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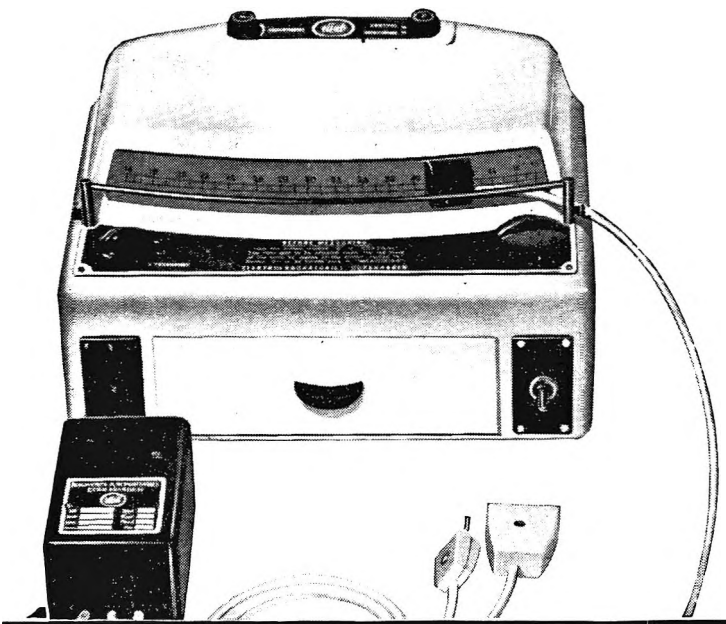

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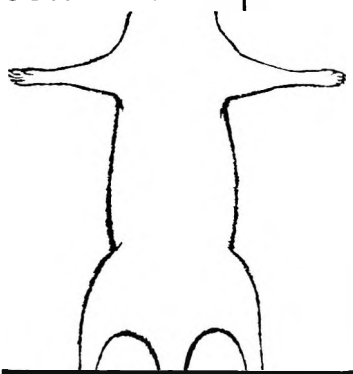

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The drug-receptor complex

A. S. V. BURGEN

The rate of association of drug and receptor when limited by diffusion alone will correspond to a basic rate of $2{\cdot}5 \times 10^9$ litres/mole sec and will have a net activation energy of 3–4 kcal/mole. This rate will not be significantly increased by attractive forces between drug and receptor but could be reduced by repulsive forces. It could be considerably reduced by the presence of bound water and ions, by the requirement of activation energy for combination and by geometrically restricted access to the receptor. Complex formation is due to the stabilisation of the drug in the force field from which it can escape only on acquisition of kinetic energy greater than the potential energy of the field; the fraction of molecules acquiring this threshold kinetic energy can be calculated from the Boltzman equation. The rate of dissociation of the complex is the rate of loss by free diffusion multiplied by the Boltzman factor. The lifetime of drug receptor complexes is long enough to enable molecules undergoing collision in the non-ideal aspect or conformation time to present in the ideal state.

CONTEMPORARY ideas of drug action and drug specificity are all based on the assumption that the initial process in drug action is the formation of a reversible complex* between the drug and a cell component generally known as the drug receptor. The idea of a specific drug-receptor complex originated with Langley and Ehrlich and was developed into a quantitative theory by Langmuir and Clark. Until comparatively recently this general basis was accepted but little work was undertaken to evaluate it critically. The seeds of doubt were sown by the realization by Stephenson (1956) that there was no necessary reason to equate the maximum physiological response of a tissue with maximal occupancy of the receptors. Obviously the reaction of the drug with the receptors can be the limiting factor, but equally other factors in the train of events leading from drug combination to the physiological response, e.g., myofibril contraction, might be determining the maximum response and influencing the slope of the dose response curve. The possibility that spare receptors exist throws into doubt much of the previous work and it is regrettable that we still lack an unequivocal tool for assessing the magnitude of receptor occupancy by agonists. However, the single most potent factor responsible for the present revival of interest in drug-receptor interactions has been the work of Paton (1961), who has questioned whether agonist action is a direct function of drug-receptor complex concentration and has produced evidence in favour of the view that it is the act of formation of the complex that is important, i.e. that agonist action is a function of the turnover of drug-receptor complexes.

This approach has naturally focussed attention on kinetic aspects of the drug receptor complex. In this paper an attempt is made to define a theoretical basis for drug-receptor kinetics based on molecular theory.

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* Even in the relatively few cases where the drug ultimately links on to the receptor through a covalent bond it is likely that an initial reversible complex is formed; a comparable situation is the Michaelis complex of an enzyme and substrate.

Complex formation

Reaction theory postulates that for two molecular species to react it is first necessary for them to approach to a collision radius at which distance intermolecular forces can act. We shall see later that intermolecular forces are of short range and diminish to negligible values when the internuclear distance of the reactants is greater than about 12 Å, i.e. they operate over 1–2 molecular diameters.

If the reactants come within a collision radius they have a probability of reacting which may vary from 1 to 0.

We can define the limiting rate of reaction as that due to transport of the reactants into a collision radius by diffusion, any diminution below this rate must be accounted for by steric or energetic barriers to complex formation. An increasing number of reactions, particularly of an ionic type, have been shown by a variety of techniques for measuring fast reactions to be diffusion limited; examples are the combination of iodine atoms to form an iodine molecule or the quenching of the fluorescence of uranin by halide ions (Caldin, 1964). By contrast ordinary chemical reactions are slower by several orders of magnitude.

The mathematical theory of diffusion limited reactions laid by Smoluchowski (1917) (see also Alberty & Hammes, 1958; Noyes, 1961; Caldin, 1964) was concerned with the kinetics of coagulation of colloids by electrolytes.

His formulation of the problem was to calculate the rate at which particles diffuse into a hemispherical cavity surrounding the target site.

The result obtained is

$$n = \frac{4\pi r_0 D_{12} NC}{1{,}000} \qquad (1)$$

Since the flux rate $n = k_1 C$, where k_1 is the collision rate constant, then

$$k_1 = \frac{4\pi r_0 D_{12} N}{1{,}000} \qquad (2)$$

(r_0 = radius of target molecule; D_{12} = relative diffusion constant of the reactants; N = Avogadro's number, i.e. $6{\cdot}02 \times 10^{23}$ molecules/mole).

In dealing with the reaction between a mobile drug and a structurally fixed receptor D_{12} becomes the simple free diffusion constant of the drug. It is a convenience to eliminate diffusion constants from the equation completely by using the Stokes–Einstein equation

$$D = \frac{kT}{6\pi\eta \cdot r_D} \qquad (3)$$

(k = Boltzman's constant; T = absolute temperatures; η = viscosity of the medium; r_D = radius of diffusing molecule).

Substituting (3) in (2) we obtain

$$k_1 = \frac{2RT}{3{,}000\eta} \cdot \frac{r_0}{r_D} \qquad (4)$$

(R = universal gas constant).

In the case of the majority of drug-receptor interactions it can be assumed that $r_o \approx r_D$ since there is a spatial correspondence of the drug and its complementary site.*

For this case $k_1 = 2{\cdot}5 \times 10^9$ litres/mole sec (37° C).

This simple equation gives values in close agreement with the experimental measurements of diffusion limited reactions and its theoretical basis appears to be justified.

The universal dependence of the rate constant on viscosity is of considerable interest and accounts for most of the temperature dependence of collision rate. Since the viscosity of dilute aqueous solutions decreases by about 20% for each 10° C rise in temperature we can expect the rate constant to increase by 25–30% for a corresponding rise in temperature. In chemical kinetics changes in rate with temperature are usually evaluated by the Arrhenius equation

$$k_1 = Ae^{-E_a/RT} \quad .. \quad .. \quad .. \quad (5)$$

where E_a is an energy of activation; the activation energy of viscosity is 3–4 kcal/mole. This energy is attributable to the cage effect, i.e. each solute molecule is effectively enclosed by solvent molecules and to become translocated must bypass a solvent molecule. The probability of the molecule doing so depends on its kinetic energy. This is the minimum activation energy associated with molecular complex formation† and only when the activation energy exceeds this value can we presume that other steps requiring activation are present. The estimation of activation energy and viscosity dependence are clearly important tools in evaluating diffusion limited reactions but a word of caution is necessary. The viscosity of solutions can be increased most readily by the addition of high polymers such as proteins, dextran, or polyvinylpyrrolidine, but it must not be expected that these substances will materially change the rate of diffusion limited reaction because the viscosity enhancing effect of these solutes is due to frictional effects between the macromolecules that have little effect on the microscopic viscosity of the solvent.

The formation of the drug-receptor complex depends on the existence of a measure of complementarity between the two structures so that potential sources of intermolecular force can co-operate to form the complex. These forces, electrostatic, dispersion and hydrophobic, are all extensive, so that there is a force-field normal to the receptor surface acting upon the drug molecules diffusing in the neighbourhood. This field will modify the rate of bombardment of the receptor and will increase the rate if the net force is attractive and decrease it if the net force is repulsive. This effect can be incorporated (the second term in the bracket) in the Fick diffusional equation (Debye, 1942).

$$\frac{dn}{dt} = DA\left[\frac{dc}{dz} + \frac{c}{kT}\frac{dU}{dz}\right] \quad .. \quad .. \quad .. \quad (6)$$

* For large drugs such as polypeptides this may not be true if only a part of the molecule is directly concerned in complex formation.

† In a few special cases the activation energy is less than 3–4 kcal/mole when chain reactions or quantum mechanical tunnelling are involved.

where U is the free energy of the drug molecule at a distance r from the receptor.

Solution of this equation shows that the rate constant k_1 of the Smoluchowski equation (2) must be corrected by a factor f dependent on the solution of equation (7)

$$f = -\frac{1}{\int_{d_e}^{\infty} \frac{e^{U/RT}}{r^2} \cdot dr} \qquad (7)$$

(d_e = separation of drug and receptor centres in the drug-receptor complex at equilibrium).

The numerical value of f will depend on two factors, firstly the way in which U varies with distance of separation of drug and receptor and, secondly, on the absolute value of U at the equilibrium distance. The dependence of most intermolecular forces on distance can be expressed by a simple inverse power, i.e.

$$U = ar^{-p} \qquad (8)$$

For ionic forces p = 3 due to the operation of dielectric polarisation and shielding by the ionic atmosphere, for dipolar forces p = 3–6, for dispersion forces p = 6 (recent work has given evidence of both shorter and larger range dispersion forces for which p = 4–8) and for van der Waals' repulsive forces p = 9–12. There is no clear understanding as yet of the distance dependence of hydrophobic interaction, but simple geometric considerations suggest p = 2–4. Fig. 1 shows the dependence of f on U for values of p = 3 and p = 9.

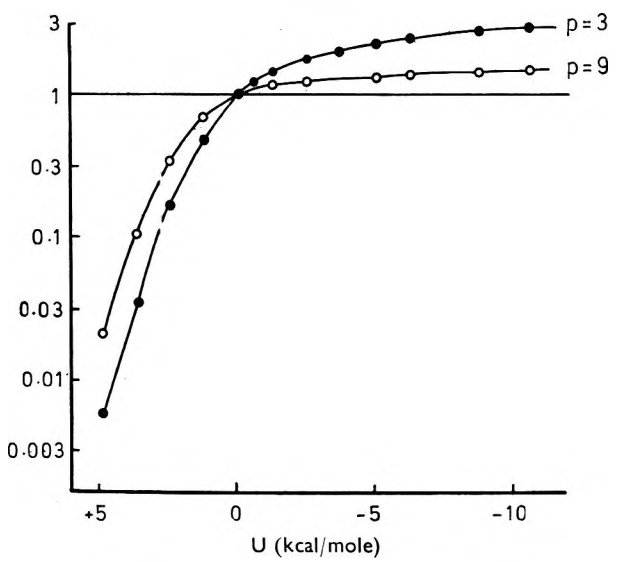

FIG. 1. The modifying effect of the force field on the rate of collision. The curves are calculated for forces varying as r^{-3} and r^{-9} respectively; intermediate values of p give intermediate values for f. The curves calculated by computer from equation (7). The ordinate gives the values for the free energy of complex formation at equilibrium.

It can be seen that the attractive force field makes a small but significant contribution to the rate of collision. For instance, in a drug interaction with the receptor whose free energy is -10 kcal/mole (equivalent to pA or pI = 7·0) in which half the interaction can be attributed to third power and half to sixth power forces, the collision rate will be increased by a factor of 2·3. On the other hand, repulsive forces can have a much more significant retarding effect. For instance, an ionic repulsive force (e.g. between groups in drug and receptor of like sign) whose magnitude at equilibrium is $+5$ kcal/mole will reduce the rate of collision by over 100-fold.

Since the net interaction between a drug and its receptor depends on the summation of fractional attractive forces of specific and complementary features of drug and receptor site, these will be fully realized only when the drug is lying in a unique rotational plane. Only if the drug molecule is presented in this optimal aspect during the approach to the receptor will the full accelerative effect of the force field be attained. In all other aspects the effect of the force field will be less and the acceleration correspondingly diminished. Our next problem is to estimate the probability of the optimal aspect of the drug being presented during approach.

Einstein, in considering Brownian motion of particles showed that the rotation of a spherical particle in solution is given (see Pollard, 1962) by

$$\bar{\theta}^2 = \frac{kTt}{4\pi\eta r^3} \qquad (9)$$

This equation for rotational diffusion may be compared with the equation for translational diffusion

$$\bar{x}^2 = \frac{kTt}{3\pi\eta r} \qquad (10)$$

Combining the two equations we find

$$\theta = \sqrt{\tfrac{3}{4}} \cdot \frac{x}{r} \qquad (11)$$

where θ is the rotation in radians and x/r, are equivalent units (Å). If a molecule is to display all aspects it must rotate through 2π radians so that the probability of displaying all aspects is

$$P = 0{\cdot}86\,\frac{x}{2\pi r} \qquad (12)$$

Now let us define as a significant acceleration of diffusion an increase of 50% over simple diffusion. The data of Fig. 1 shows this is attained at $U = -1{\cdot}4$ kcal/mole for an r^{-3} force and $U = -4{\cdot}7$ kcal/mole for an r^{-6} force. If we take the equilibrium distance to be 5 Å and $U_e = -10$ kcal/mole, we may calculate the available distance over which acceleration operates as 4·6 Å and 0·68 Å respectively and the corresponding values of P are 0·16 and 0·023 (drug radius assumed to be 4 Å). The low values of P show that the accelerative force is negligible for r^{-6} forces and of little importance even for the more extensive r^{-3} force. The exception to this rule will be in drugs showing a high degree of symmetry

(for instance, tetramethylammonium). Actually the values for P will be effectively reduced still further for the majority of drugs because they are not rigid molecules but can exist in several conformations determined by hindered rotation about valence bonds. Since only one of the set of conformations will be able to interact maximally with the force field, the overall probability must include corrections for both rotation and conformational effects.

We will assume at this point in our argument that the arrival of a drug at the receptor in an unsuitable aspect or conformation does not necessarily prevent a complex being formed although it may reduce the probability of its being formed; we will reserve until later a discussion of this effect.

The theory of drug antagonism states that if a receptor is already occupied by a drug, the complex cannot combine with another molecule. While this is not necessarily true we will assume it to be true in the next stage of our argument. We can state our rule as follows: complex formation only occurs between naked drug and receptor molecules. If the drug or receptor sites have an affinity for some ubiquitous component of biological solutions then a proportion of drug molecules and receptor sites will already be occupied and so be unable to react with each other. The obvious substances to consider are the inorganic ions and water molecules.

Consider first the case where a drug is a cation and there is a complementary anionic site on the receptor. If the anionic site is occupied by an alkali metal such as sodium, the approach of the drug will be sterically hindered and in addition the positive charge of the sodium ion will cause electrostatic repulsion. In principle, the problem is a classical one in the theory of electrolytes, i.e. the problem of ion pair formation. Bjerrum (1949) showed that for small univalent ions of radius 2 Å the degree of association is less than 10% in an ambient electrolyte concentration of 0·15 M. The same kind of argument will apply to drug molecules except that because of the larger size of the cation the potential for forming pairs is reduced. Unfortunately, we cannot be so sure about the receptor. Let us assume for the moment that the receptor is a protein. The factors determining ion binding to proteins are imperfectly understood and are certainly more complex than for simple ions. Alkali metals have little or no affinity for most proteins (for example, plasma proteins), but there are exceptions and some enzymes for instance bind alkali metals rather strongly (Steinhardt & Beychok, 1964). The existence of the metal transferring proteins, transferrin and caeruloplasmin, point to a special affinity of these proteins for the polyvalent ferric and cupric ions. We obviously cannot make a rule about cation binding. Furthermore, it is common to find that isoelectric proteins bind anions (such as chloride) more strongly than cations and that the differences in binding of different anions is quite striking. The likely explanation is that ion binding by proteins is not due solely to Coulomb attraction but is also influenced by the polarisability of both the ions and the protein. It is just not possible at present to establish any general rule for ion binding and we

must recognise that this could be a significant effect in reducing the frequency of complex formation. However, since the local peculiarities are likely to be restricted to the receptor, ion occupancy should have a uniform effect on the whole series of drugs combining with the receptor and which interact with the ionic site.

THE EFFECT OF HYDRATION

The problem of water binding is even more difficult to evaluate. We know that proteins bind quite large amounts of water of the order of several molecules per residue and there is no doubt that water is bound on drugs too. Binding by hydrogen bonding sites is the most obvious mechanism but attraction by other dipolar forces must also occur. There is also little doubt that water is eliminated when some aggregates form. For instance, water displacement has been studied quantitatively in the aggregation of collagen monomers. Indeed, it is more accurate to write the overall reaction between a drug and receptor in the form shown in equation (13).

$$\begin{array}{ccc} D \cdot mH_2O \rightleftharpoons D \cdot nH_2O + (m - n)\,H_2O & & \\ \searrow & & \\ & R \cdot D \cdot (n + y)\,H_2O \;\ldots\; \ldots & (13) \\ \swarrow & & \\ R \cdot xH_2O \rightleftharpoons R \cdot yH_2O + (x - y)\,H_2O & & \end{array}$$

In general, we can expect the number of water molecules involved in hindrance of complex formation to be a function of both the total area of molecular interaction and also to be dependent on groups with a particular affinity for water molecules.

The probability of both drug and receptor sites being optimally hydrated at the time of collision is

$$P = (1 - \pi)^{\frac{(m + x) - (n + y)}{\pi}} \quad \ldots \quad \ldots \quad \ldots \quad (14)$$

(where π is the probability of finding an average binding site occupied by a water molecule).

Let us assume, for example, that $\pi = 0{\cdot}5$ then if

$$(m + x) - (n + y) = 4,\ P = 0{\cdot}004 \text{ and if}$$

$$(m + x) - (n + y) = 8,\ P = 0{\cdot}000015.$$

It is obvious that interference by hydration can be a very serious problem.

It may help to consider the operation of the hydration effect in another way. Suppose the interaction of two groups forming the complex is by dispersion forces, the interposition of a water molecule with a diameter of about 1·9 Å will reduce the free energy of interaction to about one tenth (ignoring the possibility of three body interactions and the malpositioning effect on other group interactions).

The effect of hydration will obviously be mitigated if despite the presence of bound water a weak complex can be formed with a lifetime which is large compared with that of water binding. This possibility will be considered later.

Koshland (1959) has suggested that where substrates and inhibitors combine with enzymes, the complex may not be the result of combination between the reactants in their initial state but that it may be induced by intermolecular forces. Evidence in support of this has been found in a number of cases, and the idea is obviously applicable to drug-receptor complexes and accords with the most widely held theory of agonist action, i.e. that agonists are capable of inducing conformation changes in the receptors. If such an effect occurs it will reduce the rate of complex formation below that for diffusion limited reactions according to the activation energy of the induced change and may be evaluated by equation (5). The presence of induced fit will be improbable if the overall activation energy is not greater than that expected for diffusion alone.

