Journal of Pharmacy and Pharmacology

The Pharmaceutical Society of Great Britain

Volume 23 Number 4 April 1971

Coming into force in the United Kingdom April 5, 1971

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The Pharmaceutical Press, 17 Bloomsbury Square, London WC1A 2NN

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN
17 Bloomsbury Square, London, WC1A 2NN Telephone: 01–405 8967

Volume 23 Number 4 **April 1971**

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J. Pharm. Pharmac., 1971, **23**, 233–251

REVIEW

Emulsion stabilization by non-ionic surfactants: experiment and theory*

A. T. FLORENCE AND J. A. ROGERS†

School of Pharmaceutical Sciences, University of Strathclyde, Glasgow, U.K.

QUANTIFYING THE STABILITY OF EMULSIONS

The stability of a dispersion is frequently denoted by a rate constant which is a quantitative measure of the time required for the initial concentration of particles to be reduced to some critical value.

The flocculation of hydrophobic sols has been treated in a kinetic study by Smoluchowski (1916, 1917) and a second-order reaction process was found to hold. The kinetics of coalescence of emulsion globules has been studied by van den Tempel (1953), who reported that coalescence is a first-order reaction process which occurs only between adjacent drops in an aggregate and which is independent of the number of droplets in the aggregate.

An emulsion cannot be thermodynamically stable, but it can display a high degree of permanence in the kinetic sense. Therefore, the factors determining the kinetics of degradation of emulsion systems are of great importance.

The first elaborate study of the kinetics of emulsion breakdown was made by King & Mukherjee (1939) on emulsions of olive oil and kerosene in aqueous solutions of soaps. They surmised that emulsion instability was due to the preponderance of a large interfacial area of the disperse phase and decided that a reasonable representation of the process of coalescence would be a measure of the decrease with time in the specific interfacial area S of emulsified oil.

$$S = \frac{A}{\rho V} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (22)$$

where A is the total interfacial area of emulsified oil having a volume V and density ρ. When S was plotted as a function of time, a linear relation held, which was proportional to the initial specific interfacial area S_0, giving

$$\frac{-dS}{dt} = \frac{S_0}{k} \quad .. \quad .. \quad .. \quad .. \quad (23)$$

k is the rate constant which had an initial high value in the early stages of breakdown but which changed to a much lower value for the remaining life of the emulsion. Lotzkar & Maclay (1943) also measured changes in the specific interfacial area with emulsions of olive, cottonseed and mineral oils stabilized with pectin. The data were treated on the assumption that the rate of change of specific surface at any time is proportional to the specific surface at that time, or

$$\frac{-dS}{dt} = \frac{S}{k} \quad .. \quad .. \quad .. \quad .. \quad (24)$$

Plotting the logarithm of S against time they obtained linear relations from which values of the stability coefficient, k, were computed.

Jellinek & Anson (1950) attempted to find a suitable numerical expression which defined the stability of emulsions stabilized by α-monostearin and sodium stearate.

* The second and final part of this review: the first part appeared on pp. 153–169.

† Present address: Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada.

They plotted several statistical quantities against time for their emulsions but the best linear plots were of specific interfacial area, and stability rate constants were determined from these. In addition, curves of the reciprocal of the number of globules per gram of disperse phase were linear with time, representing a formal agreement with the Smoluchowski theory.

The relatively simple treatment of the kinetics of emulsion breakdown by these workers has provided a plausible agreement with theory for the systems studied. It does not follow that a similar agreement would occur in other systems, particularly when materials other than surfactants are used as stabilizers. By studying these less stable systems, a greater insight into the mechanisms involved might be obtained.

Lawrence & Mills (1954) have attempted to describe the stability of their systems by incorporating into Smoluchowski's theory an expression for the mean drop volume $\bar{V}_t$, given by

$$\bar{V}t = \frac{\sum_{r=1}^{r=\infty} N_r V_r}{\sum_{r=1}^{r=\infty} N_r} \quad .. \quad .. \quad .. \quad .. \quad (25)$$

(N_r is the number of aggregates of r particles and $V_r = rV_0$, the volume of the rth-sized particle composed of r primary particles each of volume V_0.) The rate of change of the mean drop volume was expressed as

$$\bar{V}t = V_0 + \beta\phi t \quad .. \quad .. \quad .. \quad .. \quad (26)$$

in which $\beta = 4\pi D_1 \bar{R}$. $\bar{R}$ is the effective radius of the emulsion particles (composed of r-mer primary particles); ϕ is the phase volume fraction of disperse phase and is equal to N_0V_0, the total oil emulsified in the unit volume of emulsion. Equation (26) must be modified to account for inefficiency of collisions, when the theory of "rapid" coagulation does not apply, i.e. in stabilized systems. Hence,

$$\bar{V}t = V_0 + p\beta\phi t \quad .. \quad .. \quad .. \quad .. \quad (27)$$

Lawrence & Mills (1954) equated p to energy in an expression of the form

$$p = X \exp\left(-\frac{E}{RT}\right) \quad .. \quad .. \quad .. \quad .. \quad (28)$$

where E represents an energy barrier to effective collisions between the emulsion globules and X is the frequency of collision. p may then be evaluated from the slope of a plot of V_t against t. In emulsions containing 1% phase volume of 3,3-ditolyl or Nujol, linear relations were obtained for both unstabilized emulsions and emulsions stabilized with 1% sodium oleate. Taking X as unity, an energy of activation, E, of 16·32 kJ mol^{-1} was evaluated from the slope of the curve for unstabilized emulsions while a value of 27·17 kJ mol^{-1} was found for sodium oleate stablized emulsions. Computation of energies of activation from Jellinek & Anson's results (1950) gave comparable values.

The use of the simple Maxwell-Boltzman factor (exp{E/RT}) for E has been disputed by Kitchener & Mussellwhite (1968) because the "activated state" of the particles is probably a function of several physical parameters, notably the flux of particles up to the central particle. The treatment of coagulation of emulsion globules by Lawrence & Mills (1954) neglects to account for a number of factors affecting the kinetics of these systems. An attempt has been made by Kharin (1956) to account for particle size variation and the deaggregating effect of emulsion particles on the coagulation rate. The rate of disappearance of n particles of all sizes is:

$$\frac{-dn}{dt} = \beta n_1^2 \exp\left[\frac{-q_1}{kT}\right] - \alpha n_2^2 \exp\left[\frac{-q_2}{kT}\right] \quad .. \quad .. \quad (29)$$

The first term on the right-hand side quantifies the coagulation process where n_1 is the number of primary particles coagulating at a rate β against a potential energy barrier q_1. The second term accounts for the deaggregation process of aggregates, n_2, opposed by a potential energy barrier, q_2. α is the coefficient of the dispersion process, whereas β is the coefficient of the coagulation process, equal to $4\pi D_1 R$, where the symbols have the same meaning as before. The influence of particle size is estimated from the magnitudes of q_1 and q_2 which are empirically related by $q = f(a)$. The activation energy for the coagulation process was found to be 6·28 kJ mol^{-1} for transformer oil emulsions, less than one-half the value obtained by Lawrence & Mills (1954) for their unstabilized o/w emulsions.

A new approach to the stability of emulsions has been presented by Hill & Knight (1965). They have employed a collision frequency based on a gas-kinetic model rather than the classical diffusion model to formulate a kinetic theory for the slow coagulation of emulsion systems. The assumption is made that probability of coalescence is proportional to the integral $\int$(area $\times$ pressure)·dt for the encounter between particles with velocities distributed according to Maxwell's distribution law. The theory accounts for the average effects of coalescence over all sizes of droplets but, electrical effects and droplet deformations on collision are ignored. It has been previously stated that for stabilized emulsions the barrier to coalescence is independent of the actual size of the drops coalescing (Reerink & Overbeek, 1954; Lawrence & Mills, 1954). The change of total interfacial area, (A), with time (t) is, according to Hill & Knight,

$$\frac{1}{A} = \frac{11\beta kT}{4\alpha} t + \text{constant} \qquad .. \quad .. \quad .. \quad (30)$$

A plot of A^{-1} versus t should be linear, the slope β being a proportionality constant characterizing the probability of rupture of unit area of common interface under unit pressure per unit time. α is defined as the available volume of dispersed oil. The theory was tested employing results of previous workers (King & Mukherjee, 1939; Lotzkar & Maclay, 1943; Lawrence & Mills, 1954). Generally, better agreement with the new theory was found, but three of the ten plots considered could be represented equally well by Smoluchowski's theory in the form $A^{-3} = Bt +$ constant. Further support of a linear relation of A^{-1} versus t for emulsions of high stability has been demonstrated by Elworthy & Florence (1967) with emulsions of anisole and chlorobenzene in aqueous cetomacrogol solutions.

The rate of coalescence of particles colliding with average violence was calculated by Hill & Knight to be 10^{-9} to 10^{-10} s^{-1} (cf. 10^{-7} s^{-1} found experimentally by Elworthy & Florence, 1967; van den Tempel, 1957), making it very unlikely that an actual coalescence would be observed in emulsions of this type. The theory is applicable to systems stabilized primarily by steric and hydrational mechanisms where electrical effects are unimportant. In some systems, however, aggregation into the secondary minimum has explained instability even when dense interfacial films are expected to be present (Prakash & Srivastava, 1967). Elastic collisions between emulsion droplets is not explained by this theory but the significance of this factor is as yet unknown.

van den Tempel (1953a,b) treated the coagulation of emulsion globules by considering the processes of flocculation and coalescence separately. Flocculation was assumed to proceed by a second-order reaction process according to the Smoluchowski theory. A convenient point in time was chosen to begin the analysis such that a "nearly stationary state" was established around each particle, i.e. the number of particles diffusing in unit time through a sphere surrounding one central particle equals the number of particles adhering to this central particle in unit time. This

state has been reached after a time $t > a^2/D$, which means that all experimental coagulation times should be large compared with a^2/D.

Measurements were made of the number of particles decreasing with time per unit volume of emulsion using an ultramicroscope which does not differentiate between single globules and aggregates. Provided the general shape of the size-frequency distribution curve does not change appreciably during coagulation, it can be shown that a 10% decrease in interfacial area is accompanied by a 27% decrease in the number of particles, making the latter a more sensitive measurement.

The total number of primary particles, n_1 at time, t, is found from

$$n = \frac{n_0}{1 + a_1 n_0 t} + \frac{a_1 n_0^2 t}{(1 + a_1 n_0 t)^2}\left[\frac{a_1 n_0}{K} + \left(1 - \frac{a_1 n_0}{K}\right)e^{-Kt}\right] \quad .. \quad (31)$$

where n_0 is the number of primary particles at $t = 0$, a_1 is a rate constant for flocculation equal to $8\pi D_1 R$, approximately evaluated as 10^{-11} $cm^3 s^{-1}$ for "rapid" coagulation, and K is the rate constant for coalescence. If a_1 is large compared to K, then equation (31) may be approximated by

$$n = \frac{n_0}{Kt}\left[1 - e^{-Kt}\right] \quad .. \quad .. \quad .. \quad (32)$$

Since the particle number is found to decrease nearly exponentially with time until Kt becomes large compared with unity, this supports the observations made by Lotzkar & Maclay (1943). In emulsions stabilized with macromolecules, the rate of coalescence can be very small. The exponential term of equation (32) may, therefore, be expanded in a power series, of which only the first two terms are used when $Kt \ll 1$. In this case

$$n = n_0\left[1 - \frac{Kt}{1 + a_1 n_0 t} + \frac{Kt}{(1 + a_1 n_0 t)^2}\right] \quad .. \quad .. \quad (33)$$

A plot 1/n against t is linear if the emulsions are very dilute or concentrated since the rate of coagulation is unaffected by the particle concentration.

van den Tempel has rigorously tested his theory for o/w emulsions (1953, 1957). In concentrated liquid paraffin emulsions stabilized by sodium dodecyl sulphate and Aerosol OT, respectively, a plot of log n against time produced two lines corresponding to an initial "fast" rate which lasted for a few hours and a second "slow" rate which characterized the further deterioration of the emulsions. The "slow" rate could be shown to be a first order coalescence rate since flocculation had been eliminated in these systems, indicating that the coalescence of two contacting oil globules does not affect the stability at the other contact points. The initial rapid decrease in particle concentration is attributed to non-equilibrium conditions at the oil-water interface or a wide particle size distribution allowing closer packing of the particles. The time to reach equilibrium at the interface is dependent on the interfacial activity of the emulsifier. Assuming that a condensed monolayer of surfactant is necessary for stability, incomplete oil surface coverage could lead to instability in the early stages (Rowe, 1965; Neiman, Lyashenko & others, 1961).

In most studies of o/w emulsions, the emulsifier is incorporated in the continuous phase. But, for a given concentration of surfactant, the rate of adsorption at the oil-water interface will be different if the emulsifier is placed in the dispersed oil phase. If the emulsifier is soluble in both phases, then the duration of the "fast" rate of coalescence will depend on the partition coefficient of the emulsifier between the two phases and, to some extent, the orientation time of the molecules at the interface. One can never equilibrate the two phases of an emulsion before mixing because of the new interface and the finite quantities which adsorb at the interface.

The adsorption of surfactants at an interface is a rapid process. Ageing is the result of migration of surfactants between the phases. Lin & Lambrechts (1969a,b) have investigated this problem. The placing of the surfactant in the aqueous phase before emulsification produces an emulsion having rheological properties, stability and particle size distribution significantly different from emulsions of the same formulation prepared with surfactant initially in the oil phase. Therefore one factor in emulsion stability will be the possibility of changes in the location of surfactant, since this may cause undesirable changes in emulsion properties during the storage of the product. Migration of Triton X-100 through the water–iso-octane interface becomes very slow (>50h) when a second component (Arlacel 83) is present in the oil phase.

The second or "slow" rate of coalescence of emulsions has been observed to vary with the degree of fineness or coarseness of the emulsion (Elworthy & Florence, 1969b). Thus, the mean globule size existing when the second rate comes into play is critical for the "life" of the emulsion. Plots of log n against time are shown in Fig. 8 for emulsions of anisole in aqueous cetomacrogol 1000 solutions. Slopes

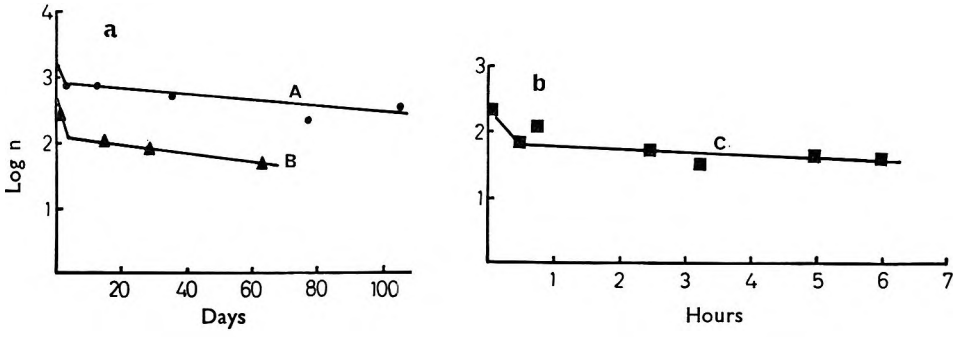


FIG. 8. Plots of log n (number of particles in arbitrary volume of emulsion, determined by the total volume of the particles sized in the initial sizing) versus *time*. A. Anisole $\phi = 0{\cdot}56$, cetomacrogol 1000 1%. B. Anisole $\phi = 0{\cdot}05$, cetomagrogol 1000 1%. C. (note time in h) Anisole $\phi = 0{\cdot}20$, cetomacrogol 0·001% (CMC in water = 0·006–0·007%).

corresponding to second rate constants of the order 10^{-6} to 10^{-7} s^{-1} compare well with the values obtained by van den Tempel (1957) for ionic stabilized emulsions, suggesting that at the coalescence stage emulsion stabilization by all surfactant types has a common mechanism.

The present authors (Elworthy, Florence & Rogers, 1971b) made a detailed investigation into the effect of cetyl alcohol on the stability of $C_{16}E_6$-stabilized chlorobenzene emulsions. Although less surface-active than the main detergent at the chlorobenzene–water interface the hexadecanol resulted in a definite increase in stability. The alcohol affected only the surface area viscosity to any great extent (and not zeta potential or interfacial tension) so it was assumed that the presence of the cetyl alcohol reduced the mobility of the emulsifier molecules so that the probability of desorption was decreased.

Prediction of particle size distribution changes in emulsions and suspensions on the basis of acceptable theories can be useful in testing the theories and serve to show how the magnitude of various forces alters the stability pattern. Recently, Suzuki, Ho & Higuchi (1969) solved the Smoluchowski flocculation rate equations for any initial particle size distribution using digital computation to obtain particle size distribution data as a function of time. It was assumed that the only rate process was the passage of the particles over an electrical barrier. The general method of analysis can be adapted to include the steric–entropic barriers which undoubtedly

exist in non-ionic-stabilized emulsions. However, until the exact role that these forces play in emulsions is elucidated, and the relevant equations to embrace all situations developed, such procedures are perhaps premature. In the cases investigated, when the electrical barrier was small any initial distribution became more polydispersed with time; when the electrical barrier was appreciable the distribution narrowed with time (Suzuki, 1969).

It is, of course, the goal to be able to predict size distributions, and hence actual stability, with a knowledge of a restricted number of experimentally accessible parameters.

DISCREPANCIES BETWEEN EXPERIMENT AND THEORY

The lack of agreement between predictions of the Smoluchowski theory employing theoretical stability ratios (W) and the observed stability of dispersions may arise from the influence of the following factors.

1. *Particle size*

The theory of Verwey & Overbeek predicts an increases in stability with an increase in particle size (Verwey & Overbeek, 1948). Studies of conventional colloids have resulted in maximum flocculation concentrations at log $W = 0$, but recent studies of dispersions of polystyrene latex particles (Matthews & Rhodes, 1968b; Higuchi, Okada & others, 1963; Swift & Friedlander, 1964) have led to minimum values of W which are a fraction of the Smoluchowski rate for rapid coagulation when particle sizes exceed the size limit of true colloids (*ca* 0·7 μm). Below about 0·5 μm in particle size, deviations from Smoluchowski theory seem to disappear.

The dependance of W on such factors as particle size, electrolyte concentration and valency has been reviewed (Verwey & Overbeek, 1948; Ottewill & Watanabe, 1960a,b,c; Somasundaran & others, 1966). The theory has also been extended to dispersions containing two particle sizes (Hogg, Healy & Fuerstenau, 1966).

2. *Polydispersity*

As emulsion systems are extremely difficult to prepare with a monodisperse particle size the influence of polydispersity on the rate of breakdown is important because most studies are made on polydisperse systems. The effect of polydispersity on the rate of coagulation of dispersions has been investigated by Müller (1926). It has been pointed out that in a dispersion consisting of equal numbers of large and small particles, the smaller particles disappear much more rapidly with time. The impression is that the smaller ones are caught by the larger ones because the collision of a small particle with a large one does not change the concentration of large particles but reduces the concentration of smaller ones. A recent study on hetero-dispersed systems has been reported by Ho & Higuchi (1968). The preferential aggregation and coalescence of small emulsion particles where moderate electrical barriers exist was determined employing equations based on the concepts of DLVO theory. It is shown that small particles may aggregate (or coalesce) with themselves or with larger particles at rates that are ten to fifty orders of magnitude faster than for particles ten times larger. These findings may explain the relatively narrow particle size distributions observed in certain aged emulsions and flocculated suspensions.

Because of the complications introduced by polydispersity, the preparation of uniformly dispersed emulsions is a desirable prerequisite for basic studies on emulsion systems, and, of course, for use for specific purposes such as parenteral emulsions. Nawab & Mason (1958) first reported an electrical dispersion method which has since been widely quoted in textbooks on emulsions as being suitable for the preparation of mono-sized emulsions with 98% by weight of the particles in the range 2·5–3·5 μm

as the original authors claimed. No other papers have appeared confirming the original findings. Many experiments have been made in these laboratories in an attempt to repeat Nawab & Mason's results using identical disperse phase and non-ionic detergent, but with little success. Stirring of the continuous surfactant phase has to be so vigorous to incorporate the falling aerosol of oil that the main emulsification appears to occur by stirring and not by the electrical dispersion, hence there is little control over particle size.

Similar experience with another technique has recently been reported by Monk, Matijevic & Kerker (1969). It had been reported by Becher (1967) that a homogenization technique similar to that employed in Brown's Emulsor, produced emulsions with modal diameters in the range 1–2 μm with a very low standard deviation. Monk & co-workers (1969), repeating the work using many variations in the technique, were unable to obtain a narrow particle size distribution and the mean particle sizes determined by light-scattering of a large number of emulsions were much smaller than those quoted by Becher. It is disappointing to find these examples and frustrating to have to test the validity of the methods by trial and error. The optimistic original reports must occur through application of inadequate sizing techniques or through chance occurrences of emulsions with the size distributions quoted for them.

Dispersal of liquid systems by ultrasonics gives fine emulsions rapidly, but not with narrow size distributions. We have used the technique to disperse oils in water in the absence of emulsifier for micro-electrophoresis, and to prepare emulsions with inefficient emulsifiers. Rajagopal (1959) found that emulsions prepared in this way had a similar size distribution to those prepared by colloid mill. The difficulty with the method is the determination of the optimum sonication time, as continuation of sonication past this optimum results in a broadening of the size distribution (Söllner & Bondy, 1935, 1936; Prakash & Ghosh, 1962). Use of the method to prepare parenteral emulsions requires control to ensure that no metal particles from the probe are retained by the product.

Orthokinetic flocculation

Variations in the rates of coagulation of dispersed systems caused by systematic movements (mechanical agitation or gravitation) are referred to as "orthokinetic" flocculation, as opposed to "perikinetic" flocculation due to Brownian motion of the particles. In a fresh suspension, when the particles are small, the coagulation is perikinetic and its rate is slow. When a certain degree of aggregation has been reached, orthokinetic coagulation comes into play and aggregation is much accelerated (Overbeek, 1952).

Refinements of the coagulation theory (Collins & Kimball, 1949; Rice & Whitehead, 1967) account for incomplete adhesion of emulsion drops on collision, reversible flocculation, delayed coalescence and orthokinetic coagulation due to sedimentation.

SPECIAL CHARACTERISTICS OF EMULSION SYSTEMS

Deviations from theory result because of some special properties of emulsion systems. As the DLVO theory explains stability by describing forces preventing the close approach of the disperse particles, where instability can arise from mechanisms other than coalescence and coagulation one can expect deviation between experiment and theory. Such a mechanism of particle growth occurs in emulsions through diffusion of minute portions of oil through the continuous phase in micellar form. The more efficient the solubilizing capacity of the non-ionic stabilizer the more important will be diffusional growth of large particles in the emulsion at the expense of smaller particles. A theory pertaining to this mechanism of emulsion breakdown has been published by Higuchi & Misra (1962). For the case where

there are n_A and n_B particles of radii a_A and a_B respectively, the rate of change of the radius of B is given by:

$$\frac{da_B}{dt} = \frac{DC_\infty K}{\rho a_B^2}\left[\frac{n_A(a_B - a_A)}{n_A a_A + n_B a_B}\right] \quad .. \quad .. \quad .. \quad (34)$$

where $K = (2\sigma M/\rho RT)$, σ = interfacial tension, D = molecular diffusion coefficient, C_∞ = miscibility of an infinitely large drop, ρ = density of disperse phase.

When the two initial particle radii are 0·5 and 1·0 μm, degradation by diffusional processes is as shown in Fig. 9. Decrease of the diffusion rate of the oil can be achieved by increasing the viscosity of the external phase, or by addition of a third component to the dispersed phase if the additive has a sufficiently low rate of diffusion.

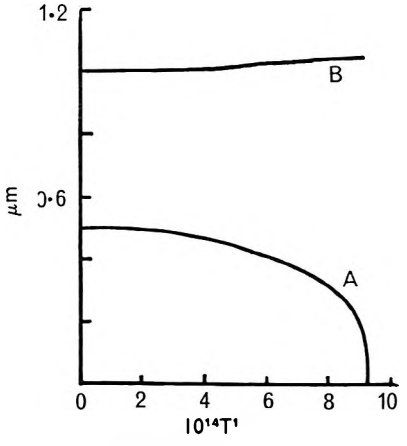


FIG. 9. The degradation of an emulsion initially composed of a mixture of 0·5 μm and 1 μm radius droplets of equal number concentration, showing the droplet diameters versus a function (T^1) of time. $T^1 = \frac{DC_\infty Kt}{\rho}$. At $T^1 = 4 \times 10^{-14}$ there is about a 10% change in a_A, after $t = 3 \times 10^7$ s (1 year), using $D = 5 \times 10^{-6}$ cm^2 s^{-1}, $\rho = 1$, even though C_∞ is only 3×10^{-8}g ml^{-1}. From Higuchi & Misra (1962) by permission of the authors and the *Journal of Pharmaceutical Sciences*.

Deformation of the globules on collision can feasibly result in the dissipation of some of the attractive energy but, if the DLVO theory reasonably predicts the behaviour of suspensions of asymmetric particles then it is unlikely that the unknown shape of the deformed particles in an emulsion will give rise to much discrepancy. More likely to be a source of deviation is the possible dissolution of the surfactant in the disperse phase when particles come together with resultant depletion of the surface concentration of stabilizer, unless the diffusion of surfactant from the continuous phase is rapid enough to counteract this tendency. Presumably in systems with high bulk concentrations of emulsifier, replenishment of the surface is rapid; on the other hand, where the solubility of the surfactant in the oil phase is high and the total concentration is high, this inhibits diffusion of the surfactant into the oil. Therefore in both cases high concentrations of surfactant will increase stability.

Spontaneous emulsification

The formation of an emulsion, without agitation or mixing, at the interface between polar oils and aqueous non-ionic solutions has been observed (Elworthy & Florence, 1967). These so-called spontaneous emulsions are undoubtedly the result of fluctuations in the interfacial region as a result of molecular motion. It can be shown that the amplitude of a spontaneous wave in the surface region is greater

by one order of magnitude than the molecular dimensions at low values of interfacial tension, e.g. $\sigma = 1$ mNm^{-1} (Sheludko, 1966). In systems of chlorobenzene or anisole emulsions spontaneously formed only with the more hydrophilic emulsifiers, i.e. those, which lowered σ to 5 mNm^{-1}. Emulsions do not form spontaneously with these surfactants and non-polar oils, an observation which can perhaps be explained by the results of Fig. 2. However, Davies & Haydon (1957) find in many systems no correlation between spontaneous emulsification and interfacial tension; they discuss a number of alternative mechanisms, including disturbances caused by diffusion of surfactant from one phase to another.

The role that supramolecular layers play in stabilizing emulsions of xylene in the presence of non-ionic detergents has been investigated by Nikitina and co-workers (Nikitina, Taubman & others, 1963; Prigorodov & others, 1965).

THE HLB SYSTEM AND EMULSION STABILITY

The arbitrary numbering system devised by Griffin (1949) which is based on the hydrophilic-lipophilic balance (HLB) of the emulsifier, has been used widely in practice as a means of selecting the most effective stabilizer for a given oil. The HLB of a non-ionic detergent is defined (Griffin, 1954) as—

$$(\text{mol } \% \text{ of hydrophilic group})/5$$

Polyoxyethylene glycols therefore have an HLB of 20. Briefly, each oil has a value of HLB which will provide a stable o/w emulsion; for example, for liquid paraffin it is 10–12. A single surfactant or mixture of surfactants which provides this HLB number will stabilize the liquid paraffin dispersion. Estimations of stability in order to assess the "required HLB" are carried out visually by observation of creaming of a series of emulsions prepared with a range of emulsifying agents. While this number has been invaluable for rapid choice of an emulsifier, its use still involves empirical standards. The HLB system neglects the concentration dependence of stability, as has been pointed out by Elworthy & Florence (1969c). Riegelman & Pichon (1962) have drawn attention to other drawbacks in its use; for example, while creaming is a criterion of instability in commercial formulations it is by no means the only one. It is imperative, they point out, to recognize that stability towards creaming is dependent on the rheological character of the emulsion far more than on the interfacial characteristics of the interfacial film. The influence of surfactants on the viscosity of the continuous phase is therefore of primary importance in this case. Richards & Whittet (1955) were able to obtain stable liquid paraffin-in-water emulsions with surfactant combinations having an HLB as low as 3·9. The stable emulsions were all thixotropic indicating that the surfactants were contributing to the structural viscosity of the system and thereby contributing to stability by preventing creaming.

Hadgraft (1954) also has obtained stable liquid paraffin emulsions with cetyl alcohol-cetyl polyoxyethylene ether combinations having HLB values as low as 1·9; this stability undoubtedly arises from the viscous nature of both interface and bulk phases. Recent rheological measurements on similar systems confirm this view (Talman & others, 1967).

The conclusion is that the HLB system will give a quick answer to a practical problem, but will offer little scope for basic improvements on the formulation. Vold (1969) has written "it is intriguing that HLB numbers of mixed non-ionics are additive according to the proportions of each present. One is left with the conviction that the HLB number has a rational interpretation and with a sense of frustration in not being able to show its origin conclusively".

MICROEMULSIONS

Microemulsions or micellar emulsions are systems, usually optically clear, which can be considered to consist of oil or water laden micelles in aqueous or oily continuous media respectively. Schulman, Stoeckenius & Prince (1969) and more recently Adamson (1969) and Tosch, Jones & Adamson (1970) have contributed a large amount to the subject. Schulman emphasized that micellar emulsions are systems in true equilibrium, it being proposed that the components of the surface films in these systems produce a negative interfacial tension at the hydrocarbon water interface (Schulman & Montagu, 1961). On mixing, a spontaneous interfacial area increase occurs until zero interfacial tension is attained. In Adamson's (1969) model for micellar w/o emulsions stability is accounted for by a balance of the Laplace pressure ΔP, related to the micellar radius r and interfacial tension σ by

$$\Delta P = 2\sigma/r \quad .. \quad .. \quad .. \quad .. \quad .. \quad (35)$$

and the osmotic pressure difference $\Delta\pi_{os}$ between the inside and outer region of the micelle which arises from the difference in ionic concentration. The osmotic pressure difference is positive hence in the presence of water an indefinite swelling of the micellar units is produced until equilibrium is reached when

$$\Delta\pi_{os} = 2\sigma/r \quad .. \quad .. \quad .. \quad .. \quad (36)$$

Microemulsions, prepared from benzene and water using 5 or 10% polysorbate 20–1% Span 20 blends, having dispersed phase volumes up to 0·406 had viscosities which could not be represented by any simple equation which merely related viscosity and dispersed phase volume (Matsumoto & Sherman, 1969). Particle diameters, measured by light-scattering, were in the range 54–125 nm. Viscosity depends on both particle size and the viscosity of the particles, the swollen micelles behaving not as rigid spheres although circulation within the micellar droplets is undoubtedly restricted.

Why should there be this range of particle sizes in these emulsions? If the non-ionic micellar species are essentially monodisperse as they would appear to be (Attwood, Elworthy & Kayne, 1968), then the microemulsion, if formed by growth of solubilized micelles, might also be expected to be reasonably monodisperse. However, this would not be so if formation of the emulsion is by random dispersion, although if the particles are thermodynamically stable, as is suggested, then it would seem that there should be only one equilibrium size.

INVERSION

In stable emulsion formulations, inversion can be induced by increasing the disperse phase volume, ϕ, to a round 0·7. This is a phenomenon not accounted for in stability theories. The exact value of ϕ at the inversion point depends on the surfactant present and its concentration. The viscosity of a series of chlorobenzene-in-water emulsions has been determined in this laboratory as a function of cetomacrogol 1000 concentration and ϕ. Some unpublished results are shown in Fig. 10 which indicate that the higher the concentration of cetomacrogol in the system the lower the phase volume at inversion. Becher (1958) obtained this trend with low HLB emulsifiers (e.g. sorbitan monoesters) but, in general, with polyoxyethylated compounds the inversion point increased with increasing concentration. In a recent paper, Shinoda & Saito (1969) described emulsification by a phase-inversion method, this being the preparation of a stable, finely dispersed emulsion by rapid cooling of an emulsion at its phase inversion temperature. This differs from the normal emulsification by inversion which involves alteration of phase volume, for example, by addition of water to a w/o emulsion to form an o/w type. It was concluded that the optimum HLB for stability of an emulsion cannot be obtained accurately from HLB-stability

data but that, as stability is sensitive to temperature near the phase-inversion temperature (PIT), the selection of an emulsifier according to the PIT may be more reliable.

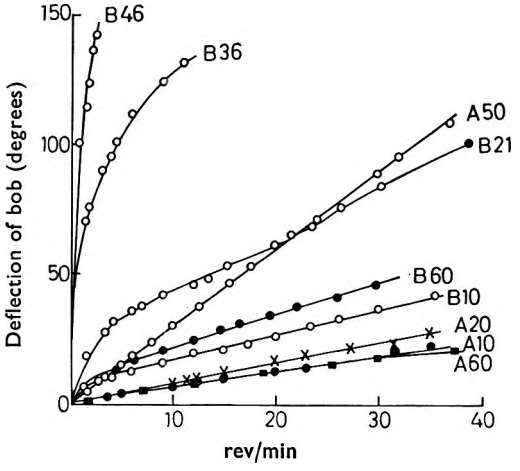


FIG. 10. Viscosity results obtained with a Couette viscometer on a series of emulsions of chlorobenzene stabilized A with 5% cetomagrogol 100 and B with 10% cetomacrogol 1000. Results are given in arbitrary units: deflection in degrees of inner bob versus rev/min of outer container. Phase volumes for both series are appended to lines as percentage oil. The diagram shows the inversion of A and B at a phase volume about 0·60 (see low viscosity of this one). B inverts before A. (Florence, A. T. & Guthrie, W., unpublished.)

