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Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins

Y. MIZUSHIMA AND M. KOBAYASHI

The interaction of clinically established anti-inflammatory drugs with some proteins has shown these drugs to strongly inhibit heat coagulation of whole serum at a concentration attainable in the sera of patients. Phenylbutazone and sodium salicylate do not inhibit the biological activity of three biologically active and labile serum proteins, namely, necrotizing factor, heterogenous serum and complement. However, they do influence the effect of heat on these proteins. The relation between this drug action *in vitro* and the possible mode of action of the proteins *in vivo* is discussed.

As reported by Mizushima & Suzuki (1965) and Mizushima (1966) many non-steroidal anti-inflammatory drugs interact with plasma proteins and especially with Cohn's fraction IV and V. All the acidic anti-inflammatory drugs stabilize serum albumin and some other fractions against heat coagulation, but paradoxically, accelerate the heat precipitation of the crude globulin fraction (fraction IV-4). It was postulated that this property of drugs may have some connection with their mode of anti-inflammatory action. It is important in discussing possible modes of drug action to know if the drug effect *in vitro* is observable in a medium similar to a body fluid and at a drug concentration which can be attained in the serum of patients. We have examined the interaction of anti-inflammatory drugs with proteins, especially with biologically active and heat labile proteins, using nearly undiluted serum and at therapeutic (serum) drug concentrations (Hollander, 1962; Rechenberg, 1962; Mizushima, unpublished observations).

Experimental

DRUGS

Salicylic acid (commercial), phenylbutazone (Geigy), flufenamic acid (synthesized), and ibufenac (Boots) were used. All compounds were dissolved in isotonic saline and neutralized with sodium hydroxide to give the appropriate concentrations for each experiment.

BIOLOGICALLY ACTIVE AND HEAT LABILE PROTEINS

Necrotizing factor. Fresh sera from active rheumatoid patients were obtained from the clotted blood, stored below −20° and used within 2 days. The serum was mixed with the drug solution and 0·1 ml of the mixture was injected intradermally into the backs of male guinea-pigs weighing 300–400 g. Necrotizing activity was recorded 4 hr after the injection as described by Mizushima, Kasukawa & Oshima (1962). To

From the Department of Physical Therapy and Medicine, Faculty of Medicine, University of Tokyo, Bunkyo, Tokyo, Japan.

compare the activity of two sera, injections were made at the same site on the left and the right side of the one animal.

Heterogenous serum. Fresh goat serum obtained from clotted blood was used. The storage of the serum, method of injection into guinea-pigs and recording of the biological activity were similar to procedures used in the experiments with necrotizing factor.

Complement. Pooled fresh sera from 10 guinea-pigs (stored below −20°) was used. Complement activity of the sera was 230 units measured by Mayer's method (Kabat & Mayer, 1961). Serum, 2·5 ml, was diluted 300 fold and the percentage of cells suffering haemolysis was recorded.

Results

Effect of drugs on the denaturation of some serum proteins, using whole serum. Normal sera were obtained from two healthy adults. The protein concentration of a mixture of the two sera was 77 mg/ml. Paper electrophoresis of the serum showed Alb. (64%), α-gl. (12%), β-gl. (11%) and γ-gl. (13%). The pH of the serum was adjusted to 6·3 by adding a small amount of N hydrochloric acid. To 0·45 ml of the serum was added 0·05 ml of a drug solution and the mixture was incubated at room temperature for 20 min. (0·05 ml of saline was added in place of the drug solution in the controls). The mixture was heated at 57° for exactly 3 min to denature (coagulate) heat-sensitive proteins. After cooling the mixture, 2·5 ml of phosphate-buffered saline (0·07 M sodium phosphate—0·15 M saline) pH 6·3 was added. The turbidity of the solution was measured spectrophotometrically at 660 mμ.

The average percentage inhibition of the heat coagulation of serum proteins by the added drugs is shown in Table 1. The results showed that

TABLE 1. INHIBITORY ACTION OF ANTI-INFLAMMATORY DRUGS ON HEAT COAGULATION OF WHOLE HUMAN SERUM*

Drugs†	Final concentration mg/ml‡	Percentage inhibition of coagulation
Salicylic acid	0·18	81
Phenylbutazone	0·12	72
Flufenamic acid	0·03	43
Ibufenac	0·04	59

* Whole human serum was heated at 57° C for 3 min.
† Drugs (neutralized with NaOH) were added *in vitro* to the serum.
‡ These concentrations of the drugs can usually be attained in the sera of patients after oral medication

all four anti-inflammatory drugs stabilized some serum protein(s) against heat coagulation when present at concentrations attainable *in vivo* and in nearly undiluted serum.

Interaction of drugs with heterogenous serum. In experiment A, 0·1 ml of a solution of phenylbutazone or 0·1 ml of saline was added to 0·9 ml of goat serum and the mixture allowed to stand at room temperature for at least 20 min. The mixture was then heated at 47° for 60 min. The purpose of this experiment was to see whether or not phenylbutazone influenced heat inactivation of the biologic activity of heterogenous

serum. In experiment B, the goat serum was first heated at 47° for 60 min and then 0·1 ml of the drug solution or saline was added to 0·9 ml of the heated serum.

The results of both experiments (Table 2) indicate that phenylbutazone

TABLE 2. INFLUENCE OF PHENYLBUTAZONE ON HEAT INACTIVATION OF THE BIOLOGICAL ACTIVITY OF GOAT SERUM*

Serum		1	2	3	4	5	6	7	8	9	10	11	12
Unheated serum		++±	++	++	+	++	++	++	++	+	+	++	+
Expt. A (Drug was added before heating)	Saline	++‡	++	++	+	+	+	+	++	+	+	+	±
	Phenyl-butazone†	+‡	+	+	+	+	±	+	+	+	+	−	−
Expt. B (Drug was added after heating)	Saline							++	++	+	±	+	+
	Phenyl-butazone†							++	++	+	±	+	+

* Whole goat serum was heated at 47° C for 60 min.
† Phenylbutazone at a final concentration of 0·1 mg/ml was added *in vitro* to the serum.
‡ ++, +, ±, − refer to the degree of inflammation of guinea-pig skin caused by goat serum.

did not alter the biologic activity of goat serum but influenced the degree of inactivation of the biologic activity obtained by mild heating.

Interaction of drugs with necrotizing factor. The experimental methods were the same as those used in experiment 2 except that fresh sera from rheumatoid patients were used in place of goat serum and these human sera were heated at 42° for 60 min.

The results (Table 3) show that phenylbutazone did not alter the

TABLE 3. INFLUENCE OF PHENYLBUTAZONE ON HEAT INACTIVATION OF NECROTIZING FACTOR*

Serum		1	2	3	4	5	6	7	8	9	10	11	12
Unheated serum		+‡	++	+++	++	+++	++	++	+++	+++	++	++	
Drug was added before heating	Saline	+‡	±	+	−	++	−	−	+	++	−	−	
	Phenyl-butazone†	+‡	±	++	+	+++	+	±	++	++	+	+	
Drug was added after heating	Saline				±	++	+	+	+	++			
	Phenyl-butazone†				−	++	+	+	+	+++			

* Whole rheumatoid serum was heated at 42° C for 60 min.
† Phenylbutazone at a final concentration of 0·1 mg/ml was added *in vitro* to the serum.
‡ +++, ++, +, ±, − refer to the degree of inflammation of guinea-pig skin caused by necrotizing factor.

necrotizing activity, while it influenced the degree of inactivation of the necrotizing factor obtained by mild heating.

Interaction of drugs with some component of complement. The method of experiment was the same as in experiment 2 except that fresh guinea-pig

serum and both sodium salicylate and phenylbutazone were used. The serum was heated at 50° for 20 min for heat inactivation.

The results (Table 4) indicated that salicylate and phenylbutazone

TABLE 4. INFLUENCE OF SALICYLATE AND PHENYLBUTAZONE ON HEAT INACTIVATION OF COMPLEMENT*

Drugs†	Final concentration‡	Percentage immune haemolysis§	
		Drugs were added before heating	Drugs were added after heating
Control	—	41a	46d
Salicylic acid	0·20 mg/ml	38b	45e
Phenylbutazone	0·12 mg/ml	38c	46f
Unheated serum	—	73	73

* Whole guinea-pig serum was heated at 50°C for 20 min.
† Drugs were added *in vitro* to the serum.
‡ These concentrations of the drugs can be attained in the sera of patients.
§ 2·5 ml serum diluted 300-fold was used.
b and c to a: significant ($P < 0·01$), e and f to d: not significant.

slightly but definitely ($P < 0·01$) influenced the degree of heat inactivation of complement.

Discussion

The mode of action of non-steroidal anti-inflammatory drugs is still obscure, but their inhibitory effects on oxidative phosphorylation (Whitehouse, 1964), some enzymic reactions (Rechenberg, 1962) and the formation of some inflammatory mediators and factors (Spector & Willoughby, 1963) are considered important. This suggests that non-steroidal anti-inflammatory drugs interact in some way with proteins. We have found that the interaction of these drugs with proteins in nearly undiluted serum takes place using drug concentrations that can be attained clinically in the sera of patients. The drugs could, therefore, interact *in vivo* with and stabilize some protein(s) in the serum or in the tissues of patients adequately treated with them. This *in vitro* property seems to be fairly specific to active non-steroidal anti-inflammatory drugs (Mizushima, 1965).

It is likely, judging from our findings and those on drug effects on enzymic systems (Rechenberg, 1962), that non-steroidal anti-inflammatory drugs at low concentration neither alter strongly the conformation of proteins directly nor inhibit strongly the specific combination of proteins, but do influence the conformational changes suffered by some proteins on heating. It is of interest that there are many reports which indicate the importance of protein denaturation as a cause of inflammation (Opie, 1962, 1963; Ishizaka, 1965).

Necrotizing factor (Lovell, Pryce & Boake, 1954), heterogenous serum and complement were used by us merely as examples of biologically active, heat-labile proteins. There is no evidence that these particular proteins are important as a cause of inflammation, and furthermore, their biological activity was not impaired by the drugs we used.

Regarding the experimental conditions of this study, these biologically active proteins were inactivated by *mild* heating, though the degree of heating was not physiological. Some denaturation of proteins could possibly occur *in vivo*. In these experiments, whole serum was coagulated at pH 6·3, a pH which could be attained in strongly inflamed tissue.

References

Hollander, J. L. (1962). *Arthritis*, 6 edn, Philadelphia: Lea & Febiger.
Ishizaka, K. (1965). *Immunological Diseases I*, p. 131, Boston: Little Brown & Company.
Kabat, E. A. & Mayer, M. M. (1961). *Experimental Immunochemistry*, p. 149. Springfield: Charles C. Thomas.
Lovell, R. R. H., Pryce, D. M. & Boake, W. C. (1954). *Br. J. exp. Path.*, **35**, 345–349. 345–349.
Mizushima, Y., Kasukawa, R. & Oshima, Y. (1962). *Acta rheum. scand.*, **8**, 183–191,
Mizushima, Y. & Suzuki, H. (1965). *Archs int. Pharmacodyn. Thér*, **157**, 115–124.
Mizushima, Y. (1966). *Lancet*, **2**, 443.
Opie, E. L. (1962). *J. exp. Med.*, **115**, 597–608.
Opie, E. L. (1963). *Ibid.*, **117**. 425–448.
Rechenberg, H. K. von (1962). *Phenylbutazone*, London: Edward Arnold.
Spector, W. G. & Willoughby, D. A. (1963). *The Salicylates*. Editors, Dixon, A. S. G., Martin, B. K., Smith, M. J. H. & Wood, P. H. N. p. 141, London: J. & A. Churchill.
Whitehouse, M. W. (1964). *Biochem. Pharmac.*, **13**, 319–336.

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Application of the analogue computer to pharmacokinetic and biopharmaceutical studies with amphetamine-type compounds

A. H. BECKETT AND G. T. TUCKER*†

The kinetics of absorption, metabolism, and excretion of (+)-amphetamine and (+)-methylamphetamine, after oral administration of "free" dosage forms to man, under controlled acidic urine conditions, have been examined using an electronic analogue computer. This device has also been used to determine the *in vivo* rate of release of the drugs from hard gelatin capsule dosage forms and prolonged-release preparations. *In vivo* drug release from the prolonged-release preparations was correlated with *in vitro* drug release data.

FOR many drugs, urinary excretion studies offer the most practical method of evaluating the *in vivo* absorption of the drug from its dosage form. Ideally, conditions are required which give maximal excretion of unchanged drug and which allow smooth curves to be drawn through urinary excretion rate versus time data points. The finding that the urinary excretion of many drugs is pH dependent and in some instances urine-volume dependent (Milne, Scribner & Crawford, 1958; Peters, 1960; Weiner & Mudge, 1964; Braun, Hesse & Malorry, 1963; Beckett & Rowland, 1965a) has important implications in this context. Since urinary pH varies between subjects and throughout the day (Elliot, Sharp & Lewis, 1959; and others), the rate of excretion of many drugs which are partially ionized over the normal range of urinary pH (4·5 to 8·0), will vary accordingly.

Maintenance of a constant acidic urinary pH with a basic drug, such as amphetamine, has therefore been advocated (Beckett & Tucker, 1966) for the *in vivo* evaluation of dosage forms. Then, the selective and passive reabsorption of the unionized drug species from the kidney tubules is minimized and meaningful results and comparisons are obtained.

The present paper is concerned with the pharmacokinetic interpretation of urinary excretion data for amphetamine and methylamphetamine after their administration to man, in various oral dosage forms, under controlled acidic urinary pH conditions. Specifically, the objective was to evaluate the *in vivo* release rates of drugs from prolonged-release formulations and to compare them with *in vitro* release rates. An analogue computer greatly facilitated the calculations.

THEORETICAL

The fundamentals and philosophy of the use of electronic analogue computers in pharmacokinetics have been discussed by Garrett & Alway

From the Department of Pharmacy, Chelsea College of Science and Technology (University of London), Manresa Road, London, S.W.3, England.

* Present address: Department of Anesthesiology, The Mason Clinic, Seattle, Washington 98101, U.S.A.

† The work forms part of a thesis by G. T. T. accepted for the degree of Ph.D. in the University of London.

(1963). Analogue computers, which use voltages to represent the variables, have been employed in pharmacokinetics either to plot the dependent variable as a continuous function of time when programmed on the basis of a specific equation (Taylor & Wiegand, 1962) or more commonly to build compartmental models by curve-fitting procedures (eg. Garrett, Johnston & Collins, 1963).

The following assumptions are made when using simple compartmental models to investigate the kinetics of the absorption and elimination of amphetamine and methylamphetamine in various dosage forms, under acidic urine conditions. (i) The rate of urinary excretion of the drug is proportional to its concentration in the plasma, which in turn is proportional to the total amount in the body, excluding the gut and metabolite compartments. (ii) Drug transfer from one compartment to another is irreversible. (iii) Transfer rate of drug from one compartment to another is directly proportional to the amount of drug in that compartment, i.e. drug release, absorption, metabolism, and excretion are apparent first-order processes with rate constants having units of reciprocal time. (iv) Compartments are uniform and homogeneous throughout the transfer processes. (v) The release of drug from dosage forms is the rate-determining step in drug absorption. (vi) There is no decomposition of the drug at the absorption site, no enterohepatic or salivary cycling, or diffusion of the drug from the blood into the stomach. (vii) The rate constant for drug absorption is unchanged along the intestinal tract. (viii) The drug is ultimately completely available for absorption, and is 100% absorbed. (ix) Excretion of unchanged drug by pathways other than via the kidney is negligible. (x) Absorption and elimination rate constants are independent of dosage form, as also are distribution processes.

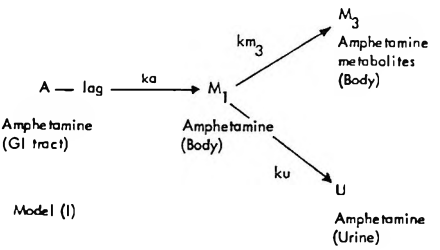
The validity of some of these assumptions will be discussed in relation to correlations observed of experimental results and computer simulations.

Although the absolute significance of kinetic data obtained under forced extremes of urinary pH is questionable, particularly as it is not known whether such conditions affect the distribution, and binding of the drug, such data is of value if used in a comparative sense, especially if the comparative performance of different drug formulations is being considered. The further assumption is made in these studies of drug formulations that the use of ammonium chloride to acidify the urine does not influence release of drug from the dosage forms.

PHARMACOKINETIC MODELS

Model (I): applicable to "free" forms of amphetamine.

Pharmacokinetic model (I) is proposed to describe the kinetics of absorption, metabolism, and excretion of (+)-amphetamine in man after oral administration of "solution" or "free pellet" forms of the drug (see Experimental for description of dosage forms) under controlled acidic-urine conditions.



Based upon this model, the following rate equations may be written (all symbols are defined in Appendix 1):

Post-lag time:

$$\frac{dA}{dt} = -\,ka.A \qquad \ldots\ldots\ 1$$

$$\frac{dM_1}{dt} = ka.A - ku.M_1 - km_3.M_1 \qquad \ldots\ldots\ 2a$$

$$= ka.A - ky.M_1 \qquad \ldots\ldots\ 2b$$

$$\frac{dM_3}{dt} = km_3.M_1 \qquad \ldots\ldots\ 3$$

$$\frac{dU}{dt} = ku.M_1 \qquad \ldots\ldots\ 4$$

The analogue computer program for the solution of model (I) is shown in Fig. 1.

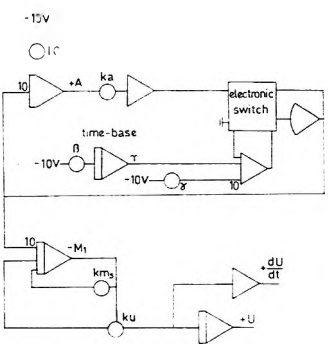
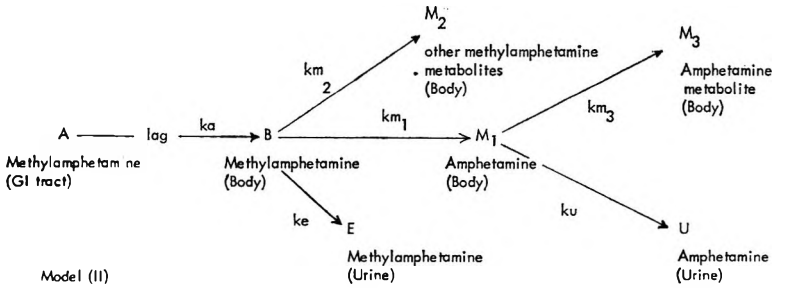


FIG. 1. Analogue computer program for Model (I). 1 sec of machine time equals 1 hr of real time.

Model (II): applicable to "free" forms of methylamphetamine.

Pharmacokinetic model (II) is proposed to describe the kinetics of absorption, metabolism, and excretion of (+)-methylamphetamine in man after oral administration of "solution" forms of the drug under controlled acidic urine conditions.



Differential equations to describe this model are:

Post-lag time:

$$\frac{dA}{dt} = -ka.A \quad .. \quad .. \quad .. \quad .. \quad 5$$

$$\frac{dB}{dt} = ka.A - ke.B - km_1B - km_2B \quad .. \quad 6a$$

$$= ka.A - kd.B \quad .. \quad .. \quad .. \quad .. \quad 6b$$

$$\frac{dM_2}{dt} = km_2.B \quad .. \quad .. \quad .. \quad .. \quad 7$$

$$\frac{dE}{dt} = ke.B \quad .. \quad .. \quad .. \quad .. \quad 8$$

$$\frac{dM_1}{dt} = km_1.B - ku.M_1 - km_3.M_1 \quad .. \quad .. \quad 9a$$

$$= km_1.B - ky.M_1 \quad .. \quad .. \quad .. \quad 9b$$

$$\frac{dM_3}{dt} = km_3.M_1 \quad .. \quad .. \quad .. \quad .. \quad 10$$

$$\frac{dU}{dt} = ku.M_1 \quad .. \quad .. \quad .. \quad .. \quad 11$$

The analogue computer program for the solution of model (II) is shown in Fig. 2.

Model (III): applicable to drug preparations of amphetamine.

Model (I) is modified to describe the kinetics of release, absorption, metabolism, and excretion of (+)-amphetamine, after administration to man of "capsule" and prolonged release preparation B forms of the drug (see Experimental for description of dosage forms), under controlled acidic urine conditions. The modification is the addition of a formulation compartment, D_m, containing the total dose at zero time and from which drug is released into the gastrointestinal tract by sequential first-order processes governed by rate constants kr_1 and kr_2.

In addition to the equations given for model (I), equation 12 also applies.

$$\frac{dD_m}{dt} = -kr.D_m \quad .. \quad .. \quad .. \quad .. \quad 12$$

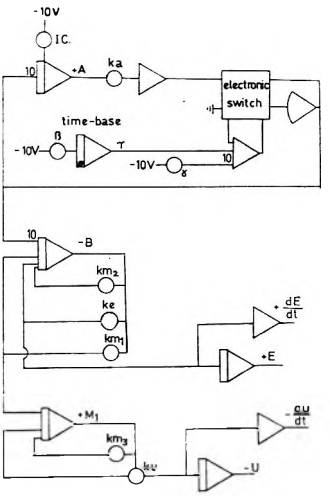


FIG. 2. Analogue computer program for Model (II). 1 sec of machine time equals 1 hr of real time.

where, kr is initially kr_1 and becomes kr_2 after the "break time". Equation 12 is modified to give equation 13.

$$\frac{dA}{dt} = kr.D_m - ka.A \qquad .. \quad .. \quad .. \quad 13$$

The analogue computer program for the simulation of model (III) is shown in Fig. 3. Since the electronic switch was required to change over from kr_1 to kr_2, lag time could not be programmed directly and was therefore estimated by manually setting the abscissa zero of the X–Y recorder.

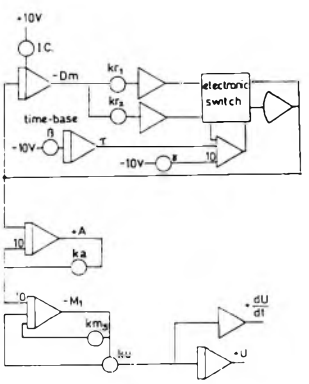


FIG. 3. Analogue computer program for Model (III). 1 sec of machine time equals 1 hr of real time.

Model (IV): applicable to drug preparations of methylamphetamine.

Model (II) was modified to describe the kinetics of release, absorption, metabolism, and excretion of (+)-methylamphetamine after administration to man of prolonged-release preparation D (see Experimental), under controlled acidic urine conditions. The basic model was extended by the addition of a formulation compartment, D_m, initially containing 80% of the total dose and from which the drug is released into the gastrointestinal tract by a first-order process governed by the rate constant kr.

In addition to the equations given for model (II), equation 12 also applies and equation 13 is substituted for equation 5. The analogue computer program required to simulate model (IV) was as shown in Fig. 2, with the addition of a formulation integrator feeding D_m into the gut integrator. An initial condition of 8 V, representing f_m, was set on the formulation integrator; similarly 2 V, representing f_l, was set on the gut integrator.

Experimental

DOSAGE FORMS

Solution: aqueous solutions of (+)-amphetamine sulphate or (+)-methylamphetamine hydrochloride. Dose: 15 mg; 5 mg. *Free pellets:* sugar pellets, coated with (+)-amphetamine sulphate. Dose: 15 mg; 3 × 5 mg (4 hrly). *Capsule:* as free pellets but pellets contained in a hard gelatin capsule. Dose: 15 mg; 3 × 5 mg (4 hrly); 5 mg. *Preparation B:* commercial prolonged-release product of (+)-amphetamine sulphate pellets each coated with a material forming a dialysing membrane and contained in a hard gelatin capsule. Dose: 15 mg. *Preparation D:* prolonged-release tablet product containing (+)-methylamphetamine hydrochloride distributed in a porous plastic matrix. Dose: 15 mg. Urinary excretion results obtained using other prolonged-release preparations (see Beckett & Tucker, 1966; Tucker, 1967) were not subjected to computer analysis since the data indicated incomplete *in vivo* availability of the drug from the dosage form).

In vitro EXPERIMENTS

Preparation B and rotating-bottle method. In vitro drug release was determined by the manufacturers of preparation B, according to Krueger & Vliet (1962). Samples equivalent to 80 mg of (+)-amphetamine sulphate were used. Release was determined in 40 ml vials containing 25 ml of digestive fluid, revolving end over end in a 37° ± 1° water bath at 30 rev/min. The amphetamine content of residues collected at appropriate time intervals was determined, after washing and extraction with alkaline chloroform, by non-aqueous titration with standard perchloric acid-glacial acetic acid, using crystal violet as the indicator.

Preparation D and rolling-bottle method. In vitro drug release was determined by the manufacturers of preparation D, according to the following procedure.

Tablets equivalent to 120 mg of (+)-methylamphetamine hydrochloride were placed in a $3\frac{1}{16}$ inch diameter bottle of 500 ml capacity containing 300 ml of distilled water. The bottle was rotated continuously at 25° ± 2·5° on rollers having a peripheral speed of 100 ± 7·5 ft/min. Aliquots were removed for analysis of released drug at appropriate time intervals.

Rotating-bottle method. Tablets equivalent to 60 mg of (+)-methylamphetamine hydrochloride were placed in a screw-cap bottle of 4 fl. oz. capacity containing 80 ml of distilled water (pH 5·2). The bottle was rotated end over end at 40 rev/min in a 37° ± 1° water bath. Samples (20 ml) of the elution fluid were removed at appropriate time intervals for analysis. At each time interval the volume of the elution fluid was maintained by addition of 20 ml distilled water from a control bottle. The 20 ml samples were allowed to cool to room temperature and diluted to 25 ml in volumetric flasks with 0·5N sulphuric acid.

An ultraviolet absorption curve was obtained from each diluted sample using a Beckman DK2 ratio recording spectrophotometer and 2 cm matched silica cells. (+)-Methylamphetamine content was determined using a calibration curve of absorption difference between a base line drawn between the minima at 255 and 262 mμ and the maximum at 257·5 mμ against methylamphetamine concentration over the range 0·1 to 1·0 mg equivalent base/ml 0·1N sulphuric acid (cf. Souder & Ellenbogen, 1958). 0·1N sulphuric acid was used as reference. The percentage of drug released up to each time interval was calculated, allowing for the amount which had been removed for analysis at earlier intervals, from the concentration of drug in each diluted sample.

Method using the BP tablet disintegration apparatus to determine dissolution rate. Tablets equivalent to 120 mg of (+)-methylamphetamine hydrochloride were placed in the glass cylinder of the apparatus containing 330 ml of distilled water (pH 5·2). The apparatus was operated at 37° ± 1°. Samples (20 ml) of the elution fluid were removed at appropriate time intervals for analysis. At each time interval the volume of the elution fluid was maintained by addition of 20 ml of distilled water from a control cylinder. The 20 ml samples were allowed to cool to room temperature and diluted to 25 ml in volumetric flasks with 0·5N sulphuric acid. Methylamphetamine was determined as described in the rotating bottle method above.

URINARY EXCRETION TRIALS

The dosage forms were given to healthy male subjects under conditions of constant acidic urinary pH (appprox. 4·7 ± 0·2). At least three subjects received each dosage form or regimen of amphetamine. Two subjects both received each form of methylamphetamine. Tables 2 and 3 show that subjects were chosen such that when comparisons were to be made between two forms or regimens, at least one subject, but usually two or three, received both forms. The protocol regarding time of

administration of drug, times of urination, measurement of urine pH, and dosage regimen for ammonium chloride has been described previously (Beckett & Tucker, 1966). Methylamphetamine and amphetamine in urine were determined by the method of Beckett & Rowland (1965b).

COMPUTER SIMULATIONS

A PACE TR20R (Electronic Associates Ltd) analogue computer was used together with an X–Y recorder (Advance Electronics Ltd) and a digital voltmeter (Roband Ltd). The appropriate pharmacokinetic model to describe absorption, metabolism, and excretion was programmed. The experimental urinary excretion data were plotted on the X–Y recorder, both as cumulative excretion and rate of excretion. In some cases, the absorption points derived from these data using the equation of Wagner & Nelson (1964) were also plotted. The settings of the rate potentiometers were then systematically varied in an effort to fit the computer generated curves to the experimental data points. When the best fit was obtained, the settings of the rate constant potentiometers were read from the digital voltmeter. The suitability of the model was judged on the basis of the fit obtained to the experimental data. Lag time was programmed using a suitable electronic switch (comparator relay). During initial curve fitting, the rate constant for elimination of unchanged drug, either kd or ky, was that estimated from the slope of the semi-logarithmic plot of rate of excretion versus time for each subject.

As well as for the fitting of single 15 mg (+)-amphetamine sulphate "free" dose data, model (I) was also used in an attempt to fit 3 × 5 mg (4 hrly) "free pellet" data obtained from two subjects. The appropriate computer program (see Fig. 1) was suitably modified; an initial condition of 3·3 V (representing the first 5 mg dose) was set on the gut integrator. At 4 hr (+ lag time) computer time, a second gut integrator, with the same initial condition, was made operational and its output fed into the body integrator. This was accomplished using the electronic switch operating between earth and the feedback of the second gut integrator. Since only one switch was available, the third dose was introduced by manually "holding" the computer at the beginning of the third dosage interval (+ lag time), plugging the output from a third gut integrator into the body integrator, then putting the computer back into the operate mode. It was also now necessary to estimate lag times manually.

