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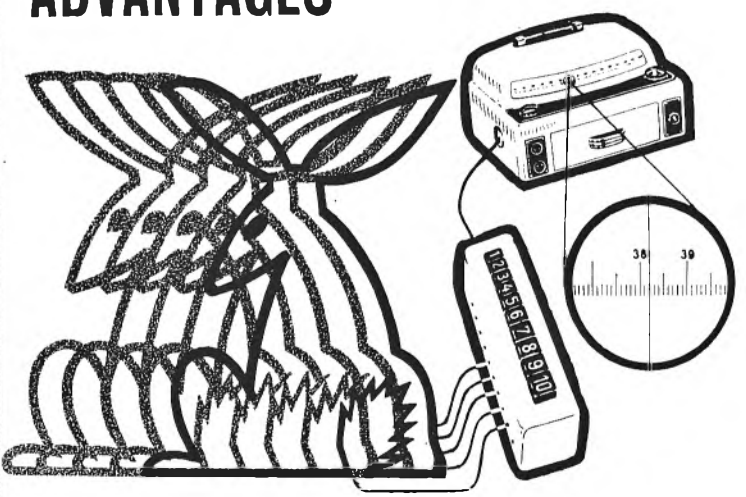

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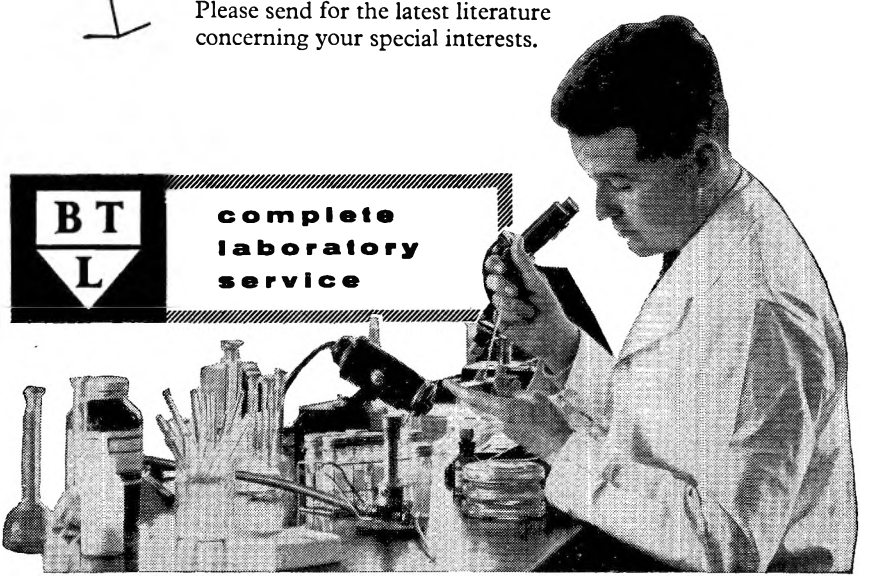

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Review Article

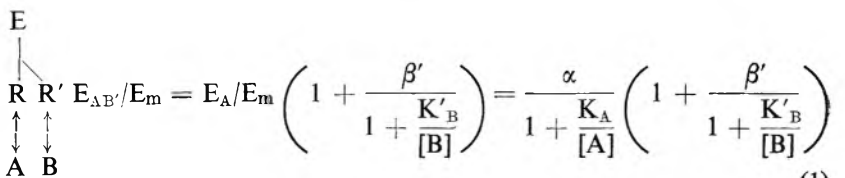
A molecular basis for drug action. The interaction of one or more drugs with different receptors*

E. J. ARIËNS AND A. M. SIMONIS

ARIENS & Simonis (1964) in the preceding review paid special attention to the action of combinations of drugs which interact with common receptors, the competitive interaction. In the following sections other kinds of drug-receptor interaction will be discussed.

NON-COMPETITIVE INTERACTION

Non-competitive interaction is the interference by a drug B with the action of a drug A, both drugs acting on different, but interrelated receptors, while drug B is inactive if applied alone and only modifies the action of a second drug, A. This type of interaction can be put into a formula based on the mass-action law (eqn 1 is an example). The second term in this equation determines the change in the effect of drug A brought about by drug B.



(1)

(compare with eqn 2, Ariëns & Simonis, 1964).

The result of the interaction of both drugs here also depends on the intrinsic activities of A and B, α and β respectively. A well-known example of the non-competitive interaction is the non-competitive antagonism (Ariëns, Simonis & de Groot, 1955; Ariëns, Van Rossum & Simonis, 1956; Ariëns & Simonis, 1961; Ariëns, 1964). Then the intrinsic activity β' of B is less than zero, e.g., -1. The effect of A ($\alpha > 0$) is reduced by the presence of B. If β' is greater than zero, B acts as a sensitiser for A. Then the effect of A is increased by the presence of B.

With a non-competitive antagonism the presence of the antagonist B results in a decline in the curves for the agonist A. An increase in the dose of A cannot overcome the influence of B. The antagonism is insurmountable. This is contrary to the case of the competitive inhibition

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* The second of two reviews on 'A molecular basis for drug action'. The first review appeared in the March, 1964 issue of the Journal, pp. 137–157.

described earlier (Ariëns & Simonis, 1964). The musculotropic spasmolytics of the papaverine-type behave as non-competitive antagonists for many spasmogens (Fig. 1).

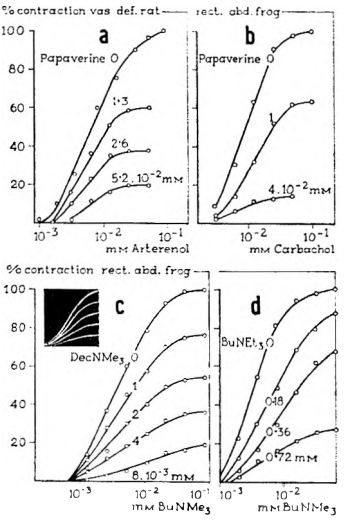


FIG. 1a–d. Cumulative log concentration-response curves for various agonists and the influence thereon of different antagonists: (a) arterenol (noradrenaline) with papaverine, (b) carbachol with papaverine, (c) $BuNMe_3$ with $DecNMe_3$ and (d) $BuNMe_3$ with $BuNEt_3$. Note the decline in the curves which indicates a non-competitive antagonistic action. Compare the experimental curves with the set of theoretical curves (calculated from eqn 1) inset.

In the earlier review (Ariëns & Simonis, 1964) the gradual change from agonist to competitive antagonist in an homologous series of compounds was described. Examples of homologous series of compounds are known in which a gradual change takes place from active compounds to "inactive" compounds which turn out behave as non-competitive antagonists of the active ones. In a series of alkyltrimethyl-ammonium derivatives ($AlkNMe_3$) tested on the isolated rectus abdominis muscle of the frog, the compounds $BuNMe_3$ and $PentNMe_3$ act as agonists while $DecNMe_3$ behaves as their non-competitive antagonist (see Fig. 1c). In the series of $BuNR_3$ derivatives $BuNMe_3$ acts as an agonist while $BuNEt_3$ acts as its non-competitive antagonist (see Fig. 1d) (Ariëns & others, 1955, 1956a; Ariëns & van Rossum, 1957; Ariëns, 1964).

The activity of the non-competitive antagonist is expressed as the pD'_2 value; this is the negative logarithm of that molar concentration of the antagonist that brings about a reduction in the effect of the agonist equal to 50% of the maximal reduction that can be brought about by high doses of the non-competitive antagonist (Ariëns & van Rossum, 1957; Ariëns, 1964).

The various non-competitive antagonists of a certain agonist do not necessarily act on the same receptors. They may interfere with the induction of the stimulus or the evocation of the effect at various points in

the sequence of events initiated by the interaction of the stimulant drug, the agonist, and its specific receptors. The changes brought about by the non-competitive antagonists in the response of the agonist simulate a change in the intrinsic activity.

Another interesting type of non-competitive interaction is the change in the affinity between the agonist and its receptors as a result of the action of a second drug on receptors different from those for the agonist. This type of non-competitive interaction, which in some ways is reminiscent of the competitive interaction, is discussed further by Ariëns & others (1956a) and Ariëns (1964).

The displacement of oxygen by carbon monoxide from the haem moiety of haemoglobin provides a model for the concept of competition for common receptors. There is another aspect: after binding of oxygen to two of the four haem groups the binding of carbon monoxide to the other haem groups is facilitated. In the same way carbon monoxide facilitates the binding of oxygen. This is an example of a change in the affinity of a compound to the receptors induced by another compound on other receptors (Pauling, 1935; Roughton & Darling, 1944; Roughton, Legge & Nicholson, 1949; Roughton, 1949). For further information the reader is referred to the literature (Ariëns, 1964).

DRUGS WITH A MULTIPLE ACTION

In the series of $AlkNMe_3$ derivatives and $BuNR_3$ derivatives tested on the rectus abdominis muscle of the frog mentioned above, the loss of the activity is not caused by a decrease in the intrinsic activity but by the introduction of non-competitive inhibitory properties as a result of the change in the chemical structure of the compounds.

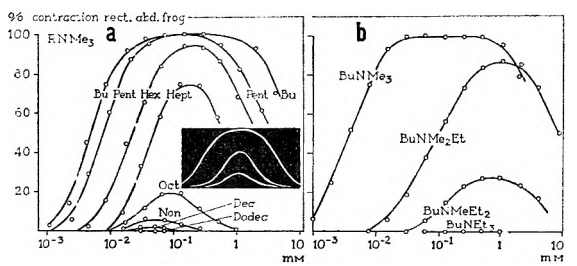
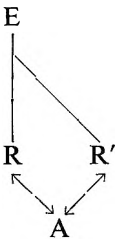


FIG. 2a, b. Cumulative log concentration-response curves for two homologous series of compounds, namely $RNMe_3$ derivatives and $BuNR_3$ derivatives. Note the gradual change from active to "inactive" compounds, the latter being non-competitive antagonists of the active compounds (see Fig. 1c and d respectively). Compare the experimental curves with the set of theoretical curves (calculated from eqn 2) inset.

In the transition from agonist to non-competitive antagonist, compounds with a multiple action are found: compounds which at lower doses behave as agonists while at higher doses, by interference of the non-competitive component in the action, the effect originally induced is abolished (see Fig. 2).

An equation for this type of non-competitive auto-inhibition (eqn 2) is obtained by substituting in eqn 1 α', K'_A and [A] for β', K'_B and [B]

respectively, while the intrinsic activities are given values of $\alpha = 1$ and $\alpha' = -1$. If in eqn 2 $\alpha = 1$ and $\alpha' > 0$, the compound A exhibits an auto-sensitisation (Ariëns & others, 1956a; Ariëns, 1964).



$$E_{AA'}/E_m = \frac{\alpha}{1 + \frac{K_A}{[A]}} \left(1 + \frac{\alpha'}{1 + \frac{K'_A}{[A]}} \right) \quad .. \quad (2)$$

In the series of $AlkNMe_3$ derivatives, the gradual elongation of the alkyl chain results in a gradual decrease of K'_A/K_A and an enhancement of the auto-inhibition. With the decrease in K'_A/K_A the auto-inhibitive properties of the drug will manifest themselves before the maximum effect of A possible with the object concerned, E_{Am}, is reached. The intermediate compounds for which this occurs may also be called partial agonists. Examples are the compounds $HexNMe_3$ and $HeptNMe_3$ in Fig. 2a and $BuNMe_2Et$ and $BuNMeEt_2$ in Fig. 2b. They differ from the agonistic compounds such as $BuNMe_3$ and $PentNMe_3$ in the slope of their curves and in the maximal effect obtained with them. These intermediate compounds interact with two different types of receptors simultaneously; they have a multiple action. They differ essentially from the type of partial agonists found on the transition from agonists to competitive antagonists (see Ariëns & Simonis, 1964). For very low values of K'_A/K_A the compounds will behave as pure non-competitive antagonists. Because of the relatively high affinity, $1/K'_A$, to the receptors, R', these compounds will saturate the receptors R' in doses which induce no effect on the receptors R. The compounds $DecNMe_3$ and $BuNEt_3$ are examples (see Fig. 1c and 1d).

If a partial agonist with a multiple action of the type just described such as $HeptNMe_3$ and $BuNMe_2Et$, is combined with a competitive

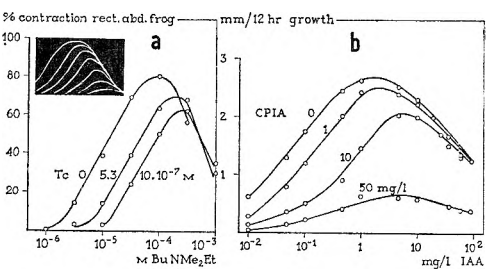
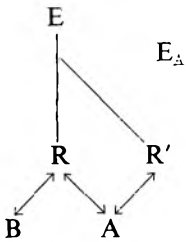


FIG. 3a, b. Cumulative log concentration-response curves for two compounds exhibiting an auto-inhibition and the influence thereon of various concentrations of a competitive antagonist: (a) $BuNMe_2Et$ and the influence of tubocurarine, and (b) 3-indolyl acetic acid (IAA) tested on the growth of Avena coleoptile sections and the influence on it of 4-chlorophenoxy-isobutyric acid (CPIA) (after Foster & others, 1955). Note the parallel shift in the ascending part of the curves. Compare the experimental curves with the set of theoretical curves (calculated from eqn 3) inset.

antagonist acting on the receptors on which the agonistic effect is induced, only the ascending part of the bell-shaped curve is expected to shift. The drug-receptor interaction responsible for the descending part is not interfered with by such a competitive antagonist. The equation for this type of interaction, eqn 3, is obtained from a combination of eqn 3, in Ariëns & Simonis (1964) and eqn 2 above. The intrinsic activities have to be substituted then as $\alpha = 1$, $\beta = 0$ and $\alpha' = -1$. Fig. 3 gives experimental curves for this type of interaction (Ariëns & others, 1955, 1956a; Foster, McRae & Bonner, 1955; Ariëns, 1964).



$$E_{ABA'}/E_m = \left(\frac{\alpha}{1+\left(1+\frac{[B]}{K_B}\right)\frac{K_A}{[A]}} + \frac{\beta}{1+\left(1+\frac{[A]}{K_A}\right)\frac{K_B}{B}}\right)\left(1+\frac{\alpha'}{1+\frac{K'_A}{[A]}}\right) \quad .. \quad .. \quad (3)$$

Another interesting combination of drugs is that of a partial agonist such as $HexNMe_3$ and $HeptNMe_3$ (Fig. 4a) or $BuNMe_2Et$ and $BuNMeEt_2$ (Fig. 4b) with a non-competitive antagonist, e.g., $DecNMe_3$ or papaverine.

