity than the physiological test. Of the three drugs used, ouabain appeared to be the most sensitive to changes in the functional integrity of the atrial tissue. Although in many instances, the muscle responded normally to isoprenaline, no positive inotropic response could be elicited by ouabain. In fact, in some instances in which the criteria of physiological responses were not met, i.e., muscle did not exhibit sustained rhythmic contractions in response to electrical stimulation during cryoprotective washout, the administration of isoprenaline stimulated the muscle to contract. In such instances, the response to ouabain was abnormal. The most obvious effect of treating heart muscle with a cryoprotective agent was the cessation of cardiac activity. This effect was demonstrated for each cryoprotective agent, but was much less rapid with 0·7 M glycerol than for any of the other agents used (Fig. 2). This shows a noticeable difference in the contractile response of the atria to the various agents. The pattern for a given cryoprotectant was reproducible and typical for that agent. Glycerol (0·7 M), dimethylsulphoxide,

TABLE 1. TOXICITY OF CRYOPROTECTANTS AT 30° ON STIMULATED RABBIT ATRIA

Cryoprotective agent	Time (hr)	Tissues which contracted after treatment	Tissues with normal pharmacological responses
Ringer	2	5/5	5/5
	20	8/8	8/8
Glycerol (0·7 M)	1	4/4	4/4
	2	2/2	2/2
	4	6/6	6/6
	6	7/10	4/10
	8	10/12	6/12
	10	6/10	3/10
	12	3/10	0/10
Glycerol (2·0 M)	0·5	0/10	0/10
Dimethylsulphoxide (2·1 M)	0·5	11/11	11/11
	1	9/9	6/9
	2	3/5	2/5
	3	2/8	0/8
Dextran (6% w/v)	1	9/9	9/9
	6	12/12	11/12
	8	2/9	1/9
$MgCl_2 \cdot 6H_2O$ ($6·6 \times 10^{-2}$ M)	1	9/9	9/9
	6	4/4	4/4
	8	8/8	4/8
	12	4/12	2/12
	16	0/16	0/10

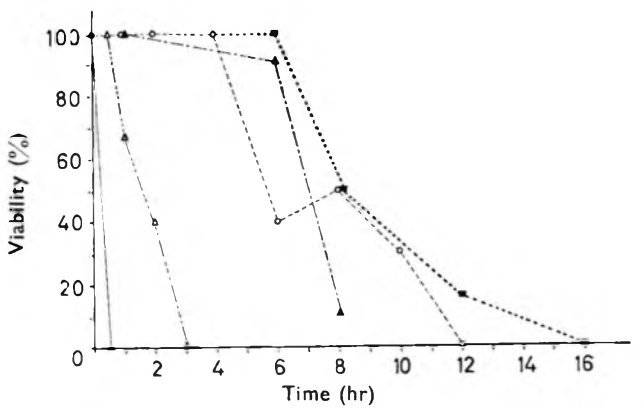

FIG. 1. Comparative toxicity of cryoprotective agents at 30° on electrically stimulated rabbit atria. Ordinate represents percent of atria viable according to pharmacological criteria. ○, 0·7 M glycerol. ●, 2·0 M glycerol. △, 2·1 M dimethylsulphoxide. ▲, 6% w/v dextran. ■, $6·6 \times 10^{-2}$ M $MgCl_2$.

magnesium and dextran all resulted in a curvilinear decrease in contraction. However, the rate of change was obviously more rapid with dimethylsulphoxide or magnesium than with either 0·7 M glycerol or dextran. Glycerol (2·0 M) caused a response that was entirely different to that produced by the other agents. With 0·7 M glycerol, atria contracted for as long as 12 hr, but with diminished contractile force. Contractions ceased within 90 min when atria were tested with dimethylsulphoxide, dextran or magnesium.

At the onset of the washout of the cryoprotectant, a difference in the behaviour of the glycerol-treated tissue was observed. Atria treated with either 0·7 or 2·0 M glycerol exhibited at this point a marked contracture which was transient with atria treated with the lower concentration, but

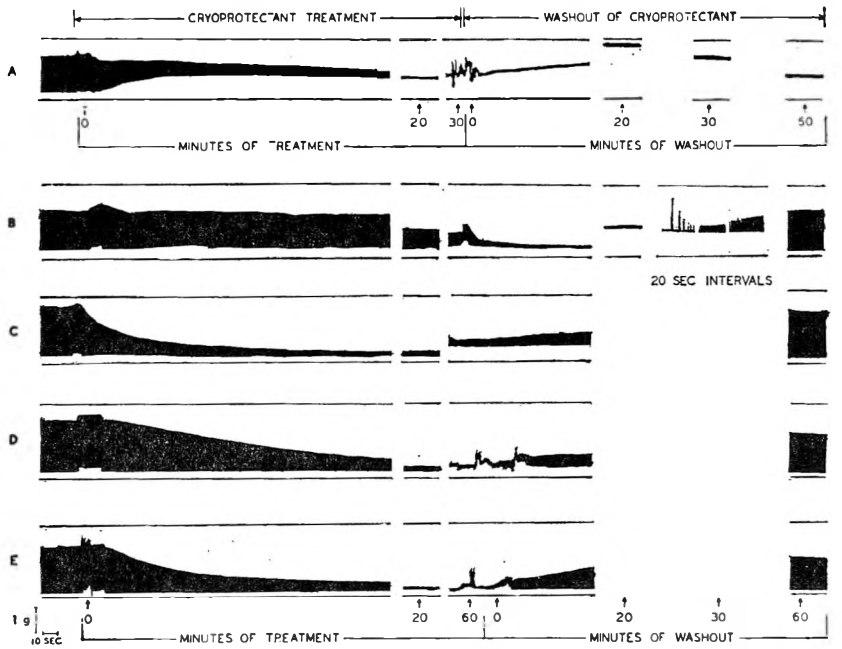

FIG. 2. Recordings illustrating effects of cryoprotective agents on rabbit atria at 30°. Panel A is typical recording obtained with 2·0 M glycerol; Panel B, 0·7 M glycerol; Panel C, 2·1 M dimethylsulphoxide; Panel D, 6% (w/v) dextran; and Panel E, $6{\cdot}6 \times 10^{-2}$M $MgCl_2$.

sustained with the higher concentration. Atria treated with 2·0 M glycerol showed no contractile activity at the end of the washout period, whereas, atria treated with 0·7 M glycerol frequently resumed contraction in the last 30 min of the washout. Tissues treated with magnesium, dextran or dimethylsulphoxide contracted within 5 min after the onset of washout.

HISTOLOGICAL RESULTS

Most atria showed no evidence of damage that would be detrimental to cardiac function. There was no necrosis, calcification, inflammation or oedema. The atrial cardiac muscle contained a rich supply or source of glycogen which implied that the muscle was healthy. However, in several instances, there was fatty infiltration of the cardiac muscle of atria from all groups, but not to the extent which would cause damage or possible abnormal cardiac (atrial) function. Also, there were areas of fibrosis within the atrial walls; these probably were spontaneous (old) changes. Three atria (one control, one from 2·0 M glycerol group and one from dimethylsulphoxide group), had severe necrosis and inflammation of the muscle. These inflammatory changes must have been pre-experimental since no source of inflammatory cells can be maintained after death. The necrotic changes probably were pre-experimental, but could have been due to the experimental procedure.

Discussion

In the present experiments, magnesium was the least toxic agent. Twenty min at −20° appears to be the maximum time heart tissue can be frozen and still survive (Smith, 1957; Karow, Webb & Stapp, 1965). Since dimethylsulphoxide and 2·0 M glycerol produced their toxic effect within the initial 20 min period of exposure, this imposes limitations on their use for tissue freezing. The other agents might also be toxic within this time period at freezing temperatures due to an increased sensitivity of some tissue constituent(s) (Almond, Anido & others 1966).

The results serve to emphasize the need for careful selection of cryoprotective agents. For many tissues, 2·0 M glycerol is an excellent cryoprotectant, but for heart muscle this concentration of glycerol is toxic (Levy, Richards & Persidsky, 1962; Karow & Webb, 1965). On the other hand, 0·7 M glycerol is relatively innocuous, but its cryoprotective ability for cardiac muscle has not been demonstrated. The pharmacological evaluation detected abnormal tissue responses even before they were detected physiologically. This suggests that the toxic manifestations of the cryoprotective agents are due to alteration of biomolecules, such as drug receptors.

The rapid loss of contractile ability of cardiac tissue treated with the cryoprotective agents at 30° is comparable to the reduction in contractile ability of intestinal and uterine smooth muscle subjected at normothermia to concentrations greater than 1·0% of dimethylsulphoxide, glycerol, methyl formamide, methyl acetamide or dimethyl acetamide. As reported by Farrant (1964) each of these cryoprotectants reduced the responses of these smooth muscle preparations to standard test doses of acetylcholine, histamine or nicotine.

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The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod

B. J. JONES AND D. J. ROBERTS

A simple modification of the standard rotarod apparatus is described which eliminates the necessity of time-consuming training and consequently gives a truer measure of motor co-ordination since any effects on memory are avoided. The sensitivity and reproducibility of the procedure are much greater than those obtained with constant speed rotarods and less animals are required to obtain statistically significant results.