ACCESS TO THE RECEPTOR

Finally, we must consider the problem of geometrically hindered access to the receptor. Suppose that the receptor lies at the bottom of a crevice on the cell membrane. Diffusional access to the site may be severely hindered and in particular it may be impossible for malpresented molecules or molecules in the inappropriate rotational conformation to gain access. It is possible to set up model situations and evaluate the reduced frequency of complex formation on the theory of rotational diffusion outlined above.

The dynamics of the drug-receptor complex

It is usual to regard the total interaction of complex molecules with each other to be the algebraic sum of the interactions of the molecular subentities. Linear combination of forces has had a striking success in molecular physics despite the implicit simplification and this is an adequate reason for continuing this approach. The range of forces operating in molecular interactions include ion-ion, ion-dipole, dipole-dipole, hydrogen bonds (which may be regarded either as a variety of dipole-dipole force or of charge-transfer complex as Mulliken & Person, 1962, have suggested), dispersion forces and hydrophobic forces* and van der Waals' repulsion. Since detailed discussions of these forces have been provided recently by Webb (1963) and Gill (1965) it is only necessary to consider here some special aspects of these forces.

Dispersion forces had their origin in studies of gas reactions and particularly in the consideration of the thermodynamic properties of the inert gases and there have long been misgivings about their magnitude in liquids. McLachlan (1965) has recently been able to calculate their magnitude in liquids by a rigorous method and similar calculations have been made by Kestner & Sinanoglu (1965). The conclusion is that these forces are little reduced by solvent. The latter authors estimate that the interaction in water is 70–85% of that in the gas phase. The view that these forces are solely related to distance as r^{-6} has also been modified as evidence of r^{-4} and r^{-8} terms has been found (Buckingham, 1965);

* Dipole-induced dipole forces are too feeble to contribute appreciably to drug interactions.

the dispersion forces are therefore not necessarily as short range as was previously believed. The nature of hydrophobic forces is due for some revision because of spectroscopic evidence by Stevenson (1965) which demonstrates that liquid water contains very little monomeric water: this necessitates a revision of the thermodynamic basis of the hydrophobic bond presented by Marchi & Eyring (1964) and by Nemethy & Scheraga (1962). This also seems to lead to a reassessment of the role of molecular association through hydrogen bonds. Recently the role of hydrogen bonds has been minimised because of hasty assumptions of the competition of water hydrogen bonds with other intermolecular hydrogen bonds. This is at variance with the evidence for strong hydrogen bond participation in protein α-helix formation produced by Linderstrom-Lang and his collaborators. The virtual non-exchangeability of helix bonding hydrogens with deuterium does not agree with the postulation of the weakness of these bonds and of active competition by solvent.

While these facts must be taken into account in assessment of the binding capacities of different parts of drug molecules with the receptor, they need not concern us in developing the theory of the drug-receptor complex and its dissociation. All the forces mentioned above are extensive in space and with certain exceptions constitute a family of attractive forces obeying equation (8) in which the value of $p = 3$–8. The universal repulsive force is that due to invasion of the van der Waals' envelope of atoms and for which p has a value of 9–12; in some interactions, presumably not in strong ones, repulsion may also be due to like sign ions and dipoles. The net result of all these forces can be represented by potential energy contours of the type illustrated in Fig. 2.

The minimum of the energy diagram corresponds to the most stable position of the drug in the composite force field of the drug and receptor and is referred to as the equilibrium position. Closer approach is restrained by the steeply rising repulsive field and loss is retarded by the attractive field.

It must not be imagined, however, that the drug is held immobile in the equilibrium position. The drug molecules are subject to thermal agitation and acquire kinetic energies distributed according to the Boltzman law.

The probability of finding a drug molecule at any particular locus within the force field can be calculated therefore from the Boltzman law and is shown in Fig. 3. The meaning of this can best be understood by considering the behaviour of a drug molecule found at $t = 0$ at the equilibrium distance. If the drug molecule acquires a kinetic energy U kcal/mole it will move away from the equilibrium position. For simplicity we will consider motion in a single plane, i.e. either towards or away from the receptor. The drug molecule will continue to move until its initial kinetic energy is balanced by the increased potential energy of its new location. It will then come to rest and the potential energy gradient will then return the molecule to the equilibrium distance (Fig. 3). The same process will apply whether the drug travels away from the receptor or towards it, although in the latter case it will travel a shorter

distance before coming to rest (for any value of the kinetic energy) because of the steeper rise of the field with distance on this side of the equilibrium position. A drug will be able to escape from the force field only if the kinetic energy is greater than the potential energy of the field (i.e. in the example shown in Figs 2–3 the kinetic energy must be greater than −10 kcal/mole). The frequency with which molecules exceed a given kinetic energy is given by the Boltzman equation

$$f = e^{U/RT} \qquad (15)$$

Inserting the value of U = −10 kcal/mole, we find that the probability of a molecule escaping is only 10^{-7} of its remaining in the field.

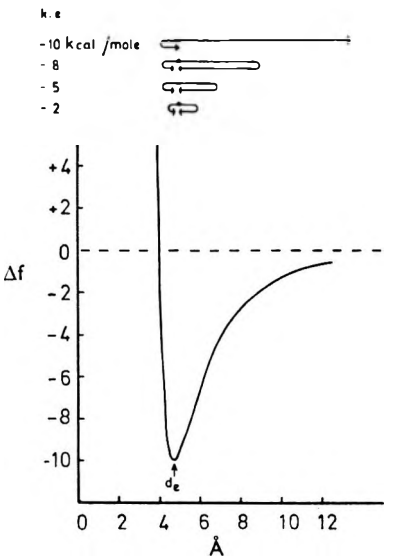

FIG. 2. Potential energy diagram for drug-receptor interaction. The curve has been calculated assuming an equilibrium distance between atomic centres of 5·0 Å and a free energy of interaction of −10 kcal/mole. The attractive forces have been divided equally among r^{-3} and r^{-6} forces, and the repulsive force has been assumed to vary as r^{-9}. Ordinate Δf in kcal/mole. The abscissa is the separation between the atomic centres (Å). In the upper part of the diagram there is shown in a schematic simplified way the behaviour of molecules initially at the equilibrium distance The arrows indicate their behaviour on acquiring kinetic energy as indicated in the direction normal to the receptor surface.

This relationship is the main determinant of the inverse dependence of the strength of drug-receptor complexes and the velocity of their dissociation. The absolute rate of dissociation will be the free diffusion rate away from the receptor multiplied by this probability factor.

The relatively prolonged residence of drug molecules in the force field is the reason for not expecting the formation of effective complexes to be seriously reduced by collision in the wrong presentation or conformation. Provided that the free energy of interaction is strong enough in these cases to ensure that the molecule remains within the field long enough to rotate to the correct presentation or conformation it can then

develop the full potentialities of the field. An idea of the time required for presentation in all aspects may be derived from equation (9) and is about 4×10^{-9} sec. The period of rotation of hindered bonds is about 10^{-9}–10^{-11} sec. The lifetime of the complex for comparison may be evaluated as follows. Let the equilibrium free energy for the correct conformation and presentation be −10 kcal/mole and let us assume that in the incorrect approach this is reduced to −2 kcal/mole. If the rate of

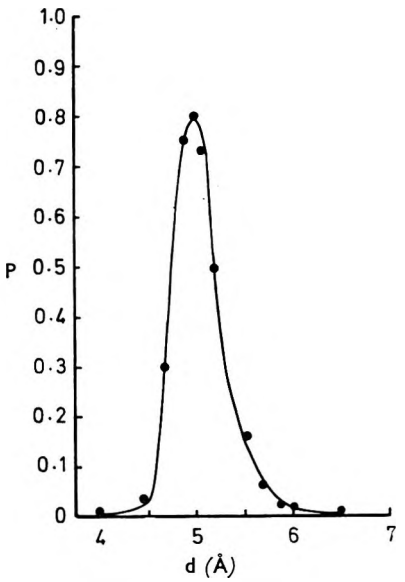

FIG. 3. From the potential energy diagram of Fig. 2 and the Boltzman distribution of energies [equation (15)] one can calculate the probability (P) of finding drug and receptor separated by a given distance (d) at any instant in the lifetime of the drug-receptor complex.

association is the maximum permitted by diffusion then the lifetime of the complex will be 10^{-8} sec; it is actually likely that the rate of association will be smaller than this (see Section 3) and the lifetime of the complex will be much longer. In any case the lifetime of the complex is long enough to reduce to minor proportions the effect of wrong presentation. An exception to this rule is likely to occur only when the receptor is buried in a crevice on the receptor surface or when the drug is very asymmetrical when adjustment of fit will be restricted.

The application of the theory of complexes to experimental results

It is unfortunate that very little work on the kinetics of drug receptor interactions or on molecular associations with proteins has been carried out so that this section must perforce be brief.

We will consider first the reaction between haptens and antibody to form complexes. Day, Sturtevant & Singer (1963) used a fast reaction technique to measure the kinetics of association of 2,4-dinitrophenyllysine

and an antibody against dinitrophenylalbumin. k_1 was found to be 8×10^7 litres/mole sec: similar rates were found with ϵ-dinitrophenyl-aminocaproate and dinitrophenylazo-1-naphthol 3,6-disulphonic acid. Similar rates have also been found by Froese, Sehon & Eigen (1962) and Froese & Sehon (1965) for nitrophenyl and phenyl arsonic haptens (Table 1). These values for association rates are between 1–10% of the maximum expected for a diffusion limited reaction. For the dinitrophenyl antibody the activation energy $E_a = 4{\cdot}1 \pm 1{\cdot}0$ kcal/mole, i.e. not significantly more than expected for a diffusion limited reaction. It is unlikely, therefore, that an induced fit operates in this case.

TABLE 1. KINETIC CONSTANTS FOR HAPTEN-ANTIBODY COMPLEXES

Antibody determinant	K litres/mole	k_1 litres/mole sec	k_2 sec^{-1}
2,4-Dinitrophenyl	$7{\cdot}3 \times 10^7$	8×10^7	1·1
4-Nitrophenyl	$2{\cdot}4 \times 10^5$	$1{\cdot}8 \times 10^8$	760
4-Phenylarsonic	4×10^5	2×10^7	50

The most reliable data for a drug receptor at present available are those of Paton (1961) and Paton & Rang (1965) obtained on the smooth muscle of guinea-pig ileum. These data, which were obtained both from studies of antagonism and by measurement of the uptake of tritiated antagonists, are summarised in Table 2. The association constants are smaller by another order of magnitude than for the haptens but are still

TABLE 2. KINETIC CONSTANTS FOR ANTAGONIST-MUSCARINIC RECEPTOR COMPLEXES

Antagonist	K litres/mole	k_1 litres/mole sec	k_2 sec^{-1}
Atropine	$0{\cdot}98 \times 10^9$	$1{\cdot}76 \times 10^6$	$1{\cdot}79 \times 10^{-3}$
N-Methylatropine	$2{\cdot}1 \times 10^6$	$3{\cdot}50 \times 10^6$	$1{\cdot}67 \times 10^{-3}$
Lachesine	$0{\cdot}70 \times 10^6$	$2{\cdot}60 \times 10^6$	$3{\cdot}73 \times 10^{-3}$

of an order expected for a diffusion limited reaction; it is regrettable that no data are available for the activation energy. The complexes formed between antagonist and the muscarinic receptor are exceptionally strong and have very low dissociation rates. Now the free diffusion rate away from a plane source into an infinite medium is given by

$$C = \frac{A}{(\pi Dt)^{\frac{1}{2}}} \cdot e^{-x^2/4Dt} \quad .. \quad .. \quad .. \quad (15)$$

We can calculate the half time for atropine to diffuse the distance of one molecular radius from the receptor by free diffusion; this is found to be 3×10^{-10} sec. The dissociation rate constant is therefore $2{\cdot}3 \times 10^9$ sec^{-1}. The actual rate constant should be this value multiplied by the Boltzman probability, in this case $1{\cdot}02 \times 10^{-9}$. The value of k_2 therefore equals 2·3 sec^{-1}. This is much higher than the experimental value. The reason for this discrepancy is to be found in the low association rate compared with the rate given by free diffusion. This low rate can be attributed most probably to either (*a*) the reduced number of effective collisions due to hydration or ion occupation effects or (*b*) geometrical restrictions to diffusion. In the first case the free energy of complex

formation is increased because it should be related to the effective concentration in the bulk solution rather than the actual concentration. This is equivalent to increasing the free energy of association by −4·5 kcal/mole. The Boltzman factor then becomes $7{\cdot}2 \times 10^{-13}$ and the value of $k_2 = 1{\cdot}65 \times 10^{-3}$ sec^{-1}, which is very close to the experimental value. The corresponding theoretical values for *N*-methylatropine and lachesine are $1{\cdot}41 \times 10^{-3}$ sec^{-1} and $3{\cdot}45 \times 10^{-3}$ sec^{-1}. The correspondence of these results is in no way remarkable but simply results from the circular argument which defines diffusion as limiting for both association and dissociation. Since the mathematical approach is slightly different in the two cases it is an internal check on their validity. The second approach, of geometrical hindrance, leads to the same result but for a slightly different reason. Clearly any geometrical hindrance to the diffusion of drug towards the receptor must apply equally to diffusion away from the receptor. The hindrance factor is equal to

$$\frac{k_1}{2{\cdot}5 \times 10^9}$$

and multiplying the value of the crude estimate of k_2 by this factor will give the corrected value of k_2.

The purpose of this paper has been to outline a theoretical basis for drug receptor kinetics and although it leaves a number of quantitative uncertainties unsettled some of these are potentially resolvable by experiment and it is the author's hope that the presence of a theoretical background will encourage further experimental work in this field.

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Some basic ketones with central nervous system depressant activity

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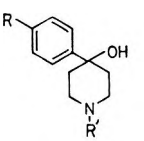
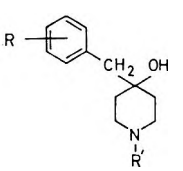
Some γ-(4-benzyl-4-hydroxypiperidino)-butyrophenones and related compounds have been prepared and screened for central nervous system depressant activity. One of the more active, γ-(4-*p*-chlorobenzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone, was selected for further study and in experimental animals was found to have effects on the central nervous system similar to both those of chlorpromazine and of haloperidol.

RECENTLY, some structural modifications of analgesics of the prodine type were prepared in an attempt to produce compounds without analgesic effects but having other potentially useful central nervous system (CNS) depressant activity (Harper & Simmonds, 1959, 1964). Although the alcohols (I; R = F or CF_3, R′ = $[CH_2]_2$·Ph. II; R = *p*-Cl, *o*-Cl or *p*-F, R′ = $[CH_2]_2$·Ph) had significant activity when assessed by a hot-plate test, they did not have the characteristic mydriatic action of the narcotic analgesics. Some of these alcohols were also found to possess a protective effect against amphetamine toxicity in aggregated mice and to block a conditioned avoidance response in rats (unpublished observations).



(I)



(II)

Janssen (1958) and Carrabateas & Grumbach (1962) showed that the introduction of a propiophenone group onto the basic nitrogen atom of analgesics of the pethidine and prodine types gave compounds with greatly increased morphine-like analgesic activity. Lengthening the chain of carbon atoms on the basic nitrogen atom to a butyrophenone group, however, gave compounds with marked activity in the hot-plate test which was not antagonised by nalorphine. The compounds were also without mydriatic activity (Janssen & Eddy, 1960). These observations led to the introduction of tranquillising compounds such as haloperidol, γ-(4-*p*-chlorophenyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone hydrochloride (I; R = Cl, R′ = $[CH_2]_3$·CO·C_6H_4·F-*p*) (Janssen & others, 1959).

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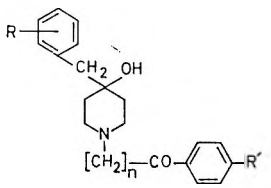
It thus became of interest to prepare propiophenones and butyrophenones of Type II and to test these for their effects on the CNS. Preliminary investigation of the pharmacological actions of these propiophenones (III; $n = 2$; R = *p*-Cl; R′ = H or F) and butyrophenones (III; $n = 3$; R = *p*-Cl; R′ = H, Br, Cl, F, OMe or Me. R = *p*-F; R′ = F. R = 2,4-Cl_2; R′ = Cl or F. R = 3,4-Cl_2; R′ = Cl or F), together with some related compounds, is now reported. These studies resulted in the selection of γ-(4-*p*-chlorobenzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone for further investigation.



(III)

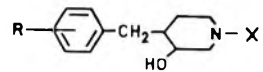
Chemistry

The compounds of type III ($n = 3$) were prepared by the addition of the appropriate Grignard reagent to 1-benzyl-4-oxopiperidine; catalytic debenzylation gave the secondary bases, which were reacted with the appropriate γ-chlorobutyrophenone by heating in toluene in the presence of sodium bicarbonate and a trace of potassium iodide. γ-(4-*p*-Chlorobenzyl-4-hydroxy-3-methylpiperidino)-*p*-fluorobutyrophenone was prepared in this way from 1-benzyl-3-methyl-4-oxopiperidine. Benzylmagnesium halides have been shown in some instances to give rise to the isomeric *o*-tolyl derivatives (Austin & Johnson, 1932). Such rearrangements did not occur in the present investigations and with compound III ($n = 3$; R = 3,4-Cl_2, R′ = F) oxidative degradation studies identified the structure of the Grignard addition product as 1-benzyl-4-(3,4-dichlorobenzyl)-4-hydroxypiperidine.

p-Fluoro-γ-[4-hydroxy-4-(pyrid-2-ylmethyl)piperidino]-butyrophenone was prepared from 1-benzyl-4-oxopiperidine and pyrid-2-ylmethyl-lithium (obtained by exchange from α-picoline and phenyl-lithium), followed by debenzylation and condensation with γ-chloro-*p*-fluorobutyrophenone.

β-(4-*p*-Chlorobenzyl-4-hydroxypiperidino)propiophenone was prepared by a Mannich base exchange reaction from 4-*p*-chlorobenzyl-4-hydroxypiperidine and the methiodide of dimethylaminoethyl phenyl ketone. The corresponding *p*-fluoropropiophenone was prepared by a Mannich reaction between 4-*p*-chlorobenzyl-4-hydroxypiperidine, paraformaldehyde and *p*-fluoroacetophenone. β-(4-*p*-Chlorobenzyl-4-hydroxypiperidino)-*p*-fluoro-α-methylpropiophenone was prepared in the same way by a Mannich reaction of 4-*p*-chlorobenzyl-4-hydroxypiperidine, paraformaldehyde and *p*-fluoropropiophenone.

