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Research Papers

A method for determining the solution rate of fine particles

I. C. EDMUNDSON AND K. A. LEES

A method is presented for determining the solution rate of fine particles, crystalline hydrocortisone acetate being used as the model substance. The method's innovation is that it takes account of the changing particle size distribution during the solution process, using a Coulter counter to observe this. The solution rate may be expressed as diameter loss per unit time ($d_t = d_o - k_2t$). The solution rate of hydrocortisone acetate under the conditions of the experiment was found to be linear and to correspond to a diameter loss of 1·68 μ/hr, equivalent to 108 $\mu g/cm^2$ of surface/hr. Corrections for the Noyes–Whitney effect were made.

IN recent years interest has been growing in the solution rate of drugs of low solubility and in the relationship between solution rate, particle size and absorption of drugs. Wagner (1961) and Lees (1963) have reviewed examples of drugs for which the clinical response to an oral dose depends on particle size and have suggested that the solution rate may be a limiting factor in the absorption of drugs of low solubility. Nelson (1959) found that the absorption of tetracycline and of some of its less soluble salts was limited by solution rate.

Edwards (1951) measured the solution rates of disintegrated aspirin tablets, but in his calculations he replaced the heterogeneous particle sizes of his samples by hypothetical spheres of uniform size; his rate constants are related to initial specific surface area rather than to individual particle sizes.

Other workers (Parrott, Wurster & Higuchi, 1955; Nelson, 1957; 1958; 1959; Higuchi, Parrott, Wurster & Higuchi, 1958) have determined the solution rates of several drugs of low solubility. Recognising the difficulties associated with ill-defined or changing surface areas, they have worked with geometric shapes, such as relatively large spheres or discs, whose surface areas either remained virtually constant or could be measured readily. On the other hand, the treatment of fine particles, as described in publications, has been theoretical rather than experimental.

We have therefore developed a direct method of determining the solution rates of fine particles that does not require the particles to be of specified size, shape or uniformity, yet discriminates between particles of different sizes.

An electronic apparatus for particle size analysis, described by Coulter (1956) and Berg (1959), has several advantages for this purpose. Because an aqueous (saline) suspension of the particles is used, the analysis can be performed while the particles are dissolving and without removing them from the suspension. Wide ranges of pH, electrolyte content and viscosity of the solvent are permissible, so that the method may be applied to a variety of liquids, including natural body fluids and their synthetic

From: Glaxo Laboratories Ltd., Greenford, Middlesex.

imitations. The method is rapid enough to keep pace with a constantly changing size distribution as the particles diminish in size by solution. Individual particles are counted, and significant results can be obtained at low concentrations (by either weight or number of particles).

Because the Coulter counter responds to particle volume rather than to particle diameter or other particle attribute (Kubitschek, 1960), the results of examining material of known density can be expressed accurately in terms of weight.

Solution is normally assumed to be a surface phenomenon, the weight of material dissolved being proportional to the total surface area of the solid and to the time during which it is exposed to the solvent.

When particles dissolve, their surface area will diminish as solution proceeds. If allowance is to be made for this, and also for possible non-uniformity in size of the particles, calculations of volume and surface become complicated (see Hixson & Crowell, 1931; Wilhelm, Conklin & Sauer, 1941; Higuchi & Hiestand, 1963). An alternative assumption is that solution is an etching process in which a given surface recedes in depth to an extent proportional to time.

Dimensional analysis shows that these two assumptions are equivalent. Let k_1 be the solution rate, d the diameter of particles (dimension L) and t the time for solution (dimension T); then—

$$\begin{aligned} k_1 &= \text{solution rate,} \\ &= \text{volume/unit time/unit area,} \\ &= L^3T^{-1}L^{-2} \\ &= LT^{-1} \qquad .. \quad .. \quad .. \quad .. \quad .. \quad (1) \end{aligned}$$

If complicating factors, such as the increasing concentration of the solution, particle shape, anisotropy of the material and the higher solubility of smaller particles, are neglected for the moment, and if variables such as temperature and degree of agitation are standardised, it can be assumed that diameters will decrease linearly with time and that the diameter of any particle at any instant in time will be given by—

$$d_t = d_o - k_2t \qquad .. \quad .. \quad .. \quad (2)$$

The dimensional analysis is valid for equidimensional isotropic particles such as spheres or cubes that dissolve equally along all axes. Equation (2) is valid for anisotropic particles such as crystals, provided that the solution rate along different axes is proportional to axis length; d then becomes the cube root of particle volume or the equivalent volume diameter (d_v).

Experimental

The method was tested on hydrocortisone acetate as an example of a sparingly soluble compound whose clinical efficacy might depend on particle size and solution rate. The sample used in this work was a fraction of narrow size-range, prepared by repeatedly settling and decanting a suspension of crystalline hydrocortisone acetate in water previously

saturated with hydrocortisone acetate. The fraction collected required 55 to 65 min to settle 20 cm. The original material had been crystallised from acetone in the presence of water. The absence of acetone from within the particles was confirmed by gas chromatography.

The photograph (Fig. 1) shows the uniformity of size and shape of the particles in the sample.

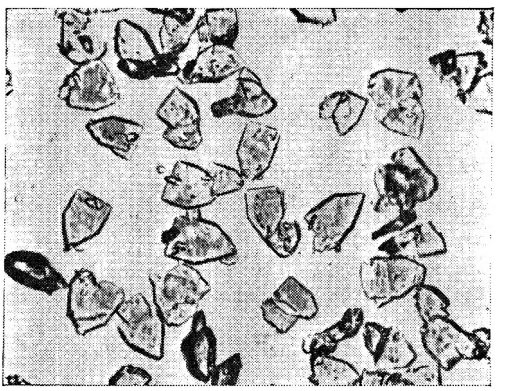

FIG. 1. Hydrocortisone acetate crystals, ×222.

The characteristic dimensions of a number of typical crystals were measured microscopically (cf. Michaels & Colville, 1960) and found to agree substantially with the crystallographic description given by Shell (1955), despite a more than ten-fold difference in the size of the crystals. Fig. 2 is an isometric projection constructed from the means of the microscopical measurements; the enclosing rectangular prism has the proportions of length, breadth and depth of the particle as usually defined in particle size microscopy (Heywood, 1947).

APPARATUS

A Coulter electronic counter, Model A, fitted with a 100 μ orifice tube and a 2 ml manometer was used in this work. The electrolyte was a 1% solution of sodium chloride, with 0·002% polyethylene glycol 600 monooleate and 0·008% propylene glycol as wetting agent; this concentration of sodium chloride was chosen as suitable for the required range of particle size and as a rough approximation to the ionic concentration of gastric juice. The threshold scale of the counter was calibrated by counting a suspension of 200 mg of the hydrocortisone acetate sample in 1 litre of electrolyte previously saturated with hydrocortisone acetate. The calibration constant, K, was calculated from the equation

$$K = \sqrt[3]{\frac{6VC}{\pi\rho\Sigma(\Delta n)\bar{t}}} \qquad (3)$$

where V is the manometer volume (μl), C is the concentration of particles in the suspension (μg/litre), ρ is the density of the hydrocortisone acetate (1·289), Δn is the count increment between successive threshold values and

$\bar{t}$ is the average threshold value for a given count increment. Particle diameter was calculated from the equation

$$d_v = K \sqrt[3]{\bar{t}} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (4)$$

where d_v is the 'volume diameter' in μ (the diameter of a sphere that has the same volume as the particle).

The aperture current settings were calibrated directly by determining the threshold settings required to give equivalent counts at different settings.

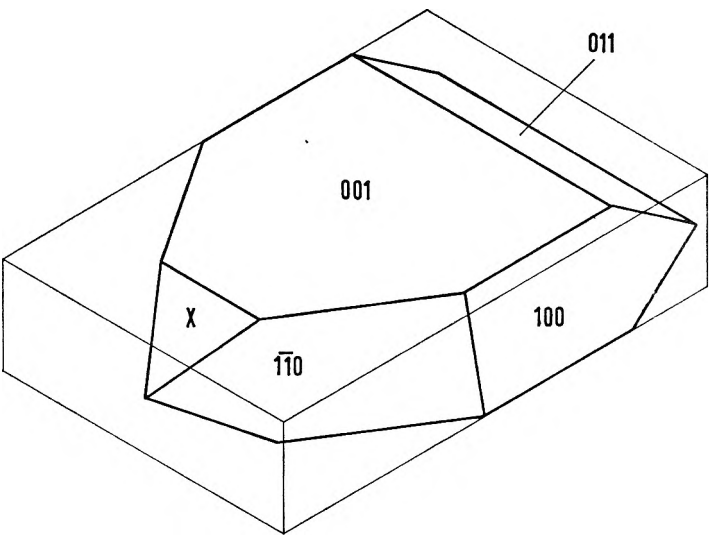

FIG. 2. Typical hydrocortisone acetate crystal in isometric projection, constructed from the means of microscopical measurements. The face numbers are as designated by Shell (1955). Face 031 was not discernible on our small crystals and face 110 was absent on most. Face X was not described by Shell. Dimensions of the enclosing rectangular prism are length, 33 μ; breadth, 22 μ; depth, 7·5 μ.

Background and coincidence corrections were made in the normal way; the former were negligible at all times, and the latter were no more than 5% of the observed count in the calibration experiment and negligible in the solution-rate experiment.

SOLUTION RATE

A suspension of particles was prepared by vigorously stirring 1·0 mg of the sample into 1 litre of electrolyte. The concentration of a saturated solution of hydrocortisone acetate in electrolyte having been found previously, by means of ultra-violet spectrophotometry, to be 10 ppm at 25° (see also Macek, Baade, Bornn & Bacher, 1952), the electrolyte would have been 10% saturated if the whole sample weight were dissolved.

Throughout the experiment the bulk suspension was maintained at 24–26° in a conical flask and transferred as required to keep the Coulter

beaker full. The suspension in the beaker was stirred by propeller at a sufficient speed to keep the particles in suspension without incorporating air bubbles. The bulk suspension was maintained in similar motion by hand swirling.

Counting was begun at an instrument setting corresponding to about the sample's initial mean particle diameter. The total count for 4 successive 2 ml portions was recorded, together with the elapsed time. After each 8 ml total had been completed, the diameter setting was reduced by 1 μ. When the count reached a maximum, counts of a second series, in descending order of diameter, were begun. Nine such series were completed in a period of 5 hr.

This method of determining solution rate depends on observing a diminishing number of particles at a given diameter setting. Therefore, to ensure that particles were not disappearing from the system because of some effect other than slow solution, the first, fourth and seventh series of counts were taken up to the maximum, to demonstrate that the same population was being counted.

Results

The initial size distribution of the sample as determined in the Coulter calibration experiment is shown in Fig. 3. The peaks of the distributions by number and by weight are at 18 μ and 20 μ respectively, the closeness

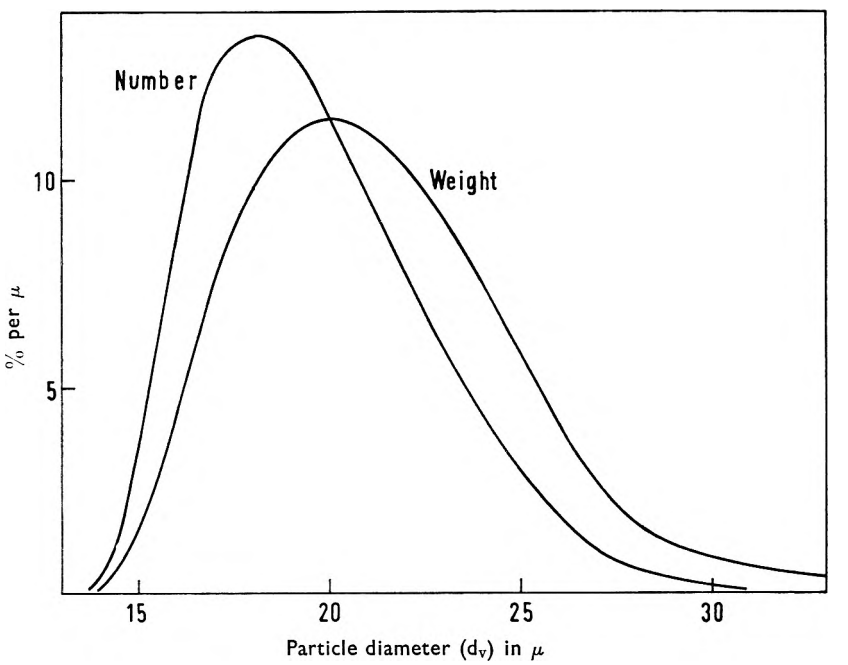

FIG. 3. Hydrocortisone acetate particle size distribution, by number and weight, determined during calibration of the Coulter counter.

of these figures indicating the narrow distribution of particle sizes. Confirmation of these results was obtained from the microscopical measurements. Calculated from the shape shown in Fig. 2, the length of a typical crystal is 1·8 times the diameter of a sphere of the same volume. The mean crystal length was found to be about 33 μ; division of this figure by 1·8 gives an equivalent volume diameter of 18·3 μ.

The experimental readings from the solution-rate experiment are shown in Fig. 4, the numbers of particles oversize in each 8 ml of suspension being plotted against time. Lines join the points representing equal diameters. Horizontal lines, corresponding to initial particle diameters of about 16 μ, 18 μ and 20 μ, cut the iso-diametric curves in their substantially straight portions. These intersections indicate the time taken for particles of about average size to be dissolved away to particles of smaller diameters.

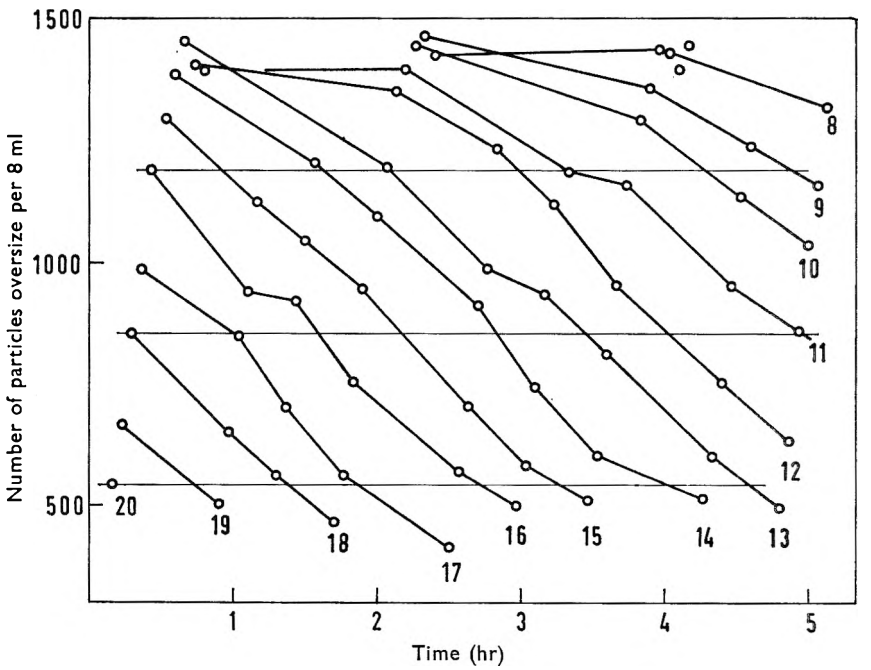

FIG. 4. Hydrocortisone acetate solution rate. The heavy lines join readings at equal diameter settings (μ). The intersections between horizontal lines and iso-diametric lines indicate the successive reductions in particle diameter.

The rate of solution is shown more clearly in Fig. 5, in which the intersections from Fig. 4 are plotted as diameter against time. The points appear to fit three parallel straight lines, as would be expected from eqn 2. However, the concentration of dissolved hydrocortisone acetate was rising during the experiment. Noyes & Whitney (1897) have shown that the rate of solution at any instant is proportional to the difference between

the concentration of a saturated solution and the concentration in solution at that instant,

$$\text{Rate of solution} = k_3(C_s - C_t) \quad .. \quad .. \quad (5)$$

This effect has been taken into account in calculating the theoretical curves that have been fitted to the experimental points in Fig. 5. The curves appear almost straight because, although 80% by weight of the sample had dissolved in 5 hr, the solution was then only 8% saturated and the solution rate at 5 hr was still 92% of the initial rate.

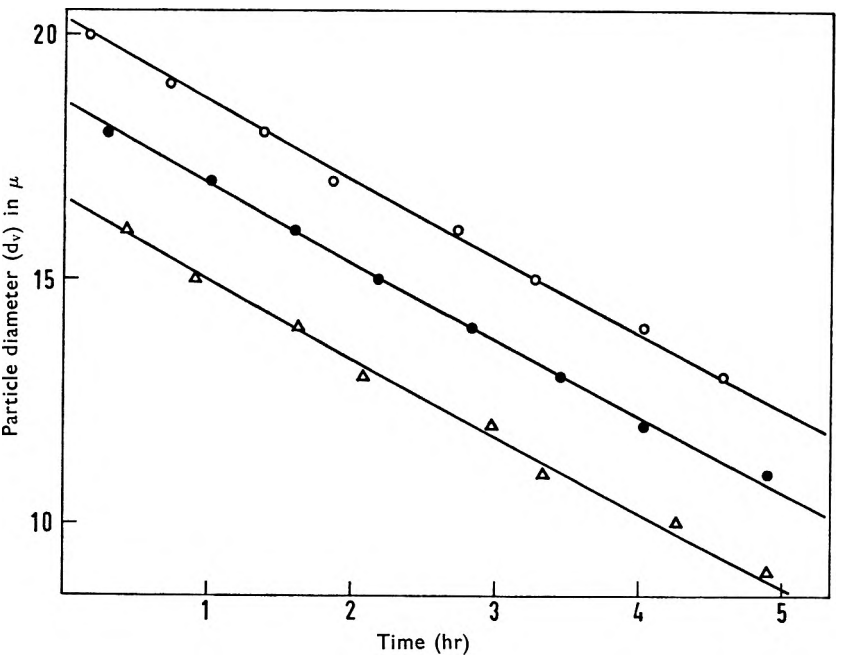

FIG. 5. Hydrocortisone acetate solution rate. Intersections from Fig. 4, at a count level of 1190, ○; at a level of 850, ●; at a level of 540, △. The theoretical curves correspond to the initial solution rate calculated as one average for all the intersections and make allowance for the increasing concentration of hydrocortisone acetate in solution.

The initial rate of solution and the theoretical curves for the Noyes–Whitney effect were calculated in stages by numerical methods. On the assumption that each size fraction lost diameter at the same rate, and with the knowledge that the sample weight was 10% of that required to saturate the electrolyte, the relative rates of solution were calculated from eqn 5 for nine successive 1 μ reductions in diameter over the whole distribution (Fig. 3).

These relative rates were calculated to relative times for each 1 μ reduction of diameter of a 20 μ particle. When the values of $d_v^{2/3}$ were plotted against relative times, the resulting curve was a straight line for the whole of its length.

In the next stage, straight lines were fitted to the experimental points of Fig. 5 by the method of least squares, with time and $d_v^{2/3}$ as co-ordinates. The slopes of these lines gave the initial rates of solution, $k_3(C_s - C_0)$, as 1·68 μ/hr at the 1,190 count level, 1·70 μ/hr at the 850 level, and 1·65 μ/hr at the 540 level, a mean initial rate of 1·68 μ/hr.

By use of this mean rate, the relative times of the previous Noyes–Whitney curve were converted to real times, thus giving the real slope of the curve. This theoretical curve, with its slope maintained, was fitted in turn to the three sets of experimental points by the method of least squares; the goodness of fit is apparent in Fig. 5.

Discussion

VALIDITY

To assess the validity of the method and its significance, one must consider critically the assumptions that have been made.

The method gives a true result in terms of weight of substance (volume × density) dissolved, provided that the Coulter instrument gives a true measure of particle volume. Kubitschek (1960) has demonstrated that the threshold scale response of the instrument is linear with volume over a wide range and has argued from earlier work by Fricke (1924) that the response is substantially independent of particle shape; the error due to the shape of the hydrocortisone acetate particles can be calculated to be less than 1% for an instrument calibrated with standard spherical particles. However, the method of calibration employed by us, together with the narrow size-range of the sample, ensures accurate volume measurement and automatic allowance for the shape factor.

Further reference to the shape factor is made in the section below on Shape and Surface Area.

The Noyes–Whitney concentration effect has been allowed for in Fig. 5 by assuming that particles of different sizes dissolve at the same rate. For the size-range involved, this assumption can be justified in three ways from the experimental evidence.

(1) Particles of average size dissolved at a linear rate until their diameter had been reduced to almost half the original diameter.

(2) The original sample contained an insignificant weight of particles having diameters less than half that of the largest (Fig. 3).

(3) A line drawn across Fig. 4 at the maximum count level, though cutting the iso-diametric curves where their shape is less well defined, does give substantially, although less reliably, the same rate constant as that given by the lines drawn at the lower count levels.

SHAPE AND SURFACE AREA

The Coulter experiment, taken alone, gives no direct information about particle shape. The expression of solution rate as diameter loss per unit time (eqn 1) therefore refers to the diameter of a hypothetical sphere having the same volume as the particle. Similarly, if the measured rate

is expressed as weight per unit surface area per unit time ($LT^{-1} = L^3T^{-1}L^{-2}$), the reference is to the surface area of the same hypothetical sphere, not to the true surface area of the particle.

The microscopical evidence of tabular shape and the demonstrated linearity of solution rate (eqn 2) together imply that the particles maintained the same shape during the course of the experiment; in other words, if the particles were originally twice as long as they were broad, the rate of reduction in length must have been double the rate of loss of breadth.

The observed solution rate at zero concentration, 1·68 μ diameter loss/hr, is equivalent, when expressed in terms of weight and surface, to a rate at a given instant of 108 μg of hydrocortisone acetate/hr/cm^2 of total surface area at that instant.

This solution rate is probably faster than that expected for pure diffusional rate control (Higuchi & Hiestand, 1963), because convection currents around the falling particles would keep the concentration low near each particle (Nielsen, 1961); from the initial gravitational settling rate of the crystals it can be calculated that each crystal of mean length 33 μ requires only 0·6 sec to settle into a zone of fresh solvent.

It is probable that the major part of the forces moving the particles relative to the solvent was due to gravity rather than to the gentle agitation that was required to keep the particles in suspension. Thus, variation in the degree of stirring, limited by the need to avoid production of air bubbles, was insufficient significantly to affect the apparent solution rate.