THE performance of untreated naive mice on the usual constant-speed rotarod (Dunham & Miya, 1957) is very variable. Accordingly some form of preliminary training (to eliminate very low times) and the introduction of a time limit for any one trial (to eliminate very high times) are necessary to obtain satisfactory measures of performance after drug administration when both graded-response (Kinnard & Carr, 1957) and quantal (Janssen, Van de Westeringh & others, 1959) assay methods are used. In addition to being time-consuming, such procedures are undesirable since (a) drugs might affect memory rather than motor co-ordination in trained mice and (b) the artificial time limit reduces the sensitivity of the method. The prototype apparatus described is an accelerating rod that eliminates these problems. It was recently demonstrated to the Joint British and German Pharmacological Societies Meeting held at Cambridge (September, 1967).

DESIGN OF THE APPARATUS

The rotating rod is a steel bar (18 inches long, $\frac{3}{4}$ inch diameter) covered by rubber tubing to give a final external diameter of $1\frac{1}{2}$ inches and divided into five 3 inch compartments by means of aluminium discs (10 inches diameter). Each end of the bar is run in a ball race. The rod is coupled *via* a 100 : 1 reduction gearbox to a permanent magnet DC motor (Pullin, 18 PM, 28 V) connected in series to a 100 ohm toroidally wound resistor (Birch and Co.). This resistor is itself driven by a kymograph motor so that a change in final speed from 2 rev/min (maximum resistance) to 50 rev/min (minimum resistance) is obtained over a period of $5\frac{1}{2}$ min. A diagram of the electrical circuit is shown in Fig. 1.

The speed-time relation in an ideal apparatus should be linear from zero to maximum speed but in the relatively cheap construction described it is not linear (Fig. 1 inset). A specially graded resistor would be required to produce a constant change in speed although the results obtained with the existing apparatus indicate that the bias introduced as a result of non-linear acceleration is not a serious problem.

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In operation mice are placed on the stationary rod with the resistor in the position of maximum resistance. When the drive to the resistor is activated, the rod slowly accelerates so that even the most agile mouse is unable to stay on the rod for more than a few minutes.

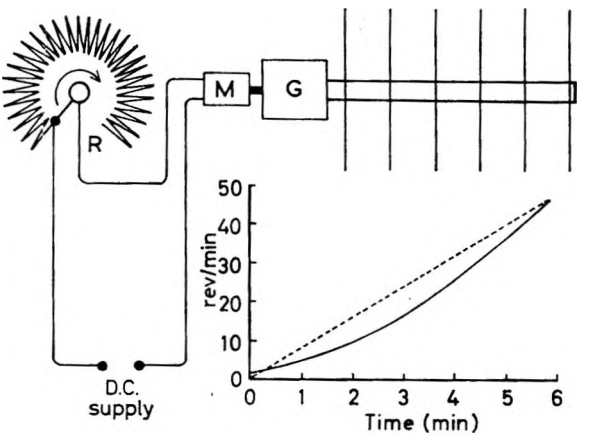

FIG. 1. Diagram of the basic arrangement for the accelerating rotarod with inset showing the actual acceleration (solid line) obtained compared with the ideal (broken line). R = variable resistance; M = motor; G = 100: 1 reduction gearbox.

TABLE 1. MEAN PERFORMANCE TIMES OF DIFFERENT GROUPS OF 25 UNTREATED NAIVE MICE TESTED ON THE ACCELERATING ROTAROD OVER A PERIOD OF 3 MONTHS. Figures are in sec ± standard errors of the means

170·4 ± 9·3	148·2 ± 10·3	164·8 ± 9·5
154·8 ± 11·4	141·0 ± 11·0	151·3 ± 11·6
158·6 ± 9·4	177·0 ± 9·9	157·9 ± 11·1
164·6 ± 7·9	135·7 ± 10·4	171·3 ± 12·5

RESULTS AND DISCUSSION

Groups of 25 male albino Swiss mice (18–23 g) were used. Table 1 shows the mean performance times (sec) of 12 such groups of untreated naive mice taken randomly over a period of several months from different stock cages. Although significant differences can be demonstrated between the lowest and highest measurements, there were no differences between groups taken from the same stock cages. Under usual experimental conditions, therefore, where control and test animals are taken from the same stock population, naive mice can be used with complete confidence in the reproducibility of performance times. As a result, the new apparatus is capable of detecting statistically significant drug-induced changes in motor co-ordination at much lower dose levels and using fewer animals than those required with the constant speed rotarod (Kinnard & Carr, 1957; Kuhn & Van Maanen, 1961; Posner, Hearst & others, 1962). The dose-effect curves for some of the drugs so far tested are shown in Fig. 2 and may be compared with equivalent curves obtained from experiments on non-accelerating rods in the literature already quoted.

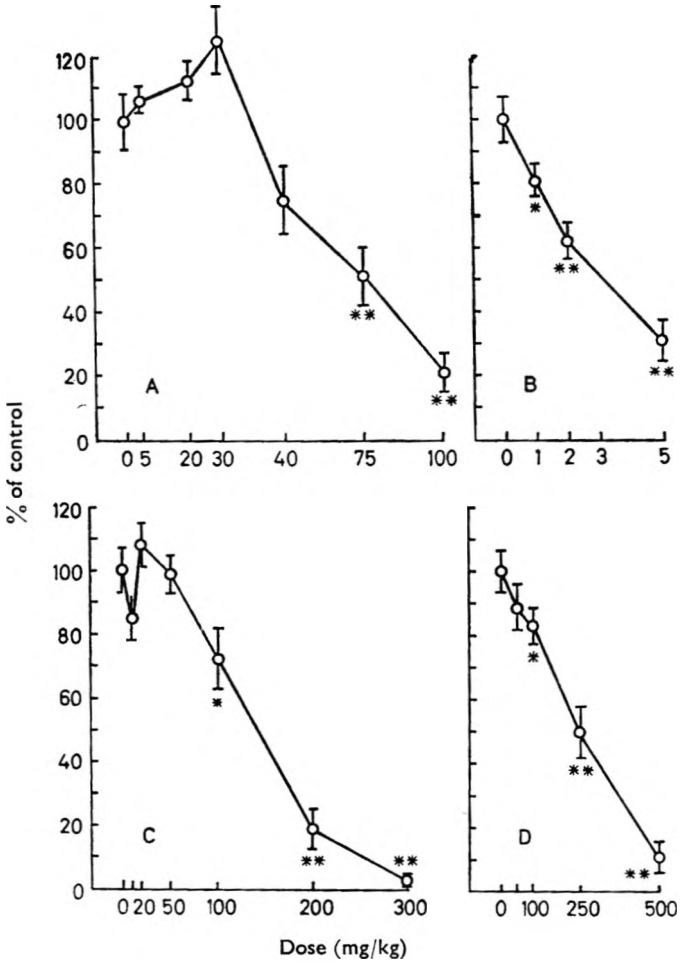

FIG. 2. The influence of phenobarbitone (A), chlorpromazine (B), meprobamate (C) and ectylurea (D) on the performance of naive mice on the accelerating rotarod. Each point represents the mean ± standard error (vertical line) of 25 observations expressed as a percentage of the performance of the control group. Doses in mg/kg administered intraperitoneally 30 min (A) and 20 min (B, C, D) before testing. A and B dissolved in saline, C and D suspended in 1·5% sodium carboxymethyl cellulose; control groups received appropriate solvent.
* $P < 0.05$. ** $P < 0.001$.

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Some aspects of the pharmacology of ibufenac, a non-steroidal anti-inflammatory agent

S. S. ADAMS, P. HEBBORN* AND J. S. NICHOLSON

When given orally to animals ibufenac has a range of potencies 2 to 4 times that of aspirin. It also suppresses thurfyl nicotinate erythema in man. Like certain other analgesic–anti-inflammatory–antipyretic drugs, when administered intravenously it suppresses bradykinin-induced bronchoconstriction in the guinea-pig. It has no glucocorticoid activity. Ibufenac, a compound chemically unrelated to existing antirheumatic drugs, can thus be classified as a non-steroidal anti-inflammatory (antirheumatic) agent.

IBUFENAC (4-isobutylphenylacetic acid) is a non-steroidal anti-inflammatory agent which has proved to be of value in the treatment of rheumatoid arthritis and other rheumatic diseases (Chalmers, 1963; Thompson, Stephenson & Percy, 1964; Hart & Boardman, 1965; Mizushima, Kikutani & others, 1965). We describe here some aspects of its pharmacology with specific reference to its anti-inflammatory, analgesic and antipyretic effects.

Experimental

METHODS

In the animal experiments, male rats (Wistar, Boots) of about 150 g weight, female guinea-pigs (Tuck) of 500–800 g weight, and male mice (Horne) of 20–25 g weight were used. Unless otherwise stated, the animals were deprived of food overnight and ibufenac and the control drugs, either aspirin, phenylbutazone or hydrocortisone sodium succinate, were administered orally in graded doses in 10% mucilage of acacia. Control animals received the same volume of acacia mucilage.

ANTI-INFLAMMATORY ACTIVITY

Ultra-violet erythema in the guinea-pig. Anti-erythemic activity in the guinea-pig was assessed by the method described by Adams & Cobb (1958), the degree of erythema being assessed on a scale 0, 1, 2, 3 or 4.

Thurfyl nicotinate erythema in man. The effect of ibufenac on thurfyl nicotinate erythema in man was determined with a Lovibond reflecting tintometer by the technique of Adams & Cobb (1963). The test was made on two successive days in a group of six volunteers. On the first (control) day, the degree of erythema was assessed by comparing the difference in redness (ΔR_{40}) of the volar surface of the forearm immediately before (R_0), and 40 min after (R_{40}), the application of 5% thurfyl nicotinate cream. On the next day each subject took 960 mg of ibufenac immediately after breakfast and the R_0 value was measured 150 min later. The thurfyl

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nicotinate cream was then applied to an area of skin different from that used for the control experiment, and the R_{40} value determined 40 min later.