Inversion of emulsion type can occur through temperature changes such as those encountered during sterilization or manufacturing procedures. Shinoda & Arai (1964) found that the more soluble a non-ionic emulsifier in a particular hydrocarbon, the lower was the phase inversion temperature of the emulsion. Hence, as Benerito & Singleton (1956) point out, emulsifiers in systems which have to withstand elevated homogenization or sterilization temperatures must be more hydrophilic than those found satisfactory at normal temperatures. However, the phase inversion temperature can be manipulated to a considerable extent by altering the composition of the oil phase. This is strikingly illustrated in Fig. 11 from the work of Arai & Shinoda (1967).

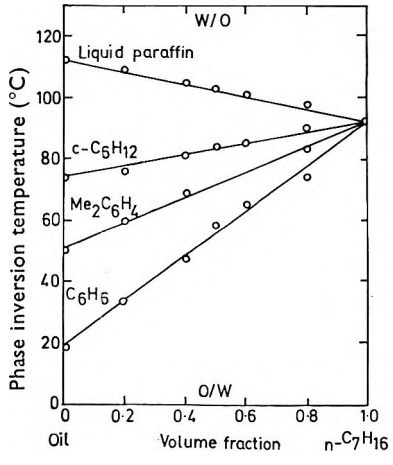


FIG. 11. The effect of the mixture of n-heptane with various oils on the phase inversion temperatures of emulsions stabilized with 3% w/w in water of polyxoyethylene (9·6) nonyl phenyl ether. Reproduced from Arai & Shinoda (1967) by permission of Academic Press.

Viscosity measurements have given information of the interaction forces operative between emulsion globules (Albers & Overbeek, 1959a,b; Doroszkowski & Lambourne, 1968) and the physical state of the continuous phase (Talman & others, 1967). The rheology of emulsions has been dealt with thoroughly by Sherman in two reviews (Sherman, 1964, 1968) and in one symposium volume (Sherman, 1963b) so there is no need to go into great detail here. It is sufficient to say that the apparent viscosity of an emulsion (η_∞) is dependent on phase volume (Fig. 10), mean particle diameter and particle size distribution (Richardson, 1953a,b), the stabilizing film and surfactant concentration. The control of the rheological properties of emulsions and creams is an important pharmaceutical problem.

Sherman (1963a) reports that multiphase globules of increasing number, size and complexity appear on increasing the concentration of sorbitan monolaurate and oil in liquid paraffin-in-oil emulsions. The multiphase globules influence η_∞ through their effect on the globule size distribution and ϕ. At $\phi = 0{\cdot}73$ and 6% surfactant concentration, inversion takes place to a w/o emulsion, the relative viscosity falling from 78·6 to 1·46 (as in Fig. 10). The resultant emulsion contains many multiple phase globules. Mulley & Marland (1970) discussed conditions in non-ionic-stabilized emulsions leading to multiple drop formation. Herbert (1965) utilized the lower viscosity of multiple emulsions to prepare parenteral mineral-oil antigen formulations. The incorporation of antigen into the aqueous phase of a w/o emulsion was proposed by Freund & Walter (1944) as an effective means of producing a prolonged antibody-response. Influenza vaccines prepared in this way, although effective (Hobson, Lane & others, 1964) have the disadvantage of high viscosity, producing problems in the syringe and *in vivo*. Herbert (1965), however, produced low-viscosity emulsions by ultrasonically redispersing w/o emulsions (containing antigen) in an aqueous phase containing a water-soluble surfactant. Samples of the multiple emulsions were stable for more than a year after storage at 56° and then at room temperature, whereas the original w/o emulsions broke down within a few months at 4°. The subcutaneous depots were diffuse and not discrete as with the viscous w/o preparation. The antibody response of mice to the multiple emulsion was superior to that of the original emulsion and the effect was well maintained. Berlin (1960) has found an inverse relation between antibody response and viscosity of w/o formulations, and there was some evidence of this trend in the system described by Herbert.

The factors affecting parenteral water-in-oil emulsions as adjuvants have been reviewed by Lazarus & Lachman (1967).

EMULSIONS AND THIN LIQUID FILMS

Because of the complexities of the real emulsion, attempts have been made to find model emulsion systems for experimental work. Latexes have been used and now that these are available commercially in monosized preparations these are very attractive systems. But, for the study of the molecular processes at work in stabilization and coalescence, foams and soap films serve as useful analogues (Sheludko, 1966, 1967). Between two emulsion globules at close approach there is left a thin film of continuous surfactant phase, the behaviour of which determines the probability of coalescence. Too often this film is neglected and the surface film of surfactant on each globule is viewed in isolation. The closest similarity to the emulsion situation is attained with aqueous soap films separating two identical oil phases (see Fig. 12), but much can be learned which has a bearing on emulsion stability from a study of soap films in air.

It is most likely that the rate of thinning of the films between the oil globules determines the coalescence probability, although thin soap films in air take minutes

to thin by a process of marginal regeneration (Mysels, Shinoda & Frankel, 1959) and vertical films of non-ionic detergents in decane thin more slowly than in air (Florence, unpublished). Undoubtedly the film between globules is under a momentary external pressure resulting from the collision of the droplets* and the suction

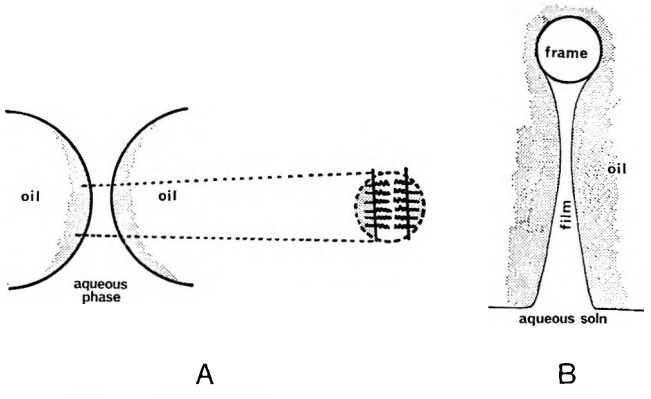


FIG. 12. A. Diagrammatic representation of the soap film between two oil globules and the arrangement of the non-ionic detergent molecules at equilibrium. B. Representation (cross section) of aqueous detergent film drawn up on a glass frame in oil for optical measurements on thickness and rates of thinning. Properties of films corresponding to w/o emulsions have been studied by Sonntag & Klare (1967).

by the Plateau borders in a film of 1 μm diameter will be much greater than in a macroscopic film. However, there is a need for the mechanisms of thinning of aqueous films in oil to be elucidated. van den Tempel (1958) measured the drainage and equilibrium thickness of the thin film between drops of liquid paraffin by an optical method and found that his thickness results agreed within experimental error with those of Derjaguin & Titjevskaja (1954) for liquid films between air bubbles (10–15 nm). van den Tempel's results appear to indicate the presence of repulsive forces other than those of electrostatic origin at distances below 125 nm.

Free aqueous films of the commercial non-ionic nonylphenyl-E_{20} (NP 20) in cyclohexane, studied by Netzel & Sonntag (1966), are thicker at equilibrium than those in air (Table 3), although at high electrolyte concentrations the thickness approaches

Table 3. *Equilibrium thicknesses of aqueous films in cyclohexane.*

Compound	Additive	Thickness (nm)	Reference
NP-20*	10^{-2} M KCl	26·0	Netzel & Sonntag (1966)
	$7{\cdot}5 \times 10^{-3}$ M KCl	28·0	Idem
	$2{\cdot}5 \times 10^{-3}$ M KCl	46·5	Idem
HD-15†	10^{-1} M NaCl	9·8	Duyvis (1962)
	10^{-2} M NaCl	29·1	Idem
	7×10^{-3} M NaCl	35·4	Idem
	3×10^{-3} M NaCl	55·3	Idem

* Length of molecule = 9·0 nm. Thickness of black film of OP-20 = 10·0 — 10·5 nm.
† Length of molecule = 7·8 nm. Thickness of black film in air = 11·0 nm.

that of the film in air (Duyvis, 1962). Sonntag & Netzel (1966) determined the critical thickness of aqueous non-ionic films between various oil phases. The thickness at rupture increases with increasing concentration of salt and varies with the organic

* A device for producing controlled collisions between pairs of droplets which might be useful in studies of this has been produced by Park & Crosby (1965).

phase, e.g. for octane $\delta_{crit} = 18{\cdot}5$ nm, chlorobenzene 19·5 nm and for cyclohexane 21·0 nm.

It is obvious that emulsion stabilizers do not act by maintaining a thick liquid film between the globules but that they function by decreasing the probability of the rupture of the film by a factor of 10^6–10^8 in "stable" systems.

Sonntag, Puschel & Strobel (1967) suggested that the emulsifying power of a surfactant for a given system is characterized by the concentration of the formation of stable black films (C_{bl}). A low value of C_{bl}, such as is given by nonyl phenol polyoxyethylene oxide adducts and cetyltrimethylammonium bromide, is indicative of good stabilizing properties.

Sheludko (1966) considers it uncertain why black films appear at C_{bl} or why low-molecular weight foaming agents should not form these films. He suggests that black "spots" appear in films in air where a dense adsorption monolayer begins to form, although just which property of the monolayer is responsible is not clear.

Derjaguin, Vorpayjeua & others (1964) has postulated a theory to account for the non-contact between dispersed particles arising from an "additional force", or "disjoining pressure" exerted at right angles to the plane of the liquid film between the particles. Thus, it acts adjacent to the surface tension which exists along the plane of the interface. Equation (7) may be modified to give

$$\frac{(\Delta\mu)_E}{V_1} = \frac{\Delta\mu - (\Delta\mu_1)_{ideal}}{V_1} = 2\left(\frac{d\gamma}{H}\right) = -P \quad .. \quad .. \quad (37)$$

where P is the disjoining pressure for particles at separation H, with surface free energy, γ. Equation (37), therefore, equates the disjoining pressure causing repulsion between the particles with the excess osmotic pressure in the overlapping volume. The thermodynamic view of disjoining pressure does not require the molecular origin of the forces to be specified. The main part of the disjoining pressure is due to forces of non-electrical origin, a conclusion supported by the fact that the pressure operates in the case of non-conducting liquid films and the absence of ions (Frenkel, 1955). A positive disjoining pressure is essential for stable films to exist. The unusual stability of thin liquid films in the presence of a stabilizer is due to their elasticity —which results from the dependence of their surface tension on their thickness, stretching depleting the surface layer and increasing tension [but see Sheludko (1966) for his views on role of elasticity. See also Kitchener & Cooper (1959)]. Frenkel (1955) suggests that polymolecular films must be still more effective owing to their higher elasticity. Unfortunately, in real emulsion systems polymolecular arrangements often lead to bridging of globules and hence to excessive creaming.

A possible mechanism for the spontaneous rupture of thin, free liquid films has been the subject of work by Vrij (1966), Vrij & Overbeek (1968) and Sheludko (1962). The latter proposed that instability of thin liquid films arises from spontaneous deformations on the surface which result in increased van der Waal's forces (due to closer approach of surface at some points) in spite of an increase in surface area. Vrij & Overbeek (1968) give as the critical thickness of a film of radius r, before rupture

$$h_{crit} = 0{\cdot}845\left[\frac{A^2r^2}{320\pi^2 f\sigma P}\right]^{\frac{1}{4}}$$

$$= 0{\cdot}267\,(A^2r^2/f\sigma P)^{\frac{1}{7}} \quad .. \quad .. \quad .. \quad .. \quad (38)$$

and the critical wavelength for rupture, Λ, as

$$\Lambda_{crit} = 2\pi\sqrt{\frac{\pi\sigma h^4}{A}} \quad .. \quad .. \quad .. \quad .. \quad (39)$$

If $A = 10^{-19}$ J, $\sigma = 10$ mNm^{-1}, and $h = 1$ μm, Λ is 0·35 cm on a macroscopic film with a linear dimension of the order of 1 mm or 1 cm. Λ_{crit} will be reached when the thickness has decreased below 1 μm. For a thin film existing between two emulsion droplets in a system of high phase volume when the droplet size is around 1 μm, its thickness has to decrease below 20 nm before fluctuations can make it unstable. There appears to be no dependence of the critical thickness on the viscosity of the film (Sonntag, 1966). Experiments have not, however, shown the dependence of h_{crit} on surface tension suggested by equation (38) when σ was varied from 4 to 16 mNm^{-1} (Sonntag, 1966).

Many investigators in considering mechanisms of film drainage have assumed that the interfaces of the film were immobile and that flow occurs between rigid "walls". Lee & Hodgson (1968) have dealt in more detail with other possibilities involving free movement, retarded mobility and complete immobility of the interface and have considered these conditions in relation to coalescence of liquid droplets. The three limiting conditions can represent the transition between sparsely covered surface and completely immobile surface having two surfactant components.

A theory of coalescence developed by Marrucci (1969) is probably applicable to non-ionic stabilized emulsions as it neglects electrical forces. The process of thinning is proposed to consist of two stages. The first involves an extremely rapid thinning of the film down to a quasi-equilibrium thickness, the time taken to reach this stage being negligible compared with the time of the second stage, thinning from quasi-equilibrium to rupture. The time of coalescence, governed by this second rate is an inverse function of the diffusion coefficient of the surfactant as diffusion at the border of the film controls the thinning rate. (The concentration in the film is different from that outside the film.)

CONCLUSIONS

It will probably be by further study of thin liquid films that progress will be made in our understanding of coalescence, the phenomenon that controls the ultimate stability of emulsions. The conditions and factors leading to flocculation and hence rapid creaming or sedimentation have been largely dealt with by the DLVO theory and theory of interaction of adsorbed layers. In an ideal situation theory should be able to predict size distribution at a specified emulsion age so that factors such as emulsifier concentration, emulsifier type and additive concentration can be altered to produce predictable distributions which change minimally with time. Until the theory has been developed that far, formulators will be able to predict less empirically from extant equations those factors which are likely to lead to stability and to measure more meaningful parameters in development stages of emulsion formulation. If stability can be successfully controlled then it is as pertinent to investigate the factors involved in the production of emulsions of a specified particle size distribution as it is to investigate the factors maintaining that distribution: work in both directions should succeed in reaching the degree of control most desirable in pharmaceutical systems.

Acknowledgement

We thank Professor P. H. Elworthy for his advice and encouragement and those publishers and authors noted in the legends for permission to use various figures.

REFERENCES

Adamson, A. W. (1969). *J. Colloid Inter. Sci.*, **29**, 261–267.
Albers, W. & Overbeek, J. Th. G. (1959a). *J. Colloid Sci.*, **14**, 501–517.
Albers, W. & Oberbeek, J. Th. G. (1959b). *Ibid.*, **14**, 510–518.

ARAI, H. & SHINODA, K. (1967). *J. Colloid Inter. Sci.*, **25**, 396–400.
ATTWOOD, D., ELWORTHY, P. H. & KAYNE, S. B. (1968). *J. Pharm. Pharmac.*, **21**, 619–620.
ATTWOOD, D. & FLORENCE A. T. (1971). *Kolloid zeitschrift.* In the press.
BAILEY, F. E. & CALLARD, R. W. (1959). *J. appl. Polymer Sci.*, **1**, 56–62.
BECHER, P. (1958). *J. Soc. cosmet. Chem.*, **9**, 141–148.
BECHER, P. (1962). *J. Colloid Sci.*, **17**, 325–333.
BECHER, P. (1963). *Ibid.*, **18**, 665–673.
BECHER, P. (1965). *Emulsions—Theory and Practice*, 2nd edn, N.Y.: Reinhold.
BECHER, P. (1967). *J. Colloid Inter. Sci.*, **24**, 91–96.
BENERITO, R. R. & SINGLETON, W. C. (1956). *J. Am. Oil Chem. Soc.*, **33**, 364–367.
BERLIN, B. S. (1960). *J. Immunol.*, **85**, 81–89.
BISWAS, B. & HAYDON, D. A. (1960). *Proc. 3rd Int. Congr. Surf. Activ.*, Cologne, **2**, 580–584.
BISWAS, N. & HAYDON, D. A. (1962a). *Kolloidzeitschrift.*, **185**, 31–38.
BISWAS, B. & HAYDON, D. A. (1962b). *Ibid.*, **186**, 57–61.
CASADIO, S. (1951). *Boll. chim. farm.*, **90**, 277–282.
CLAYFIELD, E. J. & LUMB, E. C. (1966). *J. Colloid Inter. Sci.*, **22**, 269–284.
COCKBAIN, E. G. & MCROBERTS, T. S. (1953). *J. Colloid Sci.*, **8**, 440–451.
COCKBAIN, E. G. (1956). *Ibid.*, **11**, 575–584.
COLLINGS, A. J. & SCHNEIDER, R. (1970). *Br. J. Pharmac.*, **38**, 48P–49P.
COLLINS, F. C. & KIMBALL, G. E. (1949). *J. Colloid Sci.*, **4**, 425–437.
DAVIES, J. T. & HAYDON, D. A. (1957). *Proc. 2nd Int. Congr. on Surf. Activ. I*, pp. 417–425. London: Butterworths.
DAVIES, J. T. & MAYERS, G. R. A. (1960). *Trans. Faraday Soc.*, **56**, 691–696.
DERJAGUIN, B. V. (1939). *Ibid.*, **36**, 203–915.
DERJAGUIN, B. V. (1966). *Discuss. Faraday Soc.*, **42**, 109–119; 317–321.
DERJAGUIN, B. V. & LANDAU, L. (1941). *Acta phys.-chim. URSS*, **14**, 565–565. per "*Collected Papers of L. Landau*", Pergamon Press, London.
DERJAGUIN, B. V. & TITJEVSKAYA, A. S. (1954). *Ibid.*, **18**, 24–41.
DERJAGUIN, B. V., VORPAYEUA, T. N., KABANON, B. N. & TITJEVSKAYA, A. S. (1964). *J. Colloid Sci.*, **19**, 113–135.
DOROSZKOWSKI, A. & LAMBOURNE, R. (1968). *J. Colloid Inter. Sci.*, **26**, 128–130.
DOSCHER, T. M., MYERS, G. E. & ATKINS, D. C. (1951). *J. Colloid Sci.*, **6**, 223–235.
DUYVIS, E. M. (1964). Thesis, Utrecht University.
ELIAS, H. G. & LYS, H. (1966). *Makromol. Chem.*, **92**, 1–24.
ELLIS, C. (1935). *The Chemistry of Synthetic Resins*, Vol. 1, p. 74. New York: Reinhold.
EL'SHIMI, A. F. & IZMAILOVA, V. N. (1967). *Colloid. J., U.S.S.R.*, 29, 550–552; **130**, 130–134; 326–328.
ELWORTHY, P. H. & FLORENCE, A. T. (1967). *J. Pharm. Pharmac.*, **19**, *Suppl.*, 140S–154S.
ELWORTHY, P. H. & FLORENCE, A. T. (1969a). *Ibid.*, **21**, 72–78.
ELWORTHY, P. H. & FLORENCE, A. T. (1969b). *Ibid.*, **21**, *Suppl.*, 70S–78S.
ELWORTHY, P. H. & FLORENCE, A. T. (1969c). *Ibid.*, **21**, *Suppl.*, 79S–90S.
ELWORTHY, P. H., FLORENCE, A. T. & ROGERS, J. A. (1971a). *J. Colloid Inter. Sci.*, **35**, 23–33.
ELWORTHY, P. H., FLORENCE, A. T. & ROGERS, J. A. (1971b). *Ibid.*, **35**, 34–39.
ELWORTHY, P. H. & MCDONALD, C. (1964). *Kolloidzeitschrift*, **195**, 16–23.
ELWORTHY, P. H. & MACFARLANE, C. B. (1962). *J. Pharm. Pharmac.*, **14**, *Suppl.*, 100T–102T.
ELWORTHY, P. H. & MACFARLANE, C. B. (1965). *Ibid.*, **17**, 65–82; 129–143.
ENGEL, R. H. & RIGGI, S. J. (1969). *J. pharm. Sci.*, **58**, 1352–1357.
FISCHER, E. W. (1958). *Kolloidzeitschrift*, **160**, 120–141.
FRENKEL, J. (1955). *Kinetic Theory of Liquids*, pp. 332–333. New York: Dover Publications.
FREUND, J. & WALTER, A. W. (1944). *Proc. Soc. exp. biol. med.*, **56**, 47.
GEYER, R. P. (1960). *Physiol. Rev.*, **40**, 150–186.
GLAZMAN, Yu. M. (1966). *Discuss. Faraday Soc.*, **42**, 255–266.
GLAZMAN, Yu. M. & KABYSH, G. M. (1969). *Colloid J., U.S.S.R.*, **31**, 27–33.
GLUZMAN, M. Kh. & FRIDMAN, G. M. (1968). *Ibid.*, **30**, 270–272.
GOLD, G. (1962). *Diss. Abstr.*, **22**, 3839.
GREENWALD, H. L. & BROWN, G. L. (1954). *J. phys. Chem.*, **58**, 825–828.
GREGORY, J. I. (1970). *Adv. Colloid Inter. Sci.*, **2**, 396–417.
GRIFFIN, W. C. (1949). *J. Soc. cosmet. Chem.*, **1**, 311–326.
GRIFFIN, W. C. (1954). *Ibid.*, **5**, 249.

GUTTMAN, D. E. & HIGUCHI, T. (1956). *J. Am. pharm. Ass.* (*Sci. Edn*), **45**, 659–664.
HADGRAFT, J. W. (1954). *J. Pharm. Pharmac.*, **6**, 816–829.
HAMAKER, H. C. (1937). *Physica,'s Grav.*, **4**, 1058–1072.
HAMILL, R. D. & PETERSEN, R. V 1966). *J. pharm. Sci.*, **55**, 1274–1277.
HAMMES, G. G. & SWANN, J. C. (1967). *Biochemistry*, **6**, 1591–1596.
HERBERT, W. J. (1965). *Lancet*, **2**, 771.
HIGUCHI, T. & LACH, J. L. (1954). *J. Am. pharm. Ass.* (*Sci. Edn*), **43**, 465–470.
HIGUCHI, W. I. & MISRA, J. (1962). *J. pharm. Sci.*, **51**, 459–466.
HIGUCHI, W. I., OKADA, R., STELTER, G. A. & LEMBERGER, A. P. (1963). *Ibid.*, **52**, 49–54.
HILL, R. A. W. & KNIGHT, J. T. (1965). *Trans. Faraday Soc.*, **61**, 170–181.
HO, N. F. H. & HIGUCHI, W. I. (1968). *J. pharm. Sci.*, **57**, 436–442.
HOBSON, D., LANE, C. A., BEARE, A. & CHIVERS, C. (1964). *Br. med. J.*, **2**, 271–274.
HOGG, R., HEALY, T. W. & FUERSTENAU, D. W. (1966). *Trans. Faraday Soc.*, **62**, 1633–1651.
HSIAO, L., DUNNING, H. N. & LORENZ, P. B. (1956). *J. phys. Chem.*, **60**, 657–660.
HUTCHINSON, E., SHEAFFER, V. & TOKIWA, F. (1964). *Ibid.*, **68**, 2818–2825.
ISEMURA, T. & KIMURA, Y. (1952). *Mem. Inst. sci. ind. Res.*, Osaka Univ., **9**, 137–145; through *Chem. Abstr.*, **47**, 1188 f. (1952).
JACKEL, K. (1964). *Kolloidzeitschrift*, **197**, 143–151.
JELLINEK, H. H. G. & ANSON, H. A. (1950). *J. Soc. chem. Ind.*, **69**, 229–235.
KHARIN, S. E. (1956). *Vest. Akad. Nauk B., S.S.S.R.*, No. 3, 47–59, through *Chem. Abstr.*, **51**, 13521 g. (1957).
KING, A. (1941). *Trans. Faraday Soc.*, **37**, 168–181.
KING, A. & MUKHERJEE, L. N. (1939). *J. Soc. chem. Ind.*, **58**, 243–249.
KITCHENER, J. A. & COOPER, C. F. (1959). *Q. Rev., Lond.*, **13**, 71–97.
KITCHENER, J. A. & MUSSELWHITE, P. R. (1968). In *Emulsion Science*, Chapter 2, p. 115. Editor: Sherman, P. London: Academic Press.
KURIYAMA, K. (1962). *Kolloidzeitschrift*, **181**, 144–149.
LAWRENCE, A. S. C. & MILLS, O. S. (1954). *Discuss. Faraday Soc.*, **18**, 98–104.
LAZARUS, J. & LACHMAN, L. (1967). *Bull. parent. Drug. Ass.*, **21**, 184–196.
LEE, J. C. & HODGSON, T. G. (1968). *Chem. Engng Sci.*, **23**, 1375–1397.
LEVI, S. M. & SMIRNOV, O. K. (1959). *Colloid J., U.S.S.R.*, **21**, 315, from VOYUTSKII, S. S. (1961). *Russian Chem. Revs.*, **30**, 556–568.
LIN, T. J. & LAMBRECHTS, J. S. (1969a). *J. Soc. cosmet. Chem.*, **20**, 185–198.
LIN, T. J. & LAMBRECHTS, J. S. (1969b). *Ibid.*, **20**, 627–638.
LLADSER, M., MEDRANO, C. & ARANCBIA, A. (1968). *J. Pharm. Pharmac.*, **20**, 450–455.
LONDON, F. (1930). *Z. Physik*, **63**, 245–279.
LOTZKAR, H. & MACLAY, W. D. (1943). *Ind. Engng Chem.*, **35**, 1294–1297.
LOVELL, E. L. & HIBBERT, H. (1940). *J. Am. chem. Soc.*, **62**, 2140–2143.
LUNDBERG, R. D., BAILEY, F. E. & CALLARD, R. W. (1966). *J. Polym. Sci.*, **4**, 1563–1577.
LYKLEMA, J. (1968). *Adv. Colloid Inter. Sci.*, **2**, 54–114.
LYNN, B. (1970). *J. hosp. Pharm.*, **28**, 71–86.
MACFARLANE, C. B. (1963). Ph.D. Thesis, Glasgow.
MACRITCHIE, F. (1967). *Nature, Lond.*, **215**, 1159–1161.
MACKOR, E. L. (1951). *J. Colloid Sci.*, **6**, 492–495.
MACKOR, E. L. & VAN DER WAALS, J. H. (1952). *Ibid.*, **7**, 535–550.
MALCOLM, G. N. & ROWLINSON, J. S. (1957). *Trans. Faraday Soc.*, **53**, 921–931.
MARDLES, E. W. J. & DE WAELE, A. (1951). *J. Colloid Sci.*, **6**, 42–56.
MARRUCHI, G. (1969). *Chem. Engng Sci.*, **24**, 975–985.
MATSUMOTO, S. & SHERMAN, P. (1969). *J. Colloid Inter. Sci.*, **30**, 525–536; (1970). *Ibid.*, **33**, 294–298.
MATTHEWS, B. A. & RHODES, C. T. (1968a). *J. pharm. Sci.*, **57**, 557–563.
MATTHEWS, B. A. & RHODES, C. T. (1968b). *Ibid.*, **57**, 569–573.
MEIER, D. J. (1967). *J. phys. Chem.*, **71**, 1861–1868.
MERRILL, R. C., Jr. (1943). *Ind. Engng Chem., Analyt. Edn*, **15**, 743–746.
MONK, D., MATIJEVIC, E. & KERKER, M. (1969). *J. Colloid Inter. Sci.*, **39**, 164–166.
MÜLLER, H. (1926) (1928). *Kolloidzeitschrift*, **38**, 1–2, (1926) *Kolloid-chem. Beih.* (1928), **26**, 257–311.
MÜLLEY, B. A. (1968). In *Nonionic Surfactants*, pp. 421–440. Editor: Schick, M. New York: Marcel Dekker.

MULLEY, B. A. & MARLAND, J. S. (1970). *J. Pharm. Pharmac.*, **22**, 243–245.

MUSSELLWHITE, P. R. (1964). *Proc. 4th Intern. Congr. Surface Activity*, Brussels, **2**, 947–953.

MYSELS, K. J., SHINODA, K. & FRANKEL, S. (1959). *Soap Films: A study of their thinning and a bibliography*. London: Pergamon.

NAPPER, D. H. (1968). *Trans. Faraday Soc.*, **64**, 1701–1711.

NAPPER, D. H. (1969). *J. Colloid Inter. Sci.*, **29**, 168–170.

NAWAB, M. A. & MASON, S. G. (1958). *J. Colloid Sci.*, **13**, 179–187.

NEIMAN, R. E., LYASHENKO, O. S., KIRDEEVA, A. P., EGOROV, A. K. & KISELEVA, O. G. (1961). *Colloid J. (U.S.S.R.)* (English Transl.), **23**, 618–623.

NETZEL, J. & SONNTAG, H. (1966). *Third Congr. Surf. Activ.*, Berlin, 589–596.

NEWITT, D. M., DOMBROWSKI, R. K. & KRELMAN, F. (1954). *Trans. Instn chem. Engrs.*, **32**, 244–261.

NIKITINA, S. A., TAUBMAN, A. B., KULIGINA, N. V. & SIRIDONOVA, B. A. (1963). *Dokl. Akad. Nauk, SSSR*, **149**, 905–908.

OTTEWILL, R. H. (1966). *Discuss. Faraday Soc.*, **42**, 283–284.

OTTEWILL, R. H. (1967). In *Nonionic Surfactants*, Chap. 19, pp. 627–682. Editor: Schick, M. J., New York: Marcel Dekker.

OTTEWILL, R. H. & SHAW, J. N. (1966). *Discuss. Faraday Soc.*, **42**, 154–163.

OTTEWILL, R. H. & WALKER, T. (1968). *Kolloidzeitschrift*, **227**, 108–116.

OTTEWILL, R. H. & WATANABE, A. (1960). *Ibid.*, **170**, 38–48, 132–139; **171**, 33–42; **173**, 3–14, 122–128.

OVERBEEK, J. Th. G. (1952). In *Colloid Chemistry*, Vol. 1, p. 285, p. 292. Editor: Kruyt, H. R. Amsterdam: Elsevier.

PARK, R. W. & CROSBY, E. J. (1965). *Chem. Engng Sci.*, **20**, 39–45.

PETERSEN, R. V. & HAMILL, R. D. (1968). *J. Soc. cosmet. Chem.*, **19**, 627–640.

PRINS, A. & VAN DEN TEMPEL, M. (1964). *Proc. 4th Int. Congr. Surf. Activ.*, Brussels, **2**, 1119–1131.

PRAKASH, S. & GHOSH, A. K. (1962). *Ultrasonics and Colloids*. London: Asia Publishing House.

PRAKASH, C. & SRIVASTAVA, S. N. (1967). *Bull. chem. Soc., Japan*, **40**, 1756–1965.

PRIGORODOV, V. N., NIKITINA, S. A. & TAUBMAN, A. B. (1965). *Colloid J., U.S.S.R.*, **27**, 734–737

RAJAGOPAL, E. S. (1959). *Proc. Indian Acad. Sci.*, **49A**, 333, per CA 54, 2830C (1960).

REERINK, H. & OVERBEEK, J. Th. G. (1954). *Discuss. Faraday Soc.*, **18**, 74–84.

RICE, C. L. & WHITEHEAD, R. (1967). *J. Colloid Inter. Sci.*, **23**, 174–181.

RICHARDS, R. M. E. & WHITTET, T. D. (1955). *Pharm. J.*, **175**, 141–144.

RICHARDSON, E. G. (1953a). *Flow Properties of Disperse Systems*, p. 49. Editor: Herman, J. J. Amsterdam: North Holland.

RICHARDSON, E. G. (1953b). *J. Colloid Sci.*, **8**, 367–373.

RICHTER, A. & STEIGER-TRIPPI, K. (1960). *Pharm. Acta Helv.*, **36**, 322–337.

RIEGELMAN, S. & PICHON, G. (1962). *Am. Perfum.*, **77**, 31–33.

ROSCH, M. (1956). *Kolloidzeitschrift*, **147**, 78–79, 79–81.

ROSCH, M. (1957). *Ibid.*, **150**, 153–156.

ROWE, E. L. (1965). *J. pharm. Sci.*, **54**, 260–264.

SANDERS, P. A. (1970). *J. Soc. cosmet. Chem.*, **21**, 377–391.

SATA, N. & HARISAKI, Y. (1960). *Kolloidzeitschrift*, **171**, 101–107.

SCHICK, M. J. (1962). *J. Colloid Sci.*, **17**, 801–813.

SCHICK, M. J. (1963). *Ibid.*, **18**, 378–390.

SCHICK, M. J. (1967). *Nonionic Surfactants*. New York: Marcel Dekker.

SCHICK, M. J., ATLAS, S. M. & EIRICH, F. R. (1962). *J. phys. Chem.*, **66**, 1326–1333.

SCHULMAN, J. H. & COCKBAIN, E. G. (1940a). *Trans. Faraday Soc.*, **36**, 651–661; (1940b), *Ibid.*, 661–668.

SCHULMAN, J. H. & MONTAGUE, J. B. (1961). *Ann. N.Y. Acad. Sci.*, **92**, 366–371.