Acknowledgements. We thank Mr. J. L. Martin for the gas chromatography and Mr. J. P. R. Tootill for helpful discussion.

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Different types of sympathomimetic α-receptors

J. M. VAN ROSSUM

Sympathomimetic effects have been studied on the isolated vas deferens of the rat, in the isolated jejunum of the rabbit and on the cardiovascular system of the cat. (−)-Noradrenaline (1-*R* configuration), (+)-noradrenaline (1-*S* configuration) and dopamine as well as a number of homologues were used as agonists. The adrenergic blocking drugs piperoxan, phentolamine, yohimbine, aceperone and the tranquillising drugs chlorpromazine, (−)-mepromazine, haloperidol, droperidol and spiramide as well as bulbocapnine were used as antagonists. The results obtained with both agonists and antagonists provide evidence that the structural requirements for drugs to react with and to activate α-receptors in the vas deferens and the rabbit intestine are different; epinine and dopamine have an identical mechanism of action.

IT is now generally accepted that adrenaline exerts its physiological actions by reacting with two distinct types of sympathetic receptors, which are classified as α- and β-receptors (Ahlquist, 1948).

(−)-Noradrenaline is the physiological substance acting on α-receptors. The configuration of the (essential) hydroxyl group in the side chain of (−)-noradrenaline is D according to Pratesi, LaManna, Campiglia & Ghislandi (1958) and consequently 1-*R* in terms of the sequence rule of Cahn, Ingold & Prelog (1956). Dopamine differs in not having the hydroxyl group in the side chain. Evidence is available that it has a transmitter function of its own in the central nervous system (Carlsson, Lindquist, Magnusson & Waldeck, 1958; Bertler & Rosengren, 1959; Hornykiewicz, 1963; Everett & Wiegand, 1962). In addition it has been found that dopamine in the peripheral nervous system to some degree evokes a different type of effect, as e.g. fall in blood pressure in the dog, rabbit and guinea-pig (Holtz & Credner, 1942; Burn & Rand, 1958; Holtz, Stock & Westermann, 1963; McDonald & Goldberg, 1963). Recently, Eble (1964) proposed specific receptors for dopamine in the renal and mesenteric vessels in the dog.

It is uncertain whether the peripheral effects of dopamine are caused by an action on sympathetic receptors or on separate non-sympathetic dopamine receptors. If dopamine is acting on sympathetic α-receptors the question is raised whether there is a scale of α-receptors with at one end receptors which are best fitted by noradrenaline and at the other end receptors which are best fitted by dopamine. The present study on the sympathetic action of optical isomers with known configuration and of other drugs is an attempt to clarify this point, and is also an attempt to determine if α-receptors in the rabbit intestine or the rat vas deferens could form a model for dopamine receptors in the brain.

Methods and materials

(*a*) The isolated vas deferens of the rat was used for making cumulative dose-response curves of α-sympathomimetic drugs, both in the absence and the presence of their antagonists. See Figs 1, 2 and 4. It has been

From the Department of Pharmacology, University Medical School, Nijmegen, The Netherlands.

shown that β-sympathomimetic agents have no relaxing effect on the vas deferens except in very high doses when myotropic spasmolytic actions become involved, this suggesting an absence of β-receptors in this organ. From the dose-response curves obtained, the intrinsic activity constant (i.a.c.) and the affinity constant (pD_2 or pA_2) was calculated by using (−)-noradrenaline, (−)-NA, as a reference drug (Ariëns, van Rossum & Simonis, 1957; Schild, 1949; van Rossum, 1963). The affinity is also given relative to that of (−)-noradrenaline (rel. aff. = 100).

(*b*) The isolated rabbit intestine was used for studying the inhibitory effect on pendular movements by individual doses of the α-sympathomimetic drugs, as well as the prevention of inhibition by antagonists. See Figs 3 and 5. The rabbit intestine contains both α- and β-receptors, and activation of both kinds results in inhibition of pendular movements (Ahlquist & Levy, 1959). Recently, van Rossum & Mujíc, (1965) have demonstrated that the types of inhibition induced by α- and β- drugs are essentially different. α-Sympathomimetics cause an inhibition with a rapid onset of action, whereas the onset of action of β-drugs is very slow. The β-type effect is seen only at the same time as an α-type effect when both effects occur at the same concentration. This is so with α-methyl-noradrenaline (van Rossum & Mujíc, 1965). Furthermore the indirectly acting α-sympathomimetics do not cause an inhibition of peristalsis but behave as antagonists (van Rossum & Mujíc, 1965). This appears to be a general feature of indirectly-acting sympathomimetic drugs in the rabbit intestine. From dose-response curves obtained by plotting the percentage inhibition versus the logarithm of the molar concentration, the intrinsic activity constant and affinity constant were calculated in a manner analogous to that used for the curves obtained from the vas deferens. A β-receptor activation, which may occur with adrenaline and noradrenaline in addition to their predominantly α-receptor activation, might influence the estimates of pD_2 values and relative affinities. However, β-receptor activation does not influence our results to a significant degree since β-receptor blockade has no effect on the relative affinities.

(*c*) The pentobarbitone anaesthetised cat (30 mg/kg) was used for studying the antagonistic action of adrenergic blocking drugs and tranquillisers on the effects of (−)-noradrenaline and dopamine.

(*d*) Some experiments were made on the chloralose anaesthetised rabbit (70 mg/kg) to study the antagonistic action of yohimbine, droperidol and spiramide on the pressor effects of (−)-noradrenaline, dopamine, (−)-adrenaline and epinine.

(*e*) The adrenergic blocking drugs and tranquillisers were also studied as antagonists of dexamphetamine using as the parameter the increase in locomotor activity in mice as measured by a light-beam method with continuous cumulative registration (van Rossum, 1962) (see Fig. 6).

AGONISTS AND ANTAGONISTS

The sympathomimetic agents (−)-, (+)-noradrenaline and dopamine as well as the enantiomorphs and the corresponding desoxy compounds

of adrenaline, phenylephrine and oxedrine (parasympatol) were used as agonists. Phentolamine, piperoxan, droperidol, yohimbine, aceperone, (−)-mepromazine, chlorpromazine, spiramide and bulbocapnine were used as adrenergic blocking agents.

Results

SYMPATHOMIMETIC DRUGS AND RAT VAS DEFERENS

A difference in sympathomimetic activity of stereo- and optical isomers has been found for many compounds (Luduena & others, 1962; Ariëns, 1963). However, no systematic study in which the absolute configuration has been taken into account is available. The absolute configurations of the isomers and derivatives used in the present study are known.

Fig. 1 records a typical experiment on the isolated vas deferens of a rat using (−)-, (+)-noradrenaline (NA) and dopamine (DA). (−)-noradrenaline was used as a reference drug. This procedure was also adopted

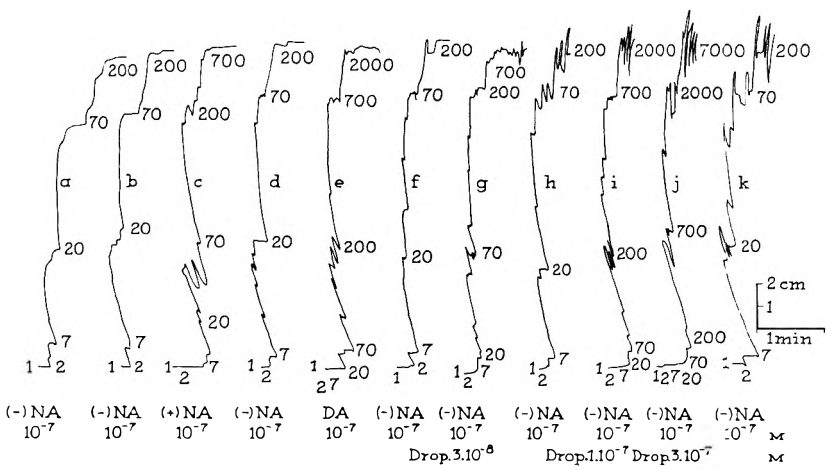

FIG. 1. Record of eleven cumulative dose-response curves obtained from a single vas deferens of a rat. The sympathomimetic stimulants (−)-noradrenaline, (−)NA, (reference drug) (+)-noradrenaline (+)NA, and dopamine, (DA), cause a similar type of response although different doses are required to produce 50% contraction. Curves were also made from results obtained in the presence of the antagonist droperidol. The doses correspond to molar concentration in the 10 ml bath. If, for instance, a dose of 20×10^{-7} M causes about 80% contraction, the concentration in the bath is actually 30×10^{-7} M (1 + 2 + 7 + 20). See curve a.

for the other drugs. Dose-response curves had a similar shape and equal height. From records such as that in Fig. 1, dose-response curves were calculated by using 100% contraction for the maximal effect obtained with (−)-noradrenaline (see Fig. 2a). From a number of such dose-response curves, usually more than 10 for each drug obtained on at least 5 preparations of the vas deferens, the average value of the negative logarrithm of the molar concentration that produced a 50% effect was calculated (see Table 1). Dose-response curves were also made with

(−)-, (+)-adrenaline (A) and epinine (DAMe) showing similar results (Fig. 2b). Since the experiment in Fig. 2b was on a single isolated organ, the dose-response curve of the reference drug, (−)NA, is also given. The average values for the affinity constants and intrinsic activity constants are in Table 1.

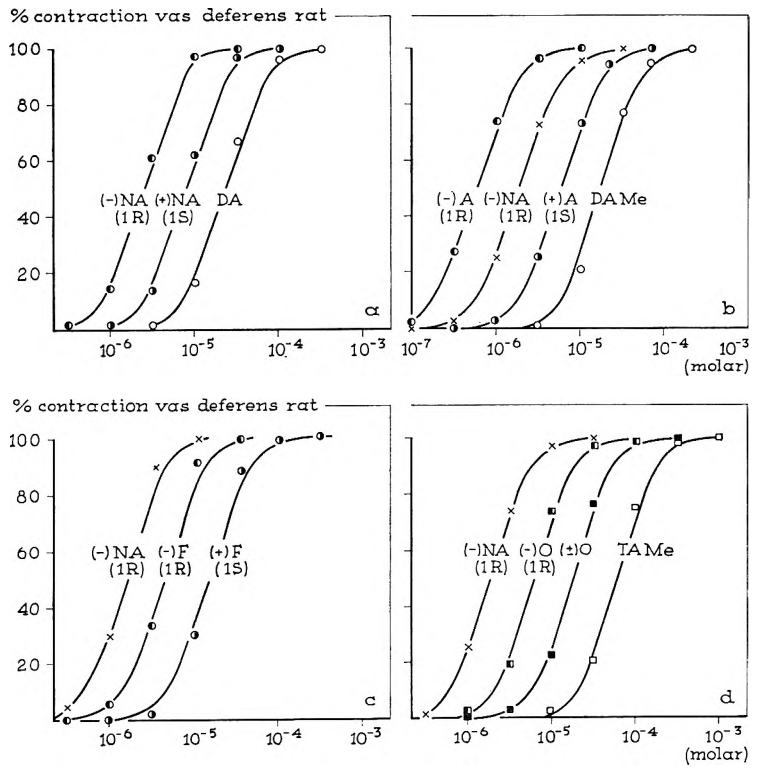

FIG. 2. Dose-response curves from several experiments given in the Fig. 1, left hand side, calculated by plotting the % contraction versus the concentration in the bath (logarithm scale). (*a*) (−)- and (+)-noradrenaline and dopamine; (*b*) (−)- and (+)-adrenaline and epinine (DAMe), reference drug (−)NA; (*c*) (−)- and (+)-phenylephrine and (−)NA; (*d*) (−)- and racemic oxedrine and *N*-methyltyramine. Note that the drugs with the OH-group in the *R*-configuration are the most potent of each group.

Dose-response curves of the enantiomorphs of phenylephrine, (−)F, and (+)F are in Fig. 2c. The corresponding desoxy derivative was not available. Curves for the (−)-isomer of oxedrine (parasympatol), (−)O, and the corresponding desoxy derivative *N*-methyltyramine, TAMe, as well as the racemic oxedrine, (±)O, are in Fig. 2d.

All drugs are agonists with equal intrinsic activity. From the average pD_2-values and the relative affinities given in Table 1 it may be seen that there is no great difference in affinity between the optical isomers and the corresponding desoxy derivatives. The 1-*R*/1-*S* affinity ratios for noradrenaline, adrenaline and phenylephrine are 4; 10 and 3 respectively.

The affinity ratios for 1-*R*-NA/DA and 1-*R*-A/epinine are 10 and 24 respectively.

TABLE 1. α-SYMPATHOMIMETIC DRUGS ON DIFFERENT TYPES OF α-RECEPTORS

	Rat vas deferens			Rabbit intestine				Affinity ratio vas/intestine
Drug	i.a.c.†	pD_2	rel. aff.‡	conf.§	i.a.c.	pD_2	rel. aff.	
(−)NA	1	5·7	100	1*R*	1	7·8	100	0·008
(+)NA	1	5·1	25	1*S*	1	6·0	6	0·13
DA	1	4·7	10	—	1	5·2	0·25	0·32
(−)A	1	6·0	200	1*R*	1	8·3	300	0·005
(+)A	1	5·0	20	1*S*	1	6·0	1·6	0·10
DAMe	1	4·6	8	—	1	5·4	0·4	0·16
(−)F	1	5·2	30	1*R*	1	7·2	22·5	0·01
(+)F	1	4·7	10	1*S*	1	4·1	0·02	4·0
(−)O	1	4·9	15	1*R*	1	4·6	0·06	2
(+)O	–	—	—	1*S*	0·8	4·4	0·04	—
(±)O	–	4·5	7	—	1	4·3	0·03	1·6
TAMe	1	4·1	2·5	—	0	4·4*	0·04	—

* antagonists so that pA_2 values were determined.
† Intrinsic activity constant.
‡ Relative affinity.
§ Configuration.

The sympathomimetic drugs containing a hydroxyl group on the side chain in the 1-*R* configuration are most potent and the desoxy derivatives, dopamine and epinine are the least potent drugs. With the aid of a competitive antagonist it could be ascertained that all the compounds in Table 1 react with common α-receptors in the isolated vas deferens of the rat.

SYMPATHOMIMETICS AND THE RABBIT INTESTINE

The various enantiomorphs and desoxy derivatives were tested as α-sympathomimetic drugs in the rabbit intestine. With the exception of *N*-methyltyramine, all compounds induced a rapid inhibition of pendular movements. This suggests a direct α-sympathomimetic action (van Rossum & Mujíc, 1965) (Fig. 3). The inhibitory action of (−)-noradrenaline and dopamine was antagonised by α-adrenergic blocking drugs, piperoxan for example, indicating a reaction with common α-receptors. Specific β-adrenergic blocking agents such as pronethalol and propranolol (inderal) did not influence the estimates of the pD_2 values of the various sympathomimetics. Only in very high concentrations ($>10^{-4}$M) did the β-adrenergic blocking drugs augment the response of the sympathomimetics and this was by a myotropic spasmolytic effect. In some experiments with (−)-noradrenaline and (−)-adrenaline a β-sympathomimetic effect was uncovered by giving an α-adrenergic blocking agent. However, no indications of β-effects were observed with the other drugs. Although the intestine contains both α- and β-receptors, the effects of the drugs were elicited only on α-receptors (Fig. 3).

The various sympathomimetics in sufficiently high concentrations cause complete inhibition of peristalsis as does (−)-noradrenaline. They have therefore an intrinsic activity constant equal to that of (−)-noradrenaline and differ only in affinity (Table 1). There are great differences

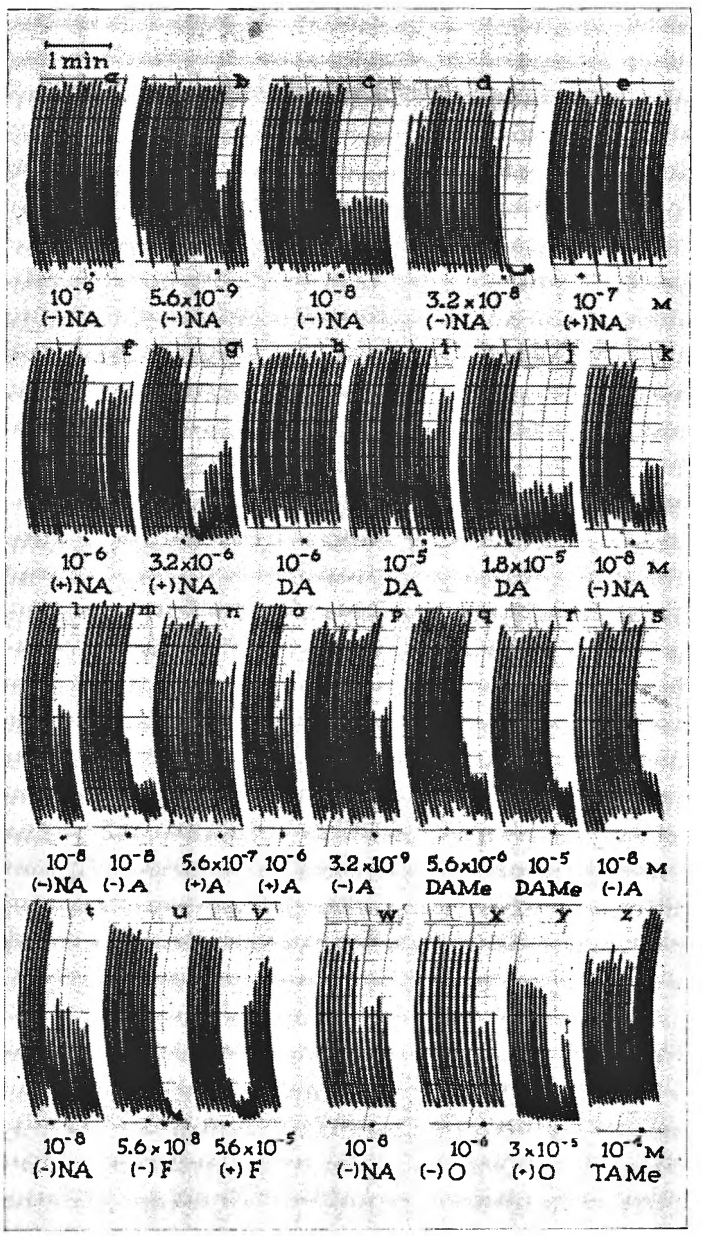

FIG. 3. Records of the inhibition of peristalsis on rabbit intestine by various α-sympathomimetic drugs related to noradrenaline and dopamines. Note the difference in potency between (−)-noradrenaline (exper. *a*, *b*, *c*, *d*, *k*) and dopamine (exper. *h*, *i*, *j*) between (−)-adrenaline (exper. *m*, *p*, *s*) and epinine, (DAMe), (exper. *q*, *r*) and between (−)-phenylephrine (exper. u) and (+)-phenylephrine (exper. *v*). Note also that *N*-methyltyramine (exper. *z*) does not cause inhibition and that the 1-*R* configuration of the OH-group in the side-chain is of great importance for α-sympathomimetic action.

between the optical isomers. (—)-Noradrenaline and (—)-adrenaline have a high affinity compared with their isomers and also with dopamine and epinine (Fig. 3; Table 1). (—)-Phenylephrine (1-*R* configuration) is almost 1,000 times more potent than its optical isomer.

The optical isomers of oxedrine differ slightly in action. The (—)-isomer is an α-sympathomimetic while the (+)-isomer is only a partial mimetic. This difference in potency is slight presumably because the affinity of (—)-oxedrine is low initially. The desoxy derivative, *N*-methyltyramine, does not inhibit but increases peristalsis (Fig. 3). At a concentration at which alone it is inactive, it antagonises noradrenaline in the same way as does tyramine and other indirectly-acting compounds (van Rossum & Mujíc, 1965). This appears to be a general feature of indirectly-acting sympathomimetics.

The potent isomers of the various series have the same absolute configuration (1-*R*) as natural noradrenaline (Table 1). The configuration of the hydroxyl group is therefore critical. The drugs in which the OH-group is lacking—dopamine, epinine and methyltyramine—are weak α-sympathomimetics or even antagonists. The 1-*R*/1-*S* affinity ratios for noradrenaline, adrenaline, phenylephrine and oxedrine are 16; 180; 1,000 and 1·5 respctively. The affinity ratios for (—)NA/DA and (—)A/epinine are 400 and 750 respectively. The *R*/*S* affinity ratios as well as the ratios of the affinity of the 1-*R* compounds and their desoxy derivatives are substantially greater in the rabbit intestine than in the vas deferens. These results indicate a difference in the α-receptor systems investigated.

SYMPATHOMIMETICS ON THE CAT BLOOD PRESSURE

(—)-Noradrenaline (1 μg/kg), dopamine (30 μg/kg), (—)-adrenaline (0·5 μg/kg) and epinine (20 μg/kg) have a pressor effect in the cat which could be antagonised to the same extent by the α-adrenergic blocking drug piperoxan. All four compounds therefore react with common α-receptors in the cardiovascular system of the cat. The affinity ratios of 1-*R*-NA/DA and 1-*R*-A/epinine are 30 and 50 respectively. Dopamine and epinine do not cause an increase in heart rate indicating that they do not activate β-receptors.

SYMPATHOMIMETICS ON THE RABBIT BLOOD PRESSURE

In the rabbit, (—)-noradrenaline (1 μg/kg), (+)-noradrenaline (10 μg/kg), (—)-adrenaline (0·7 μg/kg), (+)-adrenaline (8 μg/kg), (—)-phenylephrine (5 μg/kg) and (+)-phenylephrine (1 mg/kg) caused an increase in blood pressure, which was antagonised by α-adrenergic blocking agents such as piperoxan (0·2 mg/kg) and yohimbine (0·2 mg/kg). In contrast, dopamine (20–50 μg/kg) and epinine (10–20 μg/kg) induced a fall in blood pressure or a biphasic effect. With the fall in blood pressure there was often a fast and a slow component. We confirmed the results of Eble (1964) who found that the fall in blood pressure produced by dopamine was not antagonised by piperoxan and yohimbine. The same holds for epinine. Since no specific antagonists are yet available, it is not

possible to prove that dopamine and epinine react with common dopamine receptors. The similarity in the action of dopamine and epinine suggests that both drugs may react with common dopamine receptors.