Formation of granulation tissue in the rat. The development of granulation tissue in the rat was examined by the technique of Bush & Alexander (1960), using carrageenan-impregnated cotton wool dental pellets. Four pellets were implanted subcutaneously into the ventral region of the rat, one near each axilla. The compounds were administered daily in divided doses at 9·00 and 16·00 hr for 7 days; at the end of this period the pellets were removed and dried to constant weight.

Adjuvant arthritis in the rat. This was produced by a single intradermal injection into the tail of 0·1 ml of a 6 mg/ml suspension of killed tubercle bacilli, derived from human strains PN, DT and C, in liquid paraffin. Assessment of the severity of the arthritis in the hind paws only was made 19 days after the injection of adjuvant. Each hind paw was given a score of 0 to 4, the maximal possible score for each rat therefore being 8. The compounds were administered by mouth in divided doses at 9·00 and 16·00 hr each day.

SUPPRESSION OF BRADYKININ-INDUCED BRONCHOCONSTRICTION IN THE GUINEA-PIG

The bronchoconstrictor response of guinea-pigs to intravenous bradykinin was investigated by a technique similar to that originally described by Collier & Shorley (1960). Aspirin and ibufenac, as the soluble sodium salts, were administered intravenously and a period of 5 min was allowed to elapse before the following dose of bradykinin. Tachyphylaxis to bradykinin is usual in the guinea-pig and in most experiments only one dose of a compound could be tested in each animal.

ANALGESIC ACTIVITY

This was assessed by determining the pain reaction threshold in rats to pressure applied to the yeast-inflamed paw, using the apparatus described by Randall & Selitto (1957). Compounds were administered 30 min before the injection of 0·1 ml of a 20% suspension of dried yeast into the plantar surface of the rat paw. Two hr after the injection the pain reaction threshold was assessed, two determinations being made on each foot.

ANTIPYRETIC ACTIVITY

Pyrexia was induced in rats by means of a subcutaneous injection of 1 ml/100 g bodyweight of a 20% suspension of dried yeast. Sixteen hr later the rectal temperature of each rat was determined, animals with a temperature below 38·5° being discarded. The temperatures of the remainder were again recorded 1, 2 and 4 hr after drug administration.

GLUCOCORTICOID ACTIVITY

This was assessed by the increase of liver glycogen in the non-adrenalectomized mouse (Silber, 1959). Five hr after administration of the

compounds the mice were killed, the livers removed and the glycogen concentration estimated by means of the anthrone reaction (Carroll, Longley & Roe, 1956).

Results

ANTI-INFLAMMATORY ACTIVITY

Ultraviolet erythema in the guinea-pig. The collective results from a series of ultraviolet erythema experiments in the guinea-pig are shown in Table 1. An exact comparison of potency between ibufenac and aspirin is impossible since the dose-response slopes for the two compounds are obviously different. The results do suggest, however, that the potency of ibufenac is in the range 2 to 4 times that of aspirin. The shallower dose-response slope is not specific for ibufenac since we have found that other related phenylalkanoic acids have a similar shape.

TABLE 1. EFFECT OF IBUFENAC AND ASPIRIN ON THE DEVELOPMENT OF ULTRAVIOLET ERYTHEMA IN THE GUINEA-PIG. The degree of erythema in each animal was scored 0, 1, 2, 3 or 4.

Compound	Oral dose mg/kg	Mean erythema response	No. of animals
Aspirin	40	3·85	34
	80	1·27	33
	160	0·18	34
Ibufenac	10	3·53	34
	20	2·12	34
	40	0·97	34
	80	0·25	28
Control	—	4·00	30

TABLE 2. EFFECT OF AN ORAL DOSE OF 960 MG OF IBUFENAC ON THE ERYTHEMA PRODUCED IN MAN BY THE APPLICATION OF 5% THURFYL NICOTINATE CREAM TO THE VOLAR SURFACE OF THE FOREARM

	Tintometer readings (red units only)					
	Control: no drug			After 960 mg ibufenac		
Subject	R_0	R_{40}	ΔR_{40}	R_0	R_{40}	ΔR_{40}
A	4·3	6·2	1·9	4·2	5·1	0·9
B	4·3	5·5	1·2	4·8	5·4	0·6
C	4·2	5·7	1·5	4·1	4·9	0·8
D	4·2	5·6	1·4	4·0	5·2	1·2
E	5·4	6·4	1·0	5·4	5·6	0·2
F	4·2	5·8	1·6	4·3	5·4	1·1
Means	4·43†	5·87	1·43*	4·47†	5·27	0·80*
s.e.	0·20	0·15	0·13	0·22	0·10	0·15

* Significantly different $P < 0{\cdot}01$.

† Not significantly different.

Thurfyl nicotinate erythema in man. The effect of 960 mg of ibufenac on the development of thurfyl nicotinate erythema in man is shown in Table 2: only the figures for red units are presented. Ibufenac did not affect the basic skin colour (R_0), but the degree of erythema (ΔR_{40}) was significantly reduced. This response is similar in degree to that obtained

on another occasion with 320 mg of aspirin. This qualitative similarity of the two compounds is of particular interest since 600 mg of phenylbutazone, 400 mg of oxyphenbutazone and 650 mg of sodium salicylate did not suppress the erythema (Adams & Cobb, 1963).

TABLE 3. ACTION OF IBUFENAC AND HYDROCORTISONE SODIUM SUCCINATE ON THE FORMATION OF GRANULATION TISSUE IN THE RAT

Compound	Oral dose mg/kg/day	No. of rats	% Body weight change	Mean granulation tissue per rat in mg ± s.e.	% Inhibition
Control	—	9	+13·6	23·4 ± 1·20	0
Hydrocortisone sodium succinate	16	8	+ 6·2	18·3 ± 1·11*	21·8
	32	8	+ 3·7	16·5 ± 0·81*	29·5
	64	8	0	11·1 ± 0·57*	52·6
Ibufenac	80	9	+14·8	20·2 ± 1·04	13·7
	160	9	+14·6	19·8 ± 0·79*	15·4
	320	9	+15·5	17·2 ± 0·37*	26·5

* Differs significantly from control $P \leqslant 0{\cdot}05$.

Formation of granulation tissue in the rat. The comparative effects of ibufenac and hydrocortisone sodium succinate on the development of granulation tissue in the rat are shown in Table 3. Although doses of 160 and 320 mg/kg of ibufenac produced a significant reduction in granulation tissue compared with controls this was quite modest and the slope was shallow, in marked contrast to that produced by hydrocortisone sodium succinate. These results are in agreement with the findings of Winder, Wax & Welford (1965), who also found a lower ceiling and a shallower dose-response curve for the non-steroidal anti-inflammatory compounds mefenamic acid, flufenamic acid, meclofenamic acid and phenylbutazone than for glucocorticoids. There was a typical depression of general body growth in those animals receiving hydrocortisone sodium succinate, which did not occur with ibufenac.

TABLE 4. EFFECT OF PHENYLBUTAZONE, ASPIRIN AND IBUFENAC ON THE DEVELOPMENT OF ADJUVANT ARTHRITIS IN THE RAT. The degree of arthritis in each hind paw was scored 0 to 4.

Compound	Oral dose mg/kg/day	No. of rats	Mean arthritis score on hind feet (max. 8)	% Inhibition of arthritis
Control	—	6	5·9	0
Phenylbutazone	12·5	6	3·3	44·1
	25	6	1·8	69·5
	50	6	1·5	74·6
	100	6	0·7	88·2
Aspirin	25	6	6·5	−10·2
	50	6	5·6	5·1
	100	5	6·2	−5·1
	200	5	2·6	56·0
Ibufenac	25	6	5·5	6·8
	50	6	5·8	1·7
	100	6	2·6	56·0
	200	6	2·4	59·4

Adjuvant arthritis in the rat. From Table 4 it will be seen that ibufenac was twice as active as aspirin but less effective than phenylbutazone in suppressing the development of adjuvant arthritis. Glenn, Bowman & others (1967) found ibufenac to be approximately twice as active as aspirin and half as active as phenylbutazone in reducing established arthritis in the rat.

SUPPRESSION OF BRADYKININ-INDUCED BRONCHOCONSTRICTION IN THE GUINEA-PIG

The effective dose of a compound was considered to be one which reduced by 50% or more the bronchoconstrictor effect previously produced by a similar dose of bradykinin. In a series of experiments the effective intravenous dose for aspirin was in the range 1 to 2 mg/kg and for ibufenac 2·5 to 5 mg/kg. The results for aspirin were similar to those originally obtained by Collier & Shorley (1960, 1963) and, compared with the potencies of other compounds recorded by these workers, ibufenac is less active than mefenamic and flufenamic acid, approximately equipotent with phenylbutazone, and more active than amidopyrine. The activity of ibufenac in this sytem supports the evidence of Collier & Shorley (1960) that a relation exists between the ability of certain analgesic/antipyretic compounds to suppress bradykinin-induced bronchoconstriction and ultra-violet erythema in guinea-pigs.

ANALGESIC ACTIVITY

The results of the analgesic studies are shown in Table 5. The log dose-response curves of the two drugs are linear and parallel and the potency of ibufenac compared with aspirin is 3·4 with 95% confidence limits of 2·8 to 4·1.

