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The relation between the vapour pressure of a drug and its concentration emerging in the air stream from a nasal inhaler

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A method of determining the absolute vapour pressures of volatile drugs and drug-adjuvant mixtures is described. The absolute vapour pressures of methylamphetamine, propylhexedrine and eucalyptol have been determined over a range of temperatures. The concentrations of these drugs emerging from a nasal inhaler system have also been measured under similar experimental conditions. From the inhaler results, it has been possible to derive values for the vapour pressures of the drugs in the system and compare them with absolute vapour pressure values. The derived pressures were lower than the absolute pressures at any given temperature. Nevertheless, there seems to be reasonable agreement among the latent heats of vaporization of the three drugs using either parameter. The factors that may give rise to the low values of derived vapour pressure are discussed. Mixtures of eucalyptol and methylamphetamine examined using the same techniques, show that both with the inhaler system and with mixtures of the pure drugs a similar liquid-vapour equilibrium exists. The vapour pressure-composition diagram constructed from either set of results shows a positive deviation from Raoult's law.

Recently, Armstrong, Carless & Enever (1970) examined factors affecting the dose of a drug delivered from a conventional nasal inhaler. The drug concentration in the air stream emerging from the inhaler was measured for a variety of volatile drugs impregnated on fibrous support material. With a range of air flow rates and temperatures, it was found that, in all the systems studied, an equilibrium was established between the liquid and vapour phases. In addition, linear relations were obtained when the logarithm of the drug concentration was plotted against the reciprocal of absolute temperature. Since the drug concentration in the air stream is proportional to its vapour pressure, the relation is similar to that expressed by the Clausius-Clapeyron equation. It was therefore concluded that the partial vapour pressure exerted by the drug in the presence of other volatile constituents in an inhaler formulation is a major factor governing the dose delivered to the patient. An attempt has now been made to determine the absolute vapour pressures of small quantities of various volatile drugs and adjuvants so that these data may be correlated with the results obtained from our inhaler systems.

The techniques available for determining vapour pressure may be broadly classified into three categories. (1) Dynamic methods that involve the measurement of the boiling points of a substance at various pressures (Swietoslawski, 1953). Unfortunately, in many cases difficulty is encountered in accurately determining the point at

which the liquid boils because of superheating effects. In addition, relatively large quantities of liquid are necessary. (2) Gas saturation and effusion methods that necessitate measurement of the loss of material from the liquid to the vapour state (Halstead, 1970). Such methods are generally suitable for determination of vapour pressures of pure substances. Mixtures of volatile materials are not easily evaluated with these techniques. (3) Static methods that involve direct measurement of the pressure exerted by an outgassed sample of either a pure or multicomponent system. To this end, various pressure measuring devices have been used, including manometers, McLeod, Pirani and Bayard-Alpert gauges. For our purposes, the McLeod gauge is the most suitable since it is a primary standard of pressure measurement that can be used for a variety of substances provided condensation of the vapour does not occur in the gauge (Ede, 1947; Fleuss, 1924; Francis, 1936). The McLeod gauge which we have designed is capable of measuring pressures in the range 2×10^{-3} to 10 torr, and has been used to determine the vapour pressures of methylamphetamine, propylhexedrine, eucalyptol and methylamphetamine–eucalyptol mixtures.

Design of vapour pressure measuring system

The system (Fig. 1) consists of the McLeod gauge in a temperature controlled enclosure, a thermostatted sample tube, and an oil diffusion pump backed by a rotary vacuum pump. A cold trap surrounded by liquid nitrogen is interposed between the vacuum pumps and the remainder of the system to prevent contamination of the pump oil with organic vapours, and also to prevent the gauge and sample tube becoming contaminated with pump oil. Provision is also made for a second non-thermostatted McLeod gauge to be connected into the system for measurement of pressures in the range 5×10^{-2} to 1×10^{-4} torr.

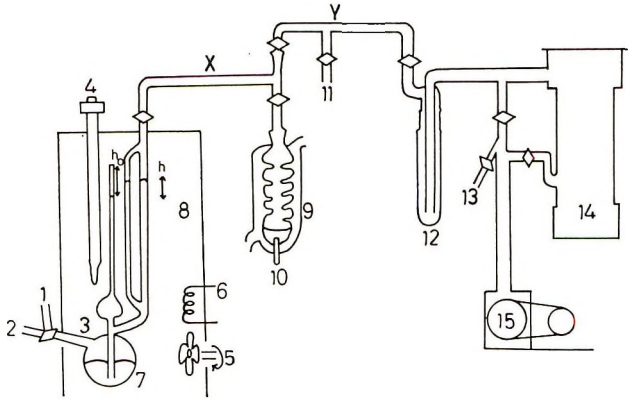

FIG. 1. Schematic diagram of apparatus used for determining vapour pressure of drugs at various temperatures. 1, 13, Airbleed; 2, water pump; 3, cut-off point; 4, mercury contact thermometer; 5, fan; 6, heater element; 7, McLeod gauge range 2×10^{-3} — 10 torr; 8, temperature controlled enclosure; 9, sample tube; 10, tungsten wire; 11, additional McLeod gauge range 1×10^{-4}–5×10^{-2} torr; 12, liquid nitrogen cold trap; 14, oil diffusion pump; 15, rotary vacuum pump.

The air thermostat is essential to maintain the gauge at a higher temperature (approximately 50°) than the sample tube and prevent condensation of the vapour in

the gauge. The air temperature around the gauge can be controlled to $\pm 1°$, and it is accurately determined at the time of vapour pressure measurement by means of a calibrated bead thermistor system.

The sample tube is designed with many indentations on its inner surface so that a large temperature-controlled surface area is presented to both the liquid and the vapour phases. This ensures that temperature equilibrium is established. The temperature is controlled by circulating water through the sample jacket from a thermostatted water bath-pump system and monitoring the inlet and outlet temperatures by means of calibrated sensitive bead thermistors. The sample tube temperature is thus maintained within $\pm 0{\cdot}1°$ of the required value. A tungsten wire, fused into the base of the sample tube, serves as a nucleus for ebullition during outgassing.

Evacuation of the system is initially achieved by use of the rotary backing pump, and subsequently, when the pressure has dropped to 0·05 torr, the oil diffusion pump is switched into the system. The oil used in the diffusion pump (Silicon 704) is of high stability and has a low vapour pressure at the pump operation temperature. This arrangement enables a vacuum pressure of 1×10^{-5} torr to be drawn in the system.

The vapour pressure of a substance introduced into the sample tube connected to the evacuated system is determined by measuring the difference in the heights of mercury, h, in the open and closed capillaries of the McLeod gauge and the distance, h_0, between the top of the closed capillary and the mercury meniscus in that capillary. A Vickers cathetometer (reading to 0·02 mm) is used for this purpose. From a knowledge of the cross-sectional area, A, of the closed capillary and the volume, V, of the bulb and closed capillary up to the cut-off point, the pressure, P, can be calculated using the relation:

$$P = \frac{h_0 A h}{[V - h_0 A]} \qquad (1)$$

With the present gauge designed for measuring pressures in the range 2×10^{-3} to 10 torr, the value of A and V are 8·720 mm^2 and 10318·6 mm^3 respectively.

The equation relating pressure to the heights of mercury in the gauge, assuming that Boyle's Law is obeyed and that the compressed gas does not condense in the bulb and capillary, has been verified for every volatile substance examined by measuring various values of h and h_0 for the same sample of trapped gas. From equation (1), assuming that h_0A is small in comparison with V, a graph of h against $1/h_0A$ at constant pressure can be constructed. This will yield a straight line passing through the origin provided that condensation does not occur.

Since the gauge is maintained at a higher temperature than the sample tube to prevent condensation of the vapour, the pressure reading obtained from the gauge has to be corrected to determine the vapour pressure of the sample. In this system the mean free path of the molecules is large compared with the dimensions of the system, and heat transfer occurs through collisions with the walls rather than intermolecular collisions. Therefore a thermal transpiration correction (Bennett & Tompkins, 1957) has to be applied in the form

$$P_1 = \left(T_1/T_2\right)^{\frac{1}{2}} P_2 \qquad (2)$$

where P_1 is the pressure at absolute temperature T_1

P_2 is the pressure at absolute temperature T_2.

MATERIALS AND METHODS

Materials

(+)-Methylamphetamine from Aldrich Chemicals Co. Inc., Milwaukee, Wisconsin.

Propylhexedrine from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin.

Eucalyptol B.P.C. from Bush Boake and Allen, London.

The purity of these materials was checked by gas-liquid chromatography using the column described below. Under these conditions each material produced a single peak.

The fibrous supports used for the inhaler studies were composed of cellulose acetate and were 22 mm long by 8 mm diameter.

Methods

Determination of absolute vapour pressure of volatile materials, Since a static method of vapour pressure measurement was used, it was essential to outgas the equipment and sample to ensure that no air or water vapour contributed to the measured vapour pressure. Before it was filled with a volatile sample, the sample tube was cleaned with chromic acid, washed with distilled water, and dried by rinsing with absolute ethanol, acetone and then placed in an hot air oven at 90°. The tube was allowed to cool to room temperature while stoppered to prevent ingress of water. Using a cannula, 1 ml of the volatile material was introduced into the base of the tube, the tube was immediately immersed in liquid nitrogen and subsequently placed in position on the equipment. The McLeod gauge was isolated from the system, and the sample tube, still immersed in liquid nitrogen, was evacuated to approximately 1×10^{-5} torr. The sample tube was then isolated from the system and allowed to reach room temperature when the entrapped gases bubbled out of the liquid. This process was repeated 15 times to achieve complete removal of gaseous impurities. The water flow through the sample jacket was then established and sufficient time allowed for temperature equilibrium to be achieved. The sample tube and McLeod gauge were then connected together and successive pressure readings taken at 15 min intervals until a constant pressure was recorded. This pressure was taken to be the saturation vapour pressure of the volatile material after the thermal transpiration correction had been applied.

The McLeod gauge measures the total pressure in equilibrium with the sample, hence, if the sample consists of more than one volatile component, then the composition of the vapour phase must also be known in order to determine the partial pressure of each component. For example, to determine the partial pressures exerted by methylamphetamine and eucalyptol in mixtures of these materials at 25·25°, the following procedure was adopted. The mixture to be analysed was outgassed as described above, and then allowed to reach equilibrium at 25·25° with the gauge and vacuum pumps isolated. After equilibration for 1 h the sample tube was isolated from the system and the vapour present in the connecting tubes X and Y (Fig. 1) was sucked into a clean outgassed cold trap. The trap was removed, and to the condensed vapour was added diethyl ether containing a known amount of internal marker so that the solution could be analysed for methylamphetamine and eucalyptol content using gas-liquid chromatography.

Determination of drug concentration emerging in the airstream from a nasal inhaler. Commercially available cellulose acetate fibrous supports of the type used in nasal inhalers were used. The apparatus for this study has previously been described

(Armstrong & others, 1970). The fibrous supports were impregnated with 0·1 ml of the pure drug or mixture of drugs being examined, and air was allowed to flow through the inhaler system for 30 s at a rate of 0·95 litre min^{-1}. The drug content in the airstream was collected in diethyl ether and analysed using a Perkin Elmer F11 gas chromatograph equipped with a column composed of 10% w/w Carbowax 6000, and 5% w/w potassium hydroxide on Celite 545. The experimental conditions for analysis have been described by Armstrong & others (1970).

With this system, the relation between temperature and the concentration of eucalyptol, propylhexedrine, methylamphetamine and eucalyptol–methylamphetamine mixtures emerging from the inhaler was investigated.

RESULTS

Fig. 2 (a) and 2 (b) show the effect of temperature upon the concentration of drug emerging from the inhaler system using methylamphetamine, propylhexedrine and eucalyptol. For any temperature selected, the order of magnitude of drug concentration in the airstream is eucalyptol > propylhexedrine > methylamphetamine. With all three drugs there is an exponential relation between temperature and concentration.

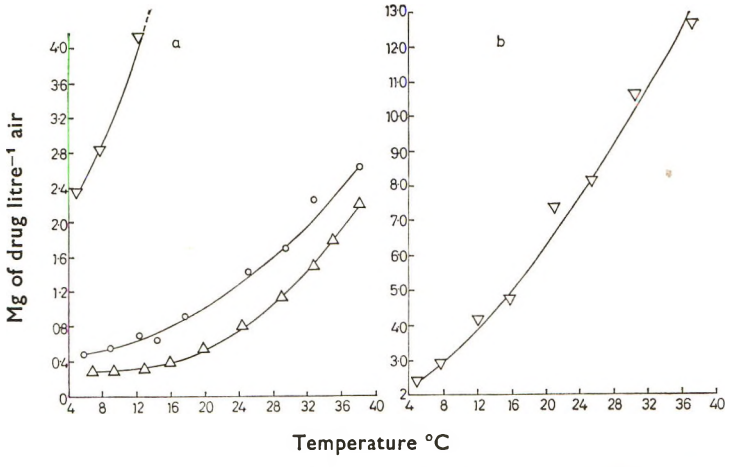

FIG. 2. Effect of temperature on amount of drug $litre^{-1}$ air emerging from inhaler system: (a) methylamphetamine Δ; propylhexedrine ○; eucalyptol ▽. (b) Eucalyptol ▽.

These results may be converted into values of vapour pressure for comparison with the absolute values by assuming that the inhaler system is analogous to that encountered when measuring the vapour pressure of a liquid by the gas saturation method. With this method, the flow of dry gas through the liquid is sufficiently slow to permit complete saturation of the airstream at the temperature of the determination. The loss in weight of the liquid after passage of a known volume of air is measured. The vapour pressure P is calculated from the equation:

$$P = \frac{G}{MV} RT \qquad (3)$$

where V is the volume of dry gas, in litres, containing G grams of material of molecular weight M. T is the absolute temperature and R the gas constant.

Equation (3) neglects the increase in volume of the gas caused by vaporization of the liquid. However, at low vapour pressures this is negligible, and the equation is sufficiently accurate for the present study where pressures of the order 2×10^{-3} to 10 torr are being considered.

Fig. 3 (a) and (b) shows the results of the absolute vapour pressure determinations of the drugs compared with the values derived from the inhaler systems. The two sets of log P *vs* 1/T data in each case for methylamphetamine, propylhexedrine and eucalyptol show that the derived values are always lower than the absolute vapour pressure values. At any given temperature the vapour pressure of the drugs is in the order eucalyptol > propylhexedrine > methylamphetamine.

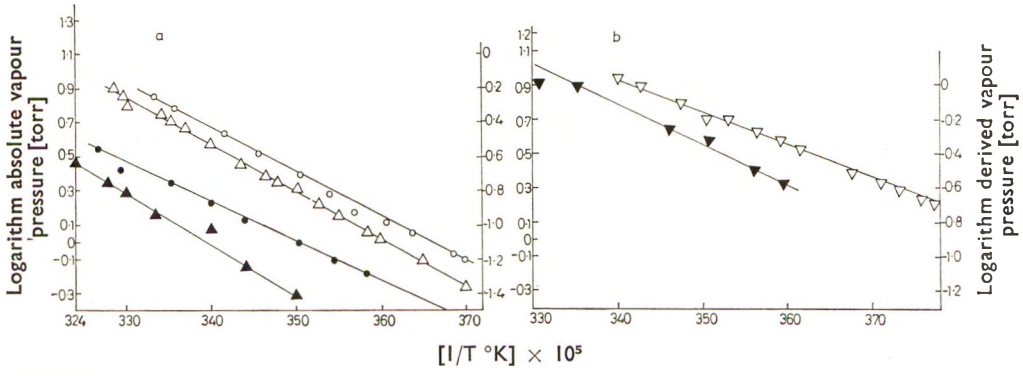

FIG. 3. Logarithm vapour pressure against reciprocal absolute temperature: (a) Methylamphetamine, Δ absolute values, ▲ derived values. Propylhexedrine, ○ absolute values, ● derived values. (b) Eucalyptol, ▽ absolute values, ▼ derived values.

The log vapour pressure reciprocal of absolute temperature relation is commonly expressed by the Clausius-Clapeyron equation, where the change in pressure P with temperature T is given by:

$$\frac{dP}{dT} = \frac{P\Delta H_{vap}}{RT^2} \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where ΔH_{vap} is the latent heat of vaporization of the material. Now ΔH_{vap} is constant over a small range of temperature, and hence eqn (4) may be integrated to yield

$$\text{Log } P = \frac{-\Delta H_{vap}}{2{\cdot}303RT} + C \quad .. \quad .. \quad .. \quad (5)$$

where C is the integration constant.

Therefore, the slopes of the lines in Fig. 3 (a) and (b) will give the latent heats of vaporization for the drugs. These values are: [kJ mol^{-1} (kcal mol^{-1})] 53·1 (12·7), 48·2 (11·5), 36·4 (8·7) from absolute vapour pressure values and 52·7 (12·6),* 41·4 (9·9),* 40·6 (9·7)* from derived vapour pressure values for methylamphetamine, propylhexedrine and eucalyptol respectively.

Fig. 4 (a) and (b) show the vapour pressure-mol fraction relation for mixtures of methylamphetamine and eucalyptol at 25·25° using absolute and derived vapour pressure values. In Fig. 4 (a), the partial vapour pressures of each component have

* Since calculation for the derived vapour pressures assumes that there is saturation of the air stream flowing through the inhaler, it is more appropriate to regard these figures as apparent ΔH_{vap} values.

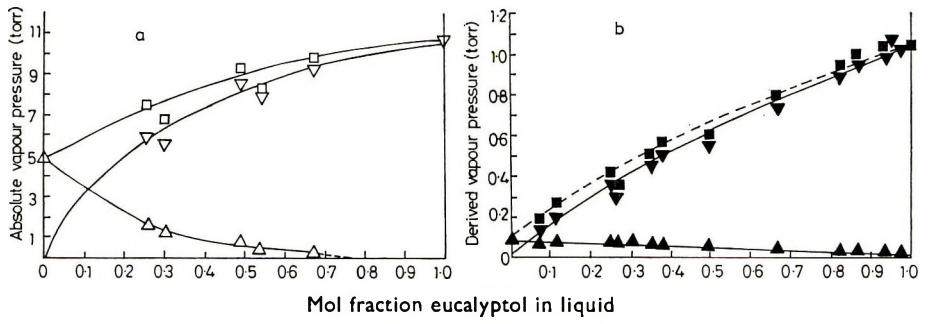

FIG. 4. Effect of mol fraction eucalyptol in methylamphetamine on total and partial vapour pressures of the mixtures at 25·25° C. (a) Absolute vapour pressure Δ partial pressure methylamphetamine, ▽ partial pressure eucalyptol, □ total pressure. (b) Derived vapour pressure values ▲ partial pressure methylamphetamine, ▼ partial pressure eucalyptol, ■ total pressure.

been calculated from a knowledge of the molar composition of the vapour phase and the total absolute pressure of the system. Fig. 4 (b) shows the partial pressures of each component as derived from the concentrations emerging from the inhaler system, together with the total pressure obtained by summation. This assumes that no other component is present that contributes to the total vapour pressure.

Fig. 4 (a) and (b) shows that the pressure exerted by either component is increasingly depressed by increasing mol fraction of the other component. In addition both graphs of total pressure show that there is a slight positive deviation from Raoult's Law.

DISCUSSION

It is evident from comparison of Fig. 3 (a) and (b) that the ratio derived vapour pressure : absolute vapour pressure at any temperature varies with the drug examined. The lowest ratio is obtained with methylamphetamine and the highest with eucalyptol. It follows therefore that, when comparing Fig. 4 (a) and 4 (b), the ratio derived total pressure : absolute total pressure will vary with the mol fraction of eucalyptol. Nevertheless, both diagrams show a similar pattern, since the binary mixtures of methylamphetamine and eucalyptol exhibit a slight positive deviation from Raoult's law. This is not an unexpected phenomenon and can be explained by hydrogen bonding between the amine groups of methylamphetamine molecules. Evidence for this has been obtained from infrared spectroscopic studies.

The partial vapour pressure-mol fraction relation for methylamphetamine as shown in Fig. 4 (a) and (b) are slightly anomalous. From the absolute values in Fig. 4 (a), methylamphetamine shows a negative deviation from Raoult's Law, whilst from the derived partial pressures in Fig. 4 (b) there appears to be no deviation from ideal behaviour. It can be shown theoretically that, when one component of a binary mixture exhibits positive deviation from ideality, the other component must do the same. The anomaly is probably the result of experimental difficulties encountered when analysing the condensed vapour phase containing a low mol fraction of methylamphetamine.

The experimental measurements of total vapour pressure of methylamphetamine-eucalyptol mixtures have been made in the region of 0·25 to 0·65 mol fraction of eucalyptol for two reasons. Firstly, it is difficult to control the composition of mixtures containing a low mol fraction of either component due to the loss of the

more volatile component during outgassing. In all instances, analysis of the bulk liquid phase was made before and after a vapour pressure determination to check that no change in liquid composition had occurred. Secondly, deviation from Raoult's law will be more easily detected in this region. As the mixture approaches either pure eucalyptol or pure methylamphetamine, the partial pressures exerted by the components approximate to the ideal values predicted by Raoult's law.

The difference in absolute and derived vapour pressures of the drugs and also the binary mixture may be due to a number of factors. A combination of some, or all, of these may explain the discrepancy.

(1) The calculation of vapour pressures from the results obtained with the inhaler system assumes that the system is analogous to a dynamic method of vapour pressure measurement. In the calculation, we assume that the air stream is saturated with vapour. At the air flow rates used in this work, saturation of the air may not have been achieved. Under these circumstances one might expect the derived vapour pressure values to vary with the flow rate of air. However, we have previously shown (Armstrong & others, 1970), that the drug concentration in the air stream, and therefore the derived vapour pressure, is constant within the range 0·3 to 2·0 litre min^{-1} at any given temperature. Hence there is no clear evidence as to the degree to which possible undersaturation of the air stream may influence the derived vapour pressure values. In any event, it is clear that there is an equilibrium between liquid and vapour phase.

(2) With the static vapour pressure measurements that have been made, the vapour is in equilibrium with a plane liquid surface. However, in the inhaler system, the drugs impregnated on a fibrous support are present in capillaries throughout the material. From the Kelvin equation (Gregg, 1961) it can be shown that the vapour pressure exerted by a liquid when confined in a finely porous medium is less than that exerted by a liquid at a plane surface.

(3) There may be some physical interaction between the impregnated drug and the cellulose acetate support material, e.g. hydrogen bonding, or solubility of support material in the drug. This could cause a depression of the vapour pressure exerted by the drug, and account for the difference in the ratio derived vapour pressure-absolute vapour pressure with the three drugs examined.

(4) Since the cellulose acetate support material used in the inhaler systems has an affinity for water, it is probable that it will contain traces of moisture. If the water is soluble in the impregnated drug, then a binary water-drug system will be produced with the pure drug, and a ternary system will result with the methylamphetamine-eucalyptol mixtures. It is then necessary to include this partial water vapour pressure in any calculation of the total pressure of such systems.

Factors (3) or (4), or both, might be expected to affect the values of the apparent latent heats of vaporization (ΔH_{vap}) of methylamphetamine, propylhexedrine and eucalyptol obtained from the inhaler experiments. In fact, the results show that there are differences between the ΔH_{vap} and the apparent ΔH_{vap} values for each drug that could be accounted for in this way. It is important to note that the ΔH_{vap} values obtained from the inhaler experiments are independent of the flow rate of air through the inhaler within the range 0·3 to 2·0 litre min^{-1}.

Despite the numerical difference between the absolute and derived vapour pressure values of the eucalyptol-methylamphetamine mixtures, the similarity of the two vapour

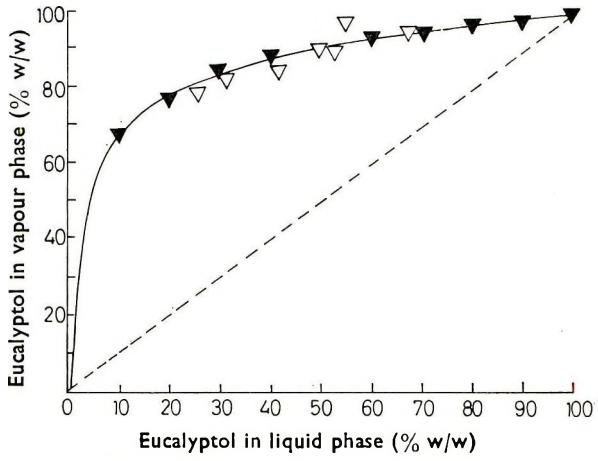

FIG. 5. Effect of composition of liquid phase on vapour phase composition using methylamphetamine, eucalyptol mixtures. ▼ from derived vapour pressure results; ▽ from absolute vapour pressure results.

pressure-composition relations is evident from Fig. 5. The relation between percentage concentration of the eucalyptol component in the vapour phase and its percentage concentration in the liquid phase is identical for the two sets of data. This therefore confirms that the same equilibrium situation occurs for the inhaler system and for the solution of the pure drugs. Fig. 5 further shows that an azeotropic mixture is not formed. If present, the composition of the azeotrope would be given by the point of intersection of the experimental curve and a straight line of slope equal to unity drawn through the origin.

As might be expected, the study of the eucalyptol-methylamphetamine mixtures shows that the concentration of methylamphetamine emerging from the inhaler is markedly reduced by the presence of the aromatic adjuvant. In order to mask the smell of the amine, it is necessary to use a mixture containing at least 0·3 mol fraction of eucalyptol, and this results in a reduction of approximately 30% in methylamphetamine concentration. Since the results presented also show that the same equilibrium situation occurs in both the inhaler system and the solutions of the pure drugs, absolute vapour pressure measurements of other volatile drug-adjuvant formulations will be of value in predicting their performance.

Acknowledgement

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Interaction of preservatives with cetomacrogol

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The interaction of a number of commonly used preservatives (benzoic acid, *p*-hydroxybenzoic acid, methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate and chloroxylenol with the non-ionic surfactant cetomacrogol was examined and a comparison made of various methods of expressing this interaction. It is suggested that the Scatchard equation is the most satisfactory equation for describing the binding data. Binding parameters determined from a Scatchard plot in the concentration range of free preservative appropriate for antimicrobial activity can be used to calculate the total concentration of preservative required in the surfactant system.

Interaction of preservatives with surfactants leads to a loss of antimicrobial activity. It is generally accepted that preservative solubilized or bound within the micelles is inactive and, although the micelles act as a reservoir of preservative, the antimicrobial activity depends largely on the concentration of unbound or free preservative (Allawala & Riegelman, 1953; Pisano & Kostenbauder, 1959; Mitchell, 1964). Hence the physicochemical parameter(s) used to express the interaction should permit calculation of the total preservative concentration required to provide a concentration of free preservative adequate to inhibit microbial growth.

The interaction of preservatives, drugs and other solutes with various macromolecules such as surfactants, polymers and proteins has been studied extensively. Methods used to express the interaction with proteins are well established (Goldstein, 1949; Klotz, 1953; Meyer & Guttman, 1970) and have been applied successfully to polymers such as methylcellulose and polyvinylpyrolidone (Eide & Speiser, 1967a,b; Cho, Mitchell & Pernarowski, 1971). Results for the interaction between solute and surfactant, however, have been presented in a variety of ways depending essentially on the particular theory adopted to explain the mechanism of interaction. In this paper, some of these methods are compared using results obtained in studies of the interaction between several commonly used preservatives and the non-ionic surfactant cetomacrogol.

MATERIALS AND METHODS

Materials. Benzoic acid, *p*-hydroxybenzoic acid, methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, chloroxylenol and cetomacrogol were as described previously (Mitchell, 1964; Mitchell & Brown, 1966; Brown, 1968).

Solubility and equilibrium dialysis. The experimental methods have been described before (Mitchell & Brown, 1966) except that a 0·0005 inch nylon membrane (Capran 77C, Allied Chemical Corporation, Morristown, New Jersey) was used in the dialysis technique and glass beads were added to each compartment to ensure continuous mixing. The interaction of cetomacrogol with benzoic acid was studied at 30° in citrate-phosphate buffer pH 3·0 and ionic strength 0·2. The amount of benzoic acid on both sides of the membrane at equilibrium was analysed spectrophotometrically at

273 nm. Data for the interaction of the other preservatives with cetomacrogol in unbuffered aqueous solution were derived from previous work (Mitchell, 1964; Mitchell & Brown, 1966; Brown, 1968).

RESULTS AND DISCUSSION

Interaction as a partition phenomenon

One of the earliest attempts to express solubilization quantitatively was due to McBain & Hutchinson (1955). They suggested that the formation of micelles, and in particular the occurrence of a hydrocarbon region in the centre of the micelles, justifies the treatment of micelle formation as a phase separation. Solubilization may be regarded therefore as the distribution of solute between water and the micellar phase. McBain & Hutchinson expressed this:

$$K_m = \frac{\text{mol micellar solute/mol micellar surfactant}}{\text{mol free solute/mol water}} \quad .. \quad .. \quad (1)$$

where K_m is the apparent partition coefficient for the distribution of solute between the micelles and aqueous phases. This approach has been used by Evans (1964) and Mitchell & Brown (1966). However (1) does not include the volumes of the aqueous or micellar phases and the values of K_m cannot therefore be compared with classical oil-water partition coefficients. An estimate of micellar volume can be made from the partial molar volume of the surfactant and K_m expressed according to equation (2) (Donbrow & Rhodes, 1963; Mitchell & Broadhead, 1967)

$$K_m = \frac{D_b/v}{D_f/(1 - v)} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where D_b is the amount of solute in the micellar phase, D_f is the amount of solute in the aqueous phase, v is the volume of the micellar phase and $1 - v$ is the volume fraction of the aqueous phase. Apparent partition coefficients calculated according to equation (2) for various preservatives in cetomacrogol solutions are shown in Fig. 1. The K_m values are not constant but depend on the free drug concentration.