SCHULMAN, J. H., STOECKENIUS, W. & PRINCE, L. M. (1959). *J. phys. Chem.*, **63**, 1677–1680.

SERRALACH, J. A. & JONES, G. (1931). *Ind. Engng Chem.*, **23**, 1016–1019.

SERRALACH, J. A., JONES, G. & OWEN, R. R. (1933). *Ibid.*, **25**, 816–819.

SHELUDKO, A. (1962). *Proc. K. ned. Akad. Wet.*, **B65**, 76.

SHELUDKO, A. (1966). *Colloid Chemistry*. Amsterdam: Elsevier.

SHELUDKO, A. (1967). *Adv. Colloid Inter. Sci.*, **1**, 391–464.

SHERMAN, P. (1963a). *J. phys. Chem.*, **67**, 2531–2537.

SHERMAN, P. (1963b). *Rheology of Emulsions*. London: Pergamon.

SHERMAN, P. (1964). *J. Pharm. Pharmac.*, **16**, 1–25.

SHERMAN, P. (1968). *Emulsion Science*, Chap. 4, p. 277. London: Academic Press.

SHINODA, K., NAKAGAWA, T., TAMAMUSHI, R. & ISEMURA, T. (1963). *Colloidal Surfactants: Some Physiochemical Properties.* New York: Academic Press.

SHINODA, K. & ARAI, H. (1964). *J. phys. Chem.*, **68**, 3483–3490.

SHINODA, K. (1967). *J. Colloid Inter. Sci.*, **24**, 4–9.

SHINODA, K. & ARAI, H. (1967). *Ibid.*, **25**, 429–439.

SHINODA, K. & SAITO, H. (1969). *Ibid.*, **30**, 258–263.

SHOTTON, E. & WHITE, R. F. (1963). In *Rheology of Emulsion*, pp. 59–71. Editor: Sherman, P. London Pergamon.

SHOTTON, E., WIBBERLEY, K. & VAZIRI. A. (1964). *Proc. 4th Int. Congr. Surf. Activ.*, Brussels, **2**, 1211–1219.

SMOLUCHOWSKI, M. (1914). In *Handbuch der Elektrizitat und des Magnetissmus*, Vol. 2, p. 366, Editor: Graetz, B. Leipzig.

SMOLUCHOWSKI, M. (1916). *Phys. Z.*, **17**, 557–585; **18**, 190 (1917), *Z. phys. Chem.*, **92**, 129–168.

SÖLLNER, E. & BONDY, I. (1935). *Trans. Faraday Soc.*, **31**, 835–846; (1966), *Ibid.*, **32**, 616–623.

SOMASUNDARAN, P., HEALY, T. W. & FUERSTENAU, D. W. (1936). *J. Colloid Inter. Sci.*, **22**, 599–605.

SONNTAG, H. & KLARE, H. (1963). *Z. phys. Chem.*, **223**, 8–16.

SONNTAG, H. & NETZEL, J. (1966). *Tenside*, **3**, 296–299.

SONNTAG, H. & KLARE, H. (1967). *Ibid.*, **4**, 104–108.

SONNTAG, H. (1968). *Ibid.*, **5**, 188–193.

SONNTAG, H., PUSCHEL, F. & STROBEL, B. (1967). *Ibid.*, **4**, 349–352.

SONNTAG, H., NETZEL, J. & UNTERBERGER, B. (1970). *Special Discussions of the Faraday Soc.*, to be published.

STANDINGER, H. (1932). *Die hochmolekularen organischen Verbindungen.* Berlin: Springer.

STEWART, G. W. (1943). *J. chem. Phys.*, **11**, 72–74.

STRASSNER, J. E. (1968). *J. Petrol. Technol.*, **20**, 303–312.

SUZUKI, A , HO, N. F. H. & HIGUCHI, W. I. (1969). *J. Colloid Inter. Sci.*, **29**, 552–564.

SWIFT, D. L. & FRIEDLANDER, S. K. (1964). *J. Colloid Sci.*, **19**, 621–647.

TALMAN, F. A. J., DAVIES, P. F. & ROWAN, E. M. (1967). *J. Pharm. Pharmac.*, **19**, 417–425.

TODAY'S DRUGS (1970). *Br. med. J.*, **1**, 352–353.

TOSCH, W. C., JONES, S. C. & ADAMSON, A. W. (1969). *J. Colloid Interface Sci.*, **31**, 297–306.

UCHIDA, T., KURITA, Y., KOISUMI, N. & KUBO, M. (1956). *J. Polym. Sci.*, **21**, 313–322.

VAN DEN TEMPEL, M. (1953a). *Recl Trav. chim. Pays-Bas Belg.*, **72**, 433–441.

VAN DEN TEMPEL, M. (1953b). *Ibid.*, **72**, 442–461.

VAN DEN TEMPEL, M. (1957). *Proc. 2nd Int. Congr. Surf. Activ.*, London, **1**, 439–446.

VAN DEN TEMPEL, M. (1958). *J. Colloid Sci.*, **13**, 125–133.

VAN DEN TEMPEL, M., LUCASSEN, J. & LUCASSEN-REYNDERS, E. H. (1965). *J. phys. Chem.*, **69**, 1798–1804.

VAN DER WAARDEN, N. (1950). *J. Colloid Sci.*, **5**, 317–325.

VAN DEN WAARDEN, N. (1958). *Kolloidzeitschrift*, **156**, 116–122.

VERWEY, E. J. W. (1940). *Trans. Faraday Soc.*, **36**, 192–203.

VERWEY, E. J. W. & OVERBEEK, J. Th. G. (1948). *Theory of the Stability of Lyophobic Colloids.* Amsterdam: Elsevier.

VERWEY, E. J. W. (1966). *Discuss. Faraday Soc.*, **42**, 314–315.

VOLD, M. J. (1961). *J. Colloid Sci.*, **16**, 1–12.

VOLD, M. J. (1969). *J. Colloid Inter. Sci.*, **29**, 181–186.

VRIJ, A. (1966). *Discuss. Faraday Soc.*, **42**, 23–33.

VRIJ, A. & OVERBEEK, J. Th. G. (1968). *J. Am. chem. Soc.*, **90**, 3074–3078.

WAN, L. S. C. & POON, P. K. C. (1969). *J. pharm. Sci.*, **8**, 1562–1567.

WATANABE, A., MATSUMOTO, M. & GOTOH, R. (1965). *Kolloidzeitschrift*, **201**, 147–154.

J. Pharm. Pharmac., 1971, **23**, 252–261 *Received December* 21, 1970

The effect of heat, pH and some buffer materials on the hydrolytic degradation of sulphacetamide in aqueous solution

B. J. MEAKIN†, I. P. TANSEY* AND D. J. G. DAVIES†

† *The Pharmaceutics Group, School of Pharmacy, Bath University of Technology, Bath, U.K. and the* **Pharmaceutical Department, Southmead Hospital Group, Bristol, U.K.*

The hydrolytic degradation of sulphacetamide has been investigated under anoxic conditions over a wide range of pH values and at different temperatures. The sole breakdown product is sulphanilamide. The reaction is essentially independent of pH (measured at 25°) over the range 5 to 11, but is subject to catalysis by buffer constituents. Below pH 4 specific hydrogen ion catalysis occurs. Calculation of activation parameters leads to the conclusion that sulphacetamide solutions can be satisfactorily autoclaved provided they are not subsequently refrigerated.

Aqueous solutions of sulphacetamide are degraded on storage by both oxidative and hydrolytic mechanisms (Clarke, 1965, 1967). Antioxidants such as sodium edetate and sodium metabisulphite have been included in official formulations of the drug to discourage colour development. Sodium metabisulphite, however, increases the hydrolytic deacetylation of sulphacetamide at pH 7·4 (Davies, Meakin & Moss, 1970) to such an extent that sulphanilamide may be deposited from strong solutions of the drug (Anderson & Maudson, 1963; Fletcher & Norton, 1963).

Little is known about the conditions which favour the hydrolysis of sulphacetamide and we report the results of a study into this aspect of the drug's stability.

MATERIALS AND METHODS

Materials

Ammonium sulphamate, *p*-dimethylaminobenzaldehyde, diethylamine, hydrochloric acid and *N*-(1-naphthyl) ethylenediamine dihydrochloride were laboratory reagent grade (BDH): acetone, citric acid, disodium hydrogen phosphate, methanol, potassium chloride, sodium chloride, sodium hydroxide, sodium nitrite were A.R. grade (BDH). Silica gel G (Merck) was used for thin-layer chromatography; oxygen free nitrogen was white spot grade (BOC); glycine and tris(hydroxymethyl)-aminomethane were biochemical grade (BDH). Sulphacetamide sodium B.P. (m.p. 183°) was recrystallized twice from ethanol, sulphanilamide B.P.C. (m.p. 163·5°) was recrystallized twice from water.

Reagent solutions

Ammonium sulphamate 2% w/v in distilled water; *N*-(1-naphthyl) ethylenediamine dihydrochloride 0·1% w/v in distilled water; sodium nitrite 0·1% w/v in distilled water; *p*-dimethylaminobenzaldehyde 1% w/v in equal volumes of concentrated hydrochloric acid and ethanol. Methanolic hydrochloric acid: 1 volume concen-

* Present address, Riker Laboratories, Loughborough, Leics.

trated hydrochloric acid added to 75 volumes of methanol and adjusted to 100 volumes with distilled water.

Buffer solutions

Buffer solutions used for determining the pH profile and temperature dependence of sulphacetamide degradation were: pH range 1·2–3·6, Sørensen's glycine, sodium chloride, 0·1M hydrochloric acid; pH range 3·6–8·0, McIlvaine's citric acid, disodium hydrogen phosphate; pH range 8·4–12·6 Sørensen's glycine, sodium chloride, 0·1M sodium hydroxide.

Buffers used for determining the effect of buffer constituents on sulphacetamide degradation at pH 7·4 were: single double and quadruple strength McIlvaine's citric, acid, disodium hydrogen phosphate (ionic strengths, 0·488, 0·976, 1·952M respectively), single strength McIlvaine's buffer adjusted to ionic strength 1·952M with potassium chloride, and Gomori's tris buffer [0·2M tris (hydroxymethyl) aminomethane, 0·2M hydrochloric acid].

The appropriate standard solutions were prepared according to Documenta Geigy (p. 315). Sulphacetamide sodium was added to the tabulated volume of glycine-sodium chloride solution, or disodium hydrogen phosphate solution and the pH adjusted to the required value by the addition of hydrochloric acid, sodium hydroxide or citric acid solutions as appropriate. All buffered sulphacetamide solutions showed pH drifts of less than 0·1 of a pH unit after heating.

pH measurement

pH measurements were made at 25° with either a Radiometer type 27 meter fitted with a Radiometer type C glass-calomel electrode system (pH 1–11; 0–70°) or a Pye Dynacap meter fitted with a Jena dual glass-calomel electrode system (pH 1–14; 20–120°). The instruments were standardized with 0·05M potassium hydrogen phthalate, 0·05M potassium tetroxalate, 0·025M potassium dihydrogen phosphate–0·025M disodium hydrogen phosphate and 0·01M borax (Schwabe, 1960).

pH measurements at elevated temperatures were made after equilibrating the solutions in a thermostatically controlled water bath (±0·5°). Apparent pK_a values for 1% solutions of sulphacetamide were determined by potentiometric titration.

Oxygen tension measurement

Oxygen tensions of sulphacetamide solutions were measured using a Radiometer type PHA 927b gas monitor fitted with a Clark type oxygen electrode, standardized with 0·1M borax containing 1% w/v sodium metabisulphite and air saturated water.

Assay method

Sulphacetamide was separated from its degradation product, sulphanilamide, before assay, by thin-layer chromatography. All solutions were then assayed for both sulphacetamide and sulphanilamide content using the following technique.

A 20 × 20 cm t.l.c. plate spread (0·25 mm) with ether-extracted silica gel G was air dried overnight and activated at 110° for 30 min. Six samples, each of nominal volume 5 μl (true volume 4·84 μl) were applied with an Agla micrometer syringe fitted with a flat tipped, metal needle. Precision of delivery had previously been checked both by direct weighing and tracer techniques and coefficients of variation were 1·22% (30 replicates) and 1·02% (10 replicates) respectively.

The chromatograms were developed in batches at room temperature (20°), using as solvent, acetone–methanol–diethylamine (9 : 1 : 1 by volume). This gave excellent separation of sulphacetamide and sulphanilamide, mean R_F values 0·28 and 0·83 respectively. For each run, spots were visualized on one reference plate with *p*-dimethylaminobenzaldehyde solution. Using this as a guide, corresponding areas containing the sulphonamides were scraped into stoppered centrifuge tubes, 5 ml of extracting solvent (methanolic hydrochloric acid for sulphacetamide; methanol for sulphanilamide) were added, the tubes shaken for 10 min and centrifuged at 3500 rev/min for 5 min. Blank areas from each plate were treated similarly.

To the supernatant (3 ml) was added sodium nitrite solution (1 ml) and water (sulphacetamide) or 0·1N hydrochloric acid (sulphanilamide) (3·3 ml). After 5 min ammonium sulphamate solution (1 ml) was added, and 10 min later *N*-(1-naphthyl) ethylenediamine dihydrochloride solution (1 ml). The solutions were adjusted to 10 ml with water and stored in the dark (15 min) to allow colour development. The extinctions of the solutions were then measured at 536 nm using an Unicam SP600 spectrophotometer. The mean values of three assays were used in calculation. Absolute concentrations of the sulphonamides were determined from the extinction coefficients of the pink dyes (sulphacetamide, E (1%, 1 cm) 2104; sulphanilamide, E (1%, 1 cm) 3000) which were calculated with reference to the original sulphonamide.

The percentage residual concentrations obtained from the heating experiments were calculated with respect to the appropriate unheated sulphacetamide solution which was assayed without prior chromatographic separation.

The efficiency of the assay process was checked by the analysis of solutions containing known compositions of sulphacetamide and sulphanilamide (90: 10; 70: 30; 50:50; 30:70); recoveries, calculated as total sulphonamide were 99·3, 99·9, 97·6, 99·9% respectively.

Heating experiments

Although 1% aqueous solutions of sulphacetamide sodium became discoloured when heated under air, the rate of disappearance of the drug was not significantly different from that obtained under anoxic conditions (Tansey, 1969). The formation of colour however, implied additional sequences in the degradation pathway when oxygen was present. Consequently, all data presented in this paper were obtained under anoxic conditions, to eliminate any possible effects of colour-forming reactions.

The effect of pH on the degradation rate of sulphacetamide at 120°. Solutions of sodium sulphacetamide (1%) in buffer over the pH range 1·6–12·6 were bubbled with nitrogen to zero oxygen tension, transferred anoxically to ampoules and sealed (Tansey, 1969). These were heated in an oil bath at 120° ± 0·5°, removed at varying time intervals, chilled in crushed ice and stored at 2° until assayed. Under all conditions, first order kinetics were obeyed, the first order rate constants (k) being computed by means of a least squares regression analysis and used as a measure of the heat sensitivity and to calculate the specific second order rate constants for the hydrogen ion catalysed reaction (k_1) and the hydroxyl ion catalysed reaction (k_5) as defined in equation 6. The values are given in Table 1, and Fig. 1 shows a plot of k against pH.

The effect of temperature. Anoxic solutions containing 1% of sodium sulphacetamide adjusted to pH 7·4 with McIlvaine's buffer were heated for varying periods at temperatures of 99·5°, 110°, 120°, 130° and 140° (all ±0·5°) and assayed as before.

The first order rate constants at each temperature were computed (being respectively 0·627, 1·588, 3·468, 6·644 and 13·370 $\times$ 10^{-2} h^{-1}) and used to calculate the activation parameters for the hydrolytic degradation of sulphacetamide (Table 3).

The effect of buffer components. Anoxic solutions containing 1% of sulphacetamide sodium were adjusted to pH 7·4 with hydrochloric acid, single, double and quadruple strength McIlvaine's buffer, ionic strengths 0·488, 0·976 and 1·952M respectively (Elving, Marcovitch & Rosenthal, 1956), single strength McIlvaine's buffer adjusted to ionic strength 1·952M with potassium chloride, and Gomori's tris buffer. The solutions were heated for varying periods at 120° assayed and the various rate constants computed (Table 2).

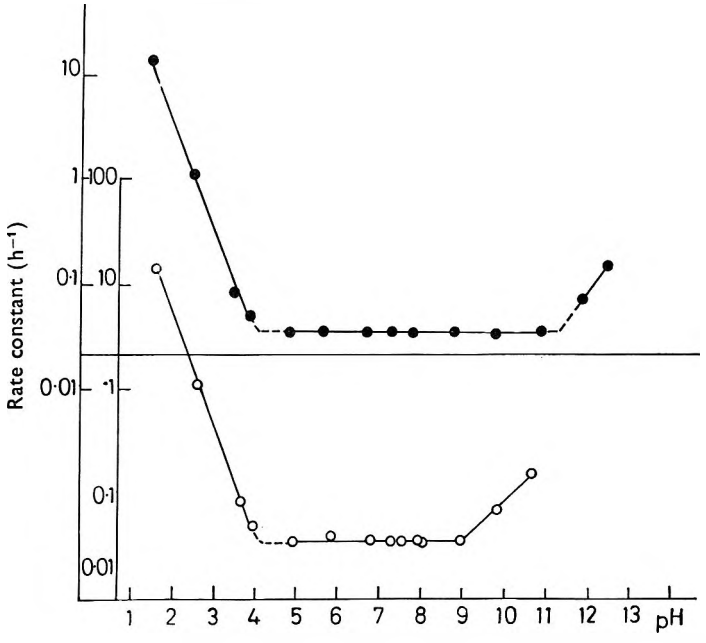


FIG. 1. The effect of pH on the observed first order rate constants for the hydrolysis of sulphacetamide to sulphanilamide at 120° (●, pH value measured at 25°; ○, calculated pH at 120°).

Table 1. *The effect of pH on the reaction rate constants at* 120°.

pH_{25}	pH_{120}	Buffer	Observed 1st order rate constant k (h^{-1}) $\times$ 10^2	2nd order rate constant (l. mol^{-1} h^{-1}) [$*k_1$ $**k_6$]
1·61	1·68	GAC	1300·0	378*
2·61	2·72	GAC	104·6	344*
3·65	3·80	GAC	8·104	332*
4·00	4·07	M	4·574	337*
4·95	5·04	M	3·267	—
5·80	5·90	M	3·459	—
6·80	6·91	M	3·364	—
7·40	7·52	M	3·468	—
7·93	8·06	M	3·281	—
8·91	7·35	GAK	3·313	—
9·98	8·22	GAK	3·174	—
11·05	9·08	GAK	3·438	16·90**
12·00	9·85	GAK	6·771	5·63**
12·64	10·37	GAK	14·650	3·74**

Table 2. *The effect of buffer strength on the reaction rate at* 120°.

pH_{25}	Buffer	Ionic strength	Observed 1st order rate constant k (h^{-1}) $\times 10^2$
7·40	Nil	—	2·837
8·36	Nil	—	2·819
7·40	M	0·488	3·468
7·40	M × 2	0·976	4·132
7·40	M × 4	1·952	5·854
7·40	M + KCl	1·952	3·521
7·40	Tris	—	7·619

(pH_{25} are pH values measured at 25° and pH_{120} are the calculated values for 120°, G_{AC}, G_{AK}, M, represent Sørensen's acid glycine, Sørensen's alkaline glycine, and McIlvaine's buffers respectively; M × 2, M × 4 represent double and quadruple strength McIlvaine's buffer.)

Table 3. *Activation parameters for the pH independent hydrolysis of sulphacetamide.*

Compound	Buffer	Activation energy (E) (kJ mol^{-1})	Activation enthalpy (ΔH^*) (kJ mol^{-1})	Activation entropy (ΔS^*) (J mol^{-1} deg^{-1})	Ref.
Sulphacetamide	Nil	≈100	—	—	Anderson, 1967
,,	M	95·9	92·5	—10·9	Data for the effect of temp. (see p. 254)
,,	M + 0·5% S.M.S.	81·6	78·7	—13·4	Davies, Meakin & Moss, 1970
Chloramphenicol	Nil	101·2	99·2	—7·1	Higuchi & others, 1954
,,	A	49·4	46·4	—21·3	,, ,,
,,	B	83·7	80·3	—12·1	Heward, Norton & Rivers, 1970

M = McIlvaine's buffer S.M.S. = sodium metabilsulphite
A = acetate buffer B = borate buffer

DISCUSSION

Throughout all the experiments, only two spots were visualized on the reference plates, at R_F values corresponding to sulphacetamide and sulphanilamide. No traces of diacetylsulphanilamide, previously suggested as a degradation product of sulphacetamide (Fletcher & Norton, 1963; Dickenson: personal communication) were found when chromatograms of the heated solutions were developed in isopropanol–concentrated ammonia–water (90:5:5), which gives good separation of the three compounds (Dickenson: personal communication). The average recovery of total sulphonamide (calculated as sulphacetamide) based on 162 experiments, was 100·2% (coefficient of variation 1·52%). It is therefore reasonable to conclude that under the experimental conditions reported here the degradation of sulphacetamide is solely one of deacetylation to form sulphanilamide.

Such a reaction is analogous to the hydrolysis of carboxylic acid amides. This is subject to acid, base and nucleophilic catalysis, following second order kinetics overall and is first order with respect to amide and catalytic species (Willens & Bruylants, 1951; Johnson, 1967; Hine, 1962; Meloche & Laidler, 1951).

For kinetic interpretation, the rate constant - pH profile should be corrected for temperature effects in view of the large differential between the heating temperatures

(120°) and that of pH measurement (25°). Any pressure effects on the value of k can be ignored, as they are considered negligible at 20 p.s.i. (Whalley, 1964).

Temperature influences the pH of a system largely by its effect on the dissociation constants (K_d) of the constituent weak acids and bases. Over a wide range of temperature the log K_d or pH versus temperature plots are generally parabolic and may be represented by the equations 1 and 2 (Bates, 1954; Robinson & Stokes, 1965).

$$\log K_d = \frac{A_1}{T} + A_2 + A_3T \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$pH = \frac{B_1}{T} + B_2 + B_3T \quad .. \quad .. \quad .. \quad .. \quad (2)$$

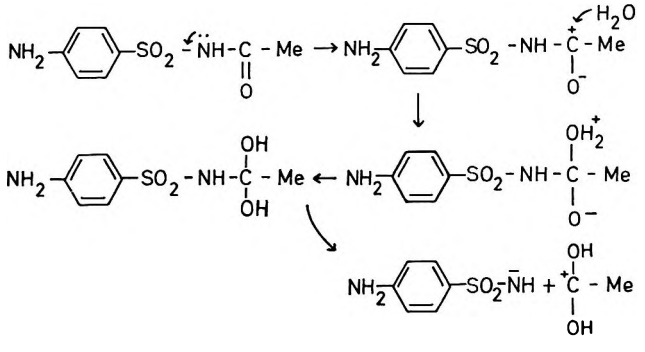
The pH values of a series of 1% sulphacetamide sodium solutions, prepared in Sørensen's acid and alkaline glycine buffers and McIlvaine's buffer, were measured at approximately 10° intervals between 25° and 95° and the data fitted to equation 2 using a multiple regression analysis (System 4 Statistic Scheme, International Computers Ltd.). Multiple correlation coefficients better than 0·8 were obtained in all cases ($r_{tab} = 0{\cdot}707$, $P = 0{\cdot}05$). The computed temperature coefficients (B_1, B_2, B_3) were used to extrapolate the data to 120°. pK_1 (2·13) and pK_2 (5·21) values for sulphacetamide at 120° were similarly obtained from equation 1. Plots of pH_{120} against pH_{25} were found to be rectilinear within a given buffer system, least squares analysis giving equations 3–5.

$pH_{120} = 1{\cdot}0382\ pH_{25} + 0{\cdot}0088$ .. (3) (Sørensen's acid glycine buffer)
$pH_{120} = 0{\cdot}8107\ pH_{25} + 0{\cdot}1251$ .. (4) (Sørensen's alkaline glycine-buffer)
$pH_{120} = 1{\cdot}0142\ pH_{25} + 0{\cdot}0174$ .. (5) (McIlvaine's buffer)

These equations were used to calculate the pH_{120} values for the heated sulphacetamide solutions (Table 1) which are also shown in Fig. 1 plotted against the apparent first order rate constants (k), and it can be seen that a plateau exists over the pH_{120} range 5 to 9.

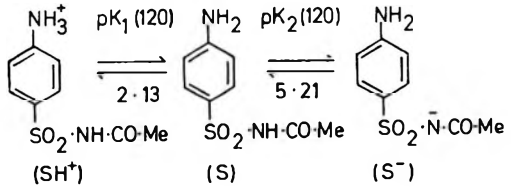
This picture differs from the pH profiles generally associated with the hydrolysis of simple acid amides, where pH independent regions are absent (Bell, 1941). This is attributable to resonance stabilization of the amido-carbonyl group which protects the compounds from attack by weak nucleophiles such as water (March, 1968).

The reactivity found between sulphacetamide and water can be related to the electron withdrawing properties of the sulphonic acid residue, decreasing the availability of the lone pair on the amide nitrogen, thus reducing resonance stabilization of the carbonyl group and facilitating nucleophilic attack.

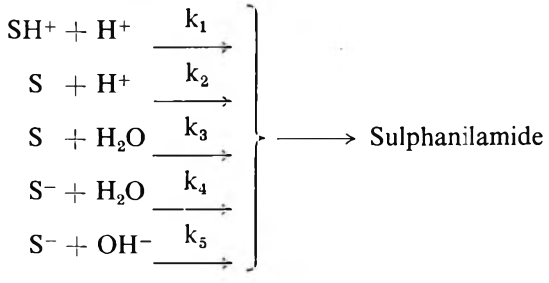


Similar reasoning involving electron withdrawing substituents on the carbon atoms, can explain the uncatalysed water reactions which occur with other pharmaceutical amides, such as chloramphenicol (Higuchi, Marcus & Bias, 1954) and nicotinamide (Finholt & Higuchi, 1962).

In aqueous solution, sulphacetamide can exist in the protonated (SH^+), neutral (S) and anionic (S^-) forms,



and inspection of Fig. 1 suggests there are five main reactions contributing to the overall hydrolysis rate of sulphacetamide, namely:



The overall rate equation (6) is then given by

$$\frac{-dS_T}{dt} = k\,[S_T] = k_1\,[SH^+]\,[H^+] + k_2\,[S]\,[H^+] + k_3\,[S] + k_4\,[S^-] + k_5\,[S^-]\,[OH^-] \quad (6)$$

where S_T represents the total concentration of sulphacetamide, $[SH^+]$, [S], $[S^-]$, $[H^+]$, $[OH^-]$ the concentrations of the various sulphacetamide and catalytic species, k the observed first order rate constant and k_1, k_2, k_3, k_4 and k_5 the corresponding second order rate constants.

The sharp rise in the value of k with decrease in pH_{120} below 4 (Fig. 1) is indicative of an acid catalysed reaction predominating in this area of the log k- pH profile. Hence only the first two terms of the general rate equation (6) will be significantly contributing to the overall reaction velocity. Least squares analysis shows that the log k — pH_{120} relation is rectilinear below pH_{120} 4 ($r = 0{\cdot}9999$ $r_{tab} = 0{\cdot}9500$; $P = 0{\cdot}05$) suggesting that the values of k_1 and k_2 are approximately equal, otherwise a discontinuity should be expected in the curve around the $pK_{1(120)}$ value of 2·13. A similar situation has been reported for the acid catalysed hyrolysis of the protonated and neutral forms of nicotinamide (Finholt & Higuchi, 1962). Under these circumstances equation 6 reduces to equation 7 (Garrett, 1967).

$$\log k = \log k_1 - pH_{120} - \log \gamma \quad .. \quad .. \quad .. \quad (7)$$

where γ is the mean ion activity coefficient. The calculated log k — pH_{120} slope of −1·03 is in good agreement with equation 7 and is indicative of specific hydrogen ion catalysis in this region. Equation 7 was also used to calculate the specific second

order rate constant (k_1) at the pH values studied experimentally and the values obtained were sufficiently similar to corroborate that specific hydrogen ion catalysis is operative (Table 1).

Above pH_{120} 9, the overall rate equation (6) reduces to equation 8,

$$\log k = \log k_5 - pK_{w(120)} + pH_{120} - \log \gamma \quad .. \quad .. \quad (8)$$

and least squares analysis indicates that it is possible to draw a reasonable straight line through the last three points on the right hand side of the $\log k - pH_{120}$ profile (Fig. 1) ($r = 0{\cdot}989$, $r_{tab} = 0{\cdot}988$, $P = 0{\cdot}10$). However, the calculated slope of 0·48 is at variance with the theoretical slope of unity required if specific hydroxyl ion catalysis is solely responsible for the observed pH effect (eqn. 8), as is the value of 0·64 obtained when only the last two points, which lie well clear of the plateau region, are considered. We believe that these figures, together with the considerable variation in the k_5 values (Table 1) point to additional hydrolytic mechanisms being operative in these areas. Similar results have been noted for the base catalysed hydrolysis of paracetamol (Koshy & Lach, 1961) and chloramphenicol (Higuchi & others, 1954). Such findings have often been attributed to the presence of buffer constituents, which can influence the kinetics, both through general acid-base or nucleophilic catalysis, and ionic strength effects. Of the buffer components used in this region glycine is the probable catalytic species since it is known to have a significant catalytic effect on other hydrolytic processes (Windheuser & Higuchi, 1962; Johnson, 1967).

Between pH 5–9, where the water reaction is considered to predominate, the presence of more powerful nucleophiles such as the buffer constituents should lead to an increase in the reaction rate (Bender, 1960). Studies at pH 7·4 (Table 2) show that k increases linearly with buffer concentration as would be expected where general acid-base or nucleophilic catalytic effects are operative, and that this is not a primary salt effect is shown by a comparison of the observed first order rate constants obtained in single strength McIlvaine's buffer ($I = 0{\cdot}488$M) with that adjusted to ionic strength 1·952M with potassium chloride ($t_{calc} = 0{\cdot}89$, $t_{tab} = 2{\cdot}31$, $P = 0{\cdot}05$). A similar finding for unbuffered sulphacetamide solution has been reported by Anderson (1966). Such observations are to be expected from theoretical considerations since one of the reacting species (water) forming the activated transition complex is a neutral molecule (Laidler, 1965).

Gomori's tris buffer has a significantly more powerful catalytic effect at pH 7·4 than the McIlvaine system. This is analogous to the effect of 'tris' on the hydrolysis of ethyl dichloroacetate and is compatible with the hypothesis that sulphacetamide undergoes direct nucleophilic attack by 'tris', rather than the latter acting as a general base (Johnson, 1967).

The effect of temperature on the water reaction was studied over the range 99·5 to 140° at pH 7·4. An Arrhenius plot of the log of the observed rate constant against the reciprocal of the absolute temperature was found to be linear ($r = 0{\cdot}9999$, $r_{tab} = 0{\cdot}8783$, $P = 0{\cdot}05$) leading to values of $95{\cdot}9 \pm 4{\cdot}5$ kJ mol^{-1} ($22{\cdot}9 \pm 1{\cdot}1$ kcal mol^{-1}) for the activation energy and $4{\cdot}9 \times 10^7$ ($1{\cdot}2 - 19{\cdot}5 \times 10^7$) s^{-1} for the frequency factor.

Anderson (1967) has reported a value of approximately 100 kJ mol^{-1} (24 kcal mol^{-1}) for the activation energy of sulphacetamide hydrolysis in water. Anderson's data are limited which may account for some of the discrepancy in the values although

the lower value reported here must, in part, be attributable to the slight catalytic effect of the McIlvaine's buffer. These values are larger than those recorded for many amide hydrolyses (Bender, 1960) which only proceed in the presence of hydrogen or hydroxyl ions, but are consistent with water reactions. Comparable values have been calculated from data available in the literature for the pH independent hydrolysis of chloramphenicol (Table 3).

The sulphacetamide data gave a marginally better fit when applied to the absolute rate equation (9), leading to values of 92·5 $\pm$ 4·5 kJ mol^{-1} (22·1 $\pm$ 1·1 kcal mol^{-1}) and $-10{\cdot}9 \pm 2{\cdot}5$ J deg^{-1} mol^{-1} ($-2{\cdot}6 \pm 0{\cdot}6$ cal deg^{-1} mol^{-1}) for the activation enthalpy and entropy respectively.

$$K_T = \frac{k_T}{h} \exp. [\Delta S^*/R] \exp. [-\Delta H^*/RT] \quad .. \quad .. \quad (9)$$

(K_T represents the rate constant at temperature T, k the Bolzmann constant, h is Planck's constant, R is the universal gas constant and ΔS^* and ΔH^* represent the entropy and enthalpy of activation, respectively).

The value of $-10{\cdot}9$ J deg^{-1} mol^{-1} for the activation entropy is at variance with the characteristically large negative values (-60 to -120 J deg^{-1} mol^{-1}) associated with the neutral water reactions of carboxylic acid derivatives (Johnson, 1967), but are similar to the figures that we calculate for the chloramphenicol water reaction (Table 3). These entropy values are comparable to that associated with the loss of freedom of a single water molecule ($\approx$22 J deg^{-1} mol^{-1}) and it would therefore appear that there is little difference in the degree of solvation between the initial and transition states in these systems (Schaleger & Long, 1963).