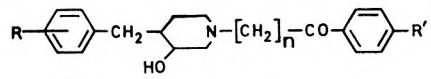
TABLE 1. 4-BENZYL-4-HYDROXYPIPERIDINES



R	X	M.p. °C	Found (%)				Formula	Required (%)			
			C	H	N	Equiv		C	H	N	Equiv
p-Cl	CH_2Ph	215	64·5	6·9	4·2	358	$C_{19}H_{22}ClNO{\cdot}HCl$	64·8	6·6	4·0	352
p-F	CH_2Ph	218	59·7	6·2	3·9	381	$C_{19}H_{22}FNO{\cdot}HBr$	60·0	6·1	3·7	380
O-Cl	CH_2Ph	219	65·2	6·3	4·1	360	$C_{19}H_{22}ClNO{\cdot}HCl$	64·8	6·6	4·0	352
†*p*-Cl	CH_2Ph	206	65·7	7·4	3·8	365	$C_{20}H_{24}ClNO{\cdot}HCl$	65·5	6·9	3·8	366
o,p-Cl_2	CH_2Ph	209	58·7	5·7	3·7	384	$C_{19}H_{21}Cl_2NO{\cdot}HCl$	59·0	5·7	3·6	387
m,p-Cl_2	CH_2Ph	213	58·6	5·8	3·7	390	$C_{19}H_{21}Cl_2NO{\cdot}HCl$	59·0	5·7	3·6	387
2-Pyr*	CH_2Ph	180	67·6	7·3	8·6	320	$C_{18}H_{22}N_2O{\cdot}HCl$	67·8	7·3	8·8	318
p-Cl	H	226	55·5	6·7	5·5	264	$C_{12}H_{16}ClNO{\cdot}HCl$	55·0	6·5	5·3	262
p-F	H	221	50·0	6·3	4·9	294	$C_{12}H_{16}FNO{\cdot}HBr$	49·7	5·9	4·8	290
†*p*-Cl	H	240	56·9	7·1	5·2	270	$C_{13}H_{18}ClNO{\cdot}HCl$	56·5	6·9	5·1	276
o,p-Cl_2	H	138	56·4	5·9	5·4	261	$C_{12}H_{15}Cl_2NO$	55·4	5·8	5·4	260
m,p-Cl_2	H	208	49·6	5·5	4·7	301	$C_{12}H_{15}Cl_2NO{\cdot}HCl$	48·6	5·4	4·7	297
2-Pyr*	H	164	56·8	7·3	12·4		$C_{11}H_{16}N_2O{\cdot}HCl$	57·8	7·4	12·3	

* Phenyl replaced by pyrid-2-yl group. † 3-Methylpiperidino derivative.

TABLE 2. γ-(4-BENZYL-4-HYDROXYPIPERIDINO)BUTYROPHENONES, PROPIOPHENONES AND RELATED COMPOUNDS



No.	R		R′	M.p. °C	Found (%)				Formula	Required (%)			
					C	H	N	Equiv		C	H	N	Equiv
1	*p*-Cl	3	H	192	65·1	6·7	3·4	408	$C_{22}H_{26}ClNO_2$·HCl	64·7	6·7	3·4	408
2	*p*-F	3	F	180	64·5	6·4	3·4	410	$C_{22}H_{25}F_2NO_2$·HCl	64·8	6·8	3·6	410
3	*p*-Cl	3	F	198	62·4	6·5	3·3	427	$C_{22}H_{25}ClFNO_2$·HCl	62·0	6·2	3·3	426
4†	*p*-Cl	3	F	205	62·3	6·5	3·4	437	$C_{23}H_{27}ClFNO_2$·HCl	62·7	6·4	3·2	440
5	*o,p*-Cl_2	3	F	191	57·4	5·6	3·1	461	$C_{22}H_{24}Cl_2FNO_2$·HCl	57·3	5·5	3·0	461
6	*m,p*-Cl_2	3	F	191	56·8	5·6	3·1	459	$C_{22}H_{24}Cl_2FNO_2$·HCl	57·4	5·5	3·0	461
7	2-Pyr*	3	F	171	64·1	6·5	7·4	388	$C_{21}H_{25}FN_2O_2$·HCl	64·1	6·7	7·2	394
8	*p*-Cl	3	Cl	188	60·4	5·9	3·3	444	$C_{22}H_{25}Cl_2NO_2$·HCl	59·7	5·9	3·2	443
9	*o,p*-Cl_2	3	Cl	191	54·6	5·7	3·0	474	$C_{22}H_{24}Cl_3NO_2$·HCl	55·4	5·3	2·9	477
10	*m,p*-Cl_2	3	Cl	147	54·9	5·0	3·1	479	$C_{22}H_{24}Cl_3NO_2$·HCl	55·4	5·3	2·9	477
11	*p*-Cl	3	Br	190	54·2	5·4	3·0	487	$C_{22}H_{25}BrClNO_2$·HCl	54·2	5·4	2·9	487
12	*p*-Cl	3	Me	218	65·8	6·9	3·5	421	$C_{23}H_{28}ClNO_2$·HCl	65·5	6·9	3·3	422
13	*p*-Cl	3	OMe	197	63·8	6·7	3·2	440	$C_{23}H_{28}ClNO_3$·HCl	63·0	6·7	3·2	438
14	*p*-Cl	3	Th‡	174	57·4	6·1	3·2	413	$C_{20}H_{24}ClNO_2S$·HCl	58·0	6·1	3·4	414
15	*p*-Cl	2	H	206	64·0	6·2	3·4	393	$C_{21}H_{24}ClNO_2$·HCl	64·0	6·4	3·5	394
16	*p*-Cl	2	F	197	61·2	5·9	3·5	406	$C_{21}H_{23}ClFNO_2$·HCl	61·2	5·9	3·4	412
17	*p*-Cl	CH_2·CHMe	F	210	62·0	6·1	3·5	428	$C_{22}H_{25}ClFNO_2$·HCl	62·0	6·2	3·3	426

* Phenyl replaced by pyrid-2-yl group. † 3-Methylpiperidino derivative. ‡ Phenyl group replaced by thien-2-yl group.

EXPERIMENTAL

1-Benzyl-4-halogenobenzyl-4-hydroxypiperidines. An ethereal solution of 1-benzyl-4-oxopiperidine (0·25 mole) was added dropwise to a stirred solution of the halogenobenzyl magnesium chloride prepared from halogenobenzyl chloride (0·5 mole) and magnesium (0·55 mole). The mixture was stirred overnight and then poured onto crushed ice and acetic acid. The ethereal layer was washed with water and the combined acidic solutions were made alkaline with ammonium hydroxide solution. Extraction with ether gave an oil which on treatment with ethanolic hydrogen chloride gave 1-benzyl-4-halogenobenzyl-4-hydroxypiperidine (yield 50–60%) (Table 1).

4-Halogenobenzyl-4-hydroxypiperidines. A solution of 1-benzyl-4-halogenobenzyl-4-hydroxypiperidine (40 g) in ethanol (1 litre) was shaken with hydrogen in the presence of palladium on charcoal (10%, 10 g) at 40° until the theoretical amount of hydrogen had been absorbed. The mixture was filtered through kieselguhr and the filtrate concentrated under reduced pressure. Dilution with ether gave crystals of the debenzylated product in almost quantitative yield (Table 1).

γ-(4-Halogenobenzyl-4-hydroxypiperidino)butyrophenones. A mixture of 4-halogenobenzyl-4-hydroxypiperidine (0·5 mole), sodium hydrogen carbonate (0·8 mole), *γ-chlorobutyrophenone* (0·55 mole) and a trace of potassium iodide in toluene (500 ml) was heated under reflux for 3 days. The mixture was then filtered, the solids washed with ether, and the combined filtrates were made acid with hydrochloric acid. Evaporation of the solvents gave γ-(4-halogenobenzyl-4-hydroxypiperidino)butyrophenone hydrochloride. Compound 14 (Table 2) was similarly prepared from γ-chloro-2-butyrylthiophen.

β-(4-Halogenobenzyl-4-hydroxypiperidino)propiophenones. The appropriate secondary amine hydrochloride (1 mole), paraformaldehyde (3 mole), a few drops of hydrochloric acid, and *p*-fluoroacetophenone (for compound 16) or *p*-fluoropropiophenone (for compound 17) (1 mole) in isopropanol were heated together under reflux for 3 hr. Evaporation of the solvent gave the Mannich bases (compounds 16 and 17) as their hydrochlorides.

β-(4-p-Chlorobenzyl-4-hydroxypiperidino) propiophenone. Dry nitrogen was passed through a mixture of 4-*p*-chlorobenzyl-4-hydroxypiperidine (extracted from its hydrochloride, 5·2 g), sodium carbonate (1 g), 2-dimethylaminopropiophenone methiodide (7 g) and formdimethylamide (25 ml) until no more trimethylamine was evolved. The mixture was diluted with water (50 ml). Extraction with ether gave an oil which on treatment with ethanolic hydrogen chloride gave β-(4-*p*-chlorobenzyl-4-hydroxypiperidino)propiophenone hydrochloride.

All salts were recrystallised from ethanol except *p*-fluoro-γ-[4-hydroxy-4-(pyrid-2-ylmethyl)piperidino]butyrophenone hydrochloride which was recrystallised from isobutyl methyl ketone. Equivalent weights of the salts were determined by titration with 0·02N perchloric acid in acetic acid in the presence of mercuric acetate.

Pharmacology

METHODS

General. Male Schofield albino mice, 18–24 g but with a maximum weight range of 4 g in any one test, male Wistar albino rats, 130–150 g, male cats, 2–4 kg, and mongrel dogs of either sex, about 10 kg, were used. Unless otherwise stated, the compounds, dissolved or suspended in 5% acacia, were administered orally to groups of five animals, the dose volume being 25 ml/kg body weight. The compounds were initially studied at a dose corresponding to about 40% of the acute LD50, those effective in all animals then being re-examined at varying dose levels in parallel with a suitable reference compound. ED50 values were calculated using Kärber's (1931) method.

Acute toxicity. Approximate LD50 values were determined by inspection from the mortalities occurring within 7 days of oral or subcutaneous administration, two animals being used at each dose level.

Hot plate test (after Eddy & Leimbach, 1953). At 30, 60 and 90 min after giving the compounds by the subcutaneous route the mice were placed in turn on a hot plate at 55–56°. "Analgesia" was considered present if the animal failed to show any signs of discomfort (as judged by raising, shaking or licking of the hind paws) within 30 sec. The ED50 was estimated from the numbers of animals showing "analgesia" at one or more observation times.

Tail pinch test (after Bianchi & Franceschini, 1954). Only mice which, in a preliminary test, made repeated attempts within 15 sec to remove the rubber covered bulldog clip were used for the test. The clip was then applied for 30 sec to each mouse in turn at 30, 60 and 90 min after the subcutaneous administration of the compounds. "Analgesia" was considered present if no attempt was made to remove the clip. The ED50 was estimated from the numbers of animals showing "analgesia" at one or more observation times.

Nalorphine antagonism. Four groups of 10 mice were injected subcutaneously with γ-(4-*p*-chlorobenzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone (5 mg/kg). The animals in three of the groups were injected at the same time with nalorphine at dose levels of 50, 100 and 200 mg/kg. 30 min later, the presence of analgesia was determined by the hot plate method as described above.

Prevention of maximal electroshock seizures (after Swinyard, Brown & Goodman, 1952). At 1, 2 and 3 hr after giving the compounds, an electroshock (25 mA, 50 cps AC for 0·2 sec, just sufficient to produce seizures characterised by tonic extension of fore and hind limbs in untreated animals) was applied to each mouse in turn via corneal electrodes. The ED50 was estimated from the numbers of animals in which the hind leg tonic extensor component of the normal seizure pattern was prevented at one or more observation times.

Prevention of maximal leptazol seizures (after Goodman, Grewal, Brown & Swinyard, 1953). Two hr after giving the test compounds, each mouse was injected intravenously with 0·2 ml/20 g body weight of a 0·6% aqueous solution of leptazol. This dose was just sufficient to

produce seizures characterised by tonic extension of fore and hind limbs in untreated mice. The ED50 was estimated from the numbers of animals in which the hind leg tonic extensor component of the normal seizure pattern was prevented.

Protection against tremorine-induced tremors (after Everett, 1956). The compounds were given to mice deprived of food for 18 hr previously. One hr later, an aqueous solution of tremorine (30 mg/25 ml/kg body weight) was injected intraperitoneally. This dose was shown to produce characteristic tremors in untreated mice. Each animal was observed for a period of 15 min after the tremorine injection and the ED50 was estimated from the numbers of animals without tremors.

Protection against amphetamine toxicity in aggregated mice (after Lasagna & McCann, 1957). The compounds were given to groups of 10 mice deprived of food for 18 hr previously. One hr later, the mice were injected subcutaneously with an aqueous solution of (±)-amphetamine sulphate, 20 mg/kg body weight. They were then placed in a constant temperature cabinet maintained at approximately 27°, food and water being provided. Most or all of a group of control animals injected with amphetamine sulphate alone and kept under similar conditions died within 24 hr. The number surviving in each treated group was recorded after 24 hr and the ED50 estimated.

Blockade of a conditioned avoidance response (after Cook & Weidley, 1957). Previously conditioned rats were given the compounds after being deprived of food for 18 hr. At 1, 2 and 3 hr after dosing, the animals were placed in the test chamber and subjected to the sound of the buzzer. Failure to climb the pole within 30 sec indicated blockade of the conditioned response. They were then subjected to the shock and buzzer together for 30 sec and if they then failed to climb the pole, the unconditioned response was also considered to be blocked. The ED50 for blockade of the conditioned response was estimated from the numbers of animals failing to respond at one or more observation times. The ED50 for blockade of the unconditioned response was estimated similarly.

Production of catalepsy. 2½ hr after giving the compounds, each rat was removed gently and quietly from its cage and placed with its feet on four corks spaced at suitable intervals. The ED50 was estimated from the numbers of animals considered cataleptic as evidence by failure to assume normal posture within 5 min.

Cardiovascular effects. The carotid blood pressure and contractions of the nictitating membrane were recorded in a cat anaesthetised with chloralose (80 mg/kg, intravenously) and in a dog anaesthetised with pentobarbitone sodium (30 mg/kg, intravenously). Responses were obtained to preganglionic stimulation of the vagus and cervical sympathetic nerves and to intravenous injection of adrenaline (both species) and noradrenaline (cat only), before and after the intravenous injection of varying amounts of γ-(4-*p*-chlorobenzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone in aqueous solution.

Behavioural studies. Varying amounts of γ-(4-*p*-chlorobenzyl-4-

hydroxypiperidino)-*p*-fluorobutyrophenone were administered by mouth in gelatin capsules to male cats (two per dose) and by stomach tube in 5% acacia to dogs (one male and one female per dose). Changes in behaviour and condition of the animals were recorded on the day of the experiment and, where necessary, on subsequent days.

Results

Table 3 summarises the results of the investigations of the CNS depressant activity of the propiophenones (III; $n = 2$; R = *p*-Cl; R′ = H or F) and butyrophenones (III; $n = 3$; R = *p*-Cl; R′ = H, Br, Cl, F, OMe or Me. R = *p*-F; R′ = F. R = 2,4-Cl_2; R′ = Cl or F), together with some related compounds. The activities of the more potent γ-(4-benzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenones relative to those of suitable reference compounds are shown in Table 4. The results of certain additional investigations on a representative compound are described below.

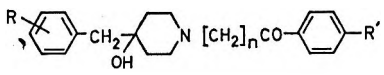
Nalorphine antagonism. The activity of γ-(4-*p*-chlorobenzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone in the hot-plate test was not antagonised by nalorphine in doses up to 200 mg/kg.

Cardiovascular effects. γ-(4-*p*-Chlorobenzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone, injected intravenously, has a blocking action on the sympathetic nervous system of both the cat and the dog at doses of 0·1 mg/kg and above. This is probably not due to ganglionic blockade for, although the responses to pre-ganglionic stimulation of the cervical sympathetic nerve were reduced, those to vagal stimulation were unaffected, even when the dose was increased to 1 mg/kg. As the pressor responses to injected adrenaline in the cat were inhibited at low and reversed at high doses, those to noradrenaline also being inhibited, it appears that the compound has α-adrenergic receptor blocking activity. The pressor responses to injected adrenaline were also inhibited in the dog.

Behavioural studies. γ-(4-*p*-Chlorobenzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone in doses of 5–40 mg/kg caused ataxia in the cat, this being due to incoordination rather than hypotonia. The animals in general then adopted a crouching position with the fore-legs extended in front of the head, with the claws also fully extended. Three animals were seen to paw repeatedly at imaginary objects immediately in front of them, after which they backed away in an apprehensive manner. This suggested a possible hallucinogenic effect. Intermittent clonic seizures ensued at the highest dose, with tremors at lower doses. All animals subsequently recovered, usually within 24 hr.

Ataxia and tremors were also observed initially in the dog at 5 and 10 mg/kg, again with no evidence of muscle weakness. The animals subsequently assumed a position similar to that adopted by the cats, with head and body on the ground and the forelegs extended forwards on either side of the head. Complete prostration, but without loss of consciousness, ensued in the male given 10 mg/kg and this animal did not fully recover until 3–4 days after dosing. Only minimal effects were observed at 2·5 mg/kg.

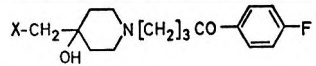
TABLE 3. CNS DEPRESSANT PROPERTIES OF γ-(4-BENZYL-4-HYDROXYPIPERIDINO)BUTYROPHENONES, PROPIOPHENONES AND RELATED COMPOUNDS



No.	R	n	R'	ED50, mg/kg								
				Block of conditioned avoidance response	Block of uncon-ditioned response	Protection against amphetamine toxicity	Prevention of tremorine-induced tremors	Hot-plate test	Prevention of maximal electroshock seizures	Prevention of maximal leptazol seizures	Production of catalepsy	Tail Pinch test
1	*p*-Cl	3	H	<100		<60	47	6·7				
2	*p*-F	3	F	4·6		0·35	1·3	3·0	Inactive	Inactive		
3	*p*-Cl	3	F	8·1, 3·1	28, 25	0·8	2·3, 2·5	1·5	Inactive	Inactive	9·2	2·2
4†	*p*-Cl	3	F	11		1·2	9·8	4·3	Inactive	Inactive		
5	2,4-Cl_2	3	F	6·2		3·5	31	6·2	Inactive	Inactive		
6	3,4-Cl_2	3	F	5·4		0·4	3·7	4·0	Inactive	8·5		
7	2-Pyr*	3	F	Inactive		4·0	13	18	Inactive			
8	*p*-Cl	3	Cl	56		Inactive	31	Inactive	Inactive	Inactive		
9	2,4-Cl_2	3	Cl	Inactive		Inactive	Inactive	Inactive	28	Inactive		
10	3,4-Cl_2	3	Cl	Inactive		<20	Inactive	Inactive	<27	Inactive		
11	*p*-Cl	3	Br	Inactive		Inactive	Inactive	Inactive	28	Inactive		
12	*p*-Cl	3	Me	Inactive		Inactive	Inactive	27	Inactive	Inactive		
13	*p*-Cl	3	OMe	Inactive		7·6	Inactive	Inactive	Inactive	Inactive		
14	*p*-Cl	3	Th‡	27		27	41	13	Inactive	Inactive		
15	*p*-Cl	2	H	Inactive		Inactive	Inactive	Inactive	45	53		
16	*p*-Cl	2	F	Inactive			Inactive	Inactive	20	Inactive		
17	*p*-Cl	CH_2·CH(Me)§	F			52						
Haloperidol				1·2	4·9	0·3	6·5	2·5				

† 3-Methylpiperidino derivative • Phenyl group replaced by pyrid-2-yl group ‡ Phenyl group replaced by thien-2-yl group § $[CH_2]n$ replaced by CH_2·CH(Me)

TABLE 4. COMPARISON OF γ-(4-BENZYL-4-HYDROXYPIPERIDINO)-*p*-FLUOROBUTYROPHENONES IN SOME CNS-DEPRESSANT TESTS



Compound No.	X	Relative activity						
		Block of conditioned avoidance response Chlorpromazine = 1·0	Block of unconditioned response Chlorpromazine = 1·0	Protection against amphetamine toxicity Chlorpromazine = 1·0	Prevention of tremorine -induced tremors Atropine = 1·0	Hot plate test Pethidine = 1·0	Tail pinch test Pethidine = 1·0	Production of catalepsy Chlorpromazine = 1·0
3	*p*-Cl.C_6H_4	1·3	0·8	2·5	3·6	10·0	4·0	1·7
5	2,4-Cl_2:C_6H_3	1·5		0·3	0·2	3·5		
6	3,4-Cl_2:C_6H_3	2·0		5·0	2·0	3·4		
4	*p*-F·C_6H_4	1·5		7·0	8·0	6·0		
7	pyrid-2-yl	Inactive		0·9	0·5	1·5		
Haloperidol ..	.. .. ..	*2·6	*5·0	16·0	*0·4	7·0	—	—
Chlorpromazine	.. .. ..	—	—	—	2·4	13·0		

* relative to Compound No. 3 = 1·0

Discussion

γ-(4-*p*-Chlorobenzyl-4-hydroxpiperidino)-*p*-fluorobutyrophenone shows effects on the CNS similar to both those of chlorpromazine and those of haloperidol. It is intermediate in activity between these compounds in protecting against amphetamine toxicity in aggregated mice and in blocking a conditioned avoidance response in rats. It appears, however, to have greater specificity with regard to the latter property in that the ratio of the ED50 for blockade of the unconditioned response to that for blockade of the conditioned response is greater than with chlorpromazine or haloperidol. It is also more active than either of these drugs in preventing tremors induced by tremorine and in the hot-plate test. Despite its high activity in the hot-plate test, and in the tail pinch test, it is probable that the compound is not a morphine-like analgesic since nalorphine does not antagonise its activity in the hot-plate test. Its resemblance to chlorpromazine has been further demonstrated by the production of catalepsy and by its action in blocking α-adrenergic receptors.