TABLE 5. EFFECT OF IBUFENAC AND ASPIRIN ON THE PAIN THRESHOLD OF THE INFLAMED FOOT OF THE RAT. 18 rats were used for each dose group.

Compound	Oral dose mg/kg	Mean pain threshold (mm/Hg)
Ibufenac	15	71·1
	30	104·2
	60	143·3
	120	166·4
Aspirin	30	54·8
	60	89·8
	120	97·1
	240	148·8
Control	—	40·4

ANTIPYRETIC ACTIVITY

The results of these tests depicted graphically in Fig. 1 indicate that ibufenac is an effective antipyretic compound having about four times the potency of aspirin.

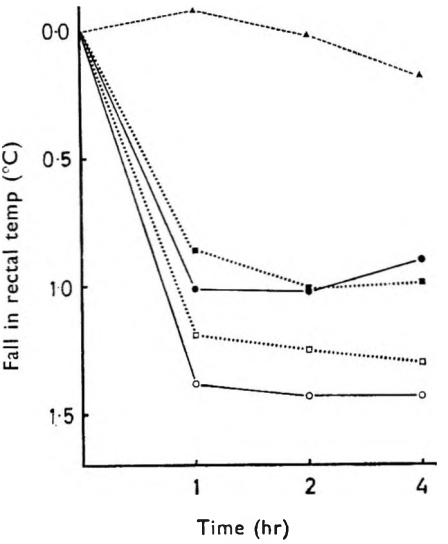

FIG. 1. Antipyretic effects of orally administered ibufenac and aspirin in the yeast-fevered rat. Each point represents the mean for 9 rats. ▲, Control; ■, aspirin 50 mg/kg; ●, ibufenac 12·5 mg/kg; □, aspirin 100 mg/kg; ○, ibufenac 25 mg/kg.

GLUCOCORTICOID ACTIVITY

From Table 6 it will be seen that, whilst 2 mg/kg of hydrocortisone sodium succinate produced a significant deposition of glycogen in the liver, 200 mg/kg of ibufenac had no effect.

TABLE 6. INFLUENCE OF IBUFENAC AND HYDROCORTISONE SODIUM SUCCINATE ON GLYCOGEN DEPOSITION IN GROUPS OF 10 MICE

Compound	Oral dose mg/kg	Mean liver glycogen mg/10 g mouse ± s.e.
Control	—	4·5 ± 0·31
Hydrocortisone sodium succinate ..	2	9·6 ± 1·30†
	4	11·2 ± 1·56†
	8	24·8 ± 4·39†
Ibufenac	50	4·0 ± 0·91*
	100	4·0 ± 1·06*
	200	3·2 ± 0·79*

* Not significantly different from control $P > 0.05$.

† Significantly different from control $P < 0.05$.

Discussion

A distinction is usually made between the acute inflammatory response of tissues to a transient stimulus, and the chronic inflammation which occurs when a stimulus is persistent. Ultraviolet erythema and thurfyl nicotinate erythema are typical of acute inflammation, whilst cotton-wool pellet granuloma and adjuvant arthritis are examples of different types of chronic inflammation.

Although ultraviolet irradiation in the guinea-pig and the application of thurfyl nicotinate in man are capable of producing oedema as well as erythema, our techniques are planned so as to measure only erythema (Adams & Cobb, 1963). Ibufenac is similar to many other non-steroidal anti-inflammatory drugs in its ability to inhibit ultraviolet erythema, but in thurfyl nicotinate erythema its action is more specific since, although aspirin is effective in very low doses, sodium salicylate, phenylbutazone and oxyphenbutazone are all inactive (Adams & Cobb, 1963).

In the granulation tissue experiments the proliferative component of inflammation predominates; whilst ibufenac, like a number of other potent non-steroidal anti-inflammatory agents, is slightly suppressive (Winder, Wax & Welford, 1965), it is markedly less effective than hydrocortisone.

Adjuvant arthritis in the rat is a complex chronic inflammatory condition of immunological origin, characterized by a persistent erythema and oedema of the paws, in some respects resembling active rheumatoid arthritis. The effect of ibufenac in this condition is probably a measure of its ability to reduce increased capillary permeability.

The overall pharmacological results indicate that ibufenac possesses anti-inflammatory, analgesic and antipyretic properties, its oral potency in animals being two to four times that of aspirin. In rheumatoid arthritis and allied conditions the compound is about twice as active as aspirin (Hart & Boardman, 1965). Thus some of the pharmacological effects of ibufenac appear to be similar to those of aspirin, but the difference between the dose-response curves of the two compounds in ultraviolet erythema indicates possible differences in their modes of action in this type of inflammation.

The failure of ibufenac to influence glycogen deposition in the mouse, or to simulate glucocorticoids in respect of both whole body growth and the formation of granulation tissue in the cotton pellet test, indicate that the compound is devoid of glucocorticoid activity.

On the basis of its pharmacological and clinical activities ibufenac can be regarded as a member of that non-steroidal group of anti-inflammatory–antirheumatic compounds which includes phenylbutazone, oxyphenbutazone, indomethacin, aspirin and the fenamic acids. This similarity of ibufenac to the other aforementioned compounds has been confirmed at the biochemical level in a variety of *in vitro* systems. It uncouples oxidative phosphorylation in rat liver mitochondria, whilst in cartilage it inhibits the incorporation of inorganic phosphate into organic phosphates, and the biosynthesis of mucopolysaccharide sulphates (Whitehouse, 1964). Skidmore & Whitehouse (1966a,b) have shown that ibufenac and other acidic anti-inflammatory drugs inhibit histamine formation by competing with pyridoxal phosphate for the coenzyme binding site, believed to be a lysyl ϵ-amino-group, on mammalian histidine decarboxylase. Mizushima & Suzuki (1965) have demonstrated that ibufenac as well as phenylbutazone, flufenamic acid and indomethacin stabilizes serum albumin (FV) against heat coagulation, whilst Gerber, Cohen & Guistra (1967) showed that ibufenac and other anti-inflammatory compounds

accelerate the disulphide interchange between serum sulphydryl groups and an aromatic disulphide.

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Effect of two non-steroidal anti-inflammatory agents on hexosamine and sialic acid contents of inflamed tissue

SIR,—It has been shown that the levels of total mucopolysaccaride, expressed as hexosamine, and the carbohydrate moiety of glycoproteins, expressed as sialic acid, are much higher in inflamed than in normal tissue (Bolognani, Coppi & others, 1961; Delaunay & Bazin, 1965; Houck & Jacob, 1965). We have explored the effect of two non-steroidal anti-inflammatory drugs, naphthipramide (Coppi, 1966; Marazzi-Uberti & Turba, 1966; Marazzi-Uberti, Turba & Erba, 1966; Turba & Marazzi-Uberti, 1966) and phenylbutazone, on hexosamine and sialic acid contents in rat tissue inflamed by kaolin and carrageenan.

Inflammation was produced in the paws of rats (Sprague-Dawley strain; male, average weight 160 g) by injecting 0·05 ml of a 10% suspension of kaolin in water or 0·1 ml of a 1% solution of carrageenan in 0·9% cold sterile saline. Naphthipramide and phenylbutazone were administered 0, 12, 24 and 36 hr after kaolin (in doses of 100 and 50 mg/kg respectively) and 0, 2·5 and 5 hr after carrageenan (in doses of 80 and 40 mg/kg respectively). Animals were killed 48 hr after kaolin and 24 hr after carrageenan and the inflamed tissue of the paw pads was homogenized in water at +4° and freeze-dried. The freeze-dried tissue was then assayed for nitrogen according to Kjeldhal, for total hexosamine (Boas, 1953; Bolognani, Coppi & Zambotti, 1958) and for sialic acid (Svennerholm, 1958). Non-inflamed paws of treated rats were similarly assayed.

The results (Table 1) show that naphthipramide and phenylbutazone have a qualitatively similar effect, both reducing hexosamine and sialic acid contents of the inflamed tissue, with a more marked effect on kaolin than on carrageenan oedema. But phenylbutazone showed an effect quantitatively greater than that of naphthipramide on both kinds of oedema.

TABLE 1. EFFECT OF NAPHTHIPRAMIDE AND PHENYLBUTAZONE ON HEXOSAMINE AND SIALIC ACID CONTENTS OF INFLAMED TISSUE OF RATS.

Treatment	Dose of drug mg/kg (oral)	Hexosamine µg/mg N			Sialic acid µg/mg N		
		Mean ± s.e.	P*	P**	Mean ± s.e.	P*	P**
Kaolin oedema (12 animals/group)							
Normal control ..	—	118·16 ± 1·75	—	—	83·46 ± 2·33	—	—
Inflammation ..	—	194·66 ± 4·27	< 0·001	—	168·67 ± 9·82	< 0·001	—
Inflammation + naphthipramide	100 × 4	172·66 ± 4·28	< 0·001	0·001 < P < 0·01	145·61 ± 6·35	< 0·001	0·02 < P < 0·05
Inflammation + phenylbutazone ..	50 × 4	162·66 ± 7·23	< 0·001	< 0·001	129·90 ± 5·22	< 0·001	< 0·001
Carrageenan oedema (10 animals/group)							
Normal control ..	—	129·60 ± 4·76	—	—	98·60 ± 3·48	—	—
Inflammation ..	—	164·20 ± 1·55	< 0·001	—	161·20 ± 6·48	< 0·001	—
Inflammation + naphthipramide	80 × 3	159·20 + 1·89	< 0·001	> 0·05	147·50 + 4·58	< 0·001	> 0·05
Inflammation + phenilbutazone ..	40 × 3	149·20 ± 1·83	< 0·001	0·02 < P < 0·05	126·00 ± 3·32	< 0·001	< 0·001

* Statistical significance of the difference between treated and normal controls.
** Statistical significance of the difference between treated and inflamed controls.