A major problem associated with the application of (2) is that the value assigned to the volume of the micelles is somewhat arbitrary since the volume could be (a) the hydrocarbon core of the micelles, (b) the entire micelle or (c) the entire micelle including bound and trapped water. Humphreys & Rhodes (1968) attempted to overcome this problem in a study of the solubilization of benzoic acid in a series of non-ionic surfactants, by extrapolating the solubility curves to 100% w/w surfactant. This value was taken to represent the solubility of the solute in the micellar phase, S_m, and

$$K_m = S_m/S_w \quad .. \quad .. \quad .. \quad .. \quad (3)$$

where S_w is the solubility in the aqueous phase.

This technique will normally entail a very large extrapolation to 100% w/w surfactant and like all methods based on solubility measurements is, in effect, a one-point method. It cannot be assumed that the value of K_m obtained from equation (3) will be applicable to under-saturated systems. Moreover it has been shown that the solubilization process of benzoic acid is not governed by the distribution law (Donbrow & Rhodes, 1964; Donbrow, Molyneux & Rhodes, 1967; Donbrow, Azaz & Hamburger, 1970).

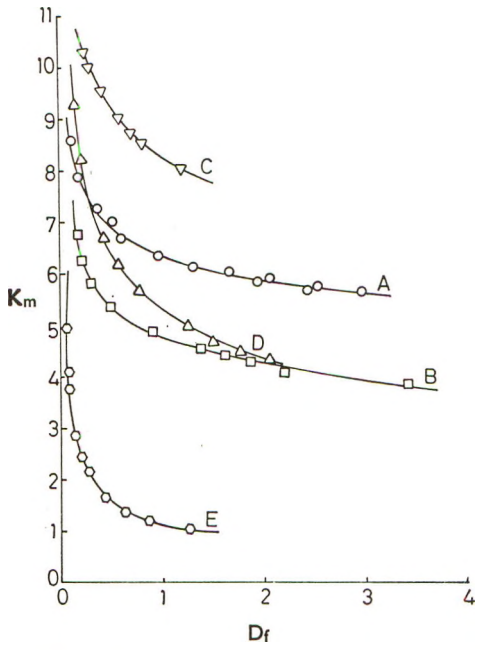

FIG. 1. Variation of apparent partition coefficient with free preservative concentration for the partition of preservative between micelles and aqueous phase of cetomacrogol. A. Benzoic acid at 30° ($D_f \times 10^2$, $K_m \times 10^{-1}$); cetomacrogol concentrations (mol/litre): 0·0077; 0·015; 0·031; B. *p*-Hydroxybenzoic acid at 25° ($D_f \times 10^2$, Km $\times 10^{-1}$); C. Methyl *p*-hydroxybenzoate at 25° ($D_f \times 10^2$, $K_m \times 10^{-1}$); D. Propyl *p*-hydroxybenzoate at 25° ($D_f \times 10^3$, $K_m \times 10^{-2}$); cetomacrogol concentrations (mol/litre): 0·01; 0·04; 0·06; 0·1 (Brown, 1968). E. Chloroxylenol at 20° ($D_f \times 10^3$; $K_m \times 10^{-3}$); cetomacrogol concentrations (mol/litre): 0·005; 0·01; 0·049; 0·096 (Mitchell & Brown, 1966).

Interaction as a "binding" phenomenon

An alternative and widely used method is to express interaction data according to equation (4) (Patel & Kostenbauder, 1958; Blaug & Ahsan, 1961 a,b; Bahal & Kostenbauder, 1964; Patel & Foss, 1965; Ashworth & Heard, 1966; Patel, 1967; Bean, Konning & Malcolm, 1969).

$$[D_t]/[D_f] = 1 + k\,[M] \quad \ldots\ldots\ldots\ldots \quad (4)$$

where $[D_t]/[D_f]$, represented by R, is the ratio of total solute concentration to the free solute concentration and [M] is the surfactant concentration. Plots of R as a function of surfactant concentration are normally presented as a single curve, the slope of which k, is taken as a measure of the binding capacity of the surfactant. The total preservative concentration is calculated by multiplying the concentration of free preservative required for antimicrobial activity by the R value at the appropriate surfactant concentration. However, as will be shown later (see Fig. 2) the R value at any given surfactant concentration is constant only under limited conditions.

Since the "partition" and "binding" approaches to solubilization are so widely used it is of interest to compare equations (2) and (4). Over a limited concentration range, the volume of the micellar phase, v, is directly proportional to the surfactant concentration, [M], i.e. $v = k'[M]$. Hence D_b/v in equation (2) can be written $[D_b]/k'[M]$ where $[D_b]$ is the concentration of solute in mol/litre. Similarly, for

relatively dilute solutions $D_f/(1-v)$ is proportional to the concentration of free solute in mol/litre, $[D_f]$, and equation (2) can be rewritten:

$$\frac{[D_b]}{[D_f][M]} = k'K_m = k \quad .. \quad .. \quad .. \quad .. \quad (5)$$

Since $[D_t] = [D_b] + [D_f]$, equation (4) can be rearranged into the same form as (5). Hence both the "partition" and simple "binding" approaches to solubilization depend on the same relation and a fit of data to either equation does not permit any assumptions to be made about the mechanism of the interaction. Although many authors have expressed solubilization in terms of a partition coefficient or as a binding constant, neither of these constants fully characterizes the interaction.

In contrast to the controversy surrounding methods used to describe the interaction of solute with surfactant, the fundamental concepts dealing with the interaction of solute with proteins are well established. The interaction can be expressed by equation (6) which is derived from the law of mass action:

$$r = \frac{nK\,[D_f]}{1 + K[D_f]} \quad .. \quad .. \quad .. \quad .. \quad (6)$$

where r is the molar ratio of bound solute to total protein $[D_b]/[P_t]$, n is the maximum number of independent binding sites on the protein and K is the association constant. Garrett (1966) suggested that the binding of preservatives to macromolecules other than protein may be treated similarly i.e. $r = [D_b]/[M]$ where [M] is the concentration of any macromolecule including surfactant. An important difference between surfactants and other macromolecules is that interaction occurs between the solute and surfactant micelles rather than monomer surfactant molecules. Theoretically [M] in equation (6) should be the concentration of micelles, n the number of binding sites per micelle and K the association constant for reaction with the micelles. From a practical viewpoint however, it is more convenient to express [M] in terms of the surfactant concentration. The critical micelle concentration of commonly used non-ionic surfactants is sufficiently low for the monomer concentration to be neglected.

Equation (6) has the same form as the Langmuir equation which has led some authors to suggest that the mechanism of interaction between solute and surfactant is one of adsorption onto the surface of the micelle or some other site within the micelle (Donbrow & Rhodes, 1964; Donbrow, Molyneux & Rhodes, 1967). However, as pointed out by Goldstein (1949) and Klotz (1953) for solute-protein interaction, although the equations are similar it is not necessarily correct to assume that binding and adsorption are identical processes.

Fig. 2 shows the results plotted as the ratio of total preservative to free preservative, R, as a function of surfactant concentration according to equation (4). Contrary to the manner in which data are normally presented for this type of plot, the results cannot be represented by a single curve. Equation (4) is in fact a special case of equation (6) and a single curve will be obtained only under two conditions: (a) when $[D_f]^{lim} \rightarrow O$ then $nK/(1 + K[D_f]) = nK$ and $R = 1 + k[M]$ where $k = nK$; (b) when $[D_f]$ is constant as in the solubility method, then $nK/(1 + K[D_f])$ = a constant, k'', and $R = 1 + k''[M]$. Hence k (or k'') does not fully characterize the interaction. A macromolecule or micelle has a limited binding capacity for solute molecules and a single value of k (or k'') will be obtained only over a limited range of free solute concentration. It is impossible to maintain $[D_f]$ constant using the equilibrium dialysis technique and Fig. 2 was constructed using calculated values of $[D_f]$. The

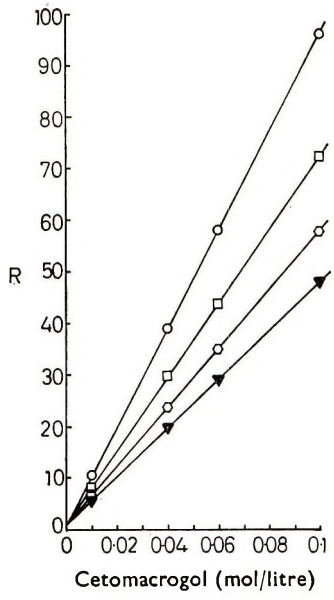

FIG. 2. Ratio of total : free propyl *p*-hydroxybenzoate as a function of cetomacrogol concentration at 25°. Concentration of free propyl *p*-hydroxybenzoate (mol/litre): ○, $0{\cdot}21 \times 10^{-3}$; □ $0{\cdot}56 \times 10^{-3}$; ⬡, $1{\cdot}23 \times 10^{-3}$; ▼, $2{\cdot}0 \times 10^{-3}$. Closed symbols represent solubility points.

slope decreases with increasing values of $[D_f]$ and the lowest limiting slope, corresponding to a solubility curve, represents the saturation-point in the Langmuir-type plot, Fig. 3.

The simplest way to express the binding data is a Langmuir-type plot of r versus $[D_f]$. Equation (6) is a segment of a rectangular hyperbola passing through the origin. If $[D_f]$ becomes infinite, the r value approaches n as a limit

$$[D_f] \xrightarrow{\lim} \infty \; r = n \qquad \ldots\ldots\ldots\ldots \quad (7)$$

and at $r = n/2$

$$[D_f] = 1/K \qquad \ldots\ldots\ldots\ldots \quad (8)$$

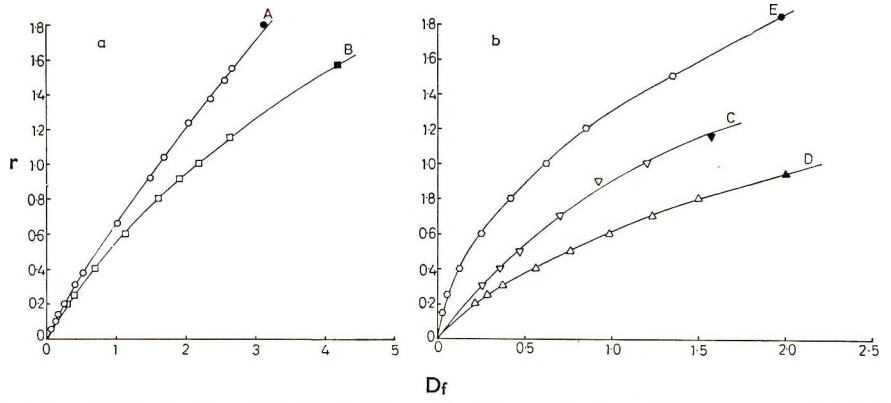

FIG. 3a, b. Langmuir-type plots for the interaction of preservatives with cetomacrogol solutions: A. Benzoic acid. B. *p*-Hydroxybenzoic acid. C. Methyl *p*-hydroxybenzoate. D. Propyl *p*-hydroxybenzoate. E. Chloroxylenol. Cetomacrogol concentrations and D_f values as in Fig. 1. Closed symbols represent solubility point.

Equations (7) and (8) indicate the importance of a wide concentration range of free solute in any binding study. Fig. 3a,b shows that at low concentrations the preservatives are more easily bound to cetomacrogol than at high concentrations. Only results obtained from solubility experiments show saturation of the binding sites. Hence binding parameters were not derived from these plots.

Equation (6) is normally rearranged into forms more convenient for graphical presentation of the results. Fig. 4a,b shows results for the interaction of some preservatives with cetomagrogol plotted according to the reciprocal form of the equation,

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK\,[D_f]} \quad .. \quad .. \quad .. \quad .. \quad (9)$$

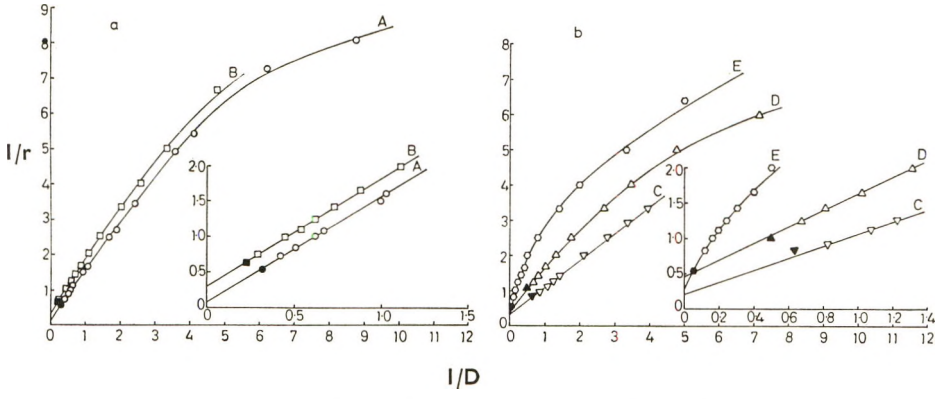

FIG. 4a, b. Double-reciprocal plot for the interaction of preservatives with cetomacrogol solutions: A. Benzoic acid ($1/D_f \times 10^{-2}$). B. *p*-Hydroxybenzoic acid ($1/D_f \times 10^{-2}$). C. Methyl *p*-hydroxybenzoate ($1/D_f \times 10^{-2}$). D. Propyl *p*-hydroxybenzoate ($1/D_f \times 10^{-3}$). E. Chloroxylenol ($1/D_f \times 10^{-4}$). Cetomacrogol concentrations as in Fig. 1. Closed symbols represent solubility points.

A line passing through the origin rather than an intercept corresponding to a limiting binding capacity has been taken as evidence that the mechanism of interaction is partitioning into the micelles rather than adsorption on a micellar surface or to specific sites on the macromolecule (Bahal & Kostenbauder, 1964). This plot, however, heavily weights those experimental points obtained at low concentrations of free drug and may lead to large errors on extrapolation to infinitely high free preservative concentrations. An alternative rearrangement of equation (6) is known as the Scatchard equation (Scatchard, 1949)

$$\frac{r}{[D_f]} = nK - rK \quad .. \quad .. \quad .. \quad .. \quad (10)$$

which on plotting gives a more even weighting to the different points on the curve. The plot for each preservative shown in Fig. 5, has a definite curvature. In protein-binding studies, this is taken as evidence for the existence of more than one type of binding site. In the case of solute-surfactant interaction the binding sites within the micelles probably do not behave independently of one another as required by equation (6). It is possible that uptake of solute into the micelles progressively alters the

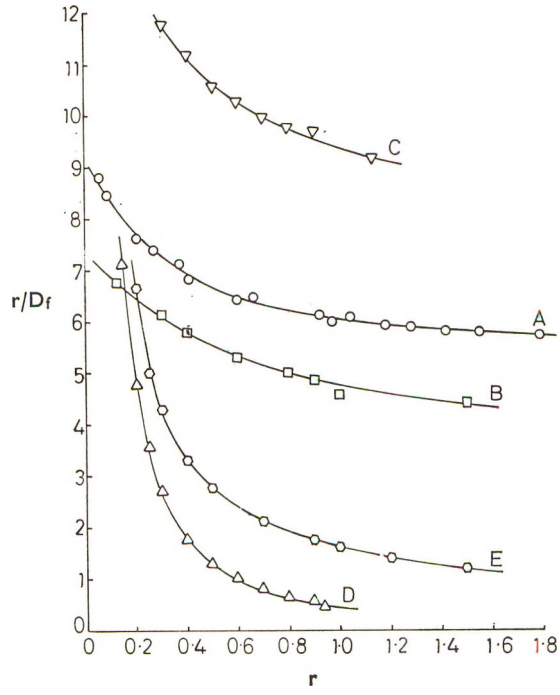

FIG. 5. Scatchard plot for the interaction of preservatives with cetomacrogol solutions: A. Benzoic acid ($r/D_f \times 10^{-1}$). B. *p*-Hydroxybenzoic acid ($r/D_f \times 10^{-1}$). C. Methyl *p*-hydroxybenzoate ($r/D_f \times 10^{-1}$). D. Propyl *p*-hydroxybenzoate ($r/D_f \times 10^{-3}$). E. Chloroxylenol ($r/D_f \times 10^{-3}$). Cetomacrogol concentrations as in Fig. 1.

interaction between the binding sites and solute leading to a change in both the number of sites available and the association constant. Hence to describe the interaction it is necessary to plot the curve over a wide range of $[D_f]$ and determine n and K values from the slope in the region of interest. In the case of preservatives, this is the concentration of free preservative required for antimicrobial activity e.g. a concentration equal to or greater than the minimum inhibitory concentration. Table 1 gives the minimum inhibitory concentrations for a number of preservatives and values of n and

Table 1. *Minimum inhibitory concentrations and binding parameters for the interaction of preservatives with cetomacrogol.*

Preservative	Minimum inhibitory concentration (%) (a)	n (Calculated using monomer molecular weight (e))	K (litre mol^{-1}) (Calculated using monomer molecular weight (e))	n (Calculated using micellar molecular weight)	K (litre mol^{-1}) (Calculated using micellar molecular weight)
Benzoic acid	0·1 (b)	4·6	16	371	16
Methyl *p*-hydroxybenzoate	0·15 (c)	5·2	22	445	22
Propyl *p*-hydroxybenzoate	0·06 (c)	2·2	343	176	341
Chloroxylenol	0·02 (d)	2·8	942	216	949

(a) Highest concentration quoted in each reference (b) Bandelin (1958) (c) Nowak (1963) (d) Aist Gucklhorn (1969) (e) taken as 1300 (f) from Attwood, Elworthy & Kayne (1969).

K for the interaction with cetomacrogol. Substitution of n, K, $[D_f]$ and [M] into equation (11)

$$[D_t] = \left[\frac{nK\,[D_f][M]}{1 + K[D_f]}\right] + [D_f] \quad .. \quad .. \quad .. \quad (11a)$$

$$= [D_f]\left[\frac{nK\,[M]}{1 + K\,[D_f]} + 1\right] \quad .. \quad .. \quad .. \quad (11b)$$

enables the required preservative concentration to be calculated.

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The drug release characteristics of various rectal suppositories as determined by specific ion electrodes

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Suppositories containing sodium phenobarbitone (200 mg) were manufactured with the following bases; cocoa butter, cocoa butter plus beeswax, Dehydag and polyethylene glycols. The release of sodium ions through a dialysis membrane was conveniently measured with a sodium-specific ion electrode. The method facilitates evaluation and comparison of suppository bases and allows continuous monitoring of the release of active ingredient.

The release of a drug from a suppository is critically dependent on the physical characteristics of the base (Lesser, 1943). It follows that base and active ingredient must be considered together. Physical factors that affect drug release from a suppository include (i) particle size of the suspended drug, (ii) the effect of surface-active agents on the mucous fluids secreted over the absorbing surface, and (iii) the binding of the drug to components of the base. Diffusion of the drug to the surface for absorption is one of the rate limiting steps (Reigelman & Crowell, 1958).

The official quality control procedures for suppository bases may be considered inadequate. The B.P. (1968) stipulates only the usual chemical assay and a melting point determination for the base. The U.S.P. XVII states that the preparation (suppository) should melt, soften or dissolve at body temperature. Other tests include deformation of the suppositories at various temperatures below the melting point of the base (Tuma, 1963); a test for the disintegration rate of suppositories (Baker & Ranson, 1934) and an evaluation of the rate of drug release by microbiological cup methods (Buchi, 1944). Determination of the amount of a drug passing out from an immersed suppository at different times into an aqueous medium has also been suggested (Gross & Becker, 1953; Peterson & Guida, 1953).

Henning (1959) and Setnikar & Fantelli (1962) have endeavoured to reproduce the mechanical and physico-chemical conditions present in the human rectum. Setnikar & Fantelli used a glass cylinder in which the water at 37° circulates around a suspended length of moistened inflated cellulose dialysis tubing containing the suppository. Liquifaction time was determined. We have modified this apparatus to permit measurements to be made with specific ion electrodes.

MATERIALS AND METHODS

Apparatus

The modified Setnikar-Fantelli apparatus used is shown in Fig. 1. This, the primary part, is a double walled glass vessel with open ends, in the interior of which is supported a length of dialysis tubing (Union Carbide 36/36 membrane) previously tied with thread 5 cm from the lower end. The ends of the dialysis tubing are folded back over the open ends of the apparatus and securely tied. A small immersible

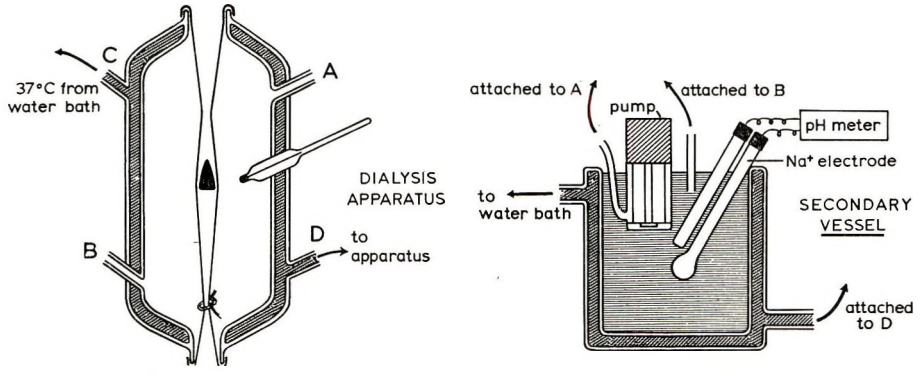

FIG. 1. Diagram of modified apparatus of Setnikar & Fantelli. See text for full description.

electric pump placed in the secondary vessel (Fig. 1) circulates the eluting fluid outside the dialysis membrane. Water at 37° is circulated through the walls of the dialysis apparatus and the secondary vessel which are interconnected (Fig. 1).

The sodium electrode (Arthur H. Thomas model 4923-L10) measuring the release was placed in the secondary vessel and a Radiometer pH meter with expanded scale was used to measure the potential difference between this and the reference electrode (a double junction model 90-01-Orion).

To provide calibration curves, solutions containing 5, 10, 50, 100, 200, 600, 1000 and 1400 mg of sodium phenobarbitone per litre were made in a tris buffer solution of composition: 0·2M tris (250 ml), 0·1N HCl (450 ml) and distilled water (to 1000 ml) adjusted to pH 6·8. All measurements were made at 37° $\pm$ 0·5°.

Suppositories

Using 1 g moulds sodium phenobarbitone (200 mg) was incorporated into the following bases: cocoa butter, cocoa butter plus 2, 3 and 5% beeswax, polyethylene glycol (PEG) type I (33% of PEG 1500, 47% PEG 6000, 20% water), PEG type II (33% PEG 4000, 47% PEG 6000, 20% water), PEG type III (30% PEG 1500, 40% PEG 4000, 30% PEG 400), PEG type IV (90% PEG 1000, 4% PEG 4000), PEG type V (70% PEG 1500, 30% PEG 6000), PEG type VI (30% PEG 1500, 50% PEG 6000, 20% water), Dehydag suppository base I and II (long chain fatty alcohols plus solid fats produced by Henkel International Germany), Suppositories for baseline measurements were made as above but without the sodium phenobarbitone.

The sodium phenobarbitone sample used was found to be 99% pure by non-aqueous titration (Beckett & Stenlake, 1964).

Method

The suppositories and suppository bases were prepared and stored for two days to allow the base to revert to the more stable crystalline state. The calibration curve was then plotted of potentiometric response in mV against the logarithm ($\log_{10}$) sodium ion concentration for sodium phenobarbitone solutions made up using tris buffer. This was done before each series of measurements to account for changing electrode characteristics such as change of junction potential. The wet dialysis tubing was secured firmly at the lower end of the apparatus and the upper end was left open until the inner chamber was filled with tris buffer solution (representing total body fluids). One litre (accurately measured) of tris buffer was used for each run;

its flow was regulated by varying the pressure of a clamp set between the electrical pump in the secondary vessel and the dialysis apparatus. By reducing the flow the dialysis tubing was made to open half way down its length. After the suppository had been dropped into the tubing, the clamp pressure was eased to refill the chamber, after which mV readings were taken every 2 min. At 5 min intervals the melted base was dispersed along the dialysis tubing by varying the clamp pressure. The melting time of each suppository base was also recorded.

RESULTS

The melting times for the various suppositories are recorded in Table 1.

Table 1. *Melting point* (°C) *and time for complete melting* (*at* 37°) *of suppository base containing* 200 *mg sodium phenobarbitone.*

Base	Melting point (° C)	Complete melting time (min) at 37° C ± 0·5°
Cocoa butter	32–34	4
Cocoa butter + 2% beeswax	35–37	35
Cocoa butter + 3% beeswax	39–44	50
Cocoa butter + 5% beeswax	42–47	80
Polyethylene glycol type 1 base	39–40	20
Polyethylene glycol type II	50–51	24
Polyethylene glycol type III	33–38	18
Polyethylene glycol type IV	33–38	20
Polyethylene glycol type V	38–43	20
Polyethylene glycol type VI	48–52	22
Dehydag base I	33–36	6–8
Dehydag base II	37·5–39·5	Does not melt at experimental temperatures

A calibration curve of log concentration of Na^+ (as mg sodium phenobarbitone per litre) versus potential gives in the linear region an experimental slope of 60 mV for each decade change of sodium ion concentration, a value approximating closely the theoretical Nernst slope (2·303 RT/F) of 61·5 mV at 37°. The concentration range recommended by the manufacturer is 1 to 10^{-5} mol of Na^+ per litre, but we found it more convenient to use standard solutions of 5 to 1400 mg/litre. Six runs of each suppository formulation were considered adequate.

Graphs of Na^+ (as mg of sodium phenobarbitone/litre) versus time were plotted and the time required for the suppository to release half of its contents to the medium was designated the T50% value, average values of which are in Table 2.

Fig. 2 shows the release of drug as assessed by Na^+ release from the cocoa butter suppositories and the Dehydag base I and base II suppositories. These are considered melting fat type bases. Fig. 3 shows the drug release characteristics of the dissolving type of suppository—the polyethylene glycol base.

No suppository base similarly treated released sodium ions.

DISCUSSION

The relation between the ion activity and electrode potential is logarithmic. Thus, the rate of release of sodium phenobarbitone from suppository bases is measured by reference to a calibration curve which is plotted before each set of measurements. This calibration curve is based on the potential produced by the electrodes as a function of sodium ion activity or $\log_{10}$ sodium ion concentration. This is expressed

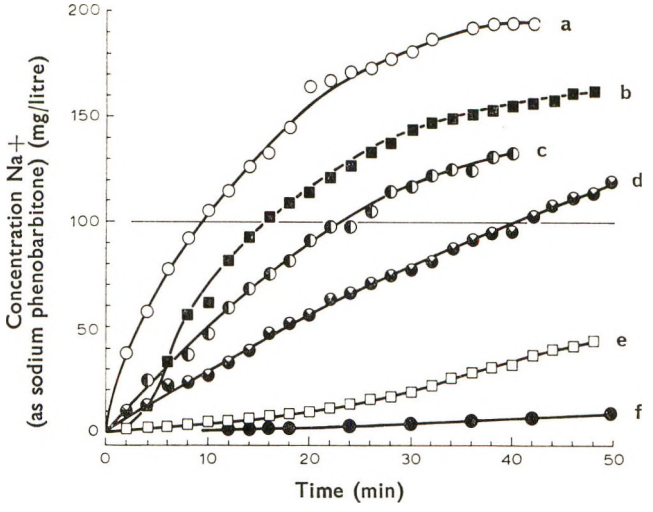

FIG. 2. The release of phenobarbitone sodium as assessed by Na^+ release from the cocoa butter and Dehydag based suppositories. a, cocoa butter Av.T50: 9·2; b, Dehydag base I Av. T50: 15·6; c, cocoa butter + 2% beeswax Av.T50 : 23; d, cocoa butter + 3% beeswax Av.T50 : 38·8; e, Dehydag base II Av. T50 : 92 min; f, cocoa butter + 5% beeswax: no T50 value.

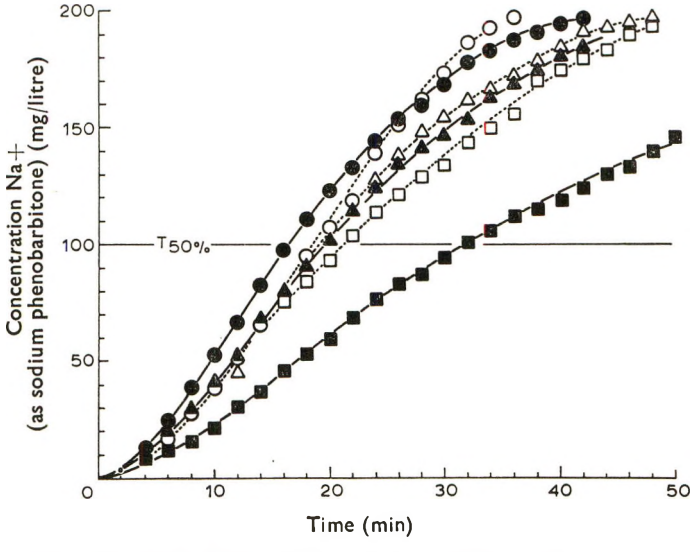

FIG. 3. The release of phenobarbitone sodium as assessed by Na^+ release from the PEG bases. ▲ PEG I T50 : 19; ○ PEG II T50 : 18·5; △ PEG III T 50 : 19; ● PEG IV T50: 16·4; ■ PEG V T50: 31·6; □ PEG VI T50 : 212.

by the Nernst equation: $E_{obs} = E_s + 2{\cdot}303\ (RT/nF) \log (A_{Na}+)$ where $2{\cdot}303R/F = 0{\cdot}1984$ (Nernst factor). At 37°, T × 0·1984 = 61·5 mV, which is the theoretical slope of E_{obs} *vs* log (A_{Na+}). For each decade change in Na^+ activity over the range of 1 to 10^{-5}M concentrations, the potential changes by approximately 61·5 mV.