A high order of activity has also been shown for other members of the series, particularly the γ-(4-benzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenones listed in Table 2. It is evident that relatively small changes in activity result from variations in the substituent in the phenyl ring of the benzyl group. Introduction of a methyl group in position 3 of the piperidyl group of γ-(4-*p*-chlorobenzyl-4-hydroxypiperidino)-*p*-fluorobutyrophenone also had little effect on overall activity. The essential nature of the *p*-fluorobutyrophenone moiety is demonstrated by the marked reduction or complete loss of activity observed on replacing the F atom by a H, Cl or Br atom or by a Me or OMe group, on replacing the whole moiety by a 2-butyrylthiophen or a *p*-fluoroisobutyrophenone moiety, and on shortening the polymethylene chain.

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Some size distributions of particulate contamination found in commercially available intravenous fluids

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The size distributions of particulate contamination found in some commercially available normal saline solutions and dextrose solutions have been determined using the Coulter Counter. Many of these solutions contain considerable numbers of particles larger than the equivalent volume diameter of erythrocytes. Earlier observations that most of the particulate contamination emanates from the rubber closure were confirmed by repeated autoclaving of saline in a rubber-plugged glass container. It is possible to prepare solutions substantially free of particles larger than 5 μ, and commercial material of this quality is available. A tentative standard is proposed and discussed.

A RECENT *Lancet* annotation (1965) discussed the particulate contamination of intravenous injection fluids and drew attention to the work of Garvan & Gunner (1963, 1964). These authors detected particles suspended in the fluids by means of a dark ground illumination technique and described a membrane filtration method for the collection of the contamination before subsequent microscopic examination and identification. They pointed out that official standards for particles in intravenous fluids are entirely inadequate, and made suggestions for the allowable limit of contamination.

Schmitt (1964) also described a similar membrane filtration technique. He concluded that the major disadvantages of the method were that it was difficult to attain and retain control standards, and that the microscopical scanning was slow and tedious.

Groves & Major (1964), discussing methods of assessing particulate contamination, suggested that the Coulter Counter was an ideal instrument for this purpose in electrolytes. Normal saline for injection from commercial sources was examined and shown to vary widely in quality. This experimental work has now been extended to other intravenous fluids and the particle size of the particulate contamination investigated.

Experimental

MATERIALS AND METHODS

The experimental method is based on that described by Groves & Major (1964). A Coulter Counter Model A (Industrial) was employed, fitted with a 70 μ orifice tube. The instrument was previously calibrated with pollens and monosize polystyrene emulsions of known particle diameter (equivalent volume diameter). Counts were made of each sample at instrument thresholds corresponding to known sizes. As the instrument counts all particles above a threshold, the threshold was progressively raised from 1·5 μ until the count fell to a value of 20 particles/ml. The results are given as cumulative over-size curves, plotting the logarithm of the count/ml as a function of the threshold (particle diameter).

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Results

NORMAL SALINE

Groves & Major (1964) reported the counts of particles above 1·3 μ. Size distributions obtained on samples from similar commercial sources are shown in Fig. 1.

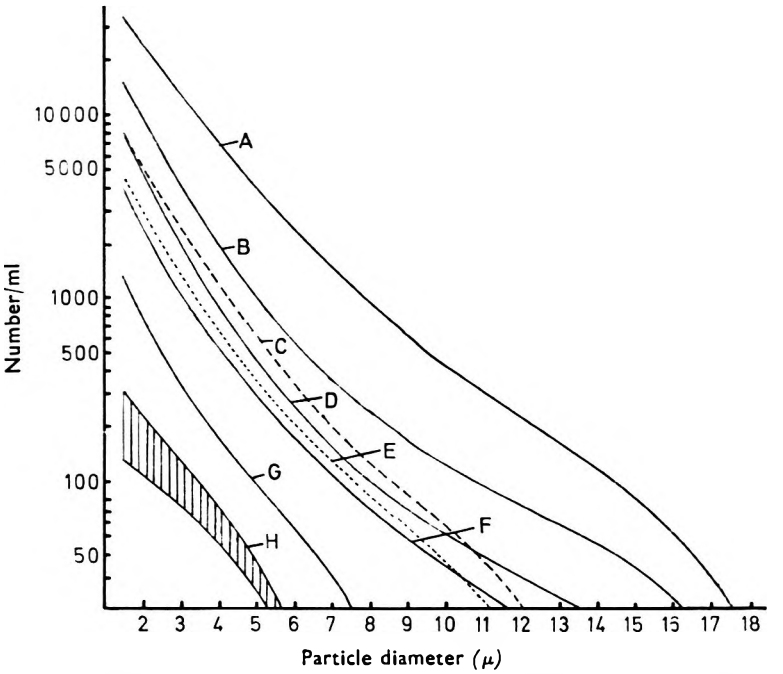

FIG. 1. Particle size distributions of particulate contamination in normal saline. A. Plastic bag, with giving set (counts of saline taken directly from bag identical to those taken after passage through the giving set). B, C, D. Rubber-plugged glass bottle, three samples from same source. E. Rubber-plugged glass bottle. F. Plastic bottle, rubber over-seal. G. Plastic bag, rubber closure. H. Range of counts found in saline from plastic ampoules (Polyfusor).

DEXTROSE (5%)

Dextrose solutions do not contain electrolyte, which must therefore be added before examination on the Counter. A 4·0% sodium chloride solution was passed repeatedly through a Millipore G.S. membrane filter and the size distribution of remaining particles measured on the Counter. This solution was diluted 1 in 4 with the dextrose solution under examination and the mixture counted on the instrument as before. If n_s = number of particles/ml above a given size and n_t = number of particles in the saline-dextrose mixture, the number of particles (n_d) present in 1 ml of undiluted dextrose solution is given by

$$n_d = 4/3\ n_t - 1/3\ n_s$$

Some results obtained on commercially available transfusion fluids are shown in Fig. 2.

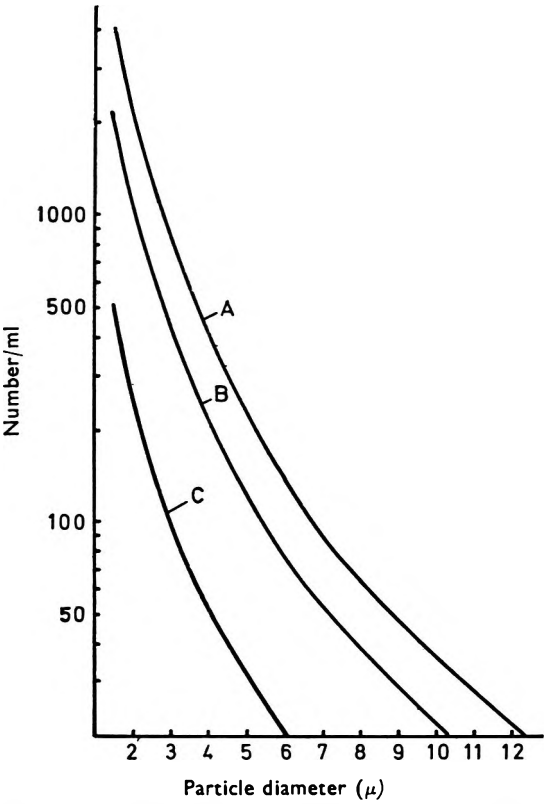

FIG. 2. Particle size distributions of particulate contamination in 5% dextrose solution. A. Rubber-plugged glass bottle (Manufacturer A). B. Rubber-plugged glass bottle (Manufacturer B). C. Plastic ampoule (Polyfusor).

OTHER TRANSFUSION FLUIDS

It was not practicable to examine more than a few of the many different transfusion fluids available. Solutions of electrolytes can be examined directly but non-electrolytes require dilution with electrolyte as described under dextrose. The Counter requires recalibration for each electrolyte system since the conductivity differs, but it was noted that the instrument calibration factors did not vary widely with 0·9% saline or Ringer–Lactate solution. For this investigation it was sufficient to regard the conductivity as being unchanged. Some direct counts, at one threshold only, illustrate the application of the method: particle counts/ml, $>$ ca. 1·5 μ, on miscellaneous intravenous injection fluids, all packed in a plastic ampoule pack (Polyfusor) were, for 1/6M sodium lactate, 696; Hartmanns solution, 398; dextrose saline, 660; sodium sulphate, 495.

THE EFFECT OF REPEATED AUTOCLAVING

New bottles and closures were cleaned by the usual methods and allowed to drain dry. A bulk quantity of filtered normal saline solution

was prepared and filled in 500 ml quantities. For control purposes similar bottles were filled and closed by rubber plugs covered with Saran film (Dow). Plastic ampoules were also filled with the same solution although these are disposable and would normally not be re-used. All the filled containers were sterilised by autoclaving (10 lb/in^2 pressure for 35 min) and total counts were recorded for samples taken from each container between each successive heating. The mean counts from four containers of each type are shown in Fig. 3 as a function of the number of sterilisation cycles.

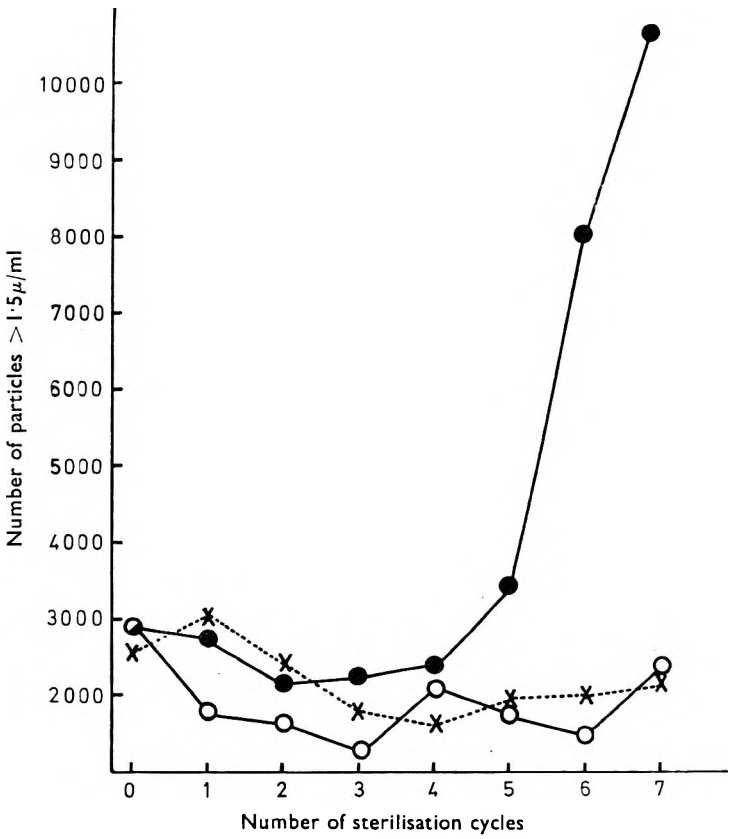

FIG. 3. The effect of repeated autoclaving on the particulate counts of normal saline. ● Rubber-plugged glass bottle. ○ Saran covered rubber-plugged glass bottle. X Plastic ampoule.

EFFECT OF FILTRATION

Groves & Major (1964) suggested that the Counter might be applicable to the routine control of the filtration process during the preparation of a transfusion solution. Sodium chloride B.P. was dissolved in freshly distilled water for injection and 1 litre passed through each filtration

medium to wash the filter before taking a sample for size analysis. The filter media were selected as being representative of types commonly used for the clarification of intravenous fluids in industrial and hospital laboratories; they were not supported by sintered glass filters to remove particles washed off the candle and asbestos pad. Results are shown in Fig. 4.

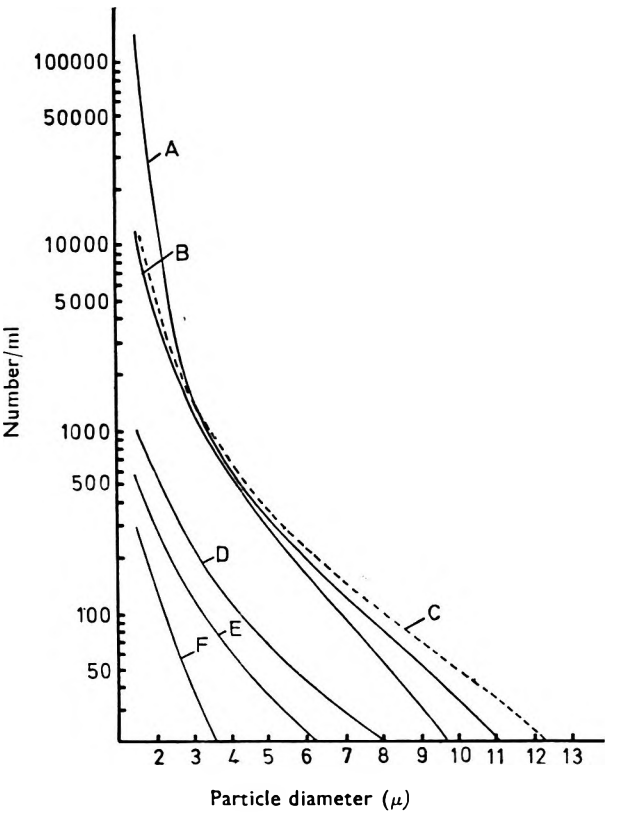

FIG. 4. The effect of filtration through various media upon the particle size distribution of material in 0·9% sodium chloride solution. A. Unfiltered. B. Sterilising asbestos pad. C. Sterilising unglazed porcelain candle. D. No. 4 sintered glass filter. E. Millipore H.A. membrane. F. Millipore G.S. membrane.

Discussion

All the samples of commercial origin examined were from reputable sources and were believed to be of recent manufacture. Although there are widely differing degrees of particulate contamination these are probably representative of material hitherto considered satisfactory for intravenous use. In most instances only one sample has been taken on a purely random basis. Nevertheless, it is possible that a patient might

sample material which is contaminated to the same degree as, say, sample A of Fig. 1.

There seem little doubt, following the work of Garvan & Gunner (1963, 1964), that most particles in an intravenous fluid emanate from the rubber closure. This is substantiated by Groves & Major (1964) and by the results shown in Fig. 3. The pack for an intravenous fluid is therefore of paramount importance. As shown in Figs 1 and 2, some commercial solutions contain very few particles above 5 μ.

In the assessment of particulate contamination, the Coulter Counter proved rapid and objective. Moreover, in one or other form, it is to be found in many hospital pathology departments and industrial laboratories. The instrument could be applied to non-electrolyte transfusion fluids by the dilution technique described for dextrose solution and seems to be ideally adapted to this problem.

However, it is necessary to determine the significance of the particulate contamination, especially the hazard presented to the patient, in order to suggest suitable and practicable standards. Groves & Major (1964) suggested that the nearer the approach to a particle-free solution the less likely is any possible hazard. Garvan & Gunner drew attention to granulomatous reactions produced in rabbits and in humans by cellulose particles. Other materials may be more or less reactive and it is obviously desirable to limit all particulate contamination as far as possible.

The significance of the particle size distribution is even less certain. Stehbens & Florey (1960) described the formation of thrombi after the injection of submicron particles into observation chambers in rabbits ears. It would also appear to be undesirable to inject large numbers of particles of the same order of size as an erythrocyte which, on the Coulter Counter, has a mean equivalent volume diameter of approximately 4·5 μ (Brecher, Schneiderman & Williams, 1956). Chances of embolic phenomena occurring clearly increase with particles above 5 μ diameter.

The data presented in Fig. 4 show that it should be possible to filter out most particles above 5 μ. In practice, much larger volumes would be passed through a filtration medium and the particle size distribution would be expected to decrease as the pores within the medium become filled. An arbitrary upper size limit of 5 μ should therefore be a workable standard for the filtered solution before packing.

If an arbitrary limit was set to exclude all particles above 5 μ this could readily be checked by all models of the Counter. A count of 20–30 particles/ml at the threshold corresponding to this size can be regarded as insignificant. With a suitable orifice tube the Model A Counter will detect material of 0·2 μ diameter but it is not certain if a practicable standard could be set at this level. It would appear from Figs 1 and 2 that any sample with a small upper particle size also contains relatively fewer particles at the lowest size-range counted. It is therefore suggested that, for the present, particulate contamination could be limited by specifying that an intravenous injection fluid should contain not more than, say, 50 particles/ml above 5 μ.

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Transformations in glycyrrhetinic acid: rearrangement of ring A

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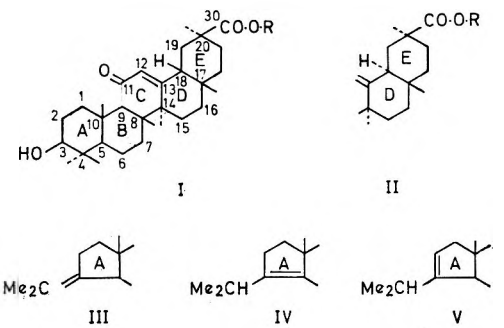
Methyl 11-oxo-18β-A-neo-oleana-3,12-dien-30-oate and the corresponding 18α-isomer have been prepared and tested for anti-inflammatory and antidiuretic activity. Relative to methyl glycyrrhetinate, both kinds of activity and toxicity are reduced by the rearrangement. Features of interest in the nuclear magnetic resonance spectra of 18α- and 18β-derivatives of glycyrrhetinic acid are recorded.

GLYCYRRHETINIC acid is known in two stereoisomeric forms: the 18 β-isomer (I; R = H) and the 18 α-isomer (II; R = H).



The 18β-isomer and a number of its derivatives are active anti-inflammatory agents (Finney & Somers, 1958; Finney & Tarnoky 1960; Kraus, 1960) and the hydrogen succinate of the 18 β-isomer has been shown to be of value in the treatment of gastric ulcer (Avery Jones, 1964). Little is known about the biological properties of the 18 α-isomer.

Methyl 18β-glycyrrhetinate was rearranged by phosphorus pentachloride in chloroform to give two compounds. The major component (methyl 11-oxo-18β-A-neo-oleana-3,12-dien-30-oate) was shown to have the structure III (R=Me) by the formation of acetone on ozonolysis and by the appearance of two methyl peaks at τ 8·21 and 8·37, absent in the nmr spectrum of the original ester, and assigned to the methyl groups of an isopropylidene group (Lehn & Ourisson, 1962). Structure IV (R=Me) has been assigned to the minor component (methyl 11-oxo-18β-A-neo-oleana-3(5),12-dien-30-oate). The methyls of the isopropyl group are not equivalent and in the nmr spectrum showed as two doublets (J = 7c/s); the position of the two methyl group resonances are 4c/s apart (see Lehn, 1962).