Our results provide further evidence that anti-inflammatory agents reduce glycoprotein levels in inflamed tissue (Houck & Jacob, 1965).

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Effects of some sympathomimetic amines on the response of the rabbit isolated ear artery to noradrenaline and electrical stimulation

SIR,—The site of uptake of noradrenaline in the rabbit ear artery has been shown to be situated on the outer perimeter of the smooth muscle layer (de la Lande & Waterson, 1967). These authors have also shown that cocaine applied to the outer surface of the artery potentiated the effects of extraluminally injected noradrenaline and had little effect on the intraluminal noradrenaline. Cocaine was also shown to potentiate the electrically induced vasoconstriction (de la Lande & Rand, 1965). Since sympathomimetic amines are known to potentiate noradrenaline in other smooth muscle (Bentley, 1965) and to block the uptake of noradrenaline in heart (Burgen & Iversen, 1965), it seemed important to investigate the effects of these amines on the vascular smooth muscle in relation to the hypothesis postulated by de la Lande & Waterson (1967).

Central ear arteries, 4–5 cm long, isolated from anaesthetized (25% urethane, i.v.) rabbits weighing 1·5–2·5 kg were perfused in a 400 ml bath by the method of de la Lande & Harvey (1965) with Krebs bicarbonate solution at 37°, aerated with 95% oxygen and 5% carbon dioxide. Perfusion pressure and perfusion rate were maintained at 20–30 mm Hg and 4–5 ml/min respectively. Intraluminal injections were given through the rubber tubing at the proximal end of the artery. In some experiments a 20 ml bath was used to facilitate a quick washout. Constriction of the artery in response to noradrenaline added intra- or extraluminally was recorded with a mercury manometer on a smoked drum. A Grass model S4-D stimulator delivering pulses of 0·5 msec duration alternatively at a frequency of 5 or 10 shocks/sec for 5 sec each 2 min was used for periarterial nerve stimulation. The drugs used were: (−)-noradrenaline bitartrate (Koch-Light); (±)-amphetamine sulphate (L. Light & Co); metaraminol bitartrate (Merck Sharp & Dohme) and tyramine hydrochloride (Calbiochem).

The results showed that the artery was much less sensitive to extraluminal than to intraluminal noradrenaline, which is in agreement with the findings of Cannell, de la Lande & Waterson (1966). Of the sympathomimetic amines

TABLE 1. EFFECTS OF SYMPATHOMIMETIC AMINES ON THE RESPONSE OF THE EAR ARTERY TO INJECTED NORADRENALINE. The figures represent the % change in presence of the drug. + (increase), — (decrease).

Drugs	Intraluminal	Extraluminal
Amphetamine 1×10^{-6}	11·3 (−)	62·1 (+)
	7·5 (−)	65·0 (+)
	12·4 (−)	135·2 (+)
Metaraminol 1×10^{-7}	11·3 (−)	72·2 (+)
	7·2 (−)	131·4 (+)
	7·3 (−)	130·5 (+)
Tyramine 1×10^{-5}	212·1 (+)	51·0 (+)
	172·3 (+)	32·6 (+)
	176·5 (+)	58·4 (+)

TABLE 2. EFFECTS OF SYMPATHOMIMETIC AMINES ON THE RESPONSE OF THE EAR ARTERY TO THE ELECTRICAL STIMULATION. The figures represent the % change in presence of the drug. + (increase), — (decrease).

Drugs	5 shocks/sec	10 shocks/sec
Amphetamine, 1×10^{-6}	28·0 (+)	48·0 (+)
	148·1 (+)	75·0 (+)
	35·0 (+)	60·4 (+)
Metaraminol, 1×10^{-7}	22·0 (+)	14·5 (+)
	108·1 (+)	105·0 (+)
	143·3 (+)	147·0 (+)
Tyramine 1×10^{-5}	12·1 (−)	26·0 (−)
	32·0 (−)	46·4 (−)
	22·2 (−)	21·4 (−)

tested, amphetamine 1×10^{-7}, 1×10^{-6}; metaraminol, 1×10^{-8}, 1×10^{-7} and tyramine 1×10^{-6} applied to both surfaces of the artery caused little or no change in the sensitivity of the artery to intraluminally injected noradrenaline, but with tyramine, 1×10^{-5}, a two-fold potentiation was noted. The application of amphetamine, 1×10^{-6}, metaraminol, 1×10^{-7} or tyramine, 1×10^{-5} to the outer surface of the artery caused a much greater potentiation of extra- than intraluminally applied noradrenaline (Table 1). Amphetamine, 1×10^{-6} and metaraminol, 1×10^{-7}, both caused approximately a two-fold increase in the response to electrical stimulation of the periarterial nerves but tyramine 1×10^{-5} caused a small depression (Table 2). From these findings it is concluded that sympathomimetic amines potentiate both extra-luminal noradrenaline and noradrenaline released by nerve stimulation by blocking the uptake mechanism in the sympathetic nerve endings in a way similar to cocaine as shown by de la Lande & Waterson (1967).

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Modified plethysmometer for measuring foot volume of unanaesthetized rats

SIR,—We have modified the plethysmometric method of measuring oedema of the rat paw (Buttle, D'Arcy & others, 1957; Harris & Spencer, 1962). Our method differs in that water replaces the two immiscible liquids, anaesthesia is unnecessary and the procedure is carried out by the experimenter.

The apparatus (Fig. 1) consists of limb G made of a 2 ml burette graduated to 0·02 ml and connected through a glass U-tube to another limb which divides into two identical chambers A and B (int. diam. 14 mm; length 4 cm) at its upper end. S(1) and S(2) are two glass stoppers and t and t′ are side-tubes which are connected by polythene tubes through stoppers to a 5 ml syringe for refilling the apparatus with water and for readjustments. Immediately above the stopper S(2) there is an hour-glass constriction.

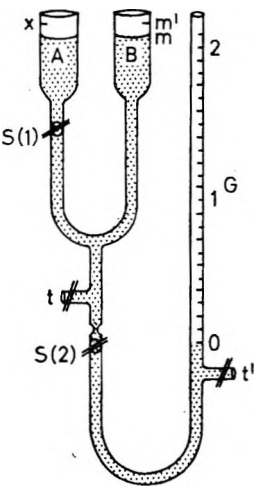

FIG. 1. Diagram of apparatus, see text for explanation.

The apparatus is filled to the mark m of the chamber B with tap water (containing a few drops of Teepol) keeping all the stoppers open and avoiding air bubbles. The side-tube t and the stopper S(2) are then closed and with the syringe the water level in G is brought to 0 and the side-tube t′ closed.

The volume of the rat paw oedema is measured by holding with one hand the unanaesthetized rat wrapped in a towel and by vertically dipping the animal's extended foot, already marked at the ankle-joint with some water-proof ink, into the chamber A till this mark coincides with the mark x. After waiting for a few sec to allow the water level in chamber B to rise (say to m′) the stopper S(1) is closed and the rat's foot withdrawn. The stopper S(2) is then slowly turned allowing the water level in chamber B to return to its initial level at m. This results in a rise in the water level above 0 in limb G. This rise is recorded and the value represents half of the total displacement produced by the animal's foot because of equal distribution of the water in chambers A and B and hence it is multiplied by 2 to give the actual volume.

Values obtained for carrageenan-induced oedema of the rat paw by the method of Harris & Spencer (1962) and by the proposed method were respectively: means 0·60 (7 exp) s.d. 0·23; 0·78 (9 exps) s.d. 0·15; coefficients of variation 38·3 and 19·2%.

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Antagonism between calcium ions and some myolytic agents on depolarized guinea-pig taenia coli

SIR,—Our previous investigations on the mechanism of action of papaverine and certain derivatives demonstrated that these drugs exert an inhibitory effect on oxidative phosphorylation and that an antagonism occurs between calcium ions and spasmolytic agents, both in polarized and in KCl- or K_2SO_4-depolarized smooth muscle preparations. According to these results, it was suggested that the mechanism of action of papaverine-like drugs could be ascribed to an impairment of the energy supply to the contractile system and to an interference with the essential function of calcium ions in muscular contraction (Santi, Contessa & Ferrari, 1963; Santi, Ferrari & Contessa, 1964; Ferrari, 1964; Ferrari & Gaspa, 1965).

Further investigations demonstrated that the inhibition of oxidative phosphorylation was elicited only by papaverine and other oxy-alkyl-benzylisoquinoline derivatives, whereas the interference with calcium ions was shared by all the spasmolytic agents tested (Tòth, Ferrari & others, 1966). In view of the importance of the latter property as a general mechanism of action of spasmolytic drugs, we have attempted to elucidate whether calcium ions and some myolytic agents behave as competitive or non-competitive antagonists.