Pharmaceutical implications

Use of the Arrhenius parameters to extrapolate the data to 18° and 25° gives mean values for the rate constants of $1{\cdot}12 \times 10^{-6}$ h^{-1} and $2{\cdot}85 \times 10^{-6}$ h^{-1} respectively leading to $t_{10\%}$ values of 10·7 and 4·2 years, indicating that solutions of sulphacetamide are stable at room temperature.

Similar calculations enable the percentage loss of sulphacetamide resulting from autoclaving solutions at 115° for 30 min to be estimated at 1·1% ($k = 0{\cdot}0223$ h^{-1} at 115°). This would result in the formation of 0·22% sulphanilamide from a 30% sulphacetamide solution. Since the reported solubility of sulphanilamide in water is 0·6% and 0·26% at 20° and 10° respectively (Merck Index, 1968), there seems little reason for not heat sterilizing sulphacetamide solutions, provided they are not subsequently refrigerated.

These conclusions cannot be directly related to the current B.P.C. eye drops of sulphacetamide however, since it has been shown that the sodium metabisulphite, present as an antioxidant has a considerable catalytic influence on the hydrolysis rate of the drug (Davies & others, 1970). As it has also been demonstrated that sodium metabisulphite accelerates photolytic colour development in sulphacetamide solutions (Anderson & Maudson, 1963; Meakin & Davies, unpublished data), the inclusion of this antioxidant in the official eye drops seems open to question.

REFERENCES

ANDERSON, R. A. (1966). *Aust. J. pharm. Sci. Suppl.*, **47**, 526–27.
ANDERSON, R. A. (1967). Ph.D. thesis, University of Sydney, Australia.
ANDERSON, R. A. and MAUDSON, J. W. (1963). *Aust. J. pharm. Sci. Suppl.*, **44**, 524.

BATES, R. G. (1954). *Electrometric pH Determination*, pp. 91–120. London: Chapman and Hall.

BELL, R. P. (1941). *Acid Base Catalysis*, pp. 8–9. Oxford University Press.

BENDER, M. L. (1960). *Chem. Rev.*, **60**, 53–113.

CLARKE, P. A. (1965). *Pharm. J.*, **194**, 375–376.

CLARKE, P. A. (1967). *Ibid.*, **199**, 414.

DAVIES, D. J. G., MEAKIN, B. J. & MOSS, S. H. (1970). *J. Pharm. Pharmac.*, **22**, *Suppl.*, 43S–52S.

ELVING, P. J., MARCOVITCH, J. M. & ROSENTHAL, T. (1956). *Analyt. Chem.*, **28**, 1179–1180.

FINHOLT, P. & HIGUCHI, T. (1962). *J. pharm. Sci.*, **52**, 655–661.

FLETCHER, G. & NORTON, D. A. (1963). *Pharm. J.*, **191**, 145–147.

GARRETT, E. R. (1967). *Adv. in Pharmaceutical Sciences*, **2**, 1–94. Editors: Bean, H. S., Beckett, A. H. & Carless, J. E. London and New York: Academic Press.

HEWARD, M., NORTON, D. A. & RIVERS, S. M. (1970). *Pharm. J.*, **204**, 386–387.

HIGUCHI, T., MARCUS, A. D. & BIAS, C. D. (1954). *J. Am. pharm. Ass., Sci. Edn*, **43**, 129–134; 530–535.

HINE, J. (1962). *Physical Organic Chemistry*, 2nd edn, p. 291. New York and London McGraw-Hill.

JOHNSON, S. L. (1967). *Adv. in Physical Organic Chemistry*, **5**, 237–325. Editor: Gold, V. London and New York: Academic Press.

KOSHY, K. T. & LACH, J. L. (1961). *J. pharm. Sci.*, **50**, 113–118.

LAIDLER, K. J. (1965). *Chemical Kinetics*, 2nd edn, p. 221. New York and London: McGraw-Hill.

MARCH, J. (1968). *Advanced Organic Chemistry, Mechanisms and Structure*, p. 313. New York and Maidenhead: McGraw-Hill.

MELOCHE, I. & LAIDLER, K. J. (1951). *J. Am. chem. Soc.*, **73**, 1712–1714.

ROBINSON, R. A. & STOKES, R. H. (1965). *Electrolyte Solutions* (2nd edn), pp. 357–358, 538. London: Butterworths.

SCHALEGER, L. L. & LONG, F. A. (1963). *Adv. in Physical Organic Chemistry*, **1**, 1–33. Editor: Gold, V. London and New York: Academic Press.

SCHWABE, K. (1960). *Electrometrische pH-Messungen unter extremen Bedingungen.* Monographien zu "Angewandte Chemie" und "Chemie-Ingenieur-Technick", No. 74, p. 34.

TANSEY, I. P. (1969). M.Sc. Thesis, Bath University of Technology, U.K.

WHALLEY, E. (1964). *Adv. in Physical Organic Chemistry*, **2**, p. 95. Editor: Gold, V London and New York: Academic Press.

WILLENS, M. & BRUYLANTS, A. (1951). *Bull. Soc. chim. Belg.*, **60**, 191–207.

WINDHEUSER, J. J. & HIGUCHI, T. (1962). *J. pharm. Sci.*, **51**, 354–364.

J. Pharm. Pharmac., 1971, **23**, 262–269 *Received October* 7, 1970

Modifications of the responses to antidiuretic hormone by hydrolytic enzymes

A. W. CUTHBERT AND M. E. PAINTER

Department of Pharmacology, University of Cambridge, Cambridge, U.K.

Proteolytic enzymes (trypsin and α-chymotrypsin) and lipolytic enzymes (phospholipases A & C) have been used to treat both surfaces of the skins of frogs and toads. The effects of the enzymes on sodium transport (frog skin) and hydraulic flow (toad skin) have been measured, together with the effects on the responses to ADH. With the experimental conditions used, proteolytic enzymes reduced resting sodium transport but had no major effect on the responsiveness to ADH. Conversely these enzymes had no effect on resting hydraulic flow but reduced or abolished the responses to ADH. Phospholipases applied to the inner surfaces of skins affected neither water nor sodium movement but abolished the responses to ADH. Phospholipases applied to the outside enhanced the effect of ADH on water flow but had no effect on the increased sodium movement caused by ADH. The results are discussed in terms of the possible removal of calcium binding sites by phospholipases.

Proteolytic and lipolytic enzymes have been used to treat the skins of frogs (*Rana temporaria*) and toads (*Bufo marinus*) to see how these agents affect the active transport of sodium and hydraulic flow down an osmotic gradient. In addition the responses of epithelia to the antidiuretic hormone, arginine vasopressin (ADH) have been tested following enzymic treatment. It was hoped to gain some insight into the nature of the permeability changes which result from hormone stimulation.

METHODS

Sodium transport was measured by the short circuit current (SCC) technique of Ussing & Zerahn (1951) using a standard procedure. The skin area was 4·5 or 7 cm^2. Experiments were performed in pairs with one skin serving as control. In some instances test and control skins were taken from the same animal. After exposure of the test skin to enzyme the effect of the hormone was tested. ADH and enzyme were added simultaneously to the appropriate side of the control skin. Amphibian skins are relatively insensitive to the mammalian hormone, arginine vasopressin, compared to the natural hormone, arginine vasotocin. Hence ADH was added only once to each tissue in high concentration so that a maximal response was obtained.

Water flow was measured volumetrically by observing the movement of a meniscus along a 1 ml graduated pipette. The pipette was sealed into an open-ended chamber which was closed off by the tissue such that the outer surface of the skin was within the chamber. The inner skin surface was bathed in aerated Ringer solution in a beaker. The solution bathing the outer skin surface was a ten times dilution of Ringer solution, thus the skin was subjected to a pressure gradient of 200 mOsm. The skin area was 6·2 cm^2.

The Ringer solution had the following composition (mM) NaCl, 111; KCl, 1·9; $CaCl_2$, 1·08; NaH_2PO_4, 0·083; $NaHCO_3$, 2·4 and glucose, 11·1. This solution had a

pH of 7·6 when bubbled with air. In the experiments on water flow Na_2HPO_4 was substituted for NaH_2PO_4 to give a pH of 8·0. The effects of ADH on water permeability are maximal at this pH.

The following enzymes were used: α-chymotrypsin (EC 3.4.4.5, salt free, 11 500 ATEE u/mg, Seravac Labs Ltd.), trypsin (EC 3.4.4.4, salt free, 10 000 BAEE u/mg, Seravac Labs Ltd.), phospholipase C (EC 3.1.4.3, α-toxin of *Cl. welchii*, phosphatidylcholine choline phosphohydrolase, Mann Research Labs Inc.) and phospholipase A (EC 3.1.1.4) *Naja naja* venom, phosphatide acyl-hydrolase, Koch-Light Labs Ltd.).

ADH was Pitressin (Parke Davis & Co.).

RESULTS

Proteolytic enzymes and sodium transport

Trypsin, EC 3.4.4.4 (0·1 mg/ml) and α-chymotrypsin, EC 3.4.4.5 (1 mg/ml) produced a progressive fall in short-circuit current (SCC) and transepithelial potential (PD) when applied to the inner surface of frog skin. Addition of ADH after this always produced an increase in SCC which was related to the ability of the skin to transfer sodium. The percentage increase in SCC has been calculated relative to the SCC which would have existed, at the time of the peak response, if ADH had not been added (Fig. 1). Table 1 gives the percentage increases in SCC in six paired experiments in which the initial SCC had fallen by 25 to 85% of its initial value after treatment with α-chymotrypsin.

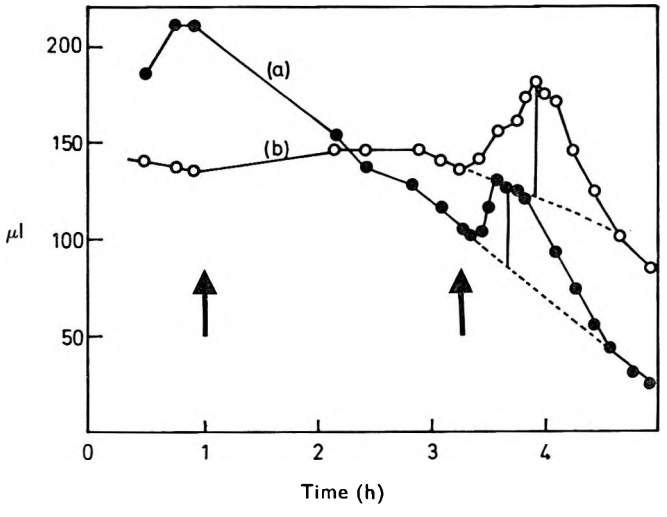


FIG. 1. SCC (μA) of two pieces of frog skin (4·5 cm²). Skin (a) was bathed on the inner surface with α-chymotrypsin (1 mg/ml) (enzyme added at first arrow). Responses to ADH (0·6 u/ml) was tested in both skins 2 h after addition of the enzyme (second arrow). The increase in SCC caused by hormone is indicated by the vertical lines on the figure at the times of the peak responses.

There was no significant difference between the responses of test and control skins. Similar results were obtained when trypsin (0·1 mg/ml) was used to treat the inner surface of skins (Table 1).

Table 1. *The effect of α-chymotrypsin* (1 mg/ml) *and trypsin* (0·1 mg/ml) *applied to the inner surface of frog skin on the response to ADH.*

% increase (SCC) to ADH (0·6 u/ml) after α-chymotrypsin (1 mg/ml)	% increase (SCC) to ADH (0·6 u/ml) in control	% reduction in SCC caused by α-chymotrypsin	Duration of exposure to α-chymotrypsin (min)
45	44	79	150
39	—	50	120
40·5	89	0	120
16	112	85	180
48	45	50	220
30	50	75	210
120	84	25	45
48·4 ± 12·6 (s.e.)	70·7 ± 11·6 (s.e.)	$0·3 > P > 0·2$	
% increase (SCC) to ADH (0·6 u/ml) after trypsin (0·1 mg/ml)	% increase (SCC) to ADH (0·6 u/ml) in control	% reduction in SCC caused by trypsin	Duration of exposure to trypsin (min)
*47	33	43	75
*36	48	55	150
34	18	57	120
39 (mean)	33 (mean)		

*Test and control skin was taken from the same animal.

Proteolytic enzymes also caused a progressive fall in SCC and PD when applied to the outer surface of frog skin. Table 2 shows the values for the percentage increases in SCC with ADH in five treated and five control skins.

The average response was greater in treated skins than in controls, however this difference was not significant due to the wide variation in values. When the SCC becomes very low, for example the last experiment in Table 2, other factors apart

Table 2. *The effects of α-chymotrypsin* (1 mg/ml for 2h) *applied to the outer surface of frog skin on the response to ADH* (0·6 u/ml)h.

% increase (SCC) to ADH (0·6 u/ml) after α-chymotrypsin (1 mg/ml)	% increase (SCC) to ADH (0·6 u/ml) in control	% reduction in SCC caused by α-chymotrypsin
120	78	50
154	43	56
29	12	60
18	0	20
0	100	90
64·2 ± 30·5 (s.e.)	46·6 ± 19·0 (s.e.)	$0·6 > P > 0·5$

from the ability of ADH to affect its receptor may be responsible for the diminished response. Fig. 2A,B illustrates this point. It can be seen that when the SCC had been reduced to 90% of its original value ADH had no effect on SCC but did cause an increase in conductance (ratio of SCC to PD) greater than that in the control skin.

Lipolytic enzymes and sodium transport

Experiments were performed with phospholipase A (naja venom) EC 3.1.1.4 and phospholipase C EC 3.1.4.3 with a protocol similar to that with proteolytic enzymes. In these experiments aeration of the Ringer solution was stopped on the side to which the enzyme was added since bubbling inactivates these enzymes (Macfarlane &

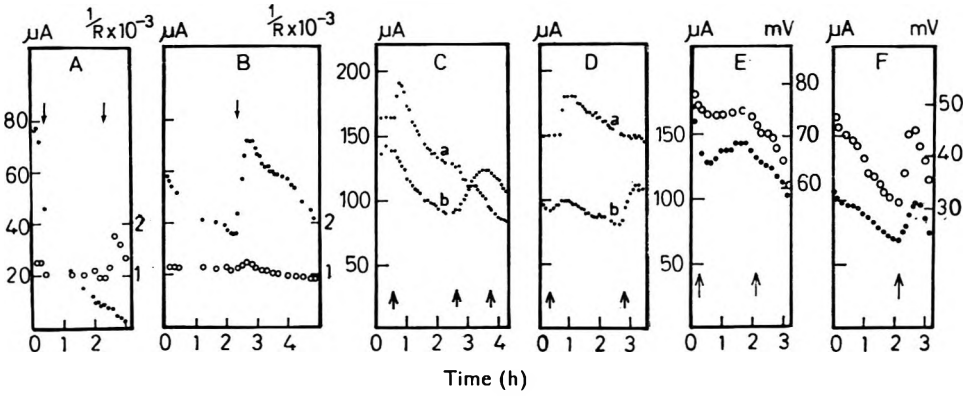


FIG. 2. A and B. SCC (μA) and conductance (ratio SCC/PD) of two pieces of frog skin (4·5 cm²). Skin (A) was bathed on the outer surface with α-chymotrypsin (1 mg/ml) (enzyme added at first arrow). Responses to ADH (0·6 u/ml) were tested in both skins (at second arrow).

C and D. SCC (μA/4·5 cm²) in two paired experiments in which naja venom (50 μg/ml) was applied to the inside of test skins (C) at the first arrow. In (C) the responses to ADH (0·1 u/ml and 0·6 u/ml) are shown at the second and third arrows. In (D) the response to ADH (0·1 u/ml) is shown at the second arrow. In (C) both skins were taken from the same animal. Note the stimulation produced by the enzyme.

E and F. SCC (μA closed circles) and PD (mV open circles) of two pieces of frog skin. In (E) the skin was exposed on the inner surface to phospholipase C (50 μg/ml) (first arrow). After 2 h ADH (0·3 u/ml) was added to inside solution bathing the test and control skins (F). Note the increase in SCC caused by the enzyme.

Left hand ordinates on B, D and F are the same as those on A, C and E respectively.

Knight, 1941). Unlike the proleolytic enzymes the lipolytic enzymes, in a concentration of 50 μg/ml, had little effect on SCC and PD when applied in the inner bathing solution apart from an initial stimulant action, after which the SCC fell at a rate comparable to the control. The fall in SCC was most probably due to the reduced oxygen tension since it fell in the control preparations not exposed to enzyme. Aeration was always stopped in control and test skins at the same time.

In spite of the high SCC's prevailing in test skins exposed to enzyme the response to ADH was abolished. This is shown in Fig. 2 C, D and Table 3. The inhibition of the response to ADH by phospholipase A was highly significant.

In six experiments with phospholipase C (50 μg/ml over 0–120 min) the development of the ADH (0·3 u/ml) inhibition was studied. Complete inhibition of the

Table 3. *The effects of phospholipase A* (50 μg/ml) *applied to the inner surface of frog skin on the response to ADH* (0·3 u/ml).

% increase (SCC) to ADH (0·3 u/ml) after phospholipase A (50 μg/ml)	% increase (SCC) to ADH (0·3 u/ml) in control	Duration of exposure to phospholipase A (min)
39	72	120
6	38	135
*0	71	120
*7	55	120
19	79	120
0	65	60
0	17	120
0	100	120
0	0	120
7·9 ± 4·4 (s.c.)	55·2 ± 10·6 (s.c.)	0·001 > *P*

* Test and control skins came from the same animal.

response was achieved with a 2 h exposure (% increase in SCC to ADH: 57, 48, 13, 26, 0, 0, to 0, 30, 60, 90, 120, 120 min exposure respectively). As with phospholipase A the response to ADH was abolished after treatment with phospholipase C at a time when the SCC remained high (Fig. 2E,F). Phospholipase C (100 μg/ml for 2 h) was used to treat the outer surface of frog skins in five paired experiments. There was no effect on the response to ADH in this series. The average % increase in SCC in the control preparations was 32·7% and in the enzyme treated preparations was 33%. In three further unpaired experiments the outer surfaces of skins from the same batch of frogs were exposed to phospholipase C 500 μg/ml for 2 h. The average percentage increase in SCC in these experiments was 24·8%. Over the period of exposure the SCC of those skins exposed on the outer surface to phospholipase C was maintained. Thus no effects of this enzyme on either resting or stimulated sodium transport were demonstrated when it was applied to the outer skin surface.

Water movement, enzymes and ADH

In initial experiments the hydraulic flow in frog skin was found to be small (5 μl/cm^{2}h^{-1}) and the increase caused by ADH was small and irregular (mean increase of 1 μl/cm^{2}h^{-1} in 17 experiments). In consequence we used the skins of toads (*Bufo marinus*) which have higher resting and stimulated rates of water flow. The size of the animals was such that test and control skins were always taken from the same animal. When the effects of enzymes were to be tested on the outer surface of the skins the skins were first exposed to enzyme in the apparatus used for SCC measurements for 1 h. After this the skins were mounted in the apparatus for water flow measurement. This procedure was necessary since ADH is added to the serosal skin surface which must, of consequence, be accessible during flow measurement. In all experiments the Ringer solution bathing the outer skin surface was diluted ten times. Table 4 gives the results of 16 paired experiments in which test and control skins were taken from the same animal.

Table 4. *The effects of enzymes* (*α-chymotrypsin* 1 mg/ml *and phospholipase C* 50 μg/ml) *on hydraulic flow in toad skin.* Numbers of experiments indicated in parentheses. ADH concentration was 0·1 μ/ml.

Enzyme treatment	Test skin	Control skin	
α-Chymotrypsin inside ..	46·3 ± 5·1 (4) (μl/cm^{2}h^{-1})	56·9 ± 14·5 (4) (μl/cm^{2}h^{-1})	0·6 > *P* > 0·5
α-Chymotrypsin inside + ADH	34·9 ± 12·2% (4)	186·9 ± 40·5% (4)	T/C = 0·19 0·01 > *P* > 0·005
α-Chymotrypsin outside ..	62·0 ± 20·1 (4) (μl/cm^{2}h^{-1})	46·6 ± 5·5 (4) (μl/cm^{2}h^{-1})	0·5 > *P* > 0·4
α-Chymotrypsin outside + ADH	35·8 ± 5·3% (4)	93·3 ± 11·2% (4)	T/C = 0·38 0·02 > *P* > 0·01
Phospholipase C inside ..	34·4 ± 2·8 (4) (μl/cm^{2}h^{-1})	40·3 ± 6·9 (4) (μl/cm^{2}h^{-1})	0·5 > *P* > 0·4
Phospholipase C inside + ADH	41·4 ± 19·8% (4)	190·8 ± 102% (4)	T/C 0·22 0·2 > *P* > 0·1
Phospholipase C outside ..	54·4 ± 7·5 (4) (μl/cm^{2}h^{-1})	46·1 ± 7·4 (4) (μl/cm^{2}h^{-1})	0·6 > *P* > 0·5
Phospholipase C outside + ADH	124·4 ± 17·8% (4)	68·2 ± 19·3% (4)	T/C 1·82 *P* > 0·001

The following features can be found in Table 4. Exposure of either surface of toad skin to either phospholipase C (50 μg/ml) or α-chymotrypsin (1 mg/ml) for 1 h had no statistically significant effect on hydraulic flow in the absence of ADH. The wide variation in flow rates (even although two pieces of skin are from the same animal) probably masks a slight increase in flow rate caused by α-chymotrypsin applied to the inner skin surface. Further evidence for this is that when α-chymotrypsin was applied to the inner surface, after a control period, there was a definite increase in flow (Fig. 3A). In all the experiments the flow rates were linear for a

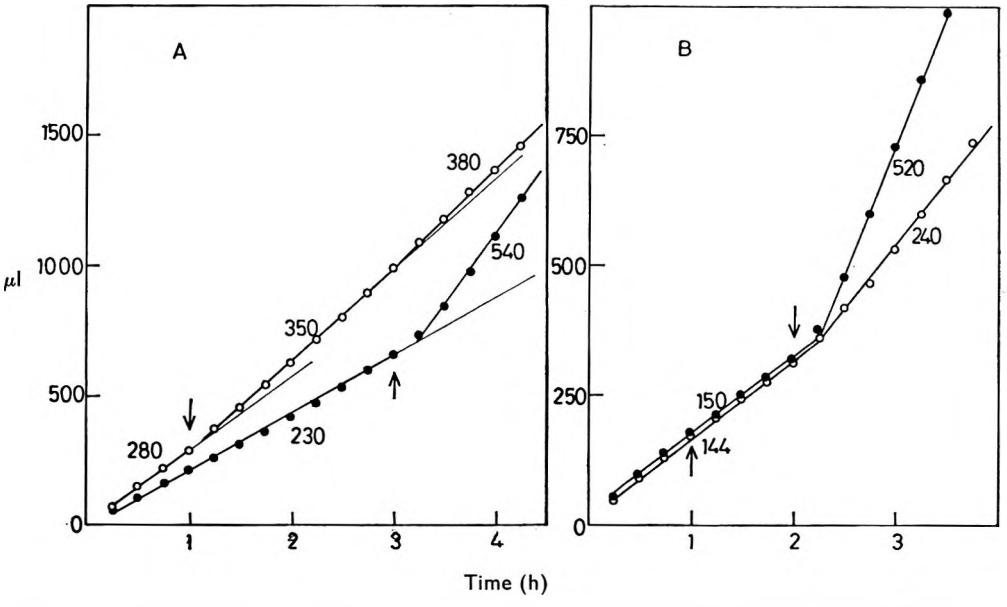


FIG. 3. Hydraulic flow across toad skin. A. α-Chymotrypsin (1 mg/ml) or, B, phospholipase C (50 μg/ml) was added to the inner bathing solution of the test skin (open circles) at the first arrow. ADH (0·1 μ/ml) added to both skins at the second arrow. The numbers on the curves indicate flow rates in μl/h.

particular condition. Fig. 3A,B are typical of all the experiments. Both types of enzyme caused a significant and similar inhibition of the ADH response when the enzymes were applied to the inside. Obviously this is a function of both concentration of enzyme and of duration of exposure. For example, phospholipase C, 50 μg/ml completely abolished the ADH response when the duration of exposure was increased to 90 min. Chymotrypsin significantly inhibited the response to ADH when the enzyme was applied to the outer surface of the skin. A striking effect was seen when phospholipase C was used to treat the outer face, when the response to ADH was almost doubled.

DISCUSSION

It was not expected that very specific lesions would result when enzymes were applied to tissues, however, in the absence of specific pharmacological antagonists efforts to discover the location, nature and diversity of receptors are made difficult. Fortunately these results show that enzymes can have differential effects on sodium transport and hydraulic flow in epithelia from which some conclusions can be made.

Phospholipases, in the concentrations used, had no inhibitory effect on either SCC

or hydraulic flow when applied to the inner surface of skins, while the responses to ADH were inhibited or abolished. The degree of inhibition was similar whether SCC or water flow was measured (when using the same enzyme concentration for the same duration) although it must be remembered that two different tissues were used. The current view of the action of ADH is that it stimulates adenylcyclase bound on the inner facing membranes to generate cyclic 3'5'-AMP which then diffuses across the cell to affect the permeability of the outer facing membranes (Orloff & Handler, 1967). The increase in SCC and water flow caused by theophylline is taken as evidence for the continual endogenous production of cyclic AMP. Normally this is converted to cyclic 5'-AMP by phosphodiesterase, an enzyme inhibited by theophylline. The inhibition of the ADH response by phospholipases may mean that adenyl cyclase is inactivated or that some ADH-receptor grouping associated with the cyclase is affected. If the former then endogenous production of cyclic AMP is not necessary for resting transport and flow. If the latter then the receptor grouping may not be obligatory for the functioning of the cyclase.

The idea of multiple specific receptor groups associated with adenyl cyclase has found much favour to explain how a variety of different hormones can work through a common system (Sutherland, Robinson & Butcher, 1968). There are, however, alternative views. Rasmussen (1970) has discussed the possibility that multiple and perhaps sequential second messengers may be involved in hormone action, for instance calcium ions. There is considerable evidence that calcium can bind to acidic groupings in phospholipids (for references see Cuthbert, 1967) and lipolysis might remove calcium on binding sites. In addition, Bentley (1959) has suggested that the ADH receptor may have an absolute requirement for calcium. Although others (Hays, Singer & Malamed, 1965) have attributed the fall in SCC following calcium removal as due to disaggregation of the epithelial layer it seems unlikely that this effect would inhibit hydraulic flow caused by ADH, as was found by Bentley. There is much confusion about whether adenyl cyclase is a calcium requiring enzyme (for references see Breckenridge, 1970) and it has been reported that ADH can mobilize bound calcium in toad bladder epithelial cells (Schwartz & Walker, 1969). The possibility remains that the prime effect of ADH may be to mobilize membrane bound calcium and the inhibition of ADH by phospholipases may be relevant to this action.

It is worth remembering that phospholipase enzymes themselves cause an initial stimulation of SCC, which might result from calcium liberation due to removal of binding sites.

Proteolytic enzymes appear to affect the active transport mechanisms rather than the ADH receptor when applied to the inner surface. The ability of ADH to stimulate SCC was retained in proportion to the ability of the skin to transport sodium. Marchesi & Palade (1967) found that trypsin treatment of red cells inactivated (Na^+–K^+) activated ATPase. Proteolytic enzymes applied to the inner surface severely inhibited the stimulation of water flow caused by ADH while the enzymes themselves caused an increase in flow. The contrasting effects of proteolytic enzymes on the SCC and flow responses to ADH may indicate separate "water" and "sodium" receptors. Others have concluded that two types of adenyl cyclase are needed to explain the functioning of these epithelia (Petersen & Edelman, 1964).

It is clear from a variety of evidence (for example, MacRobbie & Ussing, 1961; Frazier & Hammer, 1963) that the permeability changes to sodium and water follow-

ing addition of ADH occur at the outer facing membranes. The mechanism by which the permeability is changed is not clear but may result from the formation of one or more messengers within the cell, which after traversing the cell from the inner facing membranes affect the permeability of the rate limiting outer membranes. When the outer surfaces were treated with proteolytic enzymes there was no significant difference in the resting flow rate, however, the response to ADH was significantly impaired. In contrast treatment of the outer surface with proteolytic enzymes did not inhibit, and may even have enhanced, the SCC response to ADH (Table 2, Fig. 2A,B). These results would indicate that the different mechanisms are involved in the permeability increase at the outer faces following ADH and supports the idea that sodium ions and water move through separate channels (Lichtenstein & Leaf, 1965). It is not possible on the evidence to decide whether the different permeability changes are a consequence of two second messengers or a single messenger acting on different structures.

As well as the ADH receptor having an absolute requirement for calcium an excess of this ion in the outer bathing solution is known to depress the SCC and flow responses to ADH (Curran & Gill, 1961; Gill & Nedergaard, 1961). This effect of excess calcium is thought to be due to a stabilizing effect of calcium on the outer facing membranes. The enhancement of the flow response to ADH following treatment of the outer surface with phospholipase C may be connected with mobilization of membrane bound calcium by the enzyme, however the SCC responses to ADH were not enhanced by this treatment.

REFERENCES

BENTLEY, P. J. (1959). *J. Endocr.*, **18**, 327–333.

BRECKENRIDGE, B. Mch. (1970). *Ann. Rev. Pharmac.*, **10**, 19–34.

CURRAN, P. F. & GILL, J. R. (1961). *J. gen. Physiol.*, **45**, 625–641.

CUTHBERT, A. W. (1967). *Pharmac. Rev.*, **19**, 59–106.

FRAZIER, H. S. & HAMMER, E. I. (1963). *Am. J. Physiol.*, **205**, 718–722.

GILL, J. R. & NEDERGAARD, S. (1961). Quoted in *J. gen. Physiol.*, **45**, 625–641.

HAYS, R. M., SINGER, H. & MALAMED, S. (1965). *J. cell. Biol.*, **25**, 195–208.

LICHENSTEIN, N. S. & LEAF, A. (1965). *J. clin. Invest.*, **44**, 1328–1342.

MACFARLANE, M. G. & KNIGHT, B. C. J. G. (1941). *Biochem. J.*, **35**, 884–902.

MACROBBIE, E. A. C. & USSING, H. H. (1961). *Acta physiol. scand.*, **53**, 348–365.

MARCHESI, V. T. & PALADE, G. E. (1967). *Proc. natn. Acad. Sci. U.S.A.*, **58**, 991.

ORLOFF, J. & HANDLER, J. (1967). *Am. J. Med.*, **42**, 757–768.

PETERSEN, M. J. & EDELMAN, I. S. (1964). *J. clin. Invest.*, **43**, 583–594.

RASMUSSEN, H. & NAGATA, N. (1970). In *Calcium and Cellular Function*, pp. 198–213. Editor: Cuthbert, A. W. London: Macmillan.

SCHWARTZ, I. L. & WALKER, R. (1968). In *Protein and polypeptide hormones*, Part I, pp. 264-269. Editor: Margoulies, M. Excerpta Medica Foundation.

SUTHERLAND, E. W., ROBINSON, G. A. & BUTCHER, R. W. (1968). *Circulation*, **37**, 279–306.

USSING, H. H. & ZERAHN, K. (1951). *Acta physiol. scand.*, **23**, 110–127.

J. Pharm. Pharmac., 1971, **23**, 270–275 *Received September* 30, 1970

Demonstration of 3,4-dihydroxy[^{14}C]benzoic acid and [^{14}C]vanillic acid after administration of [^{14}C]noradrenaline in the rat

B. PESKAR, G. HELLMANN AND G. HERTTING

Institute of Pharmacology of the University of Vienna, A-1090 Vienna, Währingerstrasse 13a, Austria

Rats were injected with 40 μCi (±)-[1-^{3}H]noradrenaline and 10 μCi ±-[1-^{14}C]noradrenaline. Three fractions with a decreased ^{3}H:^{14}C ratio were isolated from the urine by a combined alumina adsorption-ethyl acetate extraction procedure. Two of the fractions were identified as [^{14}C]vanillic acid and 3,4-dihydroxy[^{14}C]benzoic acid, respectively. Vanillic acid represented between 1·5 and 3·0% of the total [^{14}C]activity excreted within 24 h and the contribution of dihydroxybenzoic acid was 0·2–0·5%. The third fraction with a decreased ^{3}H:^{14}C ratio has not been identified and represented about 2% of the total [^{14}C]activity excreted within 24 h. After monoamine oxidase blockade with 100 mg/kg of iproniazid, the excretion of vanillic acid, 3,4-dihydroxybenzoic acid and the unknown fraction was greatly diminished. The probability that these three substances represent those metabolites arising simultaneously with the formation of tritium water from (±)-[1-^{3}H]noradrenaline is discussed.

The metabolic fate of catecholamines in animals and man has been extensively studied ever since radioactively-labelled compounds became available. After administration of noradrenaline, only a small fraction leaves the body unchanged, most being metabolized before excretion. The two enzymes responsible for its metabolic degradation *in vivo* are catechol-*O*-methyltransferase (COMT) and monoamine oxidase (MAO). After administration of (±)-noradrenaline labelled with tritium on the C-1 (or β–C) {2-amino-1-(3,4-dihydroxyphenyl)-[1-^{3}H]ethanol; ^{3}H-NA}, tritium water (THO) was identified in the plasma of man (Gitlow, Mendlowitz & others, 1964), cats (Suko, Linet & Hertting, 1967) and rats (Pichler, Suko & Hertting, 1968). However, neither the action of COMT nor MAO can explain the loss of the tritium label at this position.