Methyl 18α-glycyrrhetinate was rearranged under similar conditions and the product crystallised to give a compound analogous to (III) (methyl 11-oxo-18α-A-neo-oleana-3,12-dien-30-oate). The mixture remaining in the mother liquors was isomerised by heating with acetic acid

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(Nowack, Jeger & Ruzicka, 1949) and gave a compound analogous to (IV) (methyl 11-oxo-18α-A-neo-oleana-3(5),12-dien-30-oate).

Rearrangement of the toluene-*p*-sulphonate of methyl 18β-glycyrrhetinate with sodium acetate and acetic acid gave a mixture which was isomerised as above to give a compound of proposed structure V (R=Me) (methyl 11-oxo-18β-A-neo-oleana-2,12-dien-30-oate). Unlike any of the above compounds this gave an nmr signal at τ 4·58 (proton at $C_{(2)}$) in addition to that at τ 4·26 (proton at $C_{(12)}$).

Table 1 lists the main nmr signals from a number of derivatives of 18α- and of 18β-glycyrrhetinic acids. For these derivatives the α-compounds can be differentiated from the β-compounds by the τ values of (*a*) the signal at highest field and (*b*) the signal from the proton at $C_{(12)}$.

During the course of this work a brief preliminary communication reporting a rearrangement of ring A of methyl 18β-glycyrrhetinate appeared (Tolstikov, Goriajev & Tolstikova, 1964). The results of these workers agree substantially with ours.

Pharmacology

The screening tests were by J. B. Dekanski, M. Khan and M. Cohen at the Pharmacology Department of Biorex Laboratories Research Division.

The cotton pellet granuloma test (Meier, Schuler & Desaulles, 1950) was used to evaluate the anti-inflammatory activity of the 18α- and 18β-isomers of methyl 11-oxo-A-neo-oleana-3,12-dien-30-oate and of the parent glycyrrhetinic acid esters from which these compounds were derived. A parallel evaluation of hydrocortisone acetate was also made.

A modification of the method described by Burn (1950) was used to determine antidiuretic activity over the 2 hr following the intraperitoneal administration of a single dose of 100 mg/kg to male rats.

In the above tests all compounds were injected as 1–3% suspensions in saline, with polysorbate 80 as suspending agent.

The LD50 was determined intraperitoneally in mice, over 7 days, the dose only being administered on the first day of the test. In these acute toxicity studies isopropyl myristate was used as the vehicle.

The results of the tests are summarised in Table 2. Thus, when ring A of methyl glycyrrhetinate is rearranged to give structure (III), anti-inflammatory activity and toxicity are reduced in both the 18α- and 18β-isomers. A similar picture seemed to emerge from the antidiuretic results.

Experimental

Melting-points are uncorrected and are taken on samples dried *in vacuo* at 100°. Optical rotations are measured on 2% solutions in chloroform, and ultraviolet spectra on solutions in chloroform. Analyses are by Mr. G. S. Crouch, School of Pharmacy, London.

Methyl 11-*oxo*-18β-*A*-*neo*-*oleana*-3,12-*dien*-30-*oate*. Methyl 18β-glycyrrhetinate (m.p. 245-248·5°, $[\alpha]_D^{20}$ + 157·4°) (8·1 g) was dissolved in alcohol-free chloroform (Vogel, 1956) (600 ml) which had been dried by

TABLE 1. NUCLEAR MAGNETIC RESONANCE SPECTRA* OF 18α- AND 18β-METHYL GLYCYRRHETINATE AND THEIR DERIVATIVES (τ VALUES)

Compound	$C_{(12)}-H$	$C_{(2)}-H$	$C_{(20)}-CO{\cdot}O{\cdot}CH_3$	C–H								
18β-Methyl glycyrrhetinate (I; R = Me)	4·23	—	6·27	8·41	—	8·58	—	—	8·82	8·95	9·14	
Methyl 11-oxo-A-homo-3*b*-aza-18β-olean-12-en-30-oate	4·25	—	6·26	8·42	—	8·59	8·66	—	8·82	—	9·16	
Methyl 11-oxo-18β-A-neo-oleana-3,12-dien-30-oate(III; R = Me)	4·23	—	6·27	8·37	—	8·61	—	—	8·83	—	9·13	
Methyl 11-oxo-18β-A-neo-oleana-2,12-dien-30-oate(V; R = Me)	4·26	4·58	6·26	8·45	—	8·61	—	—	8·82	9·03	9·14	
Methyl 11-oxo-18β-A-neo-oleana-3(5),12-dien-30-oate (IV; R = Me)	4·18	—	6·26	—	—	—	8·68	8·76	8·84 8·88	8·93 ‡ 9·05 9·00 ‡ 9·12	9·15	
Methyl 18α-glycyrrhetinate (II; R = Me)	4·41	—	6·30	8·43	—	8·63	—	8·77 8·79	8·85	8·99	9·19	9·28
Methyl 11-oxo-18α-A-neo-oleana-3,12-dien-30-oate (III; R = Me)	4·31	—	6·28	8·37	—	8·64	—	8·75	8·83	—	9·09	9·25
Methyl 11-oxo-A-homo-3*b*-aza-18α-olean-12-en-30-oate	4·36	—	6·27	8·39	8·53	8·62	8·67	8·75	8·81	—	—	9·26
Methyl 11-oxo-18α-A-neo-oleana-3(5),12-dien-30-oate (IV; R = Me)	4·28	—	6·28	8·41	8·56	—	8·71	8·78	8·83	8·93 ‡ 9·05 9·00 ‡ 9·12	—	9·25

* In $CDCl_3$ with tetramethylsilane as internal standard on Varian A-60 spectrophotometer. Main peaks occurring between τ 4 to 6·5 and τ 8·3 to 10.
‡ = J = 7c/sec.

distilling from phosphorus pentoxide. The solution was cooled to 4° in an ice-bath, with stirring, and anhydrous sodium acetate (5·4 g), dried at 100°, added. Stirring was continued and after 10 min phosphorus pentachloride (5·4 g) was added. The mixture was stirred for 35 min, sodium bicarbonate solution (3·3%, 480 ml) was added and stirring was continued for 3 hr. The chloroform layer was separated, washed with water until the washings were neutral and dried (Na_2SO_4). After removing the chloroform under reduced pressure, a white solid remained. This was dissolved in chloroform (40 ml), and methanol (309 ml) was rapidly added to the solution with rotation of the container so that a crystalline product (4·85 g) separated. This was removed by filtration and crystallised from ethyl acetate to give *methyl* 11-*oxo*-18β-*A-neo-oleana*-3,12-*dien*-30-*oate* (3·0 g) as colourless plates, m.p. 227–229° (softening at 225°), $[\alpha]_D^{20}$ + 205° (unchanged on further crystallisation from chloroform-methanol), λ_{max} 251 mμ (log ϵ 4·1). Found: C, 79·6; H, 9·8. $C_{31}H_{46}O_3$ requires C, 79·8; H, 9·9%.

Ozonolysis. The above compound (1·85 g) in methylene dichloride (120 ml) was cooled to −30° and ozonised oxygen passed into the solution for 2·5 hr. The solvent was removed at room temperature under reduced pressure and the residue heated under reflux with water (60 ml) for 50 min. The mixture was cooled and then distilled until 42 ml of distillate had been collected. A portion (3·3 ml) of the distillate was shown to contain acetone by comparison with an authentic specimen by vapour-phase chromatography on a "Pye" panchromatograph. To the remainder of the distillate, iodine solution (10%, 6 ml) was added and then sodium hydroxide solution (20%). Iodoform (0·22 g) was precipitated and was recrystallised from ethanol (m.p. and mixed m.p. 115°).

The mother liquors from the fractionation of the crude rearrangement product by chloroform and methanol were taken to dryness under reduced pressure. The residue (3·2 g) was crystallised from methanol (285 ml). The first crop of crystals was rejected and the crops formed by removing (*a*) 135 ml and (*b*) a further 100 ml of methanol were combined and twice recrystallised from methanol to give *methyl* 11-*oxo*-18β-*A-neo-oleana*-3(5),12-*dien*-30-*oate* (0·59 g) as colourless needles, m.p. 184–187°, $[\alpha]_D^{20}$ + 202°, λ_{max} 251 mμ (log ϵ 4·1). A sample further crystallised from methanol for analysis, had m.p. 186–7° Found: C, 80·0; H, 10·2. $C_{31}H_{46}O_3$ requires C, 79·8; H, 9·9%.

Methyl 11-*oxo*-18α-*A-neo-oleana*-3,12-*dien*-30-*oate*. Methyl 18α-glycyrrhetinate (m.p. 260–262°, $[\alpha]_D^{20}$ + 97°) (10 g) was dissolved in dry alcohol-free chloroform (1 litre) and the rearrangement carried out as described above using anhydrous sodium acetate (6·7 g) and phosphorus pentachloride (6·7 g). The crude product was crystallised directly from ethyl acetate (500 ml) to give *methyl* 11-*oxo*-18α-*A-neo-oleana*-3,12-*dien*-30-*oate* (6·6 g) as colourless plates, m.p. 267–270°, $[\alpha]_D^{20}$ + 139° (unchanged on further crystallisation from chloroform-methanol), λ_{max} 250 mμ (log ϵ 4·1). Found: C, 80·2; H, 9·8. $C_{31}H_{46}O_3$ requires C, 79·8; H, 9·9%.

Ozonolysis. The above compound (1·04 g) in methylene dichloride (50 ml) was ozonised as previously described and, when treated with

TABLE 2. PRELIMINARY ASSESSMENT OF ANTI-INFLAMMATORY AND ANTIDIURETIC ACTIVITY OF THE DERIVATIVES IN RATS AND THEIR ACUTE TOXICITY IN MICE

Compound	Anti-inflammatory activity			Antidiuretic activity* (Dose 100 mg/kg)		Acute toxicity (i.p.) Groups of 4 mice	
				% Effect†			
	Route	Dose (mg/rat/day for 4 days) 5 male wistar rats/dose	Mean % reduction in granulation tissue weight compared with controls	Urine volume	Na excretion	Conc. %	LD50 (approx.) mg/kg
Hydrocortisone acetate	s.c.	12	51	—	—	—	—
		3	28				
Methyl 18β-glycyrrhetinate	s.c.	12	49	59	33	4	600
		3	24				
Methyl 18α-glycyrrhetinate	s.c.	12	37	39	46	4	400
		3	27				
Hydrocortisone acetate	i.p.	10	44	—	—	—	—
Methyl 11-oxo-18β-A-neo-oleana-3,12-dien-30-oate	i.p.	30	23	77	59	5	1000
		10	12				
Methyl 11-oxo-18α-A-neo-oleana-3,12-dien-30-oate	i.p.	30	32	68	56	6	900
		20	22				
		10	8				
Control	—	—	—	124	83	—	—

* Groups of five male Wistar albino rats, approximately 200 g, were starved for about 17 hr before water-loading and during urine collection, but allowed water *ad lib*. The water-load, administered by stomach tube, was 5 ml of distilled water per 100 g body weight. Immediately after loading the animals were placed in metabolism cages and faeces-free urine was collected 2 and 6 hr later. The urines were stored below 4° until analysis. The urine volumes at 2 and 6 hr were noted and the samples analysed for sodium, potassium and chloride content. The water loading and urine collections were repeated twice weekly. Doses of the compound under test were administered intraperitoneally, simultaneous with the third water-load. The dose employed was 100 mg/kg. The drugs were administered as 1% suspensions in water, with polysorbate 80 as suspending agent. Control groups received the same volume of vehicle alone. Sodium and potassium were estimated using the E.E.L. flame photometer. Chloride was estimated by the method of Schales & Schales (1941).

† As % of mean 2 hr excretion levels determined on previous days.

iodine and sodium hydroxide, gave iodoform (0·112 g), which was crystallised from ethanol (m.p. and mixed m.p. 117–118°). A second portion (0·5 g) was ozonised as above and acetone was shown to be present in the distillate by vapour-phase chromatography.

The ethyl acetate mother liquors (500 ml above) were taken to dryness under reduced pressure and the residual solid (2·5 g) refluxed for 18 hr with glacial acetic acid (500 ml). The solution was cooled and the acetic acid removed under reduced pressure to low volume. The remaining solution was poured into water and the mixture filtered, washed with water and dried in a vacuum oven at 40°. The product (2·45 g), was three times crystallised from methanol-ethyl acetate 1 : 1 (about sixty times the weight of solid) to give *methyl* 11-*oxo*-18α-*A-neo-oleana*-3(5),12-*dien*-30-*oate* (0·49 g) as colourless plates, m.p. 237–238°, $[\alpha]_D^{20}$ + 151·6°, λ_{max} 247 mμ (log ε 4·06). Found: C, 79·4; H, 9·9. $C_{31}H_{46}O_3$ requires C, 79·8; H, 9·9%.

Toluene-p-*sulphonate of methyl* 18β-*glyrrhetinate*. Methyl 18β-glycyrrhetinate (7·1 g) was dissolved in pyridine (100 ml, distilled from KOH) and the solution cooled in an ice-bath. Toluene-*p*-sulphonyl chloride (19·6 g) was added and the solution, protected by a calcium chloride tube, set aside for three days. The solution was poured into ice and water and the mixture extracted with ether. The ethereal solution was washed with 2N hydrochloric acid (100 ml), then with saturated sodium bicarbonate solution and finally with water. The solution was dried (Na_2SO_4), the ether removed by distillation and the residue refluxed with light petroleum (b.p. 100–120°, 500 ml). The hot solution was decanted from the oily residue and allowed to cool, whereupon colourless needles of the toluene-*p*-sulphonate were deposited. The mother liquors were used to extract the residue and obtain further crops. Yield 7·73 g, m.p. 139–140° (decomp.), $[\alpha]_D^{20}$ + 110°. Found: C, 71·4; H, 8·5; S, 4·9. $C_{38}H_{54}O_6S$ requires C, 71·45; H, 8·5; S, 5·0%. The toluene-*p*-sulphonate can also be crystallised from methanol (150 ml/g).

Rearrangement of the toluene-p-*sulphonate of methyl* 18β-*glycyrrhetinate*. The toluene-*p*-sulphonate (4·1 g) and anhydrous sodium acetate (2·3g) were dissolved in acetic acid (540 ml). The solution, in a flask with condenser and calcium chloride tube, was heated in a water-bath for 6 hr at 90–95°.

Water was added to the solution to dilute the acetic acid to about 10% and the solid removed by filtration and dried. This product (1 g) was heated under reflux with acetic acid (130 ml) for 12 hr. The solution was poured into water, extracted with ether and the ethereal solution washed and dried (Na_2SO_4). Removal of the ether by distillation left a resinous product (1·08 g), which was extracted with boiling methanol (5 ml) to leave a solid (0·76 g), m.p. 161–172°. Recrystallisation from ethyl acetate and then from methanol gave crystals (0·17 g), m.p. 186–188·5°, which depressed the m.p. of methyl 11-oxo-18β-A-neo-oleana-3(5),12-dien-30-oate. $[\alpha]_D^{20}$ + 214°, λ_{max} 249 mμ (log ε 4·1). Found: C, 80·3; H, 10·0. $C_{31}H_{46}O_3$ requires C, 79·8; H, 9·9%. This compound has been tentatively assigned the constitution *methyl* 11-*oxo*-18β-*A-neo-oleana*-2,12-*dien*-30-*oate*.

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The effect of humidity and temperature on the cohesion of powders

E. SHOTTON AND N. HARB

The cohesion of wheat, maize and potato starch, acacia, tragacanth, alginic acid, lactose, dextrose and sucrose powders has been determined at different relative humidities and temperatures. For maize, wheat and potato starches, cohesion was a maximum at an intermediate moisture content, whilst for acacia, tragacanth and alginic acid it was independent of the moisture content over the range considered. Lactose, dextrose and sucrose remained free flowing except at high relative humidities.

THE cohesion of a powder is affected by the particle shape, size and size distribution. Smooth, spherical particles flow more easily than other shapes and monodisperse powders are usually free flowing and have low cohesion.

Craik & Miller (1958) obtained high angles of repose for powders which form large aggregates and which flow with difficulty, whilst low angles are obtained for powders in which the particles are smooth and flow easily. They also found that a high moisture content reduces the ease of flow of some powders, an effect very pronounced with soluble crystalline powders.

Dawes (1952) obtained a value for cohesion in a powder by measuring the force per square centimetre required to break a powder deposit and he concluded that cohesion in a powder is controlled by the particle shape, size and size range.

Eisner, Fogg & Taylor (1960) measured the cohesion of ground limestone, untreated and water-proofed. The method and apparatus were similar to those used by Dawes and the results showed a variation in the cohesion of both powders with the humidity of the air, that of the water-proofed powder being about three-quarters that of the untreated powder. The cohesion of both powders rose slightly between 15 and 31% relative humidity and thereafter remained constant until 84% when it rose steeply with increasing humidity.

Experimental

Apparatus. The cohesion apparatus used was similar to that used by Eisner & others (1960), but modified by using a complete disc fitted behind the tilting plate for the measurement of the angle of separation of the movable slide. Using suitable gearing, the disc traversed an angle four times that of the tilting plate and this improved the reading of the angle.

Materials. The materials used were wheat, maize and potato starch, acacia, tragacanth, lactose, dextrose and sucrose of the British Pharmacopoeia. Alginic acid was of food grade.

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Method. The cohesion apparatus was put inside the humidity cabinet (Shotton & Harb, 1965), and the powders brought into moisture equilibrium at each humidity. The air circulation in the cabinet was stopped during measurement of cohesion to avoid spreading of the particles or affecting the movement of the tilting plate. The table was tilted so that the graduated disc moved through 30° in 25 sec. Every 24 hr the powder was well mixed and then sprinkled uniformly from a height of 4 cm above the tilting table, onto the glass plate, using a small 16-mesh sieve so that a deposit was formed approximately 0·5 cm thick and 4 cm wide. The table was tilted until the movable slide separated. The blank angle was measured and deducted from the reading to give the actual angle of separation.

The cohesion of the powder was measured by observing the angle to the horizontal (θ) at which the powder broke in two. The cross-sectional area (A) of the broken face of the deposit was measured in cm². If W is the weight in grams of the broken away portion of the powder and slide, then the cohesion, C, is calculated according to the equation:

$$C = \frac{W \sin \theta}{A} \text{ g/cm}^2$$

The mean of three determinations was taken as a measure of cohesion. The equilibrium moisture content of the powders has been reported in a previous paper (Shotton & Harb, 1965). The results are in Table 1.