The investigations were made with guinea-pig taenia coli depolarized by immersion in calcium free, potassium rich solution, at 35°; contractions were triggered by addition of $CaCl_2$ at concentrations ranging from 0·25 to 100 mM; higher concentrations elicited auto-inhibitory effects. The experiments were made according to Rossum (1963), following the conventional dose-response method and the cumulative dose-response method. In the conventional dose-response experiments, after each contraction, the preparations were bathed in calcium-free Tyrode medium for 5 min and then washed briefly with K_2SO_4-Ringer at room temperature (Ferrari & Gaspa, 1965) to obtain rapid relaxation; finally, K_2SO_4-Ringer was substituted after 3 min by KNO_3-Ringer (Urakawa, Karaki, Ikeda, 1967) to which $CaCl_2$ was added. In this medium $CaCl_2$ induces rapid well-maintained contractions and precipitation of calcium salts is avoided. As antagonists, we employed three myolytic agents with different mechanisms of action: papaverine (hydrochloride) (1·4 and 3·2 $\times$ 10^{-5}M), eupaverin (sulphate) (1·4 and 3·2 $\times$ 10^{-5}M), KCN (2 $\times$ 10^{-4} and 10^{-3}M). These drugs were added to the bath 3 min before $CaCl_2$. In the cumulative dose-response experiments five $CaCl_2$ doses were applied at 3 min intervals in a geometric sequence of increasing doses, to a final $CaCl_2$ concentration giving the maximal response (8–12 mM). Spasmolytic drugs were added 3 min before initiating the cumulative dose-response curve, at the lowest indicated concentrations.

The results obtained both with the conventional dose-response method and with cumulative dose-response procedure demonstrate that all the drugs tested

exert myolytic activity against $CaCl_2$-induced contraction of depolarized guinea-pig taenia coli; however, their effects are fully counteracted by increasing $CaCl_2$ concentration and the dose-response curves in the presence of the inhibitors show a parallel shift compared with the controls. These facts could suggest that within the range of concentrations of the present experiments the drugs tested behave as competitive antagonists of calcium ions. The auto-inhibitory effects occurring at $CaCl_2$ concentrations exceeding 100 mM did not allow us to test the myolytic agents at higher dose levels or after a longer incubation period; such experiments could elucidate whether these drugs act by a pure competitive antagonism or have a dual mechanism, as seems likely especially with cyanide and papaverine, which exert metabolic inhibitory effects.

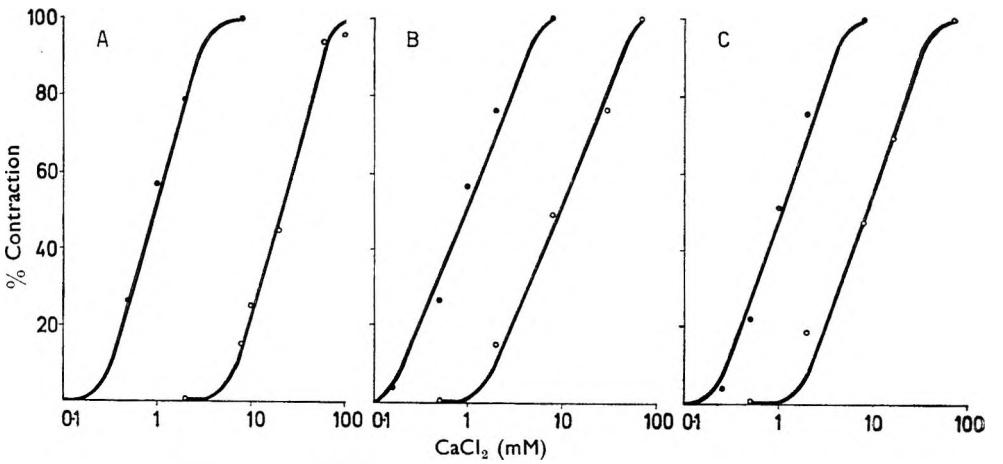

FIG. 1. Guinea-pig taenia coli suspended in KNO_3-Ringer at 35°: effects of myolytic agents on the cumulative log concentration-response curve for calcium ions. ● = controls; ○ = $1{\cdot}4 \times 10^{-5}$ M papaverine (in A), $1{\cdot}4 \times 10^{-5}$ M eupaverin (in B), 2×10^{-4} M KCN (in C). Each point represents the mean of six experiments.

Furthermore, independently of a dual action, a parallel displacement of the dose-response curve at low concentrations of the antagonists may indicate the presence of a reserve in receptors for the agonist (Rossum, 1963; Bowman, Rand & West, 1968)

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Polylysine as histamine releaser

SIR,—L-Polylysine, a synthetic polymer of L-lysine, is known to affect the such membrane of different cells. Thus it liberates a variety of small molecules, as adenylic acid and amino-acids, from Ehrlich ascites tumour cells (Kornguth & Stahmann, 1961); it also causes haemolysis (de Vries, Stein & others, 1954). I have now tested its capacity to release histamine.

Mast cells obtained from the rat peritoneum by a method described previously (Bergmann, Chaimovitz & others, 1962), were centrifuged, washed and re-suspended in saline, buffered to pH 7·4, and incubated with 50 μg/ml L-polylysine [molecular weight 3300 (Yeda Research and Development Co., Rehovoth, Israel)]. Samples were withdrawn after 15, 30 and 60 min and centrifuged; the supernatant was then assayed on the guinea-pig ileum. Controls were incubated with saline only and centrifuged simultaneously with the experimental samples.

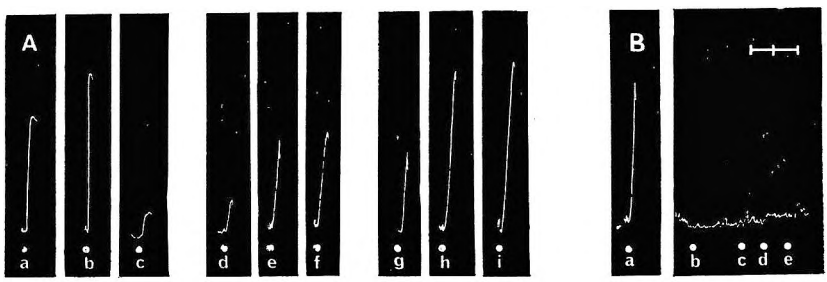

FIG. 1A. Release of smooth muscle stimulant from rat mast cells under the influence of polylysine. A loop of fresh guinea-pig ileum (3 cm length) was suspended at 37° in a 25 ml organ bath, containing Tyrode solution. The contractions of the gut were recorded with an isotonic lever, using a 10-fold magnification: a, histamine, 20 ng/ml, and b, 30 ng/ml. c, polylysine, 2 μg/ml. d–f, 0·3 ml of supernatant obtained by incubating mast cells with saline for 15, 30 and 60 min, respectively, at 37° and centrifuging. g–i, 0·15 ml of supernatant remaining after centrifugation of mast cell suspension; g, after 15 min, h, after 30 min and i, after 60 min incubation at 37° with polylysine, 50 μg/ml. Note that the final concentration of the peptide in the organ bath in experiments g–i was only 0·3 μg/ml.

B. Antagonism between diphenhydramine and the smooth muscle stimulant, released from rat mast cells. Conditions as in A. a, histamine, 30 ng/ml. b, diphenhydramine, 40 ng/ml; 2 min later, at c, without washing, addition of histamine (concentration as in a). d, 1 ml of supernatant, obtained after 30 min incubation of mast cells in saline at 37°; e, 0·5 ml of supernatant, obtained after 30 min incubation of mast cells with 50 μg/ml polylysine. Note that the volumes added in d and e are equiactive with the amount of histamine in a, when diphenhydramine is absent. Time scale 2 min.

It is evident from Fig. 1A that polylysine is a histamine releaser. Under the conditions used, a maximal action was obtained in about 30 min. The contraction was completely inhibited by diphenhydramine (Fig. 1B).

It is known that polyamines liberate histamine from tissue cells (MacIntosh & Paton, 1949); however, these compounds may—at least in part—act by penetration into the cytoplasm where they replace the histamine bound to anionic sites (Åborg, Novotny & Uvnäs, 1967). In contrast, polylysine probably attaches itself to the cell membrane, as was found to be the case with human erythrocytes (Nevo, de Vries & Katchalsky, 1955).

It now appears possible that the increased capillary permeability, seen after the intradermal injection of polylysine in rabbits (Frimmer & Schischke, 1965),

may be, in part, an indirect effect of the liberation of histamine from tissue cells.

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The influences of drugs on the uptake of 5-hydroxytryptamine by nerve-ending particles of rabbit brain stem

SIR,—Recent progress in the technique of isolation of nerve-ending particles (synaptosomes) and synaptic vesicles from brain (Whittaker, 1959; DeRobertis, Rodriguez de Lores Arnaiz & Pellegrino de Iraldi, 1962) has made it possible to investigate the detailed mechanisms of uptake, binding and release of biogenic amines at the subcellular level. Maynert & Kuriyama (1964) found that nerve-ending particles or synaptic vesicles of brain, when incubated in a medium containing noradrenaline or 5-hydroxytryptamine (5-HT), can take up these amines from the medium against the concentration gradient, and they suggested that both nerve ending particles and synaptic vesicles possessed a transport system for noradrenaline and 5-HT. Furthermore, they found that reserpine inhibited the uptake of these amines. Independently, Robinson, Anderson & Green (1965) showed that nerve ending particles and microsomes of brain can take up 5-HT and histamine *in vitro*. Little is known about the kinetics of such uptake so far.

Reserpine, cocaine, desipramine and prenylamine have been reported to inhibit the catecholamine uptake-concentrating mechanism of adrenergic neurons (Hillarp & Malmfors, 1964; Lundmar & Muscholl, 1964; Carlsson & Waldeck, 1965; Malmfors, 1965), and the present study was undertaken to investigate the influences of these drugs on the uptake of 5-HT by nerve-ending particles *in vitro*.