There are several possible transformations of ^{3}H-NA that would lead to the loss of the tritium label. (i) An unspecific exchange of labile tritium. (ii) The oxidation of noradrenaline to noradrenochrome and noradrenolutine, a mechanism that has been repeatedly discussed (Kety, 1959); here rearrangement of the noradrenochrome molecule to noradrenolutine would also cause loss of the tritium atom. Enzymic formation of adrenochrome in tissue homogenates has, indeed, been shown to occur by Axelrod (1964). (iii) The formation of vanillic acid (VA), a minor metabolic pathway demonstrated in man for adrenaline by Sandler, Ruthven & Wood (1964) and Goodall & Alton (1965), and for noradrenaline by Rosen & Goodall (1962) and Sandler & others (1964). The corresponding catechol compound 3,4-dihydroxybenzoic acid (DHBA) was found in the urine of man after administration of 3,4-dihydroxymandelic acid (DHMA) by Goodall & Alton (1969).

The double label technique was implemented, using a mixture of (±)-1-^{3}H-NA and (±)-1-^{14}C-NA in a given ratio, to investigate which of these transformations

accompanies the formation of THO in the body. Various analytical techniques were used to isolate the metabolites of noradrenaline, those fractions showing a decreased $^{3}H:^{14}C$ ratio must contain the metabolites in question. This procedure, moreover, simplifies further purification and identification of these compounds.

METHODS

Administration of drugs and urine collection

Male Wistar rats (250–350 g) were intravenously injected with a mixture of 40 μCi ^{3}H-NA (NENCO, specific activity 8·76 Ci/mmol) and 10 μCi ^{14}C-NA (CEA, specific activity 20·5 mCi/mmol). In some rats, iproniazid (100 mg/kg base) was injected intraperitoneally 16 h before the experiment to block MAO activity. Urine was collected in glass vials containing 0·1 ml of a mixture of 70% perchloric acid, 1% ascorbic acid and 5% EDTA, 2:2:1 by volume. The collection periods were 0–4, 4–8 and 8–24 h. The urine volume was 2–4 ml per sample. The collected urine was centrifuged and 50 μg of each of the following compounds added as carriers: DHBA, DHMA, 3,4-dihydroxyphenylglycol (DHPG), VA, vanillyl-mandelic acid (VMA), 4-hydroxy-3-methoxyphenylglycol (MHPG). The samples were then stored at −18°. Aliquots of urine were hydrolysed before the isolation procedure either enzymically with Glusulase (Endo) or by heating at pH 1 at 100° for 15 min.

Isolation procedure

The samples were recentrifuged after being thawed and the [^{3}H] and [^{14}C] content determined in an aliquot of the supernatant using a Packard liquid scintillation spectrometer (Okita, Kabara & others, 1957). Ascorbic acid (0·5 ml, 2%) and EDTA (0·1 ml, 5%) were then added, the samples brought to pH 5·5 and 0·7 g Al_2O_3 (Woelm, neutral) added with continuous stirring. The pH was then adjusted to 8·5. The alumina was transferred to columns, washed and eluted with 0·25N HCl. Eluates and effluents plus washings were then brought to pH 1 and processed separately as shown in Fig. 1. Aliquots of the final ethyl acetate samples were evaporated and taken up into 0·2 ml of ethyl acetate for paper chromatography. Other aliquots of the ethyl acetate extracts were evaporated directly in counting vials, 2 ml of methanol and 10 ml of toluol scintillator added and the radioactivity measured. To determine the extent of contamination of the isolated metabolite fractions by unaltered noradrenaline or by impurities present in the administered mixture of ^{3}H- and ^{14}C-NA, a diluted (1:10) solution of this mixture was subjected to the whole isolation procedure and the appropriate corrections made.

Each step of the isolation procedure was followed by paper chromatographic analysis in isopropanol–ammonia–water (8:1:1) and n-butanol–acetic acid–water (60:15:25) for effluents and n-butanol–acetic acid–water (60:15:25) and n-butanol saturated with N HCl for eluates. Distribution of radioactivity on the chromatograms was determined using a 4π-chromatogram scanner; the unlabelled reference compounds were developed by spraying the chromatograms with diazotized *p*-nitroaniline to detect *O*-methylated products and with the reagent of Goldenberg, Faber & others (1949) for catechol compounds.

In several experiments the chromatograms were cut in 1 cm strips, the activity eluted and the $^{3}H:^{14}C$ ratio of the eluates determined, whilst in other experiments the eluates of the radioactive peaks were further purified by rechromatography.

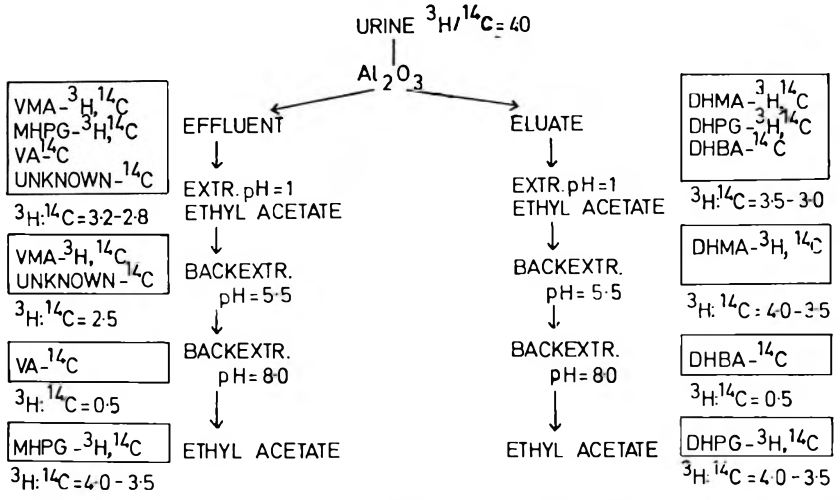


FIG. 1. Isolation procedure for various noradrenaline metabolites from rat urine. Rats were injected with 40 μCi (±)-1-^{3}H-NA and 10 μCi (±)-1-^{14}C-NA. The urine was collected and applied to alumina columns. Eluates and effluents were extracted into ethyl acetate at pH 1. The organic phases were back-extracted first into McIlvaine citrate-phosphate buffer at pH 5·5. The organic layer of the alumina effluents was then extracted with borate-HCl buffer (0·2M) at pH. 8·0, whilst the organic layer of the eluates was extracted with tris-HCl buffer (0·2M) at pH 8·0. The buffer extracts were adjusted to pH 1 and re-extracted with ethyl acetate. The metabolites found in the buffer extracts and in the remaining ethyl acetate phases are shown and the $^{3}H:^{14}C$ ratios of the various fractions given.

Identification of metabolites with a low $^{3}H:^{14}C$ ratio

Information about the identity of metabolites with a low $^{3}H:^{14}C$ ratio was initially obtained from the coincidence of the R_F values of the radioactive peaks and of the reference compounds in various chromatographic systems. Furthermore, after rechromatography, for purification, and elution, the radioactive and reference compounds behaved identically on alumina columns and on extraction as detailed in Fig. 1. For more precise identification of the radioactive peak that possessed a R_F value similar to VA, the peak was rechromatographed, eluted and chromatographed on a Dowex-1-acetate column (9 × 500 mm) according to Weise, McDonald & LaBrosse (1961), after the addition of VA and VMA as carriers. The column was eluted with a convex concentration gradient (1·5–6·0M) of ammonium formate at pH 8·0 (Fig. 2) and the eluate collected in 7 ml fractions. The VMA and VA content in the fractions was measured directly by absorption at 279 nm, whilst the radioactivity was measured in ethyl acetate extracts (pH 1).

The radioactive peak corresponding to DHBA was eluted from the paper chromatogram and *O*-methylated enzymically (Axelrod & Tomchick, 1958). After the mixture had been incubated for 2 h at 37° it was extracted at pH 1 with ethyl acetate and the identity of the *O*-methylated product formed was established by column and paper chromatography.

Recovery of vanillic acid

DHBA was *O*-methylated enzymically using methyl-[^{14}C]-*S*-adenosylmethionine by the procedure of Axelrod & Tomchick (1958). The ^{14}C-VA formed was isolated by extraction into ethyl acetate at pH 1 and further purified by back-extraction (Fig. 1) and paper chromatography. This purified ^{14}C-VA was then added to rat

urine and the whole isolation procedure applied to determine the recovery of VA; 82% of the radioactivity added to the urine was recovered in the fraction of the alumina effluent isolated by back-extraction at pH 8·0.

RESULTS

The $^{3}H:^{14}C$ ratios of the various fractions isolated are shown in Fig. 1. The $^{3}H:^{14}C$ ratio has already decreased in the organic phase obtained after extraction of the alumina effluents and eluates into ethyl acetate at pH 1.

Paper chromatography of the fraction isolated by back-extraction at pH 5·5 of the eluate extract showed one radioactive peak identical with DHMA, the $^{3}H:^{14}C$ ratio of this fraction ranging from 4 to 3·5. Back-extraction at pH 8·0 isolated a chromatographically-uniform compound which corresponded to DHBA. The $^{3}H:^{14}C$ ratio of this fraction was 0·5. Rechromatography and elution of the DHBA peak led to a further great decrease in the $^{3}H:^{14}C$ ratio to 0·01. Enzymic *O*-methylation of the eluted peak resulted in the formation of VA. The radioactivity remaining in the organic phase proved to be ascribable to DHPG, the $^{3}H:^{14}C$ ratio lying between 4 and 3·5. There was no indication of the presence of 3,4-dihydroxy[^{14}C]benzylalcohol in this fraction.

The $^{3}H:^{14}C$ ratio of the fraction isolated by back-extraction at pH 5·5 of the effluent extract was decreased to 2·5. Paper chromatography revealed the existence of two peaks. One peak had the same R_F value as the carrier VMA and possessed a $^{3}H:^{14}C$ ratio of 3·5, the second peak was more polar than VMA and had a R_F of 0·61 in the butanol–acetic acid–water system and a R_F of 0·13 in the isopropanol–ammonia–water system. The $^{3}H:^{14}C$ ratio of this latter peak was 0·5. The metabolite responsible for this activity has not yet been identified; it accounted for approximately 2% of the excreted ^{14}C total activity.

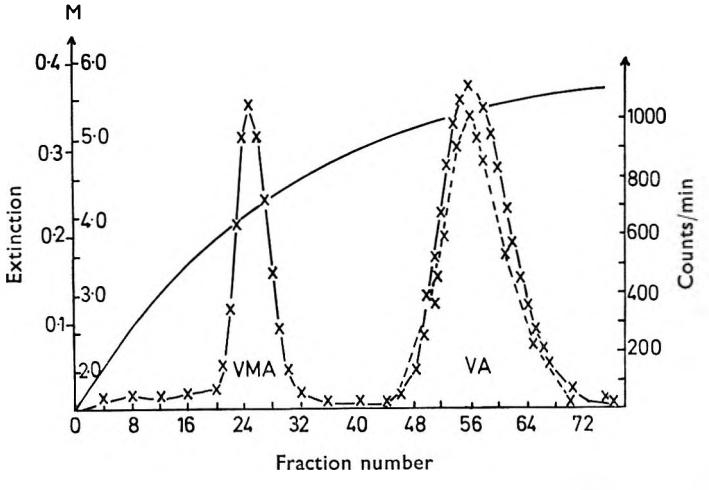


FIG. 2. Anion exchange chromatography of [^{14}C]vanillic acid isolated from the urine of rats that had received ^{3}H-NA and ^{14}C-NA. ^{14}C-VA purified from rat urine to which carrier VA and VMA (1 mg each) had been added was applied to a 500 × 9 mm Dowex-1-acetate column and eluted in fractions of 7 ml with a convex concentration gradient of 1·5–6·0M ammonium formate. The VMA and VA content was measured by absorption at 279 nm. For determination of radioactivity the fractions were acidified, extracted into ethyl acetate and the extracts measured in a liquid scintillation spectrometer. Left outer ordinate: extinction of added carrier VMA and VA, left inner ordinate: molarity of ammonium formate gradient, right ordinate: radioactivity as counts/min. Solid line represents extinction at 279nm, broken line represents radioactivity.

The fraction isolated by back-extraction at PH 8·0 of the effluent extract had a $^{3}H:^{14}C$ ratio of 0·5 and contained one radioactive peak corresponding to VA. Further chromatographic purification resulted in a $^{3}H:^{14}C$ ratio of 0·004. The pattern of the radioactivity in this fraction after elution from a Dowex-1-acetate column is shown in Fig. 2. It can be seen that the radioactivity leaves the column in the same pattern as the added carrier VA.

MHPG was found in the remaining ethyl acetate fraction, the $^{3}H:^{14}C$ ratio of which was 3·5. Vanillyl alcohol added as a carrier appeared in this fraction of our isolation procedure, but there was no radioactive peak with identical R_F values demonstrable on the paper chromatograms of this fraction.

Of the radioactivity administered, 60–70% was excreted in the urine within 24 h. After the appropriate corrections for recoveries had been made, ^{14}C-VA represented 1·5–3% and ^{14}C-DHBA 0·2–0·5% of the total [^{14}C]activity excreted. Neither enzymic nor acid hydrolysis of the urine increased the yield of ^{14}C-VA and ^{14}C-DHBA.

Blockade of MAO by iproniazid caused a decrease in the excretion of the total activity in the urine to about 50% of the administered radioactivity during the 24 h period. In these animals, ^{14}C-VA represented 0·3% of the excreted total [^{14}C]-activity, whereas ^{14}C-DHBA virtually disappeared. The unknown metabolite contained in the fraction isolated by back-extraction at pH 5·5 from the alumina effluent also disappeared after MAO-blockade.

DISCUSSION

Using the double label method, three fractions with a decreased $^{3}H:^{14}C$ ratio were isolated from the urine of rats that had received ^{3}H-NA and ^{14}C-NA. Two of these fractions were identified as VA and DHBA. The metabolite responsible for the third [^{14}C]-enriched fraction is as yet unidentified. The alcohols corresponding to VA and DHBA were not found in the urine of these animals.

During the 24 h collection period the sum of the metabolites without tritium label was between 4 and 5% of the excreted total [^{14}C] activity. In man, Rosen & Goodall (1962) found that 2·6% of the total activity, excreted within 24 h after the infusion of (±)-1-^{14}C-NA was vanillic acid. Our VA values in the rat of 1·5–3·0% of the excreted [^{14}C] activity are in the same range.

Dirscherl, Thomas & Schriefers (1962) observed the formation of VA from VMA in the perfused rat liver. The formation of DHBA in liver slices and homogenates incubated with DHMA was described by Thomas (1966). Goodall & Alton (1969) infused 1-^{14}C-DHMA in man and recovered large amounts of DHBA (7·7% of the total infused radioactivity) and smaller amounts of VA (2·0%) from the urine. These experiments indicate that the deaminated catecholamine metabolites serve as substrates for the formation of DHBA and VA. The considerable decrease in the formation of DHBA and VA after MAO blockade found in our experiments seems to indicate that deamination must take place before the removal of the 2-C-atom. Since in the rat the main deaminated metabolites of noradrenaline are the alcohols, MHPG and DHPG rather than the acids, VMA and DHMA, it can be assumed that the alcohols can also serve as precursors for DHBA and VA synthesis. If an isotope effect were involved in the formation of ^{14}C-VA and ^{14}C-DHBA, this should be reflected in an increase in the $^{3}H:^{14}C$ ratio of the precursors, VMA and DHMA. Since the $^{3}H:^{14}C$ ratios of the VMA and DHMA, as well as MHPG and DHPG fractions were close to 4, an isotope effect can be excluded.

In the rat, about 10% of administered $^{3}H:^{14}C$-NA undergoes a transformation in the body, which leads to a loss of the tritium atom attached to the side chain of the noradrenaline molecule (Pichler & others, 1968). About 50% of the THO formed from the ^{3}H-NA can be accounted for by the sum of VA, DHBA and the unknown metabolite isolated. Using our experimental procedure, noradrenochrome and noradrenolutine would be expected to appear in the alumina eluate, but there was no paper chromatographic indication for their presence. This is in accordance with the fact that the catecholamines are specifically stored within the sympathetic nerves and are, therefore, not accessible to degradation by catechol oxidase, although this enzyme is present in rat tissues. Hence, no oxidative degradation of the catecholamines by catechol oxidase occurs *in vivo* as observed in tissue homogenates (Axelrod, 1964).

Pichler & others (1968) found that treatment with 25 mg/kg of the MAO-blocker, pargyline, did not change the formation of THO from ^{3}H-NA, whereas we have found that MAO-blockade by iproniazid markedly decreased the amounts of the substances arising simultaneously with THO formation, viz. VA, DHBA and the unknown fraction, and, concomitantly, in preliminary experiments, also decreased the formation of THO. Although no conclusive explanation can be offered for the discrepancy, it seems possible that pargyline, in the dosage used, did not produce the same degree of MAO inhibition as iproniazid.

REFERENCES

AXELROD, J. (1964). *Biochim. Biophys. Acta*, **85**, 247–254.

AXELROD, J. & TOMCHICK, R. (1958). *J. biol. Chem.*, **233**, 702–705.

DIRSCHERL, W., THOMAS, H. & SCHRIEFERS, H. (1962). *Acta Endocr.*, **39**, 385–394.

GITLOW, S. E., MENDLOWITZ, M., KRUCK-WILK, E., WILK, S., WOLF, R. L. & NAFTCHI, E. (1964). *J. clin. Invest.*, **43**, 2009–2015.

GOLDENBERG, M., FABER, M., ALSTON, E. J. & CHARGAFF, E. C. (1949). *Science, N.Y.*, **109**, 534–535.

GOODALL, McC. & ALTON, H. (1965). *Biochem. Pharmac.*, **14**, 1595–1604.

GOODALL, McC. & ALTON, H. (1969). *Ibid.*, **18**, 295–302.

KETY, S. S. (1959). *Science, N.Y.*, **129**, 1528–1532.

OKITA, G. T., KABARA, J. J., RICHARDSON, F. & LEROY, G. V. (1957). *Nucleonics*, **15**, 111–114.

PICHLER, H., SUKO, J. & HERTTING, G. (1968). *Biochem. Pharmac.*, **17**, 1329–1337.

ROSEN, L. & GOODALL, McC. (1962). *Proc. Soc. exp. Biol. Med.*, **110**, 767–769.

SANDLER, M., RUTHVEN, C. R. J. & WOOD, C. (1964). *Int. J. Neuropharmac.*, **3**, 123–128.

SUKO, J., LINET, O. & HERTTING, G. (1967). *Arch. exp. Path. Pharmak.*, **256**, 439–449.

THOMAS, H. (1966). *Naturwissenschaften*, **53**, 705–706.

WEISE, V. K., McDONALD, R. K. & LABROSSE, E. H. (1961). *Clinica chim. Acta*, **6**, 79–86.

J. Pharm. Pharmac., 1971, 23, 276–279 *Received November* 18, 1970

Uptake and metabolism of β-phenethylamine and tyramine in mouse brain and heart slices

S. B. ROSS AND A. L. RENYI

Research Laboratories, Astra Läkemedel, Södertälje, Sweden

Tritium labelled β-phenethylamine and tyramine were incubated with slices of mouse brain and heart. Cocaine (3×10^{-5}M) caused a reduction of 30% in the uptake of tyramine as well as in the formation of its metabolite 4-hydroxyphenylacetic acid. Cocaine had no effect on either the uptake of phenethylamine nor its deamination to phenylacetic acid.

We have previously reported the uptake of various β-phenethylamine derivatives by slices of mouse brain and heart (Ross & Renyi, 1966; Ross, Renyi & Brunfelter, 1968). Cocaine reduces the uptake of those amines containing at least one phenolic hydroxyl group but has no effect on the uptake of amines without these hydroxyl groups. Since cocaine is known to be a potent inhibitor of the active uptake of catecholamines (Whitby, Herrting & Axelrod, 1960; Dengler, Spiegel & Titus, 1961; Hillarp & Malmfors, 1964) at the site of the neuronal membrane, it seems likely that the uptake of other phenethylamine derivatives which is sensitive to cocaine might utilize the same carrier mechanism as that used by catecholamines. The lack of inhibition by cocaine of the uptake of (+)-amphetamine, norephedrine and β-phenylethanolamine may indicate that these amines are not taken up by this mechanism (Ross & others, 1968). It has been suggested, however, that these amines are taken up by a cocaine-sensitive mechanism, but because of their non-polar nature they diffuse rapidly out of the cells while the more polar phenethylamines pass out only slowly (Thoenen, Hürlimann & Haefely, 1968). A rapid diffusion out could obscure an effect of cocaine.

To separate effects on uptake from effects on amine retention we have measured the enzymatic deamination of [4-^{3}H]β-phenylethylamine in brain and heart slices. Some of the monoamine oxidase (MAO) responsible for deamination is located in the adrenergic neurons (Roth & Stjärne, 1966; Champlain, Mueller & Axelrod, 1969). Thus, if an amine is actively taken up by cells by a cocaine-sensitive mechanism, the formation of phenylacetic acid should be decreased in the presence of cocaine. [^{3}H]Tyramine was used as a reference compound, since it is known to be actively taken up by the cocaine-sensitive mechanism (Ross & Renyi, 1966) and is a good substrate for MAO (Blaschko, 1952) yielding 4-hydroxyphenylacetic acid.

MATERIALS AND METHODS

[4-^{3}H]-β-Phenethylamine was synthetized from *p*-chloro-β-phenethylamine by catalytic exchange (Pd) of the chlorine atom with gaseous tritium (Isotoptjänst, AB Atomenergi, Studsvik, Sweden). The specific activity was 1·0 Ci/mmol. The radioactive purity was checked by paper chromatography in n-butanol–water–ethanol (40:10:10). [^{3}H]Tyramine (generally labelled; specific activity 7·3 Ci/mmol) was obtained from New England Nuclear Corp.

The incubation of brain (cerebral cortex) and heart slices from mouse with the tritium-labelled amines was as described by Ross & Renyi (1966). The incubation

mixture consisted of 0·2 nmol of the amine, 11 μmol of glucose, 100 mg of tissue slices and 0 or 60 nmol of cocaine. Incubations were in 2 ml of Krebs-Henseleit buffer, pH 7·4, in an atmosphere consisting of 6·5% carbon dioxide in oxygen. The slices were pre-incubated in the buffer for 5 min before the addition of the [^{3}H]amine. After 5 min (brain slices) or 10 min (heart slices) incubation the tissue were removed from the buffer, blotted on filter paper and homogenized in 1·0 ml of ethanol containing 100 μg of non-radioactive amine and 100 μg of the corresponding acid metabolite as carrier substances. A 1·0 ml aliquot of the incubation media was also saved. It was added to an equal volume of ethanol containing the appropriate carrier compounds.

Chromatographic separation of the amines was by means of thin-layer chromatography (PSC-Fertigplatten, Kieselgel F 254, Merck) in chloroform–formic acid–ethylacetate (35 : 10 : 55). Aliquots of 0·3 ml of the incubation media or 0·5 ml of the tissue extract were used. The compounds were localized by means of an ultraviolet light and were extracted from the silica gel in to 2 ml of Soluene-100 (Packard). Radioactivity was measured after the addition of 15 ml of scintillation liquid (PPO and POPOP in toluene).

RESULTS

Cocaine significantly reduced the rate of formation of 4-hydroxyphenylacetic acid from [^{3}H]tyramine on incubation with brain (Table 1) and heart slices (Table 2). The inhibition of 4-hydroxyphenylacetic acid formation was quantitatively similar to the inhibition of tyramine uptake. The total uptake of [^{3}H]tyramine is the sum of the amine found in the slices plus the 4-hydroxyphenylacetic acid formed. This

Table 1. *Effect of cocaine* (3×10^{-5}M) *on the formation of the* 4-*hydroxyphenylacetic acid* (4-HPAA) *from tyramine by slices from mouse brain.*

Treatment	n	Brain slices Tyramine nmol/g ± s.e.	Brain slices 4-HPAA nmol/g ± s.e.	Incubation fluid 4-HPAA nmol/g ± s.e.
None	23	0·055 ± 0·002	0·089 ± 0·002	0·345 ± 0·010
Cocaine	12	0·040 ± 0·003* (27%)	0·071 ± 0·003 (20%)	0·231 ± 0·009* (33%)

* $P < 0{\cdot}001$. 5 min incubation.

The figures are means ± standard error of mean. The percentage decrease produced by cocaine is indicated in brackets.

Table 2. *Effect of cocaine* (3×10^{-5}M) *on the formation of* 4-*hydroxyphenylacetic acid* (4-HPAA) *from tyramine by slices of mouse heart.*

Treatment	Heart slices Tyramine nmol/g ± s.e.	Heart slices 4-HPAA nmol/g ± s.e.	Incubation fluid 4-HPAA nmol/g ± s.e.
None	0·037 ± 0·002	0·119 ± 0·003	0·219 ± 0·007
Cocaine	0·026 ± 0·001* (30%)	0·084 ± 0·004* (29%)	0·145 ± 0·004* (34%)

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

10 min incubation. The figures are means ± standard error of mean from 12 determinations. The percentage decrease produced by cocaine is indicated in the brackets.

Table 3. *Effect of cocaine* (3×10^{-5}M) *on the formation of phenylacetic acid* (PAA) *from β-phenethylamine* (PEA) *by brain slices from mouse.*

Treatment	Brain slices PEA nmol/g ± s.e.	Brain slices PAA nmol/g ± s.e.	Incubation fluid PAA nmol/g ± s.e.
None	0·014 ± 0·001 (n = 18)	0·081 ± 0·003 (n = 34)	0·631 ± 0·033 (n = 16)
Cocaine	0·017 ± 0·002* (n = 18)	0·083 ± 0·004* (n = 18)	0·673 ± 0·023* (n = 16)

* $P > 0{\cdot}05$.

5 min incubation. The figures are means ± standard error of means from the number of determinations indicated in the brackets.

Table 4. *Effect of cocaine* (3×10^{-5}M) *on the formation of phenylacetic acid* (PAA) *from β-phenethylamine* (PEA) *by heart slices from mouse.*

Treatment	Heart slices PEA nmol/g ± s.e.	Heart slices PAA nmol/g ± s.e.	Incubation fluid PAA nmol/g ± s.e.
None	0·014 ± 0·001	0·123 ± 0·004	0·820 ± 0·022
Cocaine	0·012 ± 0·001*	0·121 ± 0·004*	0·888 ± 0·035*

* $P > 0{\cdot}05$.

10 min incubation. The figures are means ± standard error of mean from 12 determinations

uptake represents both active uptake and the result of passive diffusion. The assumption is made that the high level of cocaine used completely blocks active uptake. Thus, the net uptake of tyramine in the control brain slices was 0·489 nmol/g each 5 min. This value decreased to 0·342 nmol/g each 5 min in presence of cocaine, suggesting that the active uptake of tyramine amounts to 0·147 nmol/g each per 5 min at a concentration of [^{3}H]tyramine of 10^{-7}M. For heart slices under the same conditions this value is 0·120 nmol/g each 10 min.

The uptake and deamination of [^{3}H]β-phenylethylamine by brain and heart slices is shown in Tables 3 and 4. The total rate of uptake of phenethylamine in brain and heart slices was 0·726 nmol/g each 5 min and 0·957 nmol each 10 min, respectively. Cocaine had no significant effect on the uptake of this amine or on the formation of phenylacetic acid.

DISCUSSION

There are several problems that arise when one attempts to measure the uptake of a compound into a tissue, where it is being simultaneously metabolized (Ross & Renyi, 1969). In the present instance, since some of the metabolizing enzyme is located in the cells responsible for the uptake, inhibition of uptake should reduce the amount of the metabolite formed. The assumptions are made that cocaine specifically inhibits the active uptake of phenethylamines and that at a high concentration of cocaine active uptake approaches zero. The capacity of the active uptake mechanism can be estimated by summing the reduction in the formation of the metabolite with the decrease in the amount of the compound itself in the tissue.

The observation that the formation of 4-hydroxyphenylacetic acid was inhibited by cocaine to the same degree as the uptake of tyramine supports the assumption. Since neither the deamination nor the uptake of phenethylamine was inhibited by cocaine, this amine does not seem to be taken up by such a cocaine-sensitive mechanism. This observation supports the hypothesis that at least one phenolic hydroxyl group is necessary for the cocaine-sensitive uptake of phenethylamine derivatives (Ross & Renyi, 1966; Ross & others, 1968).

If the same relation between structure and active uptake described here for brain and heart tissue is also valid for uptake into the adrenergic nerves of other tissues it seems difficult to explain the antagonism by cocaine of the cardiovascular effects of indirectly acting amines, such as (+)-amphetamine and phenethylamine (Burn & Rand, 1958) as being due to an inhibition of the uptake of these indirectly-acting amines (Trendelenburg, 1961). It is possible that the cardiovascular effects are due to noradrenaline molecules released from sites located outside or at the neuron membranes for example the so called transfer site for which cocaine has high affinity (Ross & Renyi, 1966). Cocaine may also inhibit the outward passage of noradrenaline released from intraneuronal sites by the amines passively taken up. The possibility also exists that the cardiovascular effects of the indirectly acting amines are due to hydroxylated products of the amines.

Acknowledgement

We thank Dr. Perry Molinoff, Department of Biophysics, University College, London, for valuable discussion of the manuscript.

REFERENCES

BLASCHKO, H. (1952). *Pharmac. Rev.*, **4**, 415–458.
BURN, J. H. & RAND, M. J. (1958). *J. Physiol., Lond.*, **144**, 314–336.
CHAMPLAIN, J. DE, MUELLER, R. A. & AXELROD, J. (1969). *J. Pharmac. exp. Ther.*, **186**, 339–345.
DENGLER, H. J., SPIEGEL, H. E. & TITUS, E. O. (1961). *Nature, Lond.*, **191**, 816–817.
HILLARP, N. Å. & MALMFORS, T. (1964). *Life Sci.*, **3**, 703–708.
ROSS, S. B. & RENYI, A. L. (1966). *Acta pharmac. tox.*, **24**, 297–309.
ROSS, S. B. & RENYI, A. L. (1969). *Europ. J. Pharmac.*, **7**, 270–277.
ROSS, S. B., RENYI, A. L. & BRUNFELTER, B. (1968). *J. Pharm. Pharmac.*, **20**, 283–288.
ROTH, R. H. & STJÄRNE, L. (1966). *Acta physiol. scand.*, **68**, 342–346.
THOENEN, H., HÜRLIMANN, A. & HAEFELY, W. (1968). *J. Pharm. Pharmac.*, **20**, 1–11.
TRENDELENBURG, U. (1961). *J. Pharmac. exp. Ther.*, **134**, 8–17.
WHITBY, L. G., HERRTING, G. & AXELROD, J. (1960). *Nature, Lond.*, **187**, 604–605.

J. Pharm. Pharmac., 1971, **23**, 280–287 *Received September* 14, 1970

The spontaneous electrical and mechanical activity of the longitudinal smooth muscle of the rabbit duodenum and its modification by drugs and temperature changes

R. C. SMALL AND A. H. WESTON

Department of Pharmacology, University of Manchester, Manchester M13 9PL, U.K.

The electrical and mechanical activity of strips of longitudinal smooth muscle from the rabbit duodenum was recorded using the sucrose gap method. The muscle exhibited rhythmic tension changes each of which was associated with a slow potential wave of electric depolarization surmounted by a burst of spike activity (a multispike complex). Cooling the tissue reduced the frequency of tension waves and multispike complexes. Catecholamines produced a reduction in the amplitude of tension waves. This was associated with hyperpolarization and decreased spike activity. Slow wave frequency and amplitude were unaffected. Acetylcholine, methacholine and histamine produced tension wave fusion associated with depolarization and increased spike activity.

In spite of the widespread use of isolated preparations of rabbit duodenum for both research and teaching purposes, the electrical activity of the longitudinal muscle layer of this tissue has been described only briefly (Bortoff, 1961a; Gonella, 1965). This paper presents the results of experiments designed to examine the effects of drugs and temperature changes on the spontaneous electrical and mechanical activity of rabbit duodenum longitudinal muscle using the sucrose gap method.

Some of the results have been presented to the British Pharmacological Society (Small & Weston, 1969).

MATERIALS AND METHODS

Preparation of the muscle strip

New Zealand White rabbits (Hyline), 2–3 kg, of either sex, were stunned and bled. The duodenum (defined for the purposes of these experiments as the 15 cm of small intestine adjacent to the pyloric sphincter) was removed and strips of the longitudinal muscle layer were prepared according to Ambache (1954). Histologically, the strips consisted of the longitudinal muscle layer together with an adhering nerve plexus. After removal of the damaged ends, 2 cm lengths of the muscle strip were prepared for mounting in the sucrose gap apparatus.

Sucrose gap apparatus

The apparatus used was similar to that described by Bülbring & Burnstock (1960). The potential difference across the gap was recorded by means of a pair of Ag/AgCl wick electrodes attached to a Grass P17 high impedance probe. The output of the probe was recorded on one channel of a Grass polygraph. The frequency response of the recording system was such that a sine wave calibration signal of 12 Hz was reduced in amplitude by 50%.