TABLE 1. COHESION OF THE POWDERS AT DIFFERENT HUMIDITIES AND TEMPERATURES (g/cm²)

Temperature °C	Relative humidity	Maize starch	Wheat starch	Potato starch	Tragacanth	Acacia	Alginic acid	Lactose	Dextrose	Sucrose
25	34	0·27	0·03	0·18	0·13	f.f.*	0·03	f.f.	f.f.	f.f.
	55	0·33	0·03	0·42	0·1	0·12	0·08	f.f.	f.f.	f.f.
	66	0·33	0·30	0·75	0·09	0·05	0·05	f.f.	f.f.	f.f.
	87	0·28	0·28	0·67	0·11	Paste	0·05	0·179	0·14	0·06
	100	0·09	0·07	0·13	0·03	Paste	0·05	0·266	Solution	Solution
30	33	0·31	0·26	0·31	0·11	0·06	0·03	f.f.	f.f.	f.f.
	44	0·37	0·29	0·33	0·08	0·05	0·06	f.f.	f.f.	f.f.
	65	0·34	0·35	0·79	0·15	0·15	0·04	f.f.	f.f.	f.f.
	80	0·3	0·3	0·79	0·12	Caked	0·08	0·16	0·13	0·06
	86	0·25	0·16	0·52	0·07	Caked	0·05	0·15	0·10	0·06
	92·5	0·08	0·04	0·13	0·04	Gel	0·01	0·21	Solution	Solution
	100	0·05	0·04	0·04	Caked	Solution	0·03	0·29	Solution	Solution
40	32	0·25	0·27	0·37	0·10	0·06	0·04	f.f.	f.f.	f.f.
	50	0·3	0·23	0·47	0·11	0·14	0·04	f.f.	f.f.	f.f.
	63	0·25	0·23	0·7	0·11	0·13	0·07	f.f.	f.f.	f.f.
	75·5	0·22	0·21	0·54	0·11	Paste	0·05	0·16	0·13	f.f.
	89·5	0·20	0·15	0·41	0·09	Paste	0·06	0·19	Solution	Cake
	100	0·05	0·05	Caked	Caked	Solution	0·05	0·19	Solution	Solution
50	32	0·32	0·28	0·6	0·05	0·05	0·05	f.f.	f.f.	f.f.
	47	0·24	0·25	0·61	0·08	0·07	0·04	f.f.	f.f.	f.f.
	67	0·27	0·24	0·64	0·1	0·1	0·04	f.f.	f.f.	f.f.
	76	0·14	0·14	0·50	0·09	Paste	0·07	f.f.	0·11	f.f.
	87	0·2	0·21	0·35	0·08	Paste	0·06	0·21	Solution	Solution
	100	0·09	0·07	Caked	Caked	Solution	0·05	0·22	Solution	Solution

* Free flowing powder.

Discussion

The relationship between equilibrium moisture content (Shotton & Harb, 1965) and cohesion shows that starches followed the same pattern at each of the temperatures used. Potato starch had the highest measurable cohesion of all the powders. The cohesion of maize starch was slightly higher than that of wheat starch and both were lower than that of potato starch (Fig. 1).

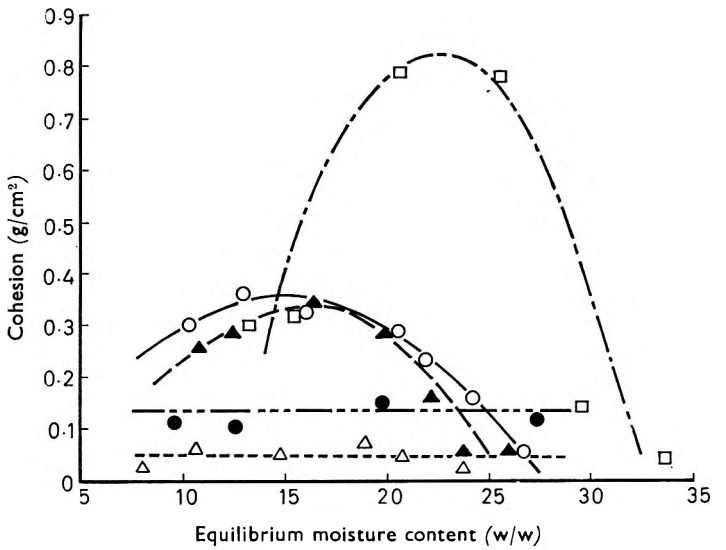

FIG. 1. Relationship between equilibrium moisture content and cohesion at 30° C. —○— Maize starch. – – ▲ – – wheat starch. – — - □ - — – – Potato starch. - - - △ - - - Alginic acid. — - - - —● — - - - — Tragacanth.

At low moisture content the less angular potato and wheat starches have lower cohesion than maize starch. With increasing moisture content the starches showed increasing adhesion up to a maximum at each temperature and this may be attributed to the capillary effect of surface moisture. This is also in agreement with Eisner & others (1960), who stated that cohesion in a dry atmosphere is due to van der Waals' forces and that cohesion rises when the asperities between most of the contacts are covered by a moisture film. The cohesion of all the starches falls at higher moisture contents, due to aggregation of the starch into granules.

The cohesion of powdered tragacanth, alginic acid and acacia were approximately the same at the different humidities and temperatures until tragacanth and acacia powders lost their powdery form, due to absorption of moisture, to form a cake which aggregated into lumps of different sizes on stirring. At saturation the cohesion could not be measured due to deliquescence.

Dextrose and sucrose were free flowing whilst free of moisture and it was impossible to measure the area of the broken surface, but when they

contained traces of moisture, measurement could be made (Table 1) until deliquescence occurred.

The maximum cohesion of maize, wheat starch, tragacanth, acacia and alginic acid was not affected by a change of temperature but that of potato starch showed a slight decrease with increase of temperature.

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A note on the determination of pilocarpine in solutions containing methylcellulose

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Low results obtained by a colorimetric method for the determination of pilocarpine in ophthalmic solutions are attributed to pilocarpine-methylcellulose interaction. Low results were not obtained with a second colorimetric method. Other factors affecting this interaction are discussed.

ANALYSIS of locally manufactured pilocarpine ophthalmic solutions by the procedure of Webb, Kelley & McBay (1952) yielded unusually low results, a phenomenon previously reported by Levine & Horrocks (1960). Examination of the manufacturer's formula suggested methylcellulose 4000 (0·33%) might be responsible, and this was confirmed in preliminary experiments, in which ingredients were omitted one by one.

Application of the Elvidge (1947) method to the same solutions gave total initial concentration of pilocarpine, whether or not methylcellulose was present. Using both the method of Webb & others, and the Elvidge method, a number of ophthalmic preparations of two manufacturers were assayed for pilocarpine content and compared with a set of control solutions of the same concentrations.

Experimental

The solutions were assayed as described by Webb & others (1952)* and by Elvidge (1947), all ophthalmic solutions and control solutions being diluted to contain 2 mg/ml of pilocarpine hydrochloride.

Results and discussion

Calibration curves, constructed using the Webb method, for (i) 1% pilocarpine hydrochloride in water and (ii) 1% pilocarpine hydrochloride in 0·33% methylcellulose 4000 solution, confirmed that the Webb method gave low results in the presence of methylcellulose. Preliminary experiments with the Elvidge procedure showed that the methylcellulose had no effect and a figure representing total initial concentration of pilocarpine hydrochloride was obtained. The methylcellulose must therefore interact with or "bind" a portion of the pilocarpine and the Webb method probably determines only the free or unbound pilocarpine.

The difference in results given by the two methods can be explained on the basis of the chemical reactions involved. Helch's (1902) test, the basis of the Webb method, causes formation of a coloured salt and does not otherwise affect the pilocarpine molecule. If the basic centre of the pilocarpine were involved in the interaction with methylcellulose, the

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* The amount of hydrogen peroxide added is critical (Levine & Horrocks, 1960) and a fresh solution was prepared each day.

relatively mild conditions of the Helch test probably would not affect that amount of the pilocarpine bound to the methylcellulose. The mechanism of the Ekkert (1925) test (the basis of the method described by Elvidge) has not been described but this type of qualitative test usually involves a specific portion of the structure. Also, since the Ekkert test takes place under conditions more likely to reverse any pilocarpine–methylcellulose interactions, total assay is achieved whether the pilocarpine is initially bound or not, or even if it remains bound to the methylcellulose through the part of the molecule remote from the site attacked by the test reagents.

Using the two methods, commercial ophthalmic pilocarpine solutions from two manufacturers and a set of control solutions prepared by ourselves were examined for pilocarpine hydrochloride content. The results are summarized in Table 1. The control solutions contained the stated concentration of pilocarpine hydrochloride in 0·33% methylcellulose solution.

TABLE 1. DETERMINATION OF PILOCARPINE IN SOLUTIONS CONTAINING METHYL CELLULOSE

Source	Labelled strength (%)	Webb method (Free pilocarpine)		Elvidge method (Total pilocarpine)		mg/ml bound
		% labelled strength	mg/ml	% labelled strength	mg/ml	
Manufacturer A	0·5	92·5	4·65	101	5·05	0·4
	1·0	93·8	9·4	103	10·3	0·9
	2·0	95·0	19·0	104	20·8	1·8
	3·0	95·0	28·5	107	32·1	3·6
	4·0	97·5	39·0	102·5	41·0	2·0
Manufacturer B	0·5	45	2·25	103·5	5·18	2·9
	1·0	71	7·1	105	10·5	3·4
	2·0	82	16·4	102·5	20·5	4·1
	3·0	75	22·5	106	31·8	9·3
	4·0	96	38·4	105	42·0	3·6
Control Solutions	0·5	82·5	4·13	99	4·95	0·8
	1·0	91·2	9·1	95	9·5	0·4
	2·0	90·0	18·0	94	18·4	0·4
	3·0	95·0	28·5	98	29·4	0·9
	4·0	99·0	39·6	95·5	38·2	−1·4

The same general trends are evident in the results from all three sets of solutions but there are some well-defined differences. The percentage labelled strength found by the Webb method increases with increase in the strength of the solution. This is to be expected since the *percentage* of pilocarpine bound by a constant amount of methylcellulose must decrease as the total pilocarpine content increases.

Manufacturer A uses a different type of methylcellulose (exact nature unknown to the authors) from manufacturer B and the results demonstrate that much less interaction takes place with the former type. The method of incorporating the methylcellulose may affect the amount of interaction in the final product.

A separate study was made to determine whether the amount of interaction changed with time. Manufacturer B supplied a number of 2%

pilocarpine preparations which had been stored for control purposes and these were again assayed by both methods. The results (Table 2) show that the amount of pilocarpine interacting with the methylcellulose does increase and reaches a constant value after approximately 12 months.

TABLE 2. EFFECT OF TIME ON THE INTERACTION BETWEEN PILOCARPINE HYDROCHLORIDE AND METHYLCELLULOSE IN SOLUTION

Age of solution in months	Webb method (Free pilocarpine)		Elvidge method (Total pilocarpine)		mg/ml bound	$[\alpha]_D^{23}$
	% labelled strength	mg/ml	% labelled strength	mg/ml		
1	82	16·4	102·5	20·5	4·1	+76·6
3	74	14·8	100·5	20·1	5·3	+62·2
8	74	14·8	100	20·0	5·2	not done
12	65	13·0	101	20·2	7·2	+61·4
19	59	11·8	108·5	21·7	9·9	+47·9
55	75	15·0	111·2	22·2	7·2	+51·8
60	70	14·0	106	21·2	7·2	+51·9
63	66	13·2	103	20·6	7·4	+52·4

The specific rotation of each solution in the time experiment was determined and the values are listed in Table 2. This value also reaches a constant value, apparently after a much longer period. The anomalous results for the nineteen month sample can be attributed to an error in manufacture since the viscosity was found to be much higher than that of the other seven solutions. The obviously high methylcellulose content accounts for the high weight per ml bound but the unexpectedly low specific rotation for this solution suggests that methylcellulose may accelerate the conversion of pilocarpine to isopilocarpine. Both assay procedures are non-specific and do not permit differentiation between pilocarpine content and the content of its closely related derivatives which may be formed during storage of solutions.

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The effect of chemical structure on chromatographic multiple spot formation by sympathomimetic amines in the presence of hydrochloric acid

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Of 22 sympathomimetic amines chromatographed on paper from solution in distilled water or concentrated hydrochloric acid, nine in the presence of hydrochloric acid produce multispots which are not apparent when they are chromatographed from distilled water. The presence or absence of the multiple spot phenomenon depends upon the chemical structure of each amine.

SYMPATHOMIMETIC catecholamines were recently shown by Roberts (1964) to form multiple spots when they were chromatographed on paper from solution in 10N hydrochloric acid using a phenol-hydrochloric acid solvent system. Since the amines must have been present as hydrochlorides under these conditions, the same acid was being used in the salt and the developing solvent, and the multispots must therefore have been different in origin from the spots previously reported (Shepherd & West, 1952; West, 1959; Beckett, Beavan & Robinson, 1960a,b). Because this phenomenon may occur with the use of hydrochloric acid during the preparation of biological extracts for chromatography (Roberts, 1966) we have examined the chromatographic behaviour of 22 sympathomimetic amines in detail.

Experimental

MATERIALS AND METHODS

The apparatus, materials and techniques used have been previously described (Roberts, 1963; 1964). The amines (50, 100, 200 μg) were chromatographed on Whatman No. 1 paper (washed with 0·01N hydrochloric acid) from freshly prepared solution (10 mg/ml) in distilled water or hydrochloric acid (10N). Each solution was then subjected to further chromatography at intervals until discrete spots were no longer obtained. The developing solvent was phenol containing 15% v/v 0·1N hydrochloric acid and chromatography was at 25–30° by the ascending technique until the solvent front had advanced at least 25 cm. Potassium ferricyanide (0·5 g) in sodium hydroxide solution (100 ml, 0·5N) or ninhydrin (0·25 g) in butanol (100 ml) followed by heating (120° for 3 min), were used to locate the amines. The Rf values were measured from the centre of each spot.

DRUGS

Phenethylamine hydrochloride, (−)-noradrenaline acid tartrate, dopamine hydrochloride, metanephrine (*m*-*O*-methyladrenaline), (±)-amphetamine

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sulphate, (−)-phenylpropanolamine (norephedrine) sulphate, (±)-phenylpropanolamine (*dl*-norephedrine), (+)-norpseudoephedrine hydrochloride (L. Light & Co. Ltd.), (−)-adrenaline acid tartrate, (±)-isoprenaline sulphate (Burroughs Wellcome & Co.), L-dopa, tyramine hydrochloride (B.D.H. Ltd.), and phenylethanolamine (Aldrich Chem. Co.) were obtained commercially. Noradrenalone acid tartrate, (±)-*N*-ethylnoradrenaline, (±)-*N*-butylnoradrenaline, (±)-*N*-isobutylnoradrenaline (Prof. A. S. V. Burgen), nordefrin (α-methylnoradrenaline; Cobefrine) hydrochloride, oxedrine (1-*p*-hydroxyphenyl-2-methylaminoethanol) tartrate, phenylephrine hydrochloride (Bayer Prod. Ltd.), metaraminol acid tartrate (Merck Sharpe & Dohme Ltd.), and Epinine (3,4-dihydroxy-*N*-methylphenethylamine) hydrochloride (Dr. H. T. Openshaw) were all gifts for which we are most grateful.

Quantities quoted in the text refer to the amount of each amine calculated as base.

Results

All the results obtained are summarised in Table 1. In the presence of hydrochloric acid the Rf value of every amine (spot d, Table 1) was always significantly higher than that obtained when chromatography was from distilled water, but only 9 of the amines showed the multiple spot phenomenon; of these, adrenaline produced the maximum number of discrete multispots over a seven day period (Table 1, Fig. 1). Thereafter all spots gradually disappeared so that chromatograms of solution 8 weeks old showed nothing more than a streak from the point of application to very near the solvent front. The same sequence of events was observed when the developed chromatograms were sprayed with ninhydrin, except that all the spots now showed pink. Over the same 8-week period of time solutions of adrenaline in distilled water produced only one spot on chromatography.

Of the other 21 amines investigated, noradrenaline, *N*-ethylnoradrenaline, isoprenaline, *N*-butylnoradrenaline, *N*-isobutylnoradrenaline, as well as nordefrin, oxedrine and metanephrine all produced four or more multispots. In each instance the phenomenon was always associated with retention of amine at the application point (spot a) and a colouring of the solutions on standing. Despite differences in Rf values, colour reactions and the number of multispots produced, it was possible to correlate each spot seen with these amines with one or other of the spots seen with adrenaline under the same conditions (a–g, Fig. 1 and Table 1). All other amines giving spot c did so after only 24 hr contact with the hydrochloric acid, in contrast to the 7 days required with adrenaline. The remaining 13 amines did not show baseline retention, did not form coloured solutions on standing and, with the exception of phenylephrine which showed spot f after 24 hr, did not produce multispots relatable to those described for adrenaline.

Epinine, metaraminol, phenylethanolamine, (−)-phenylpropanolamine and amphetamine did, however, produce two spots when chromatographed

TABLE 1. THE INFLUENCE OF HYDROCHLORIC ACID (10 N) ON THE PAPER CHROMATOGRAPHIC BEHAVIOUR OF β-PHENETHYLAMINE DERIVATIVES WHEN DEVELOPED IN PHENOL CONTAINING 15% v/v 0·1N HYDROCHLORIC ACID. LETTERS a g CORRESPOND TO THE SPOTS AS DESCRIBED IN THE TEXT AND AS ILLUSTRATED IN FIG. 1. THE ABSENCE OF Rf VALUES IN SOME COLUMNS INDICATES THAT THE RELATIVE SPOTS WERE NOT OBTAINED

Amine	Spray reagent	Rf values in distilled water	Soln colour (24 hr)		Rf values and colours of multispots in 10N hydrochloric acid						
			Water	10N HCl	a	b	c	d	e	f	g
Adrenaline acid tartrate	F	0·44 ± 0·006 Pink	Pink	Brown	0 Pink	0·30 ± 0·018 Grey	0·36 ± 0·005 Scarlet	0·48 ± 0·011 Pink	0·64 ± 0·011 Blue	0·71 ± 0·011 Turquoise	0·77 ± 0·009 Pink
Noradrenaline acid tartrate	F	0·22 ± 0·006 Pink	Pink	Blue	0 Pink	0·02 ± 0·001 Brown	0·13 ± 0·007 Scarlet	0·21 ± 0·008 Pink	0·40 ± 0·012 Blue		0·60 ± 0·008 Pink
N-Ethylnoradrenaline hydrochloride	F	0·57 ± 0·011 Pink	Pink	Brown	0 Pink	0·53 ± 0·009 Blue	0·51 ± 0·013 Scarlet	0·60 ± 0·010 Pink	0·70 ± 0·008 Blue	0·73 ± 0·009 Turquoise	0·81 ± 0·008 Pink
Isoprenaline sulphate	F	0·63 ± 0·005 Pink	Pink	Brown	0 Pink		0·61 ± 0·007 Scarlet	0·66 ± 0·008 Pink	0·75 ± 0·006 Pink	0·73 ± 0·006 Turquoise	0·84 ± 0·011 Pink
N-Butylnoradrenaline hydrochloride	F	0·63 ± 0·007 Pink	Pink	Brown	0 Pink		0·63 ± 0·009 Scarlet	0·66 ± 0·009 Pink	0·75 ± 0·011 Blue	0·75 ± 0·004 Turquoise	0·83 ± 0·012 Pink
N-Isobutylnoradrenaline hydrochloride	F	0·64 ± 0·005 Pink	Pink	Brown	0 Pink		0·64 ± 0·012 Scarlet	0·70 ± 0·004 Pink	0·78 ± 0·006 Blue	0·76 ± 0·003 Turquoise	0·85 ± 0·006 Pink
Nordefrin hydrochloride	F	0·36 ± 0·004 Orange	Pink	Brown	0 Pink	0·08 ± 0·012 Pink		0·34 ± 0·014 Orange	0·49 ± 0·020 Pink		0·69 ± 0·011 Mauve
Dopamine hydrochloride	F	0·40 ± 0·003 Dark brown	Brown	—				0·44 ± 0·008 Dark brown			
Epinine hydrochloride	F	0·38 ± 0·004 Orange 0·63 ± 0·005 Crimson	Brown	—				0·42 ± 0·004 Orange 0·66 ± 0·005 Crimson			
Noradrenalone acid tartrate	F	0·19 ± 0·007 Brown	Brown	—				0·24 ± 0·020 Brown			
L-Dopa	N	0·13 ± 0·008 Orange 0·26 ± 0·006 Yellow	Brown	—				0·15 ± 0·010 Orange			
Oxedrine acid tartrate	N	0·15 ± 0·006 Orange 0·73 ± 0·015 Mustard	—	Green	0 Pink	0·58 ± 0·008 Mauve		0·14 ± 0·008 Orange 0·75 ± 0·008 Mustard	0·83 ± 0·008 Pink		0·91 ± 0·007 Pink
Metanephrine	N	0·78 ± 0·011 Mauve	—	Brown	0 Pink	0·55 ± 0·009 Pink		0·78 ± 0·006 Mauve			0·87 ± 0·009 Pink
Tyramine hydrochloride	N	0·64 ± 0·008 Grey	—	—				0·64 ± 0·015 Grey			
Phenylephrine hydrochloride	N	0·67 ± 0·008 Mauve	—	—				0·67 ± 0·005 Mauve		0·78 ± 0·007 Orange	
Metaraminol acid tartrate	N	0·10 ± 0·002 Orange 0·40 ± 0·005 Mauve	—	—				0·16 ± 0·005 Orange 0·52 ± 0·014 Mauve			
Phenylethanolamine	N	0·59 ± 0·007 Pink 0·79 ± 0·003 Yellow	—	—				0·64 ± 0·006 Pink 0·74 ± 0·003 Yellow			
Phenethylamine hydrochloride	N	0·79 ± 0·007 Purple	—	—				0·80 ± 0·007 Purple			
(−)-Phenylpropanolamine sulphate	N	0·70 ± 0·006 Mauve	—	—				0·09 ± 0·002 Black* 0·70 ± 0·010 Mauve			
(+)-Phenylpropanolamine sulphate	N	0·68 ± 0·004 Mauve	—	—				0·08 ± 0·002 Black* 0·70 ± 0·007 Mauve			
(+)-Norpseudoephedrine hydrochloride	N	0·69 ± 0·005 Mauve	—	—				0·70 ± 0·007 Mauve			
Amphetamine sulphate	N	0·81 ± 0·013 Pink	—	—				0·10 ± 0·002 Black* 0·82 ± 0·007 Pink			

F = Ferricyanide; N = Ninhydrin; * = Char spot due to sulphate

in the presence of 10N hydrochloric acid. Of these, the first three showed similar double spots when chromatographed from solution in distilled water and no further explanation of the phenomenon was sought. The remaining three amines showed only single spots when chromatographed from distilled water, but the extra spots obtained in the presence of hydrochloric acid had identical Rf values. These spots were characterised by a charring of the paper, and were subsequently shown to be associated with the use of amine sulphates when ninhydrin followed by heating was used to detect the amines. The hydrochloric acid presumably displaces the sulphate which then undergoes chromatographic separation as sulphuric acid.