In addition to established routine quality control procedures for suppositories it is essential for effective quality control to estimate the rate and extent of release of medicament. The drug release rate of 37° is dependent on melting characteristics of the fatty suppositories so that melting point determination does give some indica-

Table 2 *T50% values (time required for suppository to release half of its contents to the medium).*

Base	T50% values (min)	Standard deviation
Cocoa butter	9·2	0·08
Cocoa butter with 2% beeswax	23	2·20
Cocoa butter with 3% beeswax	38·8	3·04
Cocoa butter with 5% beeswax	T50% value not reached at 37°	—
Dehydag base I	15·6	2·06
Dehydag base II	92	7·03
Polyethylene glycol type I	19·0	0·11
Polyethylene glycol type II	18·5	0·23
Polyethylene glycol type III	19·0	0·27
Polyethylene glycol type IV	16·4	0·77
Polyethylene glycol type V	31·6	5·76
Polyethylene glycol type VI	21·0	2·56

tion of release in this class of suppository. With water-soluble bases, quantitative measurement of release is the most desirable parameter of availiability of drug.

No easy method, as far as we are aware, is available for measuring quantitatively *in vitro* release rates of medicaments from suppository bases, nor a method to measure a continuous change with time.

The specific ion-dialysis membrane method described furnishes a means of measuring a continuous change in the amount of drug released from a suppository in conditions which approximate those *in vivo*, namely: (i) an average temperature of 36–37°, (ii) little or no peristaltic movement, (iii) minimal quantity of unbound water present in the liquid state, and (iv) a pressure of 0–50 cm of water. Furthermore this method can be easily modified to measure release under "sink" conditions.

The results obtained substantiate previous claims that the melting characteristics of fat-base suppositories influence drug release rate at 37° irrespective of other factors. This is adequately demonstrated by the T50% values, which increase as the melting point of base increases. Readings with all the water-soluble polyethylene glycol bases were recorded after 2 min but it appears—as is substantiated in the literature (Hassler & Sperandio, 1953)—that the duration of action is longer than with the fat-wax melting suppositories (Table 2). T50% values are about 20 min.

The results obtained and the ease and simplicity of operation of the apparatus indicate that specific ion electrodes can be used to evaluate drug release from dosage forms as long as the active ingredient is represented by free ion.

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The effect of drugs upon the uptake of 5-hydroxytryptamine and metaraminol by human platelets

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The abilities of some tricyclic and bicyclic antidepressive drugs and an α-receptor blocking agent, phenoxybenzamine, to inhibit the uptake of 5-hydroxytryptamine (5-HT) and (−)-metaraminol into human platelets have been compared *in vitro*. All the drugs inhibited the uptake both of 5-HT and of metaraminol into platelets. But there were differences in their abilities to inhibit the uptake of these two monoamines. The desmethylated antidepressive drugs were more potent inhibitors of metaraminol uptake than were their tertiary analogues, whereas imipramine, a tertiary amine, was by far the best inhibitor of 5-HT uptake. The order of the activities of the antidepressive drugs in inhibiting the uptake of 5-HT and metaraminol into platelets paralleled their potencies in blocking the uptake of 5-HT, and noradrenaline or metaraminol into nerve endings. It is suggested that the uptake of 5-HT and metaraminol into platelets is a useful model for the neuronal uptake of 5-HT and noradrenaline, respectively.

Uptake of monoamines into blood platelets can be inhibited by various drugs among which the most potent are the tricyclic antidepressants (Paasonen, 1965; 1968). Of these the tertiary amines are the more potent inhibitors of 5-hydroxytryptamine (5-HT) uptake (Yates, Todrick & Tait, 1964; Ahtee, Tuomisto & others, 1968; Todrick & Tait, 1969). The secondary amines are more potent as inhibitors of the uptake of noradrenaline into central and peripheral neurons (Callingham, 1966; Iversen, 1967; Carlsson, Corrodi & others, 1969b). Carlsson, Corrodi & others (1969a) recently demonstrated the tertiary amines to be more potent in blocking the uptake of 5-HT into central neurons than corresponding secondary amines. In the present work the effects of several antidepressive drugs on the uptake of 5-HT and (−)-metaraminol into human blood platelets has been examined. Metaraminol was used as a model for sympathomimetic amines since it has a high affinity for the storage sites of noradrenaline (Andén, 1964; Shore, Busfield & Alpers, 1964; Carlsson & Waldeck 1965).

MATERIALS AND METHODS

Buffy coats from 400 ml human citrated blood were diluted with one volume of modified calcium-free Tyrode solution (g/litre: disodium edetate 0·8, NaCl 7·6, KCl 0·42, $NaH_2PO_4x2\ H_2O$ 0·14, $NaHCO_3$ 2·1, glucose 2·0 and sucrose 4·5) and platelets were separated by centrifuging at about 130 *g* for 20 min at 20°. The final dilution of the platelet suspension contained $8{\cdot}86 \pm 0{\cdot}41 \times 10^8$ platelets/ml (means ± s.e. from 53 experiments) and about 1/10 of the medium was original plasma.

If not otherwise stated, duplicate samples (2 ml) of the platelet suspension were incubated for 15 min with gentle shaking at 37° with or without 10^{-5}M of 5-hydroxytryptamine creatinine sulphate (Fluka AG) or 3×10^{-6}M of (−)-metaraminol bitartrate (Merck Sharp & Dohme), in oxygen containing 4% carbon dioxide. At these extracellular concentrations of the monoamines, the platelets took up sufficient of the monoamines for them to be accurately estimated spectrophotofluorometrically in the samples taken.

The hydrochlorides of imipramine and desipramine (Geigy A.G.), protriptyline (Merck Sharp & Dohme), and 1-(3-methylaminopropyl)-1-phenyl-3,3-dimethylphthalane (Lu 3-010), and its corresponding tertiary amine (Lu 3-009) (H. Lundbeck & Co. A/S), and of phenoxybenzamine (Smith Kline & French Labs.) were added to the suspension 10 min before 5-HT or metaraminol. All drugs were dissolved in saline and added in a volume of 0·2 ml. Only polypropylene vessels and pipettes were used to handle platelets.

After incubation, the platelets were cooled immediately to below 5° and centrifuged for 20 min at 2500 *g*. The supernatant was decanted and traces remaining in the incubation tubes were removed with a filter paper. The platelet pellet was then lysed in 1·5 ml of distilled water in a vortex mixer.

The proteins were precipitated with 10% $ZnSO_4$ (0·2 ml) and N NaOH (0·1 ml). The mixture was shaken well and centrifuged for 5 min at 700 *g*. After the addition of 12N HCl (0·3 ml), the 5-HT was measured from 1 ml of the supernatant spectrophotofluorometrically. Metaraminol was measured by the *o*-phthaldialdehyde procedure of Shore & Alpers (1964). None of the drugs studied interfered with the fluorescence of 5-HT or metaraminol.

The means and standard errors (s.e.) were calculated and the statistical significance of the differences was determined by Student's *t*-test. The concentrations of the drugs which caused 50% inhibition of monoamine uptake (IC50) were determined by using straight lines obtained by the method of least squares.

RESULTS

Uptake of metaraminol into platelets

Table 1 shows the uptake of metaraminol into, and release of 5-HT from, human blood platelets as a function of extracellular metaraminol concentration. The

Table 1. *Uptake of (−)-metaraminol (MA) by and release of 5-HT from human blood platelets incubated with various concentrations of MA.* Duplicate 2-ml samples of platelet suspensions were incubated for 1 h at 37°. Means ± s.e. from 4 experiments.

Added MA (M)	MA taken up nmol/10^9 platelets	Concentration gradient*	5-HT release nmol/10^9 platelets**
10^{-6}	0·4 ± 0·04	40	0
3×10^{-6}	1·2 ± 0·2	39	0
10^{-5}	2·8 ± 0·4	28	0
3×10^{-5}	4·5 ± 0·7	15	0·24 ± 0·07
10^{-4}	8·8 ± 1·5	9	0·63 ± 0·17

* MA in 10^{11} platelets (ml packed platelets)/MA in ml of incubation medium.
** Original 5-HT content 3·94 ± 0·72 nmol/10^9 platelets.

platelets took up metaraminol against a concentration gradient which was inversely proportional to its concentration in the external medium. No release of platelet 5-HT was observed below 3×10^{-5}M metaraminol which released 6% of platelet 5-HT in 1 h.

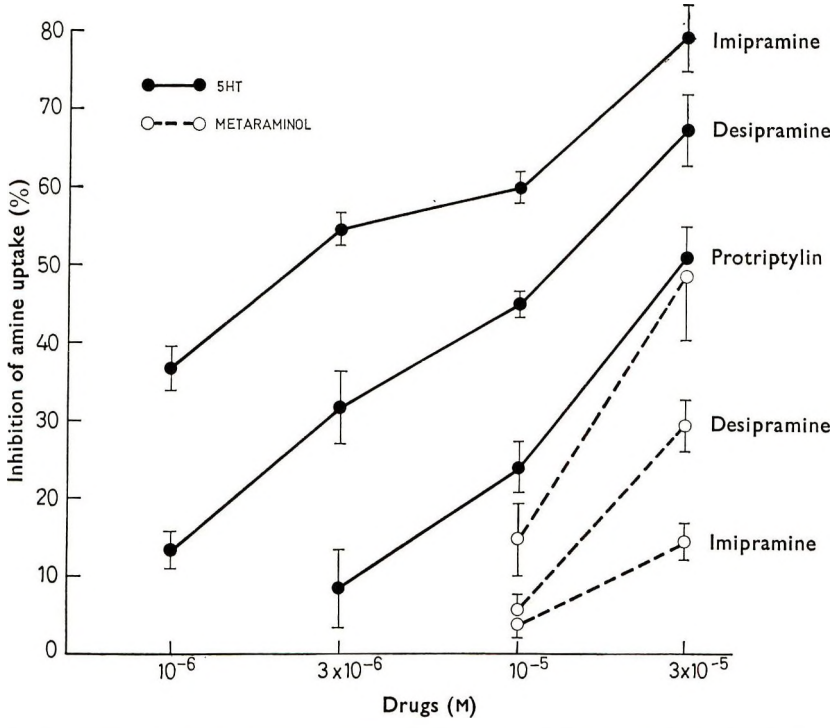

FIG. 1. Inhibition of 5-hydroxytryptamine (5-HT) and (−)-metaraminol (MA) uptake into human blood platelets by tricyclic antidepressants. Means ± s.e. from 5 to 13 experiments.

Inhibition of monoamine uptake into platelets by drugs

Fig. 1 shows the effects of imipramine, desipramine and protriptyline on the uptake of 5-HT and metaraminol into platelets. At 10^{-6}M, both imipramine and desipramine significantly inhibited 5-HT uptake ($P < 0.001$) and the inhibition increased with increasing concentrations of the compounds. Imipramine, a tertiary amine, was a more potent inhibitor than the corresponding secondary amine, desipramine ($P < 0.001$ at 10^{-6}, 3×10^{-6} and 10^{-5}M). Protriptyline inhibited 5-HT uptake even less than desipramine. The order of potency of these drugs in blocking the metaraminol uptake was protriptyline > desipramine > imipramine which is opposite to that of their inhibition of 5-HT uptake. Phenoxybenzamine inhibited the uptake of metaraminol slightly more than it did that of 5-HT (Table 2).

Table 3 shows the concentrations of different drugs that caused 50% inhibition (IC50) of 5-HT and metaraminol uptake. It also gives the ratios of IC50 of metaraminol uptake to that of 5-HT uptake. Imipramine was by far the best inhibitor of 5-HT uptake but was a relatively weak inhibitor of metaraminol uptake. To inhibit the uptake of metaraminol to the same extent as that of 5-HT, a concentration of imipramine over 300 times higher was needed. Also desipramine more effectively inhibited the uptake into platelets of 5-HT than of metaraminol.

Table 2. *Inhibition of 5-HT and MA uptake into human blood platelets by phenoxybenzamine.* Means ± s.e., number of experiments in brackets.

Phenoxybenzamine (M)	Inhibition of amine uptake (%) 5-HT uptake	MA uptake
10^{-6}	8·8 ± 2·8 (7)	0·4 ± 6·7 (3)
3×10^{-6}	16·5 ± 4·8 (7)	27·3 ± 8·5 (5)
10^{-5}	50·3 ± 4·8 (7)	58·9 ± 3·8 (7)
3×10^{-5}	62·6 ± 3·6 (7)	74·1 ± 5·8 (5)

Table 3. *Inhibition of 5-HT and MA uptake into human blood platelets by drugs.* Concentrations causing 50% inhibition (IC50) of monoamine uptake were determined from means of three to ten experiments using at least four different concentrations of drugs. Last column gives the ratios of IC50 for MA uptake to that of 5-HT uptake.

Drug	IC50 (M) 5-HT uptake	MA uptake	IC50 MA / IC50 5-HT
Imipramine	$2{\cdot}86 \times 10^{-6}$	$9{\cdot}63 \times 10^{-4}$	337
Desipramine	$1{\cdot}09 \times 10^{-5}$	$7{\cdot}80 \times 10^{-5}$	7·2
Protriptyline	$4{\cdot}64 \times 10^{-5}$	$3{\cdot}20 \times 10^{-5}$	0·7
Lu 3-009	$5{\cdot}77 \times 10^{-6}$	$4{\cdot}08 \times 10^{-5}$	0·7
Lu 3-010	$2{\cdot}12 \times 10^{-5}$	$2{\cdot}39 \times 10^{-5}$	1·2
Phenoxybenzamine	$1{\cdot}35 \times 10^{-5}$	$8{\cdot}16 \times 10^{-6}$	0·6

The secondary amine protriptyline was about thirty times more potent than imipramine and twice as potent as desipramine in inhibiting metaraminol uptake. It inhibited metaraminol uptake in concentrations approximately similar to those needed to inhibit the 5-HT uptake.

The bicyclic compound with a secondary amine group, Lu 3-010, was even more potent inhibitor of metaraminol uptake than was protriptyline. It inhibited both metaraminol and 5-HT uptake more than its tertiary amine analogue Lu 3-009. The most potent inhibitor of metaraminol uptake in these experiments was phenoxybenzamine.

DISCUSSION

Our results show that human blood platelets are able to accumulate (−)-metaraminol against a concentration gradient with a saturable process. The concentration gradient is higher than that which has been shown for (±)-noradrenaline (Abrams & Solomon, 1969) or for (±)-adrenaline (Born & Smith, 1970). As the release of 5-HT did not occur until after the platelets had taken up about as many mol of metaraminol as they contain 5-HT, there seems to be, in human platelets, a space where metaraminol is accumulated before it starts replacing 5-HT. Rabbit platelets contain about 15–20 times as much 5-HT as human platelets and from these metaraminol releases 5-HT stoichiometrically (Ahtee & Saarnivaara, unpublished results). This discrepancy could be explained by the fact that human platelets accumulate *in vitro* many times their original content of 5-HT whereas rabbit platelets are more saturated. Both in human and rabbit platelets most of the accumulated metaraminol can be released by thrombin and therefore it is not localized in the cytoplasm but in a

bound form most probably in the storage granules (Ahtee & Mills, unpublished results).

All the drugs studied inhibited both the uptake of 5-HT and metaraminol into platelets. There were, however, differences in their abilities to inhibit the uptake of these two monoamines. The most striking was the 300-fold difference in concentrations of imipramine needed to inhibit the uptake of 5-HT and metaraminol. These results agree with those of Carlsson, Corrodi & others (1969 a,b) and Carlsson, Fuxe & others (1969) who used a displacement technique to show that the abilities of several tricyclic and bicyclic compounds to block the uptake of monoamines into central and peripheral noradrenaline neurons were dissociated from their abilities to block uptake into central 5-HT neurons.

As has been previously shown (Yates & others, 1964; Ahtee & others, 1968; Todrick & Tait, 1969) the tricyclic antidepressant with a tertiary amine structure, imipramine, was a more potent inhibitor of 5-HT uptake than its *N*-desmethyl derivative, desipramine or the secondary amine, protriptyline. Of the two bicyclic compounds studied, however, the one with a secondary amine structure, Lu 3-010, inhibited 5-HT uptake in lower doses than the corresponding tertiary amine, Lu 3-009. The potency of the bicyclic compounds in inhibiting 5-HT uptake was about the same as that of desipramine and protriptyline. The uptake of metaraminol into platelets was more strongly inhibited by the secondary amines, Lu 3-010, protriptyline and desipramine, than their corresponding tertiary derivatives, Lu 3-009 and imipramine. A similar superiority of the desmethyl series of antidepressive drugs in inhibiting the uptake of noradrenaline or metaraminol into peripheral ($uptake_1$) or central neurons has been demonstrated previously (Callingham, 1966; Waldeck, 1968; Carlsson, Corrodi & others, 1969b; Carlsson & others, 1969).

In relatively high concentrations *in vitro* the tricyclic antidepressants and related drugs liberate 5-HT and other cellular components from the platelets causing membrane damage. They also haemolyse red cells. The antidepressive drugs containing a secondary amine are more potent 5-HT releasers than are the tertiary amines, although the tertiary amines cause haemolysis in lower concentrations than the secondary amines (Paasonen, 1964; Ahtee, 1966; Solatunturi, 1968; Ahtee & Paasonen, 1968a, b). The effects of these drugs on the membrane of two different cells thus differ and moreover the 5-HT uptake-inhibiting and 5-HT-releasing effects in the platelets are not parallel. Furthermore, we have now demonstrated that these drugs inhibit the uptake of the two monoamines, 5-HT and metaraminol in different ways. The monoamines are most probably transported through the cell membrane by means of carriers or through uptake sites. Several drugs, e.g. the tricyclic antidepressants, affect these transport mechanisms. Our results indicate that these drugs inhibit the uptake of 5-HT and metaraminol into the platelets in the same way as they inhibit the uptake of 5-HT and noradrenaline into neurons. It is likely that the same carriers or uptake sites (or both) are responsible for the pumping of both monoamines into platelets with the various drugs obstructing the pump in different ways, one drug leaving more room for one monoamine to pass through than for another.

Phenoxybenzamine, an α-blocking drug, was the most potent inhibitor of metaraminol uptake in our experiments. It inhibited the uptake of metaraminol slightly more than it did that of 5-HT. Similar results are reported by Bygdeman & Johnsen (1969) who found that higher concentrations of α- and β-blockers were needed to

inhibit the uptake of 5-HT than that of noradrenaline into platelets. Phenoxybenzamine is a potent inhibitor of the uptake of monoamines into granules (Euler & Lishajko, 1968). This effect might be partially responsible for its inhibitory activity in our experiments which did not distinguish between the membrane and granular part of the monoamine uptake.

Recently, Iversen & Langer (1969) showed that phenoxybenzamine usually prevents the uptake of noradrenaline competitively. However, in circumstances that resembled ours, phenoxybenzamine behaved as a non-competitive inhibitor of noradrenaline uptake into vas deferens. Iversen & Langer showed that in addition to preventing the uptake of noradrenaline into sympathetic nerve endings ($uptake_1$), phenoxybenzamine inhibited the uptake of noradrenaline into the extraneuronal cells ($uptake_2$). In the perfused rat heart these two membrane mechanisms ($uptake_1$ and $uptake_2$) are inhibited in different ways by various drugs, metaraminol possessing the highest affinity for $uptake_1$ (Iversen, 1967). Lahovaara, Neuvonen & Paasonen (1970) could demonstrate neither the inhibitory specifity of $uptake_1$ nor that of $uptake_2$ in the uptake of noradrenaline into human platelets. It is plausible, however, that the uptake of monoamines into platelets, which occurs both through the membrane and into the granules, and operates against a concentration gradient of hundreds (5-HT) or tens (metaraminol), resembles more the true neuronal uptake than the extraneuronal accumulation of monoamines.

We suggest that the effects of drugs on the uptake of different monoamines into platelets could be used as a model to study drug effects on the uptake of different monoamines into neurons. The platelet is suitable serving as a model for 5-HT uptake but more knowledge can be gained from experiments studying also the uptake of other monoamines into platelets. We have recently found a dissociation in the effects of several analgesic drugs on 5-HT and metaraminol uptake into platelets (Ahtee & Saarnivaara, 1970).

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Effects of papaverine and eupaverin on calcium uptake by isolated sarcoplasmic vesicles

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Papaverine and eupaverin increase the rate of uptake of calcium by sarcoplasmic vesicles isolated from rabbit white skeletal muscle. The degree of activity of the above drugs is clearly affected by changes of ATP, oxalate and Ca^{2+} concentrations. The results are discussed in view of present knowledge about the effects of papaverine-like drugs upon muscular contraction.

The effects of various spasmolytic agents have been reliably referred to an interference with the essential role of calcium ions in muscular function (Daniel, 1964; Ferrari, 1964; Ferrari & Carpenedo, 1965; Tóth, Ferrari & others, 1966; Ferrari, 1970). These views presumably apply not only to the activity of spasmolytics on smooth muscle, but also to the effects of these drugs on skeletal muscle, where papaverine and eupaverin exert contracture or inhibition, depending on the concentrations (Buttar, 1969; Carpenedo, unpublished observation).

However, present knowledge about the influence of papaverine-like drugs on calcium ion movements is unsatisfactory. Only indirect conclusions have been made, suggesting an interference by spasmolytic agents with Ca^{2+} influx or binding at some cellular components, or both (Imai & Takeda, 1967; Ferrari & Carpenedo, 1968; Tashiro & Tomita, 1970).

We have now examined the effects of papaverine and eupaverin on Ca^{2+} uptake by sarcoplasmic reticulum, a model which has been largely employed to study the effects of drugs on Ca^{2+} movements at membrane level (Martonosi & Feretos, 1964; Inesi, Goodman & Watanabe, 1967; Balzer, Makinose & Hasselbach, 1968).

METHODS

All experiments were made with a 10 000–50 000 *g* centrifugal fraction of rabbit white skeletal muscle precipitated in 10% sucrose-2 mM tris pH 7·3 and purified by extraction in 0·6M KCl–5mM histidine, pH 7·3. The final sediment was resuspended in 40% sucrose-2 mM tris pH 7·3 to maintain a good uptake after several days (Repke & Katz, 1969). Preparations not older than 10 days were used.

Incubation was at 26° with a standard mixture for the measurement of Ca^{2+} uptake containing: 50 mM tris–HCl pH 7·3, 1 mM $MgCl_2$, 50 μM $CaCl_2$, carrier free $^{45}CaCl_2$ 30 μM ethyleneglycolbis (2-aminoethyl)tetra-acetate (EGTA) pH 7·3; 0·5 mM ATP disodium salt and 4 mM potassium oxalate. The protein concentration (Lowry & others, 1951) was 0·1 mg/ml. The amount of calcium 45 taken up was measured by a Millipore filtration technique (Martonosi & Feretos, 1964).

RESULTS

The kinetics of calcium accumulation appear to show both papaverine and eupaverin increase the rate of Ca^{2+} uptake in the presence of oxalate (Fig. 1). Eupaverin appears to be about 10 times more active than papaverine (Fig. 2).

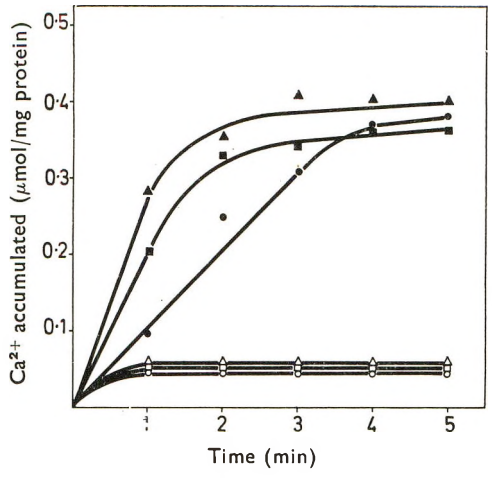

FIG. 1. Effect of papaverine and eupaverin on the velocity of Ca^{2+} uptake by sarcotubular vesicles in the absence and in the presence of 4 mM potassium oxalate. Experimental conditions are reported in the text. The reaction was started by the addition of microsomes at 0 time. (○) No potassium oxalate, (□) no potassium oxalate, 0·5 mM papaverine; (△) no potassium oxalate, 50 μM eupaverin; (●) 4 mM potassium oxalate; (■) 4 mM potassium oxalate, 0·5 mM papaverine; (▲) 4 mM potassium oxalate, 50 μm eupaverin.

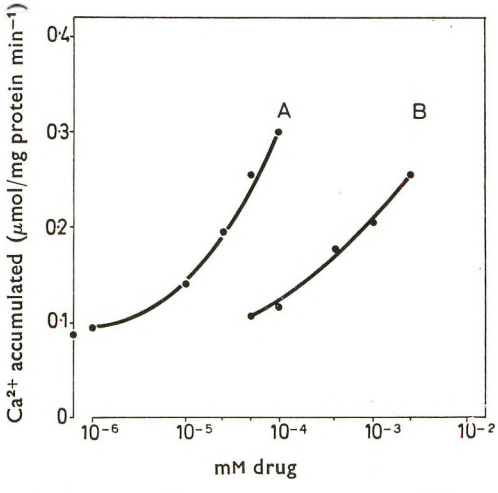

FIG. 2. Dose-response relation of Ca^{2+} uptake to papaverine HCl and eupaverin HCl in the presence of 4 mM potassium oxalate. Experimental conditions as in Fig. 1. Time of incubation 1 min. (●) eupaverin HCl; (○) papaverine HCl.

In Fig. 3 is reported the influence of various parameters affecting the rate of Ca^{2+} uptake in the absence and in the presence of 50 μM eupaverin. It appears that increasing concentrations of ATP (Fig. 3A) or oxalate (Fig. 3B) enhance the rate of Ca^{2+} uptake and that eupaverin induces a further increase of the velocity of this process. The increase of Mg^{2+} concentration reduces (Fig. 3C) the rate of Ca^{2+} uptake, in a parallel way, both in the control and in the presence of eupaverin, without significantly affecting the activity of the drug. Increasing concentrations of Ca^{2+}

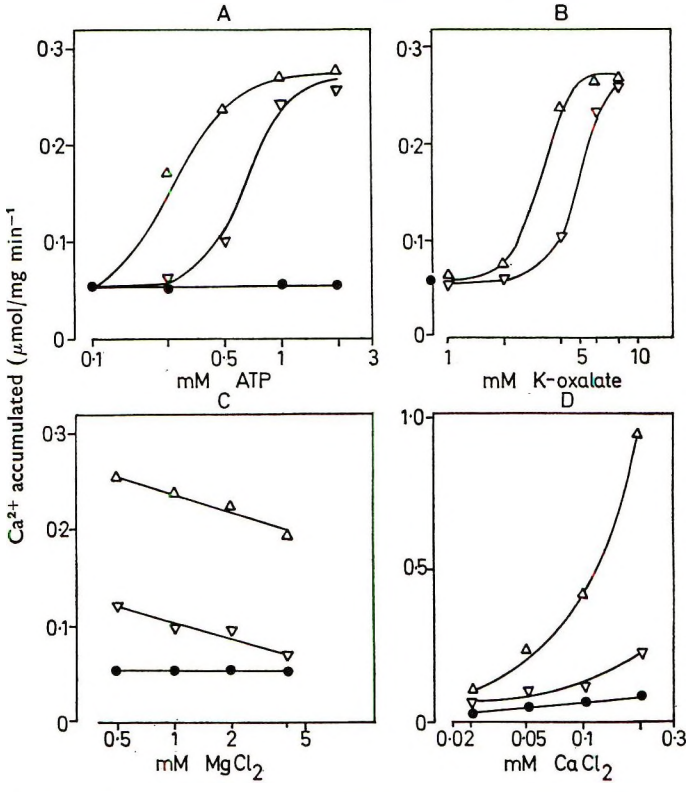

FIG. 3. Effects of varying concentrations of ATP, potassium oxalate, $MgCl_2$ and $CaCl_2$ on Ca^{2+} uptake in the absence and in the presence of 50 μM eupaverin HCl. Time of incubation: 1 min. (●) No potassium oxalate; (○) 4 mM potassium oxalate, (△) 4 mM potassium oxalate, 50 μM eupaverin.

(Fig. 3D) poorly enhance the velocity of uptake but clearly increase the activity of eupaverin.

The results obtained with papaverine 0·5 mM closely parallel those reported for eupaverin.

DISCUSSION

The results indicate that papaverine and eupaverin significantly enhance the rate of Ca^{2+} uptake by isolated sarcoplasmic vesicles and that this effect is influenced by the concentrations of ATP, oxalate and Ca^{2+}.

The degree of activity appears remarkable, especially for eupaverin, which was about ten times more effective than papaverine.

Since the sequestration of Ca^{2+} by sarcoplasmic reticulum is generally considered as the basic mechanism of relaxation in skeletal muscle (Ebashi, 1961; Hasselbach, 1964; Bianchi, 1970), the results of present investigations suggest that the increase of the rate of Ca^{2+} uptake by sarcoplasmic vesicles could play a part in the mechanism of the inhibitory effect on potassium and caffeine-induced contractures of frog skeletal muscle observed with papaverine and eupaverin (Buttar, 1969; Carpenedo: unpublished observation). No explanation can be obtained from these findings to account for the contracture elicited by high doses of these drugs.

The mechanism by which calcium is taken up, stored and released in the fibres of smooth muscle has not yet been elucidated (Hurwitz & Joiner, 1969). Also the presence of sarcotubular-like structures is still uncertain: in some types of smooth muscle it is conceivable that the surface membrane takes over the major function of the sarcoplasmic reticulum (Bianchi, 1970).

Isolated sarcoplasmic vesicles with the properties of binding calcium ions and of splitting ATP have been reported, but only for the cow uterus (Carsten, 1970). However, if, in smooth muscle, calcium sequestration and the consequent muscle relaxation develops through a process that is sensitive to papaverine and eupaverin, the enhancement of the rate of Ca^{2+} uptake by these drugs could be relevant in the mechanism of their spasmolytic activity.

No definite conclusions can be drawn about the mechanism of action of the drugs tested on calcium uptake; but cyclic AMP facilitates the uptake of Ca^{2+} by sarcoplasmic vesicles (Shinebourne & White, 1970) and papaverine and eupaverin strongly inhibit phosphodiesterase of various tissues (Kukovetz & Pöch, 1970; Markwardt & Hoffman, 1970; Triner, Vulliemoz & others, 1970).