One side of the gap was perfused with a physiological salt solution (PSS) at 37·5° equilibrated with 5% carbon dioxide in oxygen. The solution had the following composition (mM): Na^+, 143; K^+, 5·93; Ca^{2+}, 2·55; Mg^{2+}, 1·2; Cl^-, 125; HCO_3^-, 25; SO_4^{2-}, 1·2; $H_2PO_4^-$, 1·18; dextrose, 11·1. The free end of the perfused muscle strip in this side of the gap was attached by cotton thread to a force-displacement transducer (Grass FTO3C) for the simultaneous recording of mechanical activity under isometric conditions at a resting tension of 1 g.

The other side of the gap was perfused with potassium sulphate solution (100 mM) at room temperature. The sucrose solution (300 mM) perfusing the gap itself was passed through a deionizing column before use and had a specific resistance greater than 1 MΩ cm.

Constant flow rates were maintained for all solutions during an experiment and ranged from 2 to 3 ml/min.

Addition of drugs

(−)-Adrenaline, (−)-noradrenaline, (−)-isoprenaline, acetylcholine, methacholine and histamine were diluted in PSS and 0·05 ml volumes were introduced into the flow of PSS by injection.

Temperature changes

The temperature of the PSS was measured with an accuracy of 0·1° using an electric thermometer (Light Laboratories). Cooling experiments were performed by switching off the warm water circulating system.

RESULTS

Spontaneous activity

Records were made of the spontaneous mechanical and electrical activity of muscle strip preparations from a total of 42 rabbits. The mechanical activity of these strips, i.e. waves of tension of variable amplitude and frequency, was similar to that of pieces of intact duodenum mounted in a tissue bath. In general, three types of mechanical activity could be distinguished:

1. *Regular.* The waves of tension were relatively constant in their shape and amplitude (55% of the preparations, Fig. 1a).

2. *Intermittently regular.* Large, regular tension waves were interposed with periods of smaller, irregular activity (25% of preparations, Fig. 1c).

3. *Irregular.* The size, shape and frequency of the tension waves varied continuously (20% of preparations, Fig. 1b, d).

In the preparations examined, the frequency of tension waves ranged from 11 to 24/min (mean 17/min) whilst their range of amplitude (measured from the foot to the peak of the tension wave) was 0·05 to 10 g (mean 1·7 g). The pattern of electrical activity exhibited by the muscle strips also varied, but usually consisted of slow waves of depolarization with each peak surmounted by a burst of spike activity (Fig. 1a,c). Electrical events such as these have been described for the rabbit portal vein by Holman, Kasby & others (1968) who termed them multispike complexes (MSCs). The same terminology is used in this paper.

The total amplitude of the MSCs ranged from 0·2 to 6·5 mV (mean 1·6 mV). The rise time of the slow potential wave was shorter than its decay time. Indeed, the rate

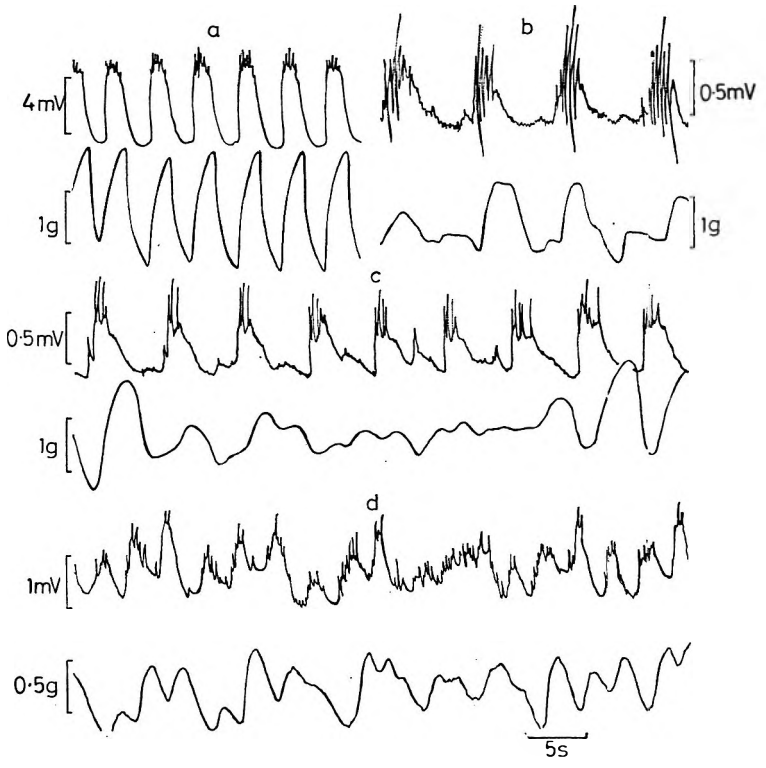


FIG. 1. Spontaneous electrical and mechanical activity at 37·5° in four different preparations. (a) regular mechanical activity; (b) irregular mechanical activity, spikes show positive undershoots; (c) intermittently regular mechanical activity; (d) irregular mechanical activity, spikes not always associated with slow waves.

of rise of the slow waves was often comparable to that of the spikes. Each MSC contained from 1 to 20 spikes whose amplitude ranged from 0·2 to 2·5 mV (mean 0·75 mV). The duration of the fastest spikes measured at half total amplitude was 30 ms. Occasionally, the spikes exhibited a rapidly developing phase of repolarization which extended below the point of initiation of the spike. This is illustrated in Fig. 1b. In most preparations, there was a discrete period between successive slow waves during which the potential remained stable or showed only a very slow rate of change compared to the repolarization phase of the slow waves.

Usually, the rising phase of the slow wave occurred either immediately before or simultaneously with the onset of a tension wave. The correlation between the frequency of MSC generation and the frequency of tension wave development is illustrated in Fig. 2.

Effects of cooling

When the warm water circulating system was switched off, the tissue cooled from 37·5° to 25° in 16 min. The effects of cooling were investigated on ten preparations. Of these, six showed an overall resting hyperpolarization of up to 5 mV. Three preparations exhibited no change in mean potential level and one was depolarized by 4 mV. Cooling also produced a reduction in the resting tension of seven preparations but the other three showed no change.

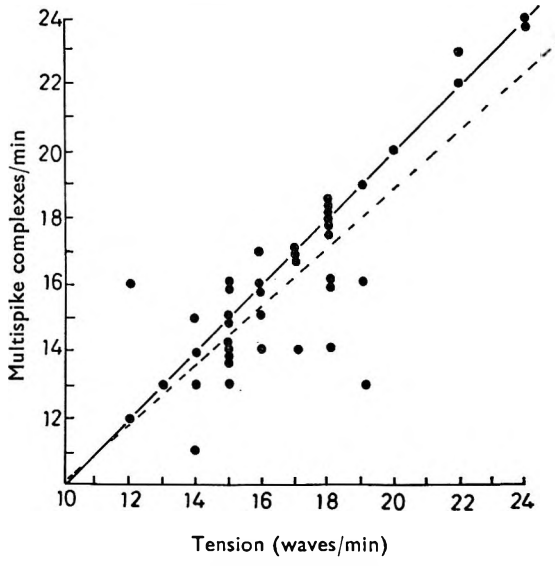


FIG. 2. The correlation between MSC frequency and tension wave frequency. Each point represents a single preparation. The broken line indicates the calculated regression line with $r = 0{\cdot}84$ ($P < 0{\cdot}001$). The continuous line represents a perfect positive correlation.

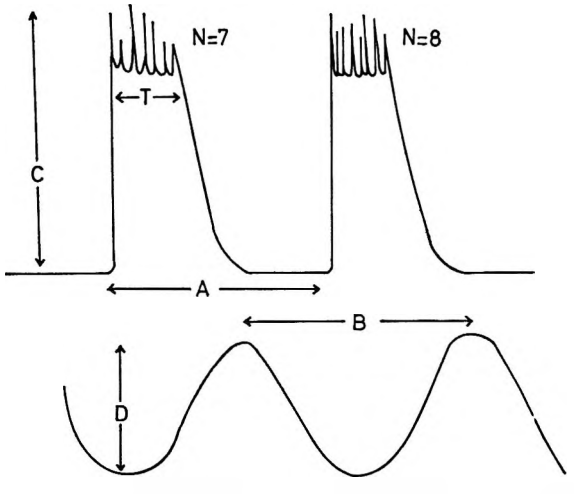


FIG. 3. Parameters of electrical and mechanical activity measured to assess the effects of cooling. A: MSC cycle time; B: tension wave cycle time; C: MSC amplitude; D: tension wave amplitude; N: number of spikes/MSC; T: duration of spiking; N/T: intracomplex spike frequency.

In order to examine the effects of cooling in detail, the MSC cycle time, tension wave cycle time, MSC amplitude, tension wave amplitude and intracomplex spike frequency were measured at 2·5° intervals between 37·5° and 25°. These parameters are defined in Fig. 3. At a given temperature, measurements of each parameter were made upon four consecutive MSCs or tension waves and the mean value was calculated. In three preparations, spike activity disappeared at temperatures above 25° thus reducing the size of the group in which intracomplex spike frequency measurements were made.

The most marked effect of cooling was a parallel increase in the MSC and tension wave cycle times. The amplitude of tension waves decreased but this was not accompanied by a corresponding reduction in MSC amplitude. However, there was a reduction in intracomplex spike frequency. These results are illustrated in Fig. 4.

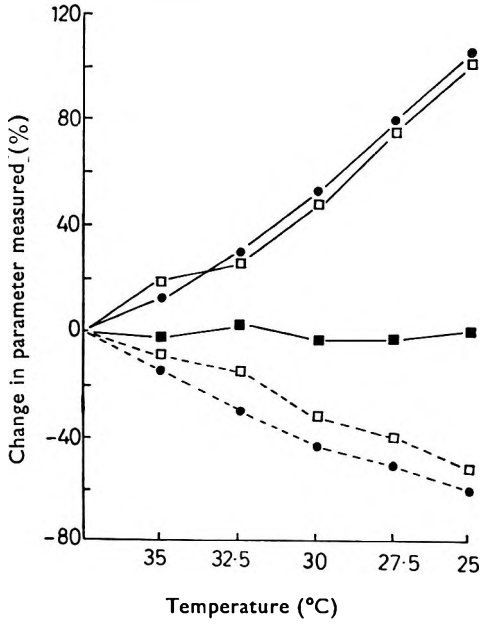


FIG. 4. The effect of cooling on MSC cycle time ●——●, tension wave cycle time □——□, MSC amplitude ■——■, tension wave amplitude □ - - - □, and intracomplex spike frequency ● - - - ●. These parameters are defined in Fig. 4. Ordinate, percentage change in the parameter measured taking the change at 37·5°C as zero. Each point represents the mean of measurements from ten preparations.

Effects of rewarming

When the warm water circulating system was switched on again, the temperature of the PSS rose from 25° to 37·5° in 3 min. Such experiments showed that the effects of cooling were reversible. In one experiment, a complete inhibition of both MSCs and tension waves was observed in the initial rewarming period.

Effects of drugs

Catecholamines such as adrenaline, noradrenaline and isoprenaline all caused a reduction in the amplitude of tension waves together with a decrease in the resting tension of the tissue. These effects were associated with an overall hyperpolarization. Spike activity was reduced or abolished by these agents but slow wave frequency and amplitude of the rhythmical electrical changes were unaffected. Moreover, the slow waves retained their fast rising phase in the absence of spike activity. The effects of increasing concentrations of isoprenaline are shown in Fig. 5.

Acetylcholine, methacholine and histamine all caused an increase in the resting tension of the preparation. With large doses of these drugs, individual tension waves were lost in a fused tension response. When tension waves remained discrete, slow waves of electrical potential change were still discernible but these were associated with increased superimposed spike activity. With the larger, fused

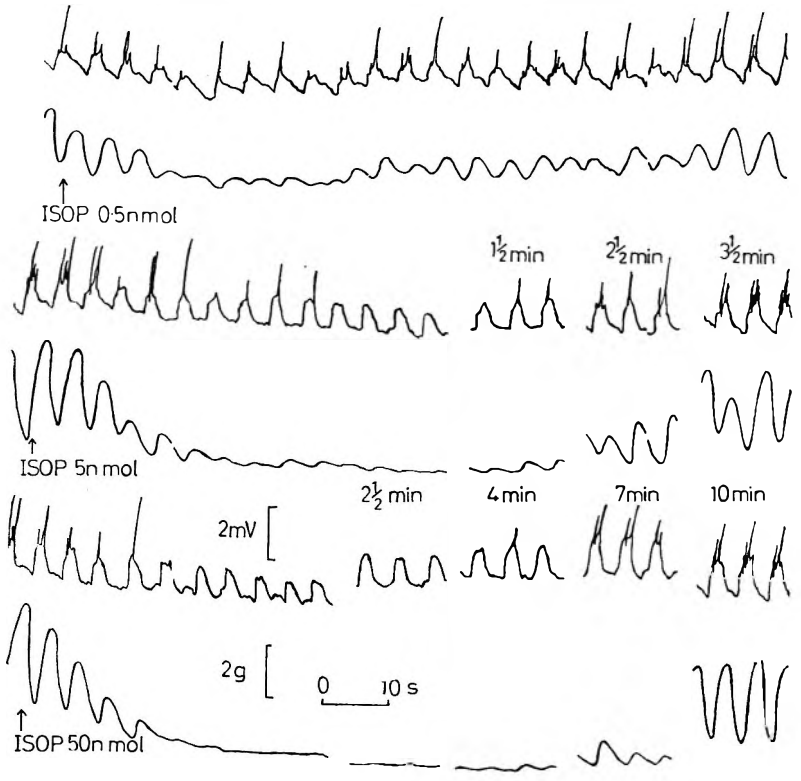


FIG. 5. The effect of increasing doses of isoprenaline on electrical and mechanical activity. All records from the same preparation.

tension responses, electrical spike activity occurred both on the crests and in the troughs of the slow waves and there was a greater overall depolarization. The effects of increasing doses of acetylcholine are shown in Fig. 6.

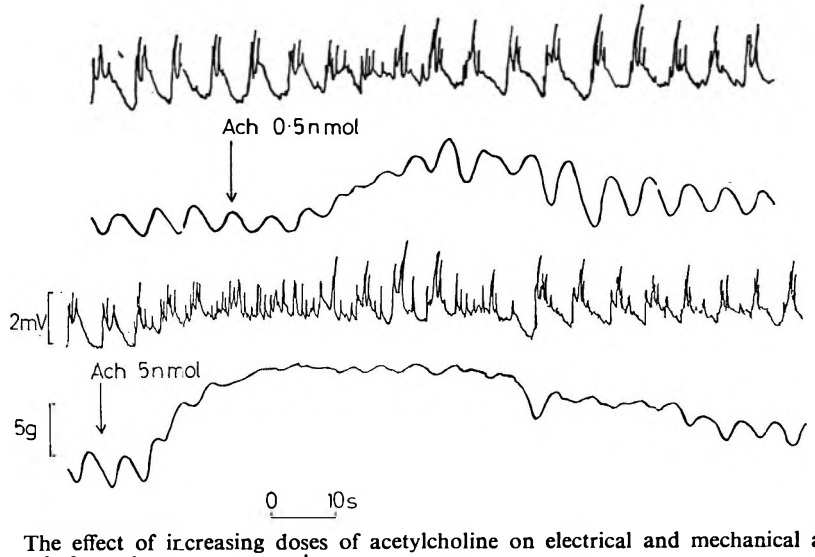


FIG. 6. The effect of increasing doses of acetylcholine on electrical and mechanical activity. Both records from the same preparation.

DISCUSSION

The electrical activity of the longitudinal smooth muscle of the rabbit duodenum was seen to consist of a series of MSCs i.e. slow waves of depolarization each surmounted by a burst of spike activity. This pattern of electrical events is similar to that obtained by the intracellular recording technique applied to this tissue by Bortoff (1961a) and Gonella (1965). Most preparations produced MSCs in a 1 to 1 relation with tension waves, suggesting a correlation between these two events. Since the rising phase of the MSC occurred immediately before or simultaneously with an increase in tension, it seems probable that the electrical events trigger the mechanical response of the tissue.

In contrast to most preparations examined, some showed no regular pattern of either electrical or mechanical activity. Such preparations may have been damaged during dissection or mounting in the apparatus, thus destroying the functional integrity of the smooth muscle syncytium and permitting random excitation of cells. This explanation might not suffice for those preparations exhibiting intermittently regular mechanical activity. Here, the electrical record maintained its regularity despite the transient loss of regular mechanical events. This might suggest that, at these times, the part of the tissue from which the electrical record was obtained contributed very little to the overall mechanical record.

The ability of catecholamines to abolish spikes and reduce the amplitude of tension waves whilst leaving slow waves intact suggests that, as in guinea-pig taenia coli, tension development is closely related to spike activity (Bülbring, 1955). Our cooling experiments and those in which the spasmogens acetylcholine, methacholine and histamine were used also demonstrate the importance of spike activity in determining the magnitude of the mechanical response.

Catecholamines did not affect slow wave frequency or amplitude. A similar observation has been made by Bortoff (1961b) for the action of adrenaline on cat jejunum. Low concentrations of spasmogens were also without effect on slow wave frequency although at higher concentrations, individual slow waves became difficult to distinguish because of the overall depolarization and continuous spiking. Cooling was the only procedure to affect slow wave frequency; this was reduced in parallel with the frequency of tension waves.

The failure of spasmogens and spasmolytics to alter MSC frequency in rabbit intestinal muscle contrasts with the observations of Holman, Kasby & others (1968) on the smooth muscle of rabbit portal vein. Although the MSCs of the latter preparation were similar in shape to those recorded from rabbit duodenum, their frequency was markedly affected by drugs; it was increased by spasmogens and decreased by spasmolytics. Furthermore, spasmolytic agents did not selectively abolish spike activity. This suggests that the slow component of the vascular MSC may be produced by the same mechanism as that responsible for spike generation. Alternatively, the slow waves of vascular smooth muscle may not represent discrete entities but merely result from residual depolarization produced by repetitive high frequency spiking (Holman, 1968).

The ability of drugs to dissociate slow waves from spikes in visceral smooth muscle supports the hypothesis of Tamai & Prosser (1966) that these two events are produced by different electrogenic processes. These authors showed that the slow waves and spikes of the cat small intestine exhibited marked differences in their sensitivity to changes in the ionic environment.

REFERENCES

AMBACHE, N. (1954). *J. Physiol., Lond.*, **125**, 53–55P.

BORTOFF, A. (1961*a*). *Am. J. Physiol.*, **201**, 203–208.

BORTOFF, A. (1961*b*). *Ibid.*, **201**, 209–212.

BÜLBRING, E. (1955). *J. Physiol., Lond.*, **128**, 200–221.

BÜLBRING, E. & BURNSTOCK, G. (1960). *Br. J. Pharmac. Chemother.*, **15**, 611–624.

GONELLA, J. (1965). *C. r. hebd. Séanc. Acad. Sci., Paris*, **260**, 5362–5365.

HOLMAN, M. E. (1968). *Handbook of Physiology*, Section 6, Volume IV, 1665–1708, Baltimore: Williams & Wilkins Co.

HOLMAN, M. E., KASBY, C. B., SUTHERS, M. B. & WILSON, J. A. F. (1968). *J. Physiol., Lond.*, **196**, 111–132.

SMALL, R. C. & WESTON, A. H. (1969). *Br. J. Pharmac.*, **37**, 545–546P.

TAMAI, T. & PROSSER, C. L. (1966). *Am. J. Physiol.*, **210**, 452–458.

J. Pharm. Pharmac., 1971, **23**, 288–294 *Received November* 11, 1970

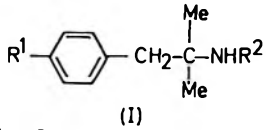
The metabolism and urinary excretion in man of phentermine, and the influence of *N*-methyl and *p*-chloro-substitution*

A. H. BECKETT AND L. G. BROOKES†

Department of Pharmacy, Chelsea College (University of London), Manresa Road, S.W.3, U.K.

The urinary excretion of phentermine, mephentermine and chlorphentermine was examined after oral administration of phentermine and chlorphentermine hydrochlorides and mephentermine sulphate to man under normal and acidic conditions of urinary pH. The rate of excretion of both phentermine and mephentermine fluctuated with changes in urine pH, a more acidic pH causing a faster rate of excretion; changes in urine flow rate had only a slight effect. The rate of excretion of chlorphentermine was affected by changes in pH and urinary flow rates. Phentermine and mephentermine were recovered almost quantitatively within 24 h from subjects under acidic urine control; only about 35% chlorphentermine was recovered under similar conditions.

Mephentermine (Ib) is the *N*-methyl, and chlorophentermine (Ic) the *p*-chloroderivative of phentermine (Ia).



(I)

Ia $R^1 = R^2 = H$ Ib $R^1 = H$ $R^2 = Me$ Ic $R^1 = Cl$ $R^2 = H$

The metabolism of mephentermine in man does not appear to have been reported hitherto. The urinary excretion of unchanged chlorphentermine after oral administration was reported to be about 70% for rats and about 25% for mice (Opitz & Weischer, 1966, 1967; Dubnick, Towne & others, 1968); only about 5% of a dose of phentermine was similarly recovered. Lower recoveries of chlorphentermine with increase in dose were recorded from rats and man but no correlation with urine volume was noted. *N*-Demethylation and *p*-hydroxylation of mephentermine was effected by dogs, rabbits and rats given the drug intraperitoneally (Walkenstein, Chumakow & Seifter, 1955). One third of the dose was in the faeces but little unchanged drug was recovered from the urine, and then only in the first hour after administration. The urinary excretion of phentermine, mephentermine and chlorphentermine by man after the drug had been taken by mouth is now reported.

MATERIALS AND METHODS

Dosage regimens for oral administration of phentermine, mephentermine and chlorphentermine

Male subjects (age 25–45), under normal (pH about 5–8), or acidic urine control were given, on separate widely spaced occasions, an oral dose of 12·45 mg of phen-

* This work forms part of a thesis by L.G.B. accepted for the degree of Ph.D. in the University of London, 1968.

† Present address: Upjohn International, Crawley, Sussex.

termine hydrochloride, 14·11 mg of mephentermine sulphate or 6·0 to 72 mg of chlorphentermine hydrochloride, in 60–80 ml aqueous solutions. Also, two subjects under acidic urine control were given 72 mg of chlorphentermine hydrochloride whilst drinking water at a rate of 700 ml/h.

Collection and examination of urine

The general procedure adopted for diet and the collection of urine was similar to that previously described by Beckett & Rowland (1965a). The induction and maintenance of an acidic urine (pH $\geq$ 5·0) was as described by Beckett & Brookes (1967). Urine samples were analysed by gas-liquid (g.l.c.) and thin-layer (t.l.c.) chromatography. Preparative t.l.c. was used to obtain samples suitable for infrared spectroscopy of phentermine excreted in the urine after oral administration of mephentermine sulphate.

The amount of the metabolite, phentermine, excreted was calculated as a percentage of the dose of mephentermine sulphate administered.

Gas-liquid chromatography

The ethereal extracts of urine were analysed using a Perkin Elmer F11 gas chromatograph with a flame ionization detector and a stainless steel column (3 ft $\times$ $\frac{1}{8}$ inch), packed with 10% KOH, 10% Apiezon L and acid washed, DMCS treated Chromosorb G (80–100 mesh) (column A). The analysis was run isothermally at 160° and using a nitrogen flow rate of 30 ml/min, the column condition and gas pressures being made optimum. A second column (B) also used has been described earlier (Beckett & Brookes, 1967).

Aletamine hydrochloride (10 μg base/ml in water), used as internal standard was added to urine at the start of the extraction procedure. Calibration curves for the amines were prepared as for amphetamine (Beckett & Rowland, 1965a). In addition, some of the ether extracts of urine were treated with acetone, acetic and propionic anhydrides, and examined by g.l.c. as described earlier (Beckett & Brookes, 1967). Several control urines from smokers and non-smokers were similarly examined because nicotine in smokers' urine interferes with the methyl orange assay procedure for the structurally similar amphetamine (Beckett, Rowland & Triggs, 1965). Phentermine, chlorphentermine and mephentermine were added (1 μg/ml) to urines at pH 4 and 9, stored at 4° and the solutions assayed periodically for two weeks.

Thin-layer chromatography

Glass plates, 20 $\times$ 20 cm coated with silica gel G (Merck) 0·25 mm thick, prepared according to Stahl, Schröter & others (1956) and dried at 90–100° for 30 min were spotted with ethereal extracts described above, together with authentic samples of phentermine, mephentermine and chlorphentermine and developed at room temperature (22–24°) with (*a*) ethanol (96%)–ammonium hydroxide (25%) (80:20); (*b*) methanol–chloroform–ammonium hydroxide (90:10:0·3); (*c*) methanol–chloroform (50:50). Mephentermine was visualized (red spot) by Dragendorff's spray (Stahl, 1962), and chlorphentermine and phentermine (pink spots) by freshly diazotized *p*-nitroaniline (Wickström & Salvesen, 1952).

Preparative t.l.c. was carried out under identical conditions using solvent system (*a*). Those sections of the plates containing the extracted phentermine and mephentermine, and the authentic samples, were scraped off and added to separate 5 ml portions of

20% w/v aqueous sodium hydroxide, well mixed and the drug content completely extracted with 3 × 2·5 ml of diethyl ether, the extracts being examined with a Unicam SP 100 infra-red spectrophotometer.

RESULTS

Identity of the metabolite of mephentermine

Analysis by g.l.c. of ethereal extracts of urines from subjects taking mephentermine sulphate gave one peak identical in retention time with that of authentic mephentermine and another with that of authentic phentermine. Identical retention times of the propionyl and acetyl derivatives of both amines and of the corresponding authentic samples (Table 1) confirmed the analysis. No acetone derivative was obtained for phentermine (or chlorphentermine) because of the steric hindrance of the second α-methyl group

Table 1. *Retention times of phentermine, mephentermine and chlorphentermine and some derivatives on g.l.c. columns.*

Column	Compound		Retention time (min)
A	Phentermine		3·2
	Mephentermine		5·2
	Chlorphentermine		9·0
B	Acetyl derivative	Phentermine	4·4
Temp. 165°		Mephentermine	3·6
		Chlorphentermine	14·6
B	Propionyl derivative	Phentermine	12·3
Temp. 140°		Mephentermine	10·1
		Chlorphentermine	41·2

T.l.c. in systems (*a*)–(*c*) gave R_F values (0·53, 0·38 and 0·26 respectively) that were identical for the metabolite and for phentermine. Preparative t.l.c. gave an oil with an infrared spectrum identical with that of phentermine.

Quantitative analysis of phentermine, mephentermine and chlorphentermine

Analysis by g.l.c. gave linear calibration curves for all drugs. No interfering peaks occurred from control urines. The drugs in acidic and alkaline urines were stable for at least two weeks at 4°.

Urinary excretion

Phentermine. About 70–80% was excreted in 24 h from subjects under acidic urine control (Table 2). The excretion rate reached a maximum 2–3 h after drug administration, and then fell exponentially, e.g. see Fig. 1.

(*b*) *Mephentermine.* The excretion rate of mephentermine and its metabolite, phentermine, varied with urine pH in a manner similar to that seen with amphetamine and methylamphetamine (Beckett & Rowland, 1965a,b) and was only slightly influenced by urine output (Fig. 2A). With a normal urine pH, subjects excreted about 30–40% mephentermine and about 7–12% phentermine over 24 h (Table 2). In subjects with acidic urine, the excretion rate was much higher and rate fluctuations were almost abolished (Fig. 2A); about 70% of mephentermine and phentermine was excreted in 24 h and there was negligible inter-subject variation (Table 2). The

Table 2. *Urinary excretion of mephentermine, phentermine or chlorphentermine after oral administration to subjects with normal (pH 5–8) urine or acidic (pH ≯5) urine control* (24 h collection).

		Mephentermine sulphate (14·11 mg) % excreted			Phentermine HCl (12·45 mg) % excreted	Chlorphentermine hydrochloride		
Urine condition	Subject	As mephentermine	As phentermine	Total	As phentermine	Subject	Dose (mg)	% excreted as chlorphentermine
	I	60·8	7·2	68·0	74·0	III	72	16·7
	II	56·3	12·1	68·4		V	72	23·6
	III (1)	59·2	13·0	72·2	73·9	III	24	45·5
Acidic	III (2)	61·8	8·8	70·6		I	12	30·0
	IV				78·9	IV	12	38·6
	V (1)				71·7	V (1)	12	32·0
	V (2)				83·6	V (2)	12	39·2
						V	6	39·4
	VI	35·0	11·5	46·5				
Normal	III	28·9	7·0	35·9				
	V	41·1	8·1	49·2				

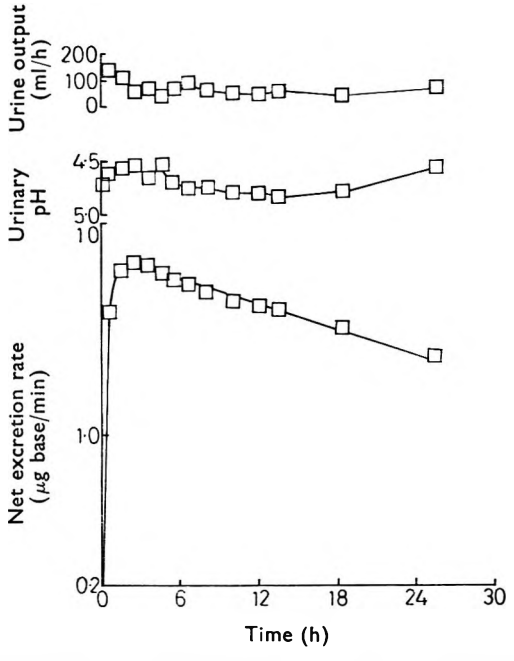


FIG. 1. Urinary excretion of phentermine with corresponding urinary pH and urine output after oral administration of 12·45 mg of phentermine hydrochloride in aqueous solution to subject III under acidic urine control.

excretion rate of mephentermine reached a maximum about 2–3 h after drug administration and then fell exponentially (Fig. 2B), while the excretion rate of phentermine reached a maximum after about 7 h.

(*c*) *Chlorphentermine.* The excretion rate was influenced by urine pH but primarily by rate of urine flow when the urine was kept acidic (Fig. 3) except when using the lowest dose (6 mg), and then the excretion rate of the drug reached a

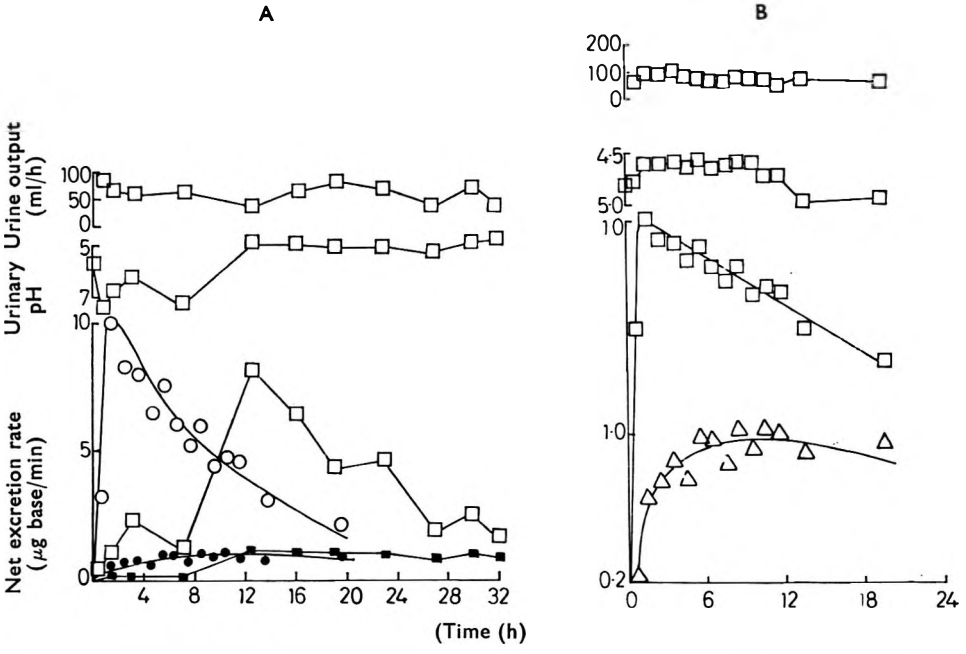


FIG. 2. A. Urinary excretion of mephentermine and its metabolite phentermine after oral administration of 14·11 mg of mephentermine sulphate with no urinary pH control (a) and acidic urine control (b). Acidic urine control: —○— mephentermine, —●— phentermine. No urinary pH control: —□— mephentermine, —■—phentermine.

B. Urinary excretion of mephentermine —□— and its metabolite phentermine —△—, with corresponding urinary pH and urine output after oral administration of 14·11 mg mephentermine sulphate in aqueous solution to subject III under acidic urine control.