In fitting the amphetamine excretion curves after the administration of methylamphetamine, it was assumed that although the ky value might be different from that determined after the administration of amphetamine, the ratio of the component rate constants, ku and km_3, would remain essentially constant for each subject.

Fitting of "capsule" and prolonged-release preparation data from appropriate subjects was made, as far as possible, using values of the rate constants for absorption, metabolism, and excretion, and the lag time, similar to those used in previous simulations of "solution" and "free pellet" data.

In vivo drug release rate was plotted by taking the output from the integrator representing the formulation compartment D. Hence, direct comparison of *in vivo* and *in vitro* drug release rates could be made.

Results

In vitro DATA

In vitro drug release data for the batches of preparations B and D supplied, are summarized in Table 1. In the spectrophotometric method

TABLE 1. PERCENTAGE DRUG RELEASED *in vitro* VERSUS TIME DATA FOR PROLONGED-RELEASE PREPARATIONS B (AMPHETAMINE) AND D (METHYLAMPHETAMINE).

Preparation	Method	Time (hr)								
		½	1	2	3	4	5	6	7	8
B (pellets)* lot BUK	Rotating bottle	—	23·2	36·1	—	56·7	63·8	—	75·8	81·1
D (tablets) lot 777-1316-21	Rolling bottle	32·1	44·5	59·9	70·6	76·6	86·3	89·4	92·4	—
	Rotating bottle†	38·0	50·8	65·5	73·6	79·0	82·7	85·8	89·1	—
	BP tablet disintegration apparatus‡	33·1	44·9	61·2	70·4	74·2	84·0	84·1	90·0	—

* Further experiments indicate that the release rate of amphetamine from this preparation is essentially independent of pH, the presence of enzymes, elution volume, and agitation rate (Dr. R. Goldman, personal communication).

† Average of three determinations } results obtained for drug release at each time in individual experiments were all within ±4% of the reported averages.
‡ Average of two determinations }

for the analysis of methylamphetamine, the calibration curves were linear over the concentration range examined. The measurements used as the basis of calibration were chosen to minimize errors due to interfering absorption from extraneous substances, i.e. other tablet ingredients also present in the elution fluid. Although not a significant problem, since the plastic matrices of the tablets did not disintegrate during the test, interference was sufficient to preclude the use of measurements employing a more conventional base-line.

Close agreement between the results using three methods for determining the release rate of methylamphetamine from preparation D was obtained (Fig. 4), when the logarithm of the percentage drug not released was plotted against time. A graph suggested that release of most of the drug from preparation D could be described as an apparent first-order rate process. Extrapolation of the plots to zero time indicates that about 20% of the dose was present in an essentially "free" form.

COMPUTER SIMULATIONS

"*Solution*" *and* "*free pellet*" *forms, i.e.* "*free*" *drug*

(+)-*Amphetamine.* Model (I) was fitted to the experimental data, and typical fitted cumulative and rate of excretion curves, along with the derived curve for the amount of amphetamine in the gastrointestinal tract, are shown in Fig. 5*a*. The fit obtained, using the same model, to the 3 × 5 mg "free pellet" data of subject 4 is shown in Fig. 5*b*; a similar result was obtained with the data of subject 5.

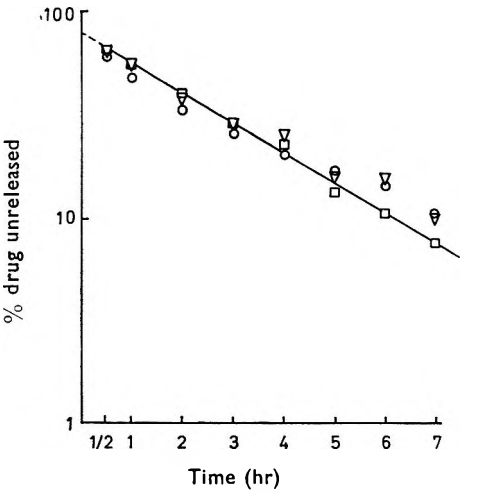


FIG. 4. Percentage methylamphetamine dose unreleased (logarithmic scale) *in vitro* from preparation D versus time, determined using three methods. □ Rolling bottle apparatus. ○ Rotating bottle apparatus. △ BP tablet disintegration test apparatus.

The kinetic parameters, obtained using the above method, for each subject are shown in Table 2. The ratio of rate constants ku/ky indicates the fraction of the dose eventually excreted unchanged in the urine, assuming there was no change in elimination half-life beyond 24 hr; the ratio km_3/ky indicates the fraction metabolized.

Excellent agreement was found among the experimental data in three of the five subjects who received the "free" forms of amphetamine, and the same data calculated by the computer. With subject 3, however, it was not possible to fit the peak hour of the rate of excretion data; data points were slightly higher (+1 to +2% dose/hr) over this period compared with the computer curve. A similar trend was observed in the

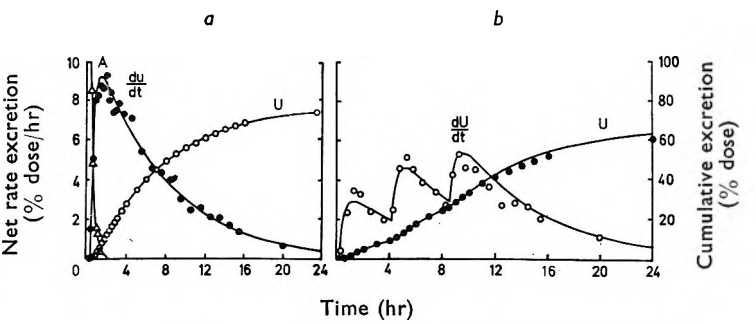


FIG. 5*a*. Computer curves and experimental data points for the urinary excretion of amphetamine, after oral administration of 15 mg (+)-amphetamine sulphate, in 'free pellet' form (Subject 5) (Model 1).

b. Computer curves and experimental data points for the urinary excretion of amphetamine, after a 3 × 5 mg dosage regimen of (+)-amphetamine sulphate orally in 'free pellet' form. (Subject 4) (Model 1).

TABLE 2. KINETIC PARAMETERS FOR THE RELEASE, ABSORPTION, METABOLISM AND EXCRETION OF (+)-AMPHETAMINE AFTER 15 MG DOSES OF THE SULPHATE IN VARIOUS DOSAGE FORMS.

Subject	Dosage form	Dose (as mg SO_4)	Lag time (lr)	kr (hr^{-1})	kr_3 (hr^{-1})	Break time	ka (hr^{-1})	ku (hr^{-1})	km_3 (hr^{-1})	ky (hr^{-1})	ku/ky	km_3/ky
1*	Solution	15	0·25	—	—	—	1·7	0·088	0·050	0·138 (5·02)	0·638	0·362
2*	"	15	0·6	—	—	—	3·0	0·117	0·024	0·141 (4·91)	0·830	0·170
3	"	15	0·25	—	—	—	2·8	0·102	0·058	0·160 (4·33)	0·638	0·362
4	"	15	0·2	—	—	—	2·8	0·107	0·044	0·151 (4·59)	0·709	0·291
4	Free pellets	15	0·3	—	—	—	2·7	0·107	0·045	0·152 (4·56)	0·704	0·296
4	"	15	0·1	—	—	—	2·8	0·108	0·031	0·139 (5·00)	0·777	0·223
5	"	15	0·4	—	—	—	2·8	0·108	0·029	0·137 (5·06)	0·788	0·212
4	"	3 × 5	0·1 0·1 0·1	— — —	— — —	— — —	2·8 2·8 2·8	0·107	0·050	0·157 (4·41)	0·682†	0·318
5	Free pellets	3 × 5	0·2 0·2 0·3	— — —	— — —	— — —	1·3 2·7 2·4	0·107	0·024	0·131 (5·29)	0·817†	0·183
6	Capsule	15	0·5	0·100	1·8	1·5	2·8	0·092	0·023	0·115 (6·03)	0·800	0·200
7	"	15	—	0·183	1·3	1·1	2·8	0·124	0·042	0·166 (4·17)	0·747	0·253
5	"	15	0·3	0·640	2·8	1·9	2·8	0·107	0·024	0·131 (5·29)	0·817	0·183
4	"	15	—	0·360	2·8	1·8	2·8	0·108	0·040	0·148 (4·68)	0·730	0·270
4	Prepn B (pellets)	15	0·6	0·074	0·339	2·0	2·8	0·107	0·041	0·148 (4·68)	0·723	0·277
4	Prepn B (capsule)	15	0·1	0·075	0·339	2·4	2·8	0·107	0·043	0·150 (4·62)	0·713	0·287
5	"	15	0·4	0·050	0·328	1·7	2·8	0·107	0·024	0·131 (5·29)	0·817	0·183
6	" ‡	15	0·2	0·163	0·285	1·6	2·8	0·092	0·023	0·115 (6·03)	0·800	0·200

* Experimental data of Beckett & Rowland (1965a).
† Values for total fraction excreted unchanged, determined by extrapolation of 24 hr experimental data to infinite time, were 0·646 and 0·762 respectively. All other values of ku/ky quoted are identical to extrapolated 24 hr experimental values.
‡ Assumed dose was 'cut-off' after 88·6% had been released.
Values in parentheses are the t/2 values, in hr, equivalent to the rate constant above.

data from subject 4. This difference was not readily apparent when fitting the cumulative excretion data and in the post-absorption period an excellent fit was obtained to both rate and cumulative plots.

Computer fits to 3 × 5 mg data were less satisfactory since trials indicated the possibility of a "dose-effect" in the distribution or elimination of the drug, or both. No significant differences in the elimination half-life were apparent when (+)-amphetamine sulphate was given in a single 15 mg dose ("solution", "free pellet", or "capsule" forms) and

when it was given in a divided 3 × 5 mg regimen (4 trials; 4 subjects) or a single 5 mg dose (3 trials; 3 subjects) in the same forms. However, a slightly smaller amount (proportionately *ca* 10% less) of unchanged amphetamine was excreted with the two latter regimens (see Table 3).

TABLE 3. RECOVERY OF UNCHANGED AMPHETAMINE FROM 3 × 5 MG AND SINGLE 5 MG DOSAGE REGIMENS RELATIVE TO RECOVERY FROM A SINGLE 15 MG DOSE.

Subject	Dose (equiv. mg SO_4)	Form	Relative recovery*
6	3 × 5	Capsule	88·3
6	5	"	92·0
7	3 × 5	"	87·0
5	3 × 5	Free pellets	95·0
5	5	Capsule	87·0
4	3 × 5	Free pellets	88·4
3	5	Solution	88·2

* Relative recovery calculated as: $\frac{\%\text{ dose excreted unchanged (total)}}{\text{mean }\%\text{ 15 mg single dose excreted unchanged (total)}} \times 100$

(+)-*Methylamphetamine.* For the two subjects, it was found that model (II) described the absorption, metabolism, and excretion of (+)-methylamphetamine, and good agreement was obtained between the experimental and the theoretical excretion curves of methylamphetamine and its metabolite, amphetamine. Fitted curves for one subject along with the derived curves for the gastrointestinal tract and body compartments, for both methylamphetamine and amphetamine, are shown in Fig. 6*a*. The solution curve 1 in Fig. 6*b* shows the computer fit to the

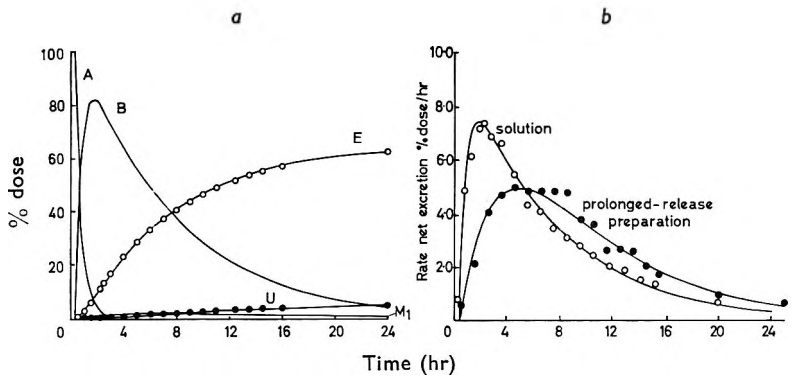


FIG. 6*a*. Computer curves and experimental data points for the elimination of methylamphetamine and amphetamine, after oral administration of a solution of 15 mg (+)-methylamphetamine hydrochloride (Subject 5) (Model II).

b. Computer curves and experimental data points for the urinary excretion of methylamphetamine, after oral administration of a solution of (+)-methylamphetamine hydrochloride and prolonged-release preparation D. (Subject 5) (Models II and IV).

rate of methylamphetamine excretion data in the same subject. A similar result was obtained for the second subject.

The individual kinetic constants are in Table 4. The ratios of rate constants, k_e/k_d, k_{m_2}/k_d, indicate the fraction of the dose present in

TABLE 4. KINETIC PARAMETERS FOR THE RELEASE, ABSORPTION, METABOLISM AND EXCRETION OF (+)-METHYLAMPHETAMINE AFTER 15 MG DOSES OF THE HYDROCHLORIDE IN DIFFERENT DOSAGE FORMS

Subject	Dosage form	Lag time (hr)	kr (hr^{-1})	ka (hr^{-1})	ke (hr^{-1})	km_1 (hr^{-1})	km_2 (hr^{-1})	kd (hr^{-1})	ke/kd	km_1/kd	km_2/kd	ku (hr^{-1})	km_3 (hr^{-1})	ky (hr^{-1})	ku/ky	km_3/ky	km_1/kd × ku/ky
5	Solution	0·4	—	2·0	0·090	0·010	0·039	0·139 (4·99)	0·648	0·072	0·281	0·201	0·044	0·245 (2·83)	0·820	0·180	0·059
4	,,	0·3	—	3·1	0·087	0·011	0·039	0·137 (5·05)	0·635	0·080	0·285	0·215	0·061	0·276 (2·51)	0·779	0·221	0·062
5	Prepn D	0·6	0·270	2·0	0·093	0·010	0·035	0·138 (5·02)	0·674	0·073	0·254	0·201	0·044	0·275 (2·83)	0·820	0·180	0·060
4	,,	0·5	0·300	2·7	0·114	0·011	0·039	0·164 (4·23)	0·695	0·067	0·238	0·300	0·085	0·385 (1·80)	0·779	0·221	0·052

Values in parentheses are the t/2 values, in hr, equivalent to the rate constants above.

compartments E and M_2 respectively, at infinite time, assuming there to be no change in elimination half-life beyond 24 hr. The ratio km_1/kd indicates the fraction of the dose which is metabolized to M_1 (i.e. amphetamine) and the fate of this fraction is controlled by the ratios ku/ky and km_3/ky. Hence, the fraction of the dose found in the urine at infinite time, as unchanged methylamphetamine and amphetamine, is given by the ratios ke/kd and km_1/kd × ku/ky respectively.

"*Capsule*" *and* "*Prolonged-release*" *drug preparations*

(+)-*Amphetamine*. Model (III) was fitted to the experimental data and typical fitted cumulative and rate of excretion curves, along with the derived curve for the amount of amphetamine in the formulation, are shown in Fig. 7*a* ("capsule" data) and Fig. 7*b* ("preparation B" data). The kinetic parameters for each individual subject are shown in

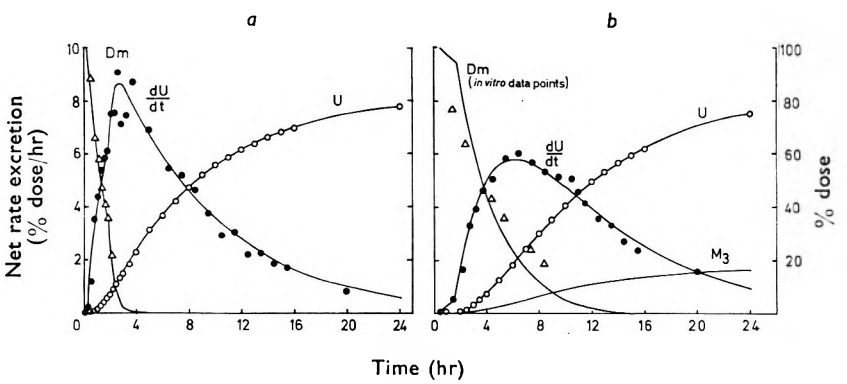


FIG. 7*a*. Computer curves and experimental data points for the urinary excretion of amphetamine, after oral administration of 15 mg (+)-amphetamine sulphate in 'capsule' form. (Subject 5) (Model III).

b. Computer curves and experimental data points for the absorption and elimination of amphetamine, after oral administration of prolonged-release preparation B. (Subject 5) (Model III).

Table 2. Since ka was relatively large, its value was not critical and was assumed to be 2·8 hr^{-1} in each instance (cf. "solution" and "free pellet" data). By assuming two sequential release rates, governed by kr_1 and kr_2, good computer fits to the data were obtained. Only for the "capsule" data in two subjects, could simulations be made using a single release rate constant.

The values for the release constants determined in fitting the preparation B data for subject 6 are arbitrary since it is assumed that the preparation ceases releasing drug after loss of 88·6% of the dose. The recovery of unchanged amphetamine in this trial, relative to that after single 15 mg "capsule" doses to the same subject, indicated that only 88·6% of the dose was available for absorption. Therefore, the output from the D_m compartment was "cut-off" (manually) after 8·86 V had passed into the gut integrator. It could also be argued that only 88·6% of the dose was available at zero time or that reduced availability was spread over a period of time, in which cases slightly different values for the release rate constants would be obtained.

(+)-*Methylamphetamine.* Model (IV) was fitted to the experimental data for "preparation D". The fitted cumulative excretion curve, for one subject, along with derived curves for the amount of methylamphetamine in the maintenance form and in the gut is shown in Fig. 8.

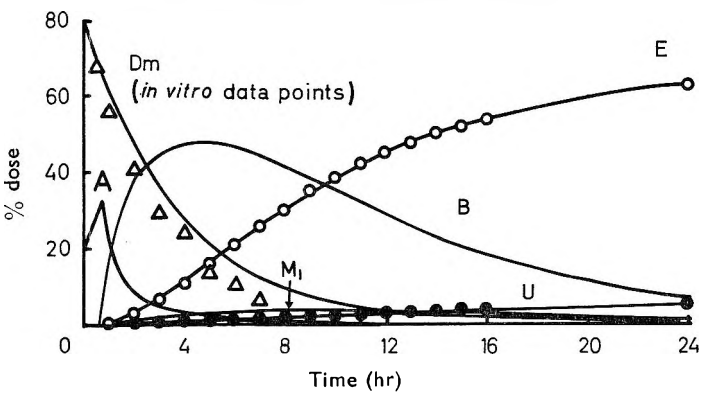


FIG. 8. Computer curves and experimental data points for the absorption and elimination of methylamphetamine and the elimination of amphetamine, after oral administration of prolonged-release preparation D. (Subject 5) (Model IV).

Predicted body levels of methylamphetamine and amphetamine are also shown. The prolonged release curve in Fig. 6*b* shows the computer fit to the rate of methylamphetamine excretion data in the same subject. A similar result was obtained for the second subject. The kinetic parameters for the two subjects used are shown in Table 4. Excellent fits to the data were obtained, assuming 20% of the dose to be in a "free" form and using a single release rate constant. This is consistent with the *in vitro* release data in Table 1.

Preliminary inspection of the semi-logarithmic plot of the methylamphetamine excretion data for preparation D in subject 4 indicated

that the elimination half-life of the drug was significantly lower than that observed after the "solution" dose in the same subject (see Table 4). In subject 5, however, the corresponding half-life was almost identical after the "solution" and prolonged-release forms.

In vivo/in vitro CORRELATIONS

In Fig. 9 the percentage of drug released *in vivo* from the maintenance forms of preparations B and D is plotted against the percentage of drug released *in vitro* after the same time intervals. The rotating bottle *in vitro* data were used for preparation B and the rolling bottle data for

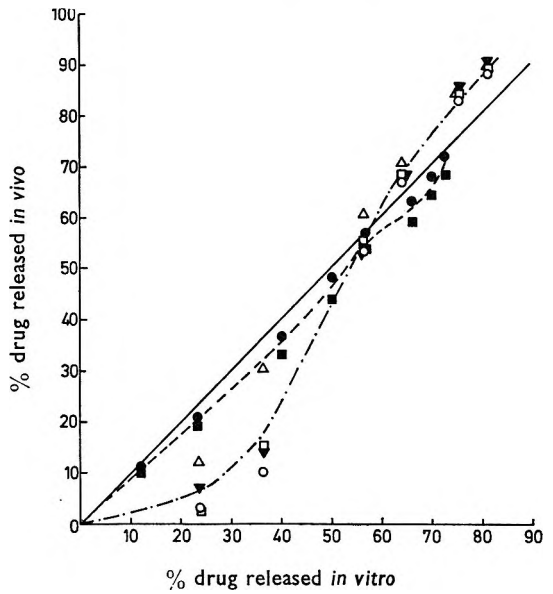


FIG. 9. *In vitro/in vivo* drug release correlations with two prolonged-release preparations: Preparation B containing amphetamine ○□▼△. Preparation D containing methylamphetamine ●■. ○▼● subject 4. □■ subject 5. △ subject 6.

preparation D (see Table 1). If complete correlation between *in vivo* and *in vitro* results were obtained the experimental points would lie on the solid line shown.

Discussion

"SOLUTION" AND "FREE PELLET" DATA

In general there was good agreement between the theoretical urinary excretion curves, based on models (I) and (II), and the experimental values. Thus, Figs 5 and 6 indicate the suitability of the models to describe the kinetics of absorption, metabolism, and excretion, in man, of the amphetamine preparations considered; they also show the usefulness of the analogue computer in analysing urinary excretion data. One

observation however, served to emphasize that although the models apparently described *in vivo* situations reasonably well, physiological reality remains much more complex. Thus, the inability to simulate peak rate of amphetamine excretion levels in two of the five subjects, despite correct computer-experimental correlation for the remaining excretion, indicates that consideration of the body as a single, homogeneous compartment may not always be justified. Rate constant, k, values determined using simple models of the type described, will always be hybrid constants including distribution as well as absorption, metabolism, and excretion. Based on the experimental data available, the use of more sophisticated models would necessitate the introduction of more variables and unknowns.

Inter- and intra-subject variation was apparent in the absorption phase parameters, lag time and ka, for both amphetamine and methylamphetamine (see Tables 2 and 4), although such variation was not great, especially when compared with effects specifically due to formulation. Lag times have previously been reported by several authors (Levy & Hollister, 1964, 1965; Moore, Portmann & others, 1965; Wilkinson, 1966; and others), and several explanations have been suggested, for example, accumulation of drug in the gastrointestinal wall before entry into the blood stream (Levy & Jusko, 1965), and stomach emptying rate limiting the absorption process. The time taken for filtered drug to pass from the kidney glomerulus to the bladder could also contribute to the observed lag time.

In the post-absorption phase, the elimination of amphetamine could be described by simultaneous first-order processes, namely excretion and metabolism. Inter-subject variation occurred in the values of the rate constants for these processes (see Table 2), but, like intra-subject variations, such variations were relatively small. Thus, in the subjects studied, the value for ku, the excretion constant, showed very little variation, i.e. about 0·1 hr^{-1}. A two to two and a half-fold variation was apparent in the value for the metabolic constant, km_3.

The total recovery of unchanged amphetamine and its elimination half-life (t/2), after single 15 mg doses (5 subjects; 11 trials, when "capsule" data are included) ranged from 63·8–81·7% of the dose (mean 74·3%) (see ku/ky values in Table 2) and from 4·17–6·03 hr (mean 4·88 hr), respectively (see Table 2). Recoveries and half-lives were reproducible within a single subject, e.g. subject 4 (4 trials, including "capsule" data) in whom total recoveries ranged from 70·4–77·7% of the dose (mean 73·0%) and the elimination half-life from 4·56–5·00 hr (mean 4·71 hr). Furthermore, no obvious progressive changes were apparent in the above parameters on repeated administration of the drug.

The elimination of methylamphetamine in the post-absorption phase was describable by three simultaneous first-order processes, namely excretion, metabolism to amphetamine, and metabolism to unmeasured metabolite(s). The total recoveries in urine of unchanged methylamphetamine and its metabolite amphetamine were similar in the two subjects used and were independent of the dosage form (see ke/kd and $km_1/kd \times ku/ky$ values in Table 4). Table 4 also shows that, with the

exception of the value obtained with preparation D in subject 4 the elimination half-life of unchanged drug was almost identical in each trial. Furthermore, the results were consistent with those of Beckett & Rowland (1965c), using solution forms of the drug. Using models(II) and (IV), the formation and elimination of amphetamine produced by *N*-demethylation of methylamphetamine, could be described by two consecutive first-order processes, with the elimination consisting of two simultaneous first-order reactions in essentially the same ratio as found after the oral administration of amphetamine itself. In the two subjects studied, the overall elimination rate constant, ky, was larger than the same value determined for (+)-amphetamine after oral administration, the increase being 75–80% (cf. Tables 2 and 4). Wilkinson (1966) reports the same effect from a similar computer analysis of urinary excretion data for ephedrine and its metabolite, norephedrine. The significance of this effect remains to be clarified.

"CAPSULE" AND "PROLONGED-RELEASE" DATA

The release, absorption, and elimination of amphetamine and methylamphetamine, after administration of the various dosage forms, was well described by pharmacokinetic models (III) and (IV), respectively. Furthermore, simulations of the experimental data were possible using parameters for excretion and metabolism essentially consistent with corresponding values obtained with the forms from which drug was rapidly available for absorption (see Tables 2 and 4). In particular, comparison of the "capsule" data in Table 2 with the data for "solution" and "free pellet" forms confirms the absence of marked inter- and intra-subject variation in the elimination of amphetamine under constant acidic urine conditions.

The present results also illustrate the dramatic effect which formulation in hard gelatin capsules can have on the rate at which drug becomes available for absorption (see kr_1 and kr_2 values in Table 2). Although effects due to variation between capsule batches, age, size, or manufacturing procedures were not systematically investigated, the results are supported by the work of Wood (1965) who has also shown, using the onset of serum salicylate levels in man, an appreciable delay (approx. 15 min) in release from hard gelatin capsules, relative to fast disintegrating, rapidly dissolving tablets.

A combination of possibilities could explain the observed "capsule-effect": thus, the gelatin may be slowly and incompletely dissolved forming an adhesive mass, around the drug-coated pellets, from which the drug is slowly released for absorption. Slow dissolution of the gelatin shells observed in the B.P. capsule disintegration test; and reports that agitation within the stomach is relatively mild (Levy, 1963; Steinberg, Frey & others, 1965) suggest this possibility. Alternatively, by preventing an initial dispersion of pellets throughout the stomach contents, the capsule may merely delay passage of the drug through to absorption sites in the small intestine.

Although the explanation of the "capsule-effect" is uncertain, its significance in terms of the pharmacological and clinical evaluation of drugs is immediately apparent. The administration of drugs in hard gelatin capsules is common practice. Consequently, in the determination of relative response times or of time of onset of a given response, the release pattern from the dosage form can be of considerable importance (Wood, 1965).

The curves in Fig. 9 indicate an excellent correlation between *in vivo* and *in vitro* drug release rate for preparation D and a fair correlation for preparation B. *In vivo* release of amphetamine from the latter preparation appears to be slower than *in vitro* release in the earlier time intervals. The reason for the sudden increase in *in vivo* release after the loss of about 10% of the dose is obscure, but does not appear to be related to a "capsule-effect" (cf. subject 4 and preparation B pellet data and also preparation B capsule data, in Table 2) or to delayed stomach emptying (cf. data in Table 2). The significance of these *in vivo/in vitro* correlations must be qualified by the following considerations.

In model (III), "lag time" is a composite of an absorption lag time and a lag time assumed in the release of drug from the formulation (cf. model (IV), where lag time refers to absorption only; the drug is assumed to release from the maintenance form immediately after administration). However, since lag times are relatively short, this factor would not appreciably influence the results.

Since prolonged-release preparations did not produce the high peak excretion rate levels, and therefore presumably lower peak blood drug levels, than solution forms, and results with divided 15 mg doses of amphetamine and doses less than 15 mg have indicated that there may be a "dose-effect" in the distribution or elimination of the drug, or both, the assumption that distribution and elimination processes are completely independent of dosage form may not be valid. Whether such a possibility has any great significance in relation to the reported correlations is doubtful.

Although good computer simulations were obtained for the complete excretion profiles after administration of preparation B to subject 4, simulations of "free" form data in the same subject were not entirely satisfactory over the short period when the excretion rate was at its peak. Therefore, in the determination of *in vivo* release of amphetamine from preparation B, in this subject, errors may have been introduced as a result of considering his "body" as a single homogeneous compartment.

It is impossible to design a laboratory device which will not contribute, by its very design, something to an *in vitro* release rate, but it is essential that its design be such that the contribution of the apparatus is minimal if *in vivo/in vitro* correlations are to be meaningful. Accordingly, release of methylamphetamine from preparation D was found to be essentially the same using three types of apparatus (see Fig. 4), and the release of amphetamine from preparation B was determined under conditions such that the observed release rate was due to the permeability of the membranes and not significantly to the conditions of the test

(Dr. R. Goldman, personal communication). The degree of agitation to be used in any *in vitro* test is usually one of the considerations of greatest concern. However, when diffusion from within a particle or matrix (as in preparations B and D respectively), assumes the controlling step in drug release, rather than a dissolution rate, then a lack of marked dependence on agitation intensity might reasonably be expected (see Wood, 1967). It is significant that other studies using matrix-type products have also demonstrated good *in vivo/in vitro* correlations (Wiegand & Taylor, 1960; Sjögren & Ostholm, 1961; Brändström & Sjögren, 1967).