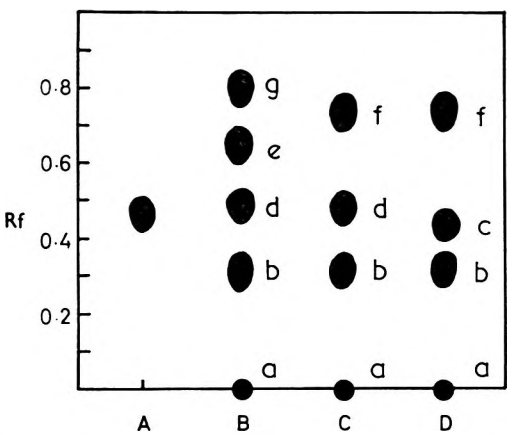

FIG. 1. Multiple spot phenomena exhibited by adrenaline acid tartrate (200 μg) when chromatographed from hydrochloric acid (10 N) immediately after (B), 24 hr after (C) and 7 days after (D), preparing the solution 10 mg/ml. At A, adrenaline acid tartrate chromatographed from solution in distilled water. Developing solvent, phenol containing 15% v/v 0·1 N HCl. The spots are labelled a - g to correspond with the text and Table 1.

Conversely, L-dopa formed two spots when chromatographed from solution in distilled water, but only one when 10N hydrochloric acid was used. A possible explanation of this phenomenon is that the dilute acid conditions prevailing in the developing solvent cause only partial conversion of the dopa in aqueous solution to the zwitterion form, in contrast to the complete conversion expected in the presence of 10N hydrochloric acid.

Discussion

Adrenaline and phenethylamine both form discrete single spots when chromatographed on paper from solution in distilled water, but whereas chromatography from 10N hydrochloric acid again results in the formation of one spot (d) only from phenethylamine, under these conditions the production of six additional spots is demonstrated with adrenaline. An

examination of Table 1 indicates that this difference in the chromatographic behaviour of the two amines results from substitution of the phenethylamine molecule.

The substituent common to all the amines showing multiple spot formation is an alcoholic β-hydroxyl group in the side-chain. When this group is removed (adrenaline to Epinine, noradrenaline to dopamine) or replaced by a ketone oxygen (noradrenaline to noradrenalone) the multiple spot phenomenon is not observed. However, the presence of an alcoholic β-hydroxyl group alone [phenylethanolamine, (−)-phenylpropanolamine] is not in itself sufficient to result in the formation of multispots, for extra spots in the presence of 10N hydrochloric acid are only seen when the benzene ring also contains an hydroxyl substituent. The position of this phenolic hydroxyl group is important. Its presence in the *para*-position (oxedrine) is associated with the production of a coloured solution in 10N hydrochloric acid, and with the formation of spots a (retention at the point of application), b, e and g. In contrast, *meta*-substitution of a phenolic hydroxyl group (phenylephrine) is associated solely with the formation of spot f from acid solutions of amine 24 hr old. The presence of spot c, on the other hand, is only evident when the alcoholic β-hydroxyl group is accompanied by both *meta*- and *para*-phenolic hydroxyl groups.

Catecholethanolamines are therefore expected to produce a total of seven spots (a–g) following chromatography from solution in 10N hydrochloric acid. Of the eight amines investigated, however, adrenaline and *N*-ethylnoradrenaline are the only ones which fulfil this expectation. The results with the other amines indicate a further set of structural requirements for the formation of individual spots which influence their formation even when phenolic and alcoholic β-hydroxyl groups are present. Spot f, for example, is not evident in the absence of substitution on the primary amino nitrogen (metaraminol, noradrenaline, nordefrin) or when the *meta*-hydroxyl group is *O*-methylated (metanephrine). On the other hand, when the *N*-alkyl substituent is greater than ethyl (isoprenaline, *N*-butylnoradrenaline, *N*-isobutylnoradrenaline) spot b is not formed. In addition, the substitution of a methyl group on the α-carbon atom, as in nordefrin, prevents it forming a spot equivalent to c although it is a catecholethanolamine with a primary amine nitrogen.

Several points of interest arise from our findings. When hydrochloric acid is used during the production of concentrated extracts of biological material for chromatography, the number of spots obtained need not necessarily indicate the number of amines present in the starting material (Roberts, 1966). This could have special significance during the investigation of the metabolism of a pure amine. Furthermore, we have found that the substances responsible for the spots corresponding to c are dibenzocycloheptatrienes of the type described by Kawazu (1958a,b). The close structural resemblances of these compounds to the antidepressive iminodibenzyl derivatives prompted us to postulate an *in vivo* synthetic route and a depressive pathological function for noradnamine, the noradrenaline dibenzocycloheptatriene (Roberts & Broadley, 1965). It may be significant that following the intracisternal administration to cats some

sympathomimetic amines caused arousal while others caused stupor (Leimdorfer, 1950); those amines which resulted in stupor are those which in our experiments formed multispots in the presence of hydrochloric acid.

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The effects of some derivatives of noradrenaline and 2-amino-1-*p*-hydroxyphenylethanol on the *in vitro* mobilisation of fat

SIR,—The structure-activity relations of sympathomimetic amines show mobilisation of fat to be associated with primary or secondary amines with a hydroxyl group on the α-carbon atom of the side-chain, and on the *para*-position of the ring (Mueller & Horwitz, 1962); alkyl substitution on the β-carbon atom of the side-chain is without marked influence on the fat mobilising action (Rudman, Garcia, Brown, Malkin & Perl, 1964). The importance of the substitution on the amino-group of catecholamines has hitherto been established only in experiments using the monoethyl and isopropyl derivatives (Mühlbachova, Wenke & Hynie, 1961a; Mueller & Horwitz, 1962; Rudman & others, 1964; Wenke, Mühlbachová, Schusterová, Elisová & Hynie, 1964). In the 2-amino-1-*p*-hydroxyphenylethanol series, only the action of 1-*p*-hydroxyphenyl-2-methylaminoethanol (oxedrine) has been studied (Mühlbachová & others, 1961b; Mueller & Horwitz, 1962).

We have now examined the effect on fat mobilisation of substitution in the amino-group in an homologous series of catechol and 2-amino-1-*p*-hydroxyphenylethanol derivatives. Epididymal adipose tissue of white rats of the Konarovice strain was incubated for 90 min at 37° in Krebs-Ringer phosphate buffer (pH 7·4) containing 5% human albumin and different concentrations of the drugs. The degree of the mobilisation of fat was measured by following the release of free fatty acids into the medium; these were estimated by the method of Dole (1956). The *N*-substituted derivatives examined were the methyl, ethyl, propyl, butyl, isopropyl, t-butyl and phenyl-t-butyl compounds. Dose-response curves to the substituted derivatives were constructed after making repeated parallel curves to noradrenaline, the drug chosen as a standard. The pD_2 value was obtained from van Rossum's tables (van Rossum, 1963). Relative values in relation to the corresponding noradrenaline standard ($\Delta pD_2 = pD_2x - pD_2$noradrenaline) were derived.

When the relative affinities of the series of catecholamines to mobilise fat are examined, remarkably little change within the series is found. Fig. 1 shows only a slight trend for the affinity to increase with increase in substituent size. Non-logarithmically expressed, the affinity differences between the derivatives with the smallest and those with the largest substituent groups is as little as three-fold. A fat mobilising affinity higher than that of noradrenaline was found only in isoprenaline ($P < 0{\cdot}001$), the *N*-t-butyl derivative of noradrenaline ($P < 0{\cdot}05$) and the *N*-phenyl-t-butyl derivative ($P < 0{\cdot}001$). Adrenaline had a lower affinity than noradrenaline.

In the 2-amino-1-*p*-hydroxyphenylethanol series, where in general the fat mobilising affinities were much lower than in the noradrenaline series, the increase in affinity is more clearly seen. The difference in affinity between the unsubstituted and the most substituted compound is about 16-fold. The decrease in affinity of the methyl derivative is striking (Fig. 1).

When the pD_2 values of the corresponding derivatives of both series were correlated, a close linear relation was obtained which could be expressed by the equation $y = 0{\cdot}42x + 0{\cdot}37$. This indicated that, in relation to the changes of the nitrogen substituent, the pD_2 values rise 2·5 times more steeply in the 2-amino-1-*p*-hydroxyphenylethanol series than in the noradrenaline series. Expressed non-logarithmically, the fat mobilising affinities of the noradrenaline series increase at only slightly more than the third root of the affinities in the corresponding 2-amino-1-*p*-hydroxyphenylethanol series. The affinity sequence —isopropyl > hydrogen > methyl—is well defined in both series.

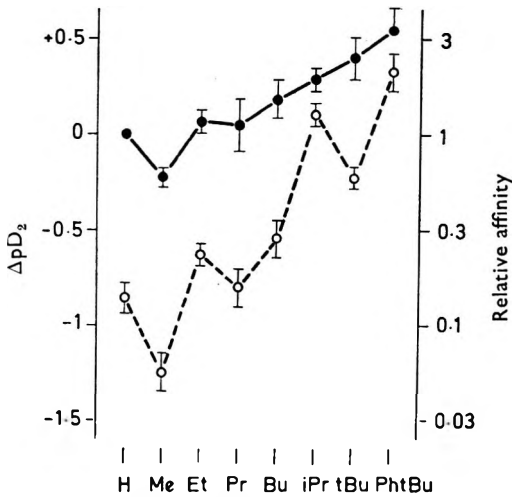

FIG. 1. ΔpD_2 values or relative affinities of both series of derivatives for their effects on fat mobilisation. Mean values ± s.e. are given. —●— Catecholamines. - - - ○ - - - 2-Amino-1-*p*-hydroxyphenylethanol derivatives.

The correlation between the slight affinity differences of the noradrenaline series and the well-graduated affinities of the parallel series, leads, therefore, to the conclusion that in the noradrenaline series there is an ascending trend of affinities towards drugs with the larger substituents, a characteristic of adrenergic reactions of the β-type.

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The relation between tracheorelaxant and fat mobilising action of some derivatives of noradrenaline and 2-amino-1-*p*-hydroxyphenylethanol

SIR,—Attempts to classify the fat mobilising action of sympathomimetic amines into Ahlquist's (1948, 1962) scheme of α- or β-adrenergic receptor functions has hitherto met with numerous difficulties. Fat mobilisation has been attributed to adrenergic actions of the α-type (Ariëns, Waelen, Sonneville & Simonis, 1963), β-type (Pilkington & others, 1962), both types (Steinberg, Vaughan & Margolis, 1960) and neither type (Wenke, Mühlbachová & Hynie, 1962; Love, Carr & Ashmore, 1963; Nickerson, 1965). We have now attempted a correlation of adrenergic fat mobilisation on the one hand and a typical β-adrenergic response, namely the relaxation of tracheal muscle, on the other.

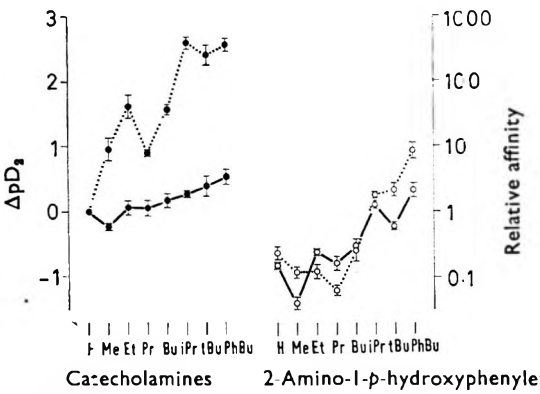

FIG. 1. ΔpD_2 values and relative affinities of the 2-amino-1-*p*-hydroxyphenylethanol and catecholamine derivatives to tracheal relaxation (●) and for fat mobilisation (○). (PhBu = phenyl-t-butyl).

Using the guinea-pig tracheal chain according to Castillo & de Beer (1947), a contraction provoked by histamine in a final concentration of $1{\cdot}1 \times 10^{-4}$M was antagonised by cumulative concentrations of sympathomimetic drugs and recorded isotonically. Homologous series of derivatives of noradrenaline and 2-amino-1-*p*-hydroxyphenylethanol substituted on the nitrogen atom were used. The derivatives were: methyl, ethyl, propyl, butyl, isopropyl, t-butyl and phenyl-t-butyl. The results were related to the effects of the same drugs on fat mobilisation from depot adipose tissue *in vitro* (Černohorský, Cepelík, Lincová & Wenke, 1966).

The affinity parameter pD_2 was derived from the dose-response curves according to van Rossum (1963). In the Figures, relative ΔpD_2 values are given ($\Delta pD_2 = pD_2x - pD_2$ noradrenaline); noradrenaline was used as the comparative standard on each occasion.

In Fig. 1, the relative affinity parameters of the two series of drugs are given for tracheal relaxation and fat mobilisation. The figures for fat mobilisation are those of Černohorský & others (1966). For the noradrenaline derivatives the affinities for tracheal relaxation show large differences from their affinities for fat mobilisation. With the 2-amino-*p*-hydroxyphenylethanol derivatives the relation between tracheorelaxant effect and fat mobilisation is closer.

The results may also be compared by following the influence of substitution by a particular radical in both parent compounds (Fig. 2). The linear correlation of the fat mobilising action of the two series of derivatives found by

Černhorský & others (1966) is again shown for comparison. In this instance, the clear linear relation of affinities differs markedly from the situation for the tracheal muscle. Here, a parabolic relation is evident. By lengthening the unbranched side-chain, the affinity of the noradrenaline derivatives rises, but the affinity of the 2-amino-1-*p*-hydroxyphenylethanol series decreases from the parent compound down to the propyl substituted compound.

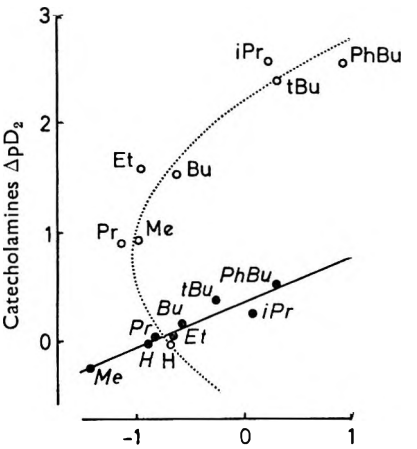

FIG. 2. Correlation of the ΔpD_2 values of both series of derivatives. Relative affinity parameters to fat mobilisation (—●—) and to tracheal relaxation (···○···) are given. (PhBu = phenyl-t-butyl).

We are well aware that it is not possible to evaluate directly all similarities and differences found by comparing the reactivity of such different material under widely different experimental conditions. In spite of this, some common features can be seen in the rising affinity in the direction of higher radicals and a marked enhancement of affinities of derivatives substituted by branched radicals compared with the unbranched ones containing the same number of carbon atoms. The pattern of response suggests that fat mobilisation, like tracheal relaxation, may be attributable to β-adrenergic activation.

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Innervation of domestic fowl and guinea-pig ventricles

SIR,—Marked changes in the force of contraction of driven ventricular strips occurred when the suprathreshold electrical stimulation used to drive the strips was increased tenfold.

A strip was cut from the wall of the right ventricle of a chick (domestic fowl) or guinea-pig heart, one end being anchored to bipolar platinum hook electrodes, and the other (apical) end to a transducer for isometric tension recording.

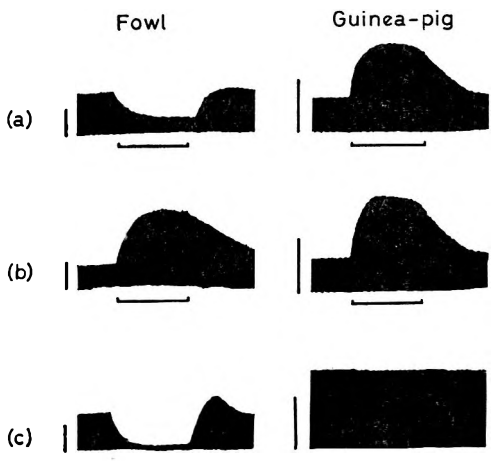

FIG. 1. Polygraph records of isometric tension of guinea-pig and chick ventricular strip preparations stimulated at constant rates. The stimulus strength was increased for periods of 2 min, indicated by the horizontal bars. Vertical calibrations 1 g. (*a*) Untreated preparations, (*b*) in the presence of 1 μg/ml atropine and (*c*) in the presence of 10 μg/ml guanethidine.

Strips were suspended in a solution of the following composition (g): NaCl 6·92, KCl 0·34, $CaCl_2$ 0·30, $MgCl_2$ 0·11, KH_2PO_4 0·16, $NaHCO_3$ 1·0, glucose 2·0, sucrose 4·5, water to 1 litre, and aerated vigorously with oxygen. The temperature was maintained at 41–42° for chick and 37–38° for guinea-pig ventricular strips, which were driven at constant rates of 4–6/sec with square wave pulses of 1–2 V and 5 msec duration.