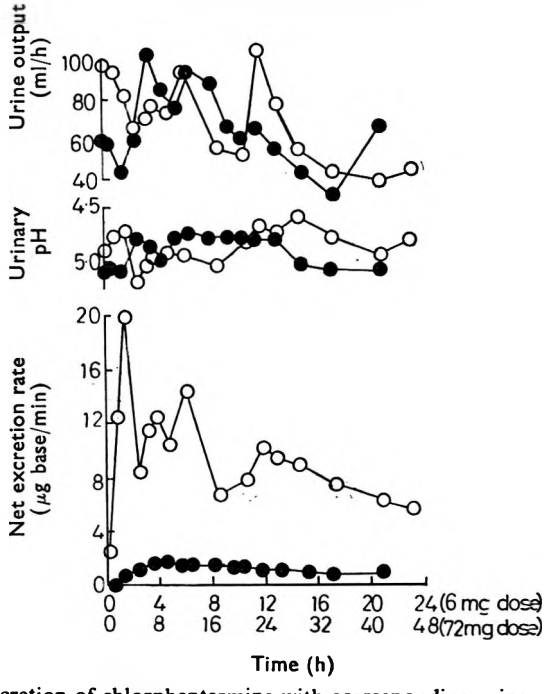


FIG. 3. Urinary excretion of chlorphentermine with corresponding urinary pH and urine output after oral administration of 6 mg (●) or 72 mg (○) of chlorphentermine hydrochloride in aqueous solution to a subject (V) under acidic urine control.

maximum 3–5 h after administration and fell exponentially. With doses of 24 mg of chlorphentermine hydrochloride or less, 24 h urine excretion accounted for about 35% drug, but this was only about 20% after doses of 72 mg (Table 2); water-loading with accompanying diuresis (about 500 ml/h) caused about double this amount of drug to be excreted unchanged.

DISCUSSION

The dealkylation of mephentermine in man to yield its major metabolite, phentermine, parallels that observed by Walkenstein & others (1955) with animals. The pH-dependent fluctuations in excretion rates of phentermine and mephentermine were expected since the pK_a values of phentermine and mephentermine are 9·84 and 10·11 respectively, so, like amphetamine and methylamphetamine (Beckett & Rowland, 1965a,b), renal tubular reabsorption of these drugs would be faster at an alkaline than at an acidic pH. Thus, the greater excretion of phentermine and mephentermine that we found when compared with the findings of Walkenstein & others (1955) and of Opitz & Weischer (1966), is because of pH control of urine. The high excretion of mephentermine and phentermine after oral administration indicates that their absorption from the gut is complete.

Our findings that the 24 h urinary excretion of phentermine and mephentermine after oral administration with acidic urine control is similar (70–80% of dose), while that of chlorphentermine is half this amount, are reflected in the elimination half lives of 7–8 h for phentermine and mephentermine and 14 h for chlorphentermine. The urinary excretion of both amphetamine and methylamphetamine in the same subjects as those used in the present work, was about 65% (Beckett & Rowland, 1965a,b; Brookes, 1968). Thus, the introduction of an α-methyl group into amphetamine has made the resulting compounds less susceptible to metabolism, most probably by steric hindrance. The ratio of drug to metabolite for both mephentermine and methylamphetamine was approximately the same, indicating that the *N*-dealkylation mechanism was not affected by the introduction of the α-methyl group into methylamphetamine. Because the excretion of mephentermine is the same as phentermine, we conclude that the *N*-methyl group has not rendered the former more susceptible to metabolism, a conclusion substantiated by drug excretion after methylamphetamine and amphetamine administration. *p*-Substitution of halogen in the molecule significantly changes the urinary excretion pattern of phentermine, not only by reducing the rate of excretion under acidic urine conditions but also by making the halogen-substituted drug susceptible to fluctuations in urine flow rate. Furthermore, compared with phentermine, there is a marked delay before chlorphentermine is excreted in the urine and reaches its maximum rate. Similar effects of such halogen substitution also occur (*a*) in the introduction of *m*-CF_3 and *p*-Cl into amphetamine to give norfenfluramine (Beckett & Brookes, 1967) and *p*-chloroamphetamine (Beckett & Salmon, to be published) respectively, and (*b*) in the introduction of *p*-Cl and *p*-Br into the pheniramine molecule to give chlorpheniramine and brompheniramine (Beckett & Wilkinson, 1965; Kabasaklian, Taggart & Townley, 1968).

In the buccal absorption test (Beckett & Triggs, 1967; Brookes, 1968), the lipid solubilities of phentermine, mephentermine, amphetamine and methylamphetamine were virtually identical, while chlorphentermine, chloramphetamine and norfenfluramine were significantly more lipid soluble, even at an acidic pH. Hence the

reduced urinary excretion, and concomitant increase in elimination half life of chlorphentermine, and probably also chloramphetamine, is the result of the greater lipid solubility of the chloro-substituted compounds which enhances their renal tubular reabsorption and redistribution in the body.

Since the completion of this work, Jun & Triggs (1970) have reported an average elimination half life for chlorphentermine in man, of 41 h, after monitoring whole blood concentrations, and suggest that the drug also undergoes multi-compartment distribution.

Acknowledgements

We are indebted to our colleagues who acted as subjects for this investigation, to W. R. Warner & Co. Ltd., for the gifts of phentermine and chlorphentermine hydrochloride, and to J. Wyeth & Brother Ltd. for the gift of mephentermine sulphate.

REFERENCES

BECKETT, A. H. & BROOKES, L. G. (1967). *J. Pharm. Pharmac.*, **19**, 42S–49S.
BECKETT, A. H. & ROWLAND, M. (1965a). *Ibid.*, **17**, 628–639.
BECKETT, A. H. & ROWLAND, M. (1965b). *Ibid.*, **17**, 109S–114S.
BECKETT, A. H., ROWLAND, M. & TRIGGS, E. J. (1965). *Nature, Lond.*, **207**, 200–201.
BECKETT, A. H. & TRIGGS, E. J. (1967). *Ibid.*, **19**, 31S–41S.
BECKETT, A. H. & WILKINSON, G. R. (1965). *Ibid.*, **17**, 256–257.
BROOKES, L. G. (1968). Ph.D. Thesis (University of London).
DUBNICK, B., TOWNE, C. A., HARTIGAN, J. M. & PHILLIPS, G. E. (1968). *Biochem. Pharmac.*, **17**, 1243–1250.
JUN, H. W. & TRIGGS, E. J. (1970). *J. pharm. Sci.*, **59**, 306–309.
KABASAKLIAN, P., TAGGART, M. & TOWNLEY, E. (1968). *Ibid.*, **57**, 621–623.
OPITZ, K. & WEISCHER, M.-L. (1966). *Arzneimittel-Forsch*, **16**, 1311–1315.
STAHL, E. (1962). *Thin-Layer Chromatography*. Berlin: Verlag-Chemi.
STAHL, E., SCHRÖTER, G., KRAFT, G. & RENZ, R. (1956). *Pharmazie*, **11**, 633–637.
WALKENSTEIN, S. S., CHUMAKOW, N. & SEIFTER, J. (1955). *J. Pharmac. exp. Ther.*, **115**, 16–20.
WEISCHER, M.-L. & OPITZ, K. (1967). *Arzneimittel-Forsch.*, **17**, 625–627.
WICKSTRÖM, A. & SALVESEN, B. (1952). *J. Pharm. Pharmac.*, **4**, 631–635.

J. Pharm. Pharmac., 1971, 23, 295.

LETTERS TO THE EDITOR

Blocking of reticuloendothelial cells by dextran

Introduction of an allyl group into the dextran molecule enables a radioactive iodine isotope to be added at the double bond (Brooks, Davies & others 1970; Ricketts, 1966). This labelled dextran is convenient for experiments *in vivo* on the permeability of membranes and has been used in an investigation of the permeability of the renal glomerulus (Hardwicke, Hulme & others, 1968).

In a preliminary experiment with rabbits, an intravenous injection of [^{125}I] dextran was found to disappear from the circulation more slowly when diluted with 6% dextran 110 (B.P. 1963, Addendum, 1966) than with saline. To investigate this phenomenon, random bred rabbits, 3–4 g, of either sex, were given NaI in the drinking water (100 mg/litre) before and during experiments to ensure that the radioactive label was not taken up by the thyroid gland. Injections of [^{125}I]dextran were given through a marginal ear vein and blood samples (about 2 ml) were taken from the opposite ear. Radio-activity was measured on lithium heparin plasma samples. Results are expressed as percentages of the radioactivity in the plasma at zero time; this value being obtained by extrapolation of the values at 10 and 20 min after injection.

To exclude the possibility that the original observation was attributable to biological variation, an experiment of "cross-over" was made in which one rabbit received 12 μCi [^{125}I]dextran (specific activity 0·8mCi/g) in 10 ml of saline and a second rabbit received the same dose of [^{125}I]dextran in 10 ml of 6% dextran 110. Seven days later the experiment was repeated in the same animals with the doses interchanged. Fig. 1A shows that there is little variation between rabbits and that the slower disappearance of radioactivity is associated with the dextran injection.

In a second group of 4 rabbits, given various amounts of 6% dextran 110, the [^{125}I]dextran left the plasma more slowly as the dextran dose increased (Fig. 1B). The [^{125}I]dextran, in all experiments, left the circulation more rapidly than dextran determined chemically after injection of 30 ml of 6% solution, shown by the dotted line in Fig. 1B. The molecular size distribution of the [^{125}I]dextran was known, from gel filtration on columns of Sephadex G-200, to be the same as that of the dextran 110 injected.

At this stage it seemed possible that the observed effect might be due to partial blocking of cells of the reticulo-endothelial system by the dextran. Experiments with mice were therefore designed to test the hypothesis that [^{125}I]dextran is taken up into cells of the reticulo-endothelial system and that this uptake can be influenced by the injection of dextran.

12 mice, 20–30 g, of either sex, were given NaI in the drinking water (50 mg/litre) before and during experiment. Each received an injection of 0·5 ml of saline, containing 0·2 μCi [^{125}I]dextran, through a tail vein, each was anaesthetized with ether, then a cardiac blood sample taken, finally the animals were killed at various times after injection. The urinary bladder was removed and discarded, the liver, spleen, remaining carcase and the blood sample were then separately assayed for radioactivity. The amounts of radioactivity taken up into the cells of the liver and spleen were obtained by subtracting the activity due to the blood in each organ from their measured activities. Standard values for the volumes of blood per gram of liver and spleen were obtained from the Handbook of Circulation (1959) and appropriate corrections made for radioactivity of included blood. The experiment was repeated with injections of 0·18, 1·8 or 6% dextran 110 in place of saline.

The percentage of the injected radioactivity found in the spleen in these experiments was always less than 1%. In four mice selected at random from the saline group, the

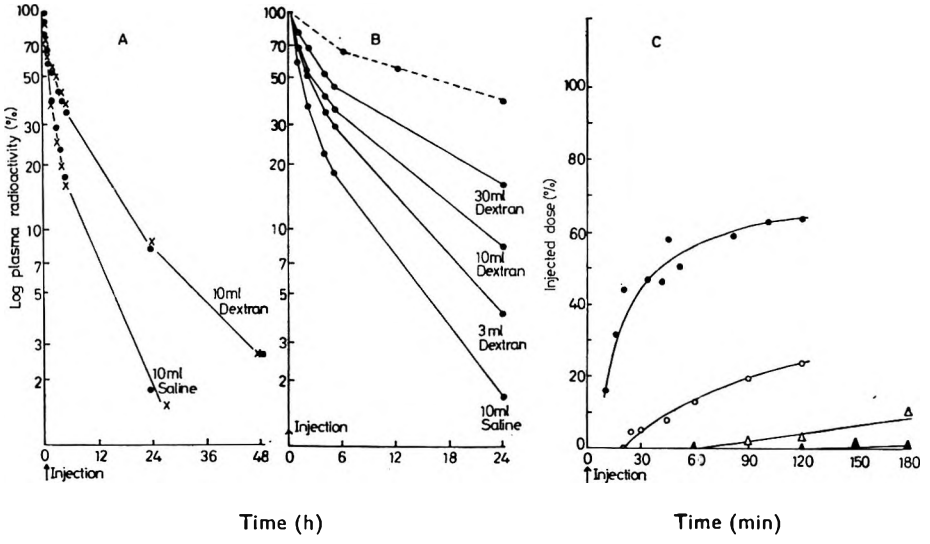


FIG. 1A. Disappearance of radioactivity from the plasma of rabbits given intravenous injections of [I^{125}]dextran. Values are expressed as percentage of the plasma activity at zero time. Rabbit I (×) received (1) [^{125}I]dextran in saline. (2) [^{125}I]dextran in 6% dextran 110 solution. Rabbit II (●) received the same injections in reverse order.

B. Disappearance of radioactivity from the plasma of rabbits given intravenous injections of [^{125}I]dextran diluted with saline or with various volumes of 6% dextran 110. The broken line shows the chemical determination of dextran in plasma after an injection of 30 ml of 6% dextran 110 solutions.

C. Uptake into the liver of [^{125}I]dextran in mice killed at various intervals after intravenous injection. The [^{125}I]dextran was diluted with saline (●), 0·18% dextran (○), 1·8% dextran (△) and 6% dextran (▲).

kidneys and lungs also contained less than 1% of the injected dose. It appears that the liver is mainly responsible for uptake of [^{125}I]dextran. Fig. 1C shows how this uptake is influenced by the inclusion of dextran 110 in the injected dose. When no dextran is present (upper curve), the liver contains some radioactivity 10 min after injection and uptake increases rapidly with time. Comparing this with the other curves in Fig. 1C, it appears that the presence of increasing amounts of dextran in the injected dose delays uptake of [^{125}I]dextran by the liver and reduces the total amount taken up.

These results support the view that dextran partially blocks the reticulo-endothelial cells of the liver responsible for the uptake of [^{125}I]dextran so that more of the labelled material is left in the circulation, as was found in the experiments with rabbits.

The presence of dextran in reticulo-endothelial cells has long been known, but these measurements of the blocking effect by graded doses *in vivo* illustrate the way in which dextran infusions may alter the uptake of bacterial antigens or therapeutic substances by reticulo-endothelial cells.

M.R.C. Industrial Burns Unit,
Birmingham Accident Hospital,
Bath Row,
Birmingham 15, *U.K.*

S. P. FARROW
C. R. RICKETTS

January 7, 1971

REFERENCES

British Pharmacopoeia (1963). *Addendum* (1966). London: Pharmaceutical Press.

BROOKS, S. A., DAVIES, J. W. L., GRABER, I. G. & RICKETTS, C. R. (1960) *Nature, Lond.*, **188**, 675–676.

Handbook of Circulation (1959). Editors: Dittmer, D. S. & Grebe, R. M. Philadelphia and London: W. B. Saunders Co.

HARDWICKE, J., HULME, B., JONES, J. H. & RICKETTS, C. R. (1968). *Clin. Sci.*, **34**, 505–514.

RICKETTS, C. R. (1966). *Nature, Lond.*, **210**, 1113–1115.

Screens for anti-inflammatory drugs

It has recently been shown by Willis (1969), Di Rosa, Giroud & Willoughby (1971), Di Rosa, Papadimitriou & Willoughby (1971) that the oedema induced in rats feet by injection of carrageenan is mediated by histamine and 5-hydroxytryptamine (5-HT) during the first hour, after which the increased vascular permeability is maintained by kinin release up to $2\frac{1}{2}$ h. From $2\frac{1}{2}$–6 h the mediator appears to be a prostaglandin, release of which is closely associated with migration of leucocytes into the inflamed site. All the mediators appear to be dependent upon an intact complement system for their activation and release (Giroud & Willoughby, 1970). Examination of the non-steroidal anti-inflammatory drugs on this model has shown that they suppress mainly the last phase of the response, namely the "prostaglandin phase." Their ability to suppress this phase correlates directly with their ability to suppress mononuclear leucocyte migration into the inflamed tissues. Thus the oedema and its suppression during the period $2\frac{1}{2}$–6 h after injection of carrageenan serves as an index of leucocyte migration. It has been suggested that this explains why the model of acute inflammation can successfully be employed in the search for new non-steroidal anti-inflammatory agents (Di Rosa, Papadimitriou & Willoughby, 1971).

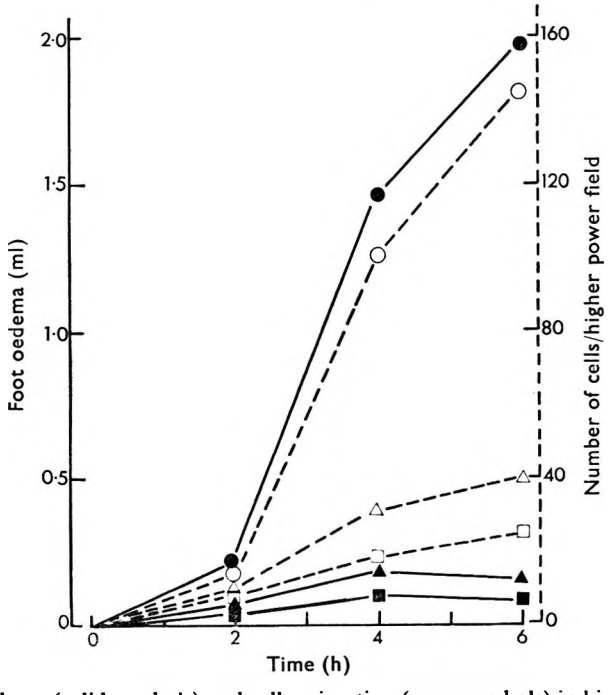


FIG. 1. Foot oedema (solid symbols) and cell emigration (open symbols) in histamine, 5-HT and kininogen-depleted rats after subcutaneous injection (0·1 ml) into the foot of either 1% carrageenan (circles), 6% dextran (triangles) or 1% formalin (squares).

In the present study rats were depleted of their tissue stores of histamine and 5-HT with compound 48/80 (Di Rosa, Giroud & Willoughby, 1971) and of kininogen with cellulose sulphate (Di Rosa, Papadimitriou & Willoughby, 1971). These depleted animals then received injections of various irritants into the sub-plantar aspect of the hind paw and the foot swelling was measured at 2, 4 and 6 h after the injection as previously described (Di Rosa, Giroud & Willoughby, 1971). In similarly treated rats cell counts were made of the total numbers of leucocytes migrating into the inflamed site.

Fig. 1 shows that 0·1 ml of 1% carrageenan provoked a good oedema despite the depletion of the earlier mediators (histamine, 5-HT and kininogen), the oedema is closely paralleled by the migration of leucocytes into the inflamed site.

In contrast, the oedema provoked by 0·1 ml of 6% dextran or 0·1 ml of 1% formalin failed to elicit either a good increase of vascular permeability or leucocyte migration. This could be interpreted as providing further support for the concept of the interrelation of leucocyte migration and activation of prostaglandins during this phase of the inflammatory response.

It has previously been shown by Winter, Risley & Nuss (1962) that suppression of 4 h carrageenan oedema in the rat paw correlates well with potential therapeutic activity of anti-inflammatory agents. It is suggested that the failure of formalin and dextran to provoke a leucocyte response indicates a failure of these injurious stimuli to serve as useful models in the search for new anti-inflammatory agents. On the other hand used with care such models can provide an indication of potential antihistamine, anti-5HT or antikinin activity.

In looking for new *in vivo* screens for non-steroidal anti-inflammatory agents it is proposed that a necessary prerequisite of the injurious stimulus should be its ability to provoke a migration of leucocytes preferably of the mononuclear type which in turn will lead to activation of the so-called prostaglandin mediated phase.

The authors would like to thank the Wellcome Trust for generous support of this work and one of us (M. Di R.) was in receipt of a Royal Society Fellowship and a N.A.T.O. Fellowship.

Department of Experimental Pathology,
St. Bartholomew's Hospital
Medical College,
*London E.C.*1, *U.K.*

M. Di Rosa*
D. A. Willoughby

December 31, 1970

REFERENCES

Di Rosa, M., Giroud, J. P. & Willoughby, D. A. (1971). *J. Path.* In the press.
Di Rosa, M., Papadimitriou, J. M. & Willoughby, D. A. (1971). *Ibid.* In the press.
Giroud, J. P. & Willoughby, D. A. (1970). *Ibid.*, **101**, 241.
Willis, A. L. (1969). *J. Pharmac. Pharmac.*, **21**, 126–128.
Winter, C. A., Risley, E. A. & Nuss, G. V. (1962). *Proc. Soc. exp. Biol. Med.*, **111**, 544–547.

* Present address: Institute of Pharmacology, University of Naples.

The influence of hypothermia on chlorpromazine-induced metabolic changes in mouse heart and brain

Disturbances in cardiac rhythm and metabolism produced by chlorpromazine have been noted in the whole animal (Huston & Bell, 1966), and in the isolated heart (Prindle, Gold & others, 1970) and Alexander & Nino (1969) were sometimes able to relate these disturbances with changes in mitochondrial structure. Chlorpromazine also decreases the incorporation of isotopically-labelled glucose into brain protein (Skinner & Spector, 1968). Furthermore, chlorpromazine is known to interfere with the regulation of body temperature (Lessin & Parkes, 1957). In view of these findings, together with the relevance of establishing the cardiac effects of chlorpromazine, we have compared the effects of chlorpromazine on total [^{14}C]glucose uptake and incorporation into the protein of heart and brain of mice; the significance of chlorpromazine hypothermia in explaining these results has also been investigated.

The experiments were done at 21° and at 38°, rectal temperatures were recorded at 10 min intervals with a thermistor probe. The experiments were made at the same time each day.

Adult female SAS/ICI albino mice, 25–40 g, had, by intraperitoneal injection, 0·9% saline, 5 ml/kg, or chlorpromazine, 20 mg/kg. Thirty min after the first injection the mice were given 5μCi[U-^{14}C]-D-glucose in 0·1 ml saline (3 mCi/mmol Radiochemical Centre, Amersham) intraperitoneally. The animals were killed by cervical dislocation 30 min after the isotope injection and the heart and cerebral hemispheres rapidly removed, washed in ice-cold saline for 2 min, blotted dry and weighed. The tissues were homogenized in 3 ml of ice-cold 10% trichloroacetic acid and then chemically fractionated (Vrba, Gaitonde & Richter, 1962).

The protein precipitate was thrice washed in trichloroacetic acid and the supernatant retained. Lipids were extracted with three 2 ml volumes of acetone. The residual precipitate was hydrolysed with 1·5 ml of 0·3M potassium hydroxide at 37° for at least 24 h and the final solution was cleared with 1·5 ml of M Hyamine-10-X in methanol. The radioactivity of the samples was measured in a Beckmann automatic liquid scintillation counter. Total uptake was expressed as counts min^{-1} mg^{-1} and incorporation into protein as a percentage of the total uptake. The results for the cerebral hemispheres were examined by an analysis of variance based on that described by Snedecor (1962) for hierarchal classifications. The results for the hearts were analysed by Students *t*-test; where variances were incompatible, a modified *t*-test was used. From the analyses it was concluded that the errors introduced by the experimental technique were less than 5%. The percentage recovery from crude homogenate in seven experiments was 97·3 $\pm$ 5·44% (mean $\pm$s.e.). Further extraction of lipid with ethanol–ethyl ether (3 : 1 v/v); chloroform–methanol (2 : 1 v/v) and ether showed that acetone failed to extract 6·3% (mean of 4) and 13·1% (mean of 7) of the total lipid from heart and brain respectively.

A dose of chlorpromazine, 20 mg/kg, reduced the mean body temperature from 37·4° to 29·2° (n = 16). In the experiment in which body temperature was maintained the mean temperatures of the test and control animals were 37·38° (n = 70) and 37·83° (n = 70) respectively.

The total uptake and percentage incorporation of ^{14}C into protein for hypothermic and normothermic mice are shown in Table 1. There was no significant change in total uptake or incorporation into protein of heart tissue after administration of chlorpromazine in either hypothermic or normothermic mice. However, a significant reduction in the percentage incorporation into cerebral protein ($P < 0{\cdot}001$) was demonstrated in hypothermic chlorpromazine-treated mice. It is therefore possible

Table 1. *The effect of chlorpromazine on the uptake of radioactivity into mouse heart and brain following administration of* [*U*-^{14}*C*]-D-*glucose*. Results are expressed as counts min^{-1} mg^{-1} wet weight and as percentage incorporation of radioactivity into protein.

	Hypothermic				Normothermic			
	Cpz	Control	Difference ±95% limits	*P*	Cpz	Control	Difference ±95% limits	*P*
	Cerebral hemispheres							
Total uptake counts mg^{-1} mg^{-1}	463	563	100 ± 193	N.S.	737	791	54 ± 38	N.S.
% incorporation into protein	1·74	3·44	1·69 ± 0·92	0·001	2·90	3·47	0·57 ± 0·83	N.S.
	Hearts							
Total uptake counts min^{-1} mg^{-1}	259	364	105 ± 107	N.S.	223	341	118 ± 141	N.S.
% incorporation into protein	4·39	4·04	0·35 ± 4·01	N.S.	7·40	5·65	1·74 ± 2·35	N.S.

Cpz = Chlorpromazine-treated animals
N.S. = Non-significant at $P = 0.05$

that there is a relation between the decreased incorporation of radioactivity into brain protein and the fall in body temperature induced by chlorpromazine. Thus the effect of chlorpromazine in decreasing the rate of cerebral protein synthesis may not be a primary effect on protein synthesizing mechanisms but could be secondary to the hypothermia induced by the drug. The results in Table 1 demonstrate that the inhibition of ^{14}C incorporation into cerebral protein by chlorpromazine is, to a large degree, dependent upon the hypothermia induced by this drug, although the total glucose uptake is not significantly affected. In the normothermic chlorpromazine-treated mice no significant decrease in the incorporation of ^{14}C into cerebral protein over the corresponding controls was noted. Similar results have been reported concerning the inhibition of brain protein synthesis from labelled amino-acids by chlorpromazine (Shuster & Hannam, 1964). Furthermore, it has been demonstrated that chlorpromazine does not inhibit the cerebral synthesis of amino-acids from glucose carbon in rats whose body temperature was maintained (Bachelard, Gaitonde & Vrba, 1966), whereas in hypothermic animals there is a decreased incorporation into α-keto-acids and α-amino-acids (Bachelard & Lindsay, 1966). It is important to note however that chlorpromazine has been shown to inhibit protein synthesis in rat brain slices maintained *in vitro* at 37° (Lindan, Quastel & Sved, 1957).

The absence of an inhibitory effect on cardiac protein synthesis by chlorpromazine is interesting because it may represent a difference in specificity of drug action-between these two excitable tissues. This is consistent with observations made on rats in which there is no effect of chlorpromazine on amino-acid synthesis from glucose by the hearts of temperature-maintained animals (Bachelard & others, 1966).

We thank Mr. F. House, Guy's Hospital Medical School for statistical advice. P. L. M. is an M.R.C. research scholar. H. J. R. is Governors' research scholar, Guy's Hospital.

Department of Pharmacology,
Guy's Hospital Medical School,
*London, S.E.*1, *U.K.*

P. L. MASON
H. J. ROGERS

December 3, 1970

REFERENCES

ALEXANDER, C. S. & NINO, A. (1969). *Am. Heart J.*, **78**, 757–769.
BACHELARD, H. S., GAITONDE, M. K. & VRBA, R. (1966). *Biochem. Pharmac.*, **15**, 1039–1043.
BACHELARD, H. S. & LINDSAY, J. R. (1966). *Ibid.*, **15**, 1053–1058.
HUSTON, J. R. & BELL, G. E. (1966). *J. Am. med. Ass.*, **198**, 134–138.
LESSIN, A. W. & PARKES, M. W. (1957). *Ibid.*, **12**, 245–250.
LINDAN, O., QUASTEL, J. H. & SVED, S. (1957). *Can. J. Biochem. Physiol.*, **35**, 1145–1150.
PRINDLE, K. H., GOLD, H. K., CARDON, P. V. & EPSTEIN, S. E. (1970). *J. Pharmac. exp. Ther.*, **173**, 133–137.
SHUSTER, L. & HANNAM, R. V. (1964). *J. biol. Chem.*, **239**, 3401–3406.
SKINNER, A. & SPECTOR, R. G. (1968). *Br. J. Pharmac. Chemother.*, **33**, 129–135.
SNEDECOR, G. W. (1962). *Statistical methods.* Iowa: University Press.
VRBA, R., GAITONDE, M. K. & RICHTER, D. J. (1962). *Neurochem.*, **9**, 465–475.

Effects of small doses of haloperidol on timing behaviour

The depressive effects of drugs of the phenothiazine and butyrophenone group are thought to be connected with the inability to block post-synaptic receptors in the central catecholamine neurons (see Andén, Carlsson & Häggendal 1969). Clinically, small doses of some phenothiazines—especially those with a propylpiperazine side chain—and some butyrophenones, e.g. triperidol, appear to have certain stimulating properties (Di Mascio, Havens & others 1961, Lingjaerde 1966). We have observed, and others have reported (Janssen, 1962; Monti & Hance, 1967) the butyrophenone derivative haloperidol to have some stimulant action in small doses.

Four male Sprague-Dawley rats were food-deprived and kept at 80% of their free-feeding weight (278 ± 6 g). The rats were trained to press a lever in standard behavioural chambers (Model E3125A, Grason-Stadler) to get food pellets (Noyes, 45 mg) on a DRL-20 schedule (Differential Reinforcement of Low rates), whereby a depression of the lever produced the pellet only if it followed the preceeding lever depression by at least 20 s. Every premature lever press (<20 s after the last response) starts the interval again.

The Inter-Response Times (IRT, interval between successive responses) were divided in 3 s categories: 0–2, 3–5, etc. Presses spaced more than 30 s apart were collected in a last category. Leverpress responses were recorded on digital counters and categorized automatically. For each session a mean IRT was calculated. The distribution was symmetrically cut (below 9–12 and above 27–30) around the optimal reinforced IRT (18–21), to avoid an open interval. A grand mean for the control and the different treatments was calculated and a 98% confidence interval determined for the differences between the means (Scheffé, 1959).

Each rat was exposed to daily sessions for 21–22 consecutive days. Experimental sessions were separated by two control sessions. The complete sessions consisted of 15 min adaptation, immediately followed by 60 min, in which responses were recorded. 15 min before the start of the experimental sessions, animals were injected with haloperidol 0·01, 0·02 or 0·03 mg/kg: each dose was tested twice on each rat. No injections were made before any of the control sessions. Drugs were freshly prepared and injected intraperitoneally in a volume of 2 ml/kg.

The effects of the doses of haloperidol on the behaviour variable used are shown in Table 1. After injection of 0·02 mg/kg of haloperidol the IRT distributions showed a statistically significant ($P < 0·02$) increase in the frequency of short IRTs compared to those in the control distributions. That is, the animals pressed the lever more frequently before the required interval had elapsed. The injections of the other doses of haloperidol, 0·01 and 0·03 mg/kg did not significantly alter the IRT distributions. As assessed by gross observation all the animals displayed a normal behaviour.

Table 1. *The effects of different doses of haloperidol on DRL-20* behaviour.* The IRT (Inter-Response Time)-values are grand means of the total IRT means for each treatment. The lower part of the Table gives a 98% confidence interval for the differences between the control value and each of the three haloperidol doses. The difference between controls and 0·02 mg/kg haloperidol is significantly different from zero.

Treatment		IRT ± s.d.	N	
I	Control	19·9 ± 0·8	61	
II	Haloperidol 0·01 mg/kg ..	19·5 ± 0·7	8	
III	Haloperidol 0·02 mg/kg ..	19·4 ± 0·9	8	
IV	Haloperidol 0·03 mg/kg	19·7 ± 0·7	8	
I–II	0·4 ± 0·5			NS
I–III	0·5 ± 0·5			$P < 0·02$
I–IV	0·2 ± 0·5			NS

* Differential reinforcement of low rates (20s)

Thus haloperidol in a narrow dose interval, <0·03 and >0·01 mg/kg, shortened the IRTs. Amphetamine induces a similar effect on timing behaviour, although more pronounced (Sidman, 1955). Amphetamine is thought to exert its stimulant effect through a release of newly synthesized transmitters (*see* Carlsson, 1970). The postsynaptic receptor blockade induced by haloperidol increases the catecholamine turnover, possibly through a negative feed-back mechanism increasing the physiological release of transmitters onto the receptors (see Andén, Carlsson & others 1969). A possible explanation of the observed stimulant effect seen after haloperidol in a dose of 0·02 mg/kg might lie in an increased physiological release of transmitters, which overcame the blockade of the receptors. When the dose of haloperidol was increased the receptor-blockade dominated. It has also been observed in this laboratory that a dose of 0·1 mg/kg haloperidol disrupts the DRL-20 behaviour.

This work was supported by grants from the Swedish Medical Research Council (B71-14X-155-07C), the Faculty of Social Sciences and the Faculty of Medicine, University of Göteborg. The valuable technical assistance of Mrs. Ulla Axelsson is gratefully acknowledged.

Department of Pharmacology,
University of Göteborg,
Göteborg, Sweden.

S. Ahlenius
J. Engel

December 22, 1970

REFERENCES

Andén, N.-E., Carlsson, A. & Häggendal, J. (1969). *Ann. Rev. Pharmac.*, **9**, 119–134.

Carlsson, A. (1970). In *Amphetamines and related compounds*, pp. 289–300, Editors: Costa, E. & Garattini, S., New York: Raven Press.

Di Mascio, A. Havens, L. L. & Snell, J. E. (1961). *Recent advances in biological psychiatry*, pp. 68–76, Editor: Wortis, J., New York: Grune and Straton Inc.

Janssen, P. (1962). *Encéphale*, **51**, 582–601.

Lingjaerde, O. (1966). *Psykofarmaka*, Oslo: J. G. Tanum Forlag.

Monti, J. M. & Hance, A. J. (1967). *Psychopharmacologia*, **12**, 34–43.

Scheffé, H. (1959). *The analysis of variance*, New York: John Wiley & Son.

Sidman, M. (1955). *Science, N.Y.*, **122**, 925.