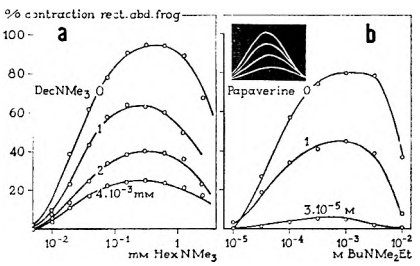
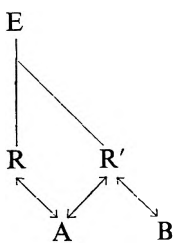


FIG. 4a, b. Cumulative log concentration-response curves for two compounds exhibiting an auto-inhibition and the influence thereon of various concentrations of a non-competitive antagonist: (a) $HexNM_3$ and (b) $BuNMe_2Et$, in the presence of $DecNM_3$ and papaverine respectively. Note the decline in the curves. Compare the experimental curves with the set of theoretical curves (calculated from eqn 4) inset.

Eqn 4, which represents this type of interaction, results from a combination of eqn 3 (Ariëns & Simonis, 1964) and eqn 2 above. In eqn 4 $\alpha = 1$, $\alpha' = -1$ and $\beta' = -1$ are substituted for the intrinsic activities. Fig. 4 gives experimental curves for this type of interaction.



$$\frac{E_{AA'B'}}{E_m} = \frac{\alpha}{1+(K_A/[A])}\left[1+\left(\frac{\alpha'}{1+(1+[B]/K'_B)K'_A/[A]} + \frac{\beta'}{1+(1+[A]/K'_A)K'_B/[B]}\right)\right] \quad .. \quad (4)$$

Attention is drawn to the Figs. 6 and 7 in the preceding review (Ariëns & Simonis, 1964) which represent the hydrolysis of various esters of choline and of combinations of such esters by acetylcholinesterase. The substrate inhibition manifested by the dose-effect curves in those figures, which was not considered then, now requires special attention. The curves in both figures represent enzymological examples of dose-effect curves which are clear-cut analogues for the curves represented in Figs 2a, 2b, and 3a and 3b of this text respectively. For most of the types of dose-response curves described in this paper analogous examples are known in enzymology (van Rossum & Hurkmans, 1962; Webb, 1963).

The experimental results presented agree well with the theory as can be seen from the equations and the theoretical curves calculated on basis of these equations.

If a compound is qualified as "inactive" it is necessary to specify the type of inactivity. The "inactive" compounds may have interesting properties such as competitive or non-competitive antagonistic actions towards related agonists. For the chemist designing new compounds it may be especially useful to know whether the loss in activity as a result of certain chemical modifications is based on a loss in affinity, a loss in intrinsic activity or the introduction of non-competitive antagonistic properties. The analysis of dose-response curves for combinations of drugs is required for this purpose.

The equations given in the various sections are presented in such a way that the application of the mass-action law to the various drug-receptor interactions involved in the actions of the drugs and drug

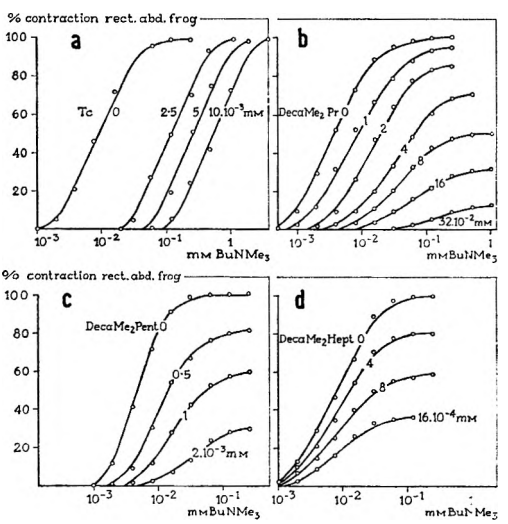
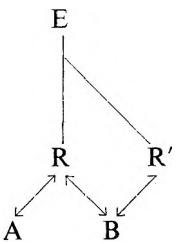


FIG. 5a–d. Cumulative log concentration-response curves for $BuNMe_3$ and the influence thereon of various concentrations of antagonistic compounds: (a) tubocurarine, (b) $DecaMe_2Pr$, (c) $DecaMe_2Pent$ and (d) $DecaMe_2Hept$. Note the purely competitive antagonistic action of tubocurarine and the gradual change from the dualism in antagonism of $DecaMe_2Pr$ to the purely non-competitive antagonistic action of $DecaMe_2Hept$.

combinations is clearly expressed. As well as the type of interaction discussed in detail here, these equations cover many other types of action of drugs and drug combinations if suitable values for the various constants, the affinities and the intrinsic activities, are introduced.

DRUGS WITH A MULTIPLE ANTAGONISTIC ACTION

In the foregoing sections, drugs which are intermediates between agonists and competitive antagonists such as pentylNMe$_2$Et (see Fig. 5, Ariëns & Simonis, 1964) and drugs which are intermediates between agonists and non-competitive antagonists such as heptylNMe$_3$ and butylNMe$_2$Et (see Fig. 2) , have been discussed. But there also exist drugs which are intermediates between competitive and non-competitive antagonists. These are compounds with a competitive antagonistic action on which a non-competitive antagonistic action is superimposed. If in eqn 5, which is closely related to eqn 3, $\alpha = 1$, $\beta = 0$ and $\beta' = -1$ is substituted for the intrinsic activities, the equation represents the interaction of an agonistic compound A with a compound B having a multiple antagonistic action such as that just described (Ariëns & others, 1956a, 1957; Ariëns & Simonis, 1961; Ariëns, 1964).



$$\frac{E_{ABB'}}{E_m} = \left(\frac{\alpha}{1 + \left(1 + \frac{[B]}{K_B}\right)\frac{K_A}{[A]}} + \frac{\beta}{1 + \left(1 + \frac{[A]}{K_A}\right)\frac{K_B}{[B]}} \right) \left(1 + \frac{\beta'}{1 + \frac{K'_B}{[B]}} \right) \quad .. \quad .. \quad (5)$$

Many examples of compounds with a multiple antagonistic action are known. This is so, for instance, in the series of the bis-*N*-alkyl-substituted decamethonium derivatives, where the propyl and the pentyl compounds (Fig. 5b and c) are intermediates between curariform drugs of the curarimimetic type, such as tubocurarine (Fig. 5a) and triethylsuccinylcholine, and curariform drugs of a practically pure non-competitive type

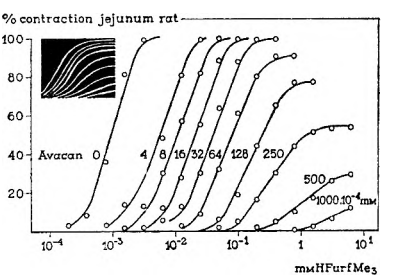


FIG. 6a. Cumulative log concentration-response curves for the agonist furtrethonium (HFurfMe$_3$) and the influence thereon of various concentrations of Avacan. Note the competitive component in the action of Avacan (the parallel shift) on which a non-competitive component (the decline in the maximal response and the slope of the curves) is superimposed. Compare the experimental curves with the set of theoretical curves (calculated from eqn 5) inset.

such as the bis-*N*-heptyl derivative of decamethonium (see Fig. 5d) (van Rossum & Ariëns, 1959a; Ariëns, 1964). Other examples are the compounds in which a parasympatholytic and a papaverine-like spasmolytic action are combined, for instance, Avacan (isopentyl α-(2-diethylamionethylamino)phenylacetate hydrochloride), and adiphenine (Goodman & Gilman, 1955; Formanek & Weis, 1963). Figs. 6a and 6b represent experimental dose-response curves obtained with Avacan.

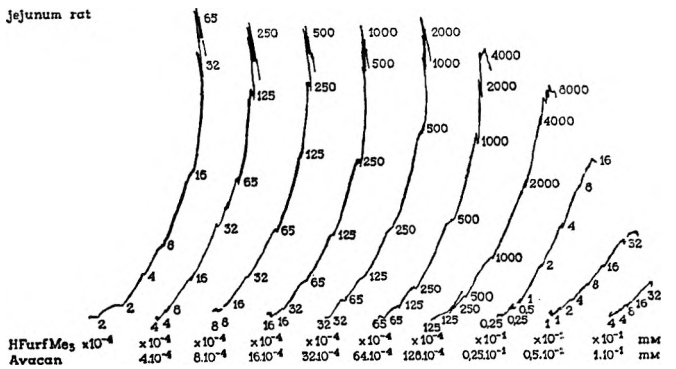


FIG. 6b. A registerogram of cumulative dose-response curves for a combination of drugs as represented in Fig. 6a.

This and the foregoing sections on non-competitive interaction dealt with the interactions of drugs with different but interdependent receptors R and R′, such that the effect induced on one type of receptor, R, is changed by the action of drugs on the second type, R′, while on the receptor R′ no primary effect can be induced. The following section will deal with the action of drugs on different but interdependent receptors R_I and R_{II} such that an effect can be induced on each of the receptors independently. This type of drug-receptor interaction can be considered as a functional interaction to distinguish it from the non-competitive interaction.

COMBINATIONS OF DRUGS WHICH INTERACT WITH DIFFERENT RECEPTORS, BUT PRODUCE THEIR EFFECT BY MEANS OF A COMMON EFFECTOR SYSTEM

In the most simple case the effect of the combination of two agonists A and B will be equal to:

$$\begin{array}{c} E \\ | \\ R_I \quad R_{II} \\ \uparrow \quad \uparrow \\ A \quad B \end{array} \quad \frac{E_A}{E_m} + \frac{E_B}{E_m}\left(1 - \frac{E_A}{E_m}\right) = \frac{E_B}{E_m} + \frac{E_A}{E_m}\left(1 - \frac{E_B}{E_m}\right) = \frac{E_A}{E_m} + \frac{E_B}{E_m} - \frac{E_A E_B}{E_m^2} \qquad (6)$$

where E_A/E_m and E_B/E_m are the effects of [A] and [B] if applied singly. The term $E_A E_B/E_m^2$ represents the mutual hindrance of the drugs as far as the use of the common effector-system is concerned. If $E_m^2 \gg E_A E_B$

the effect of the combined drugs will be close to the sum of the effects of the single drugs (Ariëns & others, 1956b, 1957; Ariëns, 1964).

Fig. 5 in the preceding review (Ariëns & Simonis, 1964) represents the combination of a partial agonist and another agonist both inducing their effects on the same receptors. The displacement of the agonist by the partial agonist results in an antagonistic effect. What then will be the consequence of combining such a partial agonist with an agonist which induces its effect on different receptors but by means of the same effector system? There is no mutual displacement of the drugs now. The partial agonists will always contribute to the effect independent of the dose of agonist used. Fig. 7 gives examples of such a combination.

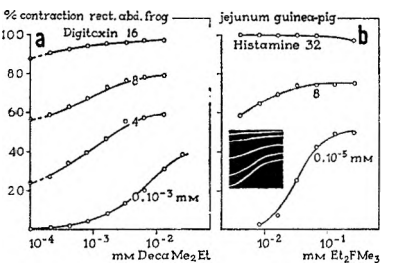


FIG. 7a, b. Cumulative log concentration-response curves for two partial agonists and the influence thereon of various concentrations of other agonists: (a) $DecaMe_2Et$ in combination with digitoxin and (b) Et_2FMe_3 in combination with histamine. Note the absence of a dualism in effect for the partial agonist which indicates a functional interaction between the compounds combined. (Compare with Fig. 5, in preceding review, p. 142). Compare the experimental curves with the set of theoretical curves (calculated from eqn 6) inset.

The spasmogen, bis-mono-ethyl-substituted decamethonium, a partial agonist, is combined with another spasmogen, digitoxin. These compounds interact with different receptors. The decamethonium derivative is antagonised in a competitive way by tubocurarine for example, while digitoxin is not antagonised by this drug. As expected, the partial agonist always acts synergistically in this combination (Fig. 7a) (Ariëns & others, 1956a). If the decamethonium derivative is combined with succinylcholine, a spasmogen which is also inhibited competitively by tubocurarine, a set of curves of the type represented in Fig. 5 of the preceding review is obtained (Ariëns & Simonis, 1964). Another example is given in Fig. 7b. Histamine acts on receptors different from those for the parasympathomimetic compound 2,2-diethyl-4-(trimethylammonium)methyl-1,3-dioxolane (Et_2FMe_3), a partial agonist. If combined with histamine, Et_2FMe_3 only acts as a synergist (van Rossum & Ariëns, 1959b).

Another example of the combination of drugs acting on different receptors but via a common effector system is the combination of an agonist with two antagonistic drugs, one of which is a competitive antagonist of the agonist while the other one is not.

As a basis for a comparison, the combination of an agonist with two competitive antagonists will be discussed first. Equation 7 represents

the competitive interaction of three compounds, A, B and C, with common receptors. If in this equation $\alpha = 1$, $\beta = 0$ and $\gamma = 0$ it represents the combination of an agonist and two competitive antagonists.

$$E_{ABC}/E_m = \frac{\alpha}{1 + \left(1 + \frac{[B]}{K_B} + \frac{[C]}{K_C}\right)\frac{K_A}{[A]}} + \frac{\beta}{1 + \left(1 + \frac{[A]}{K_A} + \frac{[C]}{K_C}\right)\frac{K_B}{[B]}} + \frac{\gamma}{1 + \left(1 + \frac{[A]}{K_A} + \frac{[B]}{K_B}\right)\frac{K_C}{[C]}} \quad .. \quad .. \quad (7)$$

If equipotent doses of the parasympatholytics lachesine and atropine are combined and tested against the parasympathomimetic furtrethonium, the effect of this combination is equal to the effect of doubling the separate doses (Fig. 8a). This is expected on basis of the theory represented by eqn 7. For the combination of a potent dose of e.g. lachesine with a less potent dose of atropine, the experimental results also agree well with the theory (Fig. 8c).

This type of co-operation between two drugs acting on common receptors, here the competitive antagonists B and C, is known as an additive action. An additive action also occurs if the drugs A and B, in eqn 3 of the preceding review (see page 141), have equal intrinsic activities, e.g. such that $\alpha = \beta = 1$; this implies that both drugs are agonists. This means that on basis of equipotencies, where $K_A/[A] = K_B/[B]$, or the effect of the dose of drug A is equal to the effect of the dose of drug B, either drug can be substituted by the other without a change in the effect. This relationship is represented in eqn 8. In case of an additive action, $q = 1$, independently of the value of n, which may vary from 1 to ∞.

$$q = \frac{\text{effect of } \left[\frac{1}{n}[A] + \left(1 - \frac{1}{n}\right)[B]\right]}{\text{effect of } [A]} \quad .. \quad .. \quad (8)$$

This can easily be seen by combining eqns 2 and 3 in the preceding review (pages 139 and 141), which gives eqn 9.

$$q = E_{AB}/E_A = \frac{(1 + K_A/[A])\,(\alpha[A]K_A n + \beta[B]/K_B - \beta[B]/K_B n)}{\alpha(1 + [A]/K_A n + [B]/K_B - [B]/K_B n)} \quad (9)$$

For the case where $[A]/K_A = [B]/K_B$ and $\alpha = \beta$, $E_{AB}/E_A = q$ becomes 1.

If $q > 1$, the effect obtained with the combination of A and B is larger than that expected in the case of an additive action, then there is potentiation. If $q < 1$ the effect obtained with the combination of A and B is smaller than in the case of an additive action. These relations hold true also for combinations of an agonist and a partial agonist acting on common receptors (Ariëns & others, 1956b; Ariëns & van Rossum, 1957a; Ariëns, 1964).

A comparison of the effect of a combination of two competitive antagonists such as atropine and lachesine, with the effect of a combination of a competitive antagonist, for instance, lachesine and an antagonist

such as isoprenaline (isopropylarterenol), often called a functional antagonist, clearly demonstrates the difference between the additive action for the first type of combination (Fig. 8a, c) and the potentiation arising from the second type (Fig. 8b, d). In the case of a combination of lachesine with isoprenaline the effect comes close to the sum of the effects of the single doses.

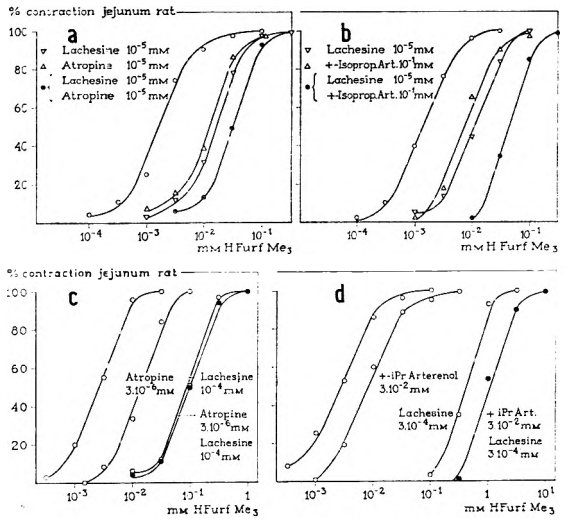


FIG. 8a–d. Cumulative log concentration-response curves for the parasympathomimetic furtrethonium ($HFurfMe_3$) and the influence thereon of the competitive antagonists lachesine and atropine and combinations of these two drugs (a and c), and the influence of the competitive antagonist lachesine and the functional antagonist +-isopropylarterenol (isoprenaline) and the combination of these two drugs (b and d). (a,b) Note, with the combination of lachesine and atropine, there is much less than a summation of the antagonistic actions of the single compounds. With the combinations of lachesine and isoprenaline there is nearly a summation of the antagonistic actions of the single compounds. (c,d) Note the doses of the antagonists have been chosen so that the differences between the combination of the two competitive antagonists and of the competitive and the functional antagonist are more emphasised than in Figs. a–b. It is clear that in (c) the low dose of atropine hardly contributes to the effect if combined with the higher dose of lachesine, while in case (d) the dose of isoprenaline equiactive with the dose of atropine in (c) gives a strong contribution to the effect if combined with lachesine; there then is nearly a summation in the antagonistic actions of the single compounds.

The differences between the actions of the competitive antagonist lachesine and of an antagonist such as isoprenaline are also manifested in the almost unrestricted shift in the dose-response curves for furtrethonium achieved by increasing doses of lachesine and the restricted shift with isoprenaline. Experiments with combinations of histamine, an antihistimine and a catecholamine such as isoprenaline give analogous results.

SEQUENTIAL BLOCKAGE

In the foregoing section, two types of combinations of blocking drugs are mentioned: the combination of two competitive antagonists and the

combination of a competitive and a functional antagonist. A special type of combination extensively discussed in the literature, especially on antibacterial chemotherapy, is the sequential blockage (Black, 1963). It can be defined as the blockage caused by a combination of two inhibitors that act on different receptors in a linear sequence of reactions (enzymes) (Black, 1963; Ariëns, 1964). Some of the most evident examples are the combination of a sulphonamide, which is a competitive antagonist of *p*-aminobenzoic acid, with a folic acid antagonist, such as aminopterin (see Fig. 9a). A combination related to the sequential blockage is

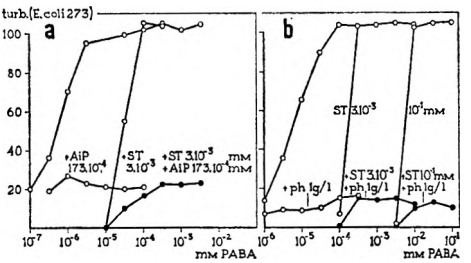


FIG. 9a, b. Log concentration-response curves for the growth of the PABA-deficient strain *E. coli* 273: (a) with PABA and the influence thereon of sulphathiazole (ST), of aminopterin (AiP) and of the combination of both antagonists; (b) with PABA and the influence thereon of sulphathiazole, of phenol and of the combination of both antagonists. Note that the effect of the combination of antagonists is practically the sum of the effects of the antagonists if applied singly.

obtained if in the last term in eqn 5, C is substituted for B, γ' for β' and K'_C for K'_B while $\alpha = 1$, $\beta = 0$ and $\gamma' = -1$. The eqn 10 then obtained represents the combination of an agonist A with a competitive antagonist B and a non-competitive antagonist C (Ariëns, 1964).

$$\frac{E_{ABC'}}{E_m} = \left[\frac{\alpha}{1 + (1 + [B]/K_B)\, K_A/[A]} + \frac{\beta}{1 + (1 + [A]/K_A)\, K_B/[B]}\right] \left[1 + \frac{\gamma'}{1 + K'_C/[C]}\right] \quad .. \quad .. \quad .. \quad (10)$$

Eqn 10 again like all the foregoing equations results from a simple application of the mass-action law to the various drug-receptor interactions involved. Fig. 10a represents an experimental example for a combination of drugs as just described. Dose-response curves are given for the spasmogen furtrethonium (a parasympathomimetic), tested in the presence of lachesine, a competitive antagonist (a neurotropic antispasmodic also called parasympatholytic); for furtrethonium tested in the presence of the musculotropic antispasmodic papaverine; and for furtrethonium in the presence of a combination of both antispasmodics mentioned. Fig. 9b gives another example. Here growth induced by the growth-factor *p*-aminobenzoic acid is inhibited by a combination of a competitive antagonist sulphathiazole and a non-competitive inhibitor phenol. Fig. 9b and 10a demonstrate that the combination of inhibitors which act independently, leads to a clear-cut potentiation, which may

be close to a summation of the inhibitive effects obtained with the drugs if applied singly.

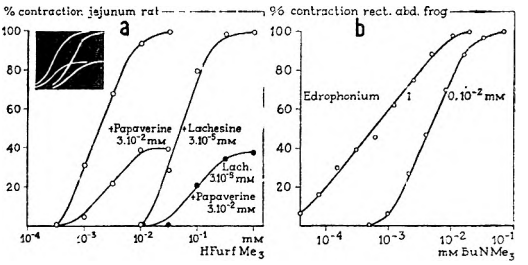


FIG. 10a, b. (a) Cumulative log concentration-response curves for the action of the parasympathomimetic furtrethonium ($HFurfMe_3$) and the influence thereon of the parasympatholytic lachesine, of the antispasmodic papaverine, and of the combination of both antagonists. Note the effect of the combination of lachesine and papaverine is practically the sum of the effects of the antagonists if applied singly. Compare the experimental curves with the set of theoretical curves (calculated from eqn 10) inset. (b) Cumulative log concentration-response curves for $BuNMe_3$ and the influence thereon of edrophonium. Note the sensitisation of the muscle to $BuNMe_3$ by edrophonium.

DESENSITISATION

Desensitisation in a general sense is a decrease in the response of a biological object to a drug as a result of the action of a previous dose of another drug or of a previous dose of the same drug. The various types of antagonism: competitive, non-competitive, and functional, described in earlier sections, can be seen as various forms of desensitisation as long as the antagonist is added first. In addition, interference with drug distribution and drug metabolism may result in a desensitisation. Take, for instance, the delay in resorption of a drug caused by a second drug, the inhibition of the bio-activation of a drug by a second one, or the enhancement of the excretion (Ariëns, 1964).

A special type of desensitisation is autodesensitisation, or tachyphylaxis (Champey & Gley, 1911). This is a decrease in the response to a dose of a drug given shortly after the effect of a previous equal dose of the same drug is abolished. After a number of doses the biological object may become fully unresponsive to the drug. Different types of tachyphylaxis can be distinguished depending on the causative mechanism. Some of the models for drug action mentioned in the preceding review imply the existence of tachyphylaxis.

(*a*) The "potential" theory says that a drug is active only as long as there is a net-flux of the drug into the cells or to the receptors, or in other words as long as there is a concentration gradient to the receptors. This implies that after removal of the drug from the extracellular phase the biological object will be desensitised to a sequential dose of the drug as long as there is an intracellular residue of the drug (Ariëns, 1964).

(*b*) The rate-theory postulates that there is a response only as long as there is a flux of drug molecules to the receptors, or, as long as there is

an association between drug molecules and receptors. After removal of the drug from the biophase, depending on the rate of dissociation, a proportion of the receptors will remain occupied for a time. During this period a desensitisation of the biological object to the drug given previously and also to related drugs will occur (Paton, 1961).

(*c*) The model for drugs acting indirectly, such as those sympathomimetics which are supposed to act by a liberation of endogenous catecholamines or liberators such as histamine liberators, requires a decrease in response after sequential dosage because the endogenous compound is depleted so the response is weakened (Axelrod, Gordon, Hertting, Kopin & Potter, 1962).

(*d*) The response induced in the biological object by a high dose of a drug may result in an exhaustion of the supply of energy or other factors necessary for the response to take place, and thus in a decrease in the responsiveness, which lasts until the system is regenerated.

In the cases (*a*), (*b*) and (*c*), the desensitisation will show a degree of specificity. It will occur only for drugs which have the same mechanism of action, i.e., drugs which intereact with the same receptors. In case (*d*) the desensitisation will occur for all drugs which make use of the effector system concerned. So, for instance, it will occur for all spasmogens where the contractile system in the biological object has reached exhaustion.

An experimental example of this type of desensitisation is given by Huidobro & Valette (1961). They described how, after high doses of acetylcholine or histamine, the isolated ileum of the guinea-pig becomes insensitive to spasmogens such as acetylcholine, histamine, barium chloride, 5-hydroxytryptamine, and also to coaxial electrical stimulation. After high doses of acetylcholine or histamine, organs, previously sensitised to specific antigens, did not respond with a contraction to these antigens.

(*e*) If the actions of sequential doses of a drug are studied *in vivo*, the response may be eliminated by the action of counter-regulating forces. The effect of a vasodepressor drug for instance may be abolished by an increase of vascular tone or heart frequency. Then the next dose may find most receptors still occupied, which may result in a desensitisation. Although many examples of the phenomenon of tachyphylaxis are given in literature (Furchgott, 1955; Blackman, 1961; Huidobro & Valette, 1961; Axelrod & others, 1962; Day & Rand, 1963), information on the mechanism is scarce. The types of autodesensitisation or tachyphylaxis described are reversible. Irreversible desensitisations also are possible although the term tachyphylaxis is not then applied.

If drug distribution and drug metabolism are also taken into account, further mechanism of autodesensitisation can be mentioned. This is, for example, where there is a decrease in sensitisation caused by an adaptive increase of degrading enzymes (Ariëns, 1964). However, relatively long times are necessary in these instances to induce the desensitisation which then lasts relatively longer; here, the term tachyphylaxis is best replaced by the term tolerance.

SENSITISATION

There are two essentially different types of sensitisation.

(1) *The sensitisation which results from repeated application of a drug or a preceding application of a related drug.* The time between applications may vary from several weeks to years. A minimum time, about 9 days, must elapse between the applications. The effects found after sensitisation differ from the effect caused by the first dose of the drug in that the reaction after sensitisation is hardly related to the type of drug used, but is closely related to the type of sensitisation. This sensitisation usually occurs because on first application, the drug acts as a hapten and this leads to the formation of antibodies against the hapten-protein complex formed. The second application of the same, or a chemically related, compound may then result in an allergic reaction.

(2) *The sensitisation which results in an increase in the response to a certain dose of a drug when applied after another, the sensitising drug.* Here, the effect after sensitisation is qualitatively of the same type as that before sensitisation, and specific for the type of drugs used. Usually, the time elapsing between the application of the compounds is restricted to minutes, hours, or, maximally, several days.

(*a*) The mechanism of the sensitisation may be based on changes in distribution or metabolism. Take, for instance, the increase in speed of absorption of drugs effected by hyaluronidase, the increased response to acetylcholine after inhibition of acetylcholinesterase, or the increase in the response to drugs as a result of the inhibition of renal excretion.

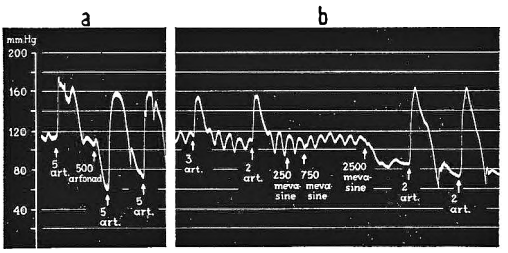


FIG. 11. Registerogram of the mean arterial blood pressure of the cat (2·5 kg) treated with noradrenaline (art.) and ganglionic blocking agents. Note that the changes induced in the blood pressure by noradrenaline (given intravenously) increase after intravenous injection of the ganglion agents trimethaphan camphorsulphonate (Arfonad) and mecamylamine (Mevasine). The vascular system is "sensitised" to noradrenaline by the ganglionic blocking agents.

(*b*) The sensitisation of the frog rectus muscle to acetylcholinomimetics such as butyltrimethylammonium ($BuNMe_3$) by acetylcholinesterase inhibitors such as neostigmine and edrophonium (Fig. 10b) should be mentioned here. Since $BuNMe_3$ is insensitive to acetylcholinesterase the sensitising action cannot be ascribed to the esterase-inhibiting properties of the sensitising drugs. Another example of a sensitisation is the sensitising action of sub-threshold doses of a stimulant drug, e.g. a spasmogen for related spasmogens (Uyldert, Tjiong & Ybema, 1955).

(*c*) An interesting example is the sensitisation of the frog rectus muscle for $BuNMe_3$ produced by an increase in the potassium concentration in the bath fluid. It works only if the potassium concentration is increased before the $BuNMe_3$ is added.

(*d*) A well-known type of sensitisation is the increase in response of a preparation to catecholamines after denervation or after depletion of the catecholamine stores by reserpine or cocaine. The decrease of the sympathetic tonus in the vascular system caused by ganglionic blocking agents also results in an increase of the response to catecholamines as is demonstrated in Fig. 11. Since, however, the maximal response to the catecholamines is hardly or not increased, it is arguable if there is a real sensitisation in this case.

(*e*) Blockade of counter-regulating processes by one drug may also result in an increase in response to a second one. The increase in the hypotensive response to β-sympathomimetic drugs such as phenyl t-butylarterenol after α-sympatholytic drugs are applied may serve as an example. The α-sympatholytic drug blocks the counter regulations based on vasoconstriction induced by endogenous noradrenaline and/or adrenaline on the α-receptors (Ariëns, Waelen, Sonneville & Simonis, 1963).

REVERSAL OF THE RESPONSE

In some instances, application of a drug may result in a reversal of the response to a second drug. Well-known examples are the change of the pressor response of adrenaline to a depressor response in the presence of α-sympatholytic drugs and the change of the depressor response of ethylarterenol to a pressor response in the presence of β-sympatholytic drugs (Ariëns & others, 1963). Reversals in response are common for drugs with a multiple action—drugs which induce effects on different types of receptors—if the effects induced on the various receptors have an opposite influence on the final effect studied. The α-sympathomimetic vasoconstriction induced on α-receptors and the β-sympathomimetic vasodilatation induced on β-receptors by adrenaline and ethylarterenol are examples. With adrenaline, the action on the α-receptors is dominant, with ethylarterenol the action on the β-receptors is dominant. The application of these drugs in the presence of compounds blocking the receptors on which the dominant action is induced, results in an unmasking of the opposite effect. Another well-known example of such a reversal is the change of the depressor action of acetylcholine on the parasympathetic division of the autonomic nervous system to a pressor response which originates from the ganglionic stimulant action in the sympathetic division, which is unmasked after parasympatholytics such as atropine.

The terms desensitisation, tachyphylaxis, sensitisation and reversal in action only indicate certain phenomena. They give no information on the specific processes of drug action involved. A closer analysis shows that various types of desensitisation, tachyphylaxis, etc., can be differentiated.

THE SLOPE OF THE DOSE-RESPONSE CURVES

It will have been seen there is a good agreement between the general characteristics of the sets of experimental dose-response curves and those of the theoretical curves which can be calculated from the equations given. However, there is a clear difference between theory and practice in the slope of the dose-response curves; in almost all cases the slope of the experimental curves is much steeper than that of the theoretical curves (eqn 1, preceding review). In exceptional cases in the literature, dose-response curves are described, in which the slope is virtually identical to that predicted by the theory (eqn 2, preceding review). In some cases this agreement is based on the plotting procedure of the dose-response curves. This is so, for instance, with the curves for acetylcholine tested on the frog rectus abdominis muscle described by van Maanen (1950) (Fig. 12a). This curve represents the dose-response relationship for a

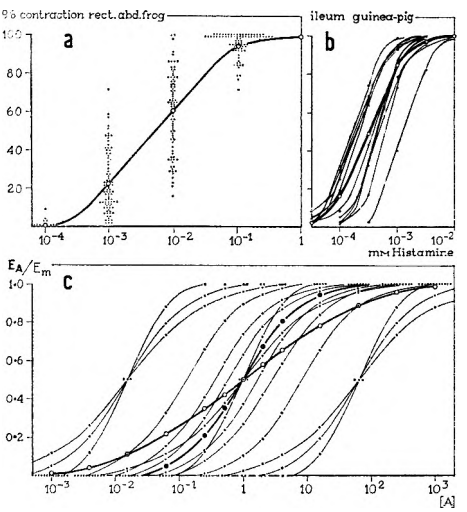


FIG. 12a, b. (a) The log dose-response curve for a group of frog rectus abdominis muscles to acetylcholine (ACh), obtained by calculating the mean response of the various muscles for different doses (van Maanen, 1950). Note that this curve is not the mean of the dose-response curves for the individual muscles. (b) Cumulative log concentration-response curves for histamine. The curves are obtained with pieces of gut of 12 different animals. Note that the slope of the mean curve for the group of organs is less steep than that of the curves for the individual organs. (c) Theoretical log concentration-response curves as calculated from eqn 2 (preceding review, p. 139) distributed symmetrically around the curve with an ED50 equal to 1. The heavy curve (○–○) represents the dose-response curve for the group of objects concerned. The steeper (bold) curve (●–●) represents the mean of the various individual dose-response curves. Note that the slope of the latter curve (●–●) is identical to the slope of the curves calculated from the theory. The slope of the other curve (○–○) is much less inclined.

group of organs; it is calculated on basis of the main response obtained with a number of doses on a number of biological objects. The slope of this curve is partially determined by the biological variation in the group of organs and is not identical with the slope of the dose-response curve for

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a single object to which in fact the theoretical dose-response curve adheres (eqn 2, preceding review) (Perry, 1950; Gaddum, 1953). The curve obtained by van Maanen runs from the lower end of the dose-response curves for the objects which are highly sensitive, to the upper end of the dose-response curves for the objects with the lowest sensitivity. The larger the variation in the sensitivity of the biological objects, the larger will be the difference between the slope of the dose-response curve for a single object and the slope of the dose-response curve obtained with a group of biological objects. Fig. 12c demonstrates schematically the difference between the mean dose-response curve obtained with a group of organs and the mean of the dose-response curves obtained with a group of organs. Fig. 12b gives experimental evidence for the difference in the slope of the mean dose-response curve obtained with a group of organs and that of the curves obtained on single organs for the case of histamine tested *in vitro* on guinea-pig gut.

The difference between the slope of the experimental and theoretical dose-response curves does not seriously undermine the theory because there are various experimentally founded possibilities to interpret this difference such as the occurrence of threshold phenomena and the receptor reserve.

THRESHOLD PHENOMENA

Without going into detail, about the possible theoretical backgrounds of threshold phenomena such as the all-or-none response or mechanical factors (Ariëns & van Rossum, 1957b), experimental evidence will be given for thresholds in the dose-response curves for some types of drugs. A threshold implies that the effect, for instance the contraction of the muscular organ, only occurs after a certain concentration or dose of the drug is reached, with the consequence that the dose-response curves plotted on a linear scale will not pass through the origin (the zero point on the effect axis) but through some virtual point below. Fig. 13 gives

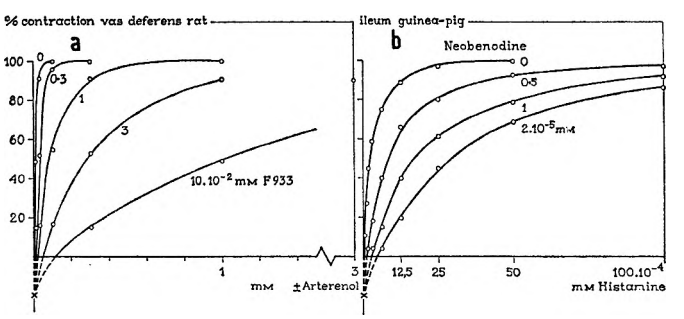

FIG. 13a, b. Cumulative concentration-response curves for the agonistic compounds noradrenaline (arterenol) and histamine and the influence thereon of various concentrations of the competitive antagonists benzodioxane (F933) and neobenodine respectively. Note how the curves intersect with the ordinate at a point below zero which indicates a threshold in the concentration-effect relation and gives the value for this threshold.

examples of dose-response curves which represent this phenomenon. Many other examples are known (Kirschner & Stone, 1951; Ariëns & Simonis, 1960; 1961). Because of the competitive relationship between the stimulant drugs noradrenaline and histamine and the antagonists benzodioxane and neobenodine (2-(*p*-methoxydiphenylmethoxy)-ethyldimethylamine), respectively, plotting the dose-response curves represented in Fig. 13 according to Lineweaver & Burk, would be expected to result in straight lines. As is shown in Fig. 14 this is not so. If, however,

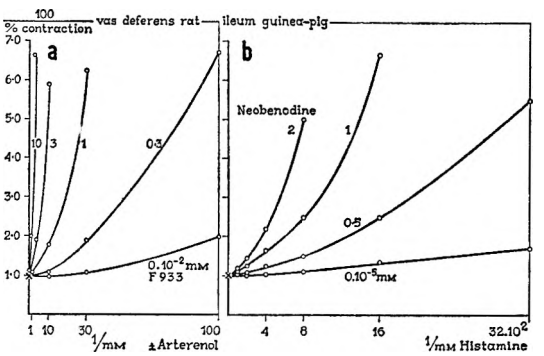

FIG. 14a, b. Cumulative concentration-response curves as represented in Fig. 13a, b, now plotted according to Lineweaver and Burk. Note that because of the competitive relation between agonist and antagonist the curves were expected to be straight.

a correction is made for the threshold according to the methods suggested by Kirschner & others (1951) the curves are straightened (see Fig. 15). These experimental results are strong evidence for a threshold in the dose-effect curves. The presence of a threshold implies that the experimental dose-response curves will differ in slope from the theoretical curves calculated from eqn 2 of the preceding review. The dose range over which the experimental curve is extended, is shortened on the side

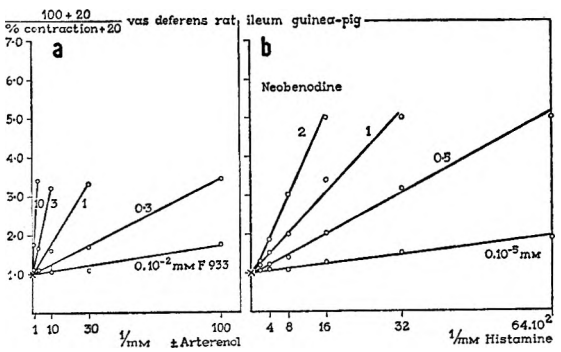

FIG. 15a, b. Cumulative concentration-response curves as represented in Figs 13a, b and 14a, b, plotted according to Lineweaver and Burk and now corrected for the threshold value as found in Fig. 13a, b. Note that after correction for the threshold value, straight lines are obtained.

of the lower concentrations and this curve will therefore be steeper than the theoretical curve which is based on the assumption of a proportionality between the fraction of the receptors occupied and the effect.

RECEPTOR RESERVE

As was emphasised by various investigators, the assumption that the effect is proportional to the fraction of receptors occupied, does not hold experimentally (Furchgott, 1954, 1955; Nickerson, 1956, 1957). In many cases a maximum effect, a 100% effect, is obtained after only a fraction of the receptors are occupied (Ariëns, van Rossum & Koopman, 1960; Ariëns, 1964). The existence of a receptor reserve can be demonstrated by making use of irreversible blocking agents of the specific receptors, such as the β-haloalkylamines of which dibenamine is an example.

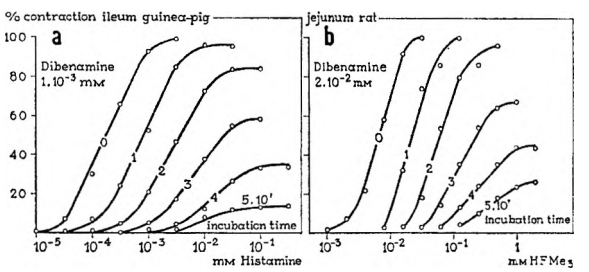

FIG. 16a, b. Cumulative log concentration-response curves for histamine (a) and $HFMe_3$ (b) after incubation of the organs with constant concentrations of dibenamine for various times. Incubation time n × 10 min. Note the shift which precedes a decline in the curves for histamine and $HFMe_3$, which indicates a receptor reserve.

Incubation of the biological object with an agent irreversibly blocking the specific receptors would be expected to lead to a decline in the dose-response curve. It nevertheless often leads to a parallel shift in the dose-response curves. This implies that after irreversible elimination

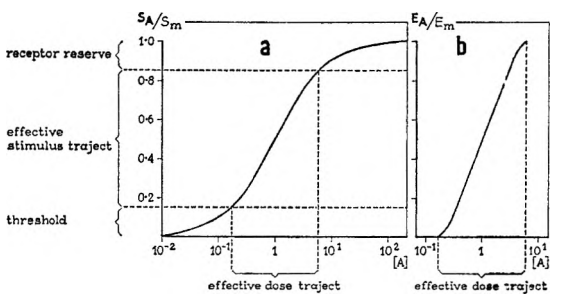

FIG. 17a, b. Theoretical curve representing the relation between the dose and the stimulus calculated from eqn 2 (preceding review, p. 139) in which S_A/S_m is substituted for E_A/E_m. (a) If a threshold and a receptor reserve as indicated are assumed—which implies no linear proportionality between stimulus and effect—a dose-effect curve as represented in (b) is obtained. Note the slope of this dose-effect curve is steeper than that of the dose-effect curve in the case where the effect is linearly proportional to the stimulus (no threshold and no receptor reserve). Then the dose-effect curve is identical to the dose-stimulus curve (a).

of a fraction of the receptors a 100% effect is still possible if higher doses of the stimulant drug are used. This means that there is a reserve in receptors. At the moment that the reserve is exhausted by the irreversible blocking agent, further elimination of receptors by this agent will lead to a decline in the slope of the dose-response curve and a decrease in the maximal effect obtained. The larger the shift in the curves that can be obtained with the irreversible blocking agent, the larger is the receptor reserve. Fig. 16a and b give experimental examples for the case of histamine and the parasympathomimetic $HFMe_3$. The existence of a receptor reserve implies that the experimental curve will be steeper than the theoretical dose-response curve calculated from eqn 2 of the preceding review. The dose range over which the experimental dose-response curve is extended is shortened on the side of the higher concentrations. Both the threshold phenomenon and the receptor reserve bring about an increase in the slope of the experimental dose-response curves, compared with that expected from theory (see Fig. 17).

RECEPTOR PROTECTION EXPERIMENTS

An interesting aspect of the irreversible blockade of specific receptors mentioned in the above section is the possibility of protecting the specific receptors against irreversible blockade by high doses of the agonist, related agonistic compounds, or reversible competitive antagonists. Their presence on the receptors will delay the irreversible blockade and thus result in a protection (Furchgott, 1955; Ariëns & others, 1960). This receptor protection will be specific, which means that a cross protection will occur for drugs which act on the same receptors. So, for instance

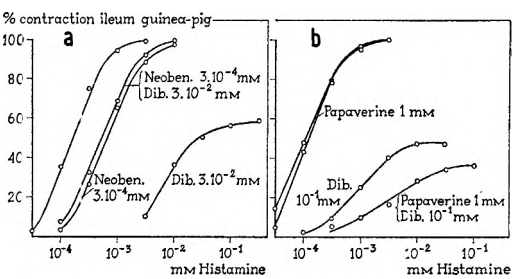

FIG. 18a, b. (a) Cumulative log concentration-response curves for the spasmogen histamine and the influence thereon of incubation of the organ with the irreversible blocking agent, dibenamine, for 10 min. and with dibenamine in the presence of the reversible blocking agent, the antihistamine neobenodine for 15 min. The reversible blocking agent was added 5 min. before dibenamine. Before adding the agonist after incubation, the organ was washed for 10 min. Note in the presence of the reversible blocking agent, a competitive antagonist, the organ is protected against the action of dibenamine. (b) Cumulative log concentration-response curves for the spasmogen histamine and the influence thereon of incubation of the organ with dibenamine for 10 min and with dibenamine in the presence of the reversible non-competitive antagonist papaverine for 15 min. Papaverine was added 5 min before dibenamine. Before adding the agonist after incubation, the organ was washed for 40 min. Note that, in contrast to the case represented in (a) the presence of the non-competitive antagonist (papaverine) does not protect the organ against the action of dibenamine.

the receptors for histamine can be protected by histamine and antihistamines (see Fig. 18a), but not by non-competitive agonists such as papaverine (see Fig. 18b) (Ariëns & Simonis, 1962a). Also here, there is an agreement between the experimental results and the theory as can be easily seen from the equations or the theoretical curves calculated on basis of them (Ariëns & others, 1960; Ariëns, 1964). The types of experiment described are by no means restricted to isolated organs such as the gut, or vas deferens. The principles of competitive and non-competitive interaction, reversible and irreversible binding hold true also in the field of microbiology, for example. The inhibition of growth of a *p*-aminobenzoic acid (PABA)-requiring strain of *Escherichia coli* 273 by specific antagonists of PABA, such as sulphathiazole and 2-methyl-PABA, may serve as an example. Fig. 19a and 19b represent the inhibition of growth if the growth factor, PABA, is added at the same time as the inhibitors. The curves argue for a competitive relationship between growth factor and inhibitor.

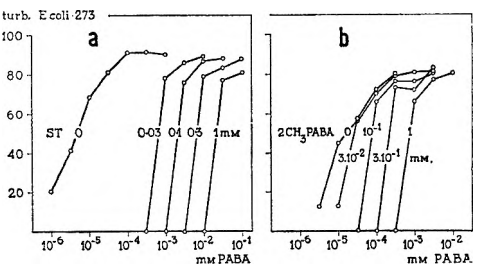

FIG. 19a, b. Log concentration-response curves for the growth (turbidity) of the *p*-aminobenzoic acid (PABA)-requiring strain *E. coli* 273 and the influence thereon of various concentrations of the antimetabolites sulphathiazole (ST) (a) and 2-CH_3-PABA (b). Metabolite and antimetabolite are added simultaneously. Note the sets of curves are characteristic for the competitive relation between PABA and the antimetabolites.

The growth factor, PABA, is assumed to serve as a precursor for folic acid and folinic acid respectively, which in its turn serves as a coenzyme F in an enzyme that plays a rôle in the transfer of one-carbon units. If the chemical structures of PABA, 2-methyl-PABA and sulphathiazole are compared it will be clear that 2-methyl-PABA could possibly substitute for PABA in the folic acid molecule, while this seems unlikely for sulphathiazole.

The incorporation of 2-methyl-PABA in folic acid and finally in coenzyme F means that this inhibitor is practically irreversibly bound. If *E. coli* 273 in the logarithmic phase of its growth is incubated with 2-methyl-PABA and sulphathiazole respectively and, after incubation, the growth factor PABA is added, the inhibition of growth by sulphathiazole can be overcome easily by the PABA while in the cultures incubated with 2-methyl-PABA no growth or only some growth after a long delay can be obtained. These experiments suggest that 2-methyl-PABA is assimilated to give an afunctional folic acid and finally an afunctional coenzyme F—while the sulphonamide competes with PABA in a strictly reversible way, possibly at the entrance of the metabolic channel for PABA.

If PABA and 2-methyl-PABA are applied simultaneously, PABA, if applied in sufficiently high concentrations, may protect the bacteria against the irreversible incorporation of 2-methyl-PABA. This is demonstrated in Fig. 20a and b (Ariëns & Simonis, 1962; Ariëns, 1964).

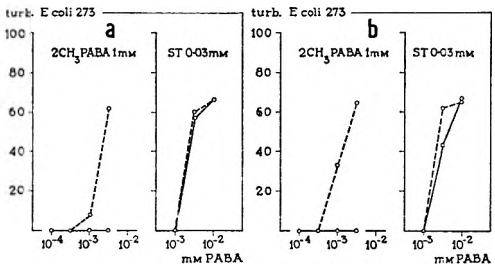

FIG. 20a, b. Log concentration-response curves for PABA as a growth factor for *E. Coli* 273. Curves – – – –: metabolite and antimetabolite are applied simultaneously. Curves ———: the same combinations of metabolite and antimetabolite, but now the metabolite is added after incubation with the antimetabolites for 15 hr (a) and for 30 hr (b). Note incubation with sulphathiazole does not change the results obtained. After incubation with 2-CH_3-PABA no further growth is obtained with PABA.

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Research Papers

Some possible causes of pharmacological activity in blank eluates following the separation of sympathomimetic catecholamines by paper chromatography

D. J. ROBERTS (WITH AN ADDENDUM BY MARTHE VOGT)

Eluates prepared from paper chromatograms at Rf values and under experimental conditions that were expected to eliminate the possibility of contamination with catecholamines were shown to inhibit the rat uterus and to cause a fall in the mean arterial blood pressures of anaesthetised rats. These isoprenaline-like actions have been shown to be due to inadequate removal of the phenolic developing solvent and to the use of sodium dihydrogen phosphate solutions as eluants. Attempts to establish qualitative and quantitative relationships between the chromatography blank and isoprenaline have failed, and it is concluded that these factors could not alone be responsible for the isoprenaline-like substance reported to occur naturally in various species of mammal.

METHODS similar to the modification of Vogt (1952) of the recommendations of Crawford & Outschoorn (1951) for the quantitative separation of catecholamines from biological extracts and tissue fluids by paper chromatography gave inconsistent answers. There were variable recoveries, deviation of the results of the biological assay from parallelism, and the presence of pharmacological activity at Rf values other than those of noradrenaline, adrenaline and isoprenaline when estimating concentrations of these amines in rabbit and cat plasma. The inconsistent results were shown to be largely due to displacement of catecholamine Rf values by lipid present in the plasma samples (Roberts, 1963b), but even after prior extraction of this interfering lipid the recoveries of small "physiological" amounts of catecholamine were inconsistent and the biological assays were still not parallel especially when the eluate volumes were in excess of 2 ml.

The results of these recovery experiments (Table 1) indicated that one or more substances were contaminating the eluates resulting in the antagonism of the actions of adrenaline and noradrenaline on the blood pressure of pithed rats. As an additional or alternative feature, the contaminant(s) potentiated the action of isoprenaline on the blood pressure of rats under pentobarbitone sodium anaesthesia. It also seemed possible that the contaminant(s) caused inhibition of the isolated tissue preparations used, with resultant apparent potentiation of the actions of all three amines on these tissues. Furthermore, pharmacological activity was still evident in blank eluates prepared from strips of paper cut at levels corresponding to Rf values other than those of the sympathomimetic catecholamines, and this

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activity (fall in mean arterial blood pressure of the rat and the inhibition of acetylcholine-induced contractions of the isolated rat uterus) seemed likely to be due to the same substance(s) as that which was interfering with the biological assays of the catecholamines.

A modified form of the paper chromatographic techniques of Vogt (1952) was used to demonstrate the presence of an isoprenaline-like substance in the adrenal glands of cat, rabbit, monkey and man (Lockett, 1954), and in cat plasma after electrical stimulation of the sympathetic chains (Lockett, 1957) and after the injection of adrenaline (Eakins & Lockett, 1961). Since the pharmacological activity of the blank eluates noticed in this laboratory on the rat blood pressure and isolated rat uterus was basically isoprenaline-like, there existed the possibility that this chromatographic blank and the isoprenaline-like substance of Lockett and her colleagues was the same substance(s). It was decided to test this possibility and to investigate the source of the activity of the blank eluates.

Methods

Whatman No. 1 papers for chromatography were washed and prepared (Roberts, 1963a, b) and were placed into tanks which were then gassed with carbon dioxide. No amines were applied to the papers. The solvent, phenol containing 15% v/v 0·1 N hydrochloric acid, was allowed to travel 25–30 cm up the paper before "chromatography" was stopped, and the papers were washed with benzene. Eluates were then prepared from strips of paper cut at levels corresponding to the estimated Rf values of noradrenaline, adrenaline and isoprenaline and from strips cut at a variety of other levels, using as eluant a solution containing 0·4 g sodium dihydrogen phosphate and 1 mg ascorbic acid per 100 ml distilled water and employing the techniques described by Crawford & Outschoorn (1952). The activity of these eluates, of initial volume before concentration, 2·2–7·6 ml, was examined on the rat mean arterial blood pressure (pithed or under pentobarbitone sodium anaesthesia) and on the rat uterus responding constantly and submaximally to acetylcholine. The following solutions were also prepared and their activity similarly examined on these biological preparations: (1) 0·01 ml of the phenolic chromatography solvent dissolved in 10 ml 0·9% w/v aqueous sodium chloride; (2) distilled water; (3) 0·9% w/v aqueous sodium chloride; (4) 1·8% w/v aqueous sodium chloride; (5) 3·6% w/v aqueous sodium chloride; (6) 1·0 ml 0·4% w/v aqueous sodium dihydrogen phosphate evaporated to dryness (1 mm Hg, bath temperature 30–35°) and redissolved in 1·0 ml 0·9% w/v aqueous sodium chloride; (7) 5·0 ml, 0·4% w/v aqueous sodium dihydrogen phosphate, dried and redissolved as in 6 above; (8) 10·0 ml 0·4% w/v aqueous sodium dihydrogen phosphate, dried and redissolved as in 6 above; (9) 0·01% w/v ascorbic acid in 0·9% w/v aqueous sodium chloride; and (10) hydrochloric acid (0·01 N).

Recovery experiments were made on 0·1, 0·25 or 0·5 μg quantities of noradrenaline, adrenaline and isoprenaline, each calculated as base, added to cat plasma (5–25 ml) which had been standing at room temperature for

24 hr. Protein and lipid-free extracts of the plasma samples were prepared and chromatographed as 10 cm bands from acetone: ethanol solution (Roberts, 1963b). Eluates were prepared using either a solution containing 0·4 g sodium dihydrogen phosphate and 1 mg ascorbic acid per 100 ml distilled water (Table 1) or distilled water alone (Table 2) as eluants, and elution times varied from 6 to 15 hr. The activity of these eluates was examined as described above.

Drugs. (—)-Noradrenaline acid tartrate (L. Light & Co. Ltd.), (—)-adrenaline acid tartrate (Burroughs Wellcome & Co.) and (±)-isoprenaline sulphate (Burroughs Wellcome & Co.) were obtained commercially.

Results

Marked pharmacological activity was demonstrated in eluates prepared from all areas of the "blank" chromatograms and the activity of eluates prepared from strips of paper representing the Rf values of noradrenaline, adrenaline and isoprenaline was in no way different from that found in eluates obtained from other areas. Solutions prepared from large volumes of eluate were, however, more active than those prepared from smaller volumes. The following results therefore apply to all areas of the paper. The numbers in brackets relate to the solutions described under "Methods" above.

BLOOD PRESSURE OF A PITHED RAT

The blank eluates usually produced an initial rapid rise, which was always immediately followed by a longer lasting fall, in the mean arterial blood pressures of pithed rats when injected intravenously. Both the rise and the fall were increased with increase in dose. Similar rapid rises in blood pressure were observed when the dilute solution of the phenolic solvent (1) was injected but these were not followed by a depressor phase (Fig. 1, left hand record). When the blank eluates and the phenolic

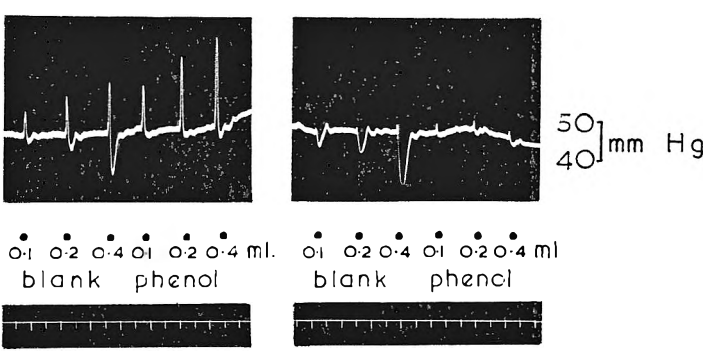

FIG. 1. The effect of blank eluate and a dilute solution of the phenolic solvent on the blood pressure of a pithed rat. Between the left hand and right hand records both solutions were washed with benzene, evaporated to dryness and redissolved. All injections i.v. Time trace in min.

solution were vigorously washed three times with equal volumes of benzene (the benzene layers after separation being sucked off through a capillary pipette), and evaporated to dryness before being redissolved in isotonic saline, the pressor activity was no longer evident in either solution when injected into the rats. The blank eluates, however, still showed marked depressor activity (Fig. 1, right hand record).

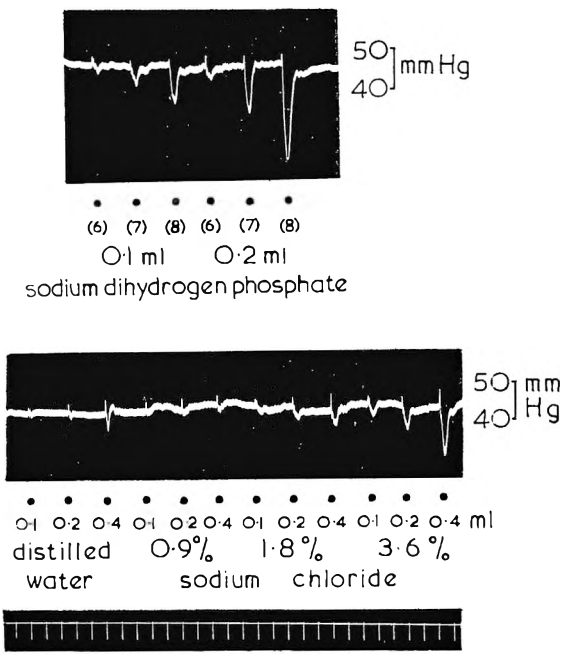

FIG. 2. Upper record. The effect of sodium dihydrogen phosphate on the blood pressure of a pithed rat. (6) = 1 ml, (7) = 5 ml, and (8) = 10 ml. 0·4% w/v $NaH_2PO_4.2H_2O$ evaporated to dryness and redissolved in 1 ml 0·9% w/v aqueous sodium chloride solution (see text).

Lower record. The effect of distilled water, and isotonic and hypertonic sodium chloride solutions on the blood pressure of a pithed rat. All injections i.v. Time trace in min.

Similar falls in blood pressure were observed on injecting solutions (6), (7) and (8), which represented the amount of sodium dihydrogen phosphate expected to be present in eluates of volume 1, 5 and 10 ml respectively. The size of fall increased with the dose volume of any one solution and with increasing concentration of sodium dihydrogen phosphate (Fig. 2, upper record). Depressor activity was also evident when hypertonic sodium chloride solutions were used (4 and 5), the size of the fall again increasing with dose and with increasing hypertonicity (Fig. 2, lower record). Small falls were occasionally seen when distilled water (2) was injected, but isotonic sodium chloride solution (3) was invariably without effect on the blood pressure (Fig. 2, lower record) as were usually ascorbic acid (9) and dilute hydrochloric acid (10).

BLOOD PRESSURE OF RAT UNDER PENTOBARBITONE SODIUM ANAESTHESIA

The blank eluates always produced a fall in blood pressure when injected intravenously into anaesthetised rats, the magnitude of the response increasing with increase in dose volume. No initial rapid rise in blood pressure was seen in these preparations. Depressor activity was also evident when the dilute solution of the phenolic solvent (1), the sodium dihydrogen phosphate solutions (6, 7 and 8) and the hypertonic sodium chloride solutions (4 and 5) were injected. The size of the response to any one solution increased with increase in dose volume and the more concentrated the solution of sodium dihydrogen phosphate or sodium chloride then the greater the fall in blood pressure (Fig. 3). The dose-

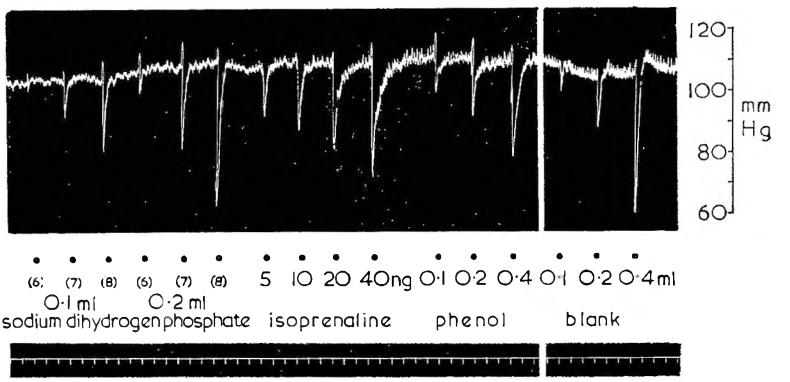

FIG. 3. Comparison of the depressor effects of sodium dihydrogen phosphate, isoprenaline, a dilute solution of the phenolic solvent and blank eluate on the blood pressure of rat anaesthetised with pentobarbitone sodium. (6) = 1 ml, (7) = 5 ml, and (8) = 10 ml 0·4% w/v $NaH_2PO_4.2H_2O$ evaporated to dryness and redissolved in 1 ml 0·9% w/v aqueous sodium chloride solution (see text). All injections i.v. Time trace in min.

response curves obtained with these solutions were steeper than that of isoprenaline and muscle tremors were evident after the administration of the solution of the phenolic solvent (1). Distilled water (2) and dilute hydrochloric acid (10) also caused the blood pressure to fall slightly, while minor fluctuations in blood pressure both above and below the resting level were observed following the administration of the ascorbic acid solution (9) which represented the amount of ascorbic acid that would be present in a 10 ml eluate. No changes in blood pressure were recorded when isotonic sodium chloride solution (3) was injected.

INHIBITION OF THE SUBMAXIMAL RESPONSES OF A QUIESCENT RAT UTERUS TO CONSTANT DOSES OF ACETYLCHOLINE

The blank eluates, the dilute solution of the phenolic solvent (1), the sodium dihydrogen phosphate solutions (6, 7 and 8) and hydrochloric acid (10), all caused inhibition of submaximal responses of a quiescent rat uterus to constant doses of acetylcholine. The degree of inhibition increased with increase in dose volume of all of the solutions and with

increase in the concentration of sodium dihydrogen phosphate. The dose volumes ranged from 0·025 to 0·2 ml and the capacity of the isolated organ bath was 5 ml. The characteristics of the inhibitions produced by these solutions were, however, different from those produced by isoprenaline. Following the initial inhibition produced by isoprenaline, several doses of acetylcholine were required before the responses were back to their original height; after the inhibition produced by the blank or solutions (1), (6), (7), (8) and (10) the responses to acetylcholine returned immediately to their original levels (Fig. 4).

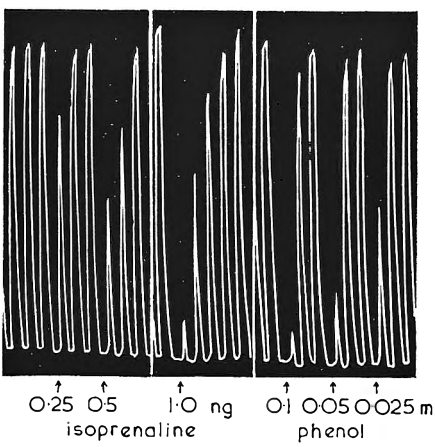

FIG. 4. Comparison of the inhibitory effects of isoprenaline and a dilute solution of the phenolic solvent on the isolated rat uterus responding to 0·5 μg acetylcholine added at 3 min intervals. Arrows mark additions of inhibitory drugs, 30 sec before the next addition of acetylcholine. Volume of organ bath = 5 ml. Contact time of acetylcholine = 45 sec.

Attempts to equate, in terms of (±)-isoprenaline sulphate, the activity of the blank, the dilute solution of the phenolic solvent (1), or the sodium dihydrogen phosphate solutions (6, 7 and 8) on the rat uterus and the rat blood pressure were unsuccessful.

When the sodium dihydrogen phosphate solutions were made up in half calcium rat uterus Ringer (de Jalon's) instead of 0·9% sodium chloride solution, the inhibitions of the rat uterus were much less marked and did not increase with increase in dose or with the amount of sodium dihydrogen phosphate. Also the pH of a 20 ml sample of the Ringer (7·3 as measured on a Pye pH meter) changed rapidly to 6·2 when 1 ml of elution fluid (0·4% w/v sodium dihydrogen phosphate in distilled water, pH 4·6) was added, and then settled down to 6·8. Further additions of elution fluid altered pH only slightly, 5 ml being required to bring the pH down to 6·2.

Distilled water (2), isotonic sodium chloride solution (3), hypertonic sodium chloride solutions (4 and 5) and ascorbic acid (9) were usually without measurable effect on the acetylcholine-induced responses of the rat uterus.

RECOVERY OF CATECHOLAMINES FROM PLASMA

When distilled water only was used as eluant the recoveries of small quantities of the amines were relatively constant (compare Tables 1 and 2) and blank activity was largely eliminated.

TABLE 1. RECOVERIES OF NORADRENALINE, ADRENALINE AND ISOPRENALINE (AS% OF THE AMOUNTS OF EACH AMINE ADDED, 0·1–0·5 μg) OBTAINED AFTER CHROMATOGRAPHIC SEPARATION ON PAPER USING 0·4% W/V AQUEOUS SODIUM DIHYDROGEN PHOSPHATE AS ELUANT

Catecholamine	Noradrenaline		Adrenaline		Isoprenaline	
Assay preparation	Rat colon	Rat blood pressure	Rat uterus	Rat blood pressure	Rat uterus	Rat blood pressure
Number of observations ..	8	8	8	8	7	7
Range of recoveries (%) ..	(47–92)	(19–33)	(48–66)	(18–28)	(51–74)	(76–113)
Mean % recoveries	72·7	23·9	59·7	22·4	61·9	91·4

TABLE 2. RECOVERIES OF NORADRENALINE, ADRENALINE AND ISOPRENALINE (AS % OF THE AMOUNTS OF EACH AMINE ADDED, 0·1–0·5 μg) OBTAINED AFTER CHROMATOGRAPHIC SEPARATION ON PAPER USING DISTILLED WATER ONLY AS ELUANT

Catecholamine	Noradrenaline		Adrenaline		Isoprenaline	
Assay preparation	Rat colon	Rat blood pressure	Rat uterus	Rat blood pressure	Rat uterus	Rat blood pressure
Number of observations ..	5	5	5	5	5	5
Range of recoveries (%) ..	(49–56)	(44–51)	(49–58)	(38–54)	(52–61)	(48–56)
Mean % recoveries	50·9	47·4	53·6	48·8	55·2	51·6

Discussion

Factors such as pH and tonicity of the solutions are shown to be liable to affect the biological assays of catecholamines after paper chromatographic separation. Traces of the phenolic solvent left in the eluates as a result of inadequate washing with benzene, and sodium dihydrogen phosphate used to prepare the elution fluid, cause a fall in the blood pressure of rats anaesthetised with pentobarbitone sodium and inhibition of the submaximal contractions of the rat uterus in response to acetylcholine.

Stimulation of the central nervous system by phenol is manifested in muscle tremors in the anaesthetised rat. The blood pressure falls probably as a result of a direct toxic action of phenol on the myocardium and on the smaller blood vessels, although the absence of such a response in pithed rats suggests that central vasomotor depression might be responsible for the fall in blood pressure in the anaesthetised animals. No explanation is offered of the brief rise in blood pressure caused by phenol in pithed rats. The toxic effects of phenol may also be responsible for the inhibition of the rat uterus.

The falls in blood pressure, in pithed and anaesthetised rats, caused by sodium dihydrogen phosphate are most likely the result of the formation

of non-isotonic solutions, since hypertonic sodium chloride solutions produce similar effects.

The sodium dihydrogen phosphate in the elution fluid remains in the flask after the water has been distilled off and is taken up in the isotonic sodium chloride solution thereby forming an hypertonic solution. It follows, therefore, that the larger the volume of eluate, then the more inherent depressor activity the eluate will possess, and the more erroneous will be the bioassay result.

Since hypertonic solutions of sodium chloride have much less inhibitory effect on the rat uterus than have the solutions of sodium dihydrogen phosphate, hypertonicity cannot be the cause of this particular effect, previously noted by Vogt (1952), of the elution fluid. The fact that the inhibitory effects of the sodium dihydrogen phosphate are markedly diminished when dissolved in rat uterus Ringer before being added to the organ bath suggests that sodium dihydrogen phosphate undergoes a reaction with one or more of the constituents of the Ringer. Solid sodium dihydrogen phosphate added to the Ringer does in fact cause a noticeable evolution of gas (presumably carbon dioxide from the bicarbonate present), and this would alter the pH of the Ringer. Similarly, the first addition of elution fluid to the Ringer causes an immediate increase in acidity followed by a fairly rapid return to near neutrality. The initial rapid reduction in pH is not apparent on further addition of elution fluid to the same sample of Ringer, but under the conditions of the biological assay the Ringer in the organ bath is changed after each dose. Acid solutions (10) do cause reduction in the responses of the rat uterus to acetylcholine, and low pH may also in part be responsible for the inhibition of this tissue caused by the dilute solution of the phenolic solvent (1).

As the depressor and inhibitory actions of the different solutions used were both qualitatively and quantitatively different from those of isoprenaline, it would seem that phenol or sodium dihydrogen phosphate present in the eluates are not by themselves responsible for the "isoprenaline-like substance" reported by Lockett with others (1954, 1957, 1961). They are however responsible for the non-parallel biological assays and inconsistent recoveries of small amounts of amines (Table 1) since the use of distilled water as the eluant prevents these effects (Table 2).

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Addendum

MARTHE VOGT

DR ROBERTS has asked me whether I would add a warning note about some of the pitfalls which should be avoided in catecholamine estimations which make use of chromatography in phenol-HCl followed by bioassay. Though these warnings have all been published, they are somewhat scattered, so that a recapitulation might be helpful.

HEAT TEST

Plasma extracts. "The phosphates and traces of other chemicals which are present in the eluates may affect the isolated uterus and mimic or mask the presence of traces of adrenaline. Thus the volume of eluate added to the uterus bath has to be kept below the threshold of such interfering substances. The threshold is established by preparing an eluate from a strip of paper not containing any adrenaline. It was usually well above the quantity of eluate required for an assay. In cases of doubt, the neutralized solution was heated for 5 min. in a boiling water bath, so that any adrenaline was destroyed, and the responses to the heated solution were examined without and with added adrenaline" (Vogt, 1952).

Tissue extracts. In extracts of brain tissue heating was extended to 10 min at pH 8 in a stoppered tube sealed with adhesive tape (Vogt, 1954).

VOLUMES OF ELUATE

To minimise effects of phosphate and hypertonic solutions, volumes of eluate should not exceed 2 or 2·5 ml 0·4% NaH_2PO_4. Elution time will control the volume of eluate. The residue is taken up in 0·5 ml distilled water which gives an approximately isotonic solution. Only if more than one tenth of the solution is needed for assay is there serious danger of effects from interfering substances.

CONTAMINATION WITH PHENOL

An eluate of, say, 2·5 ml 0·4% NaH_2PO_4, diluted to 3·5 ml with distilled water (used for rinsing the collecting tube), can be completely freed of phenol by evaporation to dryness *in vacuo*. If neither the tube containing the residue not the stopper smell of phenol the residue is perfectly safe; the stopper, however, may have taken up some phenol and may require washing before it can be used to seal the tube.

OVERLOADING OF PAPERS

An error which is obvious to any chemist but not always to the biologist is that of overloading the papers to be chromatographed. There may be overloading with the catecholamines themselves, as when adrenal medullary extracts are prepared, or with other tissue constitutents, such as the lipids

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referred to by Dr. Roberts. Thus it is not feasible to apply the extract of much more than 5 ml plasma to a paper lane 12 cm long. If lipaemia is present, the normal procedure may have to be modified.

CONTAMINATION

When very sensitive bioassays are used, particularly in the tests for isoprenaline, it is essential to cut off the strips of paper carrying control spots before washing the remainder of the paper in benzene. Traces of the control substances may otherwise contaminate the benzene and spread over the whole of the paper (Muscholl & Vogt, 1958).

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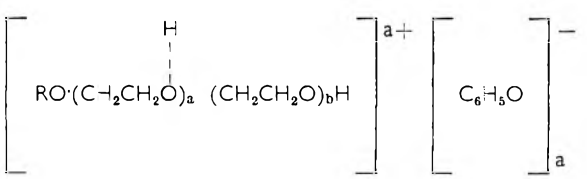
The solubilisation and inactivation of preservatives by non-ionic detergents*

W. P. EVANS

A novel potentiometric method, which depends on the pH changes which occur when an acidic material is solubilised, has been used to determine the solubilisation of the weakly acidic preservative, *p*-hydroxybenzoic acid, in a non-ionic detergent. From the pH changes observed, the partition coefficient, K_m, for the distribution of the un-ionised acid between the micellar and the aqueous phase has been calculated and found to be $2{\cdot}8 \times 10^3$. Specific interaction between the acid and the detergent, to form a complex is shown not to be important. It is suggested that the arguments against specific interaction apply generally to other preservatives and non-ionic detergents.

NON-IONIC detergents are used to an increasing extent as solubilising and emulsifying agents in cosmetic and pharmaceutical systems. They have some disadvantages, the main one being the inactivation of preservatives such as *p*-hydroxybenzoic acid and benzoic acid or their esters, which are commonly employed in such systems (de Navarre, 1953, 1956).

The cause of the inactivation has not been established, although complex formation between the preservative and the non-ionic detergent is often postulated. Higuchi & Lach (1954) state that Carbowax, a polyethylene glycol, forms complexes with benzoic, salicylic and *p*-hydroxybenzoic acid by reaction of the phenolic or carboxylic hydrogen with the ether oxygen of the glycol. Several authors have suggested that similar interactions also occur with non-ionic surface-active agents. The following formula has been proposed, but no quantitative data have been given, for a complex between phenol and a non-ionic detergent.



Protonation of the ether oxygen is, however, extremely improbable at the non-ionic detergent concentration and pH values of most cosmetic preparations; this is proved by the results described in this paper. Furthermore, it is unlikely that such interaction is the whole or even part of the inactivation, since inactivation may occur with preservatives of widely different types (Wedderburn, 1958). A more probable explanation is solubilisation of the preservative in the non-ionic micelles. Solubilisation of phenolic bactericides by micellar soap solutions is well known and it has been shown by many investigators that solubilisation results in a decrease of bactericidal activity (Alexander, 1946, 1949).

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* Part of a paper presented at a conference of "Group Rech. Prod. Superf. Actf. 5° Coll.", in Paris, 1959.

The normal method of estimating the amount solubilised by anionic and cationic detergents is to add increasing amounts of the solubilisate to the detergent solution and to equilibrate. When excess solubilisate is present the solutions are turbid and the amount solubilised can then readily be determined. Unfortunately, it is impossible in many cases to determine solubilisation by non-ionic detergents by this method because their cloud points are depressed by many solubilisates; the observed turbidity in such cases is not due to excess solubilisate but to "salted out" detergent or coacervate.

Apart from being inapplicable to many non-ionic detergents the equilibration solubilisation method is generally unsatisfactory even with anionic and cationic detergents since the results are difficult to interpret because measurements, apart from one or two exceptions (McBain, 1940), have always been made in saturated systems. The present paper describes a simple titration method which overcomes this difficulty, and which allows determination of solubilisation in unsaturated systems; it can be used with solubilisates containing a weak acidic group and depends on pH changes which result from preferential solubilisation of the un-ionised acid. It has been used in the present study to determine the solubilisation of *p*-hydroxybenzoic acid (a common cosmetic preservative) by the non-ionic detergent octyl phenol condensed with 8·5 moles of ethylene oxide.

Experimental

TITRATIONS

p-Hydroxybenzoic acid solutions (100 ml, either 0·01 or 0·03 M) containing varying concentrations of the non-ionic detergent (from 0·03 to 0·20 M) were titrated potentiometrically with 0·1 N sodium hydroxide using calomel and glass electrodes. Preliminary titrations with acetic and hydrochloric acids showed that the titration curves of the two acids were unaffected by the detergent, proving that it did not affect the potentials of the electrodes.

CLOUD POINTS

Cloud points were determined by the usual method of heating a 1% solution of the detergent alone or with additive until a faint turbidity appeared. The temperatures at which turbidity first appeared were taken as the cloud point of the detergent. These were reproducible to $\pm 0{\cdot}2°$.

Results and discussion

Fig. 1 shows a plot of the apparent "solubility" at 25° of *p*-hydroxybenzoic acid in varying concentrations of the detergent determined by the conventional turbidity method.

Up to a detergent concentration of about 3·5% (w/v), the apparent solubility of the acid in the detergent solution is less than its solubility in pure water. That the turbidity is due, not to excess solubilisate, but to a salted-out mixture of detergent plus solubilisate was confirmed by

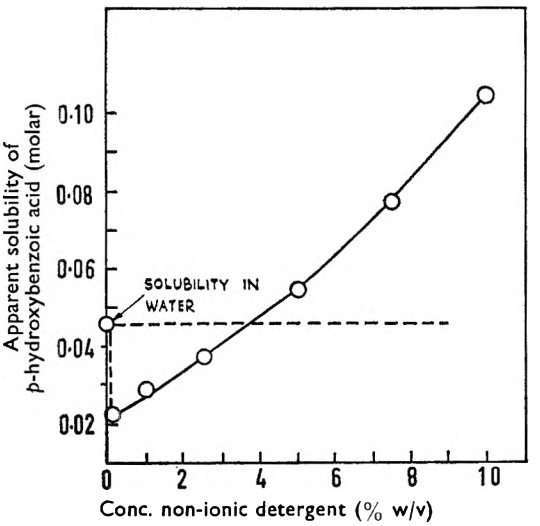

FIG. 1. Apparent solubility of p-hydroxybenzoic acid (molar) at 25° in varying concentrations of octyl phenol/8·5 moles ethylene oxide.

allowing the solutions to stand for some time, when two layers separate out, one layer being detergent rich, the other being water rich, but both layers containing the solubilisate. The separation of non-ionic detergents in this way is related to the cloud point phenomenon; a solution of the

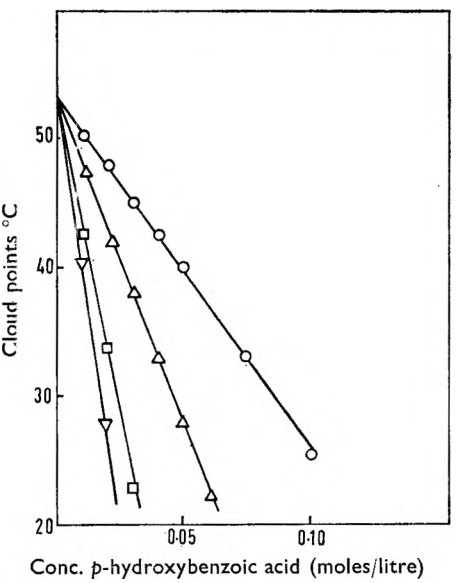

FIG. 2. Cloud points of various concentrations of octyl phenol/8·5 moles ethylene oxide in presence of *p*-hydroxybenzoic acid. ○ 10% (w/v) octyl phenol/8·5 moles ethylene oxide. △ 5% (w/v) octyl phenol/8·5 moles ethylene oxide. □ 1% (w/v) octyl phenol/8·5 moles ethylene oxide. ▽ 0·1% (w/v) octyl phenol/8·5 moles ethylene oxide.

detergent becomes cloudy when heated above a certain temperature—"the cloud point". The effect of *p*-hydroxybenzoic acid on the cloud point of different concentrations of the detergent is shown in Fig 2.

It is often stated that addition of organic additives such as phenol or hydroxybenzoic acid, results in precipitation of a complex of the non-ionic detergent and its additive. This is unlikely, however, since precipitation also occurs with additives such as benzene or sodium sulphate, with which it is difficult to visualise the formation of such a complex. The precipitate which separates in these instances is not a detergent-additive complex, but the separation of a detergent-rich layer containing the dissolved additive, i.e., a coacervate. For example, a 20% (w/v) solution of octyl phenol/8·5 mole ethylene oxide saturated with *p*-hydroxybenzoic acid separates into two layers at 25°. The analysis of the two layers is as follows:

	Cloud point °C	Surface tension dynes/cm at 25°	Acid in each layer %
Aqueous layer	>98	36·0	0·68
Non-ionic layer	—	—	19·0

The aqueous phase contains virtually no detergent as shown by the high "cloud point" and only 0·68% (w/v) of acid, while the detergent phase contains 19·0% (w/v) of the acid. [The solubility of the acid in pure water is 0·63% (w/v) and in the anhydrous detergent 26% (w/v)].

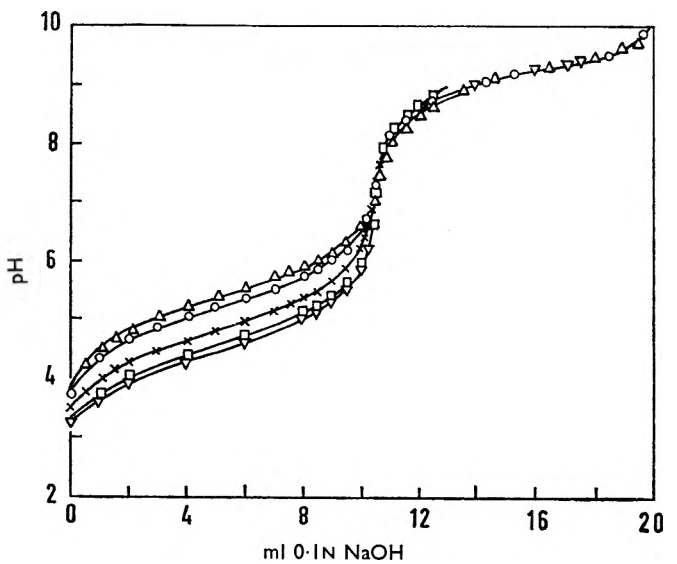

FIG. 3. Titration of 100 ml of 0·01 M *p*-hydroxybenzoic acid containing varying concentrations of octyl phenol/8·5 moles ethylene oxide with 0·1N NaOH. ▽ Acid alone. □ Acid containing 5% (w/v) ethanol. × Acid containing 0·03M octyl phenol/8·5 moles ethylene oxide. ○ Acid containing 0·10M octyl phenol/8·5 moles ethylene oxide. △ Acid containing 0·20M octyl phenol/8·5 moles ethylene oxide.

With sodium sulphate as the additive, separation into two phases again occurs, with the sodium sulphate dissolved in both phases, but, unlike the *p*-hydroxybenzoic acid, it is preferentially soluble in the aqueous phase. Further unpublished results confirm that with most additives the detergent-additive precipitate is not a definite chemical complex but a coacervate of varying stoichiometry.

Typical titration curves of *p*-hydroxybenzoic acid in the presence of a non-ionic detergent (below the cloud point) are given in Fig. 3.

The titration curves given in Fig. 3 show a pH shift to higher values in the presence of the detergent, therefore either the free concentration of the un-ionised acid has decreased as a result of some interaction with the detergent, or the detergent has changed the dielectric constant of the solution or the electrode potentials because it has been adsorbed on the glass electrode.* The titration curves of acetic acid and hydrochloric acid, however, were not affected by the presence of the detergent, so that the pH changes observed are not due to dielectric changes or to changes in the electrode potentials, but must be due to a decrease in the concentration of un-ionised acid. Furthermore, the normal titration results observed with acetic and hydrochloric acids show that protonation of the ether oxygen of the detergent as suggested by various authors does not take place, or, at least, the extent of protonation is too small to be detected by pH changes.

The titration results of *p*-hydroxybenzoic acid can be interpreted by assuming a decrease in the concentration of the un-ionised acid due either to complex formation of the type suggested by Higuchi & Lach (1954) or to solubilisation of the un-ionised acid; the results have therefore been used to calculate constants (K_c and K_m), assuming:

(1) Formation of a 1:1 complex between non-micellar detergent (i.e., single molecules) and the un-ionised acid (K_c).

(2) Solubilisation of the un-ionised acid in the detergent micelles (K_m).

The equilibria considered here are:

$$HA_{water} \rightleftharpoons H^+ + A^-$$
$$\Updownarrow$$
$$HA_{micelles}$$

The constants obtained are given in Table 1, from which it is evident that the "constant", K_c, for a 1:1 complex shows considerable drift; interaction of the acid with monomeric detergent molecules is therefore unlikely.

Furthermore, any such interaction should, at constant acid concentration, result in pH shifts which are independent of total detergent concentration provided this is above the critical micelle concentration (about $1{\cdot}7 \times 10^{-4}$ M for the detergent used here) since the concentration of the monomeric detergent molecules is constant above the CMC. Further evidence against complex formation was obtained by titrating aqueous

* Since this work was completed (Paris, 1959), Donbrow & Rhodes (1963) have published titration curves showing similar pH shifts, but no quantitative treatment was given.

solutions of *p*-hydroxybenzoic acid containing polyethylene glycol 400, a polymer of 9 ethylene oxide units, identical with the hydrophilic part of the non-ionic detergent, but, unlike the latter, not aggregating to form micelles; no pH shifts were observed.

TABLE 1. THE pH, CONCENTRATION OF THE VARIOUS SPECIES, AND SOLUBILISATION AND COMPLEXING CONSTANTS (K_mK_c) FOR VARIOUS CONCENTRATIONS OF *p*-HYDROXYBENZOIC ACID AND OCTYL PHENOL/8·5 MOLES ETHYLENE OXIDE

$[HA]_t$	[Detergent]	pH	$[A^-]$	$[HA]_{aq}$	$[HA]_m$	$K_c \times 10^{4*}$	$K_m \times 10^{-3}$
0·009902	0·02970	3·97	0·001056	0·003557	0·005289	1·2	2·3
0·009710	0·02913	4·48	0·002824	0·002803	0·004083	1·2	2·3
0·009346	0·1869	5·73	0·006269	0·000330	0·002747	0·21	2·5
0·02670	0·02678	4·59	0·01030	0·007101	0·009300	1·3	2·7
0·02577	0·1724	5·44	0·01322	0·001248	0·01130	0·19	2·9
0·009524	0·09524	5·20	0·004568	0·000836	0·004120	0·35	2·9
0·02770	0·1852	4·96	0·007109	0·002175	0·01842	0·20	2·5
0·009616	0·09616	5·05	0·003695	0·000970	0·004951	0·3	2·9
0·009854	0·02957	4·14	0·001488	0·003342	0·005024	1·1	2·8
0·02670	0·1786	5·22	0·01028	0·001666	0·01475	0·19	2·8
0·02718	0·1818	5·12	0·008718	0·001807	0·01666	0·19	2·8

$[A^-]$ = Concentration acid anion HO ·  · COO^- (moles/litre).

$[HA]_t$ = Total concentration of acid in system (moles/litre).

$[HA]_{aq}$ = Concentration un-ionised acid HO ·  · COOH in water phase (moles/litre).

$[HA]_m$ = Concentration un-ionised acid HO ·  · COOH in micelle (moles/litre).

[Detergent] = Concentration of detergent (moles/litre).

* Calculations assuming 1:2 or 1:3 complexes also gave unsatisfactory constants, K_c.

The results can, however, be interpreted satisfactorily by assuming solubilisation of the *p*-hydroxybenzoic acid in the detergent micelles, without postulating any specific interaction. McBain & Hutchinson (1955) point out that many instances of solubilisation can be quantitatively described as a partition of solute between two immiscible phases. When the solubilisate carries a polar group, as in the present study, it may orient itself on the surface of the micelle instead of passing into the hydrocarbon interior, but even in this case the solubilisation may be approximately characterised by a simple distribution coefficient. In the present work the solubilisation is also treated as a distribution phenomena of the un-ionised acid between the aqueous phase and the micellar phase and a distribution constant, K_m, calculated. The distribution constant is defined as:

$$K_m = \frac{[HA]_{micelle}}{[HA]_{water}} \quad \begin{matrix} \text{mole acid/mole detergent} \\ \text{mole acid/mole water} \end{matrix}$$

That solubilisation and not complex formation is the correct interpretation of the pH shifts is further supported by the fact that pH shifts are also observed when long-chain fatty acids are titrated in the presence of anionic detergents (e.g., lauric acid in the presence of sodium dodecyl sulphate). Again there is no evidence of specific interaction to form complexes but from the results (to be published) similar partition coefficients for solubilisation can be calculated. (From the data on page 326

a partition coefficient was also calculated and found to be $2{\cdot}3 \times 10^3$, in good agreement with that calculated from the potentiometric data).

The anions of the acid would not be expected to be solubilised to the same extent, and this is proved by the fact that there are no pH shifts when the phenolic group of the *p*-hydroxybenzoic acid is titrated. A number of authors (e.g., Maclay, 1956) have stated that solubilisation results in a decrease in the hydrophilic character of the non-ionic micelles, and any solubilised material would therefore decrease the cloud point whereas the mono-sodium salt, which is not solubilised, increases the cloud point (Fig. 4).

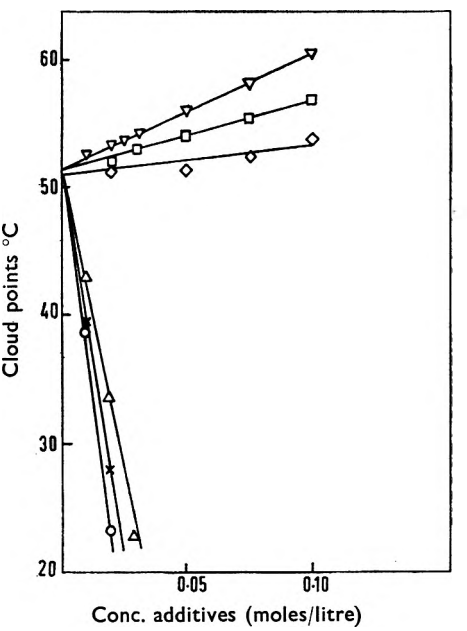

FIG. 4. Cloud points of 1% (w/v) octyl phenol/8·5 moles ethylene oxide in presence of various additives. △ *p*-hydroxybenzoic acid, □ mono sodium salt of *p*-hydroxybenzoic acid, ○ benzoic acid, ▽ sodium benzoate, × phenol, ◇ sodium phenate.

The value of the distribution constant, K_m, obtained in this work ($K_m = 2{\cdot}8 \times 10^3$) is constant over the whole concentration range examined, and is of the same order as those values reported in the literature for solubilisation by anionic and cationic detergents (McBain & Hutchinson, 1955). Few data are available for solubilisation by non-ionic detergents; Moore & Bell's results (1957) on the solubilities of phenyl ethyl alcohol and benzaldehyde in hexadecyl/14 moles ethylene oxide and in hexadecyl/24 moles ethylene oxide respectively as determined by the conventional turbidity method are given in Table 2 (in Moore and Bell's study, saturation of the solution by the solubilisate was apparently reached before the cloud point was reduced sufficiently to precipitate the detergents).

Moore and Bell's results have been used to calculate the relevant distribution constants, K_m, which are of the same order as those obtained in the present study.

TABLE 2. SOLUBILITY (g/100 ml) OF PHENYLETHYL ALCOHOL AND BENZALDEHYDE IN HEXADECYL/24 MOLES ETHYLENE OXIDE AND HEXADECYL/14 MOLES ETHYLENE OXIDE RESPECTIVELY AT 25° (Moore & Bell, 1957)

	Solubility of phenylethyl alcohol in hexadecyl/24 moles ethylene oxide			Solubility of benzaldehyde in hexadecyl/14 moles ethylene oxide		
	Concentration of detergent %					
	2·5	5·0	10·0	2·5	5·0	10·0
Total wt solubilised (g)	3·24	5·04	8·28	1·38	2·40	4·13
Wt in aqueous phase (g) ..	1·60	1·60	1·60	0·33	0·33	0·33
Wt in micellar phase (g) ..	1·64	3·44	6·68	1·05	2·07	3·8
$K_m \times 10^{-3}$	2·7	2·9	2·8	6·0	5·9	5·4

Apart from the exception noted (McBain, 1940), all previously reported solubilisation data have been obtained by examination of systems containing excess insoluble phases, where both the micellar phase and the aqueous phase are saturated with the solubilisate. By subtracting the solubility of the solubilisate in pure water from its solubility in the detergent solution, the amount of solubilisate dissolved in the micelles can be obtained. This type of calculation, however, assumes that the concentration of the solubilisate in the aqueous phase in a saturated detergent solution is the same as its solubility in pure water; this may not always be true, e.g., potassium laurate even below the CMC increases the water solubility of phenol five times. Furthermore, due to deviation from ideal behaviour, the distribution constant of a solute between two immiscible phases changes as the two phases become saturated. That the results obtained in the present paper are of the same order as those calculated from Moore and Bell's work must therefore be regarded as somewhat fortuitous.

TABLE 3. MOLAR CONCENTRATION OF UN-IONISED *p*-HYDROXYBENZOIC ACID IN THE WATER PHASE IN THE PRESENCE AND ABSENCE OF 5·8% (w/v) DETERGENT AT VARIOUS pH'S USING 0·1% (w/v) TOTAL CONCENTRATION OF *p*-HYDROXYBENZOIC ACID

pH	$[HA]_{water}$	$[HA]_{water}$ in presence of 5·8 % detergent	Total (%) (w/v) acid required in presence of 5·8 % (w/v) detergent to be equivalent to 0·1 % (w/v) acid in pure water
3·5	0·00662	0·00118	0·56
4·0	0·00559	0·00114	0·49
4·5	0·00376	0·00104	0·36
5·0	0·00220	0·000805	0·27

All the results obtained in this study can be adequately explained by solubilisation of the un-ionised *p*-hydroxybenzoic acid in the non-ionic micelles, without postulating specific interaction or formation of complexes as has been done by most authors. Since the un-ionised acid (Simon, 1952), is generally regarded as being the active preservative, and

since only the acid in the aqueous phase is effective, it is reasonable to assume, at least in the case of *p*-hydroxybenzoic acid and the detergent used, that solubilisation and not complex formation is the cause of the inactivation by non-ionic detergents. Knowing the total concentration of *p*-hydroxybenzoic acid, its dissociation constant, K_c, ($2{\cdot}95 \times 10^{-5}$), the concentration of the detergent, and the partitition coefficient, K_m, it is easy to calculate the amounts of the acid dissolved in the aqueous and the micellar phases at varying pH, detergent, and total acid concentration. The results of such a calculation, assuming a total concentration of 0·1% (w/v) *p*-hydroxybenzoic acid and 5·8% (w/v) detergent are given in Table 3.

Thus if 0·1% (w/v) is the optimum total concentration of acid needed to give the required preservative effect in water at pH 4·0, the concentration of the un-ionised acid (the 'active' species) is 0·00559 M. Addition of 5·8% of the detergent reduces this concentration of un-ionised acid to 0·00114 M (Column 3, Table 3), and Column 4 shows that at pH 4·0, 0·49% (w/v) of acid would be required in presence of 5·8% (w/v) of the detergent to give the optimum molar concentration of the un-ionised acid in the water phase—i.e., five times as much acid would be required to give the same concentration of the un-ionised acid in the aqueous phase in the presence of the detergent as in the absence of the non-ionic detergent.

Acknowledgements. The author wishes to acknowledge many valuable discussions with Dr. T. G. Jones of this Department.

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The site of action of sympathomimetic amines on the circular muscle strip from the guinea-pig isolated ileum

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The circular muscle strip of the isolated ileum of the guinea-pig, treated with the anticholinesterase mipafox (100 μg/ml) responds to doses of methacholine or carbachol with contractions that are inhibited by previous doses of noradrenaline, adrenaline or isoprenaline in that order of potency. Piperoxane antagonised the inhibitory action of adrenaline, but not that of aminophylline. Dichloroisoprenaline did not antagonise the action of adrenaline on the circular muscle strip. It is concluded that the site of action of the sympathomimetic amines on the circular muscle of the guinea-pig ileum is located at postganglionic neuro-effector junctions in the smooth muscle.

THE study of the actions of sympathomimetic amines on the circular muscle of the guinea-pig isolated ileum has been restricted to the effect of these amines on peristalsis (Pirie, 1951; McDougal & West, 1952, 1954; Kosterlitz & Robinson, 1957). Harry (1963) suggested that peristalsis does not lend itself to an accurate evaluation of drug action and introduced the circular muscle strip for this purpose. The site of action of sympathomimetic amines on the circular muscle strip is now reported.

Methods

Segments 1·5 cm in length were taken from the ileum of a guinea-pig 15 cm from the ileo-caecal junction. A segment was opened by a longitudinal incision through the wall along its mesenteric border and the resultant rectangle of ileum was pinned out under Krebs solution with the mucosal surface upwards. A strip was produced by cutting the rectangle in the direction of the circular muscle fibres (Harry, 1963). Usually five cuts produced a strip of circular muscle of sufficient length. A cotton ligature was tied around each end of the strip, one of which was tied to a glass holder and the other to a small metal hook. The holder was immersed in an organ bath containing 23 ml of Krebs solution at 37° and bubbled with 95% oxygen and 5% carbon dioxide. The metal hook was attached to an isotonic lever with a frontal writing point. The load on the tissue was 290 mg; the response of the tissue was magnified six times.

The circular muscle strip lacks a tone which sympathomimetic amines could relax, thus making it impossible to demonstrate directly the inhibitory actions of these amines. A suitable method was to obtain a series of contractions, 90% of the maximum, of the circular strip by methacholine or carbacol and to inhibit these contractions with the sympathomimetic agent. A satisfactory cycle was as follows: (1) the spasmogen was added to the bath for a contact time of 1 min; (2) the bath fluid was changed; (3) after a further min the bath fluid was changed again; (4) 1 min after the second washing the spasmogen was added to the bath

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again and the cycle repeated until a series of contractions of constant height was achieved. The effect of the sympathomimetic agent was tested by adding it to the bath immediately after the second washing at (3). In this way the sympathomimetic drug was present in the bath fluid for 1 min before the spasmogen was added. When the activity of a potential sympathomimetic antagonist was investigated, this substance was added to the bath fluid each time after it was changed.

DRUGS USED

Adrenaline acid tartrate, aminophylline, carbachol chloride, dichloroisoprenaline hydrochloride, isoprenaline sulphate, methacholine chloride, piperoxane hydrochloride, phenoxybenzamine hydrochloride.

All drugs were prepared in Krebs solution from stock solutions in distilled water and are expressed as base.

Results

THE EFFECTS OF ADRENALINE, NORADRENALINE AND ISOPRENALINE ON THE CIRCULAR MUSCLE STRIP

Adrenaline, noradrenaline and isoprenaline all produced inhibition of the contractions of the circular muscle strip caused by 1·0 μg/ml carbachol (Fig. 1). The same degree of inhibition was produced by 10 ng/ml of noradrenaline, by 20 ng/ml of adrenaline and by 4000 ng/ml of isoprenaline.

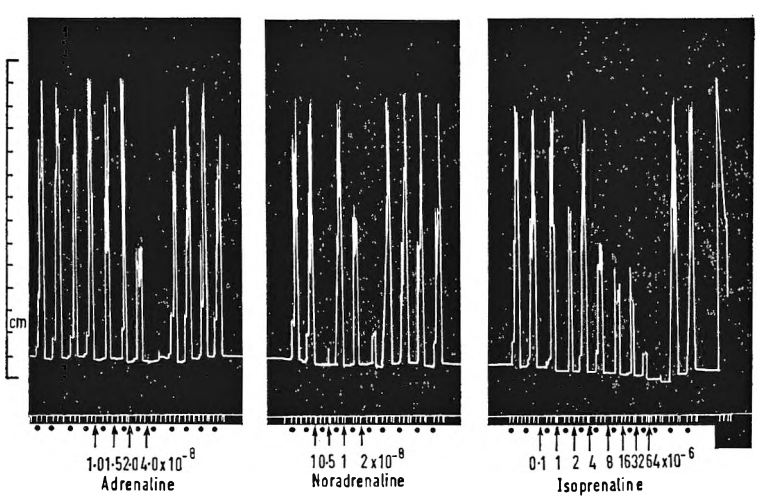

FIG. 1. The inhibitory effects of adrenaline, noradrenaline and isoprenaline on the contraction of the circular muscle strip produced by carbachol. At ● carbachol added to the bath fluid to give a concentration of 1·0 μg/ml. Sympathomimetic drugs added to bath fluid at arrows. Bath concentrations expressed as g/ml. Time marker, 30 sec.

THE EFFECT OF ADRENALINE ON THE CIRCULAR MUSCLE STRIP

Adrenaline, 0·1 μg/ml, inhibited the contractions of the circular muscle strip produced by 1·0 μg/ml of methacholine (Fig. 2). This inhibitory action of adrenaline was antagonised by piperoxane, 2·0 μg/ml, a concentration which did not affect the contraction of the strip by methacholine (central portion of Fig. 2). The inhibitory action of adrenaline returned after washing the piperoxane from the tissue (right portion of Fig. 2). Phenoxybenzamine, 0·1 μg/ml, also antagonised the action of adrenaline on the circular muscle strip.

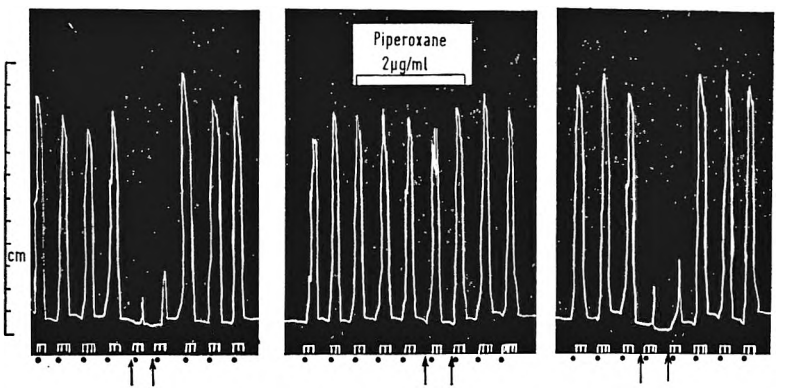

FIG. 2. The effect of piperoxane on the inhibitory action of adrenaline. At ● methacholine added to the bath fluid to give a bath concentration of 1·0 μg/ml. At arrows, adrenaline added to the bath fluid to give a bath concentration of 0·1 μg/ml. ⌐¬ shows presence of piperoxane 2·0 μg/ml in the bath fluid. Time marker, 30 sec.

THE EFFECT OF AMINOPHYLLINE ON THE CIRCULAR MUSCLE STRIP

Aminophylline, 67·5 μg/ml, inhibited the contractions of the circular muscle strip produced by methacholine, and this action of aminophylline was not influenced by piperoxane, 2·0 μg/ml, a concentration which inhibited the action of adrenaline (Fig. 3).