The process of curve-fitting using the analogue computer has an element of subjectivity. Nevertheless, considering the simplicity of the pharmacokinetic models which were applicable this method of data analysis is adequate to give meaningful results consistent with the precision of the analytical data.

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APPENDIX 1

The terms used in the equations are:

t,	time in hr after ingestion of the dose.
Lag time,	the time interval between ingestion of the dose and zero time.
Zero time,	the time at which loss of drug from the gastrointestinal tract may be described as a first-order process.
Break time,	the time after dosage at which kr_1 is changed to kr_2.
A,	the amount of drug (amphetamine or methylamphetamine) present in the gastrointestinal tract.
B,	the amount of methylamphetamine in the body.
M_1,	the amount of amphetamine in the body.
M_2,	the amount of metabolite(s) of methylamphetamine, other than amphetamine, in the body.
M_3,	the amount of metabolite(s) of amphetamine in the body.
E,	the amount of methylamphetamine in the urine.
U,	the amount of amphetamine in the urine.
D_m,	the amount of drug (amphetamine or methylamphetamine) in "maintenance" dosage form.
f_1,	the fraction of dose in "free" form.
f_m,	the fraction of dose in "maintenance" form.
ka,	the rate constant for the absorption of drug (amphetamine or methylamphetamine) from the gastrointestinal tract into the body.
ke,	the rate constant for the excretion of methylamphetamine from the body into the urine.
km_1,	the rate constant for the formation of amphetamine from methylamphetamine.
km_2,	the rate constant for the formation of metabolite(s) of methylamphetamine, other than amphetamine.
kd,	the rate constant for the elimination of methylamphetamine from the body by all processes, i.e. $kd = ke + km_1 + km_2$.
ku,	the rate constant for the excretion of amphetamine from the body into the urine.
km_3,	the rate constant for the formation of metabolite(s) of amphetamine.
ky,	the rate constant for the elimination of amphetamine from the body by all processes, i.e. $ky = ku + km_3$.

k_r, the rate constant for the release of drug (amphetamine or methylamphetamine), from "maintenance" dosage form into the gastrointestinal tract.

The term "maintenance" form refers to dosage forms from which the drug is not immediately available for absorption, i.e. formulated fractions of prolonged-release preparations *and* capsule forms.

References

Beckett, A. H. & Rowland, M. (1965a). *J. Pharm. Pharmac.*, **17**, 628–639.
Beckett, A. H. & Rowland, M. (1965b). *Ibid.*, **17**, 59–60.
Beckett, A. H. & Rowland, M. (1965c). *Ibid.*, **17**, 109S–114S.
Beckett, A. H. & Tucker, G. T. (1966). *Ibid.*, **18**, 72S–75S.
Brändström, A. & Sjögren, J. (1967). *Acta pharm. suecica*, **4**, 147-157.
Braun, W., Hesse, I. & Malorry, E. (1963). *Arch. exp. Path. Pharmak*, **245**, 457–470.
Elliot, J. S., Sharp, R. F. & Lewis, L. (1959). *J. Urol.*, **81**, 339–343.
Garrett, E. R. & Alway, C. D. (1963). In *Proc.IIIrd Intern. Congr. Chemother.*, p. 1666, Stuttgart: Georg Thieme.
Garrett, E. R., Johnston, R. L. & Collins, E. J. (1963). *J. pharm. Sci.*, **52**, 668–678.
Krueger, E. O. & Vliet, E. B. (1962). *Ibid.*, **51**, 181–184.
Levy, G. (1963). *Ibid.*, **52**, 1039–1046.
Levy, G. & Hollister, L. E. (1964). *Ibid.*, **53**, 1446–1452.
Levy, G. & Hollister, L. E. (1965). *Ibid.*, **54**, 1121–1125.
Levy, G. & Jusko, W. J. (1965). *Ibid.*, **54**, 219–225.
Milne, M. D., Scribner, B. H. & Crawford, M. A. (1958). *Am. J. Med.*, **24**, 709–729.
Moore, W. E., Portmann, G. A., Stander, H. & McChesney, E. W. (1965). *J. pharm. Sci.*, **54**, 36–41.
Peters, L. (1960). *Pharmac. Rev.*, **12**, 1–35.
Sjögren, J. & Ostholm, I. (1961). *J. Pharm. Pharmac.*, **13**, 496–503.
Souder, J. C. & Ellenbogen, W. C. (1958). *Drug Stand.*, **26**, 77–83.
Steinberg, W. H., Frey, G. H., Masci, J. N. & Hutchins, H. H. (1965). *J. pharm. Sci.*, **54**, 747–752.
Taylor, J. D. & Wiegand, R. G. (1962). *Clin. Pharmac. Ther.*, **3**, 464–472.
Tucker, G. T. (1967). Ph.D. thesis, University of London.
Wagner, J. T. & Nelson, E. (1964). *J. pharm. Sci.*, **53**, 1392–1403.
Weiner, I. M. & Mudge, G. H. (1964). *Am. J. Med.*, **36**, 743–762.
Wiegand, R. G. & Taylor, J. D. (1960). *Biochem. Pharmac.*, **3**, 256–263.
Wilkinson, G. R. (1966). Ph.D. thesis, University of London.
Wood, J. H. (1965). *J. pharm. Sci.*, **54**, 1207–1208.
Wood, J. H. (1967). *Pharm. Acta Hel.*, **42**, 129–151.

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The effect of temperature on end-plate depolarization of the rat diaphragm produced by suxamethonium and acetylcholine

J. B. HARRIS* AND G. D. H. LEACH

End-plate depolarization in the rat isolated diaphragm by suxamethonium and acetylcholine was measured at 37° and 20° using a modification of the method of Fatt (1950). At 20° the response of the motor end-plates to the depolarizing activity of acetylcholine and suxamethonium was enhanced. Since the presence of neostigmine in the bath fluid did not modify the response of the motor end-plates to the depolarizing activity of suxamethonium at 37°, the results suggest the enhanced response of the motor end-plates at 20° is due to a change in the properties of the motor end-plate, and not to a change in the activity of cholinesterases present in the tissues.

IT is well known that muscle temperature affects the activity of skeletal neuromuscular blocking agents. Holmes, Jenden & Taylor (1951), investigating the effects of tubocurarine on the rat isolated diaphragm, showed that the neuromuscular blocking activity of tubocurarine was reduced at low temperatures. Using the rat isolated diaphragm, and nerve muscle preparations in anaesthetized cats, dogs and rabbits, Bigland, Goetzee & others (1958) showed that the neuromuscular blocking activity of tubocurarine was reduced at low temperatures, whereas the neuromuscular blocking activity of suxamethonium and decamethonium was enhanced. The nature of the neuromuscular blockade produced by these drugs was qualitatively unaltered. These results suggested that cooling sensitizes the muscle end-plates to the action of depolarizing drugs. Similar results were obtained by Cannard & Zaimis (1959) in man.

Whittaker (1962a, b), using the rat isolated diaphragm, showed that the neuromuscular blocking activity of suxamethonium was enhanced at low temperatures, and also that at these temperatures the characteristics of neuromuscular blockade by suxamethonium more closely resembled pure depolarization block than at normal temperatures.

In view of these results it was of interest to investigate the ability of acetylcholine and suxamethonium to depolarize the end-plate region of the rat isolated diaphragm preparation at normal temperature (37° ± 1°) and at low temperature (20° ± 1°).

Experimental

METHOD

Segments of the right hemidiaphragm, 1 cm wide, were prepared by making parallel cuts in the tissue equidistant from the insertion of the right phrenic nerve. The preparation was immersed in Krebs solution aerated with oxygen 95%, carbon dioxide 5%, and allowed to equilibrate

From the Postgraduate School of Studies in Pharmacology, The University of Bradford, Bradford, Yorkshire, England.

**Present address*: Muscular Dystrophy Group Research Laboratories, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne, 4, England.

for 45 min before commencing the experiment. End-plate depolarization was measured by the method of Fatt (1950), suitably modified for use with mammalian tissue, using the method illustrated in Fig. 1. For recording purposes, aeration was interrupted and the fluid level lowered to dislodge any gas bubbles adhering to the preparation, thus removing a possible source of error; end-plate depolarization was measured as the fluid electrode was allowed to sweep upwards. Electrical changes were recorded from $Ag/AgCl_2$ electrodes embedded in Krebs agar, and measured directly on a Tetronix 502A oscilloscope after amplification through the direct coupled amplifier of the oscilloscope. Records of end-plate

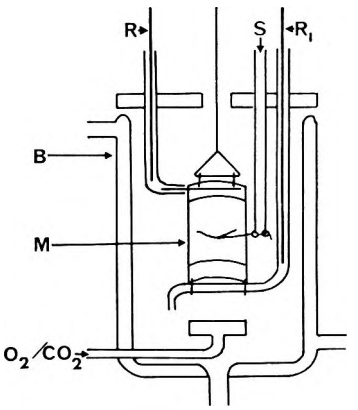

FIG. 1. Method used for measuring end-plate depolarization in the rat isolated diaphragm. B: Jacketed organ bath, 80 ml capacity. M: Segment of rat diaphragm mounted with remnants of rib cage uppermost. R: $Ag/AgCl_2$ electrode in Krebs agar. Contact to nerve-free rib cage remnants made through a pig bristle. R_1: $Ag/AgCl_2$ electrode in Krebs agar, open to Krebs solution. O_2/CO_2: Gas supply; aeration through sintered glass bubbler. S: stimulating electrodes (not used in these experiments).

depolarization were taken 1, 2, 4, 8 and 16 min after the addition of drugs. Bath temperature was maintained at normal (body) temperature (37° ± 1°) or at low (room) temperature (20° ± 1°) by means of a heat exchanger-pump unit. Preparations that exhibited injury potentials of 1 mV, or more were discarded. Drug concentrations refer to acetylcholine bromide and suxamethonium chloride. In experiments in which the end-plate depolarization induced by acetylcholine was measured, neostigmine, 1×10^{-6} g/ml was added to the Krebs solution to inhibit the action of cholinesterases.

The results are presented as end-plate depolarization in mV plotted against log time in min.

Results

At 37°, acetylcholine, $0{\cdot}5 \times 10^{-6}$ g/ml, in the presence of neostigmine, induced a depolarization of the end-plate region which remained constant

for about 4 min, and thereafter slowly declined. The peak depolarization observed was about 3 mV. At 20°, however, the same concentration of acetylcholine depolarized the end-plate region to a greater extent than at 37° and the peak depolarization (about 8 mV) was reached after about 4 min (Fig. 2a). Essentially similar results were observed when end-plate

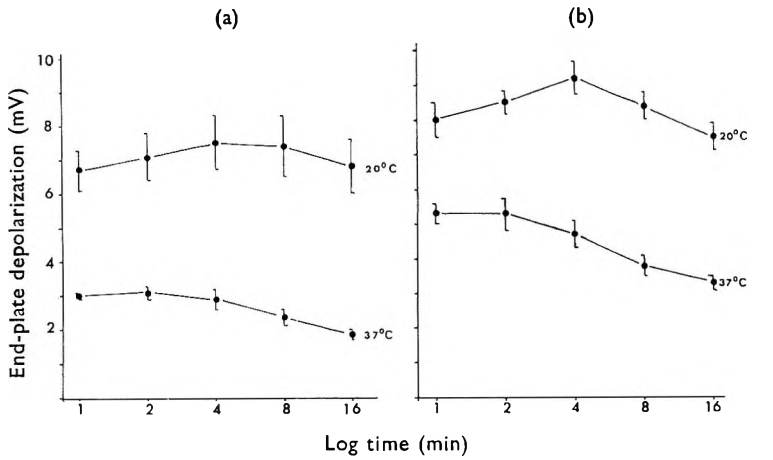

FIG. 2. End-plate depolarization in the isolated rat diaphragm at 20° and 37°. Each point mean ± s.e. of mean (n = 5 or 6). (a) Acetylcholine $0{\cdot}5 \times 10^{-6}$ g/ml in the presence of neostigmine 1×10^{-6}. Note that maximum depolarization at 37° occurs at 2 min with a value of 3·1 ± 0·2 mV. At 20°, maximum depolarization occurs at 4 min, with a value of 7·5 ± 0·8 mV. (b) Suxamethonium, 5×10^{-6} g/ml. Maximum depolarization at 37° occurs at 1–2 min, with a value of 5·3 ± 0·5 mV. At 20°, maximum depolarization occurs at 4 min with a value of 9·2 ± 0·5 mV.

depolarization was measured using suxamethonium, 5×10^{-6} g/ml, as the depolarizing agent (Fig. 2b).

Since suxamethonium is hydrolysed by cholinesterases (Bovet & Bovet-Nitti, 1955), the potentiation of end-plate depolarization by suxamethonium at 20° could be complicated by a reduction in the activity of cholinesterases at the lower temperature, which would result in a higher effective concentration of suxamethonium present in the bath under these conditions.

Fig. 3 illustrates a control experiment in which the end-plate depolarization to suxamethonium at 37° was measured in the presence of 1×10^{-6} g/ml neostigmine, the result being compared to that obtained in the absence of neostigmine. The end-plate depolarization induced by suxamethonium in the presence of neostigmine was insignificantly potentiated, although the decline in depolarization with time was slower, indicating that the hydrolysis of suxamethonium may be a causative factor in the relatively rapid decline of activity of suxamethonium. It is obvious, however, that the reduction of cholinesterase activity at low temperature cannot satisfactorily explain the potentiated end-plate depolarization induced by suxamethonium.

Discussion

The mechanism by which suxamethonium and decamethonium interrupt neuromuscular transmission is complex. In the intact cat (with the exception of certain red muscles, e.g., soleus) neuromuscular blockage is produced by a persistent depolarization of the end-plate region (Burns & Paton, 1951). In most other species, however, the mechanism of action of neuromuscular blockade induced by these drugs has overall intermediate characteristics of block by both depolarization and by competition. In these species, neuromuscular blockade by these drugs is initially of the depolarization type which later changes to a competitive type of action and has been called "dual" by Zaimis (1953). The mechanism of action of these drugs in *in vitro* preparations is confusing. Neuromuscular

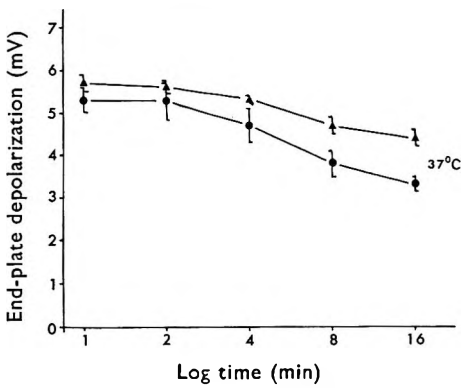

FIG. 3. End-plate depolarization in the isolated rat diaphragm at 37°. Each point mean ± s.e. of mean (n = 4 or 5). Solid circles: Suxamethonium 5 × 10^{-6} g/ml. Solid triangles: Suxamethonium, 5 × 10-6 g/ml in the presence of neostigmine, 1 × 10^{-6} g/ml. Note the insignificant potentiation of end-plate depolarization by suxamethonium in the presence of neostigmine at 37° for 2–4 min. There is some potentiation of the end-plate depolarization by suxamethonium in the presence of neostigmine after 4 min.

blockade takes place in two phases. In phase 1, the onset of block is rapid and reaches a maximum in 5–10 min. During this phase, the action of neuromuscular block appears to be by depolarization. After phase 1 neuromuscular transmission is partially restored, and then gives way to phase 2, a period of slowly developing neuromuscular block with the characteristics of block by competition with acetylcholine (Maclagan 1962). This biphasic action does not occur in *in vivo* preparations (Maclagan, 1962). Consideration of these points suggests that caution is necessary when attempts are made to transpose results obtained in one species to a situation in another species, or in attempting to transpose results obtained *in vitro* to a situation *in vivo*.

Suxamethonium is known to be hydrolysed by cholinesterases (Bovet & Bovet-Nitti, 1955). The first hydrolytic product is succinylmonocholine which has been shown to possess a weak neuromuscular blocking activity with features of a competitive type of action (Stovner, 1958). In view of

this, Whittaker (1962, b) suggested that an increase in the neuromuscular blocking activity of suxamethonium at low temperatures might be related to an inhibition of cholinesterases. However, this is unlikely to be the major factor for two reasons. Firstly, from the results here presented, the end-plate depolarizing activity of suxamethonium at 37° is hardly changed by the presence of a concentration of neostigmine known to inhibit more than 99% of total cholinesterases in the isolated homogenized rat diaphragm (Leach, unpublished observation); secondly Bigland & others (1958) have shown that the neuromuscular blocking activity of decamethonium is affected by temperature at least as much as is suxamethonium, even though decamethonium is not hydrolysed by cholinesterases (Zaimis, 1950).

Bigland & others (1958) suggested that their results might be explained by supposing that at low temperatures the end-plates are specifically sensitized to the action of agonist drugs, which would lead to an enhanced action of acetylcholine, suxamethonium and decamethonium at the neuromuscular junction. This suggestion could also be applied to the findings of Letley (1960) who showed that contracture of the isolated denervated rat diaphragm produced by a wide range of drugs including acetylcholine, suxamethonium and decamethonium was potentiated at low temperatures.

Although caution is necessary in attempting to transpose results obtained *in vitro* to a situation *in vivo*, the present results would support the supposition of Bigland & others (1958). Since suxamethonium is capable of depolarizing the end-plate region much more readily at low temperature than at normal temperatures, the obvious implication is that the drug would more readily interrupt neuromuscular transmission at low temperatures.

The present finding that acetylcholine produces larger end-plate depolarization at low temperature suggests that normal neuromuscular transmission may be similarly augmented by cooling. If this is so, it would provide an explanation for the fact that the neuromuscular blocking activity of tubocurarine is reduced at low temperature.

References

Bigland, B., Goetzee, B., Maclagan, J. & Zaimis, E. J. (1958). *J. Physiol., Lond.*, **141**, 425–434.
Bovet, D. & Bovet-Nitti, F. (1955). *Sci. Med. Italica*, English Edn., **3**, 484–513.
Burns, B. D. & Paton, W. D. M. (1951). *J. Physiol., Lond.*, **115**, 41–73.
Cannard, T. H. & Zaimis, E. J. (1959). *Ibid.*, **149**, 112–119.
Fatt, P. (1950). *Ibid.*, **111**, 408–422.
Holmes, P. E. B., Jenden, D. J. & Taylor, D. B. (1951). *J. Pharmac. exp. Ther.*, **103**, 382–402.
Letley, E. (1960). *Br. J. Pharmac. Chemother.*, **15**, 345–350.
Maclagan, J. (1962). *Ibid.*, **18**, 204–216.
Stovner, J. (1958). *Acta anaesth. scand.*, **2**, 53–67.
Whittaker, R. (1962, a). *J. Pharm. Pharmac.*, **14**, 177–181.
Whittaker, R. (1962, b). *Ibid.*, **14**, 803–807.
Zaimis, E. J. (1950). *Br. J. Pharmac. Chemother.*, **5**, 424–430.
Zaimis, E. J. (1953). *J. Physiol., Lond.*, **122**, 238–251.

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The effects of tremorine and oxotremorine in hyperthyroid mice

R. WAITE AND P. S. J. SPENCER

Hyperthyroid mice exhibit only transient low-level tremor and hypothermia in response to tremorine and oxotremorine, yet the peripheral effects of these two agents are similar in intensity and duration to those elicited in control animals. These findings support the theory that tremor and hypothermia are both central effects and separate from the peripheral muscarinic actions of tremorine and oxotremorine. Of several possible explanations for the reduction in tremor and hypothermia, the most likely appears to be a potentiation of central adrenergic mechanisms in the hyperthyroid mice.

TREMORINE(1,4-dipyrrolidino-2-butyne) produces both central and peripheral muscarinic effects in laboratory animals (Everett, 1956). These effects are mediated through its active metabolite oxotremorine, (Kocsis & Welch, 1960; George, Haslett & Jenden, 1962). In the mouse, tremorine and oxotremorine cause sustained tremors, miosis, salivation, diarrhoea and hypothermia, all of which can be prevented by centrally-acting anticholinergic drugs (Blockus & Everett, 1957), and to a large extent by centrally-acting adrenergic drugs (Spencer, 1965, 1966; Morpurgo, 1967).

For several reasons, some interaction might be expected between a hyperthyroid state and the effects of tremorine and oxotremorine. A frequent feature of hyperthyroidism in man is a tremor of the hands or of the whole body (Morgans, 1964), which suggests that the tremorgenic effects of both compounds would be enhanced in hyperthyroid animals. On the other hand, treatment with 3,5,3′-triiodothyropropionic acid reduces or prevents the hypothermia produced by chlorpromazine in the rat and ground squirrel (Hoffman, 1959). Potentiation of the cardiovascular and metabolic actions of catecholamines by thyroid hormones has been widely reported (for references see Hess & Shanfeld, 1965); it is possible that thyroid hormones would potentiate catecholamines also at central sites. If this were so, the effects of tremorine and oxotremorine might be antagonized in a way analogous to that produced by the administration of centrally-acting adrenergic drugs such as amphetamine and imipramine (Spencer, 1965, 1966; Morpurgo, 1967).

This report shows that tremorine-induced and oxotremorine-induced hypothermia and tremor are substantially reduced in hyperthyroid mice.

Experimental

Groups of 8 or 10 male adult albino mice of a TO strain, weighing 20 to 25 g, were maintained on water and a 41B cube diet, and kept under constant environmental conditions at 20 to 22°. A maximal level of hyperthyroidism was produced in the mice by the daily subcutaneous

From The Department of Pharmacy, The University of Aston, Birmingham, 4, England.

injection (neck) of thyroxine sodium, 2 mg/kg for 10 days; control animals received the vehicle, 0·01 N sodium hydroxide in 0·9% saline solution, for the same length of time. On the 11th day, 24 hr after the last pre-treatment injection, mice received a subcutaneous injection (flank) of tremorine hydrochloride, 20 mg/kg, or oxotremorine oxalate, 0·5 mg/kg, dissolved immediately before use in 0·9% saline solution. [Preliminary work with this dose of thyroxine had shown that TO mice are maximally hyperthyroid on the 11th day after commencing treatment, using the mouse anoxia test (Spencer & West, 1961) and the oxygen uptake test (MacLagan & Sheahan, 1950) to assess the level of hyperthyroidism].

The effects of tremorine and oxotremorine were determined as follows: *hypothermia* was assessed by measuring oesophageal temperatures of mice before and at intervals after the injection of the compounds, using an oesophageal thermocouple and calibrated electric thermometer (Brittain & Spencer, 1964). Group data have been expressed as the mean $\pm$ s.e., using Student's *t*-test to establish the significance of difference between groups. At the same time, each animal was observed briefly, to assess the intensity of *tremor*, using the following arbitrary scoring system: no tremor = 0, moderate or intermittent tremor = 1, and severe or continuous tremor = 2 marks. Group data have been expressed as the mean score, or "tremor index", and the significance of difference between groups calculated using a modified *t*-test. (The assumption was made that the central limit theorem held, and that the individual variances of different groups were not significantly different from one another). The measurement of oesophageal temperatures and tremor in a group of 10 mice consistently took less than 2 min, this period being spent equally before and after the nominated observation time. In addition, the mice were observed continuously for the presence or absence of peripheral muscarinic effects, particularly salivation and diarrhoea. All of these effects, tremor, hypothermia and the peripheral effects, were observed "blind", the identity of the groups being made known to the operator only at the end of the total observation period.

Results

The production of a hyperthyroid state in mice, using daily injections of thyroxine, did not induce a state of tremor; at no time was a low-level or intermittent tremor observed before the administration of a tremorgenic agent. The oesophageal temperatures of hyperthyroid mice were not significantly different to those of control mice before the administration of tremorine or oxotremorine.

Fig. 1 illustrates the levels of tremor and hypothermia produced by the compounds in control and hyperthyroid mice.

The presence of a hyperthyroid state caused a substantial reduction in the severity and duration of tremorine-induced hypothermia; at each of the three observation times after its injection, the hyperthyroid mice had significantly less hypothermia ($P = <0{\cdot}001$). Similarly, the tremor induced in hyperthyroid mice by tremorine was more transient and of

lower intensity than in controls (P = <0·05 at 60 min). This reduction in effect of tremorine was not due to an effect of thyroxine on liver enzymes, preventing the *in vivo* appearance of its metabolite, oxotremorine, since the the effects of injected metabolite were also markedly reduced in hyperthyroid mice (Fig. 1). After oxotremorine injection, there was a highly significant reduction in the level of hypothermia at each of the observation times (P = <0·001), and the tremor was almost completely inhibited; only occasional mice exhibited a low-level, intermittent tremor, and this reduction in effect was statistically significant (P = <0·05 at 30 and 60 min). When no compound was administered, a subcutaneous injection of 0·9% saline solution, 0·2 ml/20 g body weight, produced neither tremor nor a change in oesophageal temperatures of either control or hyperthyroid mice.

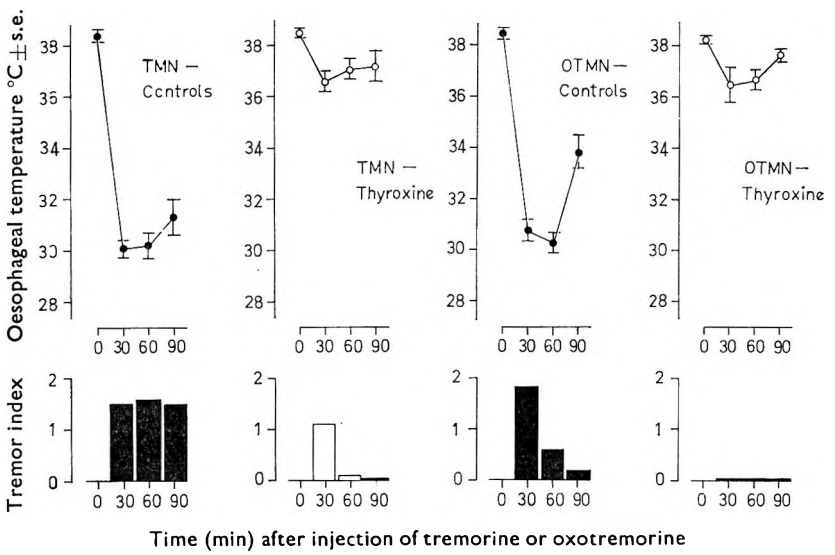

FIG. 1. Tremorine-induced (TMN) and oxotremorine-induced (OTMN) tremor and hypothermia in control and thyroxine-treated mice. Mice were pretreated with thyroxine, (2 mg/kg), or the vehicle, for 10 days; on the 11th day, each mouse received a s.c. injection of tremorine (20 mg/kg) or oxotremorine (0·5 mg/kg).

In contrast to the marked reductions in tremor and hypothermia, the production of a hyperthyroid state had no inhibitory effect upon the intensity and duration of the peripheral muscarinic effects of injections of both compounds, marked salivation and diarrhoea being produced in the hyperthyroid mice and instead of being reduced, these effects occasionally showed evidence of potentiation.

In a final experiment, oxotremorine was injected into control mice kept in an incubator at 30° for 2 hr before, and throughout the experiment. Under these conditions, these mice did not show hypothermia in response to oxotremorine, yet the intensity of tremor was as great as that present in

control mice kept at 22° and given oxotremorine. It was concluded that the absence of hypothermia at 30° in the response to the compounds would not cause a reduction in tremor similar to that observed in hyperthyroid mice.

Discussion

Previously it has been shown that peripherally-acting (quaternary) anticholinergic drugs abolish the peripheral muscarinic effects of tremorine and oxotremorine, without reducing the intensity of tremor or hypothermia. This finding led to the suggestion that hypothermia, like tremor, was centrally mediated (Spencer, 1965). The present finding, that peripheral muscarinic effects such as salivation and diarrhoea (which must be associated with marked heat losses) are not necessarily followed by the appearance of hypothermia, tends to confirm this suggestion.

In addition to there being distinct central and peripheral components to the actions of tremorine and oxotremorine, it is known that tremor and hypothermia are mediated separately. The tremor produced by oxotremorine is not a shiver brought about by the impending hypothermia; Blockus & Everett (1957) showed that if an animal's body temperature is prevented from falling, for example by conducting the experiment in an incubator, there is no reduction in the tremor. This we have confirmed. It follows that the reduction in tremor intensity observed in hyperthyroid mice is not due to the simultaneous reduction in the level of induced hypothermia. Similarly, it is not essential to produce tremor before there can be a hypothermia, since the administration of centrally-acting muscle-relaxant drugs, such as chlordiazepoxide, will abolish the tremor without affecting the hypothermia present (Spencer, 1965).

Yet it is likely that the simultaneous reductions in tremor and hypothermia observed in hyperthyroid mice are due to a similar mechanism. For example, the conversion of tremorine to oxotremorine may be hindered in some way in hyperthyroid mice, although this would not explain why the peripheral effects of tremorine are not reduced, nor why there is a reduction of tremor and hypothermia due to injected oxotremorine. Similarly, it is not likely that the biological inactivation of oxotremorine is enhanced in hyperthyroid mice, because the intensity and duration of its peripheral effects are no less than in controls. Another explanation could be that penetration of oxotremorine into nervous tissue is impaired in hyperthyroid animals; at present there is no evidence to support or refute this suggestion. A more likely explanation is that central adrenergic mechanisms are enhanced in hyperthyroid mice. For example, Prange & Lipton (1962) showed that the toxicity of imipramine is increased in hyperthyroid mice. In these laboratories, we have observed that the pharmacological effects of amphetamine are potentiated in hyperthyroid mice. Also, there is ample evidence that peripheral adrenergic mechanisms are potentiated in hyperthyroid animals (Hess & Shanfeld, 1965), and it is not unlikely potentiation also occurs centrally. Previous work (Spencer, 1965; 1966; Morpurgo, 1967) has shown that

centrally-acting adrenergic drugs, such as amphetamine and the imipramine-like drugs, antagonize the effects of tremorine and oxotremorine, whereas peripherally-acting adrenergic drugs are inactive or far less active. Whether or not the observed antagonism of the central effects of tremorine and oxotremorine in hyperthyroid mice is due to an enhancement of central adrenergic mechanisms remains to be proved.