Increasing the stimulation strength caused a decrease in the force of contraction of chick and an increase in the force of contraction of guinea-pig ventricular strips (Fig. 1a). Atropine, 1 μg/ml, had slight or no effect on the response of guinea-pig ventricular strips but in the chick the decrease was converted to a large increase in the force of contraction (Fig. 1b). Guanethidine, 2–10 μg/ml, abolished the increase in force of contraction in untreated guinea-pig strips but

the response was not converted to a depression. Guanethidine potentiated the depression observed in chick strips (Fig. 1c). In atropinised preparations from both species the increase in the force of contraction observed upon increasing the stimulus strength was abolished by the β-receptor blocking drug propranolol (0·1 μg/ml) and a slowly developing blockade was observed with the adrenergic neurone blocking drug guanethidine (2–10 μg/ml). The blockade produced by guanethidine was partially reversed by the addition of dexamphetamine, 1 μg/ml, to the bath.

Acetylcholine, 0·1 μg/ml, caused a marked depression of the force of contraction of chick ventricular strips—greater than the depression observed with 100 μg/ml of acetylcholine in guinea-pig ventricular strips. In the chick the depressions to both acetylcholine and increasing the stimulus strength could be abolished by atropine or hyoscine (1 μg/ml).

Triethylcholine, 250 μg/ml, which has been shown to compete with choline for the transport mechanisms involved in acetylcholine synthesis (Bowman & Hemsworth, 1965; Bull & Hemsworth, 1965), caused a slowly developing blockade of the depression observed upon increasing the stimulus strength driving chick ventricular strips. This blockade was reversed by the addition of choline (50 μg/ml) to the bath.

These results suggest that the decrease in the force of contraction observed upon increasing the stimulus strength driving chick ventricular strips is due to stimulation of cholinergic inhibitory fibres within the myocardium. Similarly the increase in force of contraction observed in untreated guinea-pig and atropinised chick ventricular strips is due to stimulation of adrenergic excitatory fibres within the myocardium. Thus the chick ventricle has both a parasympathetic and a sympathetic nerve supply while the guinea-pig has a sympathetic but little or no parasympathetic innervation. The latter finding is consistent with anatomical (Woollard, 1926; Nonidez, 1939) and physiological (Schreiner, Berglunde, Borst & Monroe, 1957; Sarnoff, Brockman, Gilmore, Linden & Mitchell, 1960; Vincenzi & West, 1963) evidence for the ventricles of other mammalian species.

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On the ability of desipramine to interfere with reserpine-induced noradrenaline release

SIR,—Desipramine has been shown to inhibit the depleting effect of reserpine on adrenal catecholamine content in the rat (Zbinden, 1962; Shore & Busfield, 1964). However, it fails to prevent the depletion induced by reserpine of noradrenaline stored in the mouse heart (Stone, Porter, Stavorski, Ludden & Totaro, 1964) and rat brain (Sulser, Watts & Brodie, 1962; Garattini, Giachetti Jori, Pieri & Valzelli, 1962), imipramine being slightly active at very high doses in the latter tissue (Pletscher & Gey, 1962).

In these earlier experiments the effects of the drugs were related to a single point in time so that no information on amine levels in terms of time response is available. Brodie and his colleagues have recently re-emphasised the importance of a dynamic approach to similar problems in their kinetic study of noradrenaline release by tyramine (Neff, Tozer, Hammer & Brodie, 1965).

Therefore experiments were designed to assay noradrenaline at various times after treatment with reserpine alone or in combination with desipramine.

Several hours after administration of reserpine, a fall in noradrenaline occurs which is quicker in normal than in desipramine-pretreated animals.

Female Sprague-Dawley rats, 160–200 g, after 14 hr fasting were injected in the tail vein with 2·5 mg/kg reserpine (Serpasil, Ciba). In pretreated animals desipramine dissolved in distilled water (15 mg/kg i.p.) was administered 1 hr before reserpine; all other animals received distilled water in place of desipramine.

Animals were decapitated at various times after the reserpine, organs removed, blotted on filter paper, wrapped in aluminium foil, collected in beakers embedded in broken ice-sodium chloride and preserved at −20° until assayed. Individual animals were accurately timed in all the experiments.

Noradrenaline was estimated spectrofluorimetrically (Aminco Bowman) by a sensitive method employing alumina adsorption and production of fluorescent indole derivatives (Chang, 1964) with the following modifications: 2ml of 0·01N hydrochloric acid was shaken with 4 ml limpid butanol obtained after centrifugation of tissue butanol homogenate (adjust the pH for adsorption with 2 ml 2N sodium acetate); alumina was washed with 3 m 0·2N sodium acetate after discarding supernatant. The pH at the critical steps was as follows: alumina, supernatant pH 7, wash pH 7, eluate pH 4; oxidation, after ethylenediaminetetraacetic acid pH 5·2, final pH 5·4. The range of recovery was 60–70%. Samples containing 0·008 μg noradrenaline (equivalent to approximately 0·15 μg/g tissue, i.e., 15% of heart control values) give values 3 times greater than the blank.

TABLE 1. EFFECT OF DESIPRAMINE ON THE RELEASE OF HEART NORADRENALINE INDUCED BY RESERPINE IN RATS

	Noradrenaline μg/g rat heart ± s.d. after:			
	30 min	1 hr	3 hr	4 hr
Reserpine	0·48 ± 0·1 (4)	0·19 ± 0·09 (4)*	0·06 ± 0·02 (4)**	0·06 ± 0·02 (4)
Desipramine + reserpine	0·64 ± 0·1 (4)	0·35 ± 0·05 (3)	0·14 ± 0·01 (4)	0·08 ± 0·03 (4)

Animals were killed at various times after reserpine as specified; figures in parenthesis indicate the number of observations on which the mean is based. Similar experiments on a total of 42 hearts from treated rats yielded results in agreement with these above (i.e., six pairs of significantly different means).

* $P < 0·05$ (between means at equivalent time).

** $P < 0·01$ (between means at equivalent time).

Control animals heart noradrenaline 1 ± 0·1 μg/g.

In a typical experiment (Table 1) animals were treated on the same day: each time point was later analysed separately. Single organ specimens were individually assayed.

Our results are not in disagreement with the findings previously reported indicating that desipramine is ineffective in preventing depletion of noradrenaline from sympathetic nerve endings by large doses of reserpine. However, as Table 1 illustrates, although the catecholamine stores at 4 hr are lowered in both reserpine and desipramine pretreated reserpinised animals to almost undetectable amounts, the time required to reach the depletion is prolonged by desipramine pretreatment. When rats so "protected" are killed at the times shown after reserpine administration, the noradrenaline content of their hearts is significantly higher after the same interval than that of animals receiving reserpine alone.

A similar, although probably less pronounced, tendency of desipramine to modify time response patterns of noradrenaline release induced by reserpine has been observed in the brain. But the variability and lower amine levels make the experiments on the brain of questionable significance.

Other authors (Axelrod, Whitby & Hertting, 1961; Titus & Spiegel, 1962; Glowinski & Axelrod, 1964), have shown that imipramine and desipramine impair the uptake of noradrenaline by adrenergic nerve terminals. Since the uptake is an important mechanism for restoring most of the released neurohormone in the nerve endings, our data would appear to contrast with present views. However, it is possible that the inhibition of noradrenaline uptake by desipramine creates a relatively high concentration of the catecholamine which tends to slow down the reserpine-induced outflow of it. If this is true then a concentration of noradrenaline at the receptor sites higher than after reserpine alone might be expected after the combination of desipramine and reserpine.

An example of a pharmacological correlation of this biochemical interpretation is the study describing desipramine potentiation and prolongation of the initial hyperthermia elicited by reserpine in the same animal species, doses, and times (Jori & Garattini, 1965).

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Effects of (+)-amphetamine on lactic acid concentrations in the tissues of aggregated mice

SIR,—Aggregation and other stresses markedly enhance the toxicity of amphetamine to mice. In aggregated mice, (+)-amphetamine induces tissue glycogen depletion, hypoglycaemia and tissue noradrenaline depletion (Moore, 1963; Moore, Sawdy & Shaul, 1965). The present report concerns the effects of (+)-amphetamine on lactic acid levels in the tissues of aggregated mice.

Male albino mice (Charles River Mouse Farms), 24–30 g, were injected intraperitoneally with saline or with (+)-amphetamine sulphate (10 mg/kg) and placed in groups of 4 in 9 cm square wire mesh cages. When killed, 60–90 min after injection, the saline-treated mice were quiet and often sleeping. Mice injected with (+)-amphetamine could be divided into 2 distinct groups. One group, termed "excited," exhibited constant motor activity. Mice in the other group, termed "depressed," lay quietly in their cages in an exhausted condition. They exhibited laboured breathing and some loss of motor control. It is during this depressed state that deaths occur; approximately 25% of the aggregated mice die within 4 hr of injecting 10 mg/kg of (+)-amphetamine (Moore, 1963).

Mice were killed by decapitation, and at the same time one hind leg was excised. The first few drops of blood from the trunk were collected in small heparinised beakers. In the "depressed" group blood from 2 or 3 mice had to be pooled to obtain a sufficient quantity for analysis. Glucose was determined in 0·2 ml aliquots of blood by the glucose oxidase method (Glucostat, Worthington Biochemical Corp., Freehold, N.J., U.S.A.). For lactic acid analysis a 0·1 ml aliquot of blood was added to 1·9 ml of cold water. Samples of skeletal muscle, liver and renal cortex, weighing 80–120 mg, were quickly dissected, weighed and homogenised in 1·9 ml cold water. Perchloric acid (2 ml, 0·8N) was added to all samples and the mixture centrifuged at 9,000 × *g* for 5 min. The resulting supernatant was neutralised with 10N potassium hydroxide, and after copper-calcium hydroxide precipitation lactic acid was determined in the supernatant (Barker & Summerson, 1941). These results are summarised in Table 1.

TABLE 1. BLOOD GLUCOSE AND BLOOD AND TISSUE LACTATE CONCENTRATIONS IN AGGREGATED MICE TREATED WITH (+)-AMPHETAMINE SULPHATE

Treatment	Lactic acid (μmoles/g wet weight)				Glucose (mg %)
	Skeletal muscle	Liver	Kidney	Blood*	Blood
Saline	8·49 ± ·63	3·61 ± ·55	3·76 ± ·31	1·62 ± ·03	136·6 ± 6·7
Amphetamine (excited)	10·07 ± ·58	3·43 ± ·54	4·24 ± ·43	1·85 ± ·32	116·3 ± 9·3
Amphetamine (depressed)	7·45 ± ·94	2·51 ± ·75	4·98 ± ·63	1·73 ± ·30	27·2 ± 2·9

Each value represents the mean (±1 standard error) of 10 determinations.
* The values for blood lactate represent μmoles/ml of whole blood.

The tissue and blood lactate concentrations in the control or saline treated mice were similar to those reported in the rat by Schön (1965) and by Gey & Pletscher (1961). Lactic acid levels in the tissues and blood of both excited and depressed (+)-amphetamine-treated mice were not significantly different ($P > 0·05$) from those of the saline treated controls. It should be noted that the blood glucose concentration showed the same pattern as previously reported (Moore & others, 1965). That is, following the injection of amphetamine, the depressed but not the excited mice exhibited marked hypoglycaemia.

Peterson, Hardinge & Tilton (1964) suggested that death from amphetamine may follow neuromuscular blockade. With *in vitro* studies they showed that the neuromuscular blocking action of amphetamine was enhanced by the addition of lactic acid to the bath. The concentration of lactate used by these investigators was based upon the report by Fletcher & Hopkins (1917) that, in fatigued muscle, lactate may reach a level of 0·25% (approximately equivalent to 28 μmoles/g). The relevance of these *in vitro* findings to the death of aggregated mice is questionable since, as shown here by direct measurement, the lactic acid content of skeletal muscle does not increase in the exhausted mice and is only one quarter of that used in the *in vitro* studies. However, as reported previously and substantiated here, the (−)-amphetamine treated mice which become depressed also develop a marked hypoglycaemia. This hypoglycaemia may be an important factor leading to the death of the aggregated mice (Moore & others, 1965).

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Blockade of the psychotic syndrome caused by nialamide in mice

SIR,—Inhibitors of monoamine oxidase, for example, nialamide, are known to cause a rise in noradrenaline and 5-hydroxytryptamine (5-HT) content of mouse brain whereas dopamine is hardly affected. This accumulation of amines is accompanied by a characteristic syndrome of stereotype movements of the animals involving restlessness, enhanced spontaneous motility, head movements, but no aggressiveness (Carlsson & Corrodi, 1964). These effects develop slowly after a high dose of nialamide (500 mg/kg i.p.) and are pronounced after 2½–3 hr.

By pretreating the animals with different inhibitors of the biosynthesis of noradrenaline, dopamine or 5-HT, or all three, the accumulation of these amines was blocked to discover whether the elevated level of noradrenaline or 5-HT, or of both, was responsible for the development of this syndrome.

Earlier work has shown that α-n-propyl-3,4-dihydroxyphenylacetamide (H22/54) and α-ethoxy-2,3-dihydroxyphenylacetamide (H33/07) could block this syndrome caused by nialamide (Carlsson, Corrodi & Waldeck, 1963; Carlsson & Corrodi, 1964). Both substances inhibit the hydroxylation of tyrosine and tryptophan, and so prevent the synthesis, in the animal, of dopamine, noradrenaline and 5-HT (Carlsson, & others, 1963; Carlsson & Corrodi, 1964). L-α-Methyldopa has been shown to inhibit the synthesis of

5-HT (Roos & Werdinius, 1963). The methyl ester of DL-α-methyltyrosine (H44/68) was chosen to inhibit the biosynthesis of dopamine and noradrenaline (Corrodi & Hanson, 1966).

The experiments are summarised in Table 1. Mice were treated with H22/54, H44/68 or α-methyldopa and nialamide. Three hr later the nialamide-treated control animals and the animals receiving nialamide and H44/68 had developed the characteristic syndrome, whereas the mice receiving nialamide and H22/54 or α-methyldopa looked normal. At this time the amine content in the brain was analysed fluorimetrically for noradrenaline (Bertler, Carlsson & Rosengren, 1958), dopamine (Carlsson & Lindqvist, 1962) and 5-HT (Bertler & Rosengren, 1959). In the nialamide-treated control animals there was a large increase in 5-HT (>300% of the normal value), noradrenaline was slightly elevated (~50%) whereas dopamine was unchanged. H22/54 and L-α-methyldopa blocked the rise in 5-HT and noradrenaline almost completely, whereas H44/68 blocked the rise in noradrenaline, and lowered the dopamine level. The accumulation of 5-HT, however, was not affected by H44/68. These results suggest that only substances blocking the synthesis of 5-HT are able to inhibit the development of the nialamide syndrome. Thus, the high excess of 5-HT in the mouse brain seen after nialamide treatment seems to be predominantly responsible for this model psychosis.

TABLE 1. NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE (5-HT) IN MOUSE BRAIN 3 HR AFTER NIALAMIDE (500 MG/KG I.P.) AND OTHER SUBSTANCES. The animals (9 male Albino mice per group) were killed by decapitation; 3 brains were pooled for the 5-HT-determination and 6 brains for the determination of noradrenaline and dopamine. Values are % of normal level (range)

Treatment	No. of expts.	Syndrome	5-HT	Noradrenaline	Dopamine
Untreated controls	8	—	100 (82–110)	100 (90–110)	100 (87–112)
Nialamide	5	present	340 (300–350)	160 (125–190)	112 (98–127)
Nialamide + H 22/54 (500 mg/kg i.p.)	4	blocked	130 (125–145)	115 (105–125)	95 (88–102)
Nialamide + L-α-methyldopa (500 mg/kg i.p.)	3	blocked	135 (120–150)	100 (95–105)	108 (95–120)
Nialamide + H 44/68 (250 mg/kg i.p.)	4	present	315 (270–350)	80 (70–90)	50 (38–65)

Part of the mouse brains were examined by the histochemical technique of Hillarp and Falck for the demonstration of monoamines by fluorescence microscopy (Falck, Hillarp, Thieme & Torp, 1962; Falck, 1962; Hillarp, Fuxe & Dahlström, 1965). No overflow of 5-HT to noradrenaline or dopamine neurones or uptake at these sites could be observed 3 hr after nialamide (Fuxe, personal communication).

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2-Deoxyglucose and inflammation

SIR,—2-Deoxyglucose reduces oedema formation produced by injecting dextran into rats (Goth, 1959), and also inhibits the development of erythema in guinea-pigs exposed to ultraviolet irradiation (Görög & Szporny, 1964). The anti-inflammatory activity of 2-deoxyglucose is generally supposed to be connected with its effects on carbohydrate metabolism. However, 2-deoxyglucose also stimulates the release of catecholamines from the adrenal medulla (Brown & Bachrach, 1959; Hokfelt & Bydgeman, 1961), and evidence is presented which suggests that the anti-oedematous effect of this compound is mediated through this latter mechanism.

Groups of six female rats, 140–170 g, received an intraperitoneal injection of saline or 2-deoxyglucose, 250 mg/kg, 30 min before an injection beneath the left hind paw of 0·1 ml of the supernatant fluid from a 5% suspension of Brewers yeast. Foot volumes were recorded plethysmometrically before and 3/4 hr after the injection. Adrenalectomy or adrenal demedullation was performed 1 week before the experiment, and the adrenalectomised rats were maintained on 1% saline instead of tap water. Blood was taken by cardiac puncture at the time of the second foot-volume measurement. The % inhibition of oedema and % increase in blood sugar caused by 2-deoxyglucose were, in normal 58·9 and 81·7, in adrenalectomised 6·2 and 12·2 and in adrenaldemedullated rats 1·1 and 15·7 respectively. In adrenalectomised and demedullated animals this compound has no anti-oedematous effect and its hyperglycaemic activity is reduced but not abolished. Other irritants such as formaldehyde or silver nitrate have been used and the results were similar.

Propranolol, 10 mg/kg i.m., given 1 hr before 0·1 ml of the yeast extract, antagonises the anti-oedematous activity of adrenaline, 0·5 mg/kg s.c., and 2-deoxyglucose, 250 mg/kg i.p., given 30 min before the yeast extract. Propranolol, by itself does not affect oedema formation, a % inhibition of 3·1 being obtained, nor does it modify the anti-oedematous effects of cyproheptadine, phenylbutazone or hydrocortisone (Kellett, 1966). 2-Deoxyglucose caused a 62·9% inhibition of oedema, adrenaline 66·3, deoxyglucose + propranolol 27·4 and adrenaline + propranolol 29·3% inhibition. There were six rats per group.

While the assumption that 2-deoxyglucose inhibits inflammatory reactions by an effect on carbohydrate metabolism may still be correct, it seems that a direct effect on glycolysis is unlikely to be important. It is possible, however, that an indirect effect on carbohydrate metabolism, through catecholamine release from the adrenal medulla, may be involved. Impaired disposition of a glucose load is seen after the injection of 2-deoxyglucose into normal rats, but

this effect is absent in adrenal demedullated animals (Brown & Bachrach, 1959). If, as Goth (1959) suggests, the anti-oedematous effect of 2-deoxyglucose depends on changes in the permeability of cell membranes to glucose and related macromolecules, then adrenaline may be involved in maintaining the decreased permeability to these substances which exists when 2-deoxyglucose is given to normal animals.

2-Deoxyglucose was less active in the ultraviolet erythema test than in the rat-paw test. Intraperitoneal doses of 400 mg/kg of 2-deoxyglucose were needed for consistent suppression of the erythema. Adrenaline, 1 mg/kg s.c., also inhibited the development of erythema following ultraviolet irradiation, but we have so far failed to antagonise the adrenaline or 2-deoxyglucose responses using large doses of pronethalol, propranolol or dihydroergotamine.

It is impossible to draw any conclusion from these experiments about the mode of action of 2-deoxyglucose in the ultraviolet erythema test, but it would be unwise to assume that its effect is due solely to alterations in carbohydrate metabolism (Görög & Szporny, 1964), since an effect on the adrenal medulla may also be involved.

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January 18, 1966

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