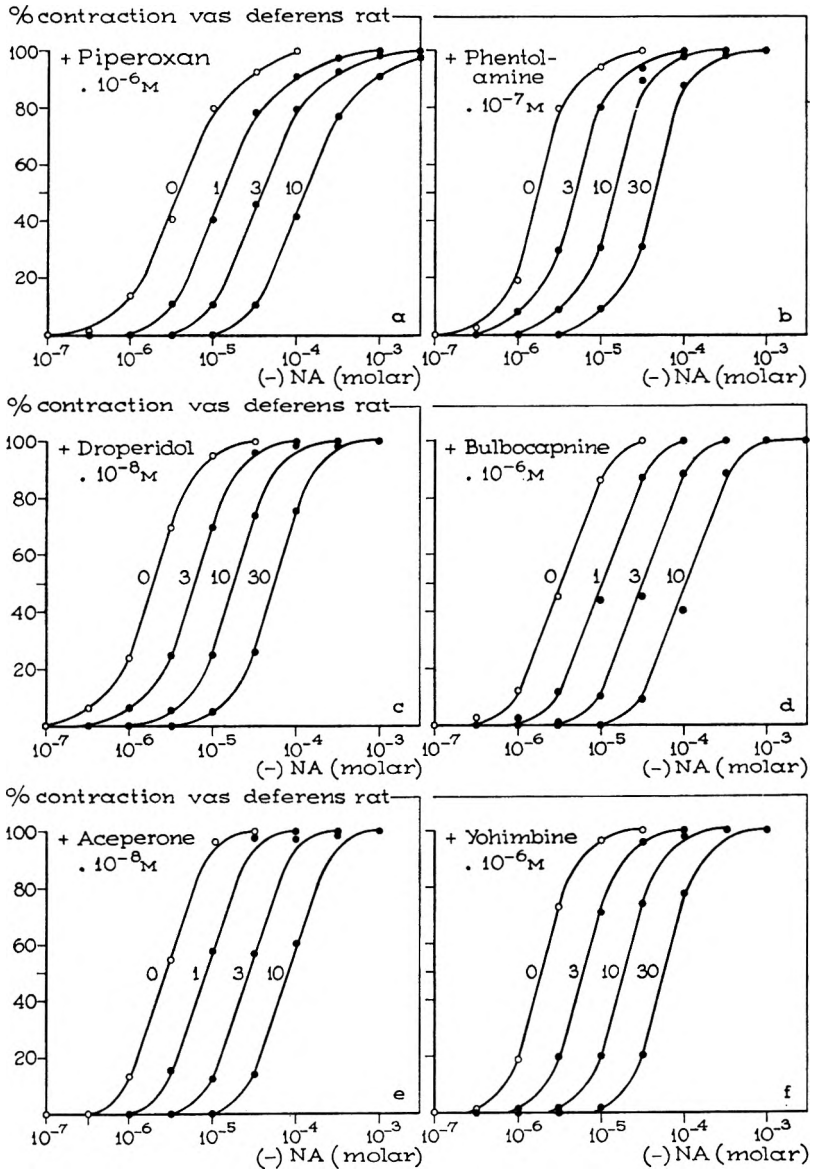

FIG. 4. Dose-response curves from experiments given in Fig. 1, at right hand side, calculated by plotting % contraction of (−)-noradrenaline versus the concentration in the bath (logarithm scale). (*a*) In combination with various concentrations of piperoxan; (*b*) with phentolamine; (*c*) with droperidol; (*d*) with bulbocapnine; (*e*) with aceperone and (*f*) with yohimbine. Note a parallel shift of the curves indicating competitive antagonism. The concentration to cause a given shift varies. The lowest concentration is required with the most potent drug, aceperone.

In a few experiments, droperidol (0·5–1 μmol/kg) and spiramide (1–10 μmol/kg) antagonised vasodepressor effects of dopamine and epinine. However, in some experiments these tranquillising drugs were ineffective themselves or caused prolonged vasodepression (see discussion).

ADRENERGIC BLOCKING DRUGS ON THE VAS DEFERENS OF THE RAT

By using (−)-noradrenaline and dopamine as agonists, cumulative dose-response curves were made on the vas deferens in the presence and in the absence of various concentrations of α-adrenergic blocking and tranquillising drugs. A typical record is given at the right-hand of Fig. 1 for droperidol. The adrenergic blocking drugs merely cause a shift of the dose-response curve to higher concentrations. From such experiments, dose-response curves were calculated as in Fig. 4. There are examples of experiments for each drug on a single organ. From a number of these experiments the affinity constants of the antagonists were calculated from the degree of antagonism and were given as pA_2-values. The average values are given in Table 2. Relative affinities are also given using piperoxan as a reference compound (rel. aff. = 1). It is interesting to see that yohimbine, which is a good adrenergic blocking agent, is the weakest of this group, while the tranquillisers droperidol and aceperone are potent α-adrenergic blocking agents.

All the compounds examined were competitive antagonists of noradrenaline and of dopamine. This can be seen from the parallel shift of the curves in Fig. 4. In some experiments, spiramide (10^{-7} M) sensitised the vas deferens to (−)-noradrenaline, but in most experiments there was a parallel shift at relatively high concentrations (Table 2). Since the isolated vas deferens contains only α-receptors, these results indicate that the tranquillisers, chlorpromazine, (−)-mepromazine, haloperidol, droperidol and spiramide, are also α-adrenergic blocking drugs like piperoxan, phentolamine, yohimbine and aceperone (see discussion).

ADRENERGIC BLOCKING DRUGS IN THE RABBIT INTESTINE

The adrenergic blocking drugs were also studied as antagonists of (−)-noradrenaline and dopamine on the rabbit intestine. A typical record of some experiments is given in Fig. 5. A dose of (−)-noradrenaline, causing about 50–80% inhibition is given first. After washing, a dose of an adrenergic blocking drug which alone does not influence the pendular movements, is given; 30 sec later noradrenaline is given in the same dose as before. The intestine is then washed and again an antagonist is given and after that a threefold higher dose of noradrenaline (Fig. 5). When the effect of this higher dose in the presence of an antagonist is equal to that of the previous dose in the absence of it, the negative logarithm of the molar concentration of the adrenergic blocking agent is equal to the pA_3 value. It is generally the custom to give the pA_2-value which is simply obtained by adding 0·3 to the pA_3-value. Due to variations in the sensitivity only a rough estimate of the pA_2-value may be obtained. On a single piece of intestine, differences in pA_2-value to the amount of 0·3 are significant.

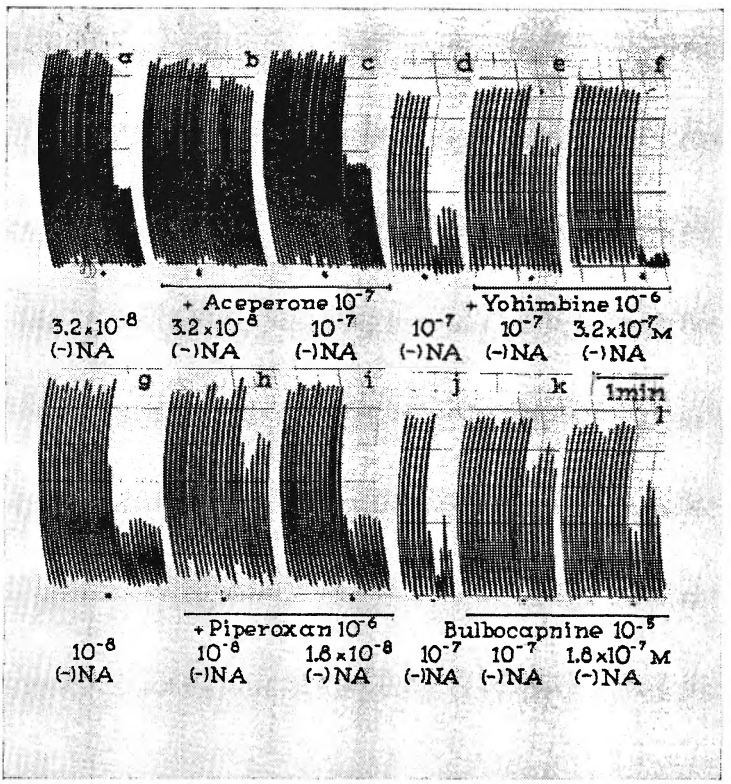

FIG. 5. Records of the inhibition of peristalsis of rabbit intestine by (−)-noradrenaline (−)NA alone and with some adrenergic blocking drugs and tranquillisers. Aceperone (exper. *a*, *b*, *c*) is the most potent antagonist of this series, yohimbine (exper. *d*, *e*, *f*) and piperoxan (exper. *g*, *h*, *i*) are relatively potent whereas bulbocapnine (exper. *j*, *k*, *l*) is a weak antagonist. Compare the order of potency with that found for the vas deferens. Note that the antagonists do not inhibit peristalsis on their own but antagonise the effect of noradrenaline, while a higher concentration of noradrenaline can break through (competition).

The affinity constants for the various antagonists (α-adrenergic blocking drugs) are given in Table 2. There is a different order of potency to that found in the vas deferens (see discussion).

TABLE 2. EFFECTS OF α-ADRENERGIC BLOCKING DRUGS AND TRANQUILLISERS ON DIFFERENT TYPES OF α-RECEPTORS

Drug	Rat vas deferens			Rabbit intestine			Affinity ratio vas/intestine
	i.a.c.*	pA_2	rel. aff.	i.a.c.	pA_2	rel. aff.	
Piperoxan	0	6·0	1	0	5·8	1	1·6
Phentolamine	0	6·9	8	0	6·3	3	4·0
Yohimbine	0	5·3	0·2	0	6·1	2	0·16
Aceperone	0	8·3	200	0	7·6	65	5·0
Chlorpromazine	0	6·8	6	0	6·1	2	5·0
Levomepromazine	0	7·3	20	0	6·0	1·6	20
Haloperidol	0	6·7	5	0	5·4	0·7	20
Droperidol	0	7·9	80	0	6·3	3	40
Spiramide	0	6·3	2	0	—	—	—
Bulbocapnine	0	6·4	2·5	0	5·2	0·2	16

* Intrinsic activity constant.

ADRENERGIC BLOCKING DRUGS ON THE BLOOD PRESSURE OF THE CAT

The various antagonists of noradrenaline were also investigated in combination with noradrenaline and dopamine on the blood pressure of the cat. Two doses of noradrenaline, 0·5 and 1 μg/cat, and 10 and 20 μg of dopamine were given. These produce the characteristic rise in blood pressure. A dose of an antagonist was then given which reduced the blood pressure response to the highest dose of agonist to about that of the lowest dose. The dose of the antagonist producing this degree of inhibition was taken as a measure of its potency. Because of a variation in the sensitivity of the cats, only a rough estimate of the potency was possible (Table 3). The most potent antagonists were aceperone, phentolamine and yohimbine; the least potent was bulbocapnine. The results correlate more with findings in the rabbit intestine than with those from vas deferens (compare Table 2 and 3 and see discussion).

TABLE 3. α-ADRENERGIC BLOCKING DRUGS AND TRANQUILLISERS AS ANTAGONISTS OF (−)-NORADRENALINE AND DEXAMPHETAMINE

Drug	Anti-NA blood pressure (cat)	Anti-amphetamine locomotive activity (mice)		Anti-amphetamine ED50*	Affinity ratio vas/anti-amphetamine
	μmoles/kg	μmoles/kg	pA_2	μmoles/kg	
Piperoxan	0·4–0·7	100†	—	—	—
Phentolamine	0·2–0·3	60†	—	—	—
Yohimbine	0·2–0·3	30†	—	—	—
Aceperone	0·1–0·2	18	5·1	>1500	1600
Chlorpromazine	—	6	5·5	3·5	20
Levomepromazine	0·8–1·5	1·6	6·2	6·0	12
Haloperidol	0·8–1·5	0·5	6·8	0·1	0·8
Droperidol	0·4–0·6	0·5	6·8	0·094	12
Spiramide	—	0·4	7·0	0·042	0·2
Bulbocapnine	2 –4	40	4·8	—	40

* Data from Janssen & others (1965).
† A competitive antagonism could not be determined.

ADRENERGIC BLOCKING DRUGS ON THE AMPHETAMINE-INDUCED LOCOMOTOR STIMULANT ACTION IN MICE

The various adrenergic blocking drugs and tranquillisers were studied as antagonists to dexamphetamine in mice. Dexamphetamine, 3·16 and 10 μmol/kg, caused a medium and a strong increase in locomotor activity. The same mice 3 or 4 days later, received the medium dose of dexamphetamine again and then a dose of antagonist. About 20 min after the antagonist a large dose of dexamphetamine was given. Doses of the antagonist were such that the effect of the larger dose of dexamphetamine was reduced to approximately that of the medium dose (Fig. 6). The average dose of antagonist in μmol/kg to produce this degree of antagonism is given in Table 3. The negative logarithm of this dose reflects a $pA_{3\cdot16}$ value when the concentration is taken in mol/kg instead of mol/litre. From the $pA_{3\cdot16}$ value, the pA_2 value is found by adding 0·34. The pA_2 values determined for the various antagonists are given in Table 3. In addition, the affinity ratio for (−)-noradrenaline antagonistic potency in the vas deferens and the anti-dexamphetamine potency is given.

Since the anti-dexamphetamine activities of a number of tranquillisers have been determined by Janssen, Niemegeers & Schellekens (1964), taking as an effect the amphetamine-induced compulsatory gnawing, these results after converting mg/kg into μmol/kg are also included in Table 3. As may be seen from Tables 2 and 3, the central anti-dexamphetamine activity does not correlate with a peripheral α-adrenergic blocking action of the various adrenergic blocking agents and tranquillisers. However, the results obtained on the vas deferens are more consistent with the central action than those obtained in the other tissues (see discussion).

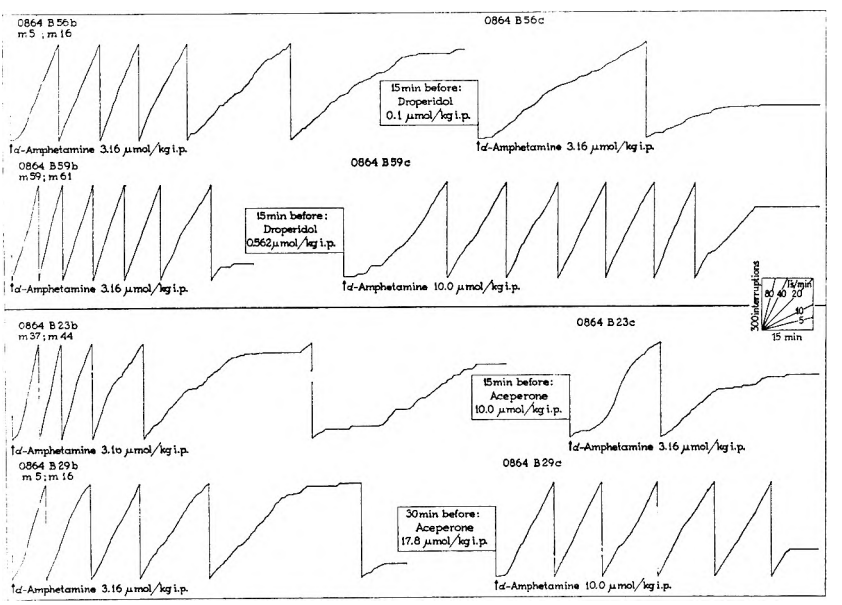

FIG. 6. Cumulative records of induction of locomotor activity by dexamphetamine, in control groups of two mice and in groups pre-treated with the tranquilliser droperidol or the adrenergic blocking drug aceperone. A higher dose of dexamphetamine can break through the inhibition, indicating a competition. Droperidol, 0·3 μmol/kg (100 μg/kg) causes the same degree of antagonism as aceperone 17·8 μmol/kg (6 mg/kg). See text. Droperidol is about 50 times more potent than aceperone in the CNS although it is about three times less potent as an antagonist of (−)-noradrenaline. Compare with Table 3.

Discussion

Dopamine like noradrenaline is known to act on sympathetic α-receptors, but there is also evidence for an action on separate dopamine receptors in peripheral tissues and in the peripheral and central nervous systems.

It has been shown that dopamine, in contrast to noradrenaline, causes a fall in blood pressure in the dog, guinea-pig and the rabbit (Holtz & Credner, 1942; Burn & Rand, 1958; Holtz & others, 1963). Dopamine causes an increase in the blood flow through the kidneys, the superior

mesenteric vessels and the coeliac vessels (Eble, 1964; McDonald, Goldberg, McNay & Tuttle, 1964). Also, in man, it differs from noradrenaline in increasing blood pressure without raising the diastolic pressure and by inducing pilo-erection (Horwitz, Fox & Goldberg, 1962; Allwood & Ginsberg, 1964). The specific dopamine effects in dogs and rabbits are not affected by specific α-blocking agents such as piperoxan, tolazoline, yohimbine and phenoxybenzamine (Holtz & others, 1963; McDonald & Goldberg, 1963; Eble, 1964; and present study) or by specific β-blocking agents (Holtz & others, 1963; Eble, 1964; Vanov, 1963). Also antihistamines and atropine-like drugs are ineffective (McDonald & Goldberg, 1963).

From the work of various authors it may be concluded that in the peripheral nervous system there are dopamine receptors and that there might be dopaminergic nerves innervating certain tissues. (Allwood & Ginsberg, 1964; Eble, 1964; Holtz & others, 1963). Dopamine on the other hand also fits sympathetic α-receptors so that depending on the relative amounts of these two types of receptors, dopamine may exert a blood pressure rise (in the cat) or a blood pressure fall (in the rabbit). Experiments on nerve transmission in lower animals also indicate specific dopamine receptors.

The rate of discharge of neurones in the stretch receptor organ in the abdominal segments of the crayfish, *Pacifastacus leniusculus*, is blocked by γ-aminobutyric acid (GABA), noradrenaline and dopamine (McGeer, McGeer & McLennan, 1961). Dopamine is 100 times more potent than noradrenaline. The inhibitory action of dopamine on the stretch receptor organ can be blocked selectively by chlorpromazine but not by picrotoxin, whereas the GABA action can be blocked by picrotoxin but not by chlorpromazine (McGeer & others, 1961). In a recent report, Gerschenfeld (1964), found receptive sites in inhibition neurones in the nervous system of the mollusc, *Cryptomphallus aspera*, which respond to dopamine in a concentration of 10^{-9} M but hardly at all to other inhibitory substances. This also points to the existence of specific dopamine receptors in nervous tissue.

It has been suggested that dopamine in the central nervous system is not only a precursor of (−)-noradrenaline, but has a "transmitter function" of its own (Carlsson & others, 1958). In reserpinised rats, the psychomotor stimulant action of dihydroxyphenylalanine (dopa) is correlated with brain dopamine levels but not with brain noradrenaline levels (Everett & Wiegand, 1961). Evidence has been provided that the central stimulant action of amphetamine may be mediated through reaction with dopamine receptors (van Rossum & Hurkmans, 1964).

These observations raise the question whether dopamine receptors are part of a range of α-receptors at one end of which are receptors best fitted by noradrenaline, and at the other end receptors which are best fitted by dopamine. This possibility has been challenged by comparing the relative activities of various α-sympathomimetic-, α-adrenergic blocking and tranquillising drugs on α-receptors in both the vas deferens of the rat and the rabbit intestine. It has been postulated by Holman & Jowett,

(1964) that there are β-receptors in the vas deferens of the guinea-pig, but in the isolated vas deferens of the rat they are absent or play an insignificant part in the study of α-sympathomimetics (Ariëns, 1963). The rabbit intestine does contain β-receptors, but the type of relaxation obtained by activation of α- or β-receptors differs substantially (van Rossum & Mujíc, 1964). In contrast to the responses obtained with β-sympathomimetics the sympathomimetics we used caused a rapid relaxation of the rabbit intestine. This is characteristic of α-sympathomimetics. Furthermore, sympathomimetics were competitively antagonised to the same degree by α-adrenergic blocking drugs. Specific β-receptor antagonists like pronethalol and propranolol did not influence the pD_2 estimates of the sympathomimetics as given in Table 1, unless used at concentrations causing myotropic spasmolytic effects.

The experiments have shown that the 1-*R* configuration of the hydroxy group in the side-chain of the various groups of mimetic drugs is essential for optimal affinity. This holds for an action on the α-receptors in the vas deferens and the rabbit intestine. Dopamine and epinine, which lacks the hydroxyl group, are relatively weak. The trend in the affinity change with alterations of the structure or configuration is the same in the vas deferens and rabbit intestine. However, there are large quantitative differences in the α-receptors of both tissues. For instance, the affinity ratios for 1-*R*-noradrenaline: dopamine and 1-*R*-adrenaline: epinine are 10 and 24, in the vas deferens but 400 and 150 respectively in the rabbit intestine (Table 1). The α-receptors of the rabbit intestine are more specific noradrenaline receptors than those in the vas deferens, but although the relative affinity of dopamine is greater for the receptors in the vas these receptors cannot be classed as dopamine receptors because they are more sensitive to noradrenaline or adrenaline.

The difference in properties of the α-receptors in the rabbit intestine and rat vas deferens is supported by a difference in the actions of antagonist drugs (Table 2). Yohimbine is about 6 times more potent as an α-adrenergic blocking agent in the rabbit intestine, whereas droperidol is 40 times more potent in the vas deferens.

Since yohimbine has a higher affinity for α-receptors best fitted by noradrenaline it may be a selective antagonist for noradrenaline receptors. Conversely it is tempting to speculate that the greater potency of droperidol on the vas deferens is related to the more pronounced affinity of the receptors in the vas for dopamine. The experiments reported here show differences between the properties of the α-receptors in the rabbit intestine and the rat vas deferens, but further work is necessary to find dopamine receptors in the peripheral tissues. A simple test organ reacting to activation of dopamine receptors would provide a valuable model and greatly facilitate the study of drugs which mimic or antagonise dopamine in the central nervous system.

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Applicability of the Ferguson principle to systems of mixed preservatives

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Using solubility and distribution data for methyl and propyl *p*-hydroxybenzoates, the degree of saturation of the aqueous phase of an oil-in-water emulsion has been calculated for various oil : water ratios. In emulsions of low oil content the propyl ester gives the higher degree of saturation of the aqueous phase, while in emulsions of high oil content the methyl ester gives the higher degree of saturation. Irrespective of the oil : water ratio, a mixture of the esters cannot give a higher degree of saturation or a higher thermodynamic activity of the aqueous phase, than either ester alone. The finding of many investigators that mixtures of the esters have higher biological activities than a single ester must therefore depend, not only on the degree of saturation, or thermodynamic activity of the aqueous phase, but also on some other unknown factors.