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References

Blockus, L. E. & Everett, G. M. (1967). *Fedn. Proc. Fedn. Am. Socs exp. biol.*, **16**, 283.
Brittain R. T. & Spencer, P. S. J. (1964). *J. Pharm. Pharmac.*, **16**, 565–567.
Everett, G. M. (1956). *Nature, Lond.*, **177**, 1238.
George, R., Haslett, W. L. & Jenden, D. J. (1962). *Life Sci.*, **1**, 361–363.
Hess, M. E. & Shanfeld, J. (1965). *J. Pharmac. exp. Ther.*, **148**, 290–297.
Hoffman, R. A. (1959). *Am. J. Physiol.*, **197**, 657–659.
Kocsis, J. J. & Welch, R. M. (1960). *Pharmacologist*, **2**, 87.
MacLagan, N. F & Sheahan, M. M. (1950). *J. Endocr.*, **6**, 456–462.
Morgans, M. E. (1964). In *Hyperthyroidism*, Vol. II of *The Thyroid Gland*, editors Pitt-Rivers & Trotter; London: Butterworths.
Morpurgo, C. (1967). *Life Sci.*, **6**, 721–731.
Prange, A. J. & Lipton, M. A. (1962). *Nature, Lond.*, **196**, 588–589.
Spencer, P. S. J. (1965). *Br. J. Pharmac. Chemother.*, **25**, 442–455.
Spencer, P. S. J. (1966). *Life Sci.*, **5**, 1015–1023.
Spencer, P. S. J. & West, G. B. (1961). *Br. J. Pharmac. Chemother.*, **17**, 137–143.

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Peripheral and central components in the hyperthermic effect of desipramine in reserpinized rats

D. BERNARDI, S. PAGLIALUNGA AND A. JORI

Pithed rats show a decrease of body temperature which is not affected by reserpine, cocaine or desipramine. Noradrenaline decreases the rate of fall of body temperature and this effect is enhanced by pretreatment with cocaine or desipramine. Reserpine with desipramine or with cocaine also produces a decreased rate of fall of body temperature. But if reserpine is given 18 hr before pithing, desipramine is without effect although noradrenaline and 2,4-dinitrophenol produce a calorigenic response. The hyperthermic effect of desipramine in reserpinized rats is also decreased by spinal transection. Lumbar transection is less effective than cervical transection. It is concluded that the integrity of the central nervous system is required for the development of the hyperthermic effect of desipramine in previously reserpinized rats.

THE possible mechanisms of the interaction between reserpine and tricyclic antidepressant drugs, such as desipramine, on the body temperature of rats have been summarized by Garattini & Jori (1967). It has been established that when imipramine-like drugs are given before reserpine, they enhance and prolong the hyperthermic phase and prevent the succeeding hypothermia (Garattini, Giachetti & others, 1962; Jori, Paglialunga & Garattini, 1967), but when they are given to hypothermic reserpinized rats they induce an increase of body temperature (Askew, 1963; Jori & Garattini, 1965).

The temperature increase is still evident when desipramine is injected directly into the brain of fully reserpinized rats (Bernardi, Jori & others, 1966; Reverski & Jori, 1968).

We now present evidence that the integrity of the central nervous system (CNS) is a necessary condition for the onset of the desipramine effect on body temperature of reserpinized rats.

Experimental

Female Sprague Dawley rats, 150–180 g, were used. Some animals were pithed under a light ether anesthesia with a needle introduced into the orbital cavity, the needle was also used to destroy the spinal cord. In other animals, spinal transections were made under ether anaesthesia at a cervical, thoracic or lumbar level. Tracheotomy and artificial ventilation were performed immediately after destruction of the CNS or after the cervical transection. After the operation, a thermistor was inserted into the rectal cavity and the body temperature continuously recorded by an automatic device (Jori & Paglialunga, 1966).

In general, the experiments were made at 22° and a relative humidity of 60%. Some animals were kept at 35° to avoid marked fall of body temperature. Drugs were injected in pithed rats, when the body temperature reached 35° ± 1°.

Drugs administered after spinal transection were generally given intravenously, to eliminate the possibility of an impaired absorption.

From the Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62-20157 Milan, Italy.

The drugs used were: noradrenaline bitartrate (Recordati), 2,4-dinitrophenol (C. Erba), reserpine (Serpasil, CIBA), desipramine (Pertrophan, Geigy), (+)-amphetamine sulphate (Recordati), cocaine hydrochloride (C. Erba).

Results

Effect of desipramine and other agents on the body temperature of pithed rats. The pithed rats were unable to regulate body temperature by any central mechanism and immediately after destruction of the CNS there was a fall in rectal temperature (Fig. 1A). The rate of fall depended on the environmental temperature and also on the initial body temperature of the rat. Consequently the drugs were administered to pithed rats when the rectal temperature was 35° ± 1°. Noradrenaline, infused for 15 min

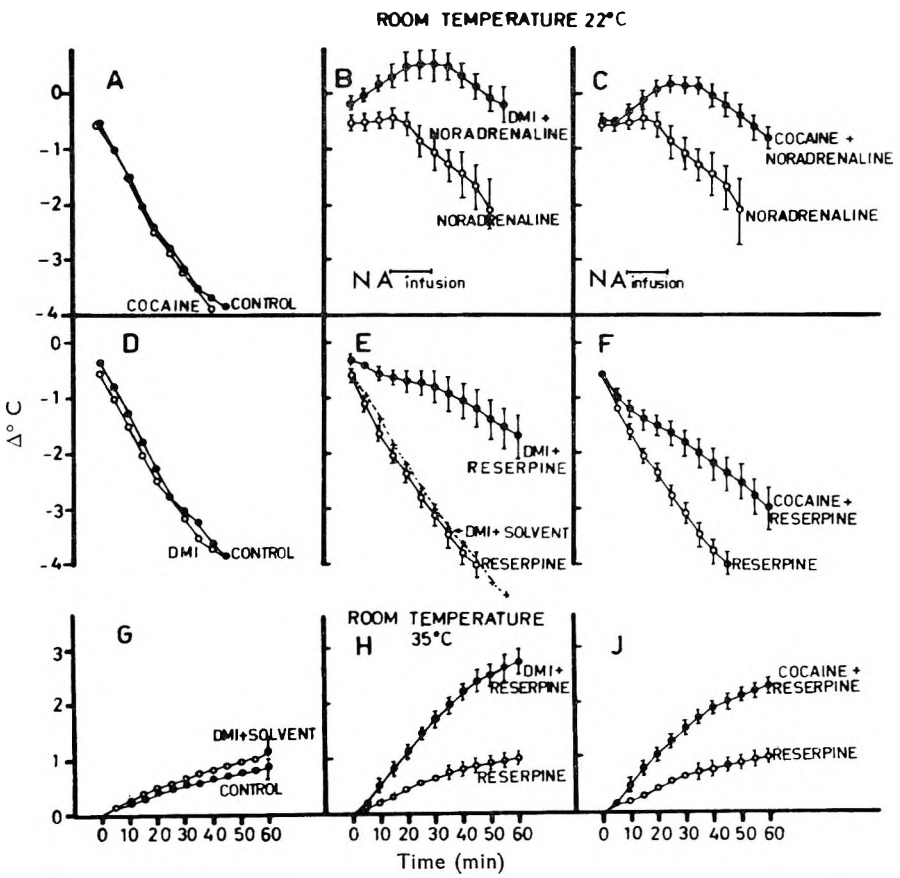

FIG. 1. Changes of body temperature in pithed rats given intravenously saline (control) or reserpine solvent (solvent), or reserpine (2·5 mg/kg) or noradrenaline (infused for 15 min at a rate of 0·1 ml/min at a concentration of 40 μl/ml). Cocaine (5 mg/kg i.v.) or desipramine (15 mg/kg i.p.) were given 5 min and 1 hr respectively before pithing.

at a rate of 0·1 ml/min (40 μg/ml), 5 min after the operation delayed the onset of the hypothermia (Fig. 1B).

Pretreatment with desipramine (15 mg/kg, i.p.) or cocaine (5 mg/kg, i.v.) much increased the noradrenaline effect (Fig. 1B and C) but the drugs themselves had no effect (Fig. 1A, D and E). Fig. 1E shows also that reserpine (2·5 mg/kg, i.v.) given at the time of pithing did not modify the fall of the body temperature. But when desipramine was given 1 hr before pithing, the reserpine injection significantly reduced the rate and the intensity of the fall of body temperature (Fig. 1E). The same results, although less marked, were obtained in animals pretreated with cocaine (Fig. 1F).

A hyperthermic effect was also present when cocaine or desipramine was given before reserpine and pithing to rats maintained at 35° (Fig. 1G, H, J).

Effect of desipramine and other drugs on body temperature of reserpinized pithed rats. Fig. 2 shows the effect of desipramine in normal and pithed

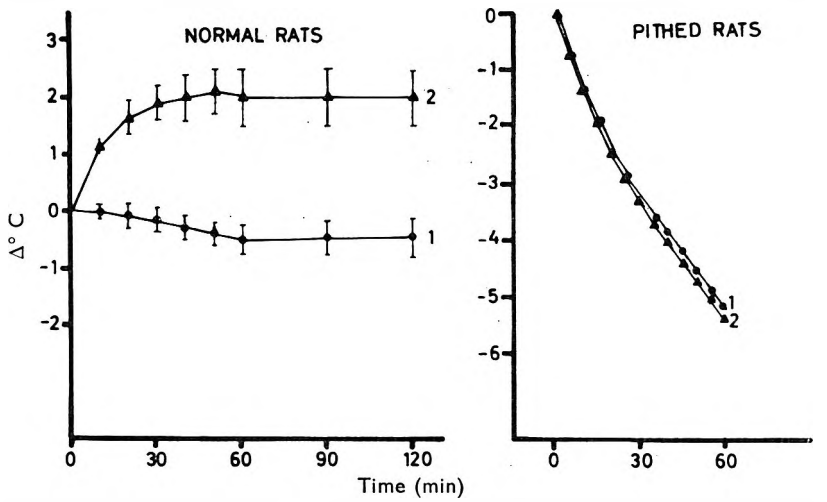

FIG. 2. Changes of body temperature induced by desipramine (1·5 mg/kg, i.v.) (curve 2) or by saline (curve 1) in rats treated 18 hr before with reserpine (2·5 mg/kg, i.v.).

rats given reserpine 18 hr previously. Desipramine produced a rapid hyperthermic effect in intact rats but no activity was apparent when the drug was injected immediately after pithing. The same results were obtained when the experiment was at 35°.

Other hyperthermic agents were given to previously reserpinized rats about 5 min after they were pitted (Table 1). Amphetamine was inactive was given before reserpine and pithing to rats maintained at 35° (Fig. but noradrenaline and dinitrophenol significantly reduced the fall in body temperature.

Effect of spinal transections. Animals were pretreated with reserpine 18 hr before spinal transection. Fig. 3 shows that the hyperthermic effect induced by desipramine progressively decreased when transections were made at the lumbar (L_4), thoracic (T_2–T_3) and cervical (C_2–C_3) level.

TABLE 1. EFFECT OF HYPERTHERMIC AGENTS ON BODY TEMPERATURE OF PITHED RATS PREVIOUSLY RESERPINIZED

No of rats	Treatment mg/kg i.v.		Body temp decrease. after 60 min (°C) ± s.e.
12	Controls		6·1 ± 0·2
6	2,4-Dinitrophenol	7·5	4·1 ± 0·1 ($P<0·01$)
6	Noradrenaline	0·24	4·0 ± 0·2 ($P<0·01$)
4	Amphetamine	7·5	5·9 ± 0·2

Reserpine (2·5 mg/kg, i.v.) was given 18 hr before pithing. Hyperthermic agents were given about 5 min after the operation. Noradrenaline (40 μg/ml, was infused at the rate of 0·1 ml/min for 15 min.

Discussion

Pithed rats, artificially respired, lost heat at an almost constant rate of about 1°/10 min when the room temperature was 22°. In these animals reserpine given immediately after pithing or 18 hr earlier did not significantly modify the onset of the hypothermia.

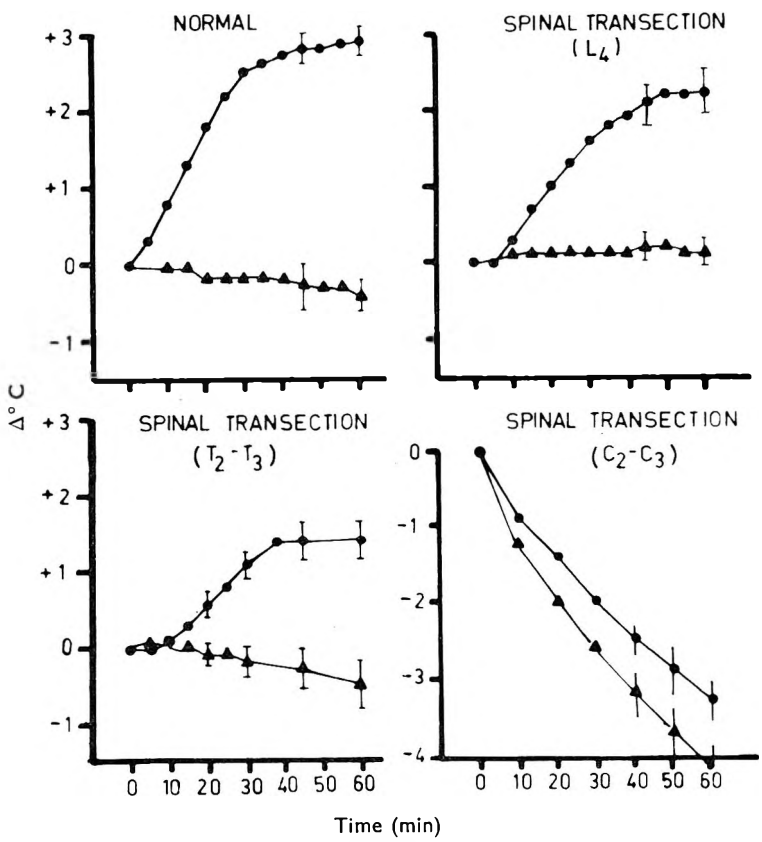

FIG. 3. Changes of body temperature induced by desipramine (—●—) (15 mg/kg, i.v.) or saline (—▲—) in rats treated 18 hr before with reserpine (2·5 mg/kg, i.v.). Spinal transection was performed 15 min before giving desipramine.

Reserpine injected intravenously into normal rats at the same dose is known to elicit a marked hyperthermia probably related to the release of central and peripheral catecholamines (Jori & others, 1967).

The difference in response to reserpine of pithed and normal rats is not due to the pithed animals losing body heat because at an ambient temperature of 35°, the pithed rats were in a phase of a slow increase of body temperature, but reserpine was still ineffective (Fig. 1). Further, pithed rats were still sensitive to the calorigenic action of noradrenaline as shown by the inhibition of the hypothermia after infusion with this amine (Fig. 1).

Reserpine is still able to release catecholamines in spinalized rats although its effect is more marked on the nerve terminals cranial than caudal to the lesion (Anden, Fuxe & Hökfelt, 1966, 1967). Probably the amount or the rate at which catecholamines are released by reserpine in pithed rats is not enough to counteract the loss of body temperature.

As expected, the calorigenic effect of noradrenaline in pithed rats is potentiated in the same way as it is in normal rats (Jori & others, 1967), by drugs such as desipramine and cocaine which inhibit catecholamine uptake (Glowinski & Axelrod, 1964; Iversen, 1965; Hillarp & Malmfors, 1964). Desipramine and cocaine, although they do not themselves affect the fall of body temperature in pithed rats, make a pre-operative injection of reserpine calorigenic. This effect is also present at 35° (Fig. 1). Desipramine given before reserpine, potentiates and prolongs the hyperthermic phase which follows reserpine (Jori & others, 1967). These results may be interpreted assuming that the catecholamines released by reserpine in pithed rats become calorigenic if the inhibition of their uptake makes them more available at the receptor sites (Garattini & Jori, 1967). Further, desipramine slows the rate at which reserpine releases catecholamines (Manara, Sestini & others, 1966; Manara, Algeri & Sestini, 1967).

A different situation is present when desipramine is given to fully reserpinized hypothermic rats (reserpine was usually given 18 hr before the experiment). While in normal animals desipramine increases body temperature, this effect is abolished if the animals are pithed before receiving desipramine.

Reserpinized pithed rats do not respond to the hyperthermic effect of amphetamine, presumably of central origin, although they are still sensitive to the calorigenic action of noradrenaline infusion and to a peripheral hyperthermic agent such as 2,4-dinitrophenol (Buffa, Carafoli & Muscatello, 1963) (see Table 1). It therefore seems logical to conclude that the hyperthermic response of desipramine in previously reserpinized rats is of central origin.

In an attempt to localize the site of action of desipramine several spinal transections were made. The hyperthermic effect of this agent in reserpinized rats tends to reappear the more caudal the lesion.

Thus it seems that desipramine is acting at a high level in the central nervous system and that the pathways carrying the stimulation to the periphery pass through the spinal cord.

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References

Anden, N. E., Fuxe, F. & Hökfelt, T. (1966). *J. Pharm. Pharmac.*, **18**, 630–632.
Anden, N. E., Fuxe, F. & Hökfelt, T. (1967). *European J. Pharmac.*, **1**, 226–232.
Askew, B. M. (1963). *Life Sci.*, **10**, 725–730.
Bernardi, D., Jori, A., Morselli, P., Valzelli, L. & Garattini, S. (1966). *J. Pharm. Pharmac.*, **18**, 278–282.
Buffa, P., Caravoli, E. & Muscatello, V. (1963). *Biochem. Pharmac.*, **12**, 769–778.
Garattini, S., Giachetti, A., Jori, A., Pieri, L. & Re, R. (1962). *J. Pharm. Pharmac.*, **14**, 509–514.
Garattini, S. & Jori, A. (1967). In *First Intern. Symposium on Antidepressant Drugs.* Editors Garattini, S. & Dukes, M. N. G. Amsterdam: Excerpta Medica Foundation, Congress Series n. 122, pp. 179–193.
Glowinski, J. & Axelrod, J. (1964). *Nature, Lond.*, **204**, 1318–1319.
Hillarp, N. H. & Malmfors, T. (1964). *Life Sci.*, **3**, 703–708.
Iversen, L. L. (1965). *J. Pharm. Pharmac.*, **17**, 62–64.
Jori, A. & Garattini, S. (1965). *Ibid.*, **17**, 480–488.
Jori, A. & Paglialunga, S. (1966). *Medna Pharmac. Exp.*, **14**, 513–514.
Jori, A., Paglialunga, S. & Garattini, S. (1967). *Archs int. Pharmacodyn. Thér.*, **165**, 384–393.
Manara, L., Algeri, S. & Sestini, M. G. (1967). In *First Intern. Symposium on Antidepressant Drugs.* Editors Garattini, S. & Dukes, M. N. G. Amsterdam: Excerpta Medica Foundation, Congress Series n. 122, pp. 51–60.
Manara, L., Sestini, M. G., Algeri, S. & Garattini, S. (1966). *J. Pharm. Pharmac.*, **18**, 194–195.
Reverski, W. & Jori, A. (1968). *Ibid.*, **20**, in the press.

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The effect of ascorbic acid on the cerebral and adrenal catecholamine content in the male rat

JUAN A. IZQUIERDO, ISABEL J. JOFRÉ AND CRISTINA ACEVEDO

The intraperitoneal administration of ascorbic acid (500 mg/kg) to rats produces a significant decrease in the concentration of dopamine, and an increase in noradrenaline, in the cerebral hemispheres, cerebellum, diencephalon, mesencephalon and medulla oblongata. The only change in the concentration of adrenaline was a decrease observed in the cerebral hemispheres. Ascorbic acid produces an increase in the concentration of ascorbic acid in the brain; this rise was not observed in the adrenal medulla where the concentrations of catecholamines were also unchanged.

ASCORBIC acid increases significantly the concentration of catecholamines, expressed as noradrenaline, in cerebral hemispheres, cerebellum and mesencephalon of rats (Izquierdo, Jofré & Dezza, 1964). We have since found that the acid decreases significantly the adrenaline concentration but does not modify the noradrenaline contents in the guinea-pig heart (Izquierdo & Jofré, 1965).

According to McLean & Cohen (1963) ascorbic acid releases adrenaline from adrenal granules *in vitro*. Levin, Levenberg & Kaufman (1960) and Levin & Kaufman (1961) considered that the acid participated in the β-hydroxylation of dopamine in the adrenal gland.

These results, together with the discrepancy between our findings on guinea-pig heart (Izquierdo & Jofré, 1965) and on rat brain (Izquierdo & others, 1964), led us to reinvestigate the effect of ascorbic acid on the cerebral and adrenal catecholamine contents in the rat.

Experimental

One hundred and thirty adult male albino rats, weighing 84–126 g, were used. They were killed by decapitation and the dopamine, noradrenaline, adrenaline and ascorbic acid contents or the amines or acid alone in brain structures and in the whole adrenal glands were estimated. There were 41 normal rats; there were 4 groups, each of 16 animals, killed 5, 10, 20 and 60 min after intraperitoneal injection of ascorbic acid (500 mg/kg). A further 4 groups, this time of 2 rats each, were injected intraperitoneally with nialamide (100 mg/kg) 4 hr before death, and pyrogallol (10 mg/kg) and ascorbic acid in separate injections 20 min before death, in which the amines only were assessed. Finally there were 4 groups of 2 rats each, and 3 groups of 3 rats each, killed 20 min after injection of ascorbic acid in which the acid alone was assessed.

Drugs used were: ascorbic acid (Roche) dissolved in twice distilled water and adjusted to pH 5·5–6·0 with 5N sodium hydroxide; nialamide (Pfizer); pyrogallol (Mallinckrodt). Solutions were prepared extemporaneously for each animal.

Brains were removed, washed immediately with cold Tyrode solution

From the Cátedra de Farmacologia Experimental, Facultad de Farmacia y Bioquímica, Junín 956, Buenos Aires, Argentina.

and dissected into component parts on ice. Adrenals and the adrenal-medulla were similarly treated. The structures were weighed and then adrenaline and noradrenaline were determined spectrofluorimetrically according to Bertler, Carlsson & Rosengren (1958), dopamine according to Laverty & Sharman (1965), and ascorbic acid by the photocolorimetric method of Roe (1954).

Results

From Table 1 it can be seen that ascorbic acid decreased dopamine and at the same time increased noradrenaline in some cerebral structures, as early as 5 min after injection. The effect increased at 10 min and reached a maximum at 20 min in all the brain structures. At 60 min there was still a decreased content of dopamine in hemispheres, mesencephalon, pons and medulla oblongata, but with no corresponding increase

TABLE 1. EFFECT OF THE INTRAPERITONEAL ADMINISTRATION OF 500 MG/KG OF ASCORBIC ACID ON THE CONTENT OF THE CATECHOLAMINES IN DIFFERENT PARTS OF THE BRAIN AND ADRENAL GLANDS OF THE RAT

		DA	NA	A	DA	NA	A	DA	NA	A
	Wt (g)	Hemispheres			Cerebellum			Diencephalon		
Normal	103	0·21	0·16	0·04	0·18	0·12	0·05	1·66	0·34	0·11
	(24)*	±0·03	±0·03	±0·002	±0·04	±0·01	±0·01	±0·12	±0·02	±0·03
	(11)†									
20 min	113	0·06	0·20	0·01	0·06	0·17	0·07	0·89	0·51	0·14
	(16) (4)	±0·01	±0·01	±0·001	±0·0054	±0·01	±0·01	±0·07	±0·005	±0·01
		P≪0·001	P<0·01	P≪0·001	P≪0·001	P<0·01	P<0·05	P≪0·001	P≪0·001	P<0·01
		73·2 (D)	22·9 (I)	83·2 (D)	69·3 (D)	41·9 (I)	54·9 (I)	46·4 (D)	48·7 (I)	25·5 (I)
60 min	104	0·15	0·16	0·04	0·14	0·13	0·05	1·44	0·35	0·12
	(16) (4)	±0·01	±0·01	±0·004	±0·01	±0·01	±0·001	±0·13	±0·019	±0·004
		P<0·02					P<0·01			
		26·4 (D)	4·4	7·3	22·7	6·5	13·7 (I)	13·1	2·8	6·0
		Mesencephalon			Pons-medulla oblongata			Adrenal glands		
Normal	103	0·80	0·31	0·05	0·72	0·42	0·09	0·03	0·13	0·52
	(24)(11)	±0·07	±0·03	±0·01	±0·06	±0·04	±0·003	±0·004	±0·02	±0·05
20 min	113	0·57	0·49	0·07	0·45	0·69	0·11	0·03	0·12	0·55
	(16) (4)	±0·03	±0·04	±0·006	±0·03	±0·02	±0·01	±0·003	±0·008	±0·02
		P<0·001	P<0·02		P≪0·001	P≪0·001				
		29·7 (D)	57·4 (I)	25·2 (I)	37·7(D)	58·5 (I)	16·4 (I)	13·4 (I)	7·5 (D)	4·5 (I)
60 min	104	0·69	0·35	0·05	0·63	0·53	0·10	0·03	0·13	0·56
	(16) (4)	±0·02	±0·03	±0·003	±0·01	±0·03	±0·003	±0·002	±0·007	±0·02
		P<0·01			P<0·01					
		13·4 (D)	12·6 (I)	8·8 (D)	12·4 (D)	21·7 (I)	5·5 (I)	14·0 (I)	2·5 (D)	7·8 (I)

Values are means and standard errors in μg/g of fresh tissue, in adrenals μg/mg.
* Number of animals
† Number of determinations
(I) % increases
(D) % decreases
DA Dopamine
NA Noradrenaline
A Adrenaline

in noradrenaline, although adrenaline was still significantly high in the cerebellum.

Adrenaline content was decreased in the hemispheres after 5 min by injection of the acid but it was not modified in the mesencephalon, pons and medulla oblongata, and was increased significantly in the diencephalon and cerebellum.

On a percent basis, only in the diencephalon, and at 10 min after injection of acid, was dopamine decreased by 44%; this corresponds to the increase of noradrenaline (29%) and of adrenaline (16%) (Table 2).

TABLE 2. EFFECT OF THE ADMINISTRATION OF ASCORBIC ACID (500 MG/KG I.P.) ON THE CONTENT OF CATECHOLAMINES IN THE DIENCEPHALON

	Dopamine	Noradrenaline	Adrenaline
Normal	1·66 ± 0·12*	0·34 ± 0·02	0·11 ± 0·03
At 10 min	0·92 ± 0·01 44·3 (D) P < 0·001	0·44 ± 0·02 28·9 (I) P < 0·02	0·13 ± 0·01 15·7 (I)

* Values are means and standard errors of 4 determinations in μg/g of fresh tissue.
(I) % increases; (D) % decreases.

After 20 min the noradrenaline increases were proportionally larger than the decreases in dopamine in the mesencephalon, pons and medulla oblongata.

The adrenal content of the three amines did not change significantly for 60 min after injection of the acid (Table 1).

Injection of ascorbic acid produced an increase in its concentration in brain structures; and even more so in the whole adrenal glands (Table 3).

TABLE 3. ASCORBIC ACID CONTENT IN BRAIN AND ADRENAL GLANDS. NORMAL AND INJECTED WITH ASCORBIC ACID 500 MG/KG I.P.

	Normal	20 min	% increase
Brain hemispheres	82·40 ± 3·65*	125·35 ± 2·40	52†
Cerebellum	80·59 ± 4·00	145·85 ± 3·46	81†
Diencephalon	66·38 ± 2·95	105·25 ± 6·39	58†
Pons and medulla oblongata	41·32 ± 2·70	85·92 ± 3·26	108†
Mesencephalon	60·52 ± 2·57	102·95 ± 7·10	70†
Adrenal glands	471·78 ± 13·00	973·60 ± 6·58	106†

* Values are means and standard errors of 4 determinations in μg/g of fresh tissue.
† $P < 0.001$.

TABLE 4. ASCORBIC ACID AND DOPAMINE CONCENTRATION OF ADRENAL GLANDS AND ADRENAL MEDULLA IN NORMAL RATS AND RATS INJECTED WITH ASCORBIC ACID 500 MG/KG I.P.

	Normal	20 min
	Ascorbic acid	
Adrenal gland	471·10 ± 12·57*	988·14 ± 14·10 109 (I) P < 0·001
Adrenal medulla	368·66 ± 12·05	402·15 ± 5·32 9·1 (I)
Cortex (deduced value)	102·44	585·99 472 (I)
	Dopamine	
Adrenal gland	0·03 ± 0·004	0·03 ± 0·003 13·4 (I)
Adrenal medulla	0·03 ± 0·001	0·04 ± 0·003 6·8

* Values are means and standard errors of 4 determinations in μg/g of fresh tissue.
(I) % increases.

This increase reached its peak at 20 min and declined at 60 min. No modification of adrenal medullary ascorbic acid was detected (Table 4), thus the increase in adrenal ascorbic acid appeared to occur only in the cortex of the gland.