Male rabbits, weighing about 2·5 kg were used. Two brain stems (*ca* 5 g) were homogenized in ice-cold 0·32 M sucrose with a Teflon pestle and made up to about 50 ml. The P_1-fraction was separated by centrifuging the homogenate at 900 *g* for 10 min. This P_1-fraction was washed twice with 0·32 M sucrose and the washings were added to the supernatant fluid from the P_1 preparation. The P_2-fraction, a crude mitochondrial fraction, was prepared by centrifuging the supernatant at 11,500 *g* for 20 min. The method of subsequent subfractionation of the P_2-fraction was similar to that described by Gray & Whittaker (1962). The P_2-fraction was resuspended in 0·32 M sucrose (2 ml/g of original tissue) and 5 ml of this suspension was laid on the top of a discontinuous density gradient consisting of 12 ml each of 0·8 M and 1·2 M sucrose per tube, and centrifuged at 53,500 *g* for 2 hr. This resulted in the subfractions A, B and C which contained predominantly myelin, nerve ending particles and mitochondria

respectively. The top fraction, A, was removed by aspiration. B-fraction, the intermediate band between 0·8 and 1·2 M sucrose, was collected and diluted to approximately 0·32 M with respect to sucrose by the addition of two volumes of ice-cold distilled water. This fraction was centrifuged again at 35,000 *g* for 20 min to yield a particulate fraction. Electron microscopic examination showed that the B-particulate fraction contained a large number of intact nerve-ending particles slightly contaminated by free mitochondria.

For 5-HT uptake experiments, the fraction containing nerve ending particles suspended in Krebs solution of pH 7·6, was mixed with a solution of monoamine oxidase inhibitor, pheniprazine (final concentration of 5×10^{-6} M) and a solution of the drug to be tested. After incubation at 37° in air for 30 min, a solution of 5-HT in phosphate buffer of pH 7·0 was added and incubation continued for a further 30 min at 37°. In another experiment the suspension of nerve ending particles was pre-incubated with pheniprazine (5×10^{-6} M) alone for 30 min at 37°, thereafter test drugs were added to the incubation medium. After 30 min incubation at 37°, 5-HT was added and incubation continued for 30 min at 37°. The mixture was then centrifuged for 20 min at 35,000 *g* and the supernatant fluid decanted. The pellet of nerve ending particle was directly (or after washing twice with Krebs solution) subjected to 5-HT estimation. 5-HT was extracted and assayed fluorimetrically as described by Snyder, Axelrod & Zweig (1965). After the experiment the fraction containing nerve ending particles was examined by electron microscopy and most of the particles were found unchanged in structure even after incubation.

Under the conditions described, the uptake of 5-HT by the particle fraction was inhibited by all drugs tested at the concentration of 20 μg/ml (Table 1). The inhibitory effects of drugs were not reduced when the particle fraction was pre-incubated with pheniprazine alone. This result eliminates the possibility that when pheniprazine and drug were added to the medium simultaneously, the drug inhibited the entry of pheniprazine into the nerve ending particles thereby enabling any 5-HT taken up into the particles to be attacked and oxidized by intact monoamine oxidase in the cytoplasm. Among the drugs tested desipramine was found to be most potent. This would coincide with the finding that it is the most effective inhibitor of noradrenaline uptake by sympathetically innervated tissue (Iversen, 1965). However this is inconsistent with the observation that desipramine does not interfere with the uptake of 5-HT perfused into a ventricle of the brain (Palaić, Page & Khairallah, 1967). Reserpine decreased

TABLE 1. EFFECTS OF DRUGS (20 μG/ML) ON THE UPTAKE OF 5-HT AT 37° BY THE FRACTION CONTAINING NERVE ENDING PARTICLES. In expt 1 the fraction containing nerve ending particles was incubated with pheniprazine and test drug, while in expt 2 the fraction was pre-incubated with pheniprazine alone before addition of test drug.

Drug	5-HT in medium (µg base/ml)	Wash	% change from control	
			Expt 1	Expt 2
Reserpine	2	0	−37·72*** (4)	
	2	2	−44·72** (8)	−47·39 (3)
Desipramine HCl	2	0	−65·55** (4)	
	2	2	−86·26* (4)	−87·79 (3)
Cocaine HCl	2	0	+12·89 (4)	
	2	2	−11·95 (4)	
	1	2	−29·86** (12)	−26·72 (8)

* $P < 0{\cdot}001$.
** $P < 0{\cdot}01$.
*** $P < 0{\cdot}05$.

5-HT uptake significantly but its effect was approximately half that of desipramine. This result might imply that there exists a reserpine-resistant uptake-concentrating mechanism in 5-HT neurons (Fuxe & Ungerstedt, 1967). In contrast to desipramine and reserpine, cocaine showed no significant effect on 5-HT uptake unless the concentration of 5-HT in the medium was decreased to 1 μg/ml.

The present findings indicate that the drugs which inhibit the uptake of catecholamine by adrenergic neurons also inhibit the uptake of 5-HT by nerve ending particles to some degree. Evidence supporting the existence of two different catecholamine uptake-concentrating mechanisms of adrenergic neurons has recently been presented (Carlsson, Hillarp & Waldeck, 1963; Hillarp & Malmfors, 1964; Malmfors, 1965; Carlsson & Waldeck, 1965; Obianwu, 1967). The first mechanism, the membrane pump, is selectively blocked by cocaine or desipramine (Hillarp & Malmfors, 1964; Lundmar & Muscholl, 1964; Carlsson & Waldeck, 1965; Malmfors, 1965), whereas the second mechanism, incorporation into a storage granule, is selectively blocked by reserpine (Hillarp & Malmfors, 1964; Carlsson & Waldeck, 1965; Malmfors, 1965). The question now arises whether similar dual mechanisms are involved in the uptake of 5-HT into 5-HT neurons, and whether these drugs act at the same site as with catecholamine uptake.

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The relation between compression force and dissolution time of tablets

SIR,— From the work of Levy, Antkowiak & others (1963) and of Ganderton, Hadgraft & others (1967) it is clear that processing variables may greatly influence the disintegration time of tablets or the dissolution rate of active ingredients. Levy & others demonstrated that the dissolution rate of salicylic acid decreases with increasing force of compression; Ganderton & others found that by increasing compression force the rate of disintegration of phenindione tablets progressively decreases, whereas the dissolution time follows a more complicated pattern. Morrison & Campbell (1965) and Wood (1967) have clearly shown that measuring the dissolution time is the method of choice since the disintegration time is less indicative of the physiological activity of tablets.

Several authors, including Lewis & Train (1965) and Lewis & Shotton (1965), have shown that at increasing compression forces the relative density of tablets becomes greater until a maximum is reached. The force at which the maximal relative density is obtained depends on the composition of the tablet concerned.

We have compared the dependence of both the dissolution time and the relative density on the force of compression. These studies were made on granulates generally used for the production of tablets containing small amounts of active ingredients, sodium chloride, 1·5%, being added to the powders before granulation so that the dissolution time could be measured conductometrically. In most experiments the granulate composition was: lactose 78·5, sodium chloride 1·5, potato starch 20%, mucilage of starch q.s. The granulate was passed through a Manesty oscillating granulator, dried in a fluidized bed dryer and passed through a 20 mesh sieve. Particles which were not retained on a 50 mesh sieve were discarded. After the addition of 3% of talc and 1% of magnesium stearate, flat tablets of 9 mm diameter were made on a single punch tabletting machine (HOKO) both punches of which are instrumented with strain gauges. The compression force was recorded on an oscilloscript (Philips PT 2104).

The dissolution time was determined by immersing the tablet in a stainless steel wire basket of 16 mesh in 75 ml of water at 25° or 37° which was circulated at a controlled speed of 8 ml/sec with the aid of a magnetic stirrer. The conductivity of the solution was measured with a Philips conductometer PR 9501 and the results were plotted against time. Recording the dissolution rate has

TABLE 1. RELATION BETWEEN COMPRESSION FORCE, RELATIVE DENSITY AND DISSOLUTION TIME

	Compression force (kg)	Relative density	Dissolution time (sec)	C.V.
1	220	0·81	1400	14·0
2	260	0·82	955	11·2
3	290	0·83	680	15·0
4	318	0·85	440	16·3
5	430	0·89	135	16·0
6	500	0·90	110	14·2
7	520	0·91	110	12·4
8	650	0·94	129	11·4
9	900	0·96	250	10·5
10	1140	0·97	380	5·0
11	1350	0·97	425	2·9
12	1640	0·97	440	3·4
13	1800	0·97	440	2·1
14	2000	0·97	440	3·6

Note: C.V. (the coefficient of variation) is only given for the dissolution time and not for the relative density since this is always less than 0·5%.

the additional advantage that comparisons can be made between the pattern of dissolution, e.g. by calculating the time required for the dissolution of 25, 50 and 90% of the sodium chloride. We chose as the criterion the time required for the dissolution of 90% of the salt.

The relative density, defined according to Lewis & Train (1965), is the ratio of compact density to density of the solid material. We always listed 10 tablets compressed at the same force for dissolution time. In Table 1 the average values for the 90% dissolution times and the relative densities at 14 different compression forces are listed. The dissolution time becomes constant at practically the same compression force at which the relative density becomes constant: the difference in dissolution time between tablets compressed at forces of 1350 and 1640 kg is still significant at the 0·95 level, whereas the difference in relative density of these tablets is no longer significant.