DETERIORATION from mould formation or bacterial action of cosmetic creams during storage occurs mainly in oil-in-water emulsions, and is rare in water-in-oil emulsions. Generally the organisms responsible for deterioration need an aqueous medium for growth, consequently cosmetic products contain preservatives which are effective in preventing growth in the aqueous phase. The most widely used preservatives are esters of *p*-hydroxybenzoic acid, the methyl and propyl esters being generally favoured.

The literature contains conflicting views (Manowitz, 1962) about whether to use these preservatives singly (Atkins, 1950) or in combination (Boehm, 1959); those who advocate using the mixture claim a broader spectrum of microbiological activity than that given by their separate constituents, and also that the mixture exhibits a greater preservative action than would be expected from the individual activity (Boehm, 1959).

It is well known from bacterial studies that the activity of the esters of *p*-hydroxybenzoic acid is related to the length of the alkyl chain; the propyl is more active than the methyl ester but increasing the chain length reduces water solubility and increases oil solubility, i.e. the oil : water distribution coefficients are markedly increased. As deterioration in cosmetic preparations occurs in the water phase, only that proportion of the added preservative which is dissolved in the aqueous phase can inhibit the growth of micro-organisms. But, is the greater bacterial activity of the propyl compared to the methyl ester more than offset by its lower water solubility and its higher oil solubility? Atkins (1950) claims that this is often so and that the methyl ester is then the more reliable preservative; Atkins' data, therefore, do not support the widely held belief that a combination of the two esters is better than either alone.

Since there is no apparent agreement in the published literature about whether a mixture of esters is better than a single ester in emulsion systems, calculations based on solubility and distribution data might throw

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further light on the problem. Such calculations, previously suggested by Manowitz (1962) would show how the degree of saturation and the thermodynamic activity of the aqueous phase of an emulsion containing a single ester or a mixture of esters varies with the oil content of the emulsion, and with the distribution coefficient of the esters. Such an approach should therefore indicate whether a mixture of esters would, a priori, be expected to have a higher biological activity than a single ester, assuming that biological activity is proportional to the degree of saturation of the aqueous phase (Ferguson's Principle).

BIOLOGICAL ACTIVITY AND DEGREE OF SATURATION

The work of Ferguson (1939) and others has shown for structurally non-specific agents that the biological activity is related to the thermodynamic activity rather than to the concentration of the active agent. This principle has been further substantiated by an examination of the relative effectiveness of a large series of narcotics by Brink & Posternack (1948), and of factors governing the permeability of the insect cuticle by Webb (1949). The thermodynamic activity of a solute will, of course, depend upon the choice of standard state; one convenient method (Allawala & Riegelman, 1954) is to express it in terms of the per cent saturation, or degree of saturation, of the aqueous phase by setting the saturated solution as the standard state of reference. Allawala & Riegelman have shown that equitoxic solutions of phenols of widely different solubilities are those in which the thermodynamic activities or the degrees of saturation, are the same, rather than solutions in which the actual or stoichiometric concentrations are the same. From a study of 23 different phenols Allawala & Riegelman (1954) showed that, while the concentrations (moles/litre) of equitoxic solutions varied by a factor of approximately 10^4, the degrees of saturation of equitoxic solutions of the phenols were all between 18 and 20%.

CALCULATIONS

The data of Atkins (1950) for the solubility of the methyl and propyl *p*-hydroxybenzoate in water and in an oil (composition not specified) at 16° are: S_w 0·31 and 0·035, S_o 1·0 and 2·0 g/100 ml for the methyl and propyl ester respectively. $K_D = 3{\cdot}2$ and $57{\cdot}1$;

where S_w, S_o, K_D = water solubility, oil solubility, and distribution coefficient respectively.

When the ester distributes itself between the water and the oil phase, the concentration in the water phase can be derived from the equation

$$C_w = \frac{\text{Total weight of ester}}{V_w + K_D V_o} \qquad (1)$$

where C_w = concentration of ester in the water phase (g/ml water)

V_w, V_o = Volume of the water and oil phase respectively.

If it is accepted that biological activity is proportional to the thermodynamic activity of the active agent, which in turn is proportional to the per cent saturation, or degree of saturation, of the water phase, it is possible to write:

Biological Activity α Thermodynamic Activity α Degree of Saturation =

$$\frac{\text{Total weight ester}}{S_w (V_w + K_D V_o)} \qquad (2)$$

where S_w = water solubility of ester (g/ml).

Using equation (2) and K_D calculated from Atkins (1950) data given above, the degree of saturation of the water phase of an emulsion at various total weights of the esters, and two different oil/water ratios have been calculated and are given in Table 1.

TABLE 1. VARIATION OF THE DEGREE OF SATURATION OF THE AQUEOUS PHASE OF AN OIL : WATER EMULSION WITH DIFFERENT AMOUNTS OF METHYL AND PROPYL *p*-HYDROXYBENZOATES AND WITH VARYING OIL WATER RATIOS

(Calculated from solubility data of Atkins (1950)

	Water : oil 95 : 5% v/v		Water : oil 60 : 40% v/v	
	Degree of saturation		Degree of saturation	
Total wt. ester (g)	Methyl ester	Propyl ester	Methyl ester	Propyl ester
0·01	0·029	0·076	0·017	0·012
0·03	0·087	0·23	0·051	0·036
0·05	0·14	0·38	0·03	0·061
0·06	0·17	0·46	0·10	0·073
0·10	0·29	0·76	0·17	0·12
0·13	0·38	1·0	0·22	0·16
0·20	0·58	>1·0	0·34	0·24

Discussion

It is clear from Table 1 that, in the system containing water : oil 95 : 5%, the propyl ester at any given total weight of the esters, gives a higher degree of saturation of the water phase than the methyl ester; consequently if biological activity is indeed proportional only to the degree of saturation, the propyl ester should be the more active in this system. However, in the system water : oil 60 : 40%, for any given weight of the ester the methyl ester gives the higher degree of saturation in the water phase implying, as before, that in this system, the methyl ester should have a higher biological activity.

Generally, for any given weight of the esters, in systems of high water : low oil content, the propyl ester will give the higher degree of saturation of the water phase; as the oil content of the system increases a certain oil : water ratio is reached at which, for any given weight of the esters, the degree of saturation will be the same for both esters. Increasing the oil content of the system above this critical level—the "cross-over" point, gives systems in which, for any given weight of the esters, the methyl ester will always give the higher degree of saturation of the water phase.

The oil : water ratio of the system at the "cross-over" point i.e., where both esters give the same degree of saturation, depends only on the values of the distribution constants, K_D and the water solubilities S_w; it can be easily shown from equation (2), at any given total weight of the esters, that the two esters will give solutions of the same degree of saturation (and hence same biological activity) when:

$$V_o = \left(\frac{S_w^{Me} - S_w^{Pr}}{S_w^{Pr} K_D^{Pr} - S_w^{Me} K_D^{Me}} \right) V_w \qquad (3)$$

where S_w^{Me}, S_w^{Pr} = Water solubility of the methyl and propyl ester respectively.

K_D^{Me}, K_D^{Pr} = Distribution coefficients of the methyl and propyl ester respectively.

For systems where the volume of the oil phase, V_o, is less than the value given by equation (3), then the propyl ester, for any given weight, will give a higher degree of saturation of the water phase than the methyl ester; similarly when V_o is greater than the value given by equation (3) then the methyl ester will give the higher degree of saturation of the water phase. It is clear that a higher degree of saturation is obtained either with one or the other of the esters depending on the oil : water ratio; under no conditions does a mixture of esters give a higher degree of saturation than either ester singly.

The above argument holds only when $K_D^{Pr} S_w^{Pr} > K_D^{Me} S_w^{Me}$; if as is possible with some oils, $K_D^{Pr} S_w^{Pr} < K_D^{Me} S_w^{Me}$ then the propyl ester will always give a higher degree of saturation of the aqueous phase, i.e., no "cross-over" point, irrespective of the oil : water ratio. The essential point is that, whatever the oil : water ratio, a mixture of esters does not give a higher degree of saturation and hence a higher thermodynamic activity of the water phase, than either ester alone.

Atkins (1950) gives no information on the relative phase volumes in the emulsions used in his study, but merely states that the propyl ester was ineffective as a preservative, whereas the methyl ester was completely effective. From equation (3) and the solubility data given by Atkins, it can be calculated that the same weight of the two esters in the emulsions studied by Atkins would give the same degree of saturation when $V_o = 0{\cdot}275\, V_w$. If biological activity is governed solely by the degree of saturation of the aqueous phase then the methyl ester would be the better preservative in the emulsions studied by Atkins provided the emulsions contained >22% of the oil phase.

The same considerations will apply when, in addition to, or instead of the oil phase, the water phase contains a surfactant which solubilises the esters; in this instance K_D in equation (3) will be the distribution coefficient of the ester between the water phase and the micellar phase, and V_o will be replaced by V_m, the micellar volume.

Such systems have been studied by Aoki, Kamata, Yoshioka & Matsuzaki (1956) whose data show the "cross-over" point clearly; these data

show that in water and in low concentrations of polysorbate (Tween) 20, the order of biological activity of the esters is butyl > propyl > ethyl > methyl, while in high concentrations of polysorbate 20, the order of biological activity is exactly the reverse—as predicted in equation 3; the "cross-over" points depend entirely on the values of K_D and S_W of the various esters and could be calculated easily from solubility data (McBain & Hutchinson, 1955) using equation (3). The data of Atkins (1950), and also those of Aoki & others (1956) are in complete agreement with equation (3), i.e., in systems of low oil or micellar content, the higher esters are more effective than the lower esters in giving higher degrees of saturation of the aqueous phase, while the reverse is true in solutions of high oil or high micellar content.

It must therefore be concluded that, for emulsion systems where a mixture of esters is claimed to be more effective than a single ester (Boehm, 1959), the biological activities of such systems are governed not only by the degree of saturation of the aqueous phase but also by some other unknown factors. Whilst it is impossible to specify what these unknown factors are, Bean (1964) and Bean & Heman-Ackah (1964) have established that, in some two-phase systems, the activity of antibacterial agents is governed, not only by the degree of saturation of the aqueous phase, but also by the concentration of the antibacterial agent at the oil-water interface. This factor may also be important in systems in which a mixture of the *p*-hydroxybenzoates have been found to be more effective than a single ester, but further work is required to establish this point.

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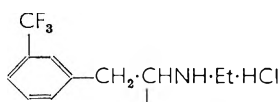
Cardiovascular and autonomic effects of fenfluramine hydrochloride*

BERNARD V. FRANKO, LEROY J. HONKOMP AND JOHN W. WARD

Fenfluramine hydrochloride (*N*-ethyl-α-methyl-3-trifluoromethylphenethylamine hydrochloride; AHR-965) administered orally or intravenously to anaesthetised or unanaesthetised dogs, caused a predominant rise in arterial blood pressure, tachycardia, an increase in myocardial contractile force and cardiac output, and an enhanced total peripheral resistance. Fenfluramine was qualitatively like dexamphetamine in its cardiovascular effects; however dexamphetamine was 10 to 20 times more potent as a pressor agent.

THE effects of sympathomimetic amines on the cardiovascular and the central nervous systems are major deterrents to their use as appetite suppressants. Numerous variations have been made on the phenethylamine moiety in attempts to overcome these liabilities. One such compound, fenfluramine hydrochloride (I; *N*-ethyl-α-methyl-3-trifluoromethylphenethylamine hydrochloride; AHR-965), has been reported by Alphin, Funderburk & Ward (1964) to reduce appetite in several laboratory species in doses that do not cause overt stimulation of the central nervous system. These findings led to the investigation of the cardiovascular and autonomic effects of this compound, the results of which are the subject of this report.



(I)

Methods

Adult mongrel dogs (either sex; 8·2 to 14·1 kg) were anaesthetised with intravenous phenobarbitone sodium, 125 mg/kg. A polygraph[1] and accessory equipment were used for recording blood pressure from a carotid artery and respiration from a tracheal cannula (24 animals); in addition, the electrocardiogram (lead II) was recorded in most of these experiments. Single doses of the drug were administered intravenously (1 to 16 mg/kg) and orally (5 and 10 mg/kg).

Alteration of cardiac dynamics was further investigated in other experiments. Cardiac rate was measured with a tachograph[2] and a myocardial force transducer[3] was sutured to the left ventricle for measuring contractile force (7 experiments); an electromagnetic flowmeter[4] was used for measuring cardiac output (flow probe placed on the ascending aorta) in two dogs.

The effect on the arterial blood pressure of trained, unanaesthetised dogs was investigated using essentially the technique of Prioli & Winbury

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[1] Model 5. Grass. [2] Grass. [3] Honeywell. [4] Medicon.

(1960). The occluding cuff was placed at the base of the tail. A microphone pickup, signal divider and electrical manometer[1] were used for detecting and measuring pressure; the signal was monitored on an oscilloscope and recorded.

A reference drug, dexamphetamine sulphate, was studied under similar conditions in each experimental procedure. Various autonomic agents and histamine acid phosphate were used in an effort to determine the mechanism by which the new drug exerted its effects. The doses of all the drugs are expressed in terms of the specified salts.

Results

ANAESTHETISED DOGS

Blood pressure. Fenfluramine usually elicited a biphasic change in arterial blood pressure, a brief decrease being followed by a more prolonged and pronounced pressor response (left panel of Fig. 1). With intravenous

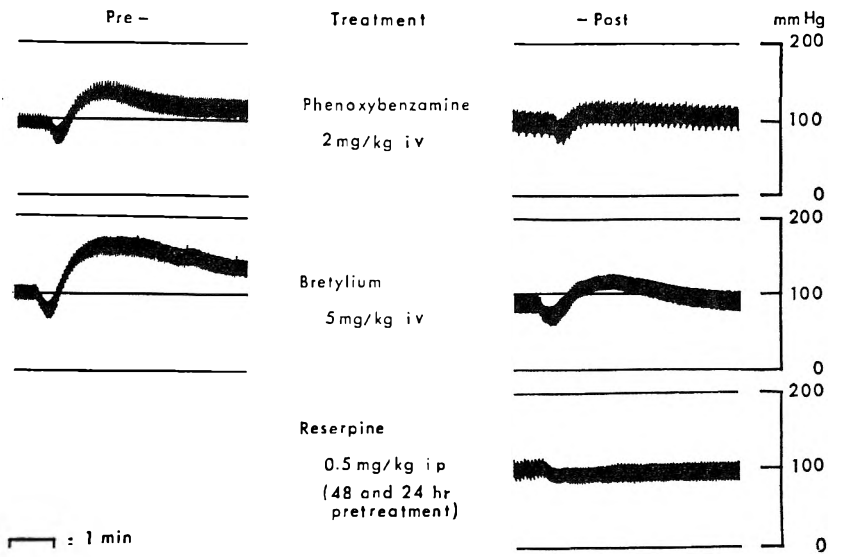

FIG. 1. Anaesthetised dogs (phenobarbitone sodium, 125 mg/kg, i.v.): effects of intravenous 4 mg/kg on carotid blood pressure; alteration of the pressor effect by adrenergic blocking agents.

doses of 1, 2 or 4 mg/kg, the pressor component was decidedly predominant; with higher doses, the depressor phase was more evident (but still not predominant) and the secondary pressor phase was much prolonged. The changes elicited by intravenous dexamphetamine (0·05 to 0·2 mg/kg) were similar to those produced by fenfluramine with the possible exception that the depressor component was seen less frequently. After fenfluramine 10 mg/kg orally, the depressor response was negligible and the pressor response reached a peak in 10 min from where it declined slowly to the control level over a period of 3 to 4 hr. An oral dose of 5 mg/kg did not alter blood pressure in the following 90 min.

[1] Beckman.

Repeated intravenous administration of fenfluramine (2 mg/kg every 6 min for 8 doses) had no apparent effect on the depressor phase of the arterial pressure response, but the pressor component became progressively less with each dose. Although the point was not investigated specifically, tachyphylaxis seemed to be more readily demonstrable with dexamphetamine than with fenfluramine.

Cardiac effects. In general, a bradycardia coincided with the hypotensive period and cardiac rate increased during the hypertensive phase. In one animal, for example, cardiac rate showed a slight decrease tc 96% of the control and then was increased to 116% of the control after intravenous fenfluramine, 8 mg/kg. A comparable tachycardia was produced with dexamphetamine, 0·02 mg/kg, given by the same route. Heart rate was not altered by fenfluramine orally, 5 mg/kg; 10 mg/kg caused a maximum increase of 23% over the control rate in another experiment.

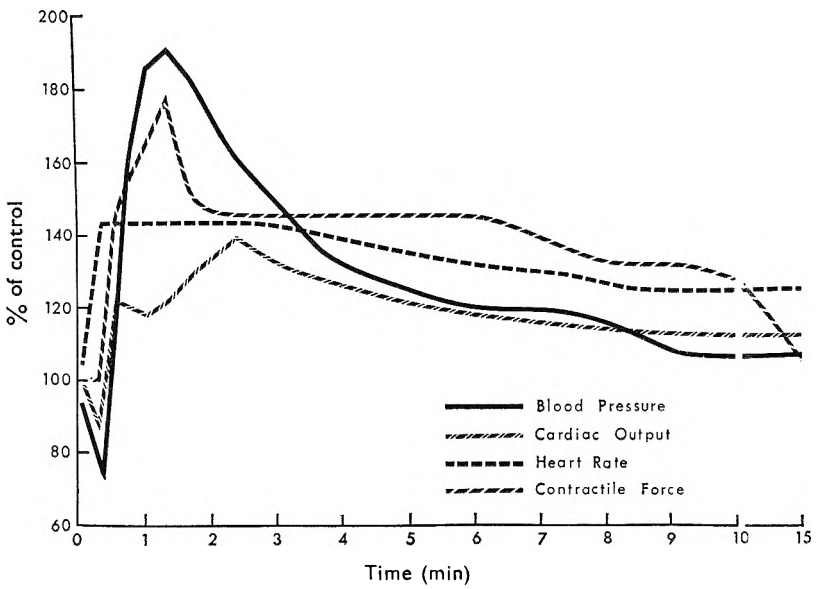

FIG. 2. Anaesthetised dogs (phenobarbitone sodium, 125 mg/kg, i.v.): effects of fenfluramine 4 mg/kg, i.v. (at ordinate) on carotid blood pressure and on cardiodynamics.

The contour of the electrocardiogram was not altered by the drug in intravenous doses below 4 mg/kg. The most distinct and frequent change after 4 or 8 mg/kg was a shortened TP interval that coincided with the tachycardia. In about 50% of the experiments, the administration of adrenaline, after treatment with fenfluramine or dexamphetamine, evoked extrasystoles throughout the adrenaline hypertension.

Force of ventricular contraction and aortic flow varied directly with arterial blood pressure alterations. These effects varied in degree among experiments, but in an individual animal there was usually a direct relationship between intensity of the response and the dose of fenfluramine. Fig. 2 illustrates these changes after intravenous fenfluramine, 4 mg/kg.

Similar changes were seen after the intravenous administration of dexamphetamine, 0·4 mg/kg.

Total peripheral resistance. Fenfluramine had the primary effect of increasing total peripheral resistance. The change was regular in occurrence and its intensity was related to dose.

Autonomic agents (and histamine). Fenfluramine had no significant effect on the arterial blood pressure responses to (−)-adrenaline hydrochloride, (−)-noradrenaline bitartrate, acetylcholine chloride, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), or histamine. Phentolamine hydrochloride, phenoxybenzamine hydrochloride, guanethidine sulphate, reserpine and bretylium tosylate reduced or eliminated the pressor phase. Some of these effects are illustrated in Fig. 1. Hexamethonium chloride had little, if any, inhibiting effect. Atropine sulphate did not prevent the depressor response after the pressor phase had been eliminated with guanethidine. Dichloroisoprenaline hydrochloride antagonised only slightly the depressor component and diphenhydramine hydrochloride had no effect on it.

UNANAESTHETISED DOGS

The effect of fenfluramine on the arterial blood pressure of unanaesthetised dogs was not unlike that in the anaesthetised preparations. As indicated in Table 1, intravenous administration caused a pressor response.

TABLE 1. EFFECT ON MEAN ARTERIAL BLOOD PRESSURE IN UNANAESTHETISED DOGS

Compound	Dose mg/kg	No. expts	Mean % increase	Post-drug time (min)	
				Max. effect	Duration*
Fenfluramine i.v.	1	4	9	10	21
	2	2	21	12	52
	4	2	28	6	60
dexamphetamine i.v.	0·05	2	0	—	—
	0·1	2	22	4	13
	0·2	2	31	3	>300

* Approximate time required to return to pretreatment level.

These data also illustrate the greater potency of dexamphetamine in producing a rise in blood pressure. Oral administration of either fenfluramine or dexamphetamine likewise produced a hypertensive effect. The method of measuring blood pressure did not permit detection of a depressor component in the intravenously-treated animals.

Discussion

The results of this study show fenfluramine to qualitatively resemble dexamphetamine on the cardiovascular system. Both compounds were pressor, caused a tachycardia, increased myocardial contractile force and cardiac output, and enhanced total peripheral resistance. Quantitatively, dexamphetamine was 10 to 20 times more potent than fenfluramine on a mg/kg basis of the respective salts. In appetite-suppressant doses, dexamphetamine caused observable central nervous system stimulation whereas fenfluramine did not. Structurally these compounds differ in that fenfluramine contains a trifluoromethyl substituent in the

aryl nucleus and an ethyl group on the nitrogen atom. Thus the question arises as to which structural difference caused the slight decrease in anorexigenic potency and the greater quantitative change in cardiovascular and central nervous system effects.