Twenty min after rats pretreated with nialamide and pyrogallol were given ascorbic acid, a significant increase in the concentration of noradrenaline and adrenaline occurred in the diencephalon alone compared with the concentration of the amines in animals not treated with nialamide or pyrogallol. This pretreatment did not modify the effect of the acid on the concentration of dopamine in the diencephalon or in the adrenals (Table 5).

TABLE 5. EFFECT OF THE ASCORBIC ACID (500 MG/KG I.P.) ADMINISTRATION ON THE DIENCEPHALON AND ADRENALS CONTENT OF CATECHOLAMINES IN RATS PREVIOUSLY TREATED WITH NIALAMIDE AND PYROGALLOL

	Diencephalon			Adrenal—gland		
	DA	NA	A	DA	NA	A
Normal	1·66 ±0·12	0·34 ±0·02	0·11 ±0·03	0·03 ±0·004	0·13 ±0·02	0·52 ±0·05
Ascorbic acid (20 min)	0·89 ±0·07 46·4 (D) P≪0·001	0·51 ±0·01 43·7 (I) P≪0·001	0·14 ±0·01 25·5 (I) P<0·02	0·03 ±0·003 11·3 (I)	0·12 ±0·01 7·5 (D)	0·55 ±0·02 4·6 (I)
Nialamide Pyrogallol Ascorbic acid (20 min)	0·92 ±0·05 44·8 (D) P<0·001	0·65 ±0·03 89·8 (I) P<0·001	0·17 ±0·01 49·8 (I) P<0·001	0·03 ±0·003 11·5 (I)	0·11 ±0·01 15·3 (D)	0·57 ±0·02 9·1 (I)

Values are means and standard errors of 4 determinations in μg/g of fresh tissue, in adrenal μg/mg.
(I) % increases.
(D) % decreases.

Discussion

The simplest explanation for the decrease in dopamine and the increase in noradrenaline produced by ascorbic acid may be the possible participation of the acid in the β-hydroxylating mechanism of dopamine (Levin & others, 1960; Levin & Kaufman, 1961). According to these authors, ascorbic acid acts as coenzyme to dopamine β-hydroxylase in adrenal gland *in vitro* (Levin & others, 1960; Kaufman, 1966). But, except in the diencephalon at 10 min after injection of the acid, close agreement between dopamine decrease and noradrenaline increase is not apparent. Furthermore, adrenaline also appears to be decreased in brain hemispheres (Table 1) and we found the acid not to affect the three amines in the adrenal gland. This suggests that, *in vivo*, factors may be involved other than those which participate in the *in vitro* effect of the acid on catecholamine concentration.

It has been suggested that the hypophyso-corticoadrenal system regulates the adrenal medullary synthesis of adrenaline (Wurtman & Axelrod, 1965; Axelrod, 1966). There is a relation between adrenal cortical function and response to catecholamines (Brodie, Davies & others, 1966). However, neither ACTH nor corticoids modify the adrenaline content of adrenal glands (Linét & Hertting, 1966). Our results

suggest the acid to have an effect independent of cortical and medullary function, inasmuch as the acid is markedly increased in cortical tissue after intraperitoneal injection, but there is no change in medullary ascorbic acid or in adrenal dopamine, noradrenaline or adrenaline. This is suggested by the fact that in brain structures, which are normally relatively low in ascorbic acid, its injection not only increases its concentration but also markedly affects catecholamine concentration.

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References

Axelrod, J. (1966). *Pharmac. Rev.*, **18** (1), 95–113.
Bertler, A., Carlsson, A. & Rosengren, E. (1958). *Acta physiol. scand.*, **44**, 273–283.
Brodie, B. B., Davies, J. I., Hyme, S., Krishna, G. & Weiss, B. (1966). *Pharmac. Rev.*, **18** (1), 273–289.
Friedman, S. & Kaufman, S. (1965). *J. biol. Chem.*, **240**, 552–554.
Izquierdo, J. A., Jofré, I. J. & Dezza, M. A. (1964). *Medna Exp.*, **10**, 45–55.
Izquierdo, J. A. & Jofré, I. J. (1965). *J. Pharm. Pharmac.*, **17**, 391–392.
Kaufman, S. (1966). *Pharmac. Rev.*, **18** (1), 61–69.
Laverty, R. & Sharman, D. F. (1965). *Br. J. Pharmac. Chemother.*, **24**, 759–772.
Levin, E. Y. & Kaufman, S. (1961). *J. biol. Chem.*, **236**, 2043–2049.
Levin, E. Y., Levenberg, B. & Kaufman, S. (1960). *Ibid.*, **235**, 2080–2086.
Linèt, O. & Hertting, G. (1966). *Archs int. Pharmacodyn. Thér.*, **159**, 407–415.
McLean, J. R. & Cohen, R. (1963). *Life Sci.*, **4**, 261–265.
Roe, J. H. (1954). In *Methods of Biochemical Analysis*, Vol. 1, p. 115. Editor Glick, D. New York: Interscience.
Weber, L. J. (1966). *Proc. Soc. exp. Biol. Med.*, **123**, 349–352.
Wurtman, R. J. & Axelrod, J. (1965). *Science, N.Y.*, **150**, 1464–1465.

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Role of pH in the coacervation of the systems: gelatin–water–ethanol and gelatin–water–sodium sulphate*

SALEH A. H. KHALIL, J. R. NIXON AND J. E. CARLESS

Phase boundary determination, coacervate volume measurements and analysis of the phases have been made to assess the influence of pH on the coacervation of gelatin solutions by ethanol and sodium sulphate. Coacervation was found to be pH dependent. In the ethanol system coacervation was noticeable only within a pH range in the vicinity of the isoionic point: at other pH values either a viscous gel phase or floccules occurred. In the sodium sulphate system, coacervation occurred at all pH values examined. The effect of pH in changing the overall charge on the gelatin molecule is explained in relation to the formation of gelatin coacervates. Finally, the role of the coacervate phase in the microencapsulation of oil and solid particulates is discussed.

THE term coacervation was introduced by Bungenberg de Jong & Kruyt (1929) to describe the various cases of partial miscibility occurring in macromolecular systems where two isotropic liquid phases co-exist. Under optimum conditions the colloid-rich phase, the coacervate, contains the bulk of the colloidal components and the equilibrium liquid contains only a negligible amount of the colloid. Two main types of coacervation have been classified by Bungenberg de Jong (1949); simple coacervation occurs as the result of a decrease in the solubility of the colloidal components caused by the addition of non-solvents, as in the systems: isoelectric gelatin–water–ethanol and isoelectric gelatin–water–sodium sulphate, first investigated by Holleman, Bungenberg de Jong & Modderman (1934). Complex coacervation, on the other hand, results from the interaction of oppositely charged colloids and has been of academic interest in relation to some biological phenomena (Bungenberg de Jong & Booij, 1956). Recently a quantitative formulation to the theory of complex coacervation was attempted by Veis (1963).

Simple coacervation of gelatin systems has received little attention as it has been assumed that the phenomenon occurs as a result of dehydration and that the charge effects play no active role.

In the past few years, attention has focussed on the application of both types of gelatin coacervation to the microencapsulation of pharmaceuticals (Luzzi & Gerraughty, 1964; Phares & Sperandio, 1964) and as a superior method for the fractionation of heterodisperse polymers (Stainsby, 1954). The present paper examines the effects of pH on the simple coacervation of gelatin by ethanol and sodium sulphate.

Experimental

MATERIALS

Gelatin. Two samples were used having the characteristics given in Table 1. The gelatins were dried in thin layers at 110° for 12 hr and

From the Department of Pharmacy, Chelsea College of Science and Technology (University of London), Manresa Road, London, S.W.3, England.

* This work forms part of a thesis presented by S.A.H.K. for the degree of Ph.D. in the University of London.

TABLE 1. CHARACTERISTICS OF GELATIN SAMPLES

Sample	Type	Source	Bloom number	pH	Viscosity (cps 6·67%)	Ash %	Isoelectric point	Isoionic point (PI)
A	Lime-pretreated	Hide	240	6·6	8·1 (40°)	1·20	5·2	4·9
B	Acid-processed	Pigskin	252	4·7	5·5 (60°)	1·11	9·2	8·9

stored in air-tight containers. *Absolute ethanol* and 20% *w/w sodium sulphate solution* were used as the coacervating agents. All *electrolytes* used were Analar and *water* was once distilled from an all-glass still (pH 5·1, specific conductivity 4·8 μ mhos cm^{-1}).

METHODS

Determination of isoionic point and preparation of ash-free gelatin. The isoionic point was determined by the mixed bed ion-exchange resin technique of Janus, Kenchington & Ward (1951) and large batches were deionized by the same technique. The maximum specific conductivity of the deionized product was 10–28 μ mhos cm^{-1} (40°) for 2–10% w/w gelatin solutions. The amount of ash estimated did not exceed 0·002%.

Adjustment of pH. Hydrochloric acid (2N) or sodium hydroxide (2N) was used to adjust the pH of the gelatin solutions. The use of buffers was avoided on account of the possible effect of electrolytes on gelatin coacervation. Measurements of pH values were made at 40° using glass/calomel electrodes.

Determination of the phase boundaries and analysis of the coacervate phase and equilibrium liquid. The methods described by Nixon, Khalil & Carless (1966) were used.

Measurement of the coacervate volume. The volume of the separated coacervate phase was directly measured using calibrated 10 ml centrifuge tubes after equilibration at 40°. When the coacervate volume was too small for direct measurement the apparatus used was a glass vessel provided with a calibrated tube and a platinum stirrer. The coacervating agent was added from a microburette attached to one side of the apparatus.

Results

The phases separating at various pH values after the minimum ethanol or sodium sulphate concentration required to produce a phase change was added to gelatin solution, are shown in Tables 2 and 3. The 10% gelatin solutions require less coacervating agent because the gelatin molecules are in a state of greater initial aggregation; also, at the higher temperatures, they required less dehydrating agent to move the dispersion into the coacervate region. Whilst the initial gelatin concentration only affected the amount of coacervating agent required to produce a phase change, the behaviour of both systems differed considerably with pH changes. In the ethanol system separation into two isotropic liquid phases (coacervation) was noted only within a pH range of 4·4 to 6·9 for

TABLE 2. EFFECT OF pH ON THE TYPE OF PHASE SEPARATING IN THE SYSTEM GELATIN–WATER–ETHANOL. Minimum ethanol concentrations (% w/w) required to initiate a phase change. Temperature: 40° ± 0·1°, 5 g samples

	240 Bloom alkali-processed gelatin					250 Bloom acid-processed gelatin					
	Before deionization % w/w initial gelatin			After deionization % w/w initial gelatin			Before deionization % w/w initial gelatin			After deionization % w/w initial gelatin	
pH	5	10	pH	5	10	pH	5	10	pH	5	10
3·0	72·1 F	63·7 F	4·1	79·9 F	73·2 F	3·2	74·7 F	66·0 F	3·8	80·5 F	77·1 F
4·2	68·2 F	60·5 F	4·2	62·6 F	59·3 F	4·3	70·4 F	62·8 F	4·4	76·9 F	75·6 F
4·3	65·0 F	58·9 F	4·3	56·7 G	54·9 G	4·7*	68·2 F	60·3 F	5·3	74·8 F	70·7 F
4·4	61·4 F	56·7 F	4·4	54·5 C	53·2 C	5·1	61·9 F	59·7 F	6·1	69·1 F	66·9 F
4·5	59·3 G	55·2 G	4·8	48·2 C	46·0 C	6·4	60·3 F	59·2 F	6·7	65·3 G	63·9 G
4·6	57·3 C	53·9 C	4·9‡	40·0 C	38·4 C	6·8	58·9 G	57·9 G	6·9	63·0 C	61·9 C
4·8	53·9 C	52·2 C	5·9	57·6 C	53·9 C	6·9	58·4 C	57·0 C	7·4	60·3 C	58·7 C
5·2†	42·1 C	41·6 C	6·3	60·3 C	57·3 C	7·1	57·6 C	56·3 C	8·1	57·0 C	55·2 C
5·9*	40·9 C	39·1 C	6·9	61·7 C	58·9 C	8·3	56·1 C	54·9 C	8·9‡	49·8 C	48·7 C
7·7	53·5 C	48·7 C	7·0	63·3 G	61·9 G	8·9	53·9 C	52·2 C	9·3	56°1 C	51·8 C
8·7	59·7 C	51·0 C	7·1	66·0 G	62·7 G	9·1†	51·0 C	50·2 C	10·2	59·7 C	55·5 C
9·0	65·6 C	55·2 C	8·1	69·9 F	66·6 F	9·7	53·2 C	51·8 C	10·8	64·9 C	61·9 C
9·2	66·9 C	55·8 C	10·6	79·0 F	70·4 F	10·1	57·6 C	54·9 C	11·0	69·1 G	65·4 G
9·3	67·4 G	54·5 G	—	—	—	10·9	59·7 C	56·7 C	11·3	72·6 G	68·2 G
9·8	68·4 F	56·4 F	—	—	—	11·3	61·9 G	60·8 G	11·7	77·7 F	73·3 F
11·1	73·9 F	61·9 F	—	—	—	11·4	66·7 G	63·0 G	—	—	—
—	—	—	—	—	—	11·8	75·8 F	71·3 F	—	—	—

Type of phase separated: F = flocculate; G = gel; C = coacervate.
‡ = isoionic point; † = isoelectric point; * = pH of gelatin solution without adjustment.

the deionized alkali-processed gelatin (pI 4·9) and pH 6·9 to 10·8 for the deionized acid-processed sample (pI 8·9). Outside these pH ranges a highly viscous gel phase separated and at extreme pH values flocculation occurred, Fig. 1. Both the gel phase and the floccules produced showed no coacervate droplets, characteristic of coacervate phase. Commercial lime-pretreated gelatin samples are less sensitive to pH changes because of the electrolytic impurities, mainly calcium salts which are bonded to the carboxyl groupings of the gelatin on the alkaline side of the isoelectric

TABLE 3. EFFECT OF pH ON THE TYPE OF PHASE SEPARATING IN THE SYSTEM: GELATIN–WATER–SODIUM SULPHATE. Minimum sodium sulphate concentrations (% w/w) required to initiate a phase change. Temperature: 40° ± 0·1°, 5 g samples

	240 Bloom alkali-processed gelatin					250 Bloom acid-processed gelatin					
	Before deionization % w/w initial gelatin			After deionization % w/w initial gelatin			Before demineralization % w/w initial gelatin			After demineralization % w/w initial gelatin	
pH	5	10	pH	5	10	pH	5	10	pH	5	10
2·1	6·35 C	6·13 C	2·4	6·38 C	6·22 C	3·2	6·80 C	6·34 C	2·4	6·70 C	6·48 C
3·8	6·61 C	6·40 C	3·7	6·51 C	6·36 C	4·1	6·93 C	6·77 C	3·3	6·93 C	6·60 C
4·3	6·92 C	6·84 C	4·4	7·11 C	6·92 C	4·7*	7·42 C	6·98 C	4·6	7·08 C	6·77 C
4·9	7·95 C	7·47 C	4·6	7·36 C	7·26 C	5·6	7·68 C	7·18 C	5·2	7·36 C	6·92 C
5·2†	8·47 C	8·11 C	4·9‡	7·89 C	7·81 C	6·4	7·85 C	7·60 C	6·7	7·48 C	7·13 C
5·9*	8·49 C	8·32 C	5·5	8·31 C	8·03 C	8·0	8·16 C	8·01 C	8·4	7·63 C	7·35 C
6·7	8·55 C	8·47 C	5·9	8·66 C	8·31 C	9·1†	8·47 C	8·24 C	8·9‡	7·80 C	7·74 C
7·7	8·91 C	8·71 C	6·6	8·90 C	8·45 C	9·7	8·68 C	8·40 C	9·7	7·86 C	7·78 C
8·0	9·46 C	9·02 C	8·3	9·27 C	9·10 C	10·4	9·11 C	8·79 C	10·8	8·40 C	8·10 C
9·1	9·48 C	9·17 C	8·9	9·45 C	9·22 C	10·7	9·24 C	8·90 C	11·3	8·78 C	8·36 C
9·3	9·51 C	9·26 C	9·1	9·63 C	9·26 C	—	—	—	—	—	—
10·2	9·54 C	9·33 C	9·6	9·70 C	9·37 C	—	—	—	—	—	—
—	—	—	10·5	9·78 C	9·48 C	—	—	—	—	—	—

* pH of the gelatin solution without adjustment; † isoelectric point; ‡ isoionic point.
C = Coacervate.

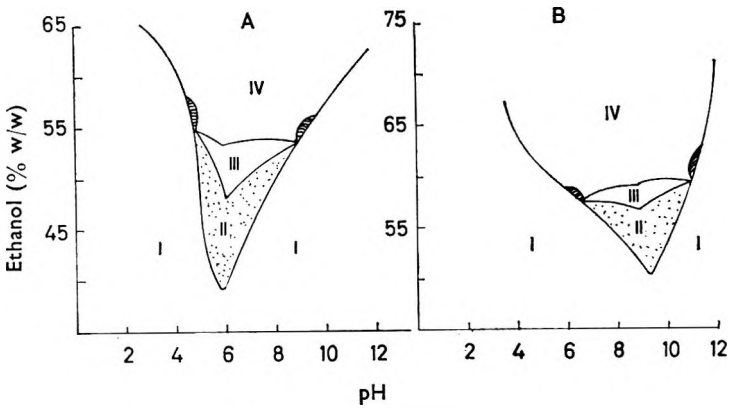

FIG. 1. Phase relationship at various pH values in the system gelatin–water–ethanol. A. Alkali-processed gelatin (I.E.P. 5·2). B. Acid-processed gelatin (I.E.P. 9.2). Initial gelatin concentration: 10% w/w Temperature: 40° ± 0.1°. I = clear isotropic phase, II = coacervation, III = Three phases, IV = Flocculation. Hatched area = gel phase.

point. For the commercial alkali-processed sample the pH range for coacervation was extended to 4·6–9·2. The addition of equivalent amounts of calcium chloride to demineralized gelatin produced a similar insensitivity to pH changes on the alkaline side of the isoelectric point. High concentrations of sodium chloride (1–2M) produced similar results.

Coacervation by sodium sulphate occurred at all pH values examined and lower electrolyte concentrations were required on the acid side of the isoionic point (Table 3).

The isothermal triangular diagrams of phase boundaries, Fig. 2 A–F, show the location of the coacervation region (II) relative to the other phases present at various pH values. At values away from the isoelectric point the boundaries move towards higher ethanol concentrations. The bandwidth of both the coacervation region (II) and the three-phase zone (III) is relatively narrow and, outside the effective pH range for coacervation, the system passes directly from the clear isotropic liquid region (I) to the flocculate region (IV). In sodium sulphate systems, all the phase boundaries are displaced away from the sodium sulphate corner at pH values on the acid side of the isoelectric point (Fig. 2 F) and the reverse occurs on the alkaline side.

Changes in coacervate volume at various pH values are shown in Figs 3 and 4. In the ethanol system, variations in the pH farther from the isoionic point not only increased the percentages of ethanol required for maximum coacervate volume, but also the volume of the separated coacervate phase at the maxima (Fig. 3A,B). In the sodium sulphate system pH values on the acid side of the isoionic point produced a displacement of the maxima in the coacervate volume plots towards the ordinate axis and also a decrease in the coacervate volume (Fig. 4A). On the alkaline side of the isoionic point the coacervate produced showed a marked increase in volume and a lower viscosity. Plots of the maximum

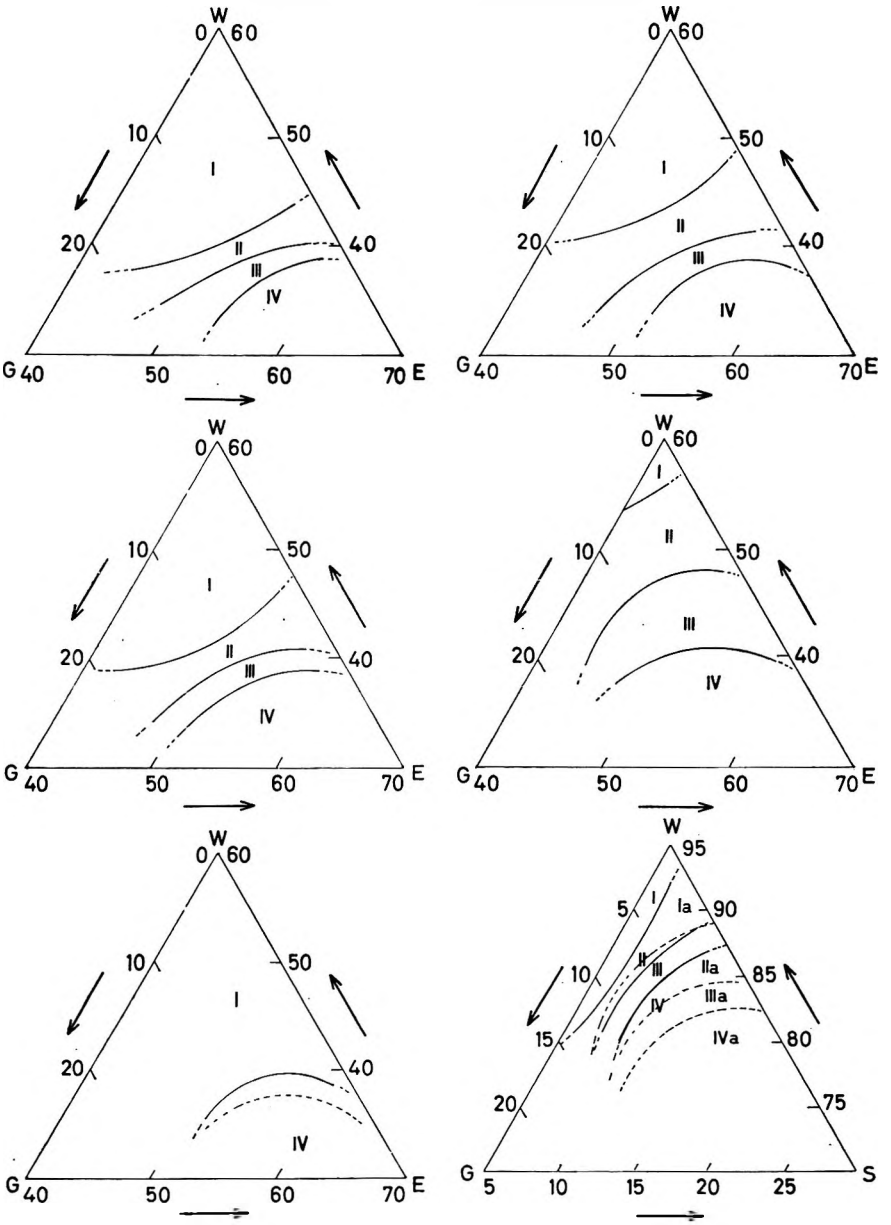

FIG. 2. A–F. Triangular diagrams of phase regions in the systems (GWE) gelatin–water–ethanol (A–E) and (GWS) gelatin–water–sodium sulphate (F) at various pH values. A (top left) pH 4·6. B (top right) pH 4·8. C (middle left) pH 5.2 (I.E.P.). D (middle right) pH 8.6. E (bottom left) solid line pH 4.4, broken line pH 9·8. F (bottom right) solid line pH 3·1 (regions I-IVa), broken line pH 8·1 (regions I–IV). I. Clear isotropic region. II. Coacervation (coacervate + equilibrium liquid). III. Three phase region (precipitated gelatin + coacervate + equilibrium liquid. IV. Flocculate region. W→G = gelatin. G→E = ethanol. E→W = water.

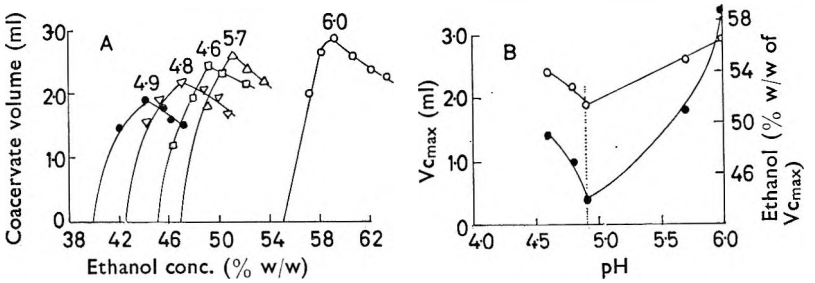

FIG. 3A. Change in coacervate volume with increasing ethanol concentrations at the pH values shown on the curves. Total gelatin concentration 4% w/v. Gelatin used: 240 Bloom pI 4.9. B. Maxima of coacervate volume (Vc $_{max}$) and their ethanol concentration plotted against pH from A. ○ Coacervate volume. ● Ethanol concentration.

coacervate volume, or the concentrations of coacervating agent which produced these maxima, against pH show minimal values at pH 4·9 in the ethanol system (the isoionic point of the alkali-processed gelatin) and pH 3·1 in the sodium sulphate system (Figs 3B, 4B). At these values the coacervates were highly viscous.

Analyses of the coacervates and their corresponding equilibrium liquids are given in Tables 4 and 5, and the values are plotted in Fig. 5 for the ethanol system. The position of the partial miscibility curves within the phase diagram, Fig. 5, depended on the pH in a manner analogous to the location of the coacervation region at a similar pH. At a fixed concentration of gelatin in the total mixture, the coacervates produced from ethanol systems showed a maximum percentage of colloid at the isoionic point which decreased at other pH values (Table 4). The corresponding equilibrium liquids contained low percentages of gelatin ($<3\%$ w/w), the minimum occurring at the isoionic point. Because of the increased concentration of ethanol required to produce coacervation at pH values away from the isoionic point, the equilibrium liquids at these pH values were relatively richer in ethanol content. Variation in the pH also produced a noticeable effect on the ethanol content of the coacervates.

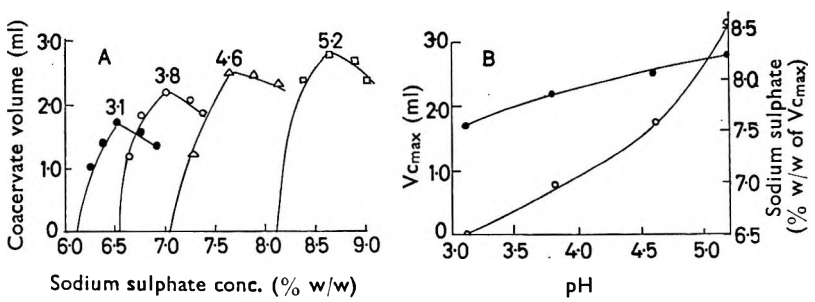

FIG. 4. A. Change in coacervate volume with increasing sodium sulphate concentrations at the pH values shown on the curves. B. Maxima of coacervate volume (Vc $_{max}$) and their sodium sulphate concentrations plotted against pH. ● Coacervate volume. ○ Sodium sulphate concentration.

TABLE 4. COMPOSITIONS OF THE COACERVATES AND EQUILIBRIUM LIQUIDS AS A FUNCTION OF pH, IN THE SYSTEM: GELATIN–WATER–ETHANOL. Gelatin used: demineralized 240 Bloom alkali-processed. Temperature: 40° ± 0·1°

	Percentage w/w compositions								
	Total mixture			Coacervate			Equilibrium liquid		
pH	Gelatin	Ethanol	Water	Gelatin	Ethanol	Water	Gelatin	Ethanol	Water
4·7	4	45	51	11·1	41·8	47·1	2·8	46·1	51·5
	4	47	49	13·8	42·3	42·9	1·6	47·9	50·5
4·8	4	44	52	11·6	39·4	49·0	2·8	45·2	52·0
	4	46	50	14·3	39·8	45·9	2·2	47·3	50·5
4·9*	4	42	54	14·1	37·1	48·8	2·2	42·6	55 2
	4	44	52	16·9	36·4	46·7	1·4	45·4	53·2
	4	46	50	19·8	36·7	43·5	0·6	47·7	51·3
5·4	4	44	52	11·3	39·5	49·2	2·5	45·7	51·8
	4	46	50	12·9	39·7	47·4	2·1	47·6	50·3
5·6	4	46	50	8·8	42·3	48·9	2·6	47·2	50·2
	4	48	48	14·9	43·1	42·0	1·3	49·4	49·3
6·0	4	57	39	14·4	51·6	34·0	1·8	58·1	40·1
	4	59	37	19·3	50·8	29·9	0·5	60·7	38·8

* The isoionic point.

At the isoionic point, demineralized gelatin coacervates had an alcohol content of about 37% w/w, similar to that found in the coacervates of isoelectric commercial gelatin (Holleman & others, 1934, and Nixon & others, 1966). Away from the isoionic point the ethanol content of the coacervates showed a progressive increase.

Similar observations were made for the coacervates and equilibrium

TABLE 5. COMPOSITIONS OF THE COACERVATES AND EQUILIBRIUM LIQUIDS AS A FUNCTION OF pH IN THE SYSTEM: GELATIN–WATER–SODIUM SULPHATE. Gelatin used: 240 Bloom, alkali-processed. Temperature: 40° ± 0·1°.