This correlation was confirmed for tablets of different compositions, although the first part of the graph was often different. In the present instance the dissolution time showed a minimum value, whereas with other tablets an initial decrease was not found: e.g. the dissolution time of tablets made from the same powder mixture but using a 10% gelatin or gum acacia solution as the granulating solution did not show the minimum. A steady increase in dissolution time was found with increasing compression force until a maximal value was reached, again at the compression force at which the relative density because constant.

Since tablets usually are compressed at a pressure lower than the critical one described, a comparison of their dissolution times seems only completely reliable if the relation with the compression force is known.

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The effect of diet on blood urea levels in the beagle

SIR,—Chance conversation between the authors revealed that, on certain occasions in the course of routine sampling of blood from beagles before or during long-term toxicity trials conducted independently at two centres (Huntingdon and Sandwich), marked variations in blood urea values had been noted. Amongst possible causes, that of dietary intake was suggested, and was investigated independently at the two centres. Our results indicate that, in the beagle, the blood urea concentration is significantly raised for a number of hours after a meal of one of various currently-used dog diets. This is particularly so if the animal receives only one main meal a day and therefore consumes virtually all its daily protein intake within 1 or 2 hr; its blood urea may then rise to levels usually considered indicative of a pathological abnormality.

Pedigree beagles of both sexes were used at both centres, those at Huntingdon (H) being 3–6 months old, whereas those at Sandwich (S) ranged from 1–5 years of age. Blood urea was determined throughout by the diacetyl-monoxime method, using a Technicon Autoanalyser (Marsh, Fingerhut & Kirsch, 1957).

TABLE 1. COMPOSITION OF BEAGLE DIETS

		Diet	Amount consumed (g)	Protein consumed (g)	n
1	(H)	Spiller P62	80–120 (mean 107)	19–28·5 (mean 25·5)	12
2	(H)	Purina chow + tinned meat preparation (Lassie) + water (100 : 120 : 80)	300	37·0	12
3	(S)	Wet meat/biscuit diet (Reinert & Smith, 1963)	500	85·0	37
4	(S)	Spur	350	87·5	26

(The amounts of diets 1 and 2 were normally fed twice daily; diets 3 and 4 were normally fed only once daily.)

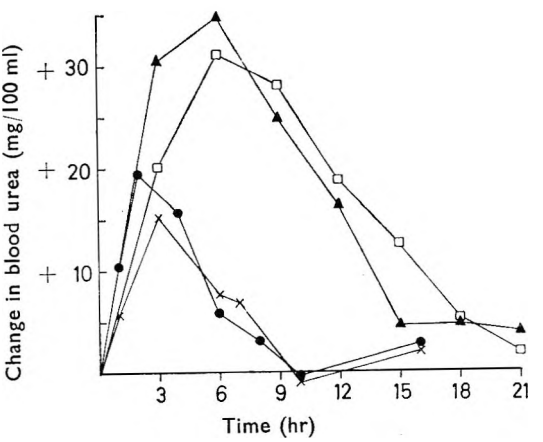

FIG. 1. Mean change from fasting level (time 0) of blood urea in dogs at various times after being fed with various diets (as Table 1): ●——● diet 1 (Spiller). ×——× diet 2 (Purina chow + Lassie). ▲——▲ diet 3 (wet meat/biscuit). □——□ diet 4 (Spur).

Blood urea levels in dogs used averaged (H) 31·2 mg/100 ml (n = 42) and (S) 29·9 mg/100 ml (n = 102), when samples were taken after a fasting period of 18–24 hr. The mean net changes from fasting level noted in the dogs fed a single meal of one of four different diets (Table 1) were calculated (Fig. 1). Rises of up to 35 mg/100 ml (diet 3) were observed, roughly proportional to the amount of protein consumed. The peak responses were reached at 2 or 6 hr after feeding, and the elevations in blood urea lasted about 9 or 21 hr, in the animals fed the smaller (diets 1 and 2) or the larger (diets 3 and 4) amounts of diet respectively.

Our findings do not agree with the published opinions of many authorities who consider that, in the dog as in man, the level of the blood urea in the normal subject reflects the general state of protein nourishment rather than the direct influence of the subject's last meal (MacKay & MacKay, 1927; Robin, 1948; Coffin, 1953; Hoe & O'Shea, 1965; Coles, 1967). McKelvie, Powers & McKim (1966) reported an increase of only 17% in the urea nitrogen values in blood taken from beagles 1–2 hr after a meal, compared with 24-hr fasting values. In a recent paper, however, Vogin, Skeggs & others (1967) have reported increases in urea nitrogen in beagles after feeding which compared closely with our results.

In view of the popularity of the blood urea determination as a guide to possible renal damage, it would appear essential that, to obtain reproducible results in dogs used for example in long-term toxicity trials, blood samples for urea determination should be taken only after a sufficient fasting period (Bloom, 1960).

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February 12, 1968

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The effect of imipramine, cocaine and neostigmine on the hyperglycaemic response to noradrenaline and adrenaline

SIR,—There are reports of an increased glycogenolytic action of adrenaline and noradrenaline in animals treated with cocaine (Schmidt & Späth, 1963; Hardman & Mayer, 1965). We could find no data about such an effect for imipramine. We have compared the activity of some drugs causing hypersensitivity to catecholamines on the hyperglycaemia caused by the catecholamines.

Rabbits of either sex, 2–3 kg weight, were fasted for 24 hr. The concentration of glucose in blood withdrawn from the ear vein was measured at the time of administering a catecholamine subcutaneously, and at 1, 2 and 3 hr afterwards. One week later the experiment was repeated but imipramine, cocaine, or atropine and neostigmine were given beforehand (see Tables for time, route and dosage). Blood glucose was estimated by the method of Hagedorn & Jensen (1923).

The adrenergic sensitizers were used in doses which caused clear-cut enhancement of the blood pressure responses to catecholamines. At these doses they did not alter the resting blood glucose level. Atropine (3 mg/kg s.c.) was also without any effect in this respect.

Cocaine, in doses of either 2 or 5 mg/kg, did not influence the effect of noradrenaline. In a dose of 5 mg/kg intravenously, it enhanced the response to adrenaline. Similarly, imipramine (2 or 5 mg/kg) did not alter the sensitivity to noradrenaline, while in a dose of 5 mg/kg it enhanced the sensitivity of the animals to adrenaline-induced hyperglycaemia.

TABLE 1. THE EFFECT OF NEOSTIGMINE ON THE BLOOD GLUCOSE CHANGES CAUSED BY NORADRENALINE

Treatments*	No. of rabbits	Blood glucose level (mg/100ml) after, hr			
		0	1	2	3
Noradrenaline 500 μg/kg + atropine 3 mg/kg s.c.	5	87·0 ± 4·2	184·8 ± 17·1	225·2 ± 10·5	203·6 ± 24·0
Noradrenaline 500 μg/kg + atropine 3 mg/kg s.c. + neostigmine 10 μg/kg i.v.	5	89·0 ± 11·6	233·2† ± 12·3	234·0 ± 5·2	234·8 ± 27·3

* Neostigmine given 1 hr and atropine 3 hr before noradrenaline.
† $P < 0.05$.

TABLE 2. THE EFFECT OF IMIPRAMINE, COCAINE AND NEOSTIGMINE ON THE BLOOD GLUCOSE CHANGES CAUSED BY ADRENALINE

Treatments*	No. of rabbits	Blood glucose level (mg/100ml) after, hr				Significance to control
		0	1	2	3	
Adrenaline 200 μg/kg s.c.	45	85·73±2·0	200·38± 6·3	235·91± 7·1	222·2 ± 6·6	
Adrenaline 200 μg/kg + imipramine 5 mg/kg i.v.	5	98·6 ±4·1	232·8†±14·0	240·0 ±21·0	241·6 ±22·9	†P<0·01
Adrenaline 200 μg/kg + cocaine 5 mg/kg i.v.	10	114·0 ±3·5	262·6†± 7·8	259·3 ± 8·1	208·9 ±25·9	†P<0·001
Adrenaline 200 μg/kg + atropine 3 mg/kg s.c.	10	101·5 ±4·3	196·4 ± 9·2	232·8 ±15·3	220·0 ±10·4	
Adrenaline 200 μg/kg + atropine 3 mg/kg s.c. + neostigmine 10 μg/kg i.v.	10	104·0 ±5·0	243·0†±13·8	275·6 ±15·7	254·8†± 9·4	†P<0·02

* See the footnote to Table 1.

The animals given neostigmine showed a definite hypersensitivity. The hyperglycaemia seen 1 hr after 500 μg/kg noradrenaline was significantly greater than the control, so too were the differences between the control and neostigmine-treated groups during the 3 hr period of the experiment when adrenaline was administered in a dose of 200 μg/kg (Tables 1 and 2).

On the basis of our results cocaine and imipramine are relatively less effective than neostigmine in enhancing the hyperglycaemic action of catecholamines. It is possible that enzymes described as destroying tropine derivatives in rabbits (Werner, 1965) could be responsible for the weak activity of cocaine.

It has been shown that neostigmine potentiates the blood pressure response to noradrenaline (Fekete, 1956). The present experiments show that this effect of neostigmine can also be demonstrated on glucose mobilization caused by catecholamines in atropinized animals.

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The origin of epileptiform seizures caused by oil of *Artemisia caerulescens* L. (Correction)

SIR,—An error has arisen in the Letter to the Editor on the above topic (Srebočan & Stern, 1968). In all instances γ-aminobenzoic acid should be replaced by γ-aminobutyric acid.

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Reference

Srebočan, S. & Stern, P. (1968). *J. Pharm. Pharmac.*, **20**, 160–161.

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Letters to the Editor

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