In recent years there have been several reports (Emele, Shanaman & Warren, 1961; Leonard, Fujita, Tedeschi & Fellows, 1961; Boxill, Ben, Hillyard & Warren, 1962; Holland, Buck & Weissman, 1963; Witoslowski, Campbell & Hanson, 1963) on primary ethylamines, having a halogen- or a haloalkyl-substituted phenyl group, that share common pharmacological characteristics with fenfluramine, a secondary amine. Thus it is suggested that the substituent on the amino-group in fenfluramine is not responsible for the reduction in pharmacologic potency. But evidence to the contrary is seen in the diminishing of effects by the *N*-ethylation of dexamphetamine (van der Schoot, Ariens, van Rossum & Hurkmans, 1962; Le Douarec, 1963). As with fenfluramine, the anorectic property of *N*-ethylamphetamine was the least affected; these compounds differ only in that fenfluramine contains a trifluoromethyl substituent in the phenyl ring.

The halo- or haloalkyl-aryl group does not seem vital for the alterations in pharmacologic effects. 2-Amino-1-cyclohexylpropan-1-one is reported to be useful in curbing the appetite without having pronounced affects on the central nervous system (Boehringer, 1963) and 1-phenoxyprop-2-ylamine is described as having anorexigenic activity free from side-effects (Zeile & Thoma, 1963). The consensus of these reports would be that among 1-arylprop-2-ylamines, cardiovascular or central nervous system effects, or both, can be eliminated or reduced without seriously sacrificing anorexigenic activity by an appropriate structure at the aryl end of the molecule or the proper substituent on the amino-group. It appears then, that both the haloalkyl-substituted aryl moiety and the *N*-ethyl group of fenfluramine contribute to the quantitative pharmacologic difference between this compound and dexamphetamine.

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The estimation of anthracene derivatives in senna and rhubarb

J. LEMLI

An assay is described for the estimation of total anthracene derivatives in senna, rhubarb or their preparations. The extracted glycosides are oxidised by ferric chloride in aqueous solution followed by hydrolysis in acid solution. The free anthraquinones are estimated colorimetrically.

A COMPARATIVE study on the estimation of anthracene derivatives in drugs has been made recently by Müller, Christ & Kühn (1962); seven methods were examined and, from these, a new method was recommended for the colorimetric estimation of anthraquinones formed after oxidation of the anthrones and dianthrones in alkaline medium.

All the existing methods use the same general principle of extraction and hydrolysis of glycosides, followed by extraction and purification of the resulting aglycones. The reduced aglycones are oxidised to anthraquinones in alkaline medium, in which they are then estimated colorimetrically. The extraction, hydrolysis and purification stages in this process are well established, but variations still result from the oxidation stage, the details of which must be precisely followed in order to obtain reproducible results. This oxidation is now studied and a method is recommended based on a new principle of oxidation.

OXIDATION OF SENNOSIDES BY FERRIC CHLORIDE

The starting point for the investigation was the ferric chloride oxidation of sennosides or sennidins A and B to rhein as published by Stoll, Becker & Kussmaul (1949), and recently applied by Fairbairn & Simic (1963, 1964) to the estimation of C-glycosides in aloes and cascara.

This method of oxidation of sennoside is attractive because, in theory, only rhein can be formed; whereas the oxidation of sennidins with oxygen or hydrogen peroxide in alkaline medium results in a mixture of products. This may be demonstrated by paper chromatography and ultraviolet spectrophotometry as used by Auterhoff & Sachdev (1962) who treated emodin dianthrone with N sodium hydroxide and found that emodin, hypericin (naphthodianthrone) and other substances of unknown constitution were formed.

Using this method, a chromatogram of the aglycones of senna, obtained after oxidation in alkaline solution with hydrogen peroxide, showed different spots with variable colours, and after acidifying this solution and extracting with ether, only 60% of the theoretical amount of aglycones was obtained. The ether insoluble part was an amorphous brown powder, probably composed of polymerised anthrones and hypericin-like compounds. The amount of these different substances formed during

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oxidation will depend on temperature, oxygen, light and concentration, thus explaining the difficulty of obtaining reproducible values for $E(1\%, 1\text{ cm})$ of the reaction product and the variability of the maxima of absorption reported by different authors.

If the sennidin could be split into two molecules of rhein then a constant $E(1\%, 1\text{ cm})$ value would be obtained. In an attempt to achieve this, ferric chloride and other metal salts (Hg, Cu, Cr, Ce) were tested in different concentrations (5–25%) in strong acid medium (>2 N). The sennosides were never completely split and the product contained rhein and sennidin together with other substances which gave a positive Bornträger reaction but with a much lower $E(1\%, 1\text{ cm})$ value. On a chromatogram these substances were located below the spot of rhein.

The incomplete conversion of sennoside to rhein may be explained by the fact that in a strong acid medium it is hydrolysed before it is oxidised, so forming the insoluble sennidin, which is not attacked by ferric chloride. The other substances formed are intermediates between sennidin and rhein, they are insoluble and remain at their respective oxidation steps, e.g. dehydrodianthrone (Hörhammer, Wagner & Kohler, 1959). Brockmann & Eggers (1955) have also shown that the oxidation of a dianthrone in the presence of light gives rise to the formation of a series of intermediates. It was therefore concluded that the oxidation must be effected before the hydrolysis of the glycosides in order to obtain a complete oxidation of sennosides into two molecules of rhein glucoside. The sugar moiety of the molecule also probably exerts a protective action on the anthraquinone structure.

The conversion of sennosides with ferric chloride in weak acid medium (<0·5 N) was tried. Although the results were better, the conversion was not complete. The complete oxidation of the sennoside to rhein glucoside appeared to be achieved quickly by the use of ferric chloride without addition of any acid. After detailed examination the concentration of $FeCl_3.6H_2O$ was found to be optimal between 5 and 10%. Finally a reaction mixture containing 6·6% of $FeCl_3.6H_2O$ was adopted because with higher concentrations it was difficult to redissolve the precipitated ferric hydroxide at the later stage in the process.

Fig. 1 gives the oxidation and hydrolysis curves of sennoside A by the action of 6·6% $FeCl_3.6H_2O$ in water at 100°. The rate of hydrolysis of sennoside to sennidin is slow and oxidation to rhein glucoside takes place before the hydrolysis. After 20 min of oxidation, 33·1% of the sennoside was split and hydrolysed into rhein, the remaining 66·9% being rhein glucoside, as shown by paper chromatography. The complete hydrolysis of this rhein glucoside was achieved in 0·4 N hydrochloric acid by further warming for 20 min at 100°. The end product was pure rhein as shown by the E (1%, 1 cm) value, paper chromatography and ultraviolet absorption curve.

The conversion of sennoside A to rhein by this method was found to be 95·0%. For pure, dry rhein E (1%, 1 cm) in N potassium hydroxide is 335. The conversion of sennosides to rhein gives a theoretical figure of 221 whilst with the proposed conversion procedure for dry sennoside A

a value of 210 was obtained. A 100% conversion cannot be expected because the sennosides are not pure substances. Thin-layer chromatography showed the presence of at least 3 spots in sennoside A and 5 spots in sennoside B; sennoside A was therefore used in this investigation.

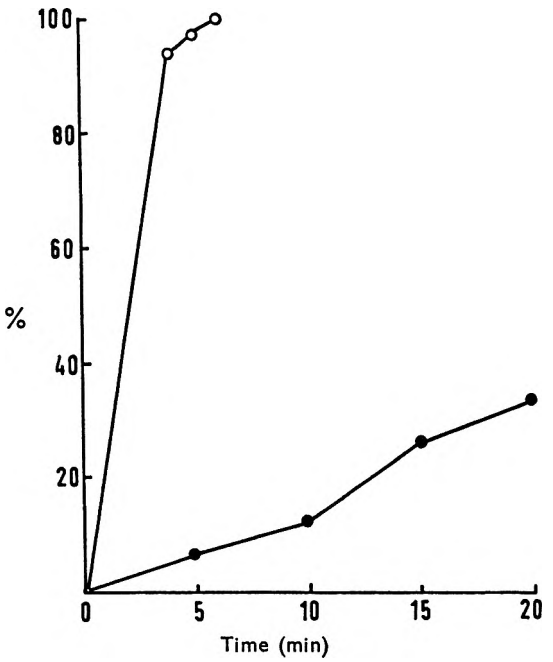

FIG. 1. Hydrolysis (●) and oxidation (○) (%) of sennoside A in water at 100° containing 6·6% $FeCl_3$ 6.H_2O.

Hence with the usual methods of oxidation the λ_{max} is variable and the E (1%, 1 cm) varies from 130 to 170. With ferric chloride in strong acid the λ_{max} is also variable with an E (1%, 1 cm) of 160–180, but using ferric chloride as described the λ_{max} is precisely 500 mμ with an E (1%, 1 cm) of 221.

APPLICATION TO SENNA AND RHUBARB

For the application of the ferric chloride oxidation method to the estimation of total anthracene derivatives in senna and rhubarb, it was necessary to test the different phases of the procedure.

Complete extraction of the glycosides from the powdered drugs can be obtained in 15 min by boiling water (Dequeker, 1962) if the drug: water ratio is no more than 1 : 200. The amount of glycosides in the oxidation step must never be more than 2 or 3 mg for each 20 ml of the 10% ferric chloride solution and exposure to light should be avoided. A reference standard was made using 5 ml (=1 mg sennoside A) of a solution of 20 mg sennoside A in 100 ml water, adding a trace of sodium bicarbonate.

During oxidation a precipitate of ferric hydroxide is formed and this must be completely redissolved by frequent shaking in the subsequent hydrolysis stage in 0·4 N hydrochloric acid over a period of 20 min. In the hydrolysis, the use of strong acids should be avoided because they react with extractive matter to produce a large quantity of amorphous material which renders the extraction of aglycones difficult. In the proposed method, no brown material is formed and the extraction of aglycones is simple, even with preparations such as syrups containing other plant extracts.

In the method the total anthracene derivatives are determined. The free forms can be removed before oxidation and hydrolysis by extraction with chloroform.

An estimation of the glycosides soluble in sodium bicarbonate has been omitted. Such an estimation has little value since I have shown the presence of the heterodianthrones sennoside C and rheidin A in the leaves and the pods of senna and the complete series of heterodianthrones in rhubarb (Lemli, 1963, 1964); several of them are only slightly soluble in sodium bicarbonate.

The percentage of glycosides is expressed as sennoside A. It may also be expressed as rhein because rhein accounts for about 80% of the total anthraquinones formed (Lemli, 1963).

For convenience, the results of the rhubarb estimation are also expressed as rhein, although different anthraquinones are present, and it has been assumed that the molecular absorptions of the different anthraquinones are approximately the same.

The chromatograms of the anthraquinones obtained when this method was applied to senna leaves and to rhubarb show only the spots of the different oxidised anthraquinone derivatives.

PROCEDURE FOR THE ASSAY OF TOTAL ANTHRACENE DERIVATIVES IN SENNA PODS AND LEAVES, RHUBARB, AND THEIR PREPARATIONS

Reagents. 10% ferric chloride solution: a solution of 10% $FeCl_3.6H_2O$ A.R. in water (w/v). Hydrochloric acid containing not less than 37% HCl. Ether. N Potassium hydroxide.

Procedure. Weigh 100 mg of the drug in moderately fine (No. 44) powder, in a 100 ml conical flask with ground joint and mix with 20 ml water. Weigh and attach a condenser and warm for 15 min in a boiling water-bath (water level must be above the liquid level in the flask). Cool, and weigh and adjust to the original weight with water. Centrifuge. Transfer 10 ml of the clear solution to a 100 ml round bottom flask with ground joint and add 20 ml of 10% aqueous solution of ferric chloride with mixing. Attach a condenser, immerse the flask to the neck in a boiling water-bath and avoiding direct illumination of the flask, warm for 20 min. Add 1 ml hydrochloric acid, mix thoroughly and continue warming for another 20 min, shaking frequently to dissolve the precipitated ferric hydroxide completely. After cooling, transfer quantitatively to a separating funnel and extract with three portions each of 25 ml ether,

rinsing the flask with each portion of ether. Wash the combined ether layers with 25 ml water. Transfer the ether to a 100 ml volumetric flask and make up to volume with ether. Evaporate 10 ml of this ether solution in a conical flask and dissolve the residue in 10 ml N potassium hydroxide, avoiding direct light. Measure the absorption of this solution at 500 mμ in a 1 cm cell, against N potassium hydroxide. Using the value E (1%, 1 cm) sennoside A = 221, or E (1%, 1 cm) rhein = 335 calculate, as equivalent of sennoside A or rhein, the percentage of total anthracene derivatives present in senna pods or leaves or their preparations. For rhubarb and preparations, calculate the percentage of total anthracene derivatives present as an equivalent of rhein.

When examining a tincture, extract or other fluid galenical preparation, the extraction step is omitted and the assay begun by mixing the preparation directly with water and ferric chloride solution.

Experimental

PAPER CHROMATOGRAPHY

The method for the separation of the anthraquinones was that described by Kinget (1963). Spots were made visible with 5% potassium hydroxide in 50% ethanol with warming at 100°.

OXIDATION AND HYDROLYSIS OF SENNOSIDE A BY FERRIC CHLORIDE

The oxidation curve was obtained as follows. To 20 ml of a solution containing 4 mg sennoside A in water was added 14 ml water and 66 ml of a 10% solution of $FeCl_3.6H_2O$. The mixture was warmed in a boiling water-bath and after 4, 5 and 6 min a sample of 20 ml was removed. To each sample was added 4 g Na_2HPO_4 (in 40 ml water) and the precipitate filtered off. The filtrate was then acidified and extracted with ether, which now contained only rhein as shown by paper chromatography. To the acidified filtrate, containing the remaining glycosides, was added sufficient sulphuric acid to obtain a 5 N solution and the glycosides were hydrolysed by heating in a boiling water-bath for 15 min. The resulting aglycones were extracted and the quantity of unoxidised sennidin determined by densitometry (Lemli, 1963). The sennidin concentrations after 4, 5 and 6 min were respectively 5·4, 3·2 and 0%.

The hydrolysis curve was established in the following manner. A mixture as described above was warmed in a boiling water-bath and 20 ml samples were taken after 5, 10, 15 and 20 min. The samples were cooled and extracted with ether to remove the liberated aglycones. Paper chromatography showed that the aglycone formed was rhein. After extraction with ether the solutions were further warmed for 20 min for complete oxidation and for another 20 min after addition of concentrated hydrochloric acid for complete hydrolysis. The aglycones were extracted and determined by colorimetry. The percentages of unhydrolysed sennoside after 5, 10, 15 and 20 min were respectively 93·1, 88·2, 73·5 and 66·9%. These results are represented graphically in Fig. 1.

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Comparison of sleeping time, immobility time and arousal time in mice

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The sodium salts of the five barbiturate hypnotics hexobarbitone, pentobarbitone amylobarbitone, secobarbitone and phenobarbitone, were studied in mice for their effect on duration of sleeping time, immobility time and arousal time. The latter measurement is described herein for the first time and is a measure of the duration of sleep with a moderate shocking stimulus to differentiate sedation and hypnosis in the mouse. The comparative potency of the drugs by these measurements is evaluated.

A standard method of measuring the depressant action of barbiturates in small animals is by the determination of what is usually, if unphysiologically called "sleeping time". This may be defined as the time from which the animal cannot right itself to that time when a righting reflex has returned. With a drug such as ethanol, this is not a useful procedure because the end-point is indistinct. The animals exhibit excitatory movements such as twitching and rolling with alternate appearance and disappearance of the righting reflex (Forney, Hulpieu & Hughes, 1962).

The present report consists of a comparison of "sleeping time," "immobility time" and "arousal time" after treatment with barbiturates. We have described immobility time as the interval between the loss of righting reflex and the time when an animal regains normal exploratory movements (Forney, Hulpieu & Hughes, 1962).

Sleeping time, in mice, comprises central nervous depression ranging from anaesthesia to hypnosis with a lingering sedative effect. An electrical stimulus might hasten the arousal of an animal from drug-induced sleep if it were strong enough to antagonise sedation. If such a current were applied continuously, the true hypnotic action of the drug might then be measured in time. Arousal time would be determined similarly to sleeping time except that the animals would be subjected to a continuous stimulating electric current. The technique for the measurement of this arousal time is described herein for the first time.

Experimental

Male Swiss albino mice, 20–30 g, were used. The five representative barbiturates selected have actions varying in duration from very short, intermediate to long; they were hexobarbitone sodium, 100 mg/kg; pentobarbitone sodium, 45 mg/kg; secobarbitone sodium, 60 mg/kg; amylobarbitone sodium, 100 mg/kg and phenobarbitone sodium, 175 mg/kg. Preliminary experiments with multiple dosages showed that, at the dosages selected, all mice slept, even though shocking stimuli were

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applied. The drugs were all administered intraperitoneally in aqueous solutions. The concentrations of the solutions were prepared so that each animal received 0·5 ml/20 g weight. Sleeping time was calculated as the time from the loss of the righting reflex to the return of the righting reflex (Brodie, Shore, & Silver, 1955; Kopmann & Hughes, 1958). Immobility time was calculated from the time of non-movement after injection to the time when the mouse was able to move beyond the border of a 6-inch diameter circle (Forney & others, 1962). The third measurement was "arousal time." For this, the "sleeping" mice were placed on a gridded platform on absorbent paper saturated with 0·85% saline. The absorbent papers were kept moistened throughout the experiment to insure maximum conduction. An electric current, supplied by a variable transformer having an input of 125V at 60 cycles and an output of 10V rated at 1·25 A, was introduced into the grid system. The arousal time was calculated, like sleeping time, as time elapsing from the loss of righting reflex to its return. Preliminary testing indicated that the stimulus provided by this circuit was the optimum necessary to arouse a mouse from a state of light sleep or near wakefulness.

Results and discussion

Table 1 gives the comparative effects of the five barbiturates on the sleeping, arousal and immobility times of the mice. The order of increasing duration of sleeping time was hexobarbitone, pentobarbitone, amylobarbitone, secobarbitone and phenobarbitone. These mean durations were reduced with a low-voltage current after all drugs. The greatest decrease in duration of sleep brought about by electrical stimulus was noted after pentobarbitone>hexobarbitone>amylobarbitone>phenobarbitone>secobarbitone. This could be a measurement of depth of sleep in mice, an increase in depth causing a decrease in the percentage reduction of sleeping time brought about by continuous stimulation. Arousal time therefore may be a measure of the true duration of hypnosis in animals.

TABLE 1. MEASUREMENT OF THE DEPRESSANT ACTION OF BARBITURATES IN MICE AS AROUSAL TIME, SLEEPING TIME, AND IMMOBILITY TIME*

Drug	Dose mg/kg	Arousal time (min)	Sleeping time (min)	Immobility time (min)
Hexobarbitone sodium ..	100	22 ± 1·8	30 ± 3·9	57 ± 2·1
Pentobarbitone sodium ..	45	34 ± 2·2	57 ± 5·7	75 ± 4·7
Amylobarbitone sodium ..	100	54 ± 5·6	66 ± 11·9	71 ± 12·2
Secobarbitone sodium ..	60	92 ± 7·6	98 ± 13·7	101 ± 13·7
Phenobarbitone sodium ..	175	135 ± 16·3	149 ± 21·2	172 ± 20·5

* Mean values of 10 mice and s.e. of mean (drugs administered i.p.).

A post-hypnotic sedative action of the drugs extends the immobility time beyond the arousal time. The order of duration of immobility time (prolonged sedation) was phenobarbitone>secobarbitone>pentobarbitone>amylobarbitone>hexobarbitone. The duration of arousal

and sleeping time after amylobarbitone was greater than that of pentobarbitone. However, as measured by immobility time, pentobarbitone had the longer duration of action (more sedative?).

If the arousal time (true hypnotic drug action) is subtracted from the immobility time, the order of post-hypnotic depression is obtained. This was found to be pentobarbitone>phenobarbitone>hexobarbitone> amylobarbitone>secobarbitone.

The techniques described should be useful in the evaluation of the hypnotic and post-hypnotic properties of barbiturates and other depressants such as ethanol. Additionally, their potentiation by drugs of the same class or of different classes, e.g., tranquillising drugs, could be quantified.

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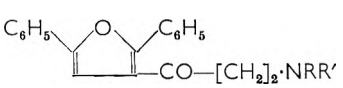
Note on derivatives of 2,5-diphenylfuran

P. M. G. BAVIN

Aminoalkyl derivatives of 2,5-diphenylfuran have been prepared and shown to be general central nervous system depressants.

SEVERAL derivatives of 2,5-diphenylfuran have been prepared and examined for tranquillising action on the central nervous system. Both groups of compounds, (I) and (II), summarised in the Table, were general central nervous system depressants and did not warrant further examination.



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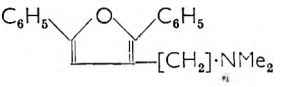

II

The obvious route to *NN*-dimethyl[3-(2,5-diphenylfur-3-yl)propyl]-amine (IIc) was thwarted by extensive decomposition which accompanied attempted reduction of the Mannich bases (I) by a variety of reagents (sodium borohydride, aluminium lithium hydride, catalytic hydrogenation, hydrazine) (cf. Williams & Day, 1952). For the preparation of the tertiary amines (II), 3-bromo-2,5-diphenylfuran was a suitable intermediate. Of several brominating agents examined, only *N*-bromosuccinimide gave, with 2,5-diphenylfuran, a product free from substitution in the phenyl groups. The bromide was converted via the nitrile to 2,5-diphenylfur-3-oic acid, identical with the product of a Kröhnke degradation (Arnold, Murai & Dodson, 1950) of the acetyl compound, 3-acetyl-2,5-diphenylfuran. The dimethylaminomethyl compound (IIa) was readily obtained by reduction of the corresponding dimethylamide. The homologues (IIb) and (IIc) were very conveniently prepared by reaction between 2,5-diphenylfur-3-ylmagnesium bromide and the appropriate ω-dimethylaminoalkyl chloride. Although the yields were low, the products were easily isolated in a high state of purity. This method has been used extensively in these laboratories (Ganellin & Ridley, 1964) and appears to be widely applicable.

Experimental

3-Bromo-2,5-diphenylfuran. 2,5-Diphenylfuran (44 g, 0·2 mol) and *N*-bromosuccinimide (39·2 g, 0·22 mol) were boiled under reflux for 2 hr with carbon tetrachloride (200 ml) and concentrated hydrobromic acid (4 drops). The mixture was chilled to complete crystallisation of the succinimide and filtered. The filtrate was washed with sodium bicarbonate solution, dried ($MgSO_4$), and evaporated to leave a yellow solid, crystallisation of which from ethanol gave pale yellow needles (49·8 g, 83%), m.p. 65–66°. Found: C, 64·4; H, 3·6. Calc. for $C_{16}H_{11}BrO$: C,

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64·2; H, 3·7%. Although this material ran as a single spot on thin-layer chromatograms and was suitable for the preparation of the Grignard reagent, chromatography from hexane on alumina gave colourless needles, m.p. 79–80° (Lutz & Reese, 1959, record m.p. 77–78° but other authors have noted the range observed by us).