	Percentage w/w composition								
	Total mixture			Coacervate			Equilibrium liquid		
pH	Gelatin	Sodium sulphate	Water	Gelatin	Sodium sulphate	Water	Gelatin	Sodium sulphate	Water
3·1	4	6·1	89·9	13·6	5·1	81·3	1·1	6·4	92·5
	4	6·9	89·1	16·1	5·0	78·9	0·6	7·6	91·8
	4	7·7	88·3	18·8	5·3	75·9	0·3	8·2	91·5
4·2	4	7·2	88·8	14·1	5·7	80·2	1·6	8·2	90·2
	4	8·0	88·0	16·3	5·7	78·0	1·0	8·8	90·2
	4	8·8	87·2	18·2	6·0	75·8	0·5	10·0	89·5
4·9*	4	8·2	87·8	10·8	7·1	82·1	1·8	9·3	88·9
	4	9·2	86·8	12·4	6·9	80·7	0·8	10·4	88·8
	4	10·2	85·8	14·2	6·9	78·9	0·6	11·5	87·9
6·6	4	8·8	87·2	11·1	7·3	81·6	2·1	9·8	88·1
	4	9·8	86·2	13·8	7·6	78·6	1·4	10·6	88·0
	4	10·8	85·2	15·5	7·5	77·0	0·9	11·8	87·3
8·9	4	9·4	86·6	12·1	8·2	79·7	2·8	9·9	87·3
	4	10·4	85·6	15·8	8·5	75·7	1·6	11·1	87·3
	4	11·4	84·6	17·7	8·1	74·2	0·9	11·9	87·2

* The isoionic point.

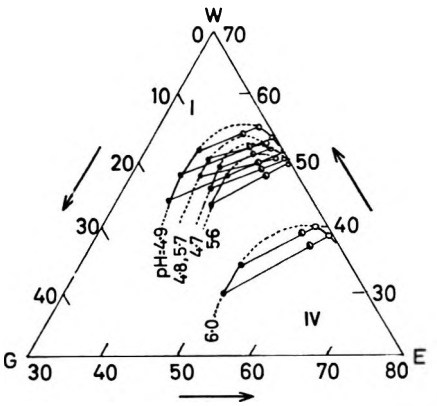

FIG. 5. Effect of pH on the composition of coacervates and the corresponding equilibrium liquids in the system: gelatin–water–ethanol. Temperature: 40° ± 0.1°. Gelatin: 240 Bloom, alkali-processed (pI 4·9). ● Coacervate. ○ Equilibrium liquid. ◑ Total mixture.

liquids of the sodium sulphate system (Table 5); the colloidal component being present exclusively in the coacervates the sodium sulphate contents of which increased on passing from an acid to an alkaline pH on both sides of the isoionic point.

Discussion

Gelatin exists in solution in a randomly coiled skein configuration. The shape of these coils is influenced by the charge on the molecules and their mutual interactions (Hermans & Overbeek, 1948). Predominance of charges of one type favours an unfolded stretched configuration. At the isoionic point the gelatin molecule attains the random coiled structure by virtue of inter- and intramolecular attractive forces. Variations in the pH result in an inbalance of charges and the force of repulsion arising causes unfolding of the coil.

Pasynskii (1958) has shown that in unbuffered solutions the shape of the gelatin molecules varied in proportion to the fourth or fifth root of the number of charges.

The role of pH in controlling gelatin coacervation in the two systems examined can be explained by the effect of pH on the two factors which appear to govern coacervation of polyelectrolyte systems: (*a*) the intra- and intermolecular attractive coulombic forces; (*b*) hydration.

The first of these favours phase separation and tends to produce floccules, whilst the second enhances redispersion of the molecular entities. The balance between these two factors gives rise to the separation of the colloid-rich isotropic liquid phase, the coacervate, which retains a certain amount of occluded liquid immobilized within the loops of the skeins. This occurs readily at the isoionic point where the hydration

effect would be balanced by the maximum attractive forces between the oppositely charged sites on the gelatin molecules.

At pH values away from the isoionic point the attractive forces decrease and, as found by Jirgenson (1946) and Czerniak & Pasynskii (1948), an increase in the hydration of the gelatin molecule takes place. Both changes tend to suppress coacervation and higher concentrations of the dehydrating agent (ethanol) will be required to restore the balance. In ethanol systems, at pH values well away from the isoionic point, flocculation occurs because the gelatin molecule is fully stretched and cannot entrap the necessary occlusion liquid. At intermediate pH values the gelatin molecules retain a certain degree of flexibility, although they are not able to entrap sufficient occlusion liquid to form coacervate. Under these conditions a viscous gel is formed. Basu & Bhattacharya (1952) reported similar changes in the phases separating in gelatin–water–ethanol systems as a function of pH, where the same sequence: viscous phase, hard gel, granular precipitate was found.

The effect of ethanol in changing the ionization constants of the various groups has been examined by Jukes & Schmidt (1934) who studied its effect on pK_E values of some amino-acids. They found an increase of about one pK_a unit of the carboxyl group compared to an increase of 0·1–0·4 pK_a units for the basic groups for ethanol concentrations between 50–72% v/v. But in the present work the effects of ethanol were not taken into account because of the difficulty in predicting accurately the changes in pK_a values caused by ethanol in the system. Table 6 shows the number of active groupings per 100,000 units of molecular weight, calculated from data by Courts (1954) and Eastoe (1955) and also the net charge produced by changes in the pH. At the isoionic point, where the net charge is zero, coacervation occurs readily, but at pH values corresponding to the limiting values for ethanol coacervation, the net charge is

TABLE 6. CALCULATION OF THE NET CHARGES PER 100,000 *M* ALKALI-PROCESSED GELATIN AS A FUNCTION OF pH

Group	No. of groups* per 100,000 *M*	pK_a†	pH	% ionized	No. of charges
Carboxyl	130	3·5–5	4·4	88·8–20·1	71(−)
			4·9	96·2–55·7	94(−)
			6·9	100–98·8	129(−)
α-Amino and imidazole ..	7	7	4·4	99·8	7(+)
			4·9	99·2	7(+)
			6·9	55·7	4(+)
ε-Amino	37	9–10	4·4	100	37(+)
			4·9	100	37(+)
			6·9	99·2–100	37(+)
Guandino	50	12	4·4	100	50(+)
			4·9	100	50(+)
			6·9	100	50(+)

* According to Eastoe (1955).
† From values obtained at 40° (Ward & Saunders, 1958).
Net charges calculated from the above data:

Zero at pH 4·9 (pI).
23(+) at pH 4·4 (lower pH limit for ethanol coacervation).
38(−) at pH 6·9 (upper pH limit for ethanol coacervation).

23(+) or 38(−) for the deionized alkali-processed gelatin. The presence of electrolytes increased the resistance of gelatin molecules towards changes in pH. The shielding effects of both the cations and anions of the electrolyte around the oppositely charged sites on gelatin weakens the tendency for repulsion between the gelatin molecules and between the segments of the same molecule. This results in an easing of the conditions for coacervation.

Because of the electrolytic nature of sodium sulphate, the effect of pH in this system differed from that in the ethanol system. Forces of repulsion arising from pH changes would have been counteracted by the shielding effect of the sodium sulphate added as a coacervating agent. At pH values on the acid side of the isoionic point, sulphate ions are preferentially fixed at the positively charged nitrogenous groups and at such pH values coacervation occurs readily. This agrees with the fact that acid solution enhances salting out of proteins where the efficiency of the salt anions followed the lyotropic series (McBain & Kellogg, 1928).

Changes in pH, within the effective pH range for coacervation, produced variations in both the composition and volume of the separated coacervates. At the isoionic point the amount of occluded liquid was at a minimum, but the amount gradually increased with variations in the pH. The effect of pH in controlling the intra- and intermolecular spaces within which occlusion occurs may be responsible for the variation of the liquid content (water + ethanol) of the coacervates. The strong attractive forces at the isoionic point produce contraction and folding of the coil, thus giving a more dense coacervate (i.e. smaller volume). At other pH values where the attractive forces diminish, the coil expands allowing more occlusion liquid to be immobilized, thus decreasing the viscosity of the separated coacervates and increasing their volume (Fig. 3A). At each pH value there is shown to be a maximum coacervate volume, e.g. at pH 4·9 the volume increases on increasing the percentage of ethanol from 42–44% and reduces when increased to 46%, while the solvent content of the coacervate is reduced over both ranges (Table 4). There must therefore be some other factor apart from solvent occlusion contributing to the coacervate volume. The initial increase in volume at a specific pH, with increase in ethanol concentration, may be due to a decrease in the dielectric constant resulting in decreasing attraction between the molecules. The decrease in volume could also be ascribed to the desolvation effect of the ethanol becoming predominant.

The recent application of coacervation techniques for microencapsulation of oil and solid particulates, is based on the unique ability of the coacervate phase to surround suspended particles, thus forming a "coacervate coat". The success of this technique will thus mainly be dependent on working within the coacervation region (II). The results presented in this paper show the effects of pH or the relative position of the coacervation region inside the triangular diagrams.

The most important consideration is the type of gelatin used and the effect of the material to be coated on the pH of the system. In a gelatin–water–ethanol system, using acid-processed samples, no coacervation

occurs unless the pH of the gelatin solution is adjusted to a value within the effective pH range for coacervation (6·9–10·8). With alkaline gelatins no prior adjustment of pH is needed. If the materials to be encapsulated produce a change in the pH, then the location of the coacervation region under the coating conditions must be predetermined, otherwise the pH change may produce a gel-like mass or one-phase system.

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References

Basu, S. & Bhattacharya, G. (1952). *Science, N.Y.*, **115**, 544–546.
Bungenberg de Jong, H. G. & Kruyt, H. R. (1929). *Proc. Sect. Sci. K. ned. Akad. Wet.*, **32**, 849–856.
Bungenberg de Jong, H. G. (1949). *Colloid Science*, Vol. 2, pp. 232–480. Editor Kruyt, H. R. Amsterdam: Elsevier.
Bungenberg de Jong, H. G. & Booij, H. L. (1956). *Biocolloids and their Interactions, Vol. 1*, in Protoplasmatologia. Berlin: Springer-Verlag.
Courts, A. (1954). *Biochem. J.*, **58**, 70–74.
Czerniak, R. S. & Pasynskii, A. G. (1948). *Kolloid Zh.*, **10**, 245–248.
Eastoe, J. E. (1955). *Biochem. J.*, **61**, 589–600.
Hermans, J. J. & Overbeek, J. Th. G. (1948). *Bull. Soc. Chim. Belg.*, **57**, 154–162.
Holleman, L. W. J., Bungenberg de Jong, M. G. & Modderman, R. S. T. (1934). *Kolloidbeihefte*. **39**, 334–420.
Janus, J. W., Kenchington, A. W. & Ward, A. G. (1951). *Research, Lond.*, **4**, 247–248.
Jirgensons, B. (1946). *J. Polym. Sci.*, **1** 475–483.
Jukes, T. H. & Schmidt, C. L. A. (1934). *J. biol. Chem.*, **105**, 359–371.
Luzzi, L. A. & Gerraughty, R. J. (1964). *J. pharm. Sci.*, **53**, 429–431.
McBain, J. W. & Kellogg, F. (1928). *J. gen. Physiol.*, **12**, 1–15.
Nixon, J. R., Khalil, S. A. H. & Carless, J. E. (1966). *J. Pharm. Pharmac.*, **18**, 409–416.
Pasynskii, A. G. (1958). *J. Polym. Sci.*, **29**, 61–68.
Phares, R. E. & Sperandio, G. J. (1964). *J. pharm. Sci.*, **53**, 515–521.
Stainsby, G. (1954). *Discuss. Faraday Soc.*, **18**, 288–294.
Veis, A. (1963). *J. phys. Chem., Ithaca*, **67**, 1960–1965.
Ward, A. G. & Saunders, P. R. (1958). *Rheology*, Vol. 2, p. 321. Editor, Eirich, F. R. New York: Acad. Press.

The action of endotoxin in mobilizing free fatty acid

SIR,—Hirsch, McKay & others (1964) have reported a rapid increase of the plasma free fatty acid level in rabbits given endotoxin intravenously.

Salmonella typhi endotoxin purified according to Boivin and Mesrobeanu, was given intravenously (10^{-3} LD50 in an 0·1 ml/kg volume) to male rabbits of 2·5–3 kg weight. Blood samples were taken by cardiac puncture from the animals starved for 18–20 hr. The serum free fatty acid content was measured photometrically (Mosinger, 1965).

The normal free fatty acid content of rabbit serum was found to be 0·46 mmole/litre (s.d. $\pm$ 0·066) in an average of 11 animals. Endotoxin was administered intravenously to 9 groups of rabbits each of 6 animals and blood samples were withdrawn 1, 2, 3, 4, 8, 16, 32, 48, 64 hr after the injection. The serum free fatty acid level exhibited an initial increase (91·3%) in the first hr (Fig. 1). Up to the end of the second hr, a maximum level of 122% of the normal was obtained. By the end of the third hour, the serum free fatty acid had returned to about its original level. This was followed by a slow and moderate increase after the sixteenth hr, which returned to the normal level after 64 hr.

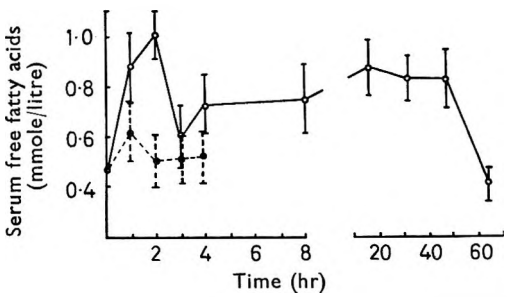

FIG. 1. Influence of *Salmonella typhi* endotoxin on the free fatty acid level of the rabbit serum. Dose: 10^{-3} LD50, 0·1 ml/kg i.v. The starting point is the average of 11, while the other points represent averages of 6 animals each.

The experiments were extended to a control group given the same volume of distilled water instead of endotoxin and in which no significant change occurred.

To examine the possible mechanism of the phenomenon, an examination of the initial response was undertaken.

The experiments were made on groups of 6 rabbits each as previously described. The free fatty acid-mobilizing effect of endotoxin was measured in groups either by transection of the spinal cord or by observing the effects of various sympathetic blocking substances.

Animals of the control groups were sham operated under hexobarbitone anaesthesia. Skin was incised above the cervical part of the vertebral column, the muscles were retracted and the spinous process removed. The wound was sutured and blood sample was withdrawn from the heart after 24 hr. The average free fatty acid level was increased to 1·01m mole/litre (Fig. 2A). This increase was identical with that observed in endotoxin-treated rabbits 2 hr after the injection. When given endotoxin 2 hr after the withdrawal of blood, these rabbits exhibited a further 60% increase of serum free fatty acid. Transection of the spinal cord resulted in a similar elevation of serum free fatty acid to that of the sham operated animals. Administration of endotoxin, to operated animals, however, failed to elicit a further increase.

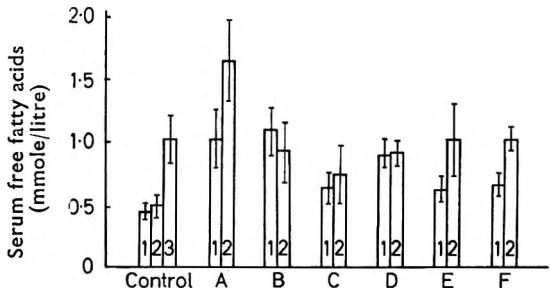

FIG. 2. Control. 1, control; 2, 120 min after 0·1 ml/kg distilled water i.v.; 3,1 20 min after endotoxin, 10^{-3} LD50 0·1 ml/kg i.v. A.1. 24 hr after sham-operation, untreated. A.2. 24 hr after sham-operation, endotoxin given 2 hr before cardiac puncture. B.1. 24 hr after spinal transection, untreated. B.2. 24 hr after spinal transection, endotoxin given 2 hr before cardiac puncture. C.1 140 min after 2 mg/kg Dibenamine i.v. C.2. 140 min after Dibenamine, endotoxin given 120 min before cardiac puncture. D.1. 140 min after 1 mg/kg yohimbine i.v. D.2. 140 min after yohimbine, endotoxin given 120 min before cardiac puncture. E.1. 140 min after 2 mg/kg pronethalol i.v. E.2. 140 min after pronethalol, endotoxin given 120 min before cardiac puncture. F.1. 140 min after 2 mg/kg Sanotensin. F.2. 140 min after Sanotensin, endotoxin given 120 min before cardiac puncture.

The effect of various drugs was studied in two groups of rabbits. Both received the same doses followed 140 min later by cardiac puncture. One of the groups was also given endotoxin 120 min before blood samples were taken.

In the first experiment, α-receptor blocking agents were tested (Fig. 2C, D). Dibenamine induced a moderate increase (0·61 mmole/litre) of serum free fatty acid. Administration of endotoxin hardly affected this value (0·72 mmole/litre). This difference was considered to be within the limits of experimental error. Yohimbine increased the free fatty acid content to 0·90 mmole/litre. Endotoxin failed to increase the free fatty acid level further.

In the next experiment (Fig. 2E), the effect of the β-receptor inhibitor pronethalol was like that of Dibenamine, in inducing a moderate elevation of serum free fatty acid. Administration of endotoxin to this group gave an average increase of 122%—similar to that of the controls; and in this pronethalol failed to inhibit the effect of endotoxin. Sanotensin (2-octahydro-1-azocinyl-ethyl-guanidine sulphate), a substance inhibiting the release of catecholamine, behaved similarly (Fig. 2F).

Our findings suggest the involvement of the central nervous system in endotoxin-induced free fatty acid mobilization.

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Department of Pathophysiology,
National Institute of Public Health,
Budapest IX, Hungary
November 11, 1967

J. TUZSON
P. KERTAI

References

Hirsch, R. L., McKay, S. G., Trayers, R. I. & Skraly, R. K. (1964). *J. Lipid Res.*, **5**, 563–568.
Mosinger, F. (1965). *Ibid.*, **6**, 157–159.

Decrease of cerebral 5-hydroxytryptamine by 3,4-dihydroxyphenylalanine after inhibition of extracerebral decarboxylase

SIR,—In rats, the increase of cerebral catecholamines (especially dopamine) caused by administration of 3,4-dihydroxyphenylalanine (dopa) is markedly enhanced by pretreatment with low doses (50 mg/kg) of Ro 4–4602, [N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)hydrazine], an inhibitor of decarboxylase of aromatic amino-acids. This action of Ro 4–4602 seems to be connected with a preferential inhibition of extracerebral decarboxylase. As a consequence, dopa accumulates in the blood and penetrates into the brain where decarboxylation into dopamine occurs, since cerebral decarboxylase is not markedly inhibited (Bartholini, Bates & others, 1967; Bartholini, Pletscher & Burkard, 1967; Bartholini & Pletscher, 1968).

Here, we demonstrate that simultaneously with the increase of cerebral catecholamines a marked decrease of endogenous 5-hydroxytryptamine (5-HT) takes place.

Albino rats weighing 80–100 g were given intraperitoneal injections of differing doses of L-dopa ½ hr after Ro 4–4602 also intraperitoneally. Controls received L-dopa alone. The endogenous 5-HT and 5-hydroxyindole-acetic acid (5-HIAA) of the whole brain were measured at various time intervals after dopa with spectrophotofluorimetric procedures (Bogdanski, Pletscher & others, 1956; Giacalone & Valzelli, 1966). To determine the uptake of 5-hydroxytryptophan (5-HTP) into the brain, 50 mg/kg of DL-[^{3}H]-5-hydroxytryptophan ([^{3}H]-5-HTP) (uniformly labelled; specific activity 20 μc/mg) was injected into animals pretreated with 500 mg/kg Ro 4–4602 + 500 mg/kg L-dopa, and the total radioactivity of the brain was measured after various time intervals with a liquid scintillation counter. Controls received 500 mg/kg Ro 4–4602 + [^{3}H]-5-HTP only. The large dose of Ro 4–4602 used, markedly inhibited the cerebral as well as the extracerebral decarboxylase (Burkard, Gey & Pletscher, 1962, 1964) preventing a major decarboxylation of [^{3}H]-5-HTP and of dopa.

In the brains of animals pretreated with 50 mg/kg Ro 4–4602, dopa caused

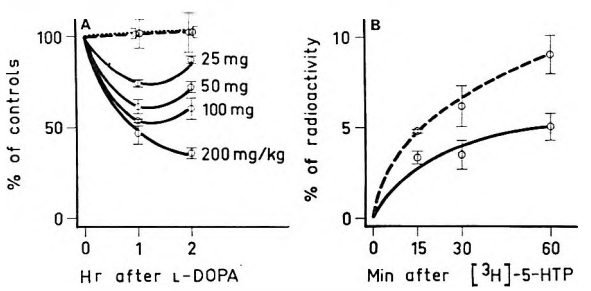

FIG. 1. A. Effect of L-dopa on the content of endogenous 5-hydroxytryptamine (5 HT) in the whole brain of rats pretreated with Ro 4–4602. – – – – 50 mg/kg Ro 4–4602 i.p. .... 200 mg/kg L-dopa i.p. ——— 50 mg/kg Ro 4–4602 i.p., followed by various doses of L-dopa i.p. after ½ hr. The values represent averages of 3–4 experiments ± s.e. and are indicated in % of controls (= 100 %). Absolute values of cerebral 5-HT in controls: 0·35 ± 0·02 μg/g.

B. Effect of L-dopa on the penetration of DL-[^{3}H]-5-hydroxytryptophan into the brain of rats. 50 mg/kg DL-[^{3}H]-5-hydroxytryptophan was injected i.p. 40 min after 500 mg/kg Ro 4–4602 i.p. 500 mg/kg L-dopa was administered i.p. 30 min after Ro 4–4602. ——— Ro 4–4602 + L-dopa + DL-[^{3}H]-5-hydroxytryptophan; – – – – Ro 4–4602 + DL-[^{3}H]-5-hydroxytryptophan. The values represent averages of two experiments ± s.e. and are expressed in % of the radioactivity injected per gram body weight.

a dose-dependent decrease of endogenous 5-HT (Fig. 1A). With 200 mg/kg dopa, the 5-HT rose again to normal levels after about 5 hr. Neither 50 mg/kg Ro 4–4602 nor dopa (200 mg/kg) alone significantly influenced cerebral 5-HT. Furthermore, 50 mg/kg Ro 4–4602 intraperitoneally followed by 200 mg/kg dopa by the same route elevated the endogenous 5-HIAA of brain by 125 ± 3%, but only for 1–1½ hr. Thereafter, 5-HIAA declines to normal levels. The increase of the total radioactivity in the brain caused by administration of [^{3}H]-5-HTP after 500 mg/kg Ro 4–4602 was diminished by dopa (Fig. 1B). Preliminary experiments with DL-[^{3}H]-tryptophan instead of [^{3}H]-5-HTP gave the same results.

These findings indicate that the decrease of brain 5-HT might be due to a combination of several mechanisms. The initial rise of 5-HIAA is probably caused by a displacement of the endogenous 5-HT, e.g. due to the cerebral accumulation of dopamine. Preliminary experiments with various doses of Ro 4–4602 + 200 mg/kg L-dopa confirm this view. Thus, with low doses of Ro 4–4602 (accumulation of cerebral dopa and dopamine formed by decarboxylation of dopa), the endogenous brain 5-HT decreases more markedly than after high doses of the inhibitor (accumulation of cerebral dopa only). On the other hand, the experiments with labelled 5-HTP or tryptophan indicate that dopa probably competes with the penetration of 5-HT precursors into the brain. Moreover, in the experiments with 50 mg/kg Ro 4–4602, competitive inhibition of the cerebral decarboxylation of 5-HTP by dopa (Bertler & Rosengren, 1959) must also be considered.

It has been demonstrated that an increase of cerebral 5-HT induced by 5-HTP (Udenfriend, Weissbach & Bogdanski, 1957), tremorine (Friedman, Aylesworth & Friedman, 1963), or intraventricular injection of 5-HT (Domer & Feldberg, 1960) is accompanied by tremor in animals. A decrease of cerebral 5-HT might therefore counteract tremor in Parkinsonism, whereas an increase of dopamine is thought to improve akinesia and rigidity (Barbeau, Sourkes & Murphy, 1962; Birkmayer & Hornykiewicz, 1964).

Research Department,
F. Hoffmann-La Roche & Co. Ltd.,
Basle, Switzerland.

G. BARTHOLINI
M. DA PRADA
A. PLETSCHER

December 14, 1967

References

Barbeau, A., Sourkes, T. L. & Murphy, G. F. (1962). In *Monoamines et Système Nerveux Central*, pp. 247–262, Genève: Georg & Cie.
Bartholini, G. & Pletscher, A. (1968). *J. Pharmac. exp. Ther.*, in the press.
Bartholini, G., Bates, H. M., Burkard, W. P. & Pletscher, A. (1967). *Nature, Lond.*, **215**, 852–853.
Bartholini, G., Pletscher, A. & Burkard, W. P. (1967). *Helv. physiol. pharmac. Acta*, **25**, CR 170–CR 172.
Bertler, Å. & Rosengren, E. (1959). *Experientia*, **15**, 382–384.
Birkmayer, W. & Hornykiewicz, O. (1964). *Arch. Psychiat. NervKrankh.*, **206**, 367–381.
Bogdanski, D. F., Pletscher, A., Brodie, B. B. & Udenfriend, S. (1956). *J. Pharmac. exp. Ther.*, **117**, 82–88.
Burkard, W. P., Gey, K. F. & Pletscher, A. (1962). *Experientia*, **18**, 411–412.
Burkard, W. P., Gey, K. F. & Pletscher, A. (1964). *Archs Biochem.*, **107**, 189–196.
Domer, F. R. & Feldberg, W. (1960). *Br. J. Pharmac. Chemother*, **15**, 578–587.
Friedman, A. E., Aylesworth, R. J. & Friedman, G. (1963). *Science, N.Y.*, **141**, 1188–1190.
Giacalone, E. & Valzelli, L. (1966). *J. Neurochem.*, **13**, 1265–1266.
Udenfriend, S., Weissbach, H. & Bogdanski, D. F. (1957). *Ann. N.Y. Acad. Sci.*, **66**, 602–608.

The effect of imipramine on central monoamine neurons

SIR,—Imipramine potentiates the action of noradrenaline in the peripheral nervous system (Sigg, 1959). This action, however, is weaker than that of desipramine, its demethylated derivative. This potentiation is because these drugs probably block the uptake-concentration mechanism at the level of the nerve cell membrane of the sympathetic adrenergic neurons (see e.g. Hillarp & Malmfors, 1964; Malmfors, 1965; Carlsson & Waldeck, 1965). The current hypothesis has been that imipramine and related drugs exert a similar blocking action on the uptake-concentration mechanism of the noradrenaline neurons in the central nervous system and that this action may be responsible for their antidepressive properties (see Klerman & Cole, 1965). Evidence (Carlsson, Fuxe & others, 1966; Hamberger, 1967) in support of this view has been given for imipramine-like drugs such as desipramine and protriptyline, but imipramine itself has been found to be only a poor blocker of the uptake-concentration mechanism of the central noradrenaline neurons (Carlsson, Corrodi, Fuxe & Hökfelt, to be published). We now present evidence that imipramine has an effect on the central 5-hydroxytryptamine (5-HT) neurons. Experiments using histochemical (Hillarp, Fuxe & Dahlström, 1966) and biochemical amine analyses have been made and they show that imipramine influences the rate of 5-HT depletion after amine synthesis inhibition (cf. Andén, Corrodi & others, 1966).

Male, Sprague-Dawley rats (200–250 g) have been used. Imipramine (10 or 30 mg/kg) was injected intraperitoneally 15 min before the injection of the tyrosine-hydroxylase inhibitor, the methylester of α-methyltyrosine (H 44/68) or the tyrosine and tryptophane hydroxylase inhibitor, α-propyldopacetamide (H 22/54). In the six histochemical experiments only the highest dose of imipramine was examined. In three experiments H 22/54 (500 mg/kg, i.p.) was used and in another three H 44/68 (250 mg/kg, i.p.). Each histochemical experiment consisted of 4 groups (untreated controls, imipramine alone, imipramine plus inhibitor, inhibitor alone) with 4 rats in each group. Thus, about 100 rats were used in the histochemical experiments. For time-intervals and other details see Table 1. The rectal temperature of the animals was regularly controlled and found to be within normal limits (37–38°).

Histochemically, it was found that in 10 rats out of 12 there was a clear retardation of amine depletion from the 5-HT nerve terminals of the brain, e.g. those of the nucleus suprachiasmaticus, after H 22/54 under the influence

TABLE 1. MONOAMINE CONCENTRATIONS IN WHOLE BRAIN 3¼ HR OR 4¼ HR AFTER IMIPRAMINE (10 OR 30 MG/KG I.P.) GIVEN 15 MIN BEFORE H44/68 (250 MG/KG, I.P.) AND H22/54 (500 MG/KG, I.P.) RESPECTIVELY. The values are given in % of controls. n = number of experiments. Each group represents 4 animals. The statistical analysis has been made according to Student's *t*-test.

	5-HT in %	Noradrenaline in %	Dopamine in %
Untreated controls (n = 4) ..	100·0 ± 3·5	100·0 ± 2·0	100·0 ± 2·5
Imipramine 30 mg/kg (n = 4) ..	96·8 ± 14·3	82·7 ± 13·4	100·6 ± 15·8
H 44/68 (n = 4) ..	—	50·1 ± 1·9	28·8 ± 2·2
Imipramine (30 mg/kg) + H 44/68 (n = 4) ..	—	45·4 ± 1·6	32·1 ± 4·0
H 22/54 (n = 6) ..	43·0 ± 2·2[1]	—	—
Imipramine (30 mg/kg) + H 22/54 (n = 4) ..	62·2 ± 4·2[2]	—	—
Imipramine (10 mg/kg) + H 22/54 (n = 4) ..	45·9 ± 3·9	—	—

* Significance between [1] and [2] $P < 0.01$.

of imipramine. In no case, however, was there any observable change in the rate of amine depletion from the central noradrenaline and dopamine nerve terminals after H 44/68. Nor was the number and intensity of the catecholamine and 5-HT nerve terminals affected by imipramine alone. In the biochemical experiments similar results were obtained (see Table 1). There was a significant ($P < 0.01$) retardation of 5-HT depletion after H 22/54 under the influence of imipramine. The effect was not present with the lowest dose used (10 mg/kg). The rate of noradrenaline and dopamine depletion after H 44/68 was not affected nor were the catecholamine or 5-HT levels (Table 1).