Provided that isolated vesicles retain adenylcyclase and phosphodiesterase activities (Rabinowitz, Desalles & others, 1965; Toson & Carpenedo, unpublished) these drugs could modify calcium uptake by increasing cyclic AMP content of microsomes.

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The effect of brocresine (NSD-1055) on the histidine decarboxylase activity in the rat gastric mucosa*

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The effect of the histidine decarboxylase inhibitor brocresine (NSD-1055) on the specific histidine decarboxylase in the gastric mucosa was investigated in rats. The inhibiting potencies of brocresine were compared after oral and intraperitoneal administration with and without 2-deoxy-D-glucose. Furthermore, different doses of brocresine were added directly to an incubation medium containing an homogenate of the gastric mucosa of untreated animals. The gastric histidine decarboxylase of the brocresine-pretreated animals was not inhibited. Addition of brocresine to the incubation medium produced a dose dependent blockade of the enzyme. 50% inhibition was accomplished by a concentration of $1{\cdot}4 \times 10^{-6}$M. The results demonstrated an inhibition of the rat stomach histidine decarboxylase *in vitro*, but not *in vivo*, indicating the inability of brocresine to interfere with the biosynthesis of histamine in the rat stomach.

4-Bromo-3-hydroxybenzyloxyamine dihydrogenphosphate (brocresine, NSD-1055) has been reported to be a potent histidine decarboxylase inhibitor. Its effectiveness *in vitro* is well established (e.g. Levine & Watts, 1966; Johnston & Kahlson, 1967; Thayer & Martin, 1967). *In vivo*, however, the effect is not unequivocal (Levine, Sato & Sjoerdsma, 1965; Levine, 1966; Johnston & Kahlson, 1967; Johnson & Burfine, 1968; Johnson, 1969).

We have investigated the effect of different routes of administration of brocresine on the histidine decarboxylase of the rat stomach and the effect of different concentrations of brocresine on the histidine decarboxylase of the rat stomach was studied *in vitro*.

METHODS

The experiments were performed in a randomized order on female rats (FW 49, 180–340 g). In the first group, 10 animals were injected with brocresine (100 mg/kg, i.p.) 4 h before the experiments, 10 control animals received 10 ml/kg phosphate buffer in the same way. In the second group, 10 animals were given an oral dose of brocresine (100 mg/kg) by gastric tube 4 h before the experiments. The control animals received 10 ml/kg phosphate buffer in the same way. Animals in the third group were treated similarly to those in group 1 with the addition of an injection of 2-deoxy-D-glucose (100 mg/kg, i.p.). Animals in group 1–3 were re-fed after pretreatment. In separate experiments, brocresine, to give final concentrations of $1{\cdot}3 \times 10^{-8}$, 4×10^{-7} and 4×10^{-6}M was added to an incubation medium ($N = 10$ for each concentration).

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Preparation of mucosa homogenates and the incubation medium. The abdominal cavity was opened under ether anaesthesia and the stomach dissected. The glandular stomach was separated from the rumen and opened along the lesser curvature. After rinsing with demineralized water the gastric mucosa (about 0·63 g) was scraped off, suspended in 20 ml phosphate buffer and homogenized in a glass homogenizer.

The incubation medium [(M) phosphate buffer (pH 7·0) 1×10^{-1}, L-histidine 5×10^{-4}, aminoguanidine sulphate $1{\cdot}3 \times 10^{-4}$, nicotinamide 1×10^{-2}; pyridoxal-5-phosphate 10 μg/ml, glucose 10 mg/ml] was preincubated for 30 min and after addition of the substrate incubated in a Warburg apparatus for 60 min at 37° under nitrogen (the main vessel contained homogenate 1·0, pyridoxal-5-phosphate 0·1, aminoguanidine sulphate 0·5, nicotinamide 0·5, glucose 0·4 ml. The side-arm contained either L-histidine or phosphate buffer. The final volume was 3 ml).

Histamine extraction and estimation. At the end of the incubation period the reaction was stopped by addition of 9 volumes 0·4N perchloric acid. Histamine was estimated fluorometrically (Shore, Burkhalter & Cohn, 1959).

Compounds. 4-Bromo-3-hydroxybenzyloxyamine dihydrogenphosphate (brocresine, NSD-1055), American Cyanamid Company, Pearl River, N.Y. and Smith & Nephew Research Ltd., Gilston; pyridoxal-5-phosphate and 2-deoxy-D-glucose, EGA-Chemie Steinheim; aminoguanidine sulphate, nicotinamide and *o*-phthaldialdehyde, Fluka, Buchs; L-histidine, Schuchardt, Munich.

The histidine decarboxylase is expressed in nmol of histamine formed per g mucosa per hour. For statistical analysis all results were treated with the *t*-test for pairs.

RESULTS

The histidine decarboxylase activity of the rat gastric mucosa of treated and untreated animals is summarized in Table 1. It is evident that brocresine has no effect on the histidine decarboxylase activity of the rat gastric mucosa when it was given by different routes of administration. There was also no brocresine effect when 2-deoxy-D-glucose was given.

Table 1. *The effect of brocresine on the rat gastric mucosal histidine decarboxylase activity.* A = amount of histamine present in the tissue or formed by decarboxylation of endogenous histidine (or both) expressed in nmol histamine per g mucosa. B = the amount of histamine present in the tissue or formed by decarboxylation of endogenous and exogenous histidine (or both) expressed in nmol histamine per g mucosa. B–A = histidine decarboxylase activity expressed in nmol histamine formed per 1 h by 1g tissue. Values mean ± s.e.

Pretreatment	n	A	B	B–A	*P*
Control	10	922·4 ± 89·1	1149·1 ± 77·0	226·5 ± 29·4	>0·5
Brocresine i.p.	10	939·0 ± 89·2	1161·0 ± 93·2	221·8 ± 31·8	
Control	10	824·9 ± 71·8	1011·8 ± 79·7	186·9 ± 52·8	>0·5
Brocresine oral	10	747·3 ± 79·2	963·5 ± 103·3	216·3 ± 40·7	
Control + 2-Deoxy-D-glucose	10	938·3 ± 82·0	1075·7 ± 91·3	157·5 ± 33·6	>0·5
Brocresine i.p. + 2-Deoxy-D-glucose ..	10	793·1 ± 60·9	928·4 ± 74·4	135·6 ± 27·7	

When brocresine was added to the incubation medium with mucosa homogenate it exhibited a dose-dependent inhibition of the histidine decarboxylase activity in the rat stomach—at brocresine concentrations of $1{\cdot}3 \times 10^{-8}$, 4×10^{-7}, 4×10^{-6}M the % enzyme activity was 90, 62 and 38% respectively.

DISCUSSION

The results demonstrate that brocresine was unable to interfere with the biosynthesis in the rat stomach *in vivo* and that it was a potent inhibitor of the gastric histidine decarboxylase when added directly to the incubation medium.

Although there is general agreement that the rat stomach histidine decarboxylase can be inhibited by brocresine *in vitro* (Levine & Watts, 1966; Johnston & Kahlson, 1967; Thayer & Martin, 1967), the concentration producing a 50% inhibition differed considerably. The one we reported ($1{\cdot}4 \times 10^{-6}$M) is the highest. This could be due to a large excess of pyridoxal-5-phosphate, since brocresine is known to compete with pyridoxal-5-phosphate as well as with histidine (Ellenbogen, Markley & Taylor, 1969).

The lack of inhibition *in vivo* is supported by Johnson & Burfine (1968) who demonstrated in female rats that brocresine had neither an effect on the histamine level of the gastric mucosa nor on the disappearance rate of exogenous [^{3}H]labelled histamine. These results were confirmed by Johnson (1969).

Even after a long-term treatment with brocresine (100–200 mg/kg, i.p.) for several days the excretion of free histamine in the urine remained constant (Johnston & Kahlson, 1967).

In contrast, Levine & others (1965) described a strong inhibition accompanied by a decline of the histamine contents in heart and stomach and the excretion of histamine in the urine with an inhibition maximum between 3 and 6 h. One year later no such an inhibition could be observed by the same author in another laboratory (Levine, 1966) suggesting a different susceptibility of nearly related strains of rats.

After oral administration of brocresine the histidine decarboxylase-inhibiting activity of the human plasma is maximal 30–45 min after the intake of the drug and declines rapidly afterwards (Wustrack & Levine, 1969). Obviously not all pharmacological activity disappears so quickly since we could demonstrate an increased histamine-stimulated gastric acid response in rats 4 h after intraperitoneal brocresine administration (Becker & Sewing, 1971). Whether these different activities can be attributed to the same compound remains to be elucidated.

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Species-dependent effects of fenfluramine on the central nervous system

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The cortical effects of fenfluramine and a metabolite, norfenfluramine, were studied in rabbits and cats. The parent compound produced slow waves in the e.e.g. which were compatible with sedation in cats. Norfenfluramine also produced slow waves and apparent sedation in cats, but neither compound produced marked changes in the cortical waves of rabbits. When cortical waves in both species were slowed with pentobarbitone, fenfluramine increased the frequency of the waves in rabbits but did not alter the frequency in cats. Both fenfluramine and the metabolite blocked cortical afterdischarges in both species. There is no apparent explanation for the differences in the action of the two drugs in the two species.

Fenfluramine, an anorectic agent, is related structurally to amphetamine but it has been shown to be without cortical stimulant action in most experimental animals and in man (Colmore & Moore, 1966; Le Douarec, Schmitt & Laubie, 1966; Hill & Turner, 1967; Ziance & Kinnard, 1967; Foxwell, Funderburk & Ward, 1969) and it occasionally produces drowsiness in man (Duncan, Hyde & others, 1965; Traherne, 1965). Fink & Shapiro (1969) found that in man fenfluramine produced e.e.g. patterns that resembled those produced by 50 mg of amobarbitone but were unlike those produced by (+)-amphetamine. The lack of stimulant action of fenfluramine, however, has been questioned. Jesperson, Bonaccorsi & Garattini (1969) have shown that it causes hyperthermia and signs of central nervous system excitation in mice treated with a combination of dopa and pheniprazine. Large overdosages in man have also resulted in convulsions (Riley, Corson & others, 1969; Campbell & Moore, 1969; and others). More recently Mayer, Southgate & Wilson (1970) reported that fenfluramine produced cortical stimulation in rabbits and this has since been confirmed (Funderburk & Ward, 1970). Since this finding contrasts sharply with the finding that fenfluramine slowed cortical waves in cats and produced other signs of sedative action in this species (Foxwell & others, 1969), the present study was undertaken to compare the actions of fenfluramine and its de-ethylated metabolite, norfenfluramine (Bruce & Maynard, 1968), in rabbits and in cats.

METHODS

Fourteen adult, mongrel cats of either sex and 15 adult, New Zealand white rabbits of either sex were used. Surgical procedures in both species were similar and were performed under ether anaesthesia. The trachea was intubated for artificial respiration and a vein was cannulated for administration of drugs. The calvarium was widely exposed and 1/4 inch stainless steel, sheet metal screws were placed in the bone of cats over the sigmoid, suprasylvian and posterior lateral gyri bilaterally. The recordings were all unipolar and a common electrode was placed

in the bone over a frontal sinus. Similar screws were also used as electrodes in rabbits and were placed in relatively similar areas. After-discharges, developed in the frontal cortex were studied in both species. The sigmoid gyrus was stimulated with 5 ms square waves at 100 Hz for 5 s with amplitudes that varied from 1 to 4·5 V depending on the threshold of the cortex. Recordings were made immediately after the stimuli were turned off. Incisions and pressure points of the animals were infiltrated with 2% procaine before the ether was withdrawn, and repeated when necessary. The animals were paralysed with gallamine triethiodide or tubocurarine hydrochloride and maintained on artificial respiration. Body temperature was maintained with thermistor-controlled heat lamps. Electrical potentials were recorded on an Offner type T electroencephalograph at least 1 h after the ether had been withdrawn. Both drugs were dissolved in distilled water and administered intravenously in doses calculated for free base.

RESULTS

Three rabbits were used to examine the effects of fenfluramine on cortical potentials. Doses of 8 mg/kg did not alter the electrical activity markedly. All three animals were alert during the control period as only low voltage fast activity was seen in the e.e.g. The administration of fenfluramine resulted in only a slight reduction in the amplitude of these waves.

To slow the cortical potentials, pentobarbitone (10 mg/kg) was given intravenously to three rabbits. Fenfluramine (8 mg/kg) given to these animals reduced the amplitude and increased the frequency of the cortical waves (Fig. 1). The electrical potentials were markedly desynchronized in all three animals.

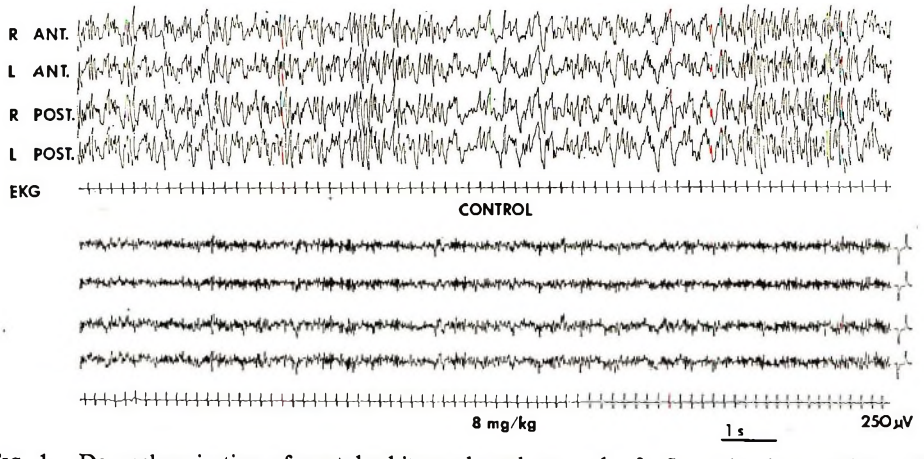

FIG. 1. Desynchronisation of pentobarbitone-slowed waves by fenfluramine in a rabbit. The cortical waves in the upper tracing have been slowed by the i.v. administration of pentobarbitone (10 mg/kg). Ten min later desynchronization of the slow waves was produced by fenfluramine (8 mg/kg) given i.v. ANT. = anterior cortex; POST. = posterior cortex. All recordings were monopolar with the common electrode in the frontal bone.

As shown by Foxwell & others (1969), cats respond to fenfluramine with slowing of the cortical waves and behavioral sleep. The findings with three cats given fenfluramine (8 mg/kg) confirmed the previous studies and, in contrast to the findings in rabbits, slow waves in the e.e.g. tracings were produced. Two other cats were given pentobarbitone (20 mg/kg) followed in 10 min by fenfluramine (10 mg/kg).

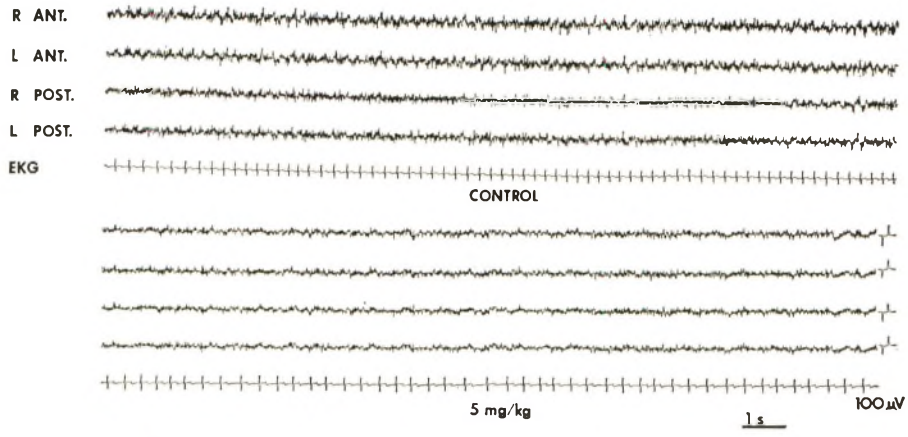

FIG. 2. Effect of norfenfluramine on cortical potentials in a rabbit. Like fenfluramine, norfenfluramine (5 mg/kg) given i.v. lowered the amplitude of the waves within 5 min without producing much change in frequency. Notations as in Fig. 1.

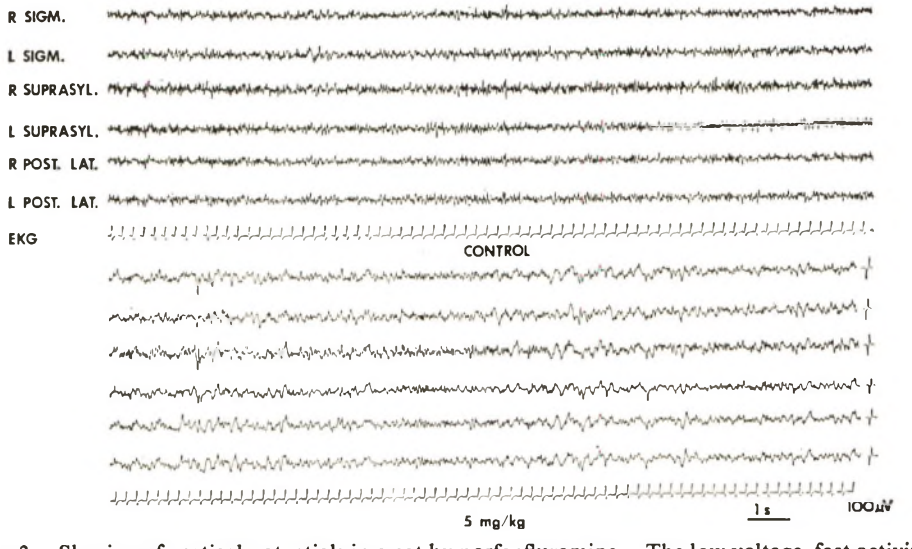

FIG. 3. Slowing of cortical potentials in a cat by norfenfluramine. The low voltage, fast activity in the control tracing was markedly slowed by the adminstration of norfenfluramine (5 mg/kg) (lower tracing). This effect was seen almost immediately and lasted for 4 h. SIGM. = sigmoid gyrus; SUPRASYL = suprasylvian gyrus; POST.LAT. = posterior lateral gyrus.

The pentobarbitone produced spindling which was not modified by the fenfluramine.

The effects of norfenfluramine (5 mg/kg) on cortical potentials were studied in three rabbits. Fig. 2 exemplifies a typical experiment. Low voltage fast activity dominated the control tracing and, after norfenfluramine, cortical activity was reduced in amplitude without apparent change in frequency of the waves although background slow waves were more prominent. In similar experiments at the same dose in three cats cortical activity was markedly slowed (Fig. 3).

The metabolite was also studied in rabbits and cats that had been given pentobarbitone (10 mg/kg). Spindling in the e.e.g. of two such rabbits was abolished and replaced by fast activity after norfenfluramine (5 mg/kg). In two cats, however, the spindling was unchanged by the same dose of the metabolite.

The effects of norfenfluramine on cortical after-discharges were studied in two rabbits. Fig. 4 shows that 5 mg/kg completely abolished this type of evoked potential without producing slow waves in the tracing. Both animals responded in the same way. Identical results were obtained in two other rabbits using fenfluramine (8 mg/kg). Norfenfluramine (5 mg/kg) in two cats produced similar responses to the rabbits in that the cortical after-discharge was completely abolished but in the cats, cortical slowing was also produced (Fig. 5). The cortical after-discharge was abolished in two other cats after the administration of fenfluramine (8 mg/kg), confirming the results of Foxwell & others (1969).

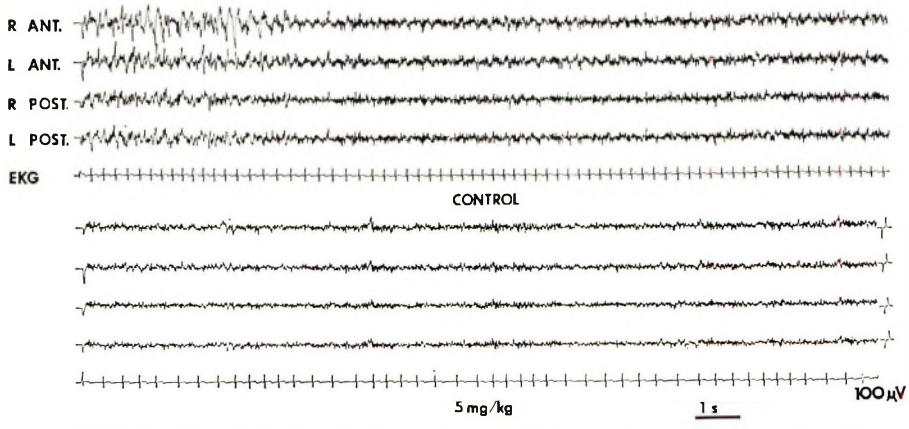

FIG. 4. Blockade of cortical after-discharges in a rabbit by norfenfluramine. After-discharges were produced in the upper tracing by stimulating the right frontal cortex with 2 V at a frequency of 100 Hz immediately before recording was made. Ten min after norfenfluramine (5 mg/kg) was given similar stimuation did not produce after-discharges (lower tracing). Similar results were obtained with fenfluramine. Notations as in Fig. 1.

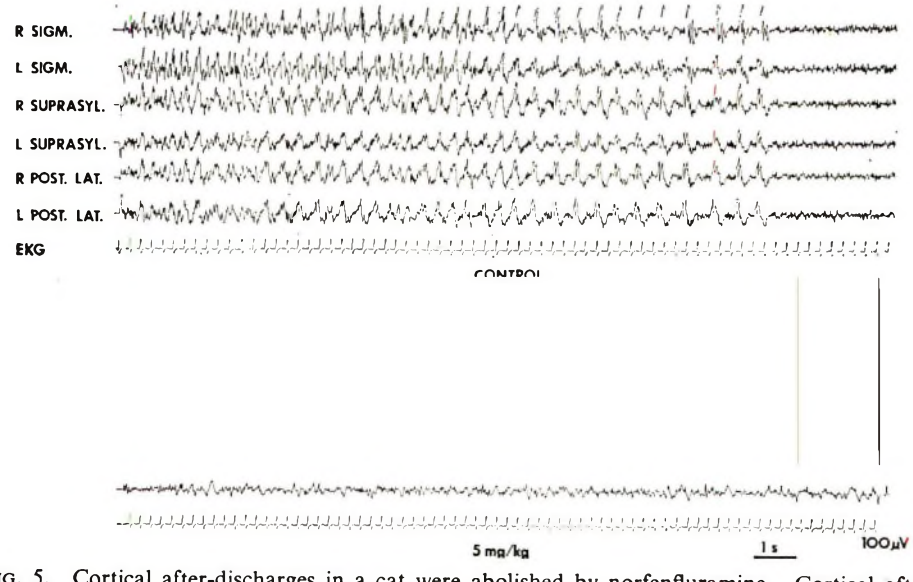

FIG. 5. Cortical after-discharges in a cat were abolished by norfenfluramine. Cortical after-discharges, produced as indicated in Fig. 4, were replaced by slow waves after the administration of norfenfluramine (5 mg/kg) (lower tracing). Similar results were obtained with fenfluramine. Notations as in Fig. 3.

DISCUSSION

The results show a species difference in the responses of rabbits and cats to fenfluramine and norfenfluramine. In general, rabbits were stimulated by both drugs and cats were depressed or sedated. The finding of a central nervous system stimulant action of fenfluramine in rabbits is in agreement with the report by Mayer & others (1970). Schmitt & Le Douarec (1965) on the other hand, found that fenfluramine produced a mixture of stimulant and sedative effects in rabbits. The only depressant action that we found in rabbits was the blockade of cortical after-discharge by both fenfluramine and norfenfluramine, but extensive subcortical studies were not done. A similar dual action of fenfluramine in cats was reported by Foxwell & others (1969) who showed that fenfluramine stimulated some subcortical structures while it depressed the cortex. The present findings show that the action of norfenflura mine on the cortex of the cat and rabbit is essentially the same as that of fenfluramine.

We have suggested previously (Funderburk & Ward, 1970) that the central nervous system stimulant action of fenfluramine in rabbits may be due to an active metabolite, norfenfluramine. Bruce & Maynard (personal communication) found that fenfluramine is metabolized to norfenfluramine in calves and rabbits and to a lesser extent in man; and Chandler, Dannenburg & others (1970) have shown that fenfluramine also has a central nervous stimulant action in calves. It was tempting to believe that cats metabolize the drug in a different manner, producing no norfenfluramine and consequently no stimulation. Bruce & Maynard (personal communication) found, however, that the cat and rabbit metabolize fenfluramine to norfenfluramine in about the same amounts and in the same time. We now have shown that norfenfluramine, like the parent compound, fenfluramine, has a predominantly stimulating action in rabbits but a predominantly sedative action in cats. The mechanism for the species difference may possibly be related to the unpublished observation of DaVanzo & Ruckart who have shown that fenfluramine significantly lowers the concentration of brain 5-hydroxytryptamine in cats but only slightly lowers the concentration of this amine in rabbits.

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The estimation of the "free" and "bound" acetylcholine content of rat brain

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Rat brains, not frozen, were homogenized in saline solution containing physostigmine, and also cupric chloride to inhibit choline acetyltransferase. When brains were homogenized for periods of up to 3 min, the amount of acetylcholine extracted was proportional to the duration of homogenization. After 3 min, there was no further significant increase in acetylcholine. The acetylcholine extracted in this way was termed "free"; whereas that remaining in the brain tissue and extracted by acid-ethanol solution was termed "bound". The total amount obtained from each brain was not significantly different from the total amount extracted by a more conventional method from the brains of rats killed by rapid freezing in liquid air. This observation applied also to brains removed from animals during anaesthesia and convulsions.

It is desirable to be able to differentiate between a change in brain acetylcholine content that has arisen due to a variation in physiological activity and that which may have arisen from changes in the biochemical systems involved in acetylcholine metabolism. A method described by Crossland & Slater (1968) has shown that drugs may differ in the extent to which they affect two fractions—"free" and "bound"—of brain acetylcholine. The term "free" referred to acetylcholine extracted by simple homogenization in a saline medium under conditions where synthesis and destruction are prevented; "bound" referred to the second component that can be released and extracted only by protein precipitation. Other attempts to measure "free" acetylcholine have been made (Stone, 1955; Kurokawa, Machiyama & Kato, 1963) but the results show wide discrepancies. A possible reason for this is that although the breakdown of acetylcholine during the extraction procedure is usually prevented, the synthesis of more acetylcholine could continue because choline acetyltransferase activity was not usually inhibited.

The method of Crossland & Slater (1968) for estimating "free" and "bound" acetylcholine is now further evaluated.

METHODS

Female Wistar rats, 80–100 g were either killed by total immersion in liquid air or decapitated. Frozen brain tissue was chipped out, crushed and extracted with acid-alcohol according to Crossland (1951) to give total brain acetylcholine.

Non-frozen brains rapidly excised from decapitated animals were homogenized with the least possible delay in physiological saline solution containing physostigmine sulphate (15 μg/ml) and cupric chloride (17 μg/ml) using 5 ml of medium for each g of brain tissue. Homogenization was effected at 0° by an M.S.E. top-drive homogenizer with a stainless steel blade (diameter 1·5 cm) rotating at 1300 rev/min in a

15 ml capacity vortex flask. The blade speed was sufficient to ensure an even disintegration of the tissue within 2 to 3 min. The brain homogenate was immediately centrifuged at 5000 rev/min for 20 min to produce a clear supernatant solution largely free from particles of cell debris. The supernatant was decanted, adjusted to pH 4·0 with 0·5N hydrochloric acid and stood for 30 min, when any slight precipitate was removed. This clear solution then contained "free" acetylcholine. The tissue residue remaining after the extraction of the "free" acetylcholine was treated with acid-ethanol solution to release the "bound" acetylcholine (Crossland, 1951). The extracts were stored at 0° until required.

The assays were performed, using a bracketing dose technique, on the frog rectus abdominis preparation sensitized with neostigmine bromide (10^{-6}M). Half of each brain extract was adjusted to pH 12 and boiled for 2–3 min to destroy acetylcholine without affecting material that increases the response of the frog rectus to acetylcholine (Feldberg, 1945). Standard solutions of acetylcholine were added to the acetylcholine-free extract, adjusted to pH 7 and used in the assay against brain extracts.

Drugs administered to rats intraperitoneally were pentobarbitone sodium (35 mg/kg) and leptazol (75 mg/kg).

RESULTS

To determine the relation between the amount of "free" acetylcholine extracted and the duration of homogenization, brains removed from decapitated rats were homogenized under standard conditions for periods of between 1 and 8 min in the saline medium containing physostigmine and cupric ion. Fig. 1 shows that there was a rapid increase in "free" acetylcholine for the first 3 min, but very little subsequent increase. Approximately 2 min of homogenization was sufficient to reduce the brain tissue to a fine, even consistency, therefore for all subsequent determinations of "free" acetylcholine 3 min was chosen as being the optimum period for the homogenization.

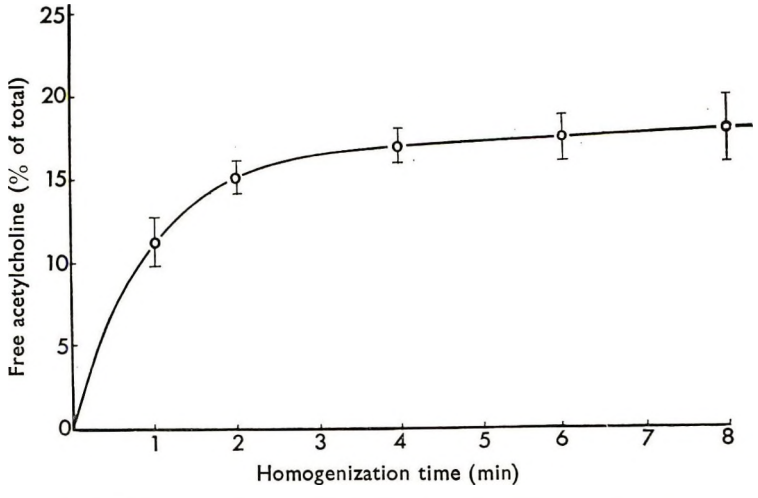

FIG. 1. The relation between the amount of "free" acetylcholine, expressed as percentage of total, obtained from rat brains subjected to saline homogenization for specified periods. The points represent the mean values from 4 determinations and each vertical line is one standard error. Further details of the homogenization procedure are given in the text.