3-*Cyano*-2,5-*diphenylfuran.* A mixture of the bromodiphenylfuran (180 g) and cuprous cyanide (60 g) was boiled under reflux for 2 hr with dimethylformamide (180 ml) and pyridine (4 drops). The mixture was cooled, poured into excess aqueous ammonia (d 0·88) and extracted with carbon tetrachloride. Evaporation of the dried (K_2CO_3) extract left a yellow solid which crystallized as white needles (88·3 g) from acetone, m.p. 115–116°. Found: C, 83·1; H, 4·5; N, 6·0. Calc. for $C_{17}H_{11}NO$: C, 83·2; H, 4·5; N, 5·7%.

TABLE 1. DERIVATIVES OF 2,5-DIPHENYLFURAN

	LD50[a]	M.p. of hydrochloride[b]	Formula of hydrochloride	Calculated				Found			
				C	H	N	Cl′	C	H	N	Cl′
a, R = R′ = Me	375	189–190°[c]									
b, R,R′ = 	450	210–212°	$C_{24}H_{26}Cl_2N_2O_2$				15·85				15·52 15·46
c, R,R′ =	1000	191–192°[d]	$C_{23}H_{24}ClNO_3$	69·42	6·08	3·52	8·91	69·15	6·01	3·46	8·80
Ia, *n* = 1	180	225–225·5°	$C_{19}H_{20}ClNO$	72·71	6·42	4·46	11·30	72·41	6·27	4·29	11·34
Ib, *n* = 2	50	212–213°	$C_{20}H_{22}ClNO$	73·27	6·77			72·84 72·81	6·77 6·94		
Ic, *n* = 3	500	135–136°	$C_{21}H_{24}ClNO$	73·80	7·08	4·09	10·29	73·28 73·19	7·23 7·40	3·82	10·29

a mg/kg, i.p. in male albino mice, mortalities being recorded for 7 days.
b corrected.
c Lutz & Rowlett (1948).
d Free base, m.p. 101·5°, Lutz & Rowlett (1948).

2,5-*Diphenylfur*-3-*oic acid.* Hydrolysis of the nitrile with potassium hydroxide in boiling diethylene glycol gave 2,5-diphenylfur-3-oic acid, m.p. 223–224° after crystallisation from ethanol, identical with the Kröhnke degradation product of 3-acetyl-2,5-diphenylfuran (Lutz & Rowlett (1948) record m.p. 217°).

NN-*Dimethyl*-2,5-*diphenylfur*-3-*amide.* The dimethylamide, prepared from the foregoing acid via the acid chloride in the usual way, crystallised from benzene-hexane as pale yellow needles, m.p. 88·5–89·0°. Found: C, 78·15; H, 5·9; N, 4·7. Calc. for $C_{19}H_{17}NO_2$: C, 78·3; H, 5·9; N, 4·8%.

NN-*Dimethyl*(2,5-*diphenylfur*-3-*ylmethyl*)*amine.* Reduction of the dimethylamide by extraction from a Soxhlet thimble with ethereal aluminium lithium hydride gave the dimethylaminomethyl compound (IIa).

NN-*Dimethyl*[2-(2,5-*diphenylfur*-3-*yl*)*ethyl*]*amine hydrochloride.* The bromodiphenylfuran (30 g) was converted to the Grignard reagent by warming and stirring with magnesium (3 g) in anhydrous benzene-ether

(1 : 1, 150 ml), the reaction being complete in approximately 15 min. A solution of 2-dimethylaminoethyl chloride (from 30 g of the hydrochloride) in toluene was added and the mixture stirred and warmed on the steam-bath for 3 hr. The mixture was poured onto ice and excess hydrochloric acid and extracted with benzene-hexane. The aqueous layer was chilled, basified with 40% aqueous sodium hydroxide solution and extracted several times with ether. The dried ($MgSO_4$) extracts were evaporated to leave an oil (IIb), the hydrochloride of which crystallised from isopropanol-ether as white needles (6·9 g), m.p. 212–213°. The dimethylaminopropyl compound was prepared similarly.

3-(2,5-*Diphenylfur*-3-*oyl*)*propionitrile.* The methiodide from (Ia) (m.p. 189–190° decomp., 12·2 g) was stirred and heated on a steam-bath for 3 hr with potassium cyanide (12·2 g) and dimethyl sulphoxide (150 ml). The mixture was cooled, diluted with water and extracted several times with ether. Evaporation of the extracts left an oil which was purified by chromatography (hexane-alumina). The pure cyano-ketone crystallised as pale yellow needles from methanol (4·1 g.) m.p. 98–99°. Found: C, 79·6; H, 5·3; N, 4·65. Calc. for $C_{20}H_{15}NO_2$; C, 79·7; H, 5·0; N, 4·65%.

The cyano-ketone decomposed during attempted reduction with ethereal aluminium lithium hydride.

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A comparison of anthelmintic and antibacterial activity of some phloroglucinol derivatives

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Some simple phloroglucinol derivatives previously reported to have anthelmintic properties have been tested for antibacterial action against *Staphylococcus aureus* and *Streptococcus pyogenes*. A comparison is made of the activities of these compounds against *Staph. aureus* and *Hymenolepis nana*.

WE report the antibacterial properties of a series of phloroglucinol compounds previously shown by Bowden, Broadbent & Ross (1965) to have anthelmintic action.

Experimental

PREPARATION OF COMPOUNDS

The synthesis of the phloroglucinol compounds was reported in the paper of Bowden, Broadbent & Ross (1965).

ANTIBACTERIAL TESTING

The compounds were tested for antibacterial activity at Smith Kline and French Laboratories, Welwyn Garden City. We thank Mr. S. G. E. Stevens for permission to publish these results and Mr. B. M. Jones for a description of the method, which is as follows.

A 10% solution or suspension of the substance under test in acetone was diluted with sterile nutrient broth (Oxoid CMI) to give concentrations of 0·01, 0·005 and 0·001%. Samples of each dilution were used in the test; uninoculated controls and nutrient broth blanks were also set up.

Each set of test dilutions was inoculated with 0·05 ml of 24 hr broth cultures of the test organisms and incubated at 37°. The samples were examined at 24 and 48 hr for growth compared with the controls. Samples found to inhibit growth in 48 hr at a concentration of 0·001% were tested at further dilutions, with incubation at 37° for 24 hr, until the minimum inhibitory concentration was reached.

ANTHELMINTIC TESTING

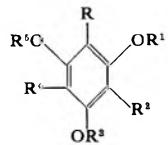
The method of testing for *in vitro* activity against *Hymenolepis nana* was that described by Sen & Hawking (1960). The *in vivo* activity against *H. nana* in mice was determined by the method of Steward (1955) and was based on the effect of a single dose of 400 mg of the substance given orally per kg body weight. The results have been given in detail by Bowden, Broadbent & Ross (1965).

Discussion

Sundman & Sundman (1961) examined the antibacterial properties of a series of phloroglucinol anthelmintics and found some relationship between activity against *Staphylococcus aureus in vitro* and anthelmintic

From the Smith Kline and French Research Institute, Welwyn Garden City, Herts.

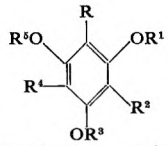
TABLE 1. ANTIBACTERIAL ACTIVITY OF COMPOUNDS

Compounds of the Type 

SK & F No.	R	R¹	R²	R³	R⁴	R⁵	Minimum inhibitory percentage concentration *in vitro* against *Staph. aureus*	Minimum inhibitory percentage concentration *in vitro* against *Str. pyrogenes*
90,525	H	H	Me·CO	H	Me·CO	H	0·0005	0·001
90,533	Me·CO	H	Me·CO	H	Me·CO	H	0·01	>0·01
90,536	Me	H	Me·CO	H	Me·CO	H	0·01	0·0005
90,540	H	H	Pr·CO	H	H	Me	0·005	>0·01
90,547	H	Me	Me·CO	Me	Me·CO	Me	>0·01	0·0005
90,562	H	H	Pr·CO	H	Pr·CO	H	0·00005	0·00005
90,567	H	H	Et·CO	H	Et·CO	H	<0·00001	0·00005
90,569	H	H	Me_2CH·CO	H	Me_2CH·CO	H	0·00005	0·00005
90,578	Me	H	Et·CO	H	Et·CO	H	0·01	0·005
90,589	Me	H	Me_2·CH·CO	H	Me_2·CH·CO	H	0·01	0·00001
90,590	H	H	Bu·CO	H	Bu·CO	H	0·00005	0·00001
90,592	Me	H	Bu·CO	H	Bu·CO	H	0·005	0·00005
90,599	H	H	Pr·CO	H	Me·CO	H	0·0005	0·0001
90,616	H	H	C_5H_{11}·CO	H	C_5H_{11}·CO	H	0·001	0·005
90,617	H	H	Et·CO	H	Me·CO	H	0·005	0·005
90,620	H	H	C_9H_{19}·CO	H	C_9H_{19}·CO	H	0·001	0·001
90,621	H	H	Me_2·CH·$(CH_2)_2$·CO	H	Me_2·CH·$(CH_2)_2$·CO	H	0·001	0·001

TABLE 1—*continued*

Compounds of the Type



SK & F No.	R	R^1	R^3	R_3	R^4	R^5	Minimum inhibitory percentage concentration *in vitro* against *Staph. aureus*	Minimum inhibitory percentage concentration *in vitro* against *Str. pyrogenes*
90,625	Me	H	C_5H_{11}·CO	H	C_5H_{11}·CO	H	0·01	0·01
90,629	Me	H	C_6H_{13}·CO	H	C_6H_{13}·CO	H	0·01	0·01
90,642	H	H	Ph·CH_2·CO	H	Ph·CH_2·CO	H	0·001	0·0001
90,644	H	H	Bu·CO	H	Me·CO	H	0·005	0·005
90,648	H	H	C_8H_{17}·CO	H	C_8H_{17}·CO	H	0·001	0·005
90,649	Me	H	Me_2·CH·CH_2·CO	H	Me_2·CH·CH_2·CO	H	0·0001	0·00005
90,651	H	Me·CO	Me·CO	Me·CO	Me·CO	Me·CO	0·00001	0·005
90,655	Me	H	C_7H_{15}·CO	H	C_7H_{15}·CO	H	>0·01	0·005
90,656	H	H	C_7H_{15}·CO	H	C_7H_{15}·CO	H	0·005	>0·01
90,657	H	H	Me_2·CH·CH_2·CO	H	Me_2·CH·CH_2·CO	H	0·0001	0·00005
90,681	H	H	Me_2·CH·$(CH_2)_2$·CO	H	H	H	0·005	0·005
90,717	Br	H	Pr·CO	H	Pr·CO	H	0·0001	0·0001

TABLE 2. COMPARISON OF *in vitro* ANTIBACTERIAL ACTIVITY WITH *in vitro* AND *in vivo* ANTHELMINTIC ACTIVITY

SK & F No.	Minimum inhibitory % concentration *in vitro* against *Staph. aureus*	Minimum inhibitory % concentration *in vitro* against *H. nana*	Activity % against *H. nana* in mice
90,547	>0·01	>0·01	0
90,589	,,	0·0002	100
90,625	,,	0·00001	98
90,655	,,	0·00002	94
90,533	0·01	0·01	0
90,536	,,	0·001	61
90,578	,,	0·0002	50
90,629	,,	0·00001	99
90,540	0·005	0·0002	0
90,592	,,	0·00001	99
90,617	,,	0·0002	14
90,644	,,	0·0001	45
90,656	,,	0·0001	92
90,681	,,	0·01	0
90,616	0·001	0·000005	100
90·620	,,	0·00001	74
90,621	,,	0·000005	99
90,642	,,	0·0001	13
90,648	,,	0·0001	90
90·525	,,	0·0002	0
90,599	,,	0·0001	87
90,649	0·0001	0·001	90
90,657	,,	0·00002	83
90,717	,,	0·0002	40
90,562	0·00005	0·0001	89
90,569	,,	0·0001	62
90,590	,,	0·000005	99
90,651	0·00001	0·002	32

activity. They suggested the antibacterial test might be a useful tool in the search for anthelmintic drugs.

In our series of phloroglucinol compounds, comparison of the *in vitro* activities of our phloroglucinol compounds against *Staph. aureus* with the *in vitro* and *in vivo* activities against *H. nana* (Table 2) shows that although antibacterial activity is often accompanied by high *in vitro* anthelmintic activity, this is not always so. An example of this is compound SK & F 90,681. When comparison is made between antibacterial and *in vivo* anthelmintic activity, more exceptions to the suggested general rule appear, for example, SK & F 90,540, 90,642, 90,525 and 90,651. In view of the possible fates of the substances in the host animal it is not surprising that this should be so. The retention of a few compounds with low anthelmintic activity by the antibacterial screening would not be a serious objection to the method but the reverse is not true. If activity against *Staph. aureus* had been used as a screen for potential anthelmintics in the series under consideration, some highly-active anthelmintics, for example SK & F 90,589, 90,625 and 90,655 would have been missed.

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Letters to the Editor

Inhibition of ^{3}H-metaraminol uptake by antidepressive and related agents

SIR,—In a previous paper we have reported a dual amine uptake mechanism of the adrenergic nerve (Carlsson & Waldeck, 1965). As an indicator of amine uptake ^{3}H-metaraminol was used. It was found that reserpine, a drug acting on the granular storage mechanism, did not inhibit the initial uptake of ^{3}H-metaraminol but greatly increased its rate of disappearance. In contrast, desipramine, a drug acting on the amine transport mechanism of the axon membrane of the adrenergic nerve, almost completely inhibited the neuronal uptake of ^{3}H-metaraminol. We have now extended our investigation to a number of other antidepressive and related agents.

Usually, 10 mg/kg of the test substances was given intravenously to mice 5 min before the intravenous administration of 0·02 mg/kg ^{3}H-metaraminol and the animals were killed 30 min later. ^{3}H-metaraminol in the heart was estimated as described earlier (Carlsson & Waldeck, 1965). The results are presented in Table 1.

TABLE 1. INHIBITION OF ^{3}H-METARAMINOL UPTAKE BY ANTIDEPRESSIVE AND RELATED AGENTS IN THE HEARTS OF MICE

Group	Compound tested	Inhibition of ^{3}H-metaraminol uptake as % of control
I	Protriptyline[1]	9
	Desipramine	9
	Ro 4-6011[2]	16
	Nortriptyline	22
	Imipramine	25
	Dibenzepin (Noveril)[3]	30
	Amitriptyline	32
II	Guanethidine	35
	Chlorpromazine	42
	Amphetamine (5 mg/kg)	46
	Cocaine	46
	Bretylium	53
	BW392C60[4] (5 mg/kg)	54
	Ph 879/4-07155[5]	57
III	Reserpine (i.p. 6 hr before)	78
	Prenylamine	62
	Prenylamine (30 min before)	111
	Trimiprimine	90
	Haloperidol	119
	Promethazine	98
	Prochlorperazine	105
	Phenoxybenzamine	110
	Azapetine	116
	Atropine	125
	Ouabain (2 mg/kg)	114
	Controls (Mean of 21 experiments = 46·3 ng/g tissue)	100 ± 14 s.d.

In general the values are the mean of two determinations.

[1] 5-(3-Methylaminopropyl)-5*H*-dibenzo[*a*,*d*]cycloheptene.
[2] 5-(3-Methylamino propylidene)-5*H*-dibenzo[*a*, *d*]cycloheptene HCl.
[3] 10-(2-Dimethylaminoethyl)-10,11-dihydro-5-methyl-11-oxo-5-dibenzo[*b*,*e*][1,4]-diazepine HCl.
[4] *N*-*o*-Chlorobenzyl-*N'N''*-dimethylguanidine.
[5] 7-Amino-5,6,8,9-tetrahydro-7*H*-benzocycloheptene.

The drugs have been divided into 3 groups according to potency. Compounds listed in group I belong to the thymoleptic series of drugs. All proved to be potent inhibitors of the cell membrane transport mechanism. This is in agreement with the finding that they potentiate the effects of noradrenaline (Sigg, 1959; Haefely, Hürlimann & Thoenen, 1964; Stone, Porter, Stavorski, Ludden & Totaro, 1964): when the neuronal uptake is inhibited more noradrenaline will reach the receptors.

Group II includes drugs with moderate activity. Some of them have been shown earlier to inhibit the uptake of noradrenaline in the heart of the rat (Axelrod, Hertting & Potter, 1962). Most of them have other, probably more important, effects.

The compounds belonging to group III showed little or no effect in this test. Some of the drugs, like reserpine, prenylamine (Segontin), phenoxybenzamine and azapetine, have previously been shown to inhibit the uptake of catecholamines by the granules of the adrenal medulla (Carlsson, Hillarp & Waldeck, 1963). Using the perfused rat heart, Iversen (1965) found reserpine and phenoxybenzamine to inhibit the uptake of ^{14}C-noradrenaline. His technique, however, does not distinguish between inhibition of uptake at the cell membrane level and inhibition of the granular storage mechanism. After the latter type of inhibition, noradrenaline can still be transported into the intracellular space but will, in contrast to metaraminol, be destroyed by the action of monoamine oxidase, and thus little or no accumulation can occur (cf. Hillarp & Malmfors, 1964; Lindmar & Muscholl, 1964).

It is interesting to note that methylation of imipramine on the second side-chain carbon—resulting in trimiprimine (Surmontil)—leads to much reduction of activity. Trimiprimine is described as a thymoleptic with strong sedative properties.

Acknowledgements. This work was supported by grants from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service (NB 04359-02) and the Swedish State Medical Research Council. The skilful technical assistance of Mrs. Inger Börjesson and Miss Ingrid Weigner is gratefully acknowledged.

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A quantitative measure of qualitative changes in blood flow

SIR,—I have been attempting to make quantitative measurements of changes in the blood flow of the cat through the hind limb caused by intravenously and intra-arterial administered vasoactive substances. The design of the experiments required answers which represented the absolute changes in flow, but it was found that qualitative differences in the responses, and the different resting flow rates observed from animal to animal, invalidated the calculations of changes in flow by the usual method. This problem may be overlooked when making measurements from actual blood flow records. Thus, Fig. 1 illustrates the records obtained of venous outflow from the hind limbs of three different cats measured with a Thorp impulse counter, in response to the same dose of vasodilator drug X.

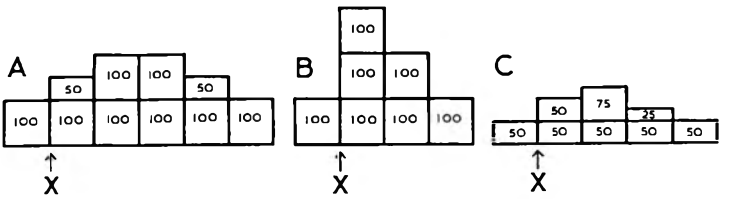

FIG. 1. Diagram of the femoral venous blood flow records obtained from three cats in response to the same dose of vasodilator drug X. The distance between consecutive vertical lines represent time intervals of 1 min and the figures indicate the number of drops flowing through the recorder. In cat A, for example, the resting flow rate is 100 drops/min and a total of 700 drops are recorded during the 4 min response to X.

In cats A and B the resting flow rate is 100 drops/min compared with a rate of 50 drops/min in cat C. An examination of the responses shows that the increased volume of blood which flowed through the drop recorder in response to X was the same in cats A and B, and that this volume was twice that measured in cat C. But, if the increase in blood flow is calculated by a usual equation in which the original flow is subtracted from the new flow and divided by the original flow, and this figure is multiplied by 100, the figures obtained for cats A, B and C are 75%, 150% and 100% respectively. The same answers are obtained by making the same calculations but using flow per unit of time; they therefore express the increases in flow as rates of flow.

It seems valid to express the vasodilation produced as the volume of blood flowing through the drop recorder in excess of the resting volume per unit of time.

$$\text{Thus, change in flow} = \frac{\text{new flow} - \text{old flow}}{\text{old flow/unit time}}$$

It might be thought that the unit of time should be that of the longest response obtained in any one series of experiments, but this has the disadvantages that all experiments must be completed before results can be calculated and that atypically long responses introduce complications. In practice, a unit of 1 min works well. Applying this equation to Fig. 1 the change in blood flow in each case is 3. This means that the extra volume of blood returning from the limb during each response is three times the amount of blood returning from the limb during 1 min before the response.

The calculation is equally applicable to decreases in blood flow or to dual responses of vasodilatation and vasoconstriction.

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The importance of bradykinin in anaphylactic shock

SIR,—Brocklehurst & Lahiri (1962) showed that during anaphylaxis in the rat, detectable amounts of bradykinin were present in the blood and they suggested that it may contribute to the anaphylactic syndrome. As 5-hydroxytryptamine and histamine do not appear to be important mediators in anaphylactic shock in the rat (Sanyal & West, 1958), the toxicity of bradykinin was examined in male Wistar albino rats after various treatments.

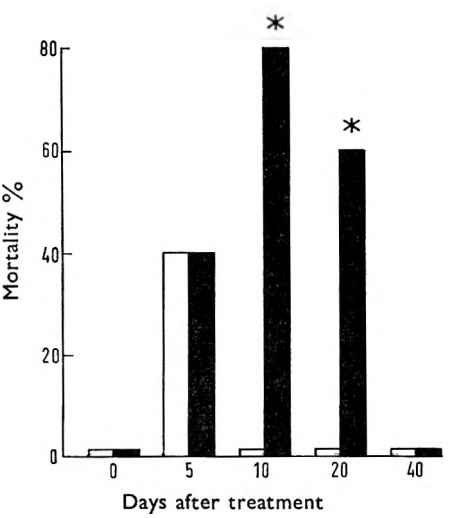

FIG. 1. Intravenous toxicity of bradykinin (2 mg/kg) in rats at varying times after treatment with *B. pertussis* vaccine and horse serum (solid columns) or *B. pertussis* vaccine (open columns). The asterisks denote the times when the specific antigen produces 100% mortality.