The present findings show that imipramine in a dose of 30 mg/kg clearly retards the rate of amine depletion from central 5-HT nerve terminals after inhibition of 5-HT synthesis (H 22/54). Since the rate of amine depletion after inhibition of synthesis is dependent on the nervous impulse flow (Andén & others, 1966; Andén, Fuxe & Hökfelt, 1966) it may be that there is a decreased nervous impulse flow in the 5-HT neurons under the influence of imipramine. If so, this change in nervous impulse flow may be related to the fact that imipramine in a dose of 30, but not 10 mg/kg, which was ineffective also in the present experiments, is probably a blocker of the reserpine-resistant uptake-concentration mechanism of the central 5-HT neurons (Carlsson, Fuxe & Ungerstedt, 1968). This blockade will increase the amounts of 5-HT reaching the postsynaptic receptors and could, thus, initiate a negative feedback on the presynaptic 5-HT neuron. This would result in a decreased impulse flow, which was, in fact, indicated from the results of the present study. It is known from previous studies that desipramine probably has no effect on the 5-HT neurons, since there is no retardation of the rate of 5-HT depletion after amine synthesis inhibition under the influence of desipramine (Corrodi, Fuxe & Hökfelt, 1967) nor is there any observable block of 5-HT uptake (Fuxe & Ungerstedt, 1967). It is proposed that an effect on the 5-HT neurons may be of importance for the antidepressant action of imipramine.

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AB Hässle, Göteborg, and
Department of Pharmacology,
University of Göteborg.

H. CORRODI

Department of Histology,
Karolinska Institutet,
Stockholm, Sweden.

K. FUXE

December 19, 1967

References

Andén, N.-E., Corrodi, H., Dahlström, A., Fuxe, K. & Hökfelt, T. (1966). *Life Sci.*, **15**, 561–568.
Andén, N.-E., Fuxe, K. & Hökfelt, T. (1966). *J. Pharm. Pharmac.*, **18**, 630.
Carlsson, A. & Waldeck, B. (1965). *Acta pharmac. tox.*, **22**, 293–300.
Carlsson, A., Fuxe, K., Hamberger, B. & Lindqvist, M. (1966). *Acta physiol. scand.*, **67**, 481–497.
Carlsson, A., Fuxe, K. & Ungerstedt, U. (1968). *J. Pharm. Pharmac.*, **20**, 150–151.
Corrodi, H., Fuxe, K. & Hökfelt, T. (1967). *European J. Pharmac.*, **1**, 363–368.
Fuxe, K. & Ungerstedt, U. (1967). *J. Pharm. Pharmac.*, **19**, 335–337.
Hamberger, B. (1967). *Acta physiol. scand., suppl.* 295.
Hillarp, N.-Å. & Malmfors, T. (1964). *Life Sci.*, **3**, 703–708.
Hillarp, N.-Å., Fuxe, K. & Dahlström, A. (1966). *Pharmac. Rev.*, **18**, 727–741.
Klerman, G. L. & Cole, J. O. (1965). *Ibid.*, **17**, 101–141.
Malmfors, T. (1965). *Acta physiol. scand.*, **64**, *Suppl.* 248.
Sigg, E. B. (1959). *Canad. Psychiat. Ass. J.*, **4**, 75–85.

The effect of prostaglandin E_1 on isolated bronchial muscle from man

SIR,—The prostaglandins are a family of closely related hydroxy unsaturated C_{20} fatty acids which have been isolated from various mammalian tissues and whose biological properties have been reviewed by Horton (1965). They were originally discovered in semen and in the prostate gland by virtue of their vasodepressor and intestinal smooth muscle stimulating properties (Goldblatt, 1933; Euler, 1934). More recently they have been shown to have inhibitory action *in vivo* on both the reproductive smooth muscle of the rabbit (Horton, Main & Thompson, 1963) and the respiratory smooth muscle of the rabbit and guinea-pig (Main, 1964). *In vitro*, prostaglandins have been shown to relax tracheal muscle from the monkey, cat, rabbit, guinea-pig and ferret (Main, 1964) and myometrial strips from non-pregnant human females (Bygdeman & Eliasson, 1963). There has been no report of the action of prostaglandins on human respiratory smooth muscle and accordingly such a study has been made using prostaglandin E_1 (PGE_1).

Human bronchi were obtained from macroscopically normal parts of human lung which had been removed in surgery for carcinoma of the lung. The lung specimens were transferred to the laboratory in ice-cold oxygenated Tyrode solution. Suitable secondary (lumen diameter 6–8 mm) or third order (lumen diameter 4–6 mm) bronchi were dissected and were used after 24 or 48 hr storage in oxygenated Tyrode solution at 4°. A bronchus was sectioned into rings, the rings cut open, and two or more of these opened rings were tied together in series to form a bronchial strip. The strip was mounted in a 10 ml isolated organ bath and attached to a lever with a magnification of 14 : 1 writing on a smoked drum. The tissue was bathed in oxygenated Krebs (1950) solution containing double strength glucose at a temperature of 37°. The tension on the tissue was about 100 mg, and the tissue was allowed to rest for 30 min before the addition of drugs.

Using the inherent tone of the bronchial strip *in vitro* it was found that PGE_1 produced inhibition of this tone. The threshold concentration was usually 0·25 μg/ml. A typical experiment is illustrated in Fig. 1, when concentrations of 2, 4 and 8 μg PGE_1/ml caused relaxations represented by falls on the tracing of 13, 15 and 25 mm respectively. For comparison, 5 and 10 ng isoprenaline/ml produced falls of 20 and 29 mm respectively.

A similar inhibitory action was seen when PGE_1 was added to the organ bath during a sustained contraction of the bronchial muscle produced by histamine acid phosphate (4 μg histamine base/ml). Fig. 2 shows a typical experiment when concentrations of 1 and 2 μg PGE_1/ml caused 36 and 100% inhibition of

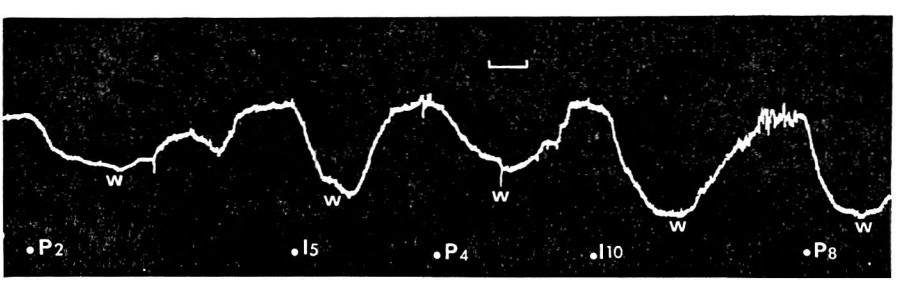

FIG. 1. The inhibitory effect of PGE_1 (P) and isoprenaline (I) on the inherent tone of isolated human bronchial muscle. Doses of P in μg/ml and of I in ng/ml. W: wash. Time scale: 5 min.

the contractile response respectively. For comparison, 2 and 5 ng isoprenaline/ml caused 32 and 100% inhibition respectively.

It was also demonstrated that although the inhibitory effect of isoprenaline was abolished in the presence of the β-adrenergic blocking agent propranolo (0·1 μg/ml), the inhibitory effect of PGE_1 was not affected. Nor did the α-adrenergic blocking agent phenoxybenzamine (10 μg/ml) affect the response to PGE_1.

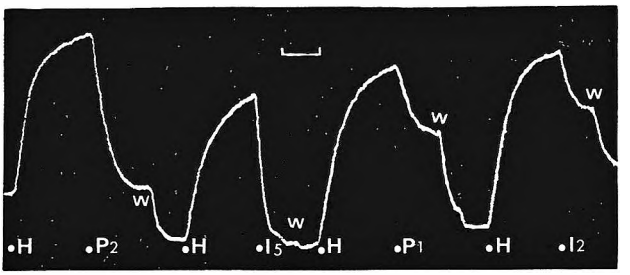

FIG. 2. The inhibitory action of PGE_1 (P) and isoprenaline (I) on contractile responses of human bronchial muscle induced by histamine (H): 4 μg/ml. Doses of P in μg/ml and of I in ng/ml. W: wash. Time scale: 5 min.

Of the isolated tracheal muscle tissues examined by Main (1964), cat preparations were the most sensitive, their tone being inhibited by concentrations as low as 1 ng PGE_1/ml. Pig and sheep preparations were least sensitive and responded irregularly only to doses of 0·25 to 3 μg/ml. In the experiments reported here, isolated human bronchial muscle was inhibited by PGE_1, but the sensitivity was low, concentrations of 0·25 μg/ml and more having to be used. Experiments with adrenergic blocking agents indicate that the inhibitory effect of PGE_1 on human bronchial muscle is not produced by an action on adrenergic receptors.

The prostaglandins found in human bronchi and lung parenchyma by Karim, Sandler & Williams (1967) were F_{2a} (1–50 ng/g) and E_2 (1–8 ng/g). Qualitatively, PGE_1 is similar in its action on cat tracheal smooth muscle to PGF_{2a} and PGE_2, while quantitatively it is at the very least equi-active (Horton, 1965). It is difficult therefore to envisage a physiological role in normal human lung from such small concentrations of prostaglandins.

Acknowledgements. I thank Mr. Gordon Jack for specimens of human lung, Drs. John E. Pike (The Upjohn Company, Kalamazoo) and D. A. van Dorp (Unilever Research Laboratories, Vlaardingen) for gifts of prostaglandin E_1, and Professor E. W. Horton for reading and commenting on this letter.

Department of Pharmacology,
Fisons Pharmaceuticals Ltd.,
Holmes Chapel, Cheshire, England.
December 6, 1967

P. SHEARD

References

Bygdeman, M. & Eliasson, R. (1963). *Medna exp.*, **9**, 409–415.
Euler, U. S. von (1934). *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **175**, 78–84.
Goldblatt, M. W. (1933). *Chemy Ind.*, **52**, 1056–1057.
Horton, E. W., Main, I. H. M. & Thompson, C. J. (1963). *J. Physiol. Lond.*, **168**, 54–55P.
Horton, E. W. (1965). *Experientia*, **21**, 113–118.
Karim, S. M. M., Sandler, M. & Williams, E. D. (1967). *Br. J. Pharmac. Chemother.*, **31**, 340–344.
Krebs, H. (1950). *Biochim. biophys. Acta*, **4**, 249–269.
Main, I. H. M. (1964). *Br. J. Pharmac. Chemother.*, **22**, 511–519.

The effects of (+)-amphetamine and (±)-phenmetrazine on the noradrenaline and dopamine levels in the hypothalamus and corpus striatum of the rat

Sir,—(+)-Amphetamine decreases and (±)-phenmetrazine increases whole rat brain noradrenaline levels (Baird & Lewis, 1964). This effect of (+)-amphetamine supports the current view that it acts by releasing noradrenaline from the brain (Stein, 1964; Carlsson, Lindqvist & others, 1965; Weissman, Koe & Tenen, 1966) while that of (±)-phenmetrazine suggests a different mechanism of action although its effects on behaviour resemble those of (+)-amphetamine (Van der Schoot, Ariëns & others, 1962; Weissman & others, 1966). However, these conclusions must be tentative as precise mechanisms of action cannot be deduced from observations on whole brain. Nevertheless, further investigation appeared warranted. Noradrenaline is unevenly distributed in the brain and as a first step, an area of the brain rich in the amine, the hypothalamus (Vogt, 1954), was used since drug-induced changes in its noradrenaline content are more likely to reflect the true action of the compounds on brain noradrenaline levels. In addition, the hypothalamus is concerned with mood and behaviour (Ingram, 1960). Therefore, the effects of (+)-amphetamine and (±)-phenmetrazine on the level of noradrenaline in the rat hypothalamus were investigated.

Drug (in 0·9% w/v NaCl solution) and control (0·9% w/v NaCl solution) solutions were injected intraperitoneally (0·2 ml/100 g body weight) into groups of 4 male rats (80–110 g). Three hr later, the animals were killed and the hypothalamic areas dissected and pooled. The pooled tissues were homogenized in ice-cold 0·4M perchloric acid, extracted and the noradrenaline adsorbed onto acid-washed alumina at pH 8·5. The amine was eluted with 0·2N acetic acid and assayed fluorimetrically by the trihydroxyindole method.

The results are shown in Table 1. As in whole brain, (+)-amphetamine reduces the hypothalamic noradrenaline level but (±)-phenmetrazine, although it increases the noradrenaline level in whole brain, does not affect it in the hypothalamus. These observations further support the view that (+)-amphetamine acts indirectly by releasing noradrenaline from the brain and suggest that

TABLE 1. THE EFFECTS OF (+)-AMPHETAMINE AND (±)-PHENMETRAZINE ON THE LEVELS OF NORADRENALINE AND DOPAMINE IN THE HYPOTHALAMUS AND CORPUS STRIATUM OF THE RAT BRAIN

Treatment	Dose mg/kg	No. of groups	Noradrenaline (μg/g fresh hypothalamus)	Dopamine (μg/g fresh corpus striatum)
Control	0	5	4·54 ± 0·34	—
(+)-Amphetamine sulphate	10	5	3·34 ± 0·22**	—
Control	0	8	6·13 ± 0·66	—
(±)-Phenmetrazine hydrochloride	40	8	7·48 ± 1·11	—
Control	0	7	5·07 ± 0·30	—
(±)-Phenmetrazine hydrochloride	80	7	4·16 ± 0·30	—
Control	0	5	—	10·68 ± 0·59
(+)-Amphetamine sulphate	10	5	—	12·38 ± 0·53
Control	0	5	—	9·51 ± 0·59
(+)-Amphetamine sulphate	20	5	—	10·28 ± 1·11
Control	0	5	—	8·94 ± 0·39
(±)-Phenmetrazine hydrochloride	80	5	—	10·61 ± 0·57*

All values are the means ± standard errors of the means. The animals (4 in each group) were killed 3 hr after the intraperitoneal injection of the drug or control solution. Significance of difference from control: * $0{\cdot}05 > P > 0{\cdot}02$; ** $0{\cdot}02 > P > 0{\cdot}01$.

noradrenaline may not be involved in the mode of action of (±)-phenmetrazine.

Since dopamine may be involved in psychomotor stimulation (Rossum, 1964), the possibility that the central actions of (±)-phenmetrazine are mediated through this amine was explored by measuring the effect of this drug on the dopamine level in the rat corpus striatum, an area rich in dopamine (Bertler & Rosengren, 1959). The experiments were made as described above for the hypothalamus except that dopamine was assayed by the method of Udenfriend (1962). The effect of (+)-amphetamine on the dopamine level in the rat corpus striatum was also examined.

The results are shown in Table 1. (±)-Phenmetrazine increases the dopamine level in the rat corpus striatum which may account for the increased motor activity it produces since increasing amounts of brain dopamine are paralleled by increasing motor activity (Everett & Wiegand, 1962). On the other hand, (+)-amphetamine does not affect the amount of dopamine in the rat corpus striatum suggesting that its central actions are not mediated through this amine.

The reported results suggest that (+)-amphetamine and (±)-phenmetrazine, in spite of their chemical and pharmacological similarity, do not have a common mode of action in the brain. A link apparently exists between (+)-amphetamine and noradrenaline, which is decreased, and between (±)-phenmetrazine and dopamine, which is increased. Therefore, it is possible that these amines are involved in the respective central mechanisms of action of the drugs.

Acknowledgements. I am grateful to Miss Frances P. Moore for skilful technical assistance, to Smith, Kline and French Laboratories for a gift of (+)-amphetamine and to Boehringer Ingelheim Ltd. for a gift of (±)-phenmetrazine.

Department of Pharmacology,
University of Glasgow,
Glasgow, W.2,
Scotland.

J. R. C. BAIRD

December 12, 1967

References

Baird, J. R. C. & Lewis, J. J. (1964). *Biochem. Pharmac.*, **13**, 1475–1482.
Bertler, A. & Rosengren, E. (1959). *Experientia*, **15**, 10–11.
Carlsson, A., Lindqvist, M, Dahlström, A., Fuxe, K. & Masuoka, D. (1965). *J. Pharm. Pharmac.*, **17**, 521–524.
Everett, G. M. & Wiegand, R. G. (1962). *Proceedings of the First International Pharmacological Meeting*, Vol. 8, editors Paton, W. D. M. & Lindgren, P., pp. 85–92, Oxford, London: Pergamon Press.
Ingram, W. R. (1960). *Handbook of Physiology*, Section 1: Neurophysiology, Vol. 2, editors Field, J., Magoun, H. W. & Hall, V. E., pp. 951–978, Washington, D.C.: American Physiological Society.
Rossum, J. M. van. (1964). *Proceedings of the Second International Pharmacological Meeting*, Vol. 2, editors Trabucchi, E., Paoletti, R. & Canal, N., pp. 115–126, Oxford. London, Edinburgh: Pergamon Press.
Stein, L. (1964). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **23**, 836–850.
Udenfriend, S. (1962). *Fluorescence Assay in Biology and Medicine*, p. 137, London: Academic Press.
Van der Schoot, J. B., Ariëns, E. J., Rossum, J. M. van & Hurkmans, J. A. Th. M. (1962). *Arzneimittel-Forsch.*, **12**, 902–907.
Vogt, M. (1954). *J. Physiol., Lond.*, **123**, 451–481.
Weissman, A., Koe, B. K. & Tenen, S. S. (1966). *J. Pharmac. exp. Ther.*, **151**, 339–352.

A device for the rapid measurement of rat foot volume

SIR,—One of the most commonly used methods for studying experimentally induced inflammation in animals is that of measuring the swelling after injection of an irritant substance into the foot of the rat. Our plethysmographic technique is based on that of Kopf & Møller Nielsen (1958), who measured the amount of fluid required to replace that displaced by immersion of the rat paw into a suitable fluid filled vessel.

The fundamental components of the apparatus are an automatic microburette (Technico Automatic 5 ml, B.W. 644, A Gallenkamp & Co.) and a glass displacement chamber (Fig. 1), the outlet tube of the burette being connected by polythene tubing to the inlet tube A of the displacement chamber. The whole is filled with water which serves as the displacement fluid. An electrically rotated tap is used to control the flow of water from the burette to the displacement chamber. The tap (P.T.F.E., "Interkey", G. Springham & Co. Ltd., Harlow New Town) is connected by a flexible spindle coupling (Eddystone Radio) to the armature shaft of a rotary solenoid (Ledex, type LX/511/16357/DHZ, N.S.F. Ltd., London) and is rotated through 90° when the solenoid is energized. The tap is turned by the solenoid either by manual operation of a biased double-pole change-over switch or automatically, depending upon the stage of the measuring procedure.

The dimensions of the components of the glass displacement chamber are fairly critical. Chamber A is 4 cm long and has an inside diameter of 1·3 cm. The inlet tube to A and the tube connecting A to B are of 0·6 cm internal diameter. The inside diameter of B is 1·7 cm and tube C (0·6 cm), is fused centrally into it extending to the same level as the brim of A. Chamber B is 1 cm longer than chamber A. The inlet tube to A and the outlet tube C pass through a Perspex base, and the whole is cemented firmly to this with Araldite [AY 103, Ciba (A.R.L.) Ltd.] moulded into the form of a block. The electrodes E1 and E2 consist of 22 S.W.G. platinum wire soldered to miniature sockets fixed firmly in a strip of Perspex. The electrodes are positioned in B on either side of C so that E2 projects at least 1 cm below E1. The electrode assembly is

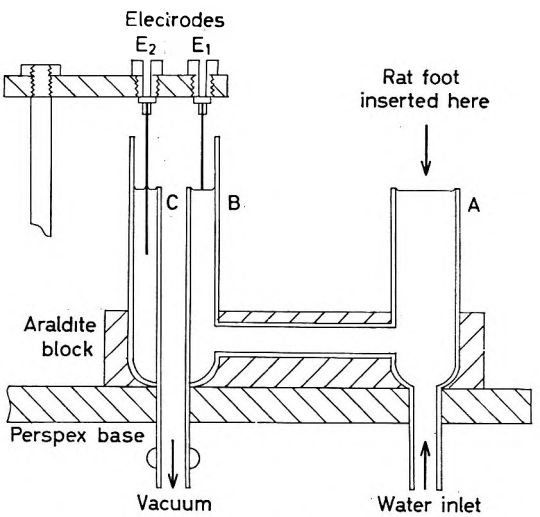

FIG. 1. Glass displacement chamber and electrode assembly.

fixed into the sleeve of a Palmer rack-work 'X' block (D36) attached to the Perspex base so that the electrodes are positioned in B and the level of the tip of E1 can be adjusted to coincide with the brim of tube C.

The glass displacement chamber contains water to the brim of tubes A and C. The rat foot is immersed into tube A to a constant anatomical level, this being indicated by a pad on the plantar surface of the hind foot. Water is displaced into tube B, and an equal volume immediately removed via tube C attached to a vacuum line through a water trap. The volume displaced by the foot is that needed to refill the displacement chamber to the original level. This is found by manually operating the double-pole change-over switch which causes the rotary solenoid to open the tap. Water is thus allowed to flow from the micro-burette into tube A until the level reaches the tip of electrode E1. A circuit is then completed by the water between E1 and E2, which causes the rotary solenoid to turn a further 90° and so cut off the water flow. The volume of water required to fill the chamber is read from the burette which is then refilled to the zero mark. The usual method of refilling this type of automatic burette is to increase the pressure in the reservoir by a hand operated rubber bulb but for convenience we use the pressure from a 5 lb/inch^2 compressed air line controlled by an electromagnetic valve.

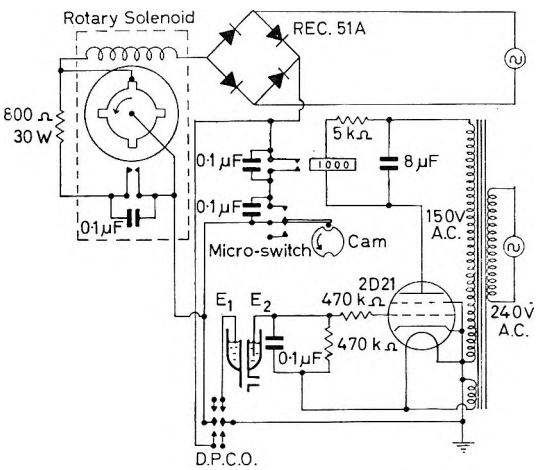

FIG. 2. Circuit diagram.

The electrical circuit (Fig. 2) uses a Thyratron 2D21 valve which is non-conducting until the grid circuit is completed by water touching the foot bath electrodes. The Thyratron then conducts and the relay in the anode circuit becomes energized. The relay contacts allow 240 V DC to pass to the rotary solenoid which rotates through 90° turning the tap to the closed position. A micro-switch, operated by a cam attached to the armature shaft of the rotary solenoid, breaks the current flow to the solenoid when it has rotated the tap to the closed position. This prevents over-heating of the solenoid coil, due to prolonged energization during the period that both electrodes are in contact with the water. The use of a biased double-pole change-over switch to operate the tap also avoids the possibility of permanent energization of the solenoid coil. One pole of this switch energizes the rotary solenoid, whilst the other opens the electrode circuit. When the switch returns to its biased position the electrode

circuit is again completed, causing the rotary solenoid to be re-energized and the water tap closed.

To allow efficient operation of the apparatus the vacuum source, assisting the removal of liquid from tube B, should be maintained at a constant level (−10 cm water) during a series of measurements. The time for immersion of the rat foot should be standardized, 5 sec is optimal. The rate of passage of water from the burette to the foot bath should be adjusted to just less than that causing an overshoot into chamber B.

The error of the measurements when the same foot of each of ten rats was measured five times in random sequence was calculated to be 3·6%.

The advantage of this apparatus over others in common use is that speed of repetitive measurement is achieved without loss of accuracy. The components cost approximately £20 and this compares favourably with commercially available equipment.

The Wellcome Research Laboratories,
Langley Court,
Beckenham, Kent, England.
December 6, 1967

L. G. GARLAND
S. J. SMITH
M. F. SIM

Reference

Kopf, R. & Møller Nielsen, I. (1958). *Arzneimittel-Forsch.*, **8**, 154–158.

The distribution of small concentrations of active ingredients in tablet granules

SIR,—It is common practice when preparing tablets containing small quantities of potent materials to add the active ingredient in solution to an inert basis granulate to obtain even distribution. After drying, coarse aggregates are broken down by sifting and the resultant granulate is tabletted.

During the preparation of a small batch of tablets containing [4-^{14}C]-lynoestrenol, an investigation into the distribution of the lynoestrenol indicated that this was uneven when the drug was applied to the tablet granulate in ethanolic solution and after drying under an infrared lamp. All assays were made using liquid scintillation counting in a Packard Tricarb Spectrometer Model 3003, with and without an internal standard for correcting quenching, on the granulate extracted quantitatively with benzene and ether. Because of the small size of the sample, it was not possible to make a particle size evaluation, therefore samples of the coarser and very fine fractions of the granule were taken and compared with the original. The results are in Table 1. A second sample of basis granulate was prepared and reduced to a fine powder of uniform appearance before the labelled lynoestrenol was added. The distribution found is in Table 2.

TABLE 1. INFLUENCE OF PARTICLE SIZE ON LYNOESTRENOL DISTRIBUTION IN GRANULES. Added amount of labelled lynoestrenol: 2·53 mg (corresponding to 21,800 disintegrations/min) per 98·0 mg granulate.

Type of granule sample	Weight of granulate (mg)	Measured radio activity (d/min)	Calculated mg drug per 98·0 mg granulate
Coarse particles	98·0	33,400	3·89
Mixed	98·0	26,800	3·11
Mixed	98·7	26,300	3·06
Mixed	98·4	24,200	2·82
Fine	98·4	14,500	1·69

TABLE 2. LYNOESTRENOL CONTENT OF GRANULATE POWDERED BEFORE THE ADDITION OF LABELLED LYNOESTRENOL SOLUTION. Drug added: 4·83 mg (corresponding to 28,900 disintegrations/min) per 100 mg granulate.

Weight of granulate (mg)	Measured radioactivity (d/min)	Calculated drug content (mg)
91·0	26,600	4·45
95·2	26,600	4·45
96·9	27,600	4·61
100·9	30,000	5·01
103·0	27,900	4·66
106·8	31,800	5·30
109·1	31,400	5·24
111·9	30,200	5·04

Residual standard deviation of drug content about the regression line: 0·21 mg = 4·4% at arithmetic mean.

From Table 1 it can be seen that the coarser granules contained more than twice the lynoestrenol content of the fine granules. If such a granule were used in tablet manufacture then it is probable that wide variations in drug content of tablets would occur. The results in Table 2 indicate a far more even distribution of lynoestrenol. When the granulate that was initially reduced to a fine powder of uniform appearance was subjected to further size reduction, by shaking with glass beads in a closed container for 30 min, the residual standard deviation for the drug distribution was reduced to 1·3%.

Lachman & Sylwestrowicz (1964) reported a concentration of active ingredient in the larger granules when preparing tablets containing a poorly water-soluble active ingredient by moist granulation using water as the granulating agent. In that instance it was suggested that the phenomenon might be due to the drug adhering to the surface of the granules and being less easily dislodged from large granules, with a small surface area, than from the smaller ones. Further there was the possibility that the larger granules would more readily encapsulate the drug than the smaller.

We were concerned with the addition of the drug in solution. The basis granule consisted of lactose with starch as disintegrant and magnesium stearate as lubricant. It might be that the larger granules had more void spaces than the smaller granules and thus on drying the larger granules contained more ingredient than the fine granules which might have had only a surface layer. A further possibility of importance when large batches of granules are prepared could be that, before drying, the granules segregated, the larger ones rising to the surface; the active ingredient would also tend to rise to the surface by capillary action during drying.

We therefore recommend caution when this kind of procedure is used to incorporate a small quantity of potent material into a tablet granule. It would appear to be preferable to have the basis granulate in the form of uniformly sized granules, to mix these further, and possibly to reduce the particle size after drying.

From the Pharmaceutical Research
and Radioisotopes Departments,
N.V. Organon,
Kloosterstraat 6,
Oss, Netherlands.

P. H. Cox
T. J. G. Ambaum
H. P. Wijnand

January 9, 1968

Reference

Lachman, L. & Sylwestrowicz, H. D. (1964). *J. pharm. Sci.*, **53**, 1234–1242.

The effect of catecholamines and β-anti-adrenergic drugs on isolated short-circuited skin of the frog

SIR,—Adrenaline (Koefoed-Johnsen, Levi & Ussing, 1952) and isoprenaline (Santi, Ferrari & others, 1967) increase the short-circuit current in frog isolated skin.

The activity of catecholamines on this preparation was compared by determining their dose-effect curves. The effect of these drugs was evident at surprisingly low concentrations (Fig. 1). Isoprenaline action started at $0{\cdot}5 \times 10^{-9}$M, while noradrenaline and adrenaline activity began from 10^{-8} to 10^{-7}M. The order of affinity* was then the following: isoprenaline $\gg$ noradrenaline $>$ adrenaline. The intrinsic activity* was the same for the three catecholamines. Auto-inhibition, developing for the higher doses of catecholamines, follows an opposite order of intensity, being the greatest for adrenaline and least for isoprenaline (Fig. 1). The skin potential variations were similar to those of the short-circuit current.