The amount of "free" and "bound" acetylcholine extracted from rat brain under these standard conditions was determined. The mean concentration of "free" acetylcholine was 0·40 ± 0·03 μg/g (mean ± s.e. from 18 determinations) and the amount of "bound" was 2·82 ± 0·15 μg/g (18 determinations). The so-called "free" acetylcholine was therefore approximately 13% of the total amount obtained. These values are similar to those of Crossland & Slater (1968). The total acetylcholine content of rat brain by this method was therefore 3·22 ± 0·14 μg/g. The mean brain acetylcholine content of a group of 14 identical rats killed by immersion in liquid air was 3·49 ± 0·02 μg/g which is 8% greater than in the non-frozen brains. When analysed by Student's *t*-test the results of the two methods were not significantly different. It may be assumed therefore, that the use of non-frozen brain under conditions where both the synthesis and destruction of acetylcholine are prevented gives a fair estimate of the true acetylcholine content of brain as measured by using the rapid freezing technique. To investigate whether this conclusion remained valid for a drug-induced change in brain acetylcholine concentration, groups of rats were killed during pentobarbitone anaesthesia and leptazol convulsions. The amount of acetylcholine obtained using both methods are shown in Table 1. There was no

Table 1. *The total brain acetylcholine content of rats killed by either rapid freezing in liquid air or decapitation.*

Treatment	Time of killing	Brain acetylcholine content (μg/g fresh tissues ± s.e.) Rapid frozen	Decapitation
Normal rats	—	3·33 ± 0·14 (9)	3·22 ± 0·14 (18)
Anaesthetized rats (pentobarbitone sodium 35 mg/kg, i.p.)	30 min	4·72 ± 0·32*(5)	5·24 ± 0·34**(11)
Convulsing rats (leptazol 75 mg/kg, i.p.)	5 min	1·65 ± 0·12** (4)	1·88 ± 0·09** (4)

Significance of difference from normal $*P < 0·02$, $**P < 0·001$.
The number of animals used is shown in parentheses.

significant difference between the values obtained in the two instances from the normal untreated rats. During anaesthesia, both methods of extraction demonstrated increased amounts of brain acetylcholine with no difference between the two sets of results. Further, chemically-induced convulsions lowered the total amount of brain acetylcholine, similarly with no statistical difference between the results obtained using the two methods. The close correspondence between the results obtained with frozen and non-frozen brain therefore reflect the actual changes occurring in the amounts of brain acetylcholine in *vivo*. The method described for the estimation of "free" and "bound" acetylcholine appears to be effective in preventing both synthesis and destruction of acetylcholine during the homogenization and extraction.

DISCUSSION

Homogenization and extraction of non-frozen brain tissue is usually accompanied by a rapid synthesis of acetylcholine (Crossland, Pappius & Elliott, 1955). This is especially evident when the medium contains an anticholinesterase, with the result that the values for the total brain acetylcholine content may bear little resemblance to

those found *in vivo*. To overcome this, the technique of killing animals in liquid air and extracting the frozen brain was introduced (Crossland, 1951). It has been widely accepted that this method with more recent modifications introduced by Aprison & Takahashi (1965), gives values very close to the acetylcholine content of brain *in vivo*. But frozen brain cannot be used to measure "free" acetylcholine, since freezing and thawing brain tissue liberates a large proportion of the "bound" acetylcholine. However, from the findings presented here, extraction of non-frozen brain in a medium that adequately prevents synthesis of acetylcholine, gives results comparable to those obtained using frozen brain. Copper has been shown by Nachmansohn & Machado (1943) and Stone (1955) to prevent the synthesis of acetylcholine.

The concept of "free" acetylcholine has found little favour and has been described as an artifact that arises during the extraction of brain tissue. This is perhaps not unreasonable since, previous estimates of the "free" acetylcholine of brain have differed widely. Almost without exception, however, no precautions were taken to prevent synthesis of acetylcholine during the homogenization.

Mann, Tennenbaum & Quastel (1938) were the first to differentiate between free and bound acetylcholine. They found that during acetylcholine synthesis *in vitro* an equilibrium was set up between free acetylcholine in the medium and bound acetylcholine in brain slices. Tobias, Lipton & Lepinat (1946) reported that normal rat brain contained 20% of free acetylcholine when extracted in eserinized saline solution while Kurokawa, Machiyama & Kato (1963) obtained 18% of free acetylcholine from homogenates of mouse brain.

There is evidence that brain acetylcholine is associated with three fractions isolated by subcellular fractionation techniques. A "labile bound" fraction occurs in the cytoplasm of isolated synaptosomes and a "stable bound" fraction is associated with the synaptic vesicles (Whittaker, 1959; 1968). These two fractions represent between 70 and 90% of the total acetylcholine (Hebb & Whittaker, 1958), while the balance, corresponding to the free fraction, is recovered from the high-speed supernatant fraction, provided that a cholinesterase inhibitor is present.

Discussion about the "free" acetylcholine extracted using the method herein described is mainly speculative. It is certainly not entirely the result of the liberation of either form of bound acetylcholine, otherwise the proportion of "free" to "bound" would always remain constant. This does not happen because drugs such as physostigmine, tremorine and atropine affect the two fractions differently (Crossland & Slater, 1968). Other workers have come to the conclusion that free acetylcholine is not a simple artifact (Whittaker, 1959; Beani, Bianchi & others, 1969). Perhaps the most likely explanation is that the "free" acetylcholine is a mixture of acetylcholine from several sources. From the available knowledge it seems that acetylcholine is synthesized in the free form and is subsequently bound into the tissue storage sites. Part of the material extracted will include this newly synthesized acetylcholine. The remainder of the "free" fraction could consist of acetylcholine liberated as a result of nervous activity and that has not been destroyed by cholinesterase. There is also the possibility that a third fraction of the "free"acetylcholine may arise from the release of some of the bound material, and in particular the "labile bound" fraction, during the homogenization.

The results reported in this paper demonstrate that the proportion of "free" acetylcholine in rat brain is approximately 13% of the total. Since the amount

involved is small, only the most sensitive frog rectus preparation can be used for the assay. The slight fluctuations in the amounts of the "free" material appear to be due in part to difficulties in assay. It must however be emphasized that the term "free" and "bound" cannot yet be identified with any specific cell constituents.

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The site of the inhibitory action of salicylate on protein biosynthesis *in vitro*

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Salicylate (10 mM) affects neither the transfer of the amino-acid from leucyl-tRNA into protein by rat liver polysomes nor the formation of the leucyl-tRNA. However, lower concentrations of salicylate inhibit the activities of other aminoacyl-tRNA synthetases. The most sensitive enzymes are those which incorporate glutamate, aspartate and histidine. It is suggested that salicylate interferes with the *in vitro* biosynthesis of protein by preferentially inhibiting the production of certain aminoacyl-tRNA species. The drug, in concentrations of 0·6 mM and above, inhibits the ATP-pyrophosphate exchange reaction promoted by individual amino-acids, including leucine.

High concentrations of salicylate (10–15 mM) inhibit the *in vitro* incorporation of radioactive glutamate and proline into the protein of rat costal cartilage (Bellamy, Huggins & Smith, 1963) and of labelled threonine into the epithelial proteins of sheep mucosal scrapings (Kent & Allen, 1968). Protein biosynthesis in the rat isolated diaphragm is more sensitive to salicylate and concentrations from 0·5 to 5 mM interfere with the incorporation of glutamate, glycine, lysine and leucine (Manchester, Randle & Smith, 1958; Dawkins, Gould & Smith, 1966). The inhibition also occurs in cell-free systems prepared from rat liver (Reunanen, Hanninen & Hartiala, 1967). It is independent of the well-known uncoupling action of salicylate on oxidative phosphorylation (Brody, 1956) since it was observed with microsomal preparations supplied with an external energy source.

This paper describes experiments designed to elucidate the site of the inhibitory action of salicylate on protein biosynthesis *in vitro*. The overall process was studied in two main stages, the formation of aminoacyl-transfer RNAs and the transfer of amino-acids by polysomes from these complexes to protein. The ATP-pyrophosphate exchange reaction was also measured in an attempt to study activation of the amino-acids. A preliminary account of the work has been published (Burleigh & Smith, 1970).

MATERIALS AND METHODS

Materials

The following radiochemicals were used: [U-^{14}C]protein hydrolysate from *Chlorella* (54 mCi/mAtom of carbon); L-[U-^{14}C]aspartic acid (208 mCi/mmol); [U-^{14}C]glycine (109 mCi/mmol); L-[U-^{14}C]glutamic acid (249 mCi/mmol); L-[U-^{14}C]glutamine (42 mCi/mmol); L-ring-[2-^{14}C]histidine (57·8 mCi/mmol); L-[U-^{14}C]leucine (311 mCi/mmol); L-[U-^{14}C]lysine monohydrochloride (310 mCi/mmol); L-methyl[^{14}C]-methionine (53·6 mCi/mmol); L-[U-^{14}C]phenylalanine (459 mCi/mmol); L-[U-^{14}C]-proline (255 mCi/mmol); L-[U-^{14}C]threonine (208 mCi/mmol); L-methylene[^{14}C]-tryptophan (54·5 mCi/mmol); L-[^{35}S]cysteine hydrochloride (18 mCi/mmol) and

[^{32}P]tetrasodium pyrophosphate. These were purchased from the Radiochemical Centre, Amersham, U.K.

ATP and GTP (sodium salts), transfer RNA (tRNA) from Bakers yeast (Type III) and ribonuclease-A from bovine pancreas (salt-free) came from the Sigma Chemical Company. Triton X-100, PPO and POPOP came from Packard Instrument Co. Inc. and Sephadex G-25 from Pharmacia Fine Chemicals Inc. The Whatman GF/A glass fibre discs were obtained from Gallenkamp and Co.

Animals

Male rats, 400–500 g, of the Wistar strain, maintained on M.R.C. cube diet no. 41, were killed by stunning and cervical fracture.

Preparation of polysomes

Polysomes were prepared by an adaptation of the method of Munro, Jackson & Korner (1964). Livers from freshly-killed rats were rinsed in ice-cold 0·25M sucrose in Medium M (20 mM tris-HCl buffer, pH 7·8 at 4°; 10 mM $MgCl_2$; 40 mM NaCl; 100 mM KCl and 6 mM mercaptoethanol). The livers were blotted, weighed and minced with scissors in fresh medium (3 volumes per g liver) and homogenized using five strokes of an all-glass, loose-fitting Potter-Elvehjem homogenizer surrounded by an ice jacket. The homogenate was centrifuged at 4° in a Spinco Model L ultracentrifuge for 20 min at 18 000 *g*, and the separated supernatant for a further 10 min at the same speed, to remove cell debris, nuclei and mitochondria. The post-mitochondrial supernatant was added to one-ninth of its volume of 10% (v/v) Triton X-100 pH 8·2, to give a final concentration of 1% Triton (Korner, 1969) and aliquots (5 ml) layered over 2·5 ml of 0·5M sucrose in medium M, which has been layered over 2M sucrose in medium M, and the whole centrifuged for 4 h at 105 000 *g*. The polysome pellet was rinsed with medium M and stored overnight at −20°. Immediately before use, it was resuspended in medium M (one-quarter of the volume of original homogenate). Polysomes were characterized by the RNA:protein ratio which was between 1·0 and 1·2 in all preparations used. RNA was determined according to Munro & others (1964) and protein to Lowry, Rosebrough & others (1951).

Preparation of 105 000 g *supernatant fraction*

Livers from freshly-killed rats were homogenized as described above, in 2·5 volumes/g liver of medium H (35 mM tris-HCl buffer, pH 7·8 at 4°; 0·25M sucrose; 75 mM KCl and 10 mM $MgCl_2$). The homogenate was centrifuged for 20 min at 20 000 *g* and the supernatant centrifuged for 90 min at 105 000 *g* to remove microsomes. The middle, fat-free portion of the 105 000 *g* supernatant was collected by Pasteur pipette.

Preparation of pH 5 enzyme fraction and pH 5 supernatant

The pH of 105 000 *g* rat liver supernatant was lowered by dropwise addition of 0·01N HCl with constant stirring at 2° until a pH of 5·2 was reached. The pH 5 precipitate was collected by centrifugation for 10 min at 12 000 *g*, resuspended in a quantity of 0·25M sucrose equal to one-third of the original volume of supernatant, and re-isolated. The final precipitate was dissolved in 0·4M tris-HCl buffer, pH 7·5 at 4° (one-tenth of the original supernatant volume). The solution was diluted with four vol Medium H and dialysed against 3 × 1-litre changes of medium H for at least 24 h to remove endogenous amino-acids (Decken & Campbell, 1962). For the preparation of pH 5 supernatant for use as a source of transfer enzymes in the polysome incubations, the pH of the 105 000 *g* rat liver supernatant was lowered to 5·05

and the pH 5 precipitate removed by centrifugation for 20 min at 12 500 *g*. Both the 105 000 *g* supernatant and the pH 5 supernatant fractions were passed through a Sephadex G-25 column at 2°, equilibrated with 35 mM Tris-HCl, pH 7·5 at 20° to remove endogenous amino-acids and low molecular-weight materials.

Preparation of aminoacyl-tRNA

Transfer RNA from yeast and rat liver was esterified with [^{14}C]leucine plus the following unlabelled L-amino-acids: alanine, arginine, aspartic acid, asparagine, cysteine, glycine, glutamic acid, glutamine, histidine, isoleucine, lysine, methionine, proline, phenylalanine, serine, threonine, tryptophan, tyrosine and valine.

The 105 000 *g* supernatant fraction (approximately 4 mg protein per ml final incubation volume), after passage through Sephadex G-25, was incubated for ten min at 37° with 100 mM tris-HCl buffer, pH 7·5 at 37°, 4 mM mercaptoethanol, 10 mM $MgCl_2$, 10 mM KCl, 0·25 mM CTP, 2·25 mM ATP, 0·25 mM each amino-acid, 1·25 μCi/ml [^{14}C]leucine (311 μCi/mmol) and 1 mg/ml yeast tRNA. The reaction mixture was cooled to 0° and an equal volume of 90% (v/v) phenol added (Hoagland, Stephenson & others, 1958). After two further extractions of the separated phenol layer, the three aqueous fractions were pooled and the RNA precipitated by the addition of one tenth volume of 20% (w/v) potassium acetate, pH 5, and 2·5 volumes of ethanol; the mixture was maintained for at least 3 h at −20° before isolating the RNA by centrifugation. The ethanol precipitation was repeated to remove all traces of phenol and the final tRNA precipitate resuspended in water to give a concentration of 1·5 mg/ml (approximately 100 000 counts/min per mg RNA) and dialysed against 3 × 1 litre distilled water for a total of 5 h at 2°.

Extraction of protein for radioactive assay

The reaction for the incorporation of [^{14}C]leucine from aminoacyl-tRNA into protein (see Fig. 1) was stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid (TCA). The precipitate was washed in 5 ml of 5% (w/v) TCA,

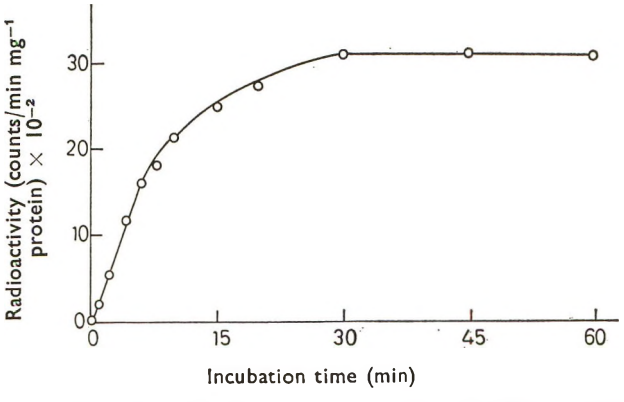

FIG. 1. Time curve for transfer of [^{14}C]leucine from aminoacyl-tRNA into protein by rat liver polysomes. Each reaction mixture contained, in 1·0 ml total volume, 3·0 μmol ATP; 0·5 μmol GTP; 300 μg aminoacyl-tRNA containing [^{14}C]leucyl-tRNA (0·015 μCi); 0·2 ml polysome suspension (140 μg RNA); 0·25 ml pH 5 supernatant (1·4 mg protein). The incubation medium was Medium M, the pH of which had been adjusted to 7·4 at 37°. The reaction was started by the addition of the polysome: pH 5 supernatant mixture and each tube was incubated at 37° with gentle shaking for an interval varying from 1 to 60 min. Counts for the zero time controls were in the range 0–5 counts/min per mg protein isolated. Each point represents the mean of at least two separate experiments.

then heated in 5 ml of 5% TCA for 20 min at 90° to remove tRNA. The precipitate was then washed successively with 5 ml quantities of 5% (w/v) TCA, ethanol–TCA (95:5 by vol), ethanol–ether–chloroform (2:2:1 by vol), acetone and ether and gently dried. The final precipitate was dissolved in 1 ml of N NaOH. All radioactive counting was performed on 0·1 ml samples dried on Whatman GF/A (2·1 cm) glass fibre discs in vials containing 5 ml of scintillation fluid [4 g 2,5-diphenyloxazole and 0·1 g 1,4-bis-(5-phenyloxazolyl-2)-benzene in 1 litre of toluene] in a Beckman LS 200B scintillation system with an efficiency of 94% for ^{14}C. Protein concentration was determined according to Lowry & others (1951) and the results expressed as counts/min per mg protein isolated.

Extraction of aminoacyl-tRNA for radioactive assay

The reaction (see Table 1) was terminated by the addition of 2 vol of ice-cold M perchloric acid (PCA) containing 20 mM tetrasodium pyrophosphate (Chambon, Ramuz & others, 1968) and the tRNA allowed to precipitate for at least 45 min at 0°. The precipitate of protein plus tRNA was washed successively with 3 ml quantities of ice-cold 0·33M PCA containing 6·7 mM pyrophosphate (two washes), ethanol–0·33M PCA (5:1 by vol) and ethanol–ether (3:1 by vol). To increase the specificity of the extraction, the aminoacyl-tRNA was extracted twice in 0·5 ml of 10% NaCl for 10 min in a water bath at 100°. The tRNA was precipitated from the pooled supernatants with two vol of ice-cold ethanol followed by standing for at least 3 h at −20°. The final aminoacyl-tRNA precipitate was dissolved in 0·5 ml of 0·1M ammonium acetate buffer, pH 9, and incubated for 10 min at 20° with pancreatic ribonuclease-A (0·05 ml, containing at least 50 μg/ml). The digestion was terminated with 0·15 ml of 2·5M acetic acid and 0·15 ml ethanol. The protein precipitate was removed by centrifugation at room temperature and the supernatant retained for counting and RNA estimation (Hurlbert, Schmitz & others, 1954). The results were expressed as counts/min mg^{-1} tRNA isolated, when necessary divided by the protein concentration in the incubation mixture.

Extraction and estimation of [^{32}P]ATP

The [^{32}P]pyrophosphate-ATP exchange reaction (see Table 3) was terminated with 2 vol of M PCA. The protein precipitate was removed by centrifugation and the supernatant was added to 0·4 ml of acid-washed Norit-A suspension (15% w/v) in water to adsorb the labelled ATP (Tsuboi & Price, 1959). After vigorous mixing and standing for 5 to 10 min, the Norit was collected and washed with three 3-ml volumes of distilled water. The [^{32}P]ATP was eluted with 3 ml of 0·3M ammonium hydroxide in 50% (v/v) ethanol and the Norit removed by filtration. The ATP concentration was estimated by measurement of the absorbance at 260 nm of sample diluted 1 in 100. The radioactivity in the samples was counted on glass fibre discs and the counts/min of the [^{32}P]ATP and of the original [^{32}P]pyrophosphate solution were corrected to zero counting time to allow for the decay of ^{32}P radioactivity. The percentage exchange (counts/min μmol^{-1} ATP $\times$ 100 $\div$ total counts/min μmol^{-1}, ATP + PP_i) was calculated by the method of Hoagland (1955).

RESULTS

Transfer of [^{14}C]leucine from aminoacyl-tRNA to polysomal protein

The time curve for the transfer of radioactivity from [^{14}C]leucyl-tRNA into protein in the presence of nineteen other non-radioactive aminoacyl-tRNAs is given in Fig. 1.

The reaction mixtures contained a polysome fraction and a pH 5 supernatant prepared from rat liver. The rate of incorporation of the radiocarbon is linear up to 6 min and reaches a steady maximum at 30 min.

Salicylate, 0·5 to 10 mM, an incubation period of 30 min, to ensure maximum incorporation of radioactivity, and incubation periods of 15 or 5 min had no effect. Nor did lower concentrations of substrate and GTP in the reaction mixtures or the exclusion of mercaptoethanol from both the polysome and pH 5 supernatant fractions.

Incorporation of radioactive amino-acids into aminoacyl-tRNAs

The effects of salicylate, in concentrations ranging from 0·5 to 10 mM, on the incorporation of radioactivity from the [^{14}C]amino-acids of a *Chlorella* protein hydrolysate into aminoacyl-tRNAs by the 105 000 *g* supernatant from rat liver are given in Table 1. In contrast to the results obtained with the transfer of amino-acid from

Table 1. *Effect of salicylate on incorporation of a radioactive protein hydrolysate into aminoacyl-tRNA.* Each reaction mixture contained, in 1·0 ml total volume, 2 μmol ATP; 10 μmol $MgCl_2$; 100 μmol tris-HCl (pH 7·5 at 37°); 500 μg yeast tRNA; 0·25 ml 105 000 *g* supernatant (0·4 mg protein); 2 μCi *Chlorella* protein hydrolysate (54 mCi/mAtom of carbon) and sufficient of a mixture of KCl and potassium salicylate to yield final salicylate concentrations ranging from 0–10 mM and a constant K^+ concentration of 20 mM. The reaction was started by the addition of the 105 000 *g* supernatant and each tube was incubated at 37° for 10 min. Counts for the zero time controls were in the range 80–130 counts/min and have been deducted from the experimental values.

Salicylate concn. (mM) ..	0	0·5	1·0	2·0	3·0	6·0	10
Counts/min* ..	10114± 493	9835± 958	9381± 457**	8786± 899**	8443± 248**	8048± 980**	7645± 404**

* Means ± standard deviations of five separate experiments.

** Statistically significant difference ($P < 0{\cdot}05$) between the control and salicylate values by t-test.

the aminoacyl-tRNA to polysomal protein, the drug caused a significant inhibition at concentrations of mM and above. The specificity of the inhibition was further studied by using individual radioactive amino-acids, representing each chemical type, either in the absence or the presence of 2 or 10 mM salicylate. The results show that the amino-acids can be divided into three groups on the basis of the effects of different salicylate concentrations on their incorporation into the corresponding aminoacyl-tRNAs. The first group, comprising aspartate, glutamate, glutamine and histidine, is sensitive to 2 mM salicylate (Table 2), the second which includes cysteine, methionine, threonine and tryptophan is affected by 10 mM but not by 2 mM drug (Table 2) and the incorporation of the remainder is not inhibited by 10 mM salicylate.

ATP-pyrophosphate exchange reaction

The effect of salicylate on the ATP-pyrophosphate exchange, which has been considered to reflect the first stage in the formation of aminoacyl-tRNAs, was studied in an attempt to further define the site of action of the drug on protein biosynthesis

Table 2. *Incorporation of aspartate, glutamate, glutamine, histidine and cysteine, methionine, threonine and tryptophan into aminoacyl-tRNA.*

Salicylate conc. (mM)	Amino-acid	Counts/min mg^{-1} tRNA isolated per mg enzyme protein†	Amino-acid	Counts/min mg^{-1} tRNA isolated per mg enzyme protein‡
0		740 ± 14		6584 ± 420
2	Aspartate	708 ± 20*	Cysteine	6940 ± 241
10		654 ± 10*		5185 ± 170*
0		1339 ± 37		873 ± 32
2	Glutamate	1232 ± 25*	Methionine	831 ± 62
10		842 ± 17*		746 ± 18*
0		2978 ± 68		3242 ± 137
2	Glutamine	2778 ± 57*	Threonine	3132 ± 60
10		2330 ± 76*		3049 ± 104*
0		295 ± 10		570 ± 18
2	Histidine	274 ± 13*	Tryptophan	575 ± 18
10		246 ± 10*		534 ± 10*

* Statistically significant difference between the control and salicylate values by the *t*-test.

† Conditions as in Table 1 except that each reaction mixture contained 0·4 ml of 105 000 g supernatant (5 mg protein) and 2·0 μCi (0·05 μmol) of the individual radioactive amino-acid. Counts for the zero time controls were in the range 0–20.

‡ Conditions as in † except that 1·7 μCi (0·1 μmol) of [^{35}S]cysteine HCl plus 1 mM mercaptoethanol were used.

Table 3. *Effect of salicylate on ATP-pyrophosphate exchange promoted by a mixture of amino-acids.* Each reaction mixture contained, in 1·0 ml total volume, 50 μmol tris-HCl (pH 7·5 at 37°); 10 μmol NaF; 10 μmol ATP; 0·5 μmol of each of the 20 amino-acids (see Methods: preparation of aminoacyl-tRNA); 4·4 × 10^6 counts/min (10 μmol) tetrasodium-[^{32}P]pyrophosphate; 0·25 ml pH 5 enzyme fraction (1 mg protein) and sufficient $MgCl_2$, KCl and potassium salicylate to yield constant Mg^{2+} and K^+ concentrations of 10 mM and 20 mM respectively and final salicylate concentrations ranging from 0–10 mM. The reaction was started by the addition of the enzyme and each tube was incubated at 37° for 40 min. Endogenous exchange, measured in the absence of the amino-acid mixture, was 0·1% .

Salicylate concn (mM)	0	0·3	0·6	1·0	2·0	3·0	6·0	10·0
% exchange*	16·00 ± 0·40	15·56 ± 0·35	15·41 ± 0·27**	15·28 ± 0·51**	15·08 ± 0·47**	14·40 ± 0·37**	13·79 ± 0·46**	12·82 ± 0·40**

* Means ± standard deviations of 6 separate experiments and calculated as percentages exchanged.

** Statistically significant difference between the control and salicylate values by the *t*-test.

in vitro. This was done using an equimolar mixture of 20 amino-acids and the results (Table 3) show that the exchange reaction was significantly inhibited by salicylate concentrations of 0·6 mM and above. In addition, the exchange promoted by certain individual amino-acids, was also studied. The results (Table 4) show that the ATP-pyrophosphate exchange supported by aspartate and histidine was significantly inhibited by 2 mM salicylate whereas that associated with leucine and phenylalanine was only sensitive to 10 mM salicylate. The addition of 10 mM glutamate did not increase the ATP-pyrophosphate exchange above the endogenous level.

Table 4. *Effect of salicylate on ATP-pyrophosphate exchange promoted by individual amino-acids.* Conditions as in Table 3 except that 10 μmol of each amino-acid was added. In the experiments with aspartate and leucine each reaction mixture contained 0·25 ml pH 5 enzyme fraction (0·72 mg protein) and $4{\cdot}7 \times 10^7$ counts/min [^{32}P]pyrophosphate but in the histidine and phenylalanine experiments each tube contained 0·25 ml enzyme (0·60 mg protein) and $7{\cdot}2 \times 10^7$ counts/min -[^{32}P]pyrophosphate. The endogenous exchange was measured in each experiment and subtracted from the corresponding experimental value. In the absence of salicylate the maximum value for the endogenous exchange was 0·094 and with 10 mM salicylate the minimum value was 0·040.

Amino-acid	Salicylate concn (mM)	% exchange
Aspartate	0	0·172 ± 0·009
	2	0·154 ± 0·009*
	10	0·089 ± 0·014*
Leucine	0	0·478 ± 0·007
	2	0·428 ± 0·094
	10	0·396 ± 0·021*
Histidine	0	0·081 ± 0·016
	2	0·166 ± 0·001*
	10	0·139 ± 0·006*
Phenylalanine	0	0·250 ± 0·013
	2	0·251 ± 0·006
	10	0·193 ± 0·007*

* Statistically significant difference between the control and salicylate values by the *t*-test.

DISCUSSION

The present results indicate that salicylate interferes with protein biosynthesis *in vitro* by inhibiting the activities of certain aminoacyl-tRNA synthetases and not by affecting the transfer of amino-acid from esterified tRNA into polysomal protein.

In the polysome experiments the transfer of only one radioactive amino-acid was followed because a single set of enzymes acts on all the aminoacyl-tRNAs (Fessenden & Moldave, 1961). The use of a pH 5 supernatant, from which the endogenous amino-acids had been removed, ensured that no esterification of tRNA could occur during the incubations. We found that 10 mM salicylate did not affect the transfer of radioactive leucine to the protein. That reduction of the aminoacyl-tRNA concentrations in the reaction mixtures made no difference, shows that the substrate concentrations were not sufficiently high to mask an inhibition due to salicylate. The GTP concentration in the incubation mixtures was also varied because it had been reported that the action of an inhibitor (Hoagland, Scornik & Pfefferkorn, 1964) present in the microsomal fraction of normal rat liver is antagonized by the nucleotide. Since salicylate remained inactive its interference with protein synthesis in the rat liver microsomal-cell sap preparation cannot be mediated via the inhibitor.

Experiments with *Chlorella* protein hydrolysate (Table 1) showed that salicylate, 1 mM and above, inhibited the formation of aminoacyl-tRNAs. This was due to a differential action of the drug on the activities of the aminoacyl-tRNA synthetases, the most sensitive being those incorporating aspartate, glutamate, glutamine and histidine (Table 2).