Groups of 5 rats weighing 200 g were used after sensitisation to horse serum. As an adjuvant (*Bordetella pertussis* vaccine) was necessary for full sensitisation, other groups were used after treatment with adjuvant only. The intravenous toxicity of bradykinin (2 mg/kg) was then measured, for which mortality rates are plotted in Fig. 1. Sensitivity to the polypeptide reached high values 10 and 20 days after sensitisation to antigen, the cause of death being characteristic haemorrhage in the jejunum and right ventricle. Similar lesions in anaphylactic shock have already been reported to be maximal at these times (Sanyal & West, 1958). In the group of rats injected previously with adjuvant only, bradykinin was not lethal at these times, although 5 days after treatment the

mortality rate was similar in both groups (see Fig. 1). Anaphylactic shock is not lethal 5 days after sensitisation so that the toxic effect of bradykinin in both groups at this time is probably due to an action of the adjuvant itself. Forty days after treatment (when anaphylactic shock is minimal), bradykinin was not toxic to either group. Konzett (1962) has already reported that the polypeptide was well tolerated by non-sensitised rats in doses of up to 10 mg/kg.

As the jejunum and heart are the tissues most damaged in both bradykinin shock and anaphylactic shock, the evidence suggests that bradykinin plays an important role in anaphylaxis in the rat.

Thanks are due to Sandoz Products Ltd. for the supply of bradykinin. W. D. is an M.R.C. Scholar.

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Modification of anaphylactic shock by mepyramine and ethanolamine

SIR,—It is evident that under different experimental circumstances the modification of anaphylactic shock by mepyramine and ethanolamine gives different answers. Herxheimer & Streseman (1965) found that ethanolamine did not improve the protection afforded by mepyramine to guinea-pigs exposed to an aerosol of antigen solution, whereas in this laboratory ethanolamine substantially improves the protective effect of mepyramine under these circumstances as originally reported by Smith (1961). It has recently been reported by Dawson, Hemsworth, & Stockham (1965) that the sensitivity of guinea-pig ileum to histamine can be influenced by dietary ascorbic acid. Since all guinea-pigs used in these laboratories receive approximately 50 mg of ascorbic acid per day in their drinking water, the discrepancy between my own findings and those of Herxheimer & Streseman might be due to this. Ascorbic acid is known to influence the metabolism and methyl donating capacity of folic acid, and the possibility that ethanolamine is dependent for its anti-anaphylactic activity upon *N*-methylation *in vivo* followed by incorporation into glycerophosphatide has been the subject of experimental investigation here for some time.

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Inhibition of noradrenaline synthesis and the pressor response to tyramine

SIR,—The hypothesis of Burn & Rand (1958), that tyramine affects the liberation of noradrenaline from sympathetic nerve endings, has received experimental confirmation from a number of laboratories. Reduction of noradrenaline stores may not parallel the reduction in response to tyramine (Bhagat, Kopin, Gordon & Booker, 1964; Bhagat, Gordon & Kopin, 1955) and the concept of an "available" or a "tyramine releasable" store of noradrenaline has been presented by a number of investigators (Trendelenburg, 1961; Kopin & Gordon, 1962; Bhagat, 1964). In atria from reserpinised guinea-pigs only 1% of the noradrenaline in the stores is required to restore about 70% of the response to tyramine (Crout, Muskus & Trendelenburg, 1962).

This letter reports the effect of an inhibition of synthesis by a tyrosine hydroxylase inhibitor on the pressor response to tyramine.

Experiments were made on mongrel dogs weighing 8–12 kg which were either anaesthetised with chloralose (80–100 mg/kg i.v.) or were spinalised. Blood pressure was recorded from the femoral artery by a mercury manometer. All drugs were injected into a cannula inserted into the femoral vein and were flushed in with 1 ml of 0·9% saline; α-methyltyrosine (60 mg/kg i.v.), a potent inhibitor of tyrosine hydroxylase (Spector, Sjoerdsma & Udenfriend, 1964), was used to block the synthesis of noradrenaline. Catecholamines were determined by the trihydroxyindole fluorometric assay of Shore & Olin (1958).

It was found that α-methyltyrosine reduced the vasopressor response to occlusion of the carotid arteries as well as to tyramine. If the inhibition of the pressor response to tyramine produced by acute administration of α-methyltyrosine were due to this compound's depleting effect on tissue stores of noradrenaline, myocardial catecholamine should be reduced. But, in a separate group of animals treated with α-methyltyrosine alone, the catecholamine concentrations in ventricles were not altered significantly. The mean value (±s.e.) for these hearts was 1·12 (0·04) μg/g. The comparable value obtained for a group of 3 untreated control dogs was 1·07 (0·05) μg/g.

Since there were no alterations in the catecholamine levels, sensitivity of the receptors to exogenous noradrenaline was tested in 4 spinalised dogs before and after the administration of α-methyltyrosine. It was observed that there was a shift of the dose-response to the right. Perhaps reduced sensitivity to noradrenaline may explain the inhibition of the response to tyramine. Tyramine may be releasing the same amount of noradrenaline from its storage site(s) in the postganglionic sympathetic nerve endings, but the amount released would be acting on the affector cells at the sites involved, the sensitivity of which to noradrenaline is reduced.

Whether the reduced sensitivity to noradrenaline is a result of blockade in synthesis or is caused by a direct action of α-methyltyrosine on the adrenergic receptors has yet to be shown.

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Effect of γ-aminobutyric acid upon brucine convulsions

SIR,—γ-aminobutyric acid (GABA) when applied to the surface of the cerebral cortex of certain mammalian species has been shown to protect the animals from electrically or chemically induced seizures (Purpura & Grundfest, 1956; Purpura, Girado & Grundfest, 1957). Furthermore it has been shown by several investigators that acute parenteral administration of GABA protects animals from electrically or chemically induced seizures (Hawkins & Sarett, 1957; McLennan, 1957; 1958). During the course of our experiments we found that, shortly after parenteral administration of GABA (3·0 g/kg) to rats, no protection from electrically induced seizures and strychnine seizures was observed. Pylkkö & Woodbury (1959) showed that the CD50 of strychnine was increased in rats pretreated with GABA 72 hr before treatment with the convulsant.

Since brucine differs from strychnine by having two methoxyl groups attached to the aromatic ring, it was of interest to study the possible protective properties of GABA against brucine seizures and to study the time course of any protective properties found.

Mature male albino Holtzman rats were pretreated with 3·0 g/kg GABA intraperitoneally and brucine alkaloid was administered after 3, 8, 15, and 30 days. The CD50 values for these animals were calculated ($CD50_1$) according to the method of Litchfield & Wilcoxon (1949). The CD50 values for brucine alkaloid ($CD50_2$) were calculated at the same time intervals for rats without GABA pretreatment. The potency ratio (P.R. = $CD50_1/CD50_2$ and the $f_{P.R.}$ were calculated by the method of Litchfield & Wilcoxon (1949).

TABLE 1. EFFECT OF GABA ON CD50 OF BRUCINE ON GROUPS OF 36 RATS

Weight of rats g	Days after GABA	$CD50_1$ with GABA	$CD50_2$ without GABA	Potency ratio	$f_{P.R.}$
83–132	3	117·0 (92·1–148·6)	71·8 (61·0–83·3)	1·63 (1·20–2·20)	1·35
112–170	8	91·0 (70–118·3)	69·8 (61·2–79·6)	1·30 (0·96–1·75)	1·35
112–218	15	88·0 (69·3°111·7)	72·0 (61·5–84·2)	1·22 (0·9–1·65)	1·35
93–220	30	82·0 (74·6–90·2)	82·0 (73·2–91·0)	1·0 (0·83–1·20)	1·2

It is evident from Table 1 that three days after GABA administration, the CD50 of brucine was elevated significantly. Thus the present observations seem to indicate that the anticonvulsant activity of GABA is not seen until three days after its parenteral administration.

Since Eccles (1956) has established that strychnine selectively blocks the inhibitory synapses in the central nervous system, it is possible that its dimethoxyl derivative, brucine, acts similarly. It is therefore of interest that the convulsant

activity of brucine is altered three days after a single dose of GABA, but whether GABA or a metabolite is responsible for this effect remains to be determined.

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Anti-anaphylactic action of water-soluble glucocorticoids

SIR,—Natural and synthetic glucocorticoids have proved to be highly active in clinical practice for the treatment of various allergic conditions and the prevention of surgical shock. But in animal experiments most authors have shown steroid treatment to be ineffective in experimental anaphylactic shock (Herxheimer & Rosa, 1952; Goadby & Smith, 1964).

We have now compared, using the intravenous route, a new water-soluble steroid Depersolon (11,17-dihydroxy-21-(4-methylpiperazin-l-yl)-pregna-1,4-diene-3,20-dione hydrochloride) (Tóth, Tuba & Szporny, 1961; Görög & Szporny, 1963) with the water-soluble prednisolone sodium hemisuccinate and dexamethasone-21-phosphate for their capacity to confer protection on guinea-pigs in shock induced by an albumin aerosol.

Anaphylactic shock was produced by the micro-shock method of Herxheimer (1952). Guinea-pigs of 250–400 g were sensitised with 5% commercial crystalline albumin solution, 75 mg per animal, injected intraperitoneally. After three weeks the animals were placed in a plastic box and 5% egg albumin aerosol was introdced into the chamber The point when the animal exhibited signs of severe dyspnoea, lying down on its side and turning its head to right and left, was taken as the preconvulsion time. If aerosol treatment is discontinued at this juncture, the animals can be saved from certain death by oxygen insufflation as recommended by Smith (1961).

Preconvulsion time was assessed initially on the 21st day after sensitisation and then twice more at weekly intervals. The average of the two latter values was taken as control. On the fourth occasion the drug was injected into the jugular vein of unanaesthetised guinea-pigs at various times before giving the albumin aerosol. The control group was given physiological saline under similar conditions.

The measure of protection was represented by the quotient of post-treatment and control preconvulsion times (Ratio = R). Protection was considered as maximal when R was 10.

The findings in Table 1 show that the new steroid exerted a strong protective influence against anaphylactic shock, the effect reaching a peak 10 min after

administration, and the protective action beginning to wane within 30 min. Prednisolone hemisuccinate on the other hand, even in massive doses, did not give any marked protection until 30 or as much as 90 min after administration.

Dexamethasone, in doses corresponding in anti-inflammatory activity to prednisolone, did not display any protection against shock.

It is worth-while to compare our results with those of Goadby & Smith (1964) who found a weak protection after subcutaneous administration from water-soluble hydrocortisone only, prednisolone proving to be completely ineffective. By the subcutaneous route Depersolon also did not avert shock.

TABLE 1. EFFECT OF WATER-SOLUBLE GLUCOCORTICOIDS ON ANAPHYLACTIC SHOCK ELICITED IN GUINEA-PIGS

Substance	Dose mg/kg i.v.	Min before shock	Protection ratio (R)	No. of animals protected of total used
Control	—	—	1·25	0/25
Depersolon	10	10	9·03	7/8
	10	30	7·60	5/8
	30	30	9·13	7/8
	60 s.c.	30	1·63	0/8
Prednisolone hemisuccinate	30	10	2·20	0/8
	30	30	5·70	4/9
	30	90	6·30	5/8
Dexamethasone phosphate ..	5	30	1·01	0/8
	5	90	1·26	0/8
	5	180	1·85	0/8

Our findings provide further evidence that solubility in water and intravenous injection bring about an essential alteration of pharmacological action, presumably by changing steroid metabolism. Applied intravenously, prednisolone hemisuccinate shows a marked protective action against shock, though its effect is much weaker and less acute than that of Depersolon. Both compounds have prednisolone structure, but the absence of the hydroxy group at position 21 and its substitution by methylpiperazine make an essential difference in the effect of Depersolon.

Our findings indicate that the shock-preventing effect of glucocorticoids can be demonstrated in animal experiments; they furthermore emphasise the importance of the metabolism of the applied steroids in the development of this effect.

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Formation and storage of dopamine in hamster mast cells following the administration of dopa

SIR,—Using the histochemical fluorescence method of Falck and Hillarp, a large proportion of the mast-cells in rabbit, cat and hamster skin have been shown to be capable of taking up L-3,4-dihydroxyphenylalanine (dopa), to contain dopa-decarboxylase activity and to have an amine storage mechanism sensitive to reserpine (Adams-Ray, Dahlström, Fuxe & Hillarp, 1964). Furthermore, the uptake and decarboxylation of dopa have been found to be specific, as practically no or very little 5-hydroxytryptophan (5-HTP) is taken up by the mast-cells (Adams-Ray, Dahlström & Sachs to be published). Since the amine content of mast-cells in the species mentioned above was much lower in untreated animals than after treatment with dopa, our working hypothesis was that the low concentrations of primary catecholamines found in the mast-cells of normal animals represented only intermediates and that the final product normally stored in the cells was a catecholamine which does not give a fluorescent product after reaction with formaldehyde. Biochemical determinations have now been made to see what primary catecholamine is present before and after the administration of dopa and to test the hypothesis.

The catecholamines were extracted from tissues and separated on ion-exchange columns as previously described (Bertler, Carlsson & Rosengren, 1958, Häggendal, 1962). Dopamine was determined fluorimetrically by the method of Carlsson & Waldeck (1958) as modified by Carlsson & Lindqvist (1962), and noradrenaline by Häggendal's method (1963). The determinations were made in the ear, heart and brain of untreated hamsters, and in hamsters treated with dopa (100 mg/kg subcutaneously) $\frac{1}{2}$, 2 and 24 hr before killing. Each determination was made on pools of tissue extracts from 5 to 7 animals. The time-course experiments were made on normal hamsters and on hamsters that had been bilaterally sympathectomised 10 to 21 days before killing. In all experiments made on the two groups of animals, the hamster ear tissue showed a unique capacity to form and store large amounts of dopamine for more than 24 hr (0·76 μg/g) with a maximum level after 2 hr (1·3 μg/g). The heart and brain tissues, on the other hand, showed a peak of dopamine as early as after 30 min (1·6 and 1·8 μg/g respectively), after which the content rapidly decreased and by 24 hr had reached normal levels (very low amounts in heart and 0·25 μg/g in brain). No significant changes were observed in the noradrenaline contents of the tissues studied after the administration of dopamine. Practically the entire noradrenaline content of the ear is in all probability stored in the adrenergic nerve terminals, since a 90% decrease in noradrenaline content was observed after bilateral sympathectomy (0·013 μg/g).

Since very few or practically no mast-cells belonging to the "monoamine" category are found in the brain or the heart and since this type of mast-cell is found in very large numbers in the ear, the marked differences observed between these tissues in the time course of the dopamine increase in ear tissue after administration of dopa, are in all probability due to the presence of abundant numbers of such mast-cells in the ear. These are able to store high concentrations of dopamine for a much longer time than the adrenergic nerve terminals after the administration of dopa. This is in complete agreement with the histochemical observations, since a marked increase in fluorescence intensity is still observed in the mast-cells 24 hr after injection of dopa. From histochemical observations in normal animals these mast-cells are known to contain low concentrations of primary catecholamines. The present biochemical data suggest that the primary catecholamine stored in the normal hamster ear is

dopamine. The results indicate furthermore that no or very low β-hydroxylase activity is present in these mast-cells, since no increase in noradrenaline content was observed. Preliminary attempts to detect tertiary catecholamines have so far proved unsuccessful.

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Drugs affecting the behaviour and spontaneous bioelectrical activity of the central nervous system in the ant, *Formica rufa*

SIR,—Recently several reports on the influence of neuro- and psychotropic drugs on various species of invertebrates have been published (Witt, Brettschneider & Boris, 1961; Fange, 1962; Katona & Woleman, 1964; Mirolli & Welsh, 1964). The ant seems to be a suitable subject for such investigation because of the relatively high degree of development of the central nervous system and its well developed social behaviour.

We have investigated the effects of certain psychotropic drugs and neurohormones on behaviour and spontaneous bioelectrical activity recorded from the lobi optici of the ant, *Formica rufa*. The ants were kept in a plastic formicarium and were given the drugs orally in honey, or injected into the abdominal cavity, or applied locally to the exposed brain. The investigations included, observations of general behaviour, phototropic reaction and records of spontaneous bioelectrical activity of the lobi optici by tungsten wire electrodes, connected to a conventional EEG apparatus. The characteristic EEG pattern of the lobi optici of the ant, obtained in conditions of normal brightness consisted of 2-5 waves/sec and of amplitude from 5–50μ V. Of the drugs investigated the most marked in their effects were reserpine, chlorpromazine and strychnine.

Reserpine, 0·1–0·5 μg of body weight, given either orally or injected into the abdominal cavity, markedly inhibited the locomotor activity of the ant without causing ataxia or disturbances of co-ordination. Simultaneous outbursts of aggressiveness of a bizarre character were observed, ants from the same population after slight stimuli or even spontaneously attacking each other, a phenomenon never observed in the controls. The EEG pattern was slightly changed with transient slowing of frequency and increase of amplitude. The phototropic reaction was significantly suppressed (Fig. 1).

Reserpine given 12 hr after nialamide, 0·1 μg/g weight, caused similar inhibition of locomotor activity with outbursts of aggressive behaviour, but the phototropic reaction remained unchanged. Nialamide itself did not alter behaviour and EEG pattern. Chlorpromazine given orally or injected into the abdominal cavity (0·1–0·5 μg/g weight) inhibited locomotor activity causing severe disturbances of motor coordination and suppressed the phototropic reaction, but did not cause any mutual aggressiveness. The EEG record compared with that of the controls did not change. After lysergic acid diethylamide both general behaviour and the phototropic reaction were unaltered, but the EEG showed a slight decrease of amplitude and increase of frequency of the waves.

Neither chlordiazepoxide nor amphetamine changed the behaviour or the bioelectrical activity.

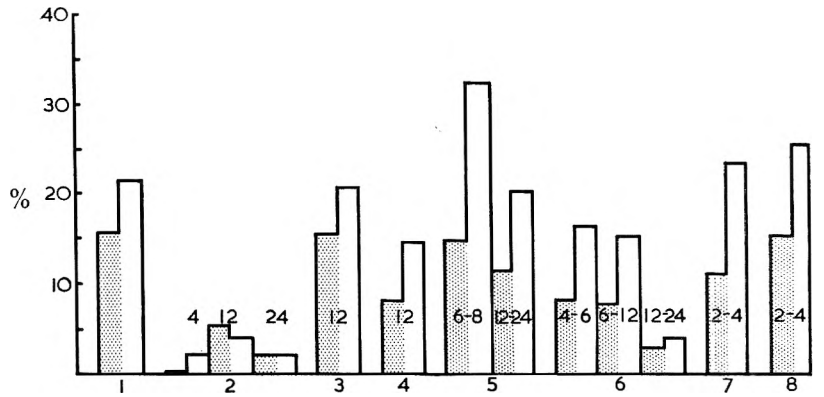

FIG. 1. Phototropic reaction of ants as a percentage of reacting insects. Shaded part of each column, percentage reacting after 5 min, and open part, after 10 min light exposure. Time (hr) after drug ingestion is marked on columns. The statistical significance of the difference between each group and control was estimated by χ^2 test. 1 = Control. 2 = Reserpine $P < 0{\cdot}005$. 3 = Nialamide $P > 0{\cdot}005$. 4 = Reserpine after nialamide $P > 0{\cdot}005$. 5 = Chlordiazepoxide $P > 0{\cdot}005$. 6 = Chlorpromazine $P > 0{\cdot}005$ (4-12 hr), $P < 0{\cdot}005$ (12-24 hr). 7 = Lysergic acid diethylamide $P > 0{\cdot}005$. 8 = Strychnine $P > 0{\cdot}005$.

Strychnine given orally in doses 0·1–0·2 μg/g weight, caused disturbances of locomotor activity, ataxia, and increased frequency and amplitude of waves in the EEG pattern. Acetylcholine, adrenaline, γ-aminobutyric acid and 5-hydroxytryptamine injected into abdominal cavity or applied locally on the exposed brain had little or no influence on bioelectrical activity of the ant brain. For instance, 5-HT caused a slight increase of amplitude and decrease of frequency of the EEG. None of the investigated neuro-hormones changed the behaviour of the ants.

The results obtained seem to indicate certain similarities between the effects of reserpine and chlorpromazine on the ant and laboratory animals. This may suggest that, despite the great differences between the subjects, the mode of action of reserpine and chlorpromazine may be similar in the different species.

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Acute toxicity of radiation-sterilised propylene glycol

SIR,—Certain drugs that are subject to hydrolysis when dispensed as an aqueous solution are prepared, for pharmaceutical purposes, as a solution in propylene glycol. Such solutions may be difficult to sterilise as many of the drugs concerned are affected by heating; bacteriological filtration is tedious due to the viscous nature of the solvent and there is always a risk that the aseptic precautions necessary for the filling and sealing of the final containers may fail. Radiation sterilisation may offer a useful alternative method: it can be applied to the preparation in its sealed container.

The present investigation was stimulated by the need to sterilise a solution of di-isopropylfluorophosphonate (dyflos, DFP) in propylene glycol (1 mg/ml). The dyflos is prepared with labelled phosphorus atoms (^{32}P) and the solution is intended for parenteral use in clinical studies of blood cell turnover. Preliminary studies indicated that the potency of the dyflos itself was only very slightly affected by radiation sterilisation (Charlton, personal communication) but no information was available on the effect of radiation sterilisation on the toxicity of propylene glycol.

A sample of redistilled propylene glycol was divided into two parts, one of which was irradiated with 2·5 Mrad of gamma radiation—the dose used for sterilisation of medical equipment (Burt & Ley, 1963).

Groups of 3 male and 3 female adult SPF albino rats were given either irradiated or unirradiated propylene glycol by intraperitoneal injection at the following doses: 9·4, 11·1, 13·0, 15·3, 18·0 and 21·1 ml/kg body weight. The LD50 values were calculated from the mortality after 5 days using the method of Finney (1952). The LD50 values, with the 95% fiducial limits were for the irradiated material 13·7 (12·5–15·1) and for the control material 14·2 (12·4–16·1) ml/kg weight; differences between groups are not significant.

Irradiated or unirradiated propylene glycol was given to groups of 5 male and 5 female mice (C3H strain) by intraperitoneal injection to a dose of 5 ml/kg weight. Before injection, 1 part of propylene glycol was diluted with 2 parts of normal saline. This dose is about half the median lethal dose in mice (Lampe & Easterday, 1953) but although all mice showed signs of intoxication (lack of co-ordination of movements followed by deep narcosis and, in a few cases, convulsions), no mice died within 7 days of injection. During the week after injection with propylene glycol no differences were observed in general health, weight changes, or food and water intake between the groups receiving irradiated or unirradiated material. All the mice were killed and examined 7 days after injection; apart from evidence of inflammation of the peritoneal cavity, presumably caused by the injection, no abnormalities were observed in mice of either group.