The antiadrenergic drugs Kö 592 [(3-methylphenoxy)-β-hydroxy-α-isopropylaminopropanol] (Engelhardt, 1965) and D(−)-INPEA (*N*-isopropyl *p*-nitrophenylethanolamine) (Somani & Lum, 1965) antagonized competitively the effect of

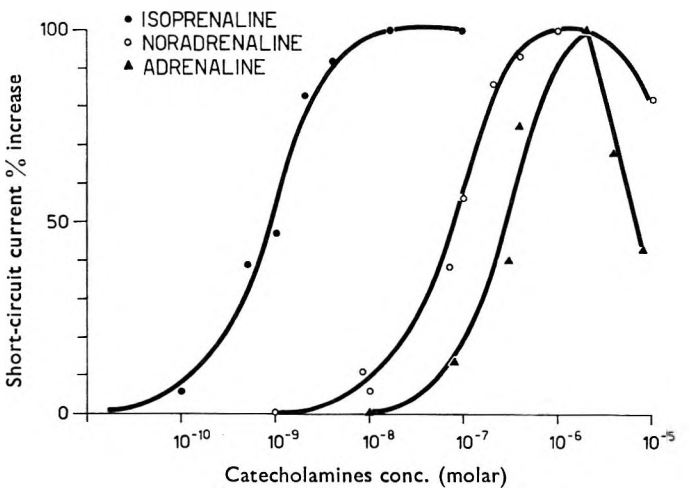

FIG. 1. Log concentration-response curves for the increase of the short-circuit current induced by catecholamines on frog isolated skin. *Abscissa:* molar conc. of isoprenaline, noradrenaline and adrenaline in the solution exposed to the inside of the skin. *Ordinate:* μA % relative increase from control (skin equilibrated for 2 to 3 hr after the dissection and mounting). The short-circuit current maximum increase, induced by isoprenaline 10^{-8}M, by noradrenaline 10^{-8}M and by adrenaline 2×10^{-6}M (263 ± 37 μA) was taken as 100% of the effect. The maximum increase was reached in 10 to 30 min. Each point represents the mean of 4 to 6 experiments. Abdominal skin of *Rana esculenta* (surface 7·06 cm²) was clamped between two lucite chambers containing frog Ringer solution. Short-circuit current and potential difference across the skin were measured according to the technique of Ussing & Zerahn (1951). Drugs were added directly to the solution contained in the chamber bathing the internal side of the skin. After each dose of catecholamine, the skin was repeatedly washed by changing the Ringer solution in the two chambers, and re-equilibrated (2–3 hr) before adding the next dose. Experiments were carried out in July, August and September, at room temperature.

* According to Ariëns (1954) nomenclature.

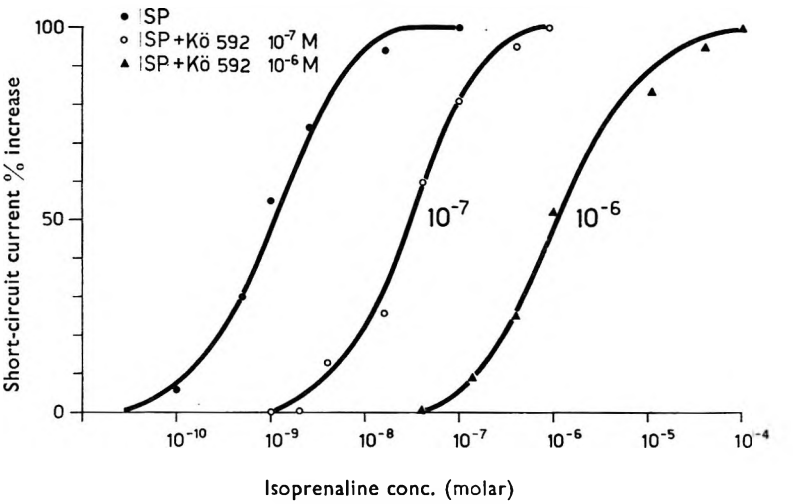

FIG. 2. Log concentration-response curves for the antagonism between isoprenaline and Kö 592 on isolated short-circuited skin of the frog. *Abscissa:* molar conc. of isoprenaline (ISP) in the solution facing the inside of the skin. *Ordinate:* μA % relative increase from control (skin equilibrated for 2 to 3 hr in the presence of frog Ringer solution, with or without addition of the stated conc. of antagonist). Experimental conditions as in Fig. 1. Isoprenaline was added cumulatively in the chamber opposite to the internal side of the skin.

isoprenaline on short-circuit current. Fig. 2 demonstrates the dose-effect curves for the antagonism between isoprenaline and Kö 592. The presence of Kö 592 alone, did not alter, at the lower concentrations (10^{-8} and 10^{-7}M), the skin potential or the resting short-circuit current; at 10^{-6}M it decreased these values.

Whether the action of catecholamines on frog skin should be regarded as one affecting permeability, sodium pump, or other functions of this organ cannot be stated, very little information being available. According to Koefoed-Johnsen & others (1952), adrenaline induces in the frog skin a transitory increase of the short-circuit current by causing an unusual active transport of Cl^- outward, probably as a result of the action on the mucous glands.

However, the action of catecholamines on isolated short-circuited frog skin shows features similar to those characterizing a typical β-tropic function, if the wide difference in the affinities between isoprenaline and noradrenaline and the specific interaction between isoprenaline and β-lytic agents are considered. On the other hand, the unusual affinity sequence isoprenaline > noradrenaline > adrenaline is a typical feature of the adrenergic lipid mobilization *in vitro* (Wenke, Mühlbachová & others, 1964; Rudman, Garcia & others, 1964; Barrett, 1965; Fain, 1967).

Isolated short-circuited frog skin could, thus, represent a useful test for adrenergic and anti-adrenergic drugs.

Institute of Pharmacology,
University of Padua,
Largo E. Meneghetti 2,
I 35100, Padua, Italy.

G. FASSINA
F. CARPENEDO
G. FIANDINI

November 15, 1967

References

Ariëns, E. J. (1954). *Archs int. Pharmacodyn. Thér.*, **99**, 32–49.
Barrett, A. M. (1965). *Br. J. Pharmac. Chemother.*, **25**, 545–556.
Engelhardt, A. (1965). *Arch. exp. Path. Pharmak.*, **250**, 245–246.
Fain, J. N. (1967). *Ann. N.Y. Acad. Sci.*, **139**, 879–890.
Koefoed-Johnsen, V., Levi, H. & Ussing, H. H. (1952). *Acta physiol. scand.*, **25**, 150–163.
Rudman, D., Garcia, L. A., Brown, S. J., Malkin, M. F. & Perl, W. (1964). *J. Lipid Res.*, **5**, 28–37.
Santi, R., Ferrari, M., Tóth, C. E., Contessa, A. R., Fassina, G., Bruni, A. & Luciani, S. (1967). *J. Pharm. Pharmac.*, **19**, 45–51.
Somani, P. & Lum, B. K. B. (1965). *J. Pharmac. exp. Ther.*, **147**, 194–204.
Ussing, H. H. & Zerahn, K. (1951). *Acta physiol. scand.*, **23**, 110–127.
Wenke, M., Mühlbachová, E., Elisová, K., Schusterová, D. & Hynie, S. (1964). *Int. J. Neuropharmac.*, **3**, 283–292.

The penetration temperature of aqueous sodium dodecyl sulphate solutions into solid long-chain alcohols

SIR,—When a surfactant, amphiphile and water are mixed, a spontaneous formation of ternary liquid crystalline phase occurs in one of two ways depending on how the components are mixed (Lawrence, 1959, 1961a, b). If a piece of surfactant-amphiphile mixture is flooded with water, myelinic tubular forms appear. These are strongly birefringent close to the original mixture but the birefringence decreases as the myelins become more elongated and finally the outer part dissolves to form an isotropic solution.

If, however, the solid amphiphile is immersed in surfactant solution, spontaneous formation of ternary mesophase again occurs, but with two differences. Firstly, true myelins are not formed but only tubular myelin-like protuberances. These are deformed spherulites which, unlike true myelins, do not possess a central core of isotropic solution (Lawrence, 1958). Secondly, there is a sharply defined temperature, T_{pen}, below which an isotropic solution occurs very slowly, while at and above T_{pen} penetration of surfactant solution into the amphiphile occurs by formation of ternary liquid crystal phase. The first step is the formation of a film of mesophase around the amphiphile, followed by myelinic-like protuberances, formed as the surfactant solution penetrates into the amphiphile. These extrude into the aqueous solution and then break up into liquid crystal spherulites as they become fluid enough for surface tension to act.

T_{pen} varies with the nature of the hydrophobic and hydrophilic groups of the surfactant and amphiphile. It has been stated that T_{pen} does not vary with concentration of surfactant over wide limits (Lawrence, 1958; Lawrence, Bingham & others, 1964) or that it varies only slightly with concentration (Lawrence, 1961a, b). We have investigated this for aqueous sodium dodecyl sulphate solutions in the range 0·5% to 20·0% w/w using the normal C_{14}, C_{16} and C_{18} alcohols. The purity of the alkyl sulphate and 1-hexadecanol was as given by Barry & Shotton (1967), 1-octadecanol was Fluka purrissimum grade, and the 1-tetradecanol was Fluka purum grade which was purified by preparative gas chromatography. Dry and wet melting points were respectively: for 1-tetradecanol 39·5, 40·0; 1-hexadecanol 49·5, 52·0; 1-octadecanol 58·5, 61·5.

A few mg of an alcohol were melted on a cavity slide and agitated with a needle whilst cooling so as to yield a thin solid layer when cold. The cavity was filled with one of the sodium dodecyl sulphate solutions, a cover slip added and the slide was placed on a Kofler micro hot stage fitted to a polarizing

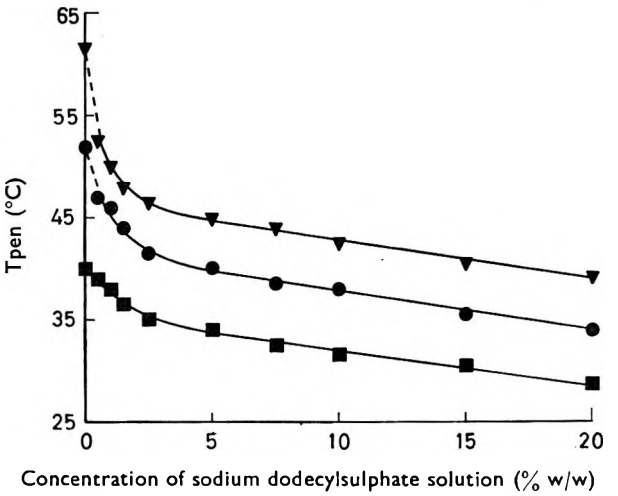

FIG. 1. Variation of the penetration temperature (T_{pen}) of aqueous solutions of sodium dodecyl sulphate into n-alkanols. ▼ 1-Octadecanol. ● 1-Hexadecanol. ■ 1-Tetradecanol.

microscope (Barry, 1967). The microscope was focussed on the edge of a crystal and the temperature at which liquid crystals first appeared was noted. The procedure was repeated at least three times for each alcohol and each solution and the average temperature found. The results shown in Fig. 1 indicate a pronounced dependence of T_{pen} on the surfactant concentration, the penetration temperature falling sharply as the concentration rises. This variation is most marked at low surfactant concentrations. The melting points in distilled water are all somewhat higher than the melting points of the dry compounds. Lawrence (1960) suggested that this is because alkanols form solid solutions with water.

Department of Pharmaceutics,
The School of Pharmacy,
29–39 Brunswick Square,
London, W.C.1, England.

B. W. BARRY*
E. SHOTTON

December 12, 1967

* Present address: School of Pharmacy, Portsmouth College of Technology Portsmouth.

References

Barry, B. W. (1967). Ph.D. thesis. University of London.
Barry, B. W. & Shotton, E. (1967). *J. Pharm. Pharmac.*, **19**, *Suppl.*, pp. 110S–120S.
Lawrence, A. S. C. (1958). *Discuss. Faraday Soc.*, **25**, 51–58, 58–80.
Lawrence, A. S. C. (1959). *Nature, Lond.*, **183**, 1491–1494.
Lawrence, A. S. C. (1960). *Membrane Transport Metab. Proc. Symp.*, Prague, Published 1961, 35–44.
Lawrence, A. S. C. (1961a). *Chemy Ind.*, 1764–1771.
Lawrence, A. S. C. (1961b). *Surface Activity and Detergency*, editor Durham, K., London: Macmillan, pp. 158–192.
Lawrence, A. S. C., Bingham, A., Capper, C. B. & Hume, K. (1964). *J. phys. Chem., Ithaca*, **68**, 3470–3476.

Greater lowering of brain and adrenal catecholamines in group-housed than in individually-housed mice administered DL-α-methyltyrosine

SIR,—We have previously interpreted differences in brain and adrenal catecholamines between mice that live in groups and their individually-housed littermates to be indicative of higher basal levels of amine metabolism in the grouped mice (Welch, 1965; 1967; Welch & Welch, 1964; 1968); support for this interpretation has now been provided by experiments with DL-α-methyltyrosine, an inhibitor of the rate-limiting enzyme in noradrenaline biosynthesis (Spector, Sjoerdsma & Udenfriend, 1965).

Male white Swiss mice born within the same two-day period were weaned at 4 weeks, and littermates weighing within 2 g of the same body weight were randomly assigned to individual housing or to live in a group of 8 to 10 mice. We have found that fighting is minimal among male mice of this strain if they are grouped together from time of weaning. Grouped mice were housed in cages 10 × 10 × 6 inches, a space slightly more liberal than that recommended for maintaining laboratory mice (U.S. Dept. of Health, Education and Welfare, 1965); individually housed mice were in cages half this size. Food and water were always accessible in abundance to the animals. The room was maintained at 75–78° F and contained no other animals. Cages were changed once each week. In preliminary experiments, 80 mg/kg of DL-α-methyltyrosine was found to reduce catecholamines as effectively as higher doses. After 14 weeks and with minimal disturbance, half of the isolated mice and half of the mice in each group were administered either DL-α-methyltyrosine, 80 mg/kg, intraperitoneally in 0·2 ml of 0·9% saline at pH 3, or vehicle alone. The mice were colour-coded and were *returned to their original cages*; exactly 6·5 hr after injection they were decapitated. The left adrenal and the whole brain, exclusive of the *bulbus olfactorius*, were weighed, frozen on dry ice, and stored at −20°C. The brains were homogenized in 0·01 N hydrochloric acid and analyzed for noradrenaline and dopamine (Welch & Welch, 1968). Duplicate internal standards of each amine were added to tissue extracts at 3 concentrations and carried through the whole procedure (% recovery = 76 ± 0·3–0·6 s.e.). The adrenal was homogenized in 0·5 N perchloric acid, titrated to pH 6 with K_2CO_3, centrifuged, and analyzed for adrenaline and noradrenaline. All manipulations were patterned to balance any diurnal or other rhythmic variations. Saline and drug pairs of isolated and grouped mice were established with the assumption that the decline of catecholamines after blockade of synthesis is proportional to their concentration (Brodie, Costa, & others, 1966), and differences between them were evaluated by Student's *t*-test.

α-Methyltyrosine lowered brain catecholamines and the adrenaline content of the adrenal medulla significantly more in the grouped mice (Table 1). Body weight was significantly less in the grouped mice, but the adrenal weight was significantly greater. The adrenaline content of the adrenal medulla was significantly greater in the grouped mice when corrected for differences in body weight by covariance. Mice that received α-methyltyrosine gradually became less active than their controls, and at the time of death they were slightly sedated, the grouped mice more so than the isolates. Deep body temperature of the drug-injected mice averaged 3°C lower than controls 3 hr after injection and 5°C lower at the time of death; grouped and isolated mice did not differ in temperature. No fighting occurred during the experiment.

Brain noradrenaline and dopamine are released by nerve stimulation (Glowinski & Baldessarini, 1966), and it is well established that adrenal catecholamines are released by stimulation of the splanchnic nerve. Further, the release of

TABLE 1. EFFECT OF TYROSINE HYDROXYLASE INHIBITION ON BRAIN AND ADRENAL CATECHOLAMINES IN MALE MICE GROUP-HOUSED AND INDIVIDUALLY HOUSED FOR 14 WEEKS SUBSEQUENT TO WEANING AT AGE OF 4 WEEKS

	Isolated (± s.e.)	Group 10 (± s.e.)	P<
Number	30	28	
Body wt.	38·1 ± 0·8	33·0 ± 0·8	0·01
Left adrenal (mg)	2·08 ± 0·09	2·44 ± 0·07	0·01*
Brain noradrenaline			
Saline control (ng/g)	402 ± 14·8	456 ± 16·7	0·025*
α-Methyltyrosine decrease (% control)	58·4 ± 1·38	64·4 ± 1·96	0·01
Brain dopamine			
Saline control (ng/g)	788 ± 30·9	748 ± 29·1	n.s.
α-Methyltyrosine decrease (% control)	53·8 ± 0·99	62·0 ± 0·89	0·001
Adrenal adrenaline			
Saline control (ug/left adrenal)	3·66 ± 0·25	4·01 ± 0·33	n.s.*
α-Methyltyrosine decrease (% control)	20·9 ± 3·25	33·3 ± 4·16	0·01
Adrenal noradrenaline			
Saline control (µg/left adrenal)	0·44 ± 0·04	0·42 ± 0·10	n.s.
α Methyltyrosine decrease (% control)	11·4 ± 10·28	14·3 ± 9·84	n.s.

* Differences are enhanced when values are adjusted to body weight by covariance analysis; on this basis, the adrenals of the grouped mice contain significantly more adrenaline ($P < 0.001$.)

catecholamines after pharmacological inhibition of tyrosine hydroxylase depends upon nervous stimulus (Dahlstrom, Fuxe, & others, 1965; Andén, Corrodi, & others, 1966). Our results, therefore, suggest that the grouped mice experienced a higher general level of activation of central and peripheral catecholamine-containing neurons than their isolated littermates, even though they lived under conditions contrived to minimize fighting. Further, inasmuch as catecholamine stores were maintained at similar or at higher relative levels in the grouped than in the isolated controls, it is implicit that normal *de novo* catecholamine biosynthesis must have been proceeding more rapidly in the former.

The rate constant of exponential lowering of catecholamines after intravenous administration of 200–300 mg/kg of the L-isomer of α-methyltyrosine to rats has been used as a direct indication of the rate of catecholamine "turnover" (Brodie, Costa, & others, 1966; Costa & Neff, 1966). However, intraperitoneal doses of 150–200 mg/kg of the L and DL-isomers have been found lethal to a high percentage of rats injected in three laboratories (Hanson, 1965; Weissman & Koe, 1965; Moore, Wright & Bert, 1967). Further, two violations of the underlying assumption that the drug does not change the metabolic rate of the catecholamines (Brodie & others, 1966) are unavoidable. First, doses of 150–200 mg/kg of the DL-isomer cause a rapid fall in body temperature in rats (Udenfriend, 1966), just as 80 mg/kg in our laboratory invariably causes mice to become hypothermic. Second, impairment of catecholamine availability by inhibition of biosynthesis causes some sedation, impairs behavioural performance (Hanson, 1965; Moore & Rech, 1967), and generally decreases responsiveness to stimuli. It follows that the reduction of catecholamine stores after inhibition of tyrosine hydroxylase must slow with passing time simply because of the lowered body temperature and the decreased responsivity of the animal to its stimulus environment. It seems appropriate, therefore, to view rates of catecholamine "turnover", which are based upon their rate of disappearance following inhibition of tyrosine hydroxylase, as *minimal* rates.

In view of the fact that the grouped mice in our experiment became mildly sedated and reduced their activity earlier than the isolates, the differences between our grouped and individually-housed mice treated with α-methyl-

tyrosine were probably minimal estimates of the differences in the rate of catecholamine release from the brain and the adrenal medulla of their non-drug controls.

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Memorial Research Center and Hospital,
University of Tennessee,
Knoxville, Tennessee, U.S.A.
October 27, 1967

BRUCE L. WELCH
ANNEMARIE S. WELCH

Andén, N.-E., Corrodi, H., Dahlstrom, A., Fuxe, K. & Hökfelt, T. (1966). *Life Sci.*, **5**, 561–568.

Brodie, B. B., Costa, E., Dlabac, A., Neff, N. H. & Smookler, H. H. (1966). *J. Pharmac. exp. Ther.*, **154**, 493–498.

Costa, E. & Neff, N. H. (1966). In *Biochemistry and Pharmacology of the Basal Ganglia.* Editors, Costa, E., Côte, L. & Yahr, M. D., pp. 141–156. New York: Raven.

Dahlstrom, A., Fuxe, K., Kernell, D. & Sedvall, G. (1965). *Life Sci.*, **4**, 1207–1212.

Glowinski, J. & Baldessarini, R. J. (1966). *Pharmac. Rev.*, **18**, 1210–1238.

Hanson, L. C. F. (1965). *Psychopharmacologia*, **8**, 100–110.

Moore, K. E. & Rech, R. H. (1967). *J. Pharmac. exp. Ther.*, **156**, 70–75.

Moore, K. E., Wright, P. F. & Bert, J. K. (1967). *Ibid.*, **155**, 506–515.

Spector, S., Sjoerdsma, A. & Udenfriend, S. (1965). *Ibid.*, **147**, 86–95.

Udenfriend, S. (1966). In *Mechanisms of Release of Biogenic Amines.* Editors, Euler, U. S. von, Rosell, S. & Uvnas, B., pp. 103–108. New York: Pergamon.

U.S. Dept. Health, Ed. and Welfare. (1965). Guide for Laboratory Animal Facilities and Care. Washington, D.C.: U.S. Govt. Print. Off.

Weissman, A. & Koe, B. K. (1965). *Life Sci.*, **4**, 1037–1048.

Welch, A. S. & Welch, B. L. (1968). *Biochem. Pharmac.*, in the press.

Welch, B. L. & Welch, A. S. (1964). In *Prog. in Brain Res.*, Vol. 8: *Biogenic Amines.* Editors, Himwich, H. E. & Himwich, W. A., pp. 201–206. Amsterdam: Elsevier.

Welch, B. L. (1965). In Proc. Symp. *Medical Aspects of Stress in the Military Climate* (April 22–24, 1964). pp. 39–99. Walter Reed Army Inst. Res. Washington. D.C.: U.S. Govt. Print. Off.

Welch, B. L. (1967). In *U.C.L.A. Forum in Medical Sciences.* Editors, Clemente, C. D. & Lindsley, D. B., Vol. 5, pp. 150–170. California: U.C.L.A. Press.

Disulfiram and the drug-induced effects on motility

SIR,—We have given disulfiram (50 or 100 mg/kg) intraperitoneally to white mice of R_3 strain, or to rats, once or three times at 2-hrly intervals and have found the level of noradrenaline in the brain to be decreased without affecting or increasing only slightly, the level of dopamine (Table 1). Determinations were made spectrophotofluorimetrically. One and a half hr after the last injection of disulfiram, cocaine hydrochloride was given subcutaneously 30 mg/kg to mice or 40 mg/kg to rats. After $\frac{1}{2}$ hr the activity was registered by the photocell method during $\frac{1}{2}$-hr sessions.

Disulfiram prevented the increase of activity induced by cocaine (Table 1).

A similar blocking effect was found by Maj & Przegaliński (1967) in mice with amphetamine-induced hyperactivity [5 mg/kg s.c., (±)-amphetamine sulphate].

Disulfiram was also given to reserpinized mice (1 mg/kg i.p.) in which sedation had been reversed by nialamide (100 mg/kg, i.p.) and DL-dopa (200 mg/kg, i.p.). Reserpine was injected 9 hr, disulfiram (50 mg/kg, i.p.) 6, 4 and 2 hr, nialamide 2 hr and dopa $\frac{1}{2}$ hr before the test. Activity was assessed, as before, in groups of 10 mice.

Disulfiram prevented the stimulation induced by nialamide and dopa. The number of movements in reserpinized animals was $6 \pm 5{\cdot}1$; in reserpinized animals treated with nialamide and dopa it was $158 \pm 22{\cdot}9$, and in animals given disulfiram as well, it was $11 \pm 4{\cdot}2$.

Analogous experiments were made with α-methyltyrosine methylester (50 mg/kg, three times at 3-hrly intervals, i.p.). Disulfiram was given as above, but the third injection being given with the third injection of α-methyltyrosine methylester. Pargyline (100 mg/kg, i.p.) was given $1\frac{1}{2}$ hr, dopa (500 mg/kg, i.p.) $\frac{1}{2}$ hr before the test. Activity was recorded in groups of 10 mice. The activity count in control mice was $183 \pm 15{\cdot}4$ and in mice treated with α-methyltyrosine methylester it was $47 \pm 11{\cdot}3$. After pargyline and dopa, stimulation was seen in mice given α-methyltyrosine methylester ($196 \pm 13{\cdot}2$), but not in mice pretreated with the ester-disulfiram combination ($21 \pm 12{\cdot}8$). All the results were significant ($P < 0{\cdot}001$).

We have already reported that a similar preventive effect is obtained with disulfiram in mice given butyrophenones, and in these animals nialamide and dopa counteract sedation (Maj & Wielosz, 1967).

TABLE 1. THE EFFECT OF DISULFIRAM ON THE COCAINE-INDUCED HYPERACTIVITY IN MICE AND RATS

Animal	Disulfiram i.p. mg/kg	Activity counts	% of the control	P	Catecholamines % of normal values*	
					Dopamine	Noradrenaline
Mouse	—	386 (±32·3)	100·0	—	100·0	100·0
,,	1 × 50	355 (±71·1)	91·9	>0·6	92·7	61·9**
,,	1 × 100	272 (±62·4)	70·4	>0·1	101·0	54·7**
,,	—	340 (±41·5)	100·0	—	100·0	100·0
,,	3 × 50	212 (±31·4)	62·3	<0·05	126·8**	42·8**
,,	3 × 100	94 (± 9·7)	27·6	<0·001	126·8**	38·1**
Rat	—	193 (±23·5)	100·0	—	100·0	100·0
,,	1 × 50	198 (±49·2)	102·5	>0·9	96·5	82·8**
,,	3 × 50	49 (±14·8)	25·3	<0·001	114·0**	51·4**

Disulfiram was injected 2 hr or 6, 4 and 2 hr, and cocaine hydrochloride (30 mg/kg s.c. in mice and 40 mg/kg s.c. in rats) 30 min before the experiment. The activity was recorded in single mice during 30 min sessions. Figures represent the means of 10 animals.

* Content of catecholamines in brain expressed as a % of normal values from animals receiving disulfiram only (means of 3–7 experiments).

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

The experiments reported seem to indicate that noradrenaline is essential for the changes in activity seen in these experiments. A contrary view exists ascribing significance to dopamine (Everett & Wiegand, 1962; Rossum & Hurkmans, 1964).

It is pertinent to consider whether it can be assumed that the described action of disulfiram is due to its influence on the level of the brain catecholamines and not to a direct influence on the receptors. Disulfiram is known not to affect the amphetamine stereotyped behaviour in rats (Maj & Przegaliński, 1967), which is ascribed to the release of dopamine in the extrapyramidal system (Ernst, 1967; Scheel-Krüger & Randrup, 1967). We were able to show that it does not block the stereotyped behaviour after apomorphine (2·5 mg/kg, s.c.) which arises from its direct action on the dopamine receptor (Anden, Rubenson & others, 1967; Ernst, 1967). An additional observation which contradicts the hypothesis that the action arises from blockade of the receptor is that in mice, in which increased spontaneous motility is induced by nialamide and dopa (271 ± 33), disulfiram administered after nialamide only partly prevents the stimulation (131 ± 54), but stimulation is prevented ($4 \pm 1{\cdot}9$) if disulfiram is administered before nialamide. This observation seems to contradict the view about the role of dopamine in these conditions. Disulfiram, in doses inducing the above-mentioned changes in the level of the brain catecholamines, likewise potentiates the convulsive action of leptazol given in subthreshold doses in mice.

Of course, the possibility of a direct action of disulfiram on the receptor has not yet been excluded, nor can we exclude the possibility of still another mechanism on enzymes other than dopamine-β-hydroxylase.

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Department of Pharmacology,
Medical Academy,
Lublin,
Poland.

J. Maj
E. Przegaliński
M. Wielosz

October 11, 1967

References

Anden, N., Rubenson, A., Fuxe, K. & Hökfelt, T. (1967). *J. Pharm. Pharmac.*, **19**, 627–629.

Ernst, A. (1967). *Psychopharmacologia*, **10**, 316–323.

Everett, G. & Wiegand, R. (1962). In *Proceedings of the First International Pharmacological Meeting*, editors Paton, W. & Lindgren, P., Vol. 8, pp. 85–92, London: Pergamon.

Maj, J. & Przegaliński, E. (1967). *J. Pharm. Pharmac.*, **19**, 341–343.

Maj, J. & Wielosz, M. (1967). *First Meeting of Polish Pharmac. Soc.*, Kraków, in the press.

Rossum, J. M. van & Hurkmans, J. A.Th.M. (1964). *Int. J. Neuropharmac.*, **3**, 227–239.

Scheel-Krüger, J. & Randrup, A. (1967). *Life Sci.*, **6**, 1389–1398.

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Letters to the Editor

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