It has been suggested that the biosynthesis of aminoacyl-tRNA occurs in two stages, activation of the amino-acids and their transfer to the tRNA (Hoagland, Zamecnik & Stephenson, 1957). The ATP-pyrophosphate exchange reaction has been considered to reflect the first stage. The results in Table 3 suggest that salicylate interferes with this proposed stage. However, the ATP-pyrophosphate exchange promoted by phenylalanine and leucine (Table 4) was significantly inhibited by 10 mM salicylate whereas the formation of the corresponding aminoacyl-tRNAs was not affected by this concentration of the drug. A possible explanation for the discrepancy is that a rat liver supernatant and a pH 5 fraction respectively, were used to study the aminoacyl-tRNA synthetase reaction and the ATP-pyrophosphate exchange. An experiment in which the pH 5 enzyme preparation was used to measure the formation of leucyl-tRNA showed that no inhibition occurred in the presence of 10 mM salicylate.

These results suggest that salicylates interfere with the biosynthesis of proteins *in vitro* by preferentially inhibiting the formation of certain aminoacyl-tRNA species. A deficiency of any one aminoacyl-tRNA would be expected to block translation on the polysome at the corresponding messenger codon (Anderson, 1969). Thus, an impaired synthesis of glutamyl-, aspartyl- and histidinyl-tRNA in the presence of salicylate could explain the reported effects of the drug in inhibiting the incorporation of leucine into the protein of cell-free systems and of other amino-acids into the protein of a variety of animal tissues *in vitro*. The observation that salicylate inhibits the incorporation of phenylalanine by a poly U-directed system from rat liver ribosomes (Reunanen & others, 1967) is less explicable. One possibility is that salicylate could inhibit the binding of the poly U to the ribosomes. This binding is qualitatively different from the binding of natural messenger RNA (Brawerman & Eisenstadt, 1966) which only occurs to a negligible extent in cell-free systems.

The concentration of salicylate (2 mM) which significantly inhibits the activities of the aminoacyl-tRNA synthetases utilizing aspartate, glutamate, glutamine and histidine (Table 2) is within the range of the concentrations of the drug attained and maintained in animal tissues for some hours after the injection of single doses of sodium salicylate of 400 mg/kg and above (Sturman, Dawkins & others, 1968; McArthur, Dawkins & Smith, 1970). An inhibitory action of salicylate on the formation of one or more of the aminoacyl-tRNAs could therefore explain the reported effects of the drug in protein synthesis *in vivo*. It has been shown that high doses of the drug interfere with the synthesis of collagen in carrageenan granuloma in the rat (Fukuhara & Tsurufuji, 1969) and with the incorporation of radioactive histidine into liver proteins in the intact mouse (Dawkins, McArthur & Smith, 1971). The daily administration of 200–300 mg/kg of salicylate caused lower rates of weight gain and impaired skeletal growth in immature rats (Limbeck, Conger & others, 1966) and in growing chicks (Thomas, Nakaue & Reid, 1967).

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Anti-acetylcholinesterase activity of some stereoisomeric aminobornanes

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Bornyl analogues of choline and acetylcholine with eclipsed *cis*- and *trans*-configurations displayed low inhibitory activity towards acetylcholinesterase catalysed hydrolysis of acetylcholine; *cis*-isomers were slightly better inhibitors than their *trans*-counterparts. (+)-Bornan-2-one-3-*endo*-yltrimethylammonium bromide with its oxygen atom and the quaternary ammonium group fixed rigidly in the gauche configuration is much more potent inhibitor than its enantiomorph.

Semi-rigid analogues of acetylcholine have been used to investigate its active conformation in its relation to acetylcholinesterase (AChE); the *cis*-alcohols of (±)-2-dimethylaminocyclohexanol methiodide and (±)-dimethylaminocyclopentanol methiodide were stated to have a slightly better fit to the catalytic surface than their *trans*- counterparts, and similarly, the *cis*-acetates were better substrates (Baldridge, MacCarville & Friess, 1955; Friess & Baldridge, 1956). However, Kay, Robinson & others (1970) recently reported that the *cis*- enantiomers of the acetate of 2-dimethylaminocyclohexanol methiodide were inactive as substrates, and the *trans*-enantiomers were hydrolysed at a much slower rate than previously reported. This appears to agree with the observations of Smissman, Nelson & others (1966) that the *trans*-isomer of 3-dimethylamino-2-acetoxydecalin methiodide is a substrate of AChE, while the *cis*-isomers are not. As these analogues of acetylcholine are only semi-rigid, and in the absence of conformational data, conclusions from these studies require cautious appraisal.

We have reported previously (Beckett, Ngiam & McDonough, 1969a, b) the syntheses of three bornyl analogues of choline, acetylcholine, and their corresponding tertiary amino-esters. Unlike the acetylcholine analogues cited above, the N^+–C–C–O segments of these compounds are *rigidly* held in *cis*-(eclipsed), *trans*-(eclipsed) or gauche configurations. Their absolute configurations, based on the absolute configuration of (+)-camphor (Northolt & Palm, 1966) are known (see Table 1). The *cis*-(eclipsed) analogues have a torsion angle τN^+–C–C–O of about 0°, and that of the *trans*-(eclipsed) analogues is about +120°; some slight deviation from these values would be expected because of steric interference between adjacent groups. The spatial relations of the nitrogen and oxygen atoms of (+)-bornan-2-one-3-*endo*-

METHODS

The syntheses of the three diastereoisomers of 2-acetoxy-bornan-3-yltrimethylammonium bromide (I, II and III); 2-hydroxybornan-3-yltrimethylammonium bromide (IV, V and VI), and 2-acetoxy-3-dimethylaminobornane hydrochloride

(VII, VIII and IX) has been reported (Beckett, Ngiam & McDonough, 1969a, b). (+)-Bornan-2-one-3-*endo*-yltrimethylammonium bromide (X), m.p. 218·5° (sealed tube), and its enantiomer XI, m.p. 218° (sealed tube) were synthesized by quaternization of the corresponding 3-*endo*-dimethylaminobornan-2-one with CH_3Br in methanol.

The procedure of Kay & Robinson (1969) for enzyme kinetic studies was followed. Bovine erythrocyte acetylcholinesterase (Sigma Chemicals) was used. Acetylcholine iodide was used as substrate and the rates of hydrolysis were measured manually employing a PYE Dynacap pH meter, glass electrode and calomel half-cell and an Agla micrometer syringe for delivering volumes of 0·02N NaOH solution to the incubation solution *via* a fine plastic tubing. The titration vessel was a glass beaker with a plastic cover and an inlet for nitrogen just above the liquid level. Because of these precautions, the background acid production from absorption of CO_2 and acid fumes was negligible. The reaction mixture was stirred with a magnetic stirrer, and maintained at $25 \pm 0{\cdot}1°$. All incubations were with 20 ml of enzyme solution previously made 0·04M in $MgCl_2$ and 0·05M in NaCl. With the addition of measured volumes of inhibitor and substrate, the total titration volume was 25 ml. All inhibitors were pre-incubated with the enzyme for 3 min and the reaction was started by the addition of acetylcholine solution. The pH was maintained just above 7·4 with 0·02N NaOH. The time, accurate to the nearest second, was recorded when the pH fell to 7·4. A titration curve of micrometer readings against time (s) was drawn. The velocity of the reaction was calculated from the average slope of the curve during the second and third minutes of the incubation and expressed as mol/min. The velocities were corrected for aqueous hydrolysis. The K_i values for the inhibitors were calculated from Lineweaver-Burk plots using a four-fold range of substrate concentrations and approximately two-fold range of inhibitor concentrations. The K_m value for acetylcholine was determined each time and was found to be consistently $4{\cdot}45 \times 10^{-4}$ throughout the investigation, in agreement with Kay & Robinson (1969).

RESULTS AND DISCUSSION

The three diastereoisomers of 2-acetoxybornan-3-yltrimethylammonium bromide (I, II and III), the acetylcholine analogues, were found either not to be substrates of bovine AChE or alternatively the rate of enzymatic hydrolysis was too slow to be detected by the apparatus over 30 min. The inability of the enzyme to hydrolyse these acetylcholine analogues shows either (*a*) the steric requirements for such a reaction to take place were not fulfilled or (*b*) if the mechanism of such a reaction involves conformational changes of the substrate molecule the rigidity of the N^+–C–C–O segment of these compounds prohibited enzyme hydrolysis.

For optimum hydrolysis, Chothia & Pauling (1969a) proposed that the substrate molecule should assume an ideal conformation (see Fig. 1) in relation to the enzyme receptor, the essential feature of which is that τN^+–C–C–O = +150°. The corresponding torsion angle for the *trans*-analogue II in the present series is +120°, which is near to this ideal value, while the torsion angle for the *cis*-analogues I, and III, being 0°, is widely different. The failure of II to be hydrolysed by AChE may be due only in part to the eclipsed configuration of its N^+–C–C–O segment; the acetyl group, which is conformationally labile, may, because of steric interaction with the C(10) methyl group (IIa), move into a position (IIb) unfavourable for enzyme hydrolysis (compare IIb with Fig. 1).

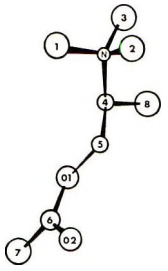
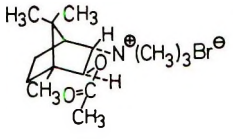
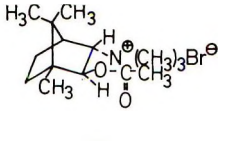


FIG. 1. The conformation of L(−)-acetyl-α-methylcholine in crystals of iodide according to Chothia & Pauling (1969a). τN⁺-C4-C5-01 = +148° and τC6-01-C5-C4 = 180°.



IIa



IIb

From their K_i values (Table 1), these acetylcholine and choline analogues (I, II, III, IV, V and VI) appear to be weak competitive inhibitors of the AChE catalysed hydrolysis of acetylcholine, and their affinity for the free enzyme would appear to be not much greater than that of the trimethylammonium ion ($K_i = 4{\cdot}0 \times 10^{-3}$; Wilson & Alexander, 1962). Comparison within each group of compounds, however, shows a difference between the *cis*- and the *trans*-isomers in their affinity for the enzyme receptor. The *cis*-alcohols (IV, VI) offer a better fit to the catalytic surface than their *trans*-counterpart (V), and this is also true with the *cis*- and *trans*-esters (I and II respectively). The only exception is ester III. The presence of the bridge C(8) methyl group probably prevents the onium and acetoxy group from acquiring a better fit on the receptor. The overall low inhibitory activities of these compounds, however, do not at this stage justify any inference about the topographical feature of the enzyme receptor, except perhaps that an eclipsed arrangement of the N⁺–C–C–O segment of cholinergics is probably not ideal for optimum interaction with the AChE receptor.

Another interesting observation is that the tertiary amino-esters (VII, VIII and IX) exhibit non-competitive inhibition of the AChE-catalysed hydrolysis of acetylcholine—a deviation from the behaviour of their quaternary ammonium analogues. But with such high K_1 values, especially those of VIII and IX, the borderline between non-competitive and mixed inhibition as shown by the Lineweaver-Burk plots is often unclear.

The optical antipodes of bornan-2-one-3-*endo*-yltrimethylammonium bromide, the only two compounds in this series with the nitrogen and oxygen atoms gauche to each other, are of interest. The (+)-isomer (X) has a K_i value of $1{\cdot}7 \times 10^{-4}$ showing a better fit to the enzyme receptor than choline and acetylcholine ($K_i = 4{\cdot}5 \times 10^{-4}$, $K_m = 4{\cdot}45 \times 10^{-4}$ respectively). The (−)-isomer (XI) on the other hand, is a weak AChE inhibitor ($K_i = 2{\cdot}5 \times 10^{-3}$). The stereoselectivity in their inhibitory action is remarkable when the absolute configuration of their N⁺–C–C–O segments, compared with the absolute conformation of the N⁺–C–C–O segments of

Table 1. *Enzyme-inhibitor dissociation constants (K_i) of some aminobornanes.*

	Compounds	Absolute configurations	N^+-C(3)-C(2)-O	K_i	Nature of inhibition
I	1(*R*),2(*R*),3(*S*),4(*S*)-2-*endo*-acetoxybornan-3-*endo*-yltrimethylammonium bromide	A	*ca* 0°	$1{\cdot}5 \times 10^{-3}$	Competitive
II	1(*R*),2(*S*),3(*S*),4(*S*)-2-*exo*-acetoxybornan-3-*endo*-yltrimethylammonium bromide	B	*ca* +120°	$2{\cdot}0 \times 10^{-3}$	Competitive
III	1(*R*),2(*S*),3(*R*),4(*S*)-2-*exo*-acetoxybornan-3-*exo*-yltrimethylammonium bromide	C	*ca* 0°	$2{\cdot}7 \times 10^{-3}$	Competitive
IV	1(*R*),2(*R*),3(*S*),4(*S*)-2-*endo*-hydroxybornan-3-*endo*-yltrimethylammonium bromide	D	*ca* 0°	$1{\cdot}4 \times 10^{-3}$	Competitive
V	1(*R*),2(*S*),3(*S*),4(*S*)-2-*exo*-hydroxybornan-3-*endo*-yltrimethylammonium bromide	E	*ca* +120°	$2{\cdot}6 \times 10^{-3}$	Competitive
VI	1(*R*),2(*S*),3(*R*),4(*S*)-2-*exo*-hydroxybornan-3-*exo*-yltrimethylammonium bromide	F	*ca* 0°	$2{\cdot}35 \times 10^{-3}$	Competitive
VII	1(*R*),2(*R*),3(*S*),4(*S*)-2-*endo*-acetoxy-3-*endo*-dimethylaminobornane hydrochloride	G	*ca* 0°	$2{\cdot}15 \times 10^{-3}$	Non-competitive
VIII	1(*R*),2(*S*),3(*S*),4(*S*)-2-*exo*-acetoxy-3-*endo*-dimethylaminobornane hydrochloride	H	*ca* 120°	$5{\cdot}3 \times 10^{-3}$	Non-competitive
IX	1(*R*),2(*S*),3(*R*),4(*S*)-2-*exo*-acetoxy-3-*exo*-dimethylaminobornane hydrochloride	I	*ca* 0°	$8{\cdot}8 \times 10^{-3}$	Non-competitive
X	1(*R*),3(*S*),4(*S*)-bornan-2-one-3-*endo*-yltrimethyl ammonium bromide ..	J	*ca* +60°	$1{\cdot}7 \times 10^{-4}$	Competitive
XI	1(*S*),3(*R*),4(*R*)-bornan-2-one-3-*endo*-yltrimethyl-ammonium bromide ..	K	*ca* −60°	$2{\cdot}5 \times 10^{-3}$	Competitive

[I] = 4·0 − 4·8 × 10^{-4}M.

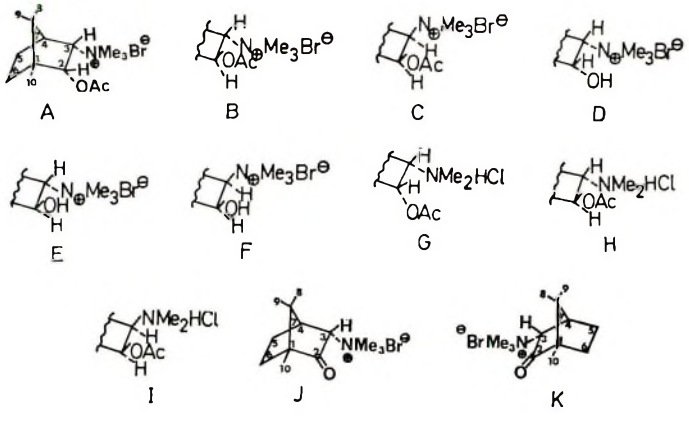


Table 2. *Relation between conformations and muscarinic activities of some cholinergic compounds.*

Compounds	τN^+-C-C-O	K_i	*Muscarinic activities
Acetylcholine	77°		1
X	+60°	$1{\cdot}7 \times 10^{-4}$	
XI	−60°	$2{\cdot}5 \times 10^{-3}$	
L(+)-Muscarine	+73°		0·4
D-(−)-Muscarine	−73°		330
L(+)-Acetyl-β-methylcholine	+85°		1
D(−)-Acetyl-β-methylcholine	−85°		240

* Number of molecules equivalent to 1 molecule of acetylcholine.

L(+)- and D(−)-muscarine, L(+)- and D-(−)-acetyl-β-methylcholine in the solid state (Canepa, Pauling & Sörum, 1966; Chothia & Pauling, 1969b) (see Table 2). The enantiomers of muscarine and acetyl-β-methylcholine are known to be stereoselective in their muscarinic activities (Waser, 1961; Beckett, Harper & Clitherow, 1963). The spatial arrangement of the N^+–C–C–O segment of the potent AChE inhibitor X resembles that of the potent muscarinics L(+)-muscarine and L(+)-acetyl-β-methylcholine in that their N^+–C–C–O torsions angles are all positive and vary between +60° to +85°. Their enantiomers, which have negative N^+–C–C–O torsion angles, are weak AChE inhibitors (XI) or weak muscarinics [D(−)-muscarine and D(−)-acetyl-β-methyl choline], suggesting incorrect fit on the enzyme or tissue receptors.

Previous comparative studies of optically active substrates or inhibitors of the enzyme AChE with their cholinomimetic or cholinolytic activity at the muscarinic receptor have suggested that a close structural similarity exists between the reactive site of the enzyme and of the muscarinic receptor (see references cited in Kay & others, 1970). The present work is an example in which stereoselective activities are shown by a pair of enantiomers with a rigid N^+–C–C–O segment, thus affording unambiguous interpretation of the topographical features of part of the enzyme receptor, and by inference, the muscarinic receptor.

If substrates for hydrolysis by AChE have to assume a *trans*-conformation, with τN^+–C–C–O = +150°, as postulated by Chothia & Pauling (1969), the fact that the AChE enzyme receptor can bind compound X well suggests that it has also the necessary structural features to accommodate cholinergics in the gauche (*cis*)-conformation, a conformation associated with potent muscarinics (Chothia, 1970). This may well explain the parallelism between the muscarinic receptors and AChE receptors in their identical pattern of absolute and relative stereoselectivities towards the dioxolane and muscarine series of stimulants (Belleau & Lacasse, 1964) and which has given rise to the contention that AChE and muscarinic receptor are structurally very similar if not the same.

A single type of enzyme receptor can accommodate the gauche- and *trans*-conformations of acetylcholine either (*a*) by being conformationally labile, and assuming two conformation arrangements corresponding to the two conformations of acetylcholine, or (*b*) by being conformationally non-labile, in which case duplicate sets of receptor sites sharing a common anionic site would be necessary.

To account for the stereoselectivity of these drug-receptor reactions, there must be more than two points of interaction between the drug and the receptors. Compound X and XI have only two recognizable points of attachment with the AChE enzyme receptor, i.e. the onium head and the carbonyl oxygen (corresponding to the ether oxygen of acetylcholine), and yet they also show stereoselectivity in their affinity to the enzyme receptor. There can be only one of two explanations:

(*a*) beside the N^+–C–C–O segment, there is a third point of interaction between the bornane molecule and the receptor, or (*b*) the N^+–C–C–O segment has more than two points of attachment with the receptor, a possibility hitherto unexplored.

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LETTERS TO THE EDITOR

Aspirin dissolution: polymorphism, crystal habit or crystal defects

Pfeiffer (1971) has questioned the evidence for the existence of polymorphs of aspirin and has asked that future publications on the subject should clarify a number of questions. We should like to clarify some points in our work.

Mitchell & Saville (1967) demonstrated that various samples of aspirin had different dissolution rates. However the samples examined were obtained from commercial sources and not recrystallized, as stated by Pfeiffer. Moreover since no differences were detected in X-ray diffraction powder patterns, infrared spectra and attenuated total reflectance of infrared we concluded that the variation in dissolution rates was *not* a result of polymorphism. Alternative causes such as differences in size and habit were considered at this time but there was no apparent correlation between these factors and dissolution rate. Hence it was suggested (Saville, 1968) that the variation in dissolution rate may result from differences in the type and number of crystal defects. Subsequently, Tawashi (1968) reported the recrystallization of two polymorphic forms of aspirin. Further studies by Mitchell & Saville (1969), using two samples of commercial aspirin that showed a large difference in dissolution rate, again failed to detect evidence for polymorphism but it seemed possible, assuming the existence of polymorphs, that some commercial samples are likely to be a mixture of polymorphs and hence their X-ray diffraction patterns may be indistinguishable from that of pure polymorphs. The effects of agitation and temperature on dissolution rates showed that the samples had different thermodynamic activities and that the metastable form was capable of rapid reversion to a more stable form. Continued investigation (Griffiths & Mitchell, 1971) has shown that the reversion occurs in the surface layers of the crystal only and that the bulk of the crystal is unchanged. It is not possible to identify the nature of the surface transformation from a kinetic and thermodynamic analysis and X-ray and attenuated total reflection (ATR) measurements failed to reveal any changes after exposure to the dissolution solvent. However, in view of Tawashi's work and the similarity of our results with other studies of transformation accompanying dissolution, it was suggested that the surface-change to a less-soluble form could be due either to the crystallization of a hydrate or a more stable polymorph.

Hydrate formation can now be ruled out since a dissolution pattern indicative of a surface transformation has been demonstrated in a non-aqueous dissolution medium (Mitchell & Milaire, unpublished work). Moreover we have been unable to produce aspirin crystals showing differences in X-ray diffraction powder patterns, infrared spectra (Nujol mull) or ATR using the recrystallization procedures described by Tawashi (1968). The crystals ranged from needles to prisms and it is likely therefore, as suggested by Pfeiffer, that the differences in X-ray diffraction pattern and infrared spectra found by Tawashi are not due to polymorphism but are orientation effects which could result from failure to subdivide the crystals to a sufficiently fine powder.

Summers, Carless & Enever (1970) claim to have found six polymorphs of aspirin, but since these were distinguished only by differences in melting point and density and exhibited only minor differences in X-ray diffraction pattern, we agree with Pfeiffer that this claim is not warranted.

It is well known that crystal faces dissolve at different rates. Hence failure to achieve a requisite degree of size-reduction may be responsible for the variation in dissolution rates of compressed discs prepared from crystals of different habit (Wood; personal communication). It is also conceivable that the phenomenon of reversion can be attributed to dissolution from a rapidly dissolving face and recrystallization onto a more slowly dissolving face. Nevertheless, the single crystal studies of Tawashi show that dissolution from the needle form is much greater than from any face of the prismatic form, and in our work, intrinsic dissolution rates, from a compressed disc of a given aspirin sample, were independent of particle size. Hence differences in crystal habit are unlikely to be the only factor involved in the observed variation in dissolution rates.

Further work to clarify the effect of size and habit is necessary but it is suggested that the effects of crystal defects on dissolution rate should be considered. Line defects, or dislocations, are thermodynamically unstable and Thomas (1970) has emphasized the important, often crucial, role played by dislocations in the reactivity of crystalline solids including such properties as crystal growth and dissolution. Variation in the conditions of crystal growth will affect the type and number of defects in a crystal. It is suggested therefore that the differences in the thermal properties of aspirin recrystallized by Tawashi (1968, 1969) and Summers & others (1970) may also be explained in terms of crystal defects.

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Interpretation of dissolution rate maxima: dependence upon tablet compression force

The relation between the dissolution rates of drugs from tablets and the force used to compress the tablets has not been well defined. Studies in this area have provided a variety of results which have not led to a simple or straightforward interpretation. Jacob & Plein (1968) demonstrated that the dissolution rates of several phenobarbitone tablets decreased with increasing tablet hardness. In a study of phenindione tablets by Ganderton, Hadgraft & others (1967) at least one maximum in the dissolution rate as a function of compression force was reported for each formula studied. Yen (1964) studied several formulations of Triamterene tablets. Both maxima and minima in the dissolution rates as a function of tablet hardness may be noted in his data. Armstrong & Griffiths (1970) measured the specific surface area of compacts of several different granulations as a function of compression force and found that maxima existed in this function for each of these granulations. This demonstrates the existence of a non-linear relation between force and the ratio of bonding to cleavage occurring during the compression event.

Non-linear data of this type are frequently described as complex because they do not lend themselves to a simple mechanistic interpretation. We suggest that one of the more logical interpretations of these data is in terms of dissolution dependency upon changes in particle size or specific surface area during tablet compression. When particle bonding is the predominating phenomenon during the compression event, dissolution rates should diminish. When, at another compression force, particle cleavage is predominant, the dissolution rate would increase. Further, the ratio of bonding to cleavage would not be linearly related to the compression force. Because of the scarcity of data to support this thesis, and of the possibility that some other phenomena occurring during compression might be responsible for the observed dissolution behaviour, there has been no general recognition of this possible explanation.

We have set out to directly assess the roles of tablet hardness, compression force used and resulting dissolution characteristics using Li_2CO_3 as the drug of choice with various concentrations of polyvinylpyrrolidone (PVP) as binder.

Tablets were prepared by fluid granulating the solid with six concentrations of PVP in ethanol. The basic formula was 300 mg of Li_2CO_3, 80 mg of lactose, 60 mg of corn starch and 5 mg of magnesium stearate per tablet. The PVP solutions were of a strength that provided tablet granulations containing from 4 to 16 mg of PVP per tablet. Each of the granulations was compressed using standard concave 7/16 inch punches on a Model F Stokes Machine maintaining constant tablet weight over a range of compression settings. This produced a series of tablets with constant weights and varying thicknesses for each of the binder concentrations. Tablet thickness is used as an inverse measure of tablet compression force.

Dissolution rates of batches of six tablets for each of the tablet series were determined in 0·1N HCl according to the USP XVIII. The rotational speed was 100 rev/min. Samples were collected every 2 min for 2 h and assayed for lithium. Thickness was measured with a micrometer. Hardness was determined as the crushing force with the Stokes Hardness Tester.

Dissolution curves, obtained by plotting the amount of Li_2CO_3 dissolved as a function of time, resulted in smooth lines for all tablets. The mg of Li_2CO_3 dissolved after 20 min was used as a measure of the initial dissolution rate. These rates were plotted as a function of tablet thickness (a measure of compression force) for tablets containing each of the six levels of PVP. The typical plot for the tablets containing 8 mg per tablet of PVP is given in Fig. 1.

These plots exhibited two maxima when sufficient data were available for completely characterizing them between thickness of 0·19 and 0·24 inches. One at lower compression forces (greater thickness) and a second at higher compression forces. It is suggested that the low compression force maxima are related to the structure and crushing characteristics of the granules and, therefore, to the concentration of the PVP binder. Fig. 2 is a plot of the PVP concentration as a function of the tablet

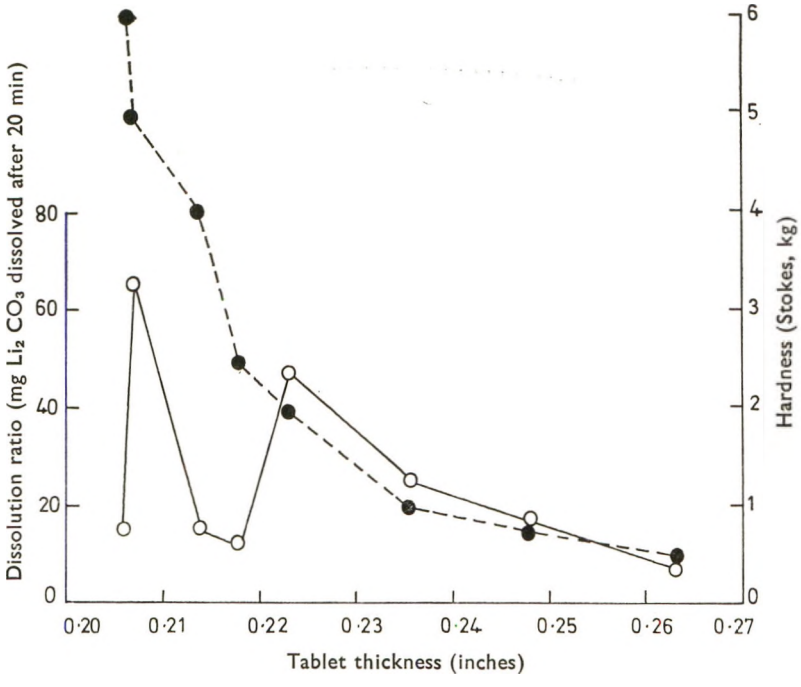
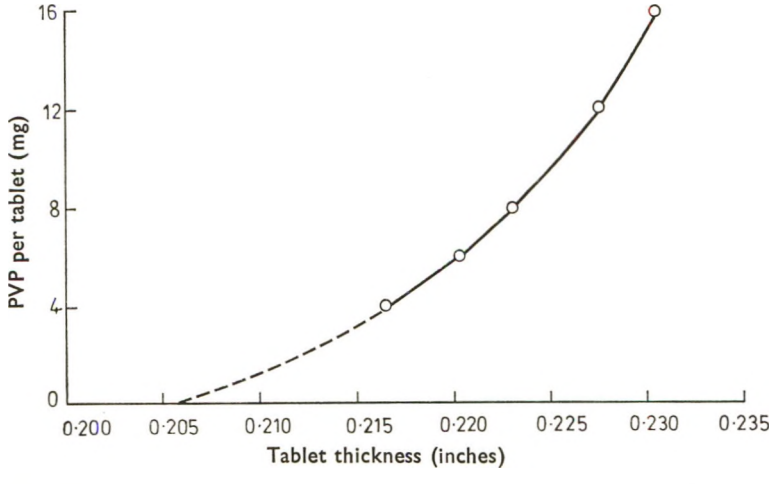


FIG. 1. Dissolution rate (○—○) of Li_2CO_3 tablets containing 8 mg per tablet of PVP as a function of tablet thickness for tablets of constant weight (left-hand scale). Hardness (●—●) of same tablets with varying thickness (right-hand scale),



FIG, 2. Tablet thickness for maximum in dissolution rate (low compression force) for Li_2CO_3 tablets with varying quantities of PVP for tablets of constant weight.

thickness at which the low compression force maxima occurred. Up to this maximum, fission of the granular structure results in increased dissolution release rate with increasing pressure. Further pressure results in a compact fusion or bonding which markedly reduces dissolution rate. At pressures higher again, there is an increased dissolution rate, indicating a new relative dominance of a cleavage component. It is further suggested that the high compression force maxima are related to the inherent compression characteristics of the basic formulation components and, therefore, should be essentially independent of the binder concentration. This holds for the values for the high compression maxima since at concentrations of PVP of 4, 6, 8, 12, 14 and 16 mg/tablet, dissolution rate maxima (expressed as thickness in inches for tablets of constant weight) are respectively: $\leqslant$0·205, $\leqslant$0·203, 0·208, $\leqslant$0·205, 0·208, 0·203 (mean 0·205). It should follow that the value for the low compression maximum should coalesce with that of the high compression maximum at zero concentration of PVP. The dotted line which is extrapolated to zero percent of PVP in Fig. 2 indicates this to be a reasonable assumption.

From Fig. 1 it is apparent that hardness increases as the tablet thickness decreases. The increases are slow in that area of compression that has been cited above as predominately cleavage rather than fusion. Where dissolution rates decrease as a result of fusion becoming the primary process, the hardness rises more rapidly.

From this and, by implication from the cited references, it is apparent that a full compressional study for a given granulation formulation is essential to determine the compression parameters that will assure adequate bioavailability.

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Inhibition of 5-hydroxyindoleacetic acid transport from the spinal fluid by probenecid

It is generally accepted that 5-hydroxyindoleacetic acid (5-HIAA) in brain ventricular fluid derives from the metabolism of 5-hydroxytryptamine (5-HT) in the brain (Guldberg & Yates, 1968). Probenecid blocks active transport of 5-HIAA from ventricular fluid to blood and this is followed by an increase of 5-HIAA in ventricular and consequently in cisternal fluid (Guldberg, Ashcroft & Crawford, 1966). This active transport mechanism could be located in the choroid plexus (Ashcroft, Dow & Moir, 1968) or in the ependyma of the ventricles. We have observed that 5-HIAA in the fluid of spinal subarachnoid space (spinal fluid) derives from metabolism of 5-HT in the spinal cord. Since neither ependymal tissue nor structures similar to choroid plexus are present in the spinal subarachnoid space, the question arises of how 5-HIAA is removed from the spinal fluid. In these preliminary experiments we report that transport of 5-HIAA from spinal fluid can be inhibited by probenecid.

Experiments were made on adult cats lightly anaesthetized with thiopentone sodium. After thoraco-lumbar laminectomy, an extradural ligature of the spinal cord was placed at the T_{11} segment and saline (control) or probenecid (200 mg/kg) were administered intraperitoneally. Two h later, a sample of the cisternal fluid, obtained by percutaneous puncture of the cisterna magna, as well as a sample of the spinal fluid below the ligature were taken for analysis of 5-HIAA (Ashcroft & Sharman, 1962). Although mixing of cisternal and spinal fluid is negligible (Davson, 1967), the ligature was made to prevent any potential access of 5-HIAA from cisternal into spinal fluid below the ligature, as well as withdrawal of cisternal fluid into the syringe during the sampling of the spinal fluid. The ligature itself did not influence normal concentrations of 5-HIAA in cisternal and spinal fluid.

Table 1. *Concentrations of* 5-HIAA *in cisternal and spinal fluid* (ng/ml) *2 h after intraperitoneal application of probenecid* (200 mg/kg) *or saline (control).*

	5-HIAA (ng/ml)			
	Saline	Probenecid	Increase %	*t*-Test
Cisternal fluid	120 ± 10* (4)	250 ± 7 (3)	207	$P < 0.001$
Spinal fluid	103 ± 5 (4)	226 ± 11 (4)	221	$P < 0.001$

*Mean ± standard error of the mean.
Figures in parentheses represent number of experiments.

Table 1 shows that 2 h after probenecid, 5-HIAA significantly increases in both the cisternal and spinal fluid. Increase of 5-HIAA in the cisternal fluid after probenecid (Table 1) supports previous finding that 5-HIAA is actively transported from ventricular fluid to blood (Guldberg & others, 1966). Concomitant augmentation of 5-HIAA in the spinal fluid (Table 1) indicates that 5-HIAA is also actively transported out of the spinal fluid. Our results, therefore, suggest that the active transport system for the removal of 5-HIAA out of cerebrospinal fluid is not exclusively located in the choroid plexus or ependyma, since such a mechanism may exist in the spinal subarachnoid space. It has been shown recently that radioactive iodide may also be actively transported from spinal fluid into blood (Lorenzo, Hammerstad & Cutler, 1970).

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"Free" and "bound" acetylcholine concentrations in rat brain: variability in determination of "free" acetylcholine fraction

Crossland & Slater (1968) have reported a method for simple fractionation of the brain acetylcholine into "free" (extracted with eserinized saline) and "bound" (extracted with acid-ethanol) components and have described the effect of some drugs on these fractions. Although the identity and the physiological significance of these fractions was not clear, they were, nevertheless, differentially affected by various groups of drugs.

We now describe our experience with determination of the "free" acetylcholine fraction using a modified approach.

Male Sprague-Dawley rats, 180–220 g were killed by dipping into liquid nitrogen for 10 s ("near-freezing" method of Takahashi & Aprison, 1964). Freezing and subsequent thawing of brain tissue under this condition does not occur. After decapitation the brains were carefully removed from the skull and weighed rapidly. The "free" and "bound" acetylcholine from the whole brain (without cerebellum, pons and medulla) were then extracted according to the procedure of Crossland & Slater (1968), or in other experiments, the "total" fraction from whole brain was extracted with acid-ethanol using the method of Crossland (1961). Assays were performed using the frog (*Rana temporaria*) rectus abdominis muscle sensitized with eserine sulphate ($1{\cdot}6 \times 10^{-5}$ M). Samples of tissue extracts were tested in a double-bracketed assay against standard solution of acetylcholine iodide prepared in alkali-inactivated parts of the same extracts (Feldberg, 1945). The recovery of acetylcholine added to the tissue homogenate was 90%.

During the bioassays we consistently noticed that the response of the frog rectus to the "free" acetylcholine samples declined after the first exposures of the muscle to this extract. This was not so when the acid-ethanol extracted samples of "bound" or "total" acetylcholine were assayed. This observation suggested to us that the direct estimation of the "free" fraction from the supernatant, obtained after extraction of the brain tissue with eserinized saline may yield false low values. We therefore decided to estimate directly the amount of "total" and "bound" fractions, both extracted with acid-ethanol from pooled opposing halves of the brains of a pair of animals ($R_1 + L_2$ for "total", $R_2 + L_1$ for "bound"). The values for the "free" fractions were then calculated by subtracting the values of "bound" from the values of "total" acetylcholine. The results are in Table 1.

Table 1. *Comparison of the values of "free", "bound" and "total" acetylcholine in whole rat brain.* Method 1: Direct estimation of "free" and "bound" acetylcholine and calculation of the value for "total" acetylcholine. Method 2: Direct estimation of "total" and "bound" acetylcholine and calculation of the value for "free" acetylcholine.

	"Free" ACh	"Bound" ACh	"Total" ACh	Ratio (%)		
	nmol/g ± s.e.			F/T	F/B	B/T
Method 1	3·38 ± 0·33 (7)	11·74 ± 0·87 (7)	15·12 ± 0·88[a] (7)	22·3	28·8	77·6
Method 2	6·98 ± 0·45[b] (12)*	10·16 ± 0·48 (12)	17·75 ± 0·48 (16)	39·3	68·7	57·2

[a] Calculated value obtained by adding the individual values of "free" and "bound" acetylcholine.
[b] Calculated values obtained by subtracting the individual values of "bound" from the values of "total" acetylcholine.
* Significantly different from the value obtained by method 1; $P < 0.001$.
In parentheses: number of animals.
Ratio F/T—"free"/"total": F/B—"free"/"bound": B/T—"bound"/"total" acetylcholine.

The discrepancy between the values of "free" acetylcholine estimated directly and by using the second approach are obvious. The same applies for the ratio "free"/"total" fractions. Lower values of directly estimated "free" fraction may account for lower values of calculated "total" as compared with directly measured "total" amounts. The latter values are in good agreement with those reported by Crossland, Pappius & Elliot (1955), Stone (1955) and Takahashi & Aprison (1964) using the same method of extraction and assay. The reported "free"/"total" ratio of rat brain varies. Crossland & Slater (1968) found it to be in the range 9·2–15%, Milosevic (1970) found 30% and Richter & Goldstein (1970) 25%. We found 22·3% Using a different approach (calculating the values for "free" from values of "total" and "bound" acetylcholine) we obtained a ratio of 39·3%.

Direct determination of the "free" fraction is subject to high degree of variability due presumably to varying efficiency of extraction procedure (Richter & Goldstein, 1970). Our experiments further emphasize problems involved in attributing physiological significance to the fractionation of brain acetylcholine to "free" and "bound" portions. Such fractionation has, however, shown some utility in studying the effect of drugs on brain concentrations of acetylcholine.

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Amphetamine toxicity in genetically aggressive and non-aggressive mice

Amphetamine and isoprenaline in high doses produce a fatal myocardial necrosis in mice and rats (Chappel, Rona & others, 1959; Halpern, Drudi-Baracco & Bessirard, 1962). This susceptibility is increased by rearing the animals in isolation, or by grouping the animals while under the influence of the drugs during the experiment, as the LD50 for these drugs shows (Balazs, Murphy & Grice, 1962; Consolo, Garattini & Valzelli, 1965; Welch & Welch, 1966; Moore, 1968). Isolation of mice also renders them more aggressive (Allee, 1942; Scott & Fredericson, 1951), and aggressiveness has been associated with increased amphetamine toxicity (Consolo & others, 1965; Welch & Welch, 1966).

Whether increased amphetamine toxicity is invariably associated with aggressiveness, or whether it is an outcome of isolation only, and thus not a necessary condition of aggressiveness, has now been tested with strains of mice selectively bred for aggressiveness or non-aggressiveness (Lagerspetz, 1961, 1964, 1969).

Male mice from the 16th–19th generations of offspring from selective breeding for aggressiveness (strain TA) or non-aggressiveness (strain TNA), about 30 g, had (+)-amphetamine sulphate intraperitoneally in doses ranging from 29 to 85 mg/kg calculated as (+)-amphetamine. Mice of the 16th to 18th generations were isolated after weaning, and reared in isolation for 5 to 6 months, save for occasional aggressiveness tests, the last of which was made in the 16th generation not later than 3 weeks before the experiment, in the 17th generation 3 days before it, and in the 18th generation 3 months earlier. The mice of the 19th generation had lived together with their male siblings in groups of at least 5 animals for 6 months after weaning. During the experiment, which lasted for 24 h, the animals were kept in isolation at 23°. Altogether, 65 isolated TA mice and 81 isolated TNA mice were used, as well as 20 of each strain that had been living grouped.

The aggressiveness of the test mice had been earlier measured using the 7-point rating scale for aggressiveness in male mice (Lagerspetz, 1961, 1964). The mean aggressiveness scores for the isolated TA and TNA mice were 6·0 and 3·2, respectively. This difference is significant at the level of $P < 0{\cdot}002$ (Mann-Whitney U-test; Siegel, 1956). The aggressiveness scores of the grouped mice were low (1·5) and equal in both strains.

The LD50-values for (+)-amphetamine were 58 and 59 mg/kg for the isolated animals of the two strains and 69 and 68 mg/kg for the grouped animals. The values did not differ significantly from each other, but did differ between isolated and grouped animals.

In the non-aggressive strain, in each generation there appear a few individuals with a high aggressiveness score (Lagerspetz, 1961, 1964). The survival times of these individuals after the administration of lethal doses of (+)-amphetamine, and the doses after which they survived were not different from those of the TNA animals with a low aggressiveness score.

The genetically aggressive and non-aggressive mice show no overt aggressive behaviour and an equal amphetamine toxicity when reared in groups. When reared in isolation, the aggressiveness is increased much more in mice of the aggressive strain, but the amphetamine toxicity is increased to the same degree in both strains. Thus, high amphetamine toxicity is not invariably linked with high aggressiveness in mice. It seems more probable that increased amphetamine toxicity is associated with changes in other variables than aggressiveness, produced by isolation.

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Some effects of butoxamine on glycolysis in the mouse brain

N-t-Butylmethoxamine (butoxamine) prevents the rise in blood free fatty acids, glucose and lactate after the administration of adrenaline or isoprenaline (Burns & Lemberger, 1965; Salvador & April, 1965). The drug also produces a selective blockade of some, but not all, β-adrenergic receptor sites in the anaesthetized dog (Levy, 1966) and can therefore be clearly distinguished from such β-adrenoceptor blocking drugs as dichloroisoprenaline and pronethalol (Pilkington, Lowe & others, 1962) and propranolol (Salvador, April & Lemberger, 1967).

The effects of butoxamine on brain glycolysis are now reported.

Groups of 5 specific pathogen free mice (18–22 g) of either sex and of the Alderley Park strain were given the drug intraperitoneally and the animals transferred to a room maintained at $38 \pm 1°$. At different times after injection, groups were killed by total immersion into liquid nitrogen. The brains were removed while still frozen and triturated with cold 10% trichloracetic acid. "Bound" glycogen was estimated in the acid insoluble material by the method of Russell & Bloom (1958). Lactate, pyruvate, glucose and "free" glycogen were estimated in aliquots of the acid soluble fraction by enzymatic methods (Leonard, 1971).

The relation between the effect on some parameters of brain glycolysis are shown in Fig. 1. At 20 mg/kg, butoxamine produced a significant decrease in glucose and "free" glycogen and a significant rise in "bound" glycogen and pyruvate. Brain lactate did not change appreciably. Doses of butoxamine lower than 20 mg/kg did not produce any noticeable change in the concentrations of these substances. The changes produced were not correlated with any change in behaviour; the mice were not unduly affected by this dose of drug compared with those given physiological saline alone.

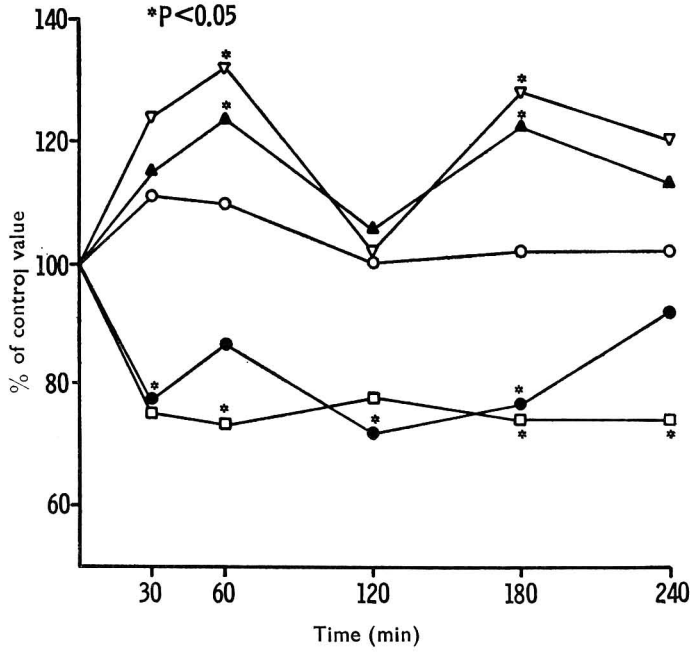

FIG. 1. Effect of butoxamine on mouse brain glycolysis. Butoxamine was administered in a dose of 20 mg/kg i.p. Each point represents the mean of at least 5 animals. All values are compared with controls (100%). The absolute values (μmol/g wet wt; mean $\pm$ s.e.) for the individual parameters were: "Bound" glycogen: 1·68 $\pm$ 0·109 (as glucose). "Free" glycogen: 0·72 $\pm$ 0·063 (as glucose)· Pyruvate: 0·113 $\pm$ 0·02. Lactate: 2·17 $\pm$ 0·23. Glucose: 0·430 $\pm$ 0·002. Significance of difference from controls shown by $*P < 0{\cdot}05$.

It is apparent that butoxamine, unlike β-adrenoceptive blocking drugs such as propranolol, has a stimulant type of neurochemical profile in that it increases brain glycolysis. In this respect it more closely resembles the action of sotalol (MJ 1999) than propranolol (Leonard, 1971). It seems that the nature of the β-adrenergic receptor(s) in the brain is unlike those found peripherally.

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The effect of angiotensin II on the release of catecholamines in the sheep

Numerous investigations have been concerned with a possible relation between the renin-angiotensin system and the secretion of catecholamines from the adrenal medulla and autonomic nerve endings (McGiff & Fasy, 1965; Palm, 1968; Pals, Fulton & Masucci, 1968; Peach & Ford, 1968). The present investigations were concerned with the effect of large intravenous doses of angiotensin II, and of a mixture of adrenaline and noradrenaline, on plasma levels of vanilmandelic acid and on urinary concentrations of the catecholamines and their metabolites.

The angiotensin II was synthetic asparaginyl[1]-valyl[5]-angiotensin II (Hypertensin, Ciba); the adrenaline was supplied by Gale Baiss and Co. Ltd., and the noradrenaline acid tartrate (Levophed) by Bayer Products. Saline solutions of the three hormones were made to the required strength and constant infusions (0·7 ml/min from a 50 ml capacity syringe) were made with a slow injection apparatus (C. F. Palmer, London, Ltd.).

A Kerry Hill ewe, 35 kg, was prepared as described by Osborn, Hughes & others (1969). A 20 ml blood sample was taken from the femoral artery 45 min after preparation, and 15 min before the start of an infusion of 2 μg/kg min^{-1} of angiotensin II into the jugular vein. The blood was added to a 25 ml polystyrene bottle, containing 5 ml of an EDTA-thiosulphate solution (Weil-Malherbe, 1961). Similar samples were prepared from femoral artery blood collected 1, 2, 3, 5, 15, 29 and 90 min after the start of the infusion of angiotensin which lasted 30 min. Urine was collected from the bladder *via* an indwelling catheter for 60 min before (Control, Table 1), during, and for 15 min (Angiotensin II infusion, Table 1) after the infusion, then for a further 75 min (Resting, Table 1).

An infusion of a mixture of adrenaline and noradrenaline (2 μg/kg min^{-1} of each) for 30 min was begun 90 min after the end of the infusion of angiotensin. Arterial blood samples were taken 5 min before the end of the infusion of the catecholamines and urine was collected during the infusion and for 15 min afterwards (Catecholamine infusion, Table 1).

The mean resting blood pressure of about 90 mm Hg rose to 145 mm Hg during the initial 10 min of the angiotensin II infusion; thereafter the pressure gradually fell so that it was about 110 mm Hg at the end of the infusion. A similar initial rise resulted from the administration of the catecholamines but it was much better maintained.

Duplicate estimations of vanilmandelic acid were made (O'Gorman, 1968) in plasma prepared from the blood samples by centrifugation at 800 *g* for 10 min at 4°; the plasma was subsequently stored at −20° before analysis. The urine was made approximately 0·2N with concentrated HCl immediately after collection. One-fifth aliquots were transferred to polystyrene bottles and stored at −20°. These were subsequently analysed for vanilmandelic acid (O'Gorman, 1968), and for metadrenaline and normetadrenaline (Gjessing, 1964). Aliquots (40 ml) were analysed for adrenaline, noradrenaline and dopamine by adsorption onto alumina at pH 8·4 using Gout's method as described by Udenfriend (1962) after a preliminary hydrolysis by boiling under reflux for 10 min. Adsorption onto Amberlite columns was at pH 6·1; the subsequent elution was with N HCl. Aliquots of the eluates were analysed by the trihydroxyindole method (von Euler & Lishakjo, 1961) at 3·5 and 6·5 and by the ethylenediamine condensation method (Weil-Malherbe, 1961). The sum of the three catecholamines is given by the condensation method while adrenaline and noradrenaline are estimated by the trihydroxyindole procedure. The difference between the two is the amount of dopamine.

Table 1. *Urinary excretion of catecholamines and metabolites* ($\mu g/min$). The amounts of urine collected for the successive periods were 114, 90, 88 and 344 ml respectively.

	Control	Angiotensin II infusion	Resting	Catecholamine infusion
Adrenaline	0·01	0·01	0·02	0·75
Noradrenaline	0·09	0·09	0·04	0·51
Metadrenaline	0·05	0·05	0·02	0·15
Normetadrenaline	0·07	0·04	0·02	0·18
Vanilmandelic acid	0·18	1·1	0·18	4·44
Dopamine	0·02	0·06	0·09	0·20

The average level of plasma vanilmandelic acid of 155 ng/100 ml (s.d = $\pm$ 10 ng/100 ml) during the infusion of angiotensin II was similar to the level before the infusion (165 ng/100 ml). The plasma sampled immediately before the infusion of the catecholamines contained 190 ng/100 ml; the concentration had risen to 260 ng/100 ml towards the end of this infusion.

The results of the urinary analyses are shown in Table 1. The output of urine was reasonably constant before, during, and after the infusion of angiotensin II. However it was much increased during that of the catecholamines.

The findings show that the infusion of catecholamines caused a rise in plasma vanilmandelic acid and this was reflected in an approximate 25-fold increase in the urinary excretion of this compound. In addition there was a significant increase of several other catecholamine metabolites. By contrast, angiotensin II, which produced a comparable rise of blood pressure, caused no detectable change in plasma vanilmandelic acid; however its infusion was associated with a significant rise in urinary vanilmandelic acid excretion. This is in keeping with previous suggestions that angiotensin II is associated with catecholamine release and our results suggest that measurement of urinary vanilmandelic acid is an acceptable index of this action.

The experiment was made at the Dr. Leonard West Research Laboratory, Sully Hospital, Sully, Glamorgan, Wales, and the expert technical assistance of Mr. J. Wilson is gratefully acknowledged.

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The effect of angiotensin I on renal blood flow in sheep

Injections of angiotensin I or angiotensin II into the renal artery of the dog are immediately effective in reducing renal blood flow (Halvorsen, Fasciolo & Calvo, 1959; Barac, 1962). But Ng & Vane (1967, 1968) found angiotensin I to have no immediate constrictor effect on the renal vessels of the dog, and to require conversion to angiotensin II in the pulmonary circulation to be effective. We now report the action of angiotensin I on renal blood flow in the sheep.

The angiotensin I used was synthetic asparaginyl^{1}-valyl^{5}-angiotensin I (Osborn, Pickens & others, 1970) which is the angiotensin I equivalent of the angiotensin II (Hypertensin). Both hormones were supplied by Ciba Ltd., Basle. When the angiotensin I was tested against the angiotensin II using the rat isolated colon (Regoli & Vane, 1964) in Tyrode solution in twelve experiments, the material was shown to contain less than 1% of angiotensin II.

Five Kerry Hill ewes 35 kg (s.d. = $\pm$ 2 kg) (Osborn, Hughes & others, 1969), had renal blood flow measured (Cohn & Gombos, 1965). Renal vein blood was withdrawn by a 105 A Gilford constant withdrawal pump, through a 103 IR Gilford cuvette densitometer. The dye curves were recorded on a Moseley 710 BM recorder and the areas under the curves estimated after extrapolation of the down stroke. Calibration was effected *in vitro* by adding various amounts of indocyanine green to renal vein blood.

The indocyanine green (0·25 mg in 2 ml saline) was injected into the renal artery over 1 s. As the internal volume of the catheter was 0·2 ml, 1·8 ml of the dye was injected into the artery. Blood was sampled from the renal vein at a constant rate before, during and after the injection of the dye until the extinction of the blood remained constant for 10 s.

The validity of the approach was tested with the angiotensin II in an experiment in which doses of 0·02–1·0 μg of the hormone were injected into the renal artery. Injections in all experiments were made as 5 ml solutions in saline followed immediately by a wash with 2 ml of saline over 2 s. Each injection of the hormone was followed 5 min later by 7 ml of saline given as a divided dose of 5 ml (over 5 s) and 2 ml (over 2 s).

The duration of the effect of 0·20 μg of angiotensin II was estimated by injecting the indocyanine green 6, 10, 15, 20, 30, 45 s and 1, 2 and 3 min after the end of the injection of the hormone. This dose and these times were chosen since about 10% of the cardiac output flows through each kidney and because previous studies in sheep had shown that 2 μg of angiotensin II injected into the left ventricle usually increased the blood pressure by 15–20 mm Hg, the pressor effect lasting for about 3 min. The results demonstrated that angiotensin II caused a significant reduction in renal blood flow within 10 s of the conclusion of the injection of the hormone; the effect was maximal at + 15 s and was well maintained for a further 30 s. Thereafter it gradually declined so that at + 3 min the flow had returned to normal.

These results indicated that a suitable time at which to inject the indocyanine green was 20 s after the administration of the hormone; further experiments were therefore made in the same animal in which the dye was injected 20 s after doses of 0·02, 0·05,

0·10, 0·20, 0·50 and 1·0 μg of angiotensin III. These amounts reduced the average control flow of 280 ml/min by 5, 10, 25, 40, 45 and 55% respectively.

Both angiotensins were used in the four other animals. The effect of angiotensin I on renal blood flow was shown to be dose-dependant in a similar manner to that for angiotensin II, in the first of these animals; a dose of 0·50 μg of angiotensin I produced a similar reduction in flow to that given by 0·20 μg of angiotensin II and the duration of its effect was similar. Six injections of 0·50 μg of angiotensin I and six of 0·20 μg of angiotensin II were therefore made in this animal and also in the three others; the injections were made in the sequence saline, angiotensin I, saline, angiotensin II and the indocyanine green was injected 20 s after the administration of the angiotensin. The control blood flow varied from 10–12 ml/kg min^{-1} and the average reduction in flow was comparable for the hormones used in these doses (40% after angiotensin I and 45% after angiotensin II; the s.d. was $\pm$ 5% of the mean in both cases). Analysis of variance (Fisher & Yates, 1948; Moroney, 1951) showed that both hormones gave a highly significant reduction in flow ($P < 0{\cdot}001$ in every experiment).

Our findings show that angiotensin I reduces renal blood flow in sheep within less than one circulation time which is 16 s but it is less effective than angiotensin II in this respect. From previous studies we have shown that only about 15% of a given dose of the angiotensins into the renal artery of sheep escapes into the general circulation. Thus, apart from its direct effect, a dose of 0·5· μg of angiotensin I would have produced little or no reduction in renal blood flow even had the portion which escaped immediate removal by the kidney been subsequently fully converted to angiotensin II in the pulmonary circulation.

The expert technical assistance of Mr. J. Wilson and Mr. O. F. Mason is gratefully acknowledged. The investigations were carried out in the Dr. Leonard West Research Laboratory of Sully Hospital.

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5-Hydroxytryptamine and monoamine oxidase in adult and foetal sheep blood vessels

5-Hydroxytryptamine (5-HT) is a potent constrictor of sheep umbilical blood vessels (Dyer, 1970a). Cocaine potentiated the contractions to 5-HT in sheep isolated umbilical artery and vein and inhibited its uptake into these blood vessels (Dyer, 1970b). We now report the presence of monoamine oxidase and 5-HT in sheep umbilical, foetal and maternal blood vessels. Blood vessels were removed from pregnant sheep at term under pentobarbitone (i.v.) or spinal anaesthesia (lignocaine) (Dyer, 1970a).

Monoamine oxidase activity in 10% tissue homogenates was measured (Kraml, 1965) by the formation of 4-hydroxyquinoline from kynuramine (Century & Rupp, 1968). 5-HT was analysed by the method of Udenfriend, Weissbach & Brodie (1958).

The umbilical vein had about three times the 5-HT concentration of the umbilical artery. Monoamine oxidase activity was found in all blood vessels examined (Table 1). The foetal and umbilical blood vessels contained similar amounts of monoamine oxidase. Although only a limited number of maternal blood vessels were examined, it appears that the maternal monoamine oxidase activity was greater than in foetal or umbilical blood vessels.

Table 1. *5-Hydroxytryptamine concentration and monoamine oxidase activity in sheep tissues.*

	Umbilical vein	Umbilical artery	Foetal portal vein	Foetal aorta	Maternal portal vein	Maternal aorta	Whole cotyledon
			5-HT concentration (μg/g wet tissue)				
N	7	7	5	5	3	2	7
$\bar{X}$	0·38	0·13	0·32	0·17	0·25	0·41	0·22
s.d.±	0·22	0·10	0·06	0·09	0·10	0·18	0·05
			Monoamine oxidase activity (μg 4-hydroxyquinoline/g h^{-1})*				
N	9	8	6	4	4	3	5
$\bar{X}$	13·3	11·5	13·4	11·3	18·0	40·0	4·9
s.d.±	6·7	6·4	6·4	3·3	5·9	15·6	1·5

* Activity expressed as μg 4-hydroxyquinoline formed from kynuramine per gram of wet weight of tissue per hour at 37°, with air as the gas phase.

Our experiments provided evidence for the presence in sheep of endogenous 5-HT and monoamine oxidase in foetal maternal and umbilical blood vessels. Monoamine oxidase may play a role in the termination of the action of 5-HT in sheep umbilical blood vessels as well as the previously described uptake mechanism.

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Potentiation by desipramine of the pressor and depressor effects of dopamine

Imipramine and desipramine are known to potentiate the peripheral effects of noradrenaline and other amines. These facts supported the hypothesis relating the antidepressant action of imipramine to the activation of adrenergic mechanisms in the brain (Sigg, 1959).

The demonstration of the inhibition of catecholamine uptake in peripheral tissues and in the brain by imipramine-like drugs (Dengler & Titus, 1961; Glowinski & Axelrod, 1964) has provided a biochemical basis for the suggested mechanism of their antidepressant effect (Sulser, Bickel & Brodie, 1964) and the interactions with endogenous amines.

Tricyclic antidepressants potentiate the inhibitory effects of noradrenaline at the synaptic level (Cairncross, McCulloch & others, 1967; Kądzielawa, Gawęcka & Kądzielawa, 1967, 1968; Kądzielawa & Widy-Tyszkiewicz, 1969). We have found that imipramine and desipramine activate the depressor action of dopamine and we now report an analysis of this effect.

Male rats, 250–350 g; guinea-pigs, 400–500 g; rabbits, 3–3·5 kg, and cats, 2·8–3·5 kg were anaesthetized with urethane (25% soln) at doses of 0·7 ml/100 g, subcutaneously for rats and 0·9 ml/100 g for guinea-pigs; 1·7 g/kg, intraperitoneally for rabbits and 1·0/kg for cats. Blood pressure was recorded from cannulated carotid common artery by means of a mercury manometer. Drugs were dissolved in normal saline and injected through a polythene cannula in the femoral vein. In rats and guinea-pigs the amount of injected solution did not exceed 0·1–0·2 ml/100 g. Desipramine hydrochloride and dopamine hydrochloride was used in these experiments. The doses refer to the salts. In guinea-pigs, rats and rabbits dopamine (2·5–30 μg/kg) produced an acute fall in blood pressure, with gradual recovery to normal values in few minutes, depending upon the dose used. In cats anaesthetized with urethane, dopamine (10–30 μg/kg) induced a biphasic response; an acute and short lasting increase in blood pressure followed by a decrease in pressure lasting for 2–5

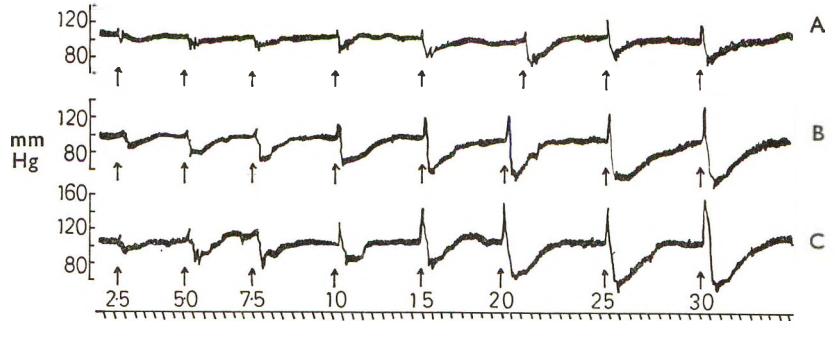

FIG. 1. The influence of a sequence of doses of dopamine (in μg/kg) on the blood pressure in a cat anaesthetized with urethane. A, Before, B, 30 min and C, 90 min after i.v. desipramine (6 mg/kg). Time marker in min.

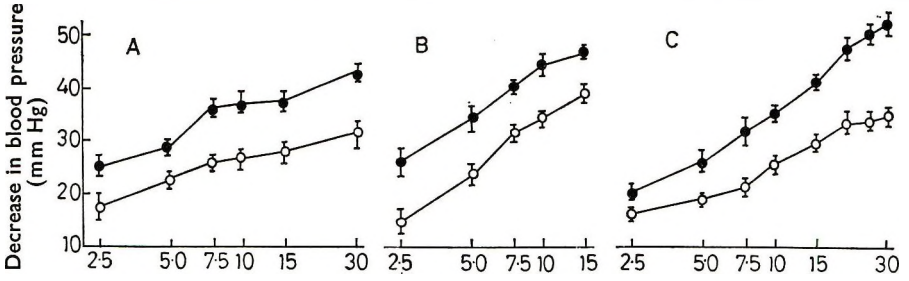

FIG. 2. Potentiation by desipramine (6 mg/kg) of the depressor effect of dopamine in animals anaesthetized with urethane: A. Rabbits (15 exp.). B. Guinea-pigs (25 exp.). C. Cats (14 exp.). —○—, Normal response to dopamine, —●—, the effect of a sequence of dopamine doses applied 30 min following desipramine. Abscissa: doses of dopamine (μg/kg) in log scale.

min (Fig. 1A). In cats anaesthetized with chloralose (80 mg/kg, i.p.) dopamine (10–30 μg/kg) induced a pressor response, which was dominant, with a secondary depressor effect, this action is similar to that seen after adrenaline.

Desipramine (3–6 mg/kg) potentiated both the pressor and depressor effects of dopamine (Fig. 1). The potentiation of the depressor effects of dopamine in the three species anaesthetized with urethane is illustrated in Fig. 2. The near parallel shift of the dose-response curves indicates a true potentiation and the results are statistically significant with $P < 0{\cdot}01$ for 7·5–30 μg/kg doses of dopamine.

Bonaccorsi & Garattini (1966) noted a potentiation by desipramine (3 mg/kg) of the pressor response to dopamine (10 and 20 μg/kg) in pithed rats. Previously only Eble (1964) has mentioned the augmentation by imipramine (2 mg/kg) of both the systemic pressor and depressor effects of dopamine (10 and 20 μg/kg) in dogs anaesthetized with pentobarbitone.

According to Løvtrup (1967) imipramine is a membrane stabilizer comparable to chlorpromazine. Present results suggest that both the inhibitory and excitatory effects of dopamine (or other amines) in the brain can be accentuated by imipramine and desipramine. This may be a factor in the mechanism of action of tricyclic antidepressants as well as in the modification in electrophysiological responses to monoamine precursors (Kądzielawa & Widy, 1970).

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Rapid accumulation of acetylcholine in nerve above a crush

An accumulation of axoplasmic material in crushed or cut axons has been attributed to interruption of a proximo-distal movement of material in the axons (Weiss & Hiscoe, 1948). The transmitter noradrenaline has been found to accumulate rapidly after ligation of nerves containing adrenergic fibres (Dahlström & Häggendal, 1966; Kappeller & Mayor, 1966). The noradrenaline is probably stored in amine granules that are transported distally in the axon. These neurons have been extensively studied with respect to this transport, which is of the "fast" type (several mm per hour, Dahlström & Häggendal, 1970). In cholinergic nerves, Sastry (1956) observed that acetylcholine increased above a nerve section. Further studies by Evans & Saunders (1967) revealed an increase in acetylcholine proximal to a crush made in nerves 3–25 days earlier. The present report describes some results from studies of acetylcholine accumulation in nerves up to 24 h after crushing, with an approach similar to that used by Dahlström & Häggendal (1966, 1970).

Sciatic nerves of rats (Sprague-Dawley, 200–230 g males) were used. Axons in both sciatic nerves were crushed by applying pressure on the nerve with a fine thread (diameter 0·1 mm) pulled against a metal rod for 5 s (Dahlström & Häggendal, 1966). One crush was placed at a level 1–2 mm below the *foramen infra-piriformis*. In some experiments a second crush was made at the same time 15 mm distal to the first. Control nerves were either uncrushed or crushed just before removal ("0 h crush").

The nerves were divided into 10 mm lengths above and below a single crush (Fig. 1) or into 5 mm lengths relative to double or single crushes as shown in Fig. 2. In the experiments of Fig. 1, 10 mm lengths were pooled from both sciatic nerves of 2 rats; in experiments of Fig. 2, 5 mm lengths from both sciatic nerves of 5 rats were pooled. Acetylcholine was extracted (MacIntosh & Perry, 1950), and the activity estimated on the guinea-pig ileum in the presence of antihistamine using a modification of the method of Blaber & Cuthbert (1961).

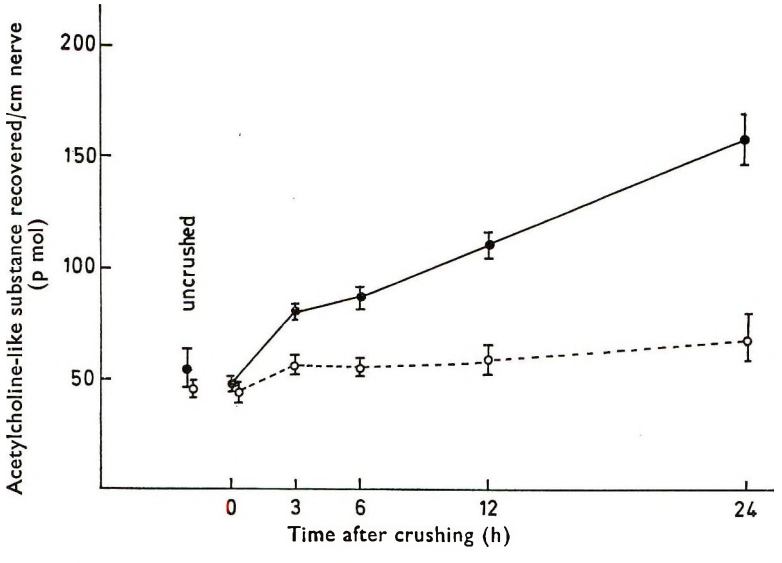

FIG. 1. The amount of acetylcholine (ACh)-like substance in the 1 cm of sciatic nerve of rat just above (●——●) and just below (○ - - - ○) a single crush. Means ± one s.e. are given, n = 4 in all cases. The ordinate indicates the amount of ACh-like substance in pmol per 1 cm of nerve (uncorrected for losses). The abscissa indicates the time interval between crushing and removal for extraction. "Uncrushed" indicates the values for the uncrushed nerve parts above (●) and below (○) the level of the crush.

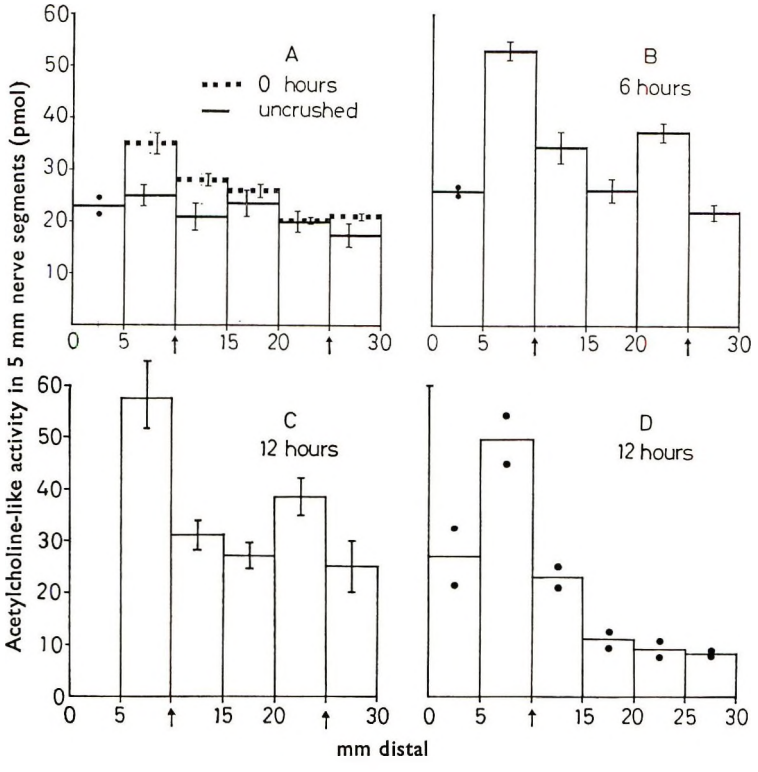

FIG. 2. The amount of acetylcholine (ACh)-like substance in 5 mm nerve segments of rat sciatic nerves. Means ± one s.e. are given for experiments, where n = 4–6. Where n = 2 the individual values are given. The ordinate shows the amount of ACh-like substance in pmol per 5 mm segments (uncorrected for losses). The abscissa indicates the distance along the nerve from the highest level 8–9 mm above *foramen infrapiriformis*. The arrows show the sites of nerve crush made 0, 6 or 12 h before dissection. In A the solid lines are uncrushed nerves. In A–C the total amount of ACh between the crushes is: A, Oh, Crush 63·2; Uncrushed 73·1; B, 98·0; C, 96·1 pmol.

The extract was inactivated by boiling in alkali or by incubating with cholinesterase. The active principle was thus shown to be "acetylcholine-like" but is referred to as acetylcholine.

The acetylcholine content in the 1 cm part of nerve just proximal to a single crush increased with time after crushing (Fig. 1). During the first 3 h the increase appeared to be greater than in later periods. The maximum value was about thrice normal at the longest time investigated (24 h) and was still rising. Below the crush there was only a slight increase. The increase above the crush was probably restricted to a region within 5 mm of nerve just above the lesion, as indicated by the results from the second experiment (Fig. 2B and D). The level of acetylcholine in the 5 mm of nerve distal to the crush was similar to that of the control, but in the more distal parts it dropped to about 40% of control by 12 h (Fig. 2D).

The increase in acetylcholine above the crush could have resulted from (a) interrupted proximo-distal bulk flow of axoplasm giving a local increase in cholinergic axon volume, (b) interrupted specific transport of acetylcholine or some factor (or both) responsible for its synthesis or storage, or (c) increased local synthesis of acetylcholine following crushing. Regarding (c), Feldberg (1943) has shown that damage to cholinergic axons causes increase in their acetylcholine content. This would be expected to

produce a symmetrical increase about the crush. But the accumulation of acetylcholine was always much greater central to a crush (Figs 1 and 2): thus it seems that damage-induced local synthesis is not an important factor in this accumulation. However, increased local synthesis may make a small contribution since the total amount of acetylcholine between two crushes (Figs 2B and C) was approximately 30% higher than in the same length of control nerve.

The rate of bulk flow of axoplasm has been estimated to be about 1–2 mm/day (Weiss & Hiscoe, 1948). This is far too slow to account for the rapid increase in acetylcholine that we found proximal to a crush. It seems more probable that crushing a nerve interrupts a specific cholinergic transport mechanism, since the peripheral loss of acetylcholine, and its accumulation central to a crush described above, would both be accounted for by a distal movement of acetylcholine of about 20 mm/day. However, this is likely to be an under-estimate for two reasons. Firstly, the rate of accumulation in the first 3 h appeared to be faster than in the subsequent period investigated (Fig. 1) Secondly, a proportion of the acetylcholine appears to be relatively immobile since at 12 h below the crush there was still some 40% of the control amount; also it has been found that even after several days the level does not fall below 20% of control (Evans & Saunders, 1967). Allowing for these factors would give a rate of transport which is probably several mm/h.

The distribution of noradrenaline (Dahlström & Häggendal, 1970) and acetylcholinesterase (Lubińska, Niemierko & others, 1964) have previously been studied in single and double crushed nerves. Qualitatively, noradrenaline, acetylcholinesterase and acetylcholine all behave similarly, and their rates of transport are all appreciably faster than the rate of bulk flow of axoplasm (Dahlström & Häggendal 1970 and Lubińska & Niemierko, 1971). Since redistribution of all three substances occurs *between* two crushes, it seems that their transport could be independent of continuity with the perikarya. Accumulation of intra-axonal organelles has been described after crushing (Martinez & Friede, 1970), but further studies are needed to elucidate the possible correlation between transport of acetylcholine and organelles such as vesicles.

Thus we have found that crushing a nerve produces a rapid accumulation of acetylcholine above a crush. We suggest that this is probably due to interference with a fast transport mechanism.

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A novel thin-layer chromatography system for lysergide (LSD)

A continually increasing demand for the rapid identification of substances suspected of containing lysergide (LSD) prompted an investigation into the possibilities of faster and more reliable forensic methods. Screening tests have already been used which eliminate many "innocent" samples from further investigation. The use of ultraviolet induced fluorescence (Government Chemist, 1969) together with a modified field test (Alliston, Bartlett & others, 1971) can restrict the problem to certain erganes and tryptamines within a few minutes.

Thin-layer chromatography has been widely employed for the final stage of the identification of lysergide. Phillips & Gardiner (1969) investigated a number of systems and recommended that of Genest & Farmilo (1964), which employs development with chloroform–methanol (9 : 1) on NaOH treated silica gel plates, for the separation of a number of natural and synthetic erganes. This is not convenient for commercial silica gel coated polyester sheets such as Eastman 'Chromagram' on which it is necessary to spot 2 μl 0·1N NaOH at the origin. Moreover, with certain common (and licit) preparations of the natural ergot base ergotamine, coextraction of the other ingredients in compound preparations may cause mobility to be modified to such an extent that, in some cases, ergotamine is confusible with lysergide. A favoured alternative—development with chloroform–methanol (1 : 4) on silica plates (Martin & Alexander, 1967)—is not an improvement in practice; low mobility and heavy streaking are observed. Separation can be achieved with 1,1,1-trichloroethane–methanol (96 : 4) on alumina plates (dal Cortivo, Broich & others, 1966) but mobilities are low and "eyebrow" shaped spots (indicating movement on a secondary solvent front) are observed. This reduction in the proportion of the plate available for separation occurs with many other systems, especially where more than two solvents are employed.

In seeking an entirely new system for use with "Chromagram" sheets, incorporation of an organic base into the mobile phase seemed desirable. Development with systems containing varying proportions of diethylamine, aniline, quinoline, morpholine, picoline and ethanolamine in a series of solvents was investigated. In most cases the substances examined did not move in the system or were carried with the solvent front. Where separation did occur, the ergotamine spot had a large tail (even after an equilibrium between the epimers had been established) or, in the case of 5% diethylamine in chloroform, 8β-ergotamine and 8α-lysergide were resolved but 8α-ergotamine and 8β-lysergide moved with the solvent front. Morpholine appears to be the best base and 1 : 9 the optimum admixture with toluene. Morpholine–xylene (1 : 4) or morpholine–benzene (1 : 9) are also suitable developing solvents but the extra drying time required for xylene and the health hazard of benzene would preclude them from routine use.

The following procedure is recommended. Place 15–20 mg of the crushed sample in an ignition tube and add 1–2 drops of reagent grade methanol. Stir with a pointed glass rod, stopper and allow to stand for 5 min. Spot 0·5 μl of the supernatant liquor onto a 8 cm $\times$ 4 cm "Chromagram 6060" sheet (silica gel with fluorescent indicator).

Table 1. *R_F values ($\times 100$) for erganes and some tryptamine derivatives.*

	††	Solvent I	Solvent II	Visualization (*a*) 360 nm	Visualization (*a*) 254 nm	Visualization (*b*) colour
Psilocybin	+	00	01	—	A	blue-grey
Lysergic acid		00	02	F	F	purple
8β-Ergometrine		11	25	F	F	purple
8β-Lysergamide	+	12	24	F	F	purple
Methylergometrine		14	31	F	F	purple
Dihydroergotamine		15	50	—	A	violet
8α-Ergometrine		22	39	F	F	purple
8β-Ergotamine		22	58	F	F	purple
Bufotenine	+	22	07	—	A	purple
Methysergide		33	51	F	F	violet
8α-Ergotamine		36	76	F	F	purple
8α-Lysergide	+	42	34	F	F	purple
8β-Ergocristine		44	70	F	F	purple
Psilocin	+	46	—	—	A	blue-grey
8β-Lysergide	+	51	60	F	F	purple
NN-Dimethyltryptamine	+	54	16	—	A	violet
NN-Diethyltryptamine	+	61*	16	—	A	violet
1-Acetyl-lysergide		61*	74	—	A	faint purple
Ibogaine		71**	—	—	A	none

T.l.c. systems: Eastman "Chromagram 6060" sheets —0·1 mm silica gel layer incorporating a fluorescent indicator.
Solvents: I = morpholine–toluene (1 : 9); II = methanol–chloroform (1 : 9).
Visualization: *a*: ultraviolet induced fluorescence (F) or absorbance (A) at 254 and 360 nm. *b*: spraying with 5% w/v 4-dimethylaminobenzaldehyde in methanol–HCl (1 : 1).

Notes: †† indicates control under the Drugs (Prevention of Misuse) Act, 1964—Modification Order 1970.

* substance travels on secondary solvent front.

** substance travels in phase ahead of secondary solvent front.

The spot area should not exceed 1 mm in diameter. Develop the sheet with morpholine–toluene (1 : 9) using an unequilibrated tank (a tall beaker is suitable). When the solvent front reaches the top of the sheet, remove the sheet and, before it is completely dry (2–3 min), observe under 360 and 254 nm ultraviolet radiation. Allow the plate to dry (about 5 min) and then observe the sheet under 254 nm ultraviolet radiation again. Spray the sheet with a solution of 5% w/v 4-dimethylaminobenzaldehyde in methanol–hydrochloric acid (1 : 1). To preserve the sheet, place it inside a re-sealable polythene bag to prevent yellowing due to oxidation.

Table 1 shows the data obtained from a range of erganes and certain tryptamine derivatives after development with morpholine–toluene (1 : 9) on "Chromagram 6060" sheets. Previous results (Phillips & Gardiner, 1969) with methanol–chloroform (1 : 9) are included for comparison.

The only compounds found to have mobilities comparable with the epimers of lysergide are 8β-ergocristine, *NN*-dimethyltryptamine (DMT) and psilocin. Ergocristine is so rarely encountered alone that the problem of resolving 8α-lysergide and 8β-ergocristine is not likely to occur. Moreover, we have never encountered 8α-lysergide without the 8β epimer (although 8β-lysergide often occurs by itself) so the absence of a spot due to 8β-lysergide would suggest the presence of 8β-ergocristine. If any doubt still remains, 8α-lysergide and 8β-ergocristine can be readily resolved using methanol–chloroform (1 : 9) (Phillips & Gardiner, 1969). Although 8β-lysergide,

DMT and psilocin have similar mobilities, the latter pair are easily identified by their lack of fluorescence at 360 nm whereas they absorb 254 nm radiation. Differences in colour response to the chromogenic agent are also observed. As with most other t.l.c. systems investigated, morpholine–toluene (1 : 9) exhibits a secondary solvent front. However, only 1-acetyl-lysergide and *NN*-diethyltryptamine travel on this front (R_F 0·61) and ibogaine, an hallucinogen with a slight history of abuse, runs in the solvent phase ahead of this front. The enhanced mobility of tryptamine derivatives in this new t.l.c. system may have some advantages over the four systems investigated by Phillips and Gardiner; this aspect is being pursued.

The system was tried with conventially prepared plates as well as other commercially available sheets. None of these gave acceptable results. The uniformity and fineness of texture of "Chromagram" sheets appears to be essential for the success of this system.

Using a covered tank prepared 24 h previously, the spread of mobilities for the series of compounds investigated became progressively smaller throughout the day. However, a tank newly prepared each day with these low volatility solvents i.e. essentially unequilibrated, gives results that are extremely reproducible. de Faubert Maunder (1969) and de Zeeuw (1970) have also reported good reproducibility in unsaturated t.l.c. chambers.

The risk of modification of the relative mobilities of the bases in compound preparations was investigated. Cafergot "Q"—a proprietary product containing 1 mg ergotamine tartrate B.P. and 100 mg caffeine B.P. within a chocolate flavoured shell and outer sugar coating—was completely ground (although in normal practice the inner, active tablet would have been separated physically before grinding), and a methanol extract was spotted on a "Chromagram 6060" sheet; a methanol extract of 8β-ergotamine tartrate was spotted on the same sheet. On development with morpholine–toluene (1 : 9) no differences in mobility were observed. The extracts were retained until some epimerization of the 8β-ergotamine had occurred and then rechromatographed: there were no changes in the mobility of 8α-ergotamine either.

The use of this new system facilitates rapid analysis of materials suspected of containing lysergide without problems of interference from compound preparations. Good separation can be obtained with an 8 cm development. Time and costly full scale t.l.c. apparatus are saved. The limit of detection for lysergide using this system was found to be 4 nanograms.

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The tetrahydrocannabinol content of cannabis leaf

Korte (1970) examined a number of cannabis plants of different origin and reported that the vegetative parts contained cannabidiol, as the cannabidiol-acid, but not Δ^1-*trans*tetrahydrocannabinol (Δ^1-THC*). Nielsen (1970), however, reported significant amounts of Δ^1-THC in the leaves of mature male and female plants of a South African strain grown in Denmark (1·4 to 12 mg/g dry weight compared with 9·6 to 17·1 mg/g in the flowering tops of female plants). However he used Korte and Sieper's thin-layer chromatographic method of assay (1964) which estimates free THC but not the low R_F value THC-acid (Merkus, 1971). In the fresh plant most of the THC occurs as the acid and this is slowly decarboxylated on storage of harvested plant material, but even after two months at 35° only about one-third is decarboxylated (Yamauchi, Shoyama & others, 1967). It is likely, therefore, that Nielsen's plant material contained significant amounts of the THC-acid, and since smoking converts the latter into THC (Mechoulam, Ben-Zvi & others, 1969), an assay method which includes this acid as well as the free THC should be used. The method of choice is, therefore, gas-liquid chromatography in which the higher temperatures involved simulate the decarboxylating effect of smoking. Kimura & Okamoto (1970) used a g.l.c. method and found up to 12 mg/g dry weight of THC-acid in the vegetative parts.

We have determined the total THC content of plants grown in England, using a g.l.c. method of assay based on that of Lerner (1969). Examination by t.l.c. confirmed that both THC and THC-acid were present in the active samples. The preliminary results are given in Table 1. Although we used the same strain of South African cannabis as Nielsen did, he only found up to 12 mg/g of THC in the leaves of female flowering plants, whereas we found about 33 mg/g (when our results are converted to mg/g dry weight); our higher figures are almost certainly due to the g.l.c. method of estimating both THC and the THC-acid.

Our results therefore confirm those of De Faubert Maunder (1970) that in active strains of cannabis significant amounts of THC occur in the leaves, especially at the flowering stage. The amounts reported in the above investigations vary from about 2 to 30 mg/g dry weight; the figures should be compared with the fact that a marihuana reefer weighs about $\frac{1}{3}$ g and a normal dose of THC would be 5 mg (Isbell, Gorodetzsky & others, 1967).

These figures have important legal consequences as the Dangerous Drugs Act (1965) defines Cannabis as "The flowering or fruiting tops of any plant of the genus

Table 1. *Total* Δ^1-THC *content of cannabis plants grown in England during* 1970.

Strain	Conditions of growth	Total Δ^1-THC content mg/g fresh weight*
(a) South African from UN/S1 Seeds	Greenhouse (vegetative phase)	
	Leaves	5·12
	Out-of-doors (flowering phase) Female plants.	
	Flowering tops	10·15
	Leaves	8·54
(b) Nepalese	Out-of-doors (flowering phase) Female plants	
	Flowering tops	3·40
	Leaves	7·63
(c) Turkish	Greenhouse (vegetative phase)	
	Leaves	traces
(d) English	Greenhouse(vegetative phase)	
	Leaves	0·34

* Values for dry weight approximately 4 × figures given.

Cannabis", implying that only the flowering tops are active. In fact a person using the leaves only would probably have a legally sound defence against prosecution, although, as the above facts indicate, they may contain active material.

We would like to thank the Medical Research Council for a grant towards part of the cost of the work; Dr. R. Mechoulam for a sample of pure THC; Dr. L. D. Kapoor for Nepalese seed and the U.N. Division of Narcotic Drugs for South African seed.

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* Numbered Δ^9 according to IUPAC rules.

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Isolation of speciogynine from the leaves of *Mitragyna inermis* (Willd.), O. Kuntze

It was reported by Shellard & Sarpong (1969) that the leaves of *Mitragyna inermis* (Willd), O. Kuntze contained 8 oxindole alkaloids together with the indole alkaloid mitraciliatine and traces of a second indole alkaloid. It was anticipated that this alkaloid might be speciogynine as the alkaloids then isolated would fit into a hypothesis proposed for oxindole biogenesis outlined by these authors. The alkaloid, designated Sp4, was, however, not speciogynine.

We have subsequently isolated from another batch of leaves of *M. inermis* small quantities of indolic substances, one of which is speciogynine. The alkaloids were isolated according to Shellard & Sarpong (1969), Fraction B being subjected to further column chromatography using chloroform–methanol (2:1) and to preparative thin-layer chromatography. Six alkaloidal substances were isolated, one corresponding to mitraciliatine (the bulk of which was in a different fraction), four were present in traces too small to characterize or identify with certainty, and the other was speciogynine. This was identified by comparing the hR_F values obtained on several thin-layer chromatographic systems and its spectral data ultraviolet, infrared and nmr with authentic speciogynine obtained from *Mitragyna speciosa* (Beckett, Shellard & others, 1966).

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'Metabolism' of 'amphetamines' to oximes as a route to deamination

Recently, a preliminary report of the metabolism of amphetamine to benzylmethyl ketoxime (isomer not specified) appeared (Hucker, Michniewicz & Rhodes, 1970). We have found that after injection of the (+) or (−)-isomers or the racemates of amphetamine, methylamphetamine and ethylamphetamine to rabbits or guinea-pigs, *syn* and *anti*-benzylmethylketoximes are present in urine in free and conjugated forms. The metabolic conversion of the parent drugs to oxime and the stereoisomeric composition of the oxime varied with the species and with the enantiomorphs administered; in general, the *anti*-isomer predominated. In some instances, more combined oxime isomers than unchanged drug were excreted, indicating the importance of the metabolic route.

The identity and the stereoisomeric content of the oxime mixture from metabolism was established by comparison with authentic samples by mass spectrometry, polarography, nuclear magnetic resonance, t.l.c. and g.l.c. using several columns, e.g. 5% Carbowax 20 M on Chromasorb W 3 m; 140°; gas 40 ml/min *anti* 25 min, *syn* 27 min (ref *p*-chloropropiophenone 8 min): 8% Apiezon L plus 4% Carbowax 20 M on Chromosorb G, 1 m; 130°; gas 25 ml/min *anti* 34 min, *syn* 36 min (ref 16 min): 3% Carbowax 20 M on Chromosorb G 1 m; 180°; gas 30 ml/min *anti* and *syn* 6 min (ref 2·5 min).

In t.l.c. the *syn*-oxime hydrolysed to benzylmethyl ketone more rapidly than did the *anti*-isomer. In aqueous solution at pH 1, the oxime isomers were rapidly hydrolysed to benzylmethyl ketone but little hydrolysis occurred at alkaline pH values. Thus, in metabolic studies, the amount of oxime converted to ketone and its stereoisomeric content will depend upon the pH of the urine, storage time, procedures used to isolate the isomers and the method of analysis. This instability of oxime in solution may account for the fact that despite many reports of the identification of benzylmethyl ketone as a metabolite of 'amphetamines', Hucker & others (1970) were the first to record the metabolism of amphetamine to benzylmethyl ketoxime.

The 'metabolism' of 'amphetamines' to oximes which are relatively unstable in weakly acidic solutions to yield benzylmethyl ketone constitutes a route to the deamination of 'amphetamines' (cf. Hucker & others for (+)-amphetamine).

We were unable to find norephedrine recently reported (Caldwell, Dring & Williams, 1971) in urine after normal doses of methylamphetamine to guinea-pigs.

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