These results show that sterilisation of propylene glycol with a dose of 2·5 Mrad of gamma radiation does not increase its acute toxicity and this information may be of interest to others who may be contemplating radiation sterilisation of other pharmaceutical preparations formulated with propylene glycol.

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Influence of urine pH and flow rate on the renal excretion of chlorpheniramine in man

SIR,—The renal excretion of amphetamine and methylamphetamine has been shown to be dependant upon the pH of the urine and is sufficiently pH-sensitive to reflect the diurnal rhythm of urinary pH (Beckett & Rowland, 1964, 1965; Beckett, Rowland & Turner, 1965). We now report that, using a specific assay for unchanged drug based upon gas liquid chromatography (Beckett & Wilkinson, to be published) the renal excretion of the antihistamine, chlorpheniramine and its (+) and (−) isomers, shows a dependance not only upon urinary pH but also upon the rate of urine flow.

The oral administration to normal male subjects, of an aqueous solution of 10 mg chlorpheniramine base as the maleate, resulted in a fluctuating excretion rate. The total amount of unchanged chlorpheniramine excreted in 24 hr was 4·5–11·5%. In contrast to the results reported for amphetamine and methylamphetamine (Beckett & Rowland, 1964; 1965; Beckett & others, 1965), maintaining the urine acid (pH 5·00 ± 0·50), or alkaline (pH 8·00 ± 0·50), by administration of ammonium chloride or sodium bicarbonate, respectively, did not abolish the fluctuations (see Fig. 1), although there was a difference in the total amount of drug excreted. When the urine was acid, 20·0–26·5% unchanged drug was excreted in 24 hr, whereas only 0·3–0·4% was excreted when the urine was alkaline.

Under constant acid urine conditions the fluctuations in the rate of excretion appeared to be related to changes in the rate of urine flow; a high flow rate resulted in a high excretion rate (see Fig. 1). The volume-dependent fluctuations were abolished when the urine flow rate was maintained above 150 ml/hr by water loading the subjects (see Fig. 1). Under these conditions the excretion rate decreased exponentially except for a rise 10–15 hr after administration of the dose; the reason for this departure from exponential excretion is under investigation. The excretion pattern of both the (+)- and (−)-isomers was similar to that of the racemate.

These results may be explained by assuming that the tubular epithelium of the distal convoluted kidney tubules is selectively permeable to the unionised base (Schanker, 1962). The rate of reabsorption of the drug from the tubular fluid will thus depend on the ratio of concentration of unionised base within the tubules and that in the peritubular fluid (Milne, Scribner & Crawford, 1958; Weiner & Mudge, 1964). The concentration of unionised base in the tubules may be altered by a change in the pH of the tubular fluid, as the pK_a of chlorpheniramine is 9·16 (Marshall, 1955), or by alteration in the fluid volume.

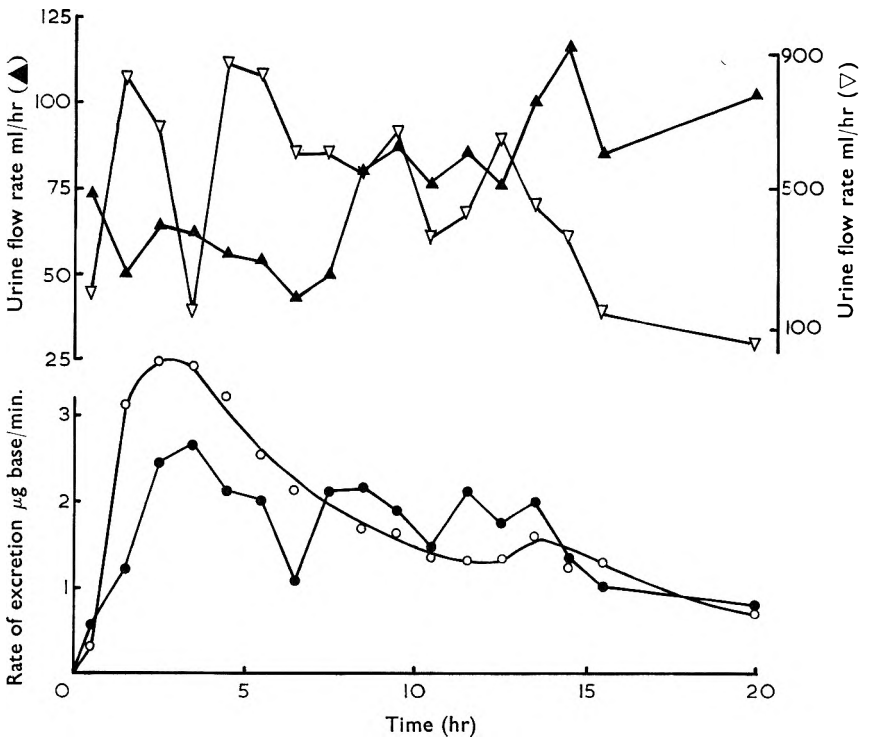

FIG. 1. The rate of excretion of chlorpheniramine and corresponding urine flow rates; conditions of normal flow rates (solid symbols) and high flow rates (open symbols). Subject G.R.W., urine pH 5·00± 0·50.

Abolition of the volume-dependent fluctuations in the excretion rate by water loading of the subjects suggests that there is a limiting ratio of concentration of unionised base in the tubular and peritubular fluids below which reabsorption is negligible.

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Solubilisation of preservatives by non-ionic agents

SIR,—Evans (1964) has recently reported a method for the solubilisation and inactivation of preservatives by non-ionic agents in which he has titrated *p*-hydroxybenzoic acid (100 ml 0·01 or 0·03M) with sodium hydroxide (0·1N) and showed that the pH is displaced to higher values in the presence of octylphenol/8·5 moles ethylene oxide. He assumed solubilisation of the acid in the detergent micelles and calculated a partition coefficient for the distribution of the acid between the detergent and water. Without giving details of the calculation, Evans indicated how the partition coefficient could be used to calculate the distribution of acid between detergent and water at various pH values.

We have evolved independently a similar potentiometric method in which displacements of the pH of titration curves of sodium benzoate with hydrochloric acid towards higher values in the presence of a non-ionic surface-active agent (cetomacrogol) were shown to be characteristic of acids which were solubilised in the micelles (Donbrow & Rhodes, 1963a). These pH displacements were interpreted using distribution theory, and the distribution coefficient

$$K_D \text{ or } [HA_m]/[HA_w] \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

was calculated from the equation:

$$\text{antilog } \Delta \text{ pH} = K_D.V_m/V_w + 1 \quad .. \quad .. \quad .. \quad .. \quad (2)$$

in which V_m, V_w are the volumes of the micellar and aqueous phases and Δ pH is the difference in pH between the titrations in the presence and absence of the surfactant. Phase volumes (estimated from density measurements) were used in this formula to enable comparison to be made with literature K_D values of benzoic acid in organic solvents, from which it appeared that the micelles were behaving as polar rather than non-polar organic solvents, which was taken as evidence that solubilisation was occurring in the "palisade" layer rather than in the micellar core (Donbrow & Rhodes, 1963b). The same formula is applicable for the calculation of the partition coefficient per unit weight of phase by substituting phase weights for phase volumes. Attention was also drawn to the significance of the partition coefficient in pharmaceutical formulation, since its value governs the relationship between the total amount of drug in a surfactant solution (the "capacity" of the system) and the concentration, or activity, in the aqueous phase. The phenomenon was further shown to be a general one, occurring with a variety of acids, amines, or phenol and a variety of non-ionic and ionic surface-active agents (Donbrow & Rhodes, 1963; 1965; Rhodes, 1964). Our method differs from that of Evans in some important respects that involve fundamental points of theory and interpretation to which we would like to draw attention.

We have throughout our work titrated the sodium salt of the acid with hydrochloric acid (or the hydrochloride of the base with sodium hydroxide) in order to keep the ionic strength relatively constant. The Henderson equation may be applied to the titrations in the form (Donbrow & Rhodes, 1963b):

$$\text{pH} = \text{p}K_a + \log[A^-]/[HA_w] + \log f_{\pm} \quad .. \quad .. \quad .. \quad (3)$$

where pK_a is the thermodynamic dissociation constant exponent, $[A^-]$ is the concentration of the ionised (salt) form of the acid, $[HA_w]$ is the concentration of the unionised "free" acid (i.e. the acid not bound by the surfactant), and $f_{\pm}$ is the appropriate activity coefficient correction. Since the total free acid in the system, HA_t, is known from the percent neutralisation, the amount of bound or micellar acid, HA_m, is calculated by difference:

$$HA_m = HA_t - HA_w \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where HA_w is the amount of unbound unionised acid in the aqueous phase. It is evident that the errors in $[HA_w]$ and $[HA_m]$ introduced by the use of uncorrected data do not cancel out, hence the partition coefficient will not be independent of the activity coefficient.

There are two methods of dealing with the salt effects: either the thermodynamic dissociation constant, pK_a, may be used together with the appropriate activity coefficient, if known, or a comparative method may be used. With the comparative method, it is convenient to define an apparent dissociation constant exponent pK'_c by the equation:

$$pK'_c = pK_a + \log f_{\pm} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (5)$$

Its value may be determined from a "blank" titration in which a solution containing the same concentration of the salt of the acid is titrated under identical conditions (except for the absence of surfactant) as in the "surfactant" titration. If the acid is back-titrated from the salt form, as in our procedure, the ionic strength of the solution and hence the value of log $f_{\pm}$ will vary only to a small extent, provided the volume of titrant added is small compared with the total volume of the system, and the pK'_c term will be virtually constant (Albert & Sergeant, 1962). Change of anion does not seem to be of importance for 1:1 electrolytes of relative short chain-length. The comparative method has the advantage of not requiring the estimation of an activity coefficient, and is preferable for establishing the operative distribution coefficient in a formulated product, provided the comparative titrations can be made at a concentration and under conditions simulating those in the formulated product. Obvious limitations occur when the acid is too insoluble in water for a comparable "blank" titration to be made. For insoluble acids, we have used the thermodynamic constant and estimated the activity coefficient.

Evans has titrated free acid to salt throughout his work; thus the ionic strength varies continuously in individual titrations and the variation through the neutralisation range may be large. Moreover, there will be large differences in ionic strength at corresponding points in titrations in which the initial concentration of acid is not the same, as in his work at 0·01 and 0·03M, so that comparisons made at arbitrarily chosen points are invalid. The effect of ionic strength on the activity coefficients and pH values observed may be predicted by means of the Deybe–Hückel equation and it would appear that near the end-point of the 0·03M titration, for example, the pH value may differ by 0·05 to 0·1 units from the value at a low ionic strength. In addition, changes in salt concentration affect micellar properties and distribution coefficients of "semi-" polar solutes. Evans's method of calculation is not given, but since he quotes a single dissociation constant of constant value (K_c $2{\cdot}95 \times 10^{-5}$) without reference to correction for ionic strength, it would appear that no correction was applied, though pH values were recorded to $\pm 0{\cdot}01$ units. This procedure can lead to errors in partition coefficient values and aqueous phase concentrations of unionised acid, particularly if the method is applied to formulation of preservatives in the presence of concentrations of salts. Unfortunately, Evans has not presented his results in a way that permits recalculation from his data, and though his partition coefficients are of qualitative interest, they are not in good agreement with the values calculated from cloud point data, and are probably insufficiently accurate for bactericidal or pharmacodynamic studies.

Evans states that, with the exception of some work by McBain "all previously reported solubilisation data have been obtained by examination of systems containing excess insoluble phases." This is not correct, for in addition to our

own publications on the potentiometric method, Kostenbauder and his collaborators have successfully applied equilibrium dialysis to a number of unsaturated surfactant systems (Patel & Kostenbauder, 1958; Pisano & Kostenbauder, 1959; Miyawaki, Patel & Kostenbauder, 1959; Deluca & Kostenbauder, 1960; Hurwitz, Deluca & Kostenbauder, 1963; Patel & Foss, 1964).

Evans has interpreted his results as we had in the earlier publications in terms of partition between an aqueous and a micellar phase. It is often more convenient to treat aqueous solutions of amphiphilic colloids, such as non-ionic surfactants, as uni-phase systems (Kruyt, 1952; Martin, 1960). The potentiometric evidence does not support the use of a two-phase model. Evans found that the presence of surfactant did not affect the activity of either hydrochloric or acetic acids. If a surfactant solution was a two-phase system one would expect the activity of these acids to be increased by the presence of surfactant by reason of the reduction of the volume of the aqueous phase. Such "concentration" effects would lead to a pH reduction of about 0·1 units in the stronger surfactant solutions, which should have been detectable.

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Effect of formaldehyde-induced periarthritis upon the composition of cotton-pellet granuloma in rats

SIR,—It is known that a local inflammatory reaction can be inhibited by prophylactic counter irritation. Laden, Blackwell & Fosdick (1958) found that injection of an irritant into the knee of a rat reduced the experimental pleuritis in the same animal. Intraperitoneal injection of substances that induce inflammatory oedema also inhibit the local inflammation of the rat paw (Büch & Wagner-Jaurregg, 1962; Benitz & Hall, 1963; Horáková, 1964). The local necrosis of the rat skin elicited the loss of dermal-insoluble collagen in apparently uninjured skin distal to the site of injury. The changes were similar to those in the necrotic area (Houck, 1962). We have now examined the influence of formaldehyde-induced periarthritis of the hind paw on the composition of experimental cotton-pellet granuloma in rats.

Experimental periarthritis of the right hind paw was produced in Wistar rats, of 150 g, by the injection of 0·2 ml of 2% formaldehyde under the plantar aponeurosis. Three days after this injection, two sterile cotton-pellets weighing 10 mg each were implanted under the skin of the back. After 7 and 21 days the induced granulomas were dissected, the pellets removed, and the tissue dried at 105° for 20 hr and then weighed. The dry tissue was analysed for its content of hydroxyproline, which was used as an indicator of fibrilar proteins (Stegemann, 1958) and also for the content of deoxyribonucleic acid (Schneider, 1945). The inflammation of the paw showed a typical course towards a chronic fibro-productive phase, with occasional necrosis. The results were compared with a control group without experimental periarthritis.

TABLE 1. DRY WEIGHTS OF GRANULOMAS AND THEIR HYDROXYPROLINE AND DEOXYRIBONUCLEIC ACID CONTENTS

		Dry weight of granulomas, mg	Hydroxyproline, µg/100 mg dry wt	DNA, µg/100 mg dry wt
7 Days	Controls	39·41 ±9·10	1354·5 ±134·8	1840·5 ±356·0
	Formaldehyde periarthritis	40·34 ±11·16	1933·3* ±247·1	1991·4 ±219·2
21 Days	Controls	35·11 ±9·32	1332·9 ±382·9	2047·6 ±339·9
	Formaldehyde periarthritis ..	37·29 ±13·29	1747·8* ±396·7	3062·1* ±280·9

* Statistically significant difference $P < 0{\cdot}02$.

Table 1 shows that the weights of the dried granulomas were not different in the group with formaldehyde-induced periarthritis compared with the control group. The contents of hydroxyproline and deoxyribonucleic acid, on the other hand, were changed. There was elevation of the hydroxyproline content on the 7th and 21st day of the experiment and the deoxyribonucleic acid level elevated significantly on the 21st day. The local injury of the hind paw may thus be paralleled, according to these biochemical indicators, by an enhanced fibroproliferative reaction in the cotton-pellet granuloma. This is different to the oedematous reactions. Whether this is a result of a change in the reactivity of the connective tissue in general remains to be proved.

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The nature of the inhibition of the rat uterus by relaxin

SIR,—Wiqvist (1959) presented evidence that the inhibitory action of relaxin on the spontaneous contractions of the isolated uterus of the rat was not blocked either by phentolamine or by dihydroergotamine in concentrations which blocked the inhibition of the these contractions by adrenaline. He suggested that relaxin acted otherwise than by the adrenergic mechanism suggested by Miller & Murray (1959). However, a fuller investigation of these actions by Rudzik & Miller (1962a, b) added weight to their original contention that the mechanism was

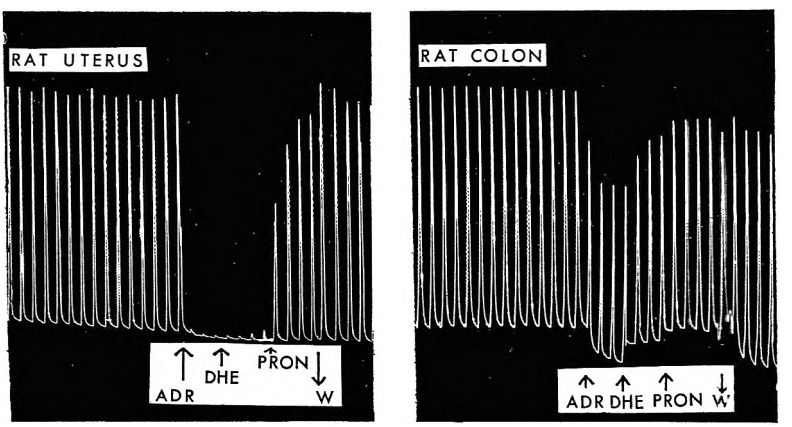

FIG. 1. Effects of pronethalol and dihydroergotamine on inhibitory actions of adrenaline. Isotonic contractions of the electrically stimulated rat uterus. At ADR 5×10^{-9} (-)-adrenaline, at DHE 5×10^{-7} dihydroergotamine and at PRON 5×10^{-7} pronethalol were added to the bath. The drugs were washed out at W. Isotonic contractions of the electrically stimulated rat colon. Legend as for uterus except for ADR, where 5×10^{-8} (-)-adrenaline was added to the bath.

adrenergic. The blocking drugs used by both groups, dihydroergotamine and phentolamine caused adrenergic block at α-receptors, but are now recognised to lack specificity (Ahlquist & Levy, 1961; Birmingham & Wilson, 1963). Also, the action of adrenaline on the rat uterus is known to be almost entirely at β-receptors (Levy & Tozzi, 1963; Levy, 1964). With the advent of a β-receptor blocking agent of high specificity, pronethalol (nethalide) (Black & Stephenson,

1962), the opportunity arose to investigate its action on the inhibition of the electrically stimulated rat uterus by adrenaline and by relaxin.

Isolated uterine horns from adult albino rats weighing 140 to 180 g were suspended at 37° in 75 ml Krebs solution gassed with 95% oxygen and 5% carbon dioxide. The preparation was mounted in a Perspex channel between two parallel platinum wires arranged along the length of the muscle. This allows electrical stimulation of the preparation in the way described for the guinea-pig vas deferens by Birmingham & Wilson (1963). The distance between the platinum wires was 4 mm so that the uterus could move unimpeded within the channel. The preparation was stimulated electrically with square wave impulses of 0·3 msec duration, 120 V at a frequency of 25 sec, for 10 sec every 2 min. No consideration will be given here to the identity of the structures which are being stimulated in the preparation, but preliminary experiments indicate that these are neuronal and cholinergic in nature. The contractions caused by the stimulation were recorded isotonically on a smoked drum with a frontal writing lever. The load on the preparation was 1g.

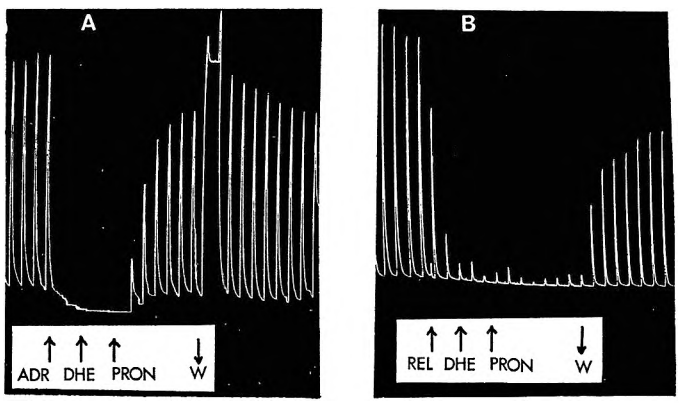

FIG. 2. Effects of pronethalol and dihydroergotamine on the inhibitory actions of adrenaline and relaxin on isotonic contractions of the electrically stimulated rat uterus. The two preparations were from the same animal and set up in identical conditions. (A) At ADR 2×10^{-8} (-)-adrenaline, at DHE 10^{-6} dihydroergotamine and at PRON. 10^{-6} pronethalol were added to the bath. The drugs were washed out at W. (B) Legend as for 2 (A) except that at REL 5×10^{-7} relaxin was added to the bath.

Experiments were also made with 3 cm lengths of rat colon prepared in the same way as for the uterus and stimulated electrically using the same parameters of stimulation. Drugs used were (–)-adrenaline bitartrate, dihydroergotamine methanesulphonate, pronethalol hydrochloride, and relaxin (Releasin). Except for relaxin the concentrations of drugs are expressed in terms of final bath concentration of the base. The concentration of relaxin is in terms of w/v of Warner-Chilcott Relaxin Standard.

Fig. 1 shows the inhibition of contractions of the rat uterus and colon by adrenaline. In the uterus the inhibition is seen to be reversed by pronethalol (5×10^{-7}) but not by dihydroergotamine (5×10^{-7}). This concentration of dihydroergotamine does, however, reverse the inhibition by adrenaline of the contractions of the rat colon. Similar results were obtained when the agonist-antagonist sequence was reversed, so that the antagonists were given 5 min before the adrenaline. The interpretations from these experiments, that adrenaline acts

at β-receptors in the rat uterus, are in agreement with the conclusions drawn by Levy & Tozzi (1963). When the antagonists were tested in the same way on the uterus against relaxin (Fig. 2), the inhibition caused by relaxin was not affected, whereas on a second horn from the same rat the action of adrenaline was reversed. Exposure of the uterus to pronethalol 10^{-6} for 5 min blocked the inhibition by adrenaline, but not that of relaxin. Contractions of the colon were not inhibited by concentrations of relaxin up to 5×10^{-6}.

These experiments do not support the hypothesis that relaxin inhibits contractions of the rat uterus by releasing adrenaline.

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