Role of L-ascorbic acid in the reversal of the monoamine oxidase inhibition by caffeine

A possible role of ascorbic acid in protecting the central nervous system from the toxic effects of endogenous psychotomimetic compounds was discussed by Galzigna, (1970) and by Galzigna & Rizzoli (1970). Also, ascorbic acid deficiency has been indicated as one of the more important symptoms of an altered mental behaviour (Pauling, 1968). We now report the results of a study on the inhibitory effect of caffeine on rat liver mitochondrial monoamine oxidase (MAO) and its reversal by L-ascorbic acid.

The oxidation of catecholamines was followed by measuring the oxygen uptake with a Clark electrode connected via a voltage divider to a Sargent RE recorder. The assay mixture contained in a total volume of 2 ml 170 μmol of Na phosphate buffer pH 7·0, 1·7 μmol of KCN and about 10 mg of mitochondrial protein. The addition of substrates and inhibitors was made by Hamilton syringes and a correction for the volume was introduced after each addition. Mitochondria were obtained from rat liver (Schneider & Hogeboom, 1951) and the protein content measured (Gornall, Bardawill & David, 1949).

The apparent dissociation constants with rat liver MAO for different substrates (all $\times 10^{-3}$M; n = 6) were: tyramine 0·3, 5-HT 1·0, dopamine 1·1, adrenaline 10·0. In each case the proportionality of the reaction velocity and enzyme concentration was verified.

Ascorbic acid (8×10^{-4}M) increased the initial velocity of oxidation with all substrates (1×10^{-4}M), without being itself oxidized, the initial velocity without acid and with acid measured as n At. O_2 min^{-1} mg^{-1} protein being respectively: tyramine 2·6, 3·9; 5-HT 2·0, 3·04; dopamine 2·3, 4·9; adrenaline 0·7, 2·1; noradrenaline 0, 0·5; histamine 0,0. Noradrenaline, which was not utilized under normal conditions, was oxidized when ascorbic acid was present in the incubation mixture. In the presence of 2×10^{-3}M ascorbic acid the apparent dissociation constant for noradrenaline was $2{\cdot}5 \times 10^{-3}$M.

Caffeine appeared to act as a competitive inhibitor of MAO activity with all the tested substrates and a 1/v *versus* caffeine plot with two tyramine concentrations ($S_1 = 1 \times 10^{-4}$M and $S_2 = 4 \times 10^{-4}$M) yielded an apparent K_i value of $1{\cdot}5 \times 10^{-3}$ according to the Dixon & Webb method (1960).

Uric acid behaved similarly to caffeine and an inhibition was verified with well-known MAO inhibitors such as ephedrine and iproniazid. Ascorbic acid reversed the inhibition by caffeine and also the effect of the other inhibitors tested. Fig. 1 shows a typical experiment.

The experiments were repeated with mitochondria isolated from the brain of the monkey (*Cercopitecus aetiops*) with the sucrose gradient technique of Marchbanks (1968) and the results paralleled those obtained with rat liver mitochondria. The possibility of a binding of labelled L-ascorbic[1-^{14}C]acid to the mitochondrial membrane in the presence of MAO substrates was studied parallel to the oxygraph experiments as a function of substrate concentration. The results of the L-ascorbic [1-^{14}C]acid binding and the stoichiometry between the amount of ascorbic acid bound and the extra amount of substrate oxidized in the presence of ascorbic acid are reported in Table 1. The inhibitory effect of caffeine on the binding is evident.

Chemical interaction between caffeine and ascorbic acid was tested in the partially hydrophobic solvent tris-dioxane (1:10) described previously by Galzigna (1970). A marked hypochromic effect induced by ascorbic acid on the 275 nm absorption band of caffeine was found which is consistent with a donor-acceptor type interaction

(Galzigna, 1969). An affinity constant of ascorbic acid for caffeine was determined spectrophotometrically (Galzigna & Rizzoli, 1970) and turned out to be 2×10^{3}M.

Burton (1951) recognized caffeine as an amino-acid oxidase inhibitor, and explained the inhibition in terms of an association of caffeine with the flavine coenzyme of the

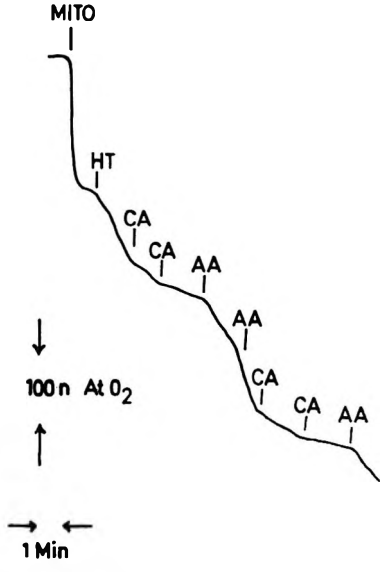


FIG. 1. Oxygraph tracing with MAO activity, caffeine inhibition and ascorbic acid reversal. 5-HT 1×10^{-3}M was used as a substrate and its addition is indicated with MT. Caffeine 5×10^{-5}M indicated as CA and ascorbic acid indicated as AA were added where shown. Protein was 10·2 mg.

Table 1. *Binding of* L-*ascorbic* [1-^{14}C]*acid and stoichiometry between ascorbic acid bound and the extra amount of substrate oxidized in the presence of ascorbic acid.* Rat liver mitochondria were incubated at 22° in conditions similar to those used for the oxygraph experiments in the presence of L-ascorbic [1-^{14}C]acid. The purity of the sample was checked by t.l.c. in an amount corresponding to about 10 000 counts/min. After 10 min incubation the mitochondria were spun down at 6000 *g* for 15 min and 0·25 ml taken from the supernatant and transferred to 10 ml of the scintillation liquid (7 g of PPO, 0·6 g of DMPOPOP and 150 g of naphthalene in 1000 ml of dioxane). The residual radioactivity was then measured with a Packard liquid scintillation spectrometer (N.3320). Ascorbic acid average values from four experiments are reported.

Substrates	L-Ascorbic[1-^{14}C]acid bound (nmol min^{-1} mg^{-1})	Extra amount of substrate oxidized* (nmol min^{-1} mg^{-1})
Ascorbic acid	0	—
Ascorbic acid + caffeine	0	0
„ „ + tyramine (1 : 1)	9·1	8·7
„ „ + „ (1 : 2)	12·7	11·5
„ „ + „ (1 : 5)	17·5	16·8
„ „ + „ (1 : 5) + caffeine	0	0
„ „ + 5-HT (1 : 1)	12	12·2
„ „ + „ (1 : 2)	16	15·3
„ „ + „ (1 : 5)	21	22·4
„ „ + „ (1 : 5) + caffeine	0	0

* Calculated from parallel MAO activity measurements.

oxidase. The same author gave the affinity constant of caffeine for flavine as $0{\cdot}1 \times 10^{3}$M. MAO has been recognized as a flavine bound enzyme (Erwin & Hellerman, 1967; Tipton, 1968) and therefore its inhibition by caffeine can be explained. In fact caffeine has been shown to be able to interact with aromatic electron donors by acting as an electron acceptor (Hanna & Sandoval, 1968) and a reduced flavine is known to be formed as a first step in the reaction sequence which constitutes the overall MAO activity:

$$R\text{-}CH_2NH_2 + \tfrac{1}{2}O_2 + \text{Flavine} \longrightarrow R\text{-}CH{=}NH + \text{Flavine } H_2$$
$$\text{Flavine } H_2 + O_2 \longrightarrow \text{Flavine} + H_2O_2.$$

Caffeine might compete with the oxygen for the reduced flavine, and ascorbic acid might reverse this effect by interacting with caffeine. In fact the affinity of ascorbic acid for caffeine is 20 times higher than the affinity of caffeine for flavine and 3 times higher than the affinity of caffeine for the mitochondrial MAO. The activating effect of ascorbic acid cannot be explained on the basis of a stimulation of the catalase system, since its action is not mimicked by reduced dichlorophenolindophenol or by uric acid which are also substrates for the catalase activity.

The binding of the labelled ascorbic acid in the presence of MAO substrates, its inhibition by caffeine and the stoichiometry between ascorbic acid bound and extra amount of substrate oxidized point to an effect of ascorbic acid as a true co-substrate of MAO or to its involvement in an allosteric regulation of the enzyme.

These results confirm that ascorbic acid must influence catecholamine metabolism at large and support its function in maintaining the normal metabolism of tyrosine (Knox, 1955). At molecular level ascorbic acid might influence the control mechanism involved in the binding and release of substrates from MAO (Hellerman & Erwin, 1968) and possibly protect MAO from the effects of a number of endogenous and exogenous toxic compounds which, by altering MAO activity, could upset (i.e. stimulate or depress) the normal functioning of the brain.

The financial support of Professor Linus Pauling is gratefully acknowledged.

Department of Medical Biochemistry,
University of Nairobi,
P.O. Box 30197, *Nairboi, Kenya.*

LAURO GALZIGNA
GODFREY MAINA
GIDEON RUMNEY

December 18, 1970

REFERENCES

BURTON, K. (1951). *Biochem. J.*, **48**, 458–467.
DIXON, M. & WEBB, E. C. (1970). *Enzymes*, p. 22. Academic Press: New York.
ERWIN, V. G. & HELLERMAN, L. (1967). *J. biol. Chem.*, **242**, 4230–4238.
GALZIGNA, L. (1969). *Biochem. Pharmac.*, **18**, 2485.
GALZIGNA, L. (1970). *Nature, Lond.*, **225**, 1058–1059.
GALZIGNA, L. & RIZZOLI, A. A. (1970). *Clinica chim. Acta*, **30**, 5.
GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1950). *J. biol. Chem.*, **177**, 751–766.
HANNA, M. W. & SANDOVAL, A. (1968). *Biochim. Biophys. Acta*, **155**, 433–436.
HELLERMAN, L. & ERWIN, V. G. (1968). *J. biol. Chem.*, **243**, 5234–5243.
KNOX, W. E. (1955). *A Symposium on amino acid metabolism.* Editors: McElroy, W. D. & Glass, H. B. Johns Hopkins Press: Baltimore.
MARCHBANKS, R. M. (1968). *Biochem. J.*, **110**, 533–541.
PAULING, L. (1968). *Science, N.Y.*, **160**, 265–271.
SCHNEIDER, W. C. & HOGEBOOM, G. H. (1950). *J. biol. Chem.*, **183**, 123–128.
TIPTON, K. F. (1968). *Biochim. Biophys. Acta*, **159**, 451–459.

The significance of the relative potencies of noradrenaline and α-methylnoradrenaline for the mode of action of α-methyldopa

It is commonly agreed that the hypotensive action of α-methyldopa is mediated through an interference with the sympathetic system, but the site of action has not been established. Opinions differ on the function of the peripheral sympathetic nerves after treatment with α-methyldopa (Muscholl, 1966, Holtz & Palm, 1967; Sourkes & Rodriguez, 1967; Stone & Porter, 1967; Kopin, 1968). It has been proposed that the amines (α-methylnoradrenaline and α-methyldopamine) formed on metabolism of α-methyldopa take over the function of the normal transmitter noradrenaline in the brain (Carlsson & Lindqvist, 1962) and in the peripheral sympathetic nerves (Day & Rand, 1963; 1964).

The observation that α-methylnoradrenaline was less potent as a pressor agent than noradrenaline (Day & Rand, 1964) led to the hypothesis that the formation of such less active amines from α-methyldopa may act as "false transmitters". This theory was challenged by the experiments of Henning & van Zwieten (1968) and Henning (1969a, b). Moreover, Brunner, Hedwall & others (1967) failed to find any hypotensive effect with α-methylnoradrenaline, even though the heart was depleted of noradrenaline by 50%, without a concomitant depletion in the brain. Furthermore, adrenergic neuronal function was only slightly impaired at the time of maximal fall in blood pressure (Henning & Svensson, 1968).

I have compared the potency of noradrenaline with that of α-methylnoradrenaline on blood pressure and heart rate of rabbits and rats, and both these parameters and also myocardial contractility and cardiac output in dogs.

Dogs of either sex (10 to 15 kg) were anaesthetized with pentobarbitone (35 mg/kg) and artificially ventilated with oxygen from a positive pressure respirator through a cuffed endotracheal tube. Arterial blood pressure was recorded by a Statham P23 Db pressure transducer and the heart rate by means of a cardiotachometer triggered by the electrical activity of the QRS complex of the electrocardiograph. The force of cardiac contraction was measured using a Walton-Brodie strain gauge arch (Boniface, Brodie & Walton, 1953) sutured to the right ventricle. Cardiac output was obtained by placing a well-fitting probe for a square wave electromagnetic flow meter (Carolina Medical Electronics) around the ascending aortic arch. Zero flow was obtained by clamping the aorta proximally. All the above-named parameters were recorded on a multi-channel Sanborn thermal recorder.

Albino rats (180 to 220 g) were pithed by the method of Shipley & Tilden (1947) and ventilated by a small animal respirator. Mean arterial pressure was recorded from a carotid artery by a Statham pressure transducer coupled to an Offner Dynograph penrecorder.

Rabbits weighing 2 to 3 kg were anaesthetized with 35 mg/kg of pentobarbitone. Systemic pressure was recorded as outlined for rats. Drugs (−)-noradrenaline bitartrate and (−)-α-methylnoradrenaline were diluted in normal saline and injected intravenously. Standard error of the mean (s.e.) and paired *t*-tests were used to analyse the results.

In dogs, noradrenaline and α-methylnoradrenaline at doses of 0·3, 0·6 and 1·2 μg/kg were equi-active in increasing blood pressure, force of cardiac contraction, cardiac output and in decreasing the heart rate reflexly. Table 1 gives a summary of these data.

Pressor responses to 10, 20, 30 and 40 ng of noradrenaline and α-methylnoradrenaline were equipotent on the blood pressure of pithed rats. This is shown in Table 2. In rabbits α-methylnoradrenaline (0·5, 1·0 and 2 μg/kg) appeared to be less potent than

noradrenaline, although it was only unequivocal at 0·5 and 2 μg/kg ($P < 0{\cdot}01$ and $<0{\cdot}05$ respectively).

The comparison of the potencies of α-methylnordrenaline and noradrenaline was determined by taking the responses to a number of doses of each amine. The results are shown in Table 3. In all the preparations tested, α-methylnoradrenaline and noradrenaline were equiactive with the exception of blood pressure responses in the rabbit where α-methylnoradrenaline was only marginally less potent.

Table 1. *Comparison of the cardiovascular effects of noradrenaline with α-methylnoradrenaline in dogs* (n = 9).

Dose	Drug	Blood pressure (mm Hg)	Contractility (g force)	Heart rate (beats/min)	Cardiac output ml/min)
0·3 μg/kg	NA	+33±3	+22·6±2·1	−37±4	+ 810±100
	αMeNA	+39±4	+24·8±3·8	−37±6	+ 880±300
0·6 μg/kg	NA	+54±4	+28·3±3·0	−43±7	+ 620±250
	αMeNA	+43±3	+22·8±1·1	−43±8	+1000±100
1·2 μg/kg	NA	+70±6	+36·7±8·5	−57±12	+1050±170
	αMeNA	+61±5	+36·6±8·7	−64±3	+1100±200
		NS	NS	NS	NS

Table 2. *Comparison of pressor responses to noradrenaline and α-methylnoradrenaline in rats and rabbits.*

Dose	No. of animals	Mean increase in blood pressure mm Hg (±s.e.) Noradrenaline	α-Methylnoradrenaline	*P* value
Rats (ng)				
10	10	29·1±2·7	28·1±2·7	NS
20	10	41·7±3·2	38·6±3·2	
30	10	50·1±5·4	53·1±2·8	
40	10	64·0±3·5	59·0±2·3	
Rabbits (μg/kg)				
0·5	6	49±2	39±3	<0·01
1	6	55±4	44±3	>0·05
2	6	74±5	52±5	<0·05

Table 3. *Relative potency of noradrenaline and -methylnoradrenaline.*

	Preparation	No. of experiments	Mean potency ratio
Dog	blood pressure		1·05
	heart rate		1·0
	cardiac output	9	0·8
	myocardial contractility		1·0
Rat (pithed)	blood pressure	10	1·03
Rabbit	blood pressure	6	1·3

It is known that α-methylnoradrenaline is taken up (Malmfors, 1965), stored in granules (Lundborg & Stitzel, 1967) in adrenergic nerve fibres and released by nerve stimulation (Muscholl & Maître, 1963). These findings indicate that the amine metabolites of α-methyldopa function as pseudo-transmitters.

Basic to this concept is that the new transmitter has less receptor activity than the natural substance. Day & Rand found α-methylnoradrenaline to be 2 to 8 times less potent than noradrenaline in increasing arterial blood pressure in several laboratory animal species. Brunner & others (1967) and Holtz & Palm (1967) also found α-methylnoradrenaline to have less pressor activity. In contrast, Muscholl & Maître (1963), Maître & Staehelin (1963) and Krzysztof (1967) found the two amines to be largely equipotent.

In my experiments, both amines were equipotent on all cardiovascular parameters in the dog and on the pressor responses in the rat over a wide dose range. Only in the rabbit, the pressor activity of α-methylnoradrenaline was slightly less than that for noradrenaline, the mean potency ratio being merely 1·3 which is in marked contrast to the eightfold difference obtained by Day & Rand (1964) in this species.

Despite these conflicting results, sufficient evidence from the present experimental findings and those of other workers (Henning, 1969a,b; Henning & van Zwieten, 1968) has been obtained to conclude that the replacement of noradrenaline by α-methylnoradrenaline at peripheral sympathetic nerve terminals does not substantially contribute to the hypotensive action of α-methyldopa.

Baker Medical Research Institute,
Melbourne, Australia.

F. R. TRINKER

December 17, 1970

REFERENCES

BONIFACE, K. F., BRODIE, O. J. & WALTON, R. P. (1953). *Proc. Soc. exp. Biol. Med.*, **84**, 263–266.

BRUNNER, H., HEDWALL, P. R., MAÎTRE, L. & MEIER, M. (1967). *Br. J. Pharmac. Chemother.*, **30**, 108–122.

CARLSSON, A. & LINDQVIST, M. (1962). *Acta physiol. scand.*, **54**, 87–94.

DAY, M. D. & RAND, M. J. (1963). *J. Pharm. Pharmac.*, **15**, 221–224.

DAY, M. D. & RAND, M. J. (1964). *Br. J. Pharmac. Chemother*, **22**, 72–86.

HENNING, M. (1969a). *Acta pharmac. tox.*, **27**, 135–148.

HENNING, M. (1969b). *Acta physiol. scand., Suppl.* 322, 3–37.

HENNING, M. & SVENSSON, L. (1968). *Acta pharmac. tox.*, **26**, 425–436.

HENNING, M. & VAN ZWIETEN, P. A. (1968). *J. Pharm. Pharmac.*, **20**, 407–419.

HOLTZ, P. & PALM, D. F. (1967). *Life Sci.*, **6**, 1847–1867.

KOPIN, I. J. (1968). *Ann. Rev. Pharmac.*, **8**, 377–394.

KRZYSZTOF, K. (1967). *Int. J. Neuropharmac.*, **6**, 435–462.

LUNDBORG, P. & STITZEL, R. E. (1967). *Br. J. Pharmac. Chemother.*, **29**, 342–349.

MAÎTRE, L. & STAEHELIN, M. (1963). *Experientia*, **19**, 573–575.

MALMFORS, T. (1965). *Acta physiol. scand., Suppl.* 248, 64.

MUSCHOLL, E. (1966). *Ann. Rev. Pharmac.*, **6**, 107–128.

MUSCHOLL, E. & MAÎTRE, L. (1963). *Experientia*, **19**, 658–659.

SOURKES, T. L. & RODRIGUEZ, H. R. (1967). In *Antihypertensive Agents*, pp. 151–189. Editor: Schittler, E. New York & London: Academic Press.

SHIPLEY, R. E. & TILDEN, J. T. (1947). *Proc. Soc. exp. Biol. N.Y.*, **64**, 453–455.

STONE, C. A. & PORTER, C. C. (1967). *Adv. Drug Res.*, **4**, 71–93.

Marihuana: importance of the route of administration

Conventionally marihuana is used, or rather abused, by inhalation. In this respect it more closely resembles tobacco than alcohol with which it has most often been compared from a behavioural point of view. Yet, surprisingly enough, most experiments on the pharmacological, neurochemical, or behavioural effects of marihuana have relied on an intraperitoneal administration of extracts of cannabis or pure 1-Δ^9-tetrahydrocannabinol (Δ^9-THC), the major psychoactive constituent.

In working with synthetic Δ^9-THC (Idänpään-Heikkilä, Fritchie & others, 1969) we were impressed by its great water insolubility which led us to question just how well it might be absorbed *via* the various conventional routes of administration. Tritiated-Δ^9-THC with a specific activity of 250 μCi/mg was synthesized (Idänpään-Heikkilä, Fritchie & others, 1969) and administered to rats in Tween-80 as a suspension intraperitoneally and intravenously. Animals were killed at various times and autoradiographs prepared using the technique previously described (Ullberg, 1968; Idänpään-Heikkilä, Vapaatolo & Neuvonen, 1968). ^{3}H-Δ^9-THC administered intraperitoneally remains in the abdominal cavity, with little absorption and distribution to other tissue, including the CNS (Fig. 1A and B). The same dose given intravenously was distributed throughout the body, including the CNS, within 5 min (Fig. 1C). Preliminary experiments also indicated good absorption and distribution after inhalation (Ho, Fritchie & others, 1970). A discrepancy exists between the known effective dose in man (100–250 μg/kg range inhaled) (Isbell, Gorodetzsky & Jasinski, 1967; Weil, Zinberg & Nelsen, 1968), and that used in most animal studies—10–25 mg/kg, and even as high as 00 mg/kg (intraperitoneally).

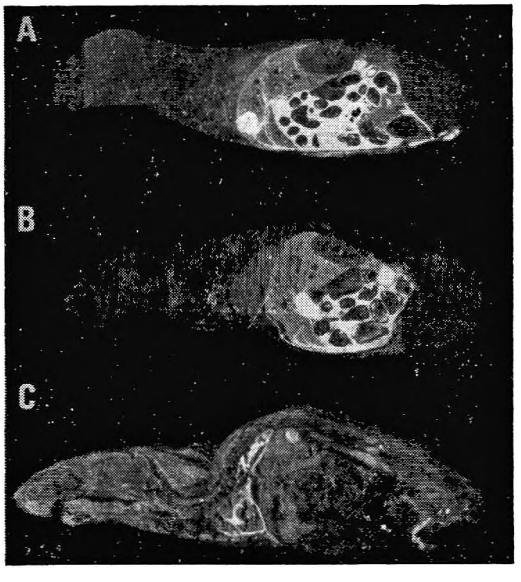


FIG. 1. Distribution of radioactivity (light areas) in mice 15 min (A) and 2 h (B) after intraperitoneal injection and 5 min (C) after intravenous injection of ^{3}H-Δ^9-THC.

Although measurable pharmacological and behavioural effects do occur after intraperitoneal administration of relatively large doses, this obviously is not the best route for the study of the compound.

This work was supported in part by PHS Grant MH-12959.

Texas Research Institute of Mental Sciences
Houston, Texas 77025, *U.S.A.*

Department of Pharmacology, University of Helsinki, Siltavuorenpenger 10, *Helsinki* 17, *Finland.*

BENG T. HO
G. EDWARD FRITCHIE
LEO F. ENGLERT
WILLIAM M. MCISAAC
J. E. IDÄNPÄÄN-HEIKKILÄ

November 10, 1970

REFERENCES

HO, B. T., FRITCHIE, G. E., KRALIK, P. M., ENGLERT, L. F., MCISAAC, W. M. & IDÄNPÄÄN-HEIKKILÄ, J. E. (1970). *J. Pharm. Pharmac.*, **22**, 538–539.

IDÄNPÄÄN-HEIKKILÄ, J. E., FRITCHIE, G. E., ENGLERT, L. F., HO, B. T. & MCISAAC, W. M. (1969). *New Engl. J. Med.*, **281**, 330.

IDÄNPÄÄN-HEIKKILÄ, J. E., VAPAATALO, H. J. & NEUVONEN, P. J. (1968). *Psychopharmacologia*, **13**, 1–13.

ISBELL, H., GORODETZSKY, C. W. & JASINSKI, D. (1967). *Ibid.*, **11**, 184–188.

ULLBERG, S. (1958). "*Proceedings of the second international conference on the peaceful uses of atomic energy*, Geneva." United Nations: New York.

WEIL, A. T., ZINBERG, N. E. & NELSEN, J. M. (1968). *Science, N.Y.*, **162**, 1234–1242.

Injectible dispersion of Δ^9-tetrahydrocannabinol in saline using polyvinylpyrrolidone

The intravenous administration of Δ^9-tetrahydrocannabinol (Δ^9-THC), an active principle of marihuana, is complicated by the extreme insolubility of the compound in aqueous media. We wish to report a preparation which appears to be well tolerated physiologically and is stable physically and chemically over extended periods of time.

This medium consists of a dispersion of Δ^9-THC in normal saline using polyvinylpyrrolidone PVP) as a carrier.

A suspension in the amount of 100 ml containing 1 mg Δ^9-THC/ml is prepared as follows: 40 ml of an ethanol solution containing 100 mg Δ^9-THC (a standard preparation of synthetic Δ^9-THC as distributed by the National Institute of Mental Health) is mixed with 30 ml of a 10% ethanol solution of PVP (polyvinylpyrrolidone K-30, average mol wt 40 000, Matheson, Coleman and Bell). The ethanol is removed by heating to 60° under a stream of dry nitrogen, or, alternatively, by vacuum rotary evaporator. Normal saline (sodium chloride injection, U.S.P., Abbot Laboratories) is then added with thorough mixing to bring the volume to 100 ml.

The resulting milky white dispersion when stored under refrigeration and in the absence of light shows no deterioration or isomerization of the Δ^9-THC, as determined by gas chromatographic analysis, over at least two months.

The PVP concentration should be at least 20 times that of the Δ^9-THC to produce a homogeneous suspension, thus there is some limitation to the amount of Δ^9-THC that can be effectively suspended without raising the viscosity of the medium to impractical levels. Preparations containing 2·5 mg/ml have been administered intravenously to rats without difficulty, and use of these dispersions in infusion pump administration to catheterized animals has been successful.

Texas Research Institue of Mental Science, 1300 *Moursund*, *Houston, Texas* 77025, *U.S.A.*

DAVID C. FENIMORE
PHILIP R. LOY

January 13, 1971

The temperature dependence of the critical micelle concentrations of cationic surface-active agents

The temperature dependence of the critical micelle concentrations, CMC values, of dodecyl pyridinium bromide (Adderson & Taylor, 1964), tetradecyl pyridinium, trimethyl ammonium and benzyl dimethyl ammonium bromides (Adderson & Taylor, 1967) and alkyl α-picolinium bromides (Adderson & Taylor, 1970) has been previously reported and an explanation of the micellization process advanced. The present data are complementary and reinforce this explanation.

Dodecyl benzyl dimethyl ammonium bromide, decyl and dodecyl trimethyl ammonium bromides were prepared by methods previously reported. CMC values were determined by conductance technique and are recorded in Table 1.

The fraction of theoretical charge, p/N has been calculated both for these salts and from the conductance-concentration data for the tetradecyl homologues (Adderson & Taylor, 1967), using the method of Evans, (1956). Aggregation numbers, N, of $C_{10} = 36$, $C_{12} = 50$ & $C_{14} = 75$ (Debye 1949) have been used throughout (Table 2).

As observed for alkyl picolinium bromides, (Adderson & Taylor, 1970) the degree of dissociation increases with temperature and decreases with increase in length of the principal alkyl chain. The dissociation is less in the alkyl trimethyl salts than in the corresponding compounds containing a cyclic structure, suggesting that the benzyl screens the charged nitrogen to a greater extent than a methyl group.

Thermodynamic parameters, Fig. 1a and b, have been calculated as previously, using the uncharged phase-change model and standard states of a mol fraction of unity for the monomeric species and the micelle itself for its species, and assuming that the heats of dilution from standard state to CMC are negligible compared to the heat of micellization. Hence, $\Delta G^0_m = 2RT \ln \text{CMC}$, and $\Delta H_m = \Delta H^0_m = -2RT^2 (\partial \ln \text{CMC}/\partial T)_p$.

Table 1. *Critical micelle concentrations, mol fractions.*

	Trimethyl ammonium bromides		Benzyldimethyl ammonium bromides
Temperature °C	Decyl	Dodecyl	Dodecyl
5	$1{\cdot}19 \times 10^{-3}$	$2{\cdot}76 \times 10^{-4}$	—
10	1·16	2·70	$1{\cdot}01 \times 10^{-4}$
15	1·15	2·64	0·989
20	1·13	2·63	0·983
25	1·12	2·64	0·994
30	1·13	2·69	1·02
35	1·14	2·75	1·06
40	1·15	2·82	1·11
45	1·18	2·89	1·15
50	1·22	3·01	1·22
55	1·25	3·15	1·28
60	1·28	3·33	1·38
65	1·31	3·48	1·51
70	1·35	3·71	1·62

Table 2. *The fraction of the theoretical charge, p/N.*

	Trimethyl ammonium bromides			Benzyldimethyl ammonium bromides	
Temperature °C	C_{10}	C_{12}	C_{14}	C_{12}	C_{14}
5	0·26	0·16	—	—	—
15	0·27	0·17	0·14	0·21	—
25	0·28	0·18	0·16	0·22	—
35	0·29	0·19	0·17	0·23	0·19
45	0·30	0·20	0·18	0·24	0·20
55	0·31	0·21	0·19	0·25	0·20

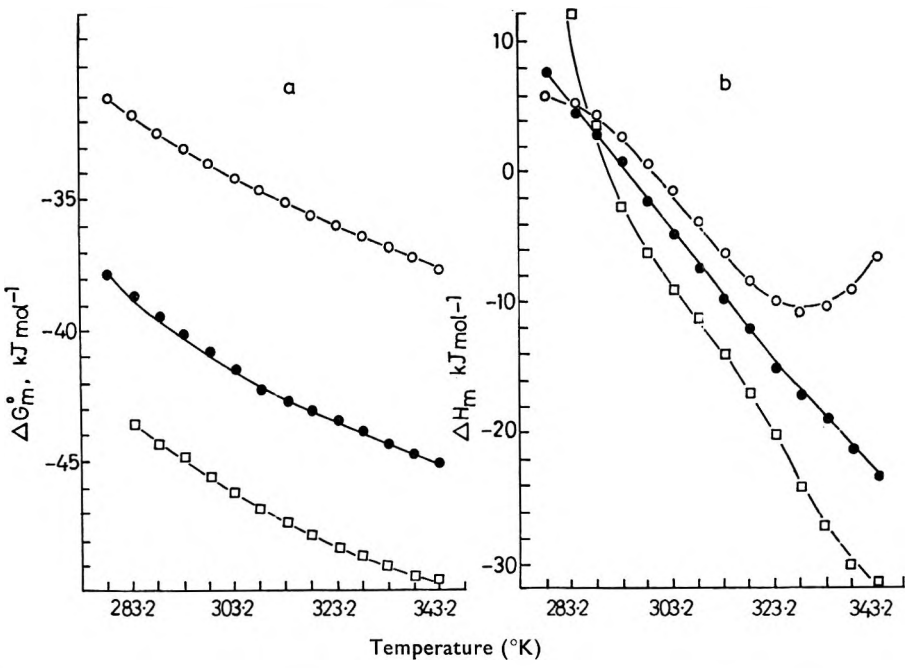


FIG. 1. a. Energy changes accompanying micellization. ○ Decyl trimethylammonium bromide; ● dodecyl trimethylammonium bromide; □ dodecyl benzyl dimethylammonium bromide.
b. Heats of micellization. Symbols as in a.

These derived enthalpy changes are very similar to those formerly reported and are in accord with the previously suggested energy changes accompanying micellization.

At least three factors contribute to the enthalpy changes, namely those associated with head-group aggregation, those with breakdown of hydrocarbon-stabilized water-structure and changes due to transfer of hydrocarbon from water to micelle. The first two are endothermic, the latter exothermic. The temperature at which micellization changes from an endo to an exothermic reaction, shown by the chain-length dependence of temperature of minimum CMC, varies with the summation of these effects. The major variable between members of a homologous series is the area of hydrocarbon-water interface associated with the alkyl chain. Increase in the associated exothermic component of micellization accompanying increase in chain length depresses the temperature at which a favourable enthalpic change first occurs.

Postgraduate School of Studies in Pharmacy, University of Bradford, Bradford, Yorks, U.K. — J. E. ADDERSON

Central Institute of Technology, Petone, New Zealand. — H. TAYLOR

January 14, 1971

REFERENCES

ADDERSON, J. E. & TAYLOR, H. (1964). *J. Colloid Sci.*, **19**, 495–499.

ADDERSON, J. E. & TAYLOR, H (1967). *Proceedings of the IVth Congress of Surface Activity* 1964, 613–620, New York: Gordon and Breach.

ADDERSON, J. E. & TAYLOR, H. (1970). *J. Pharm. Pharmac.*, **22**, 523–530.

DEBYE, P. (1949). *Ann. N. Y Acad. Sci.*, **51**, 575–592.

EVANS, H. C. (1956). *J. chem. Soc.*, 579–586.

Printed by W Heffer & Sons Ltd Cambridge England

Journal of Pharmacy and Pharmacology

Volume 23 Number 4 April 1971

Review

Original Papers

Letters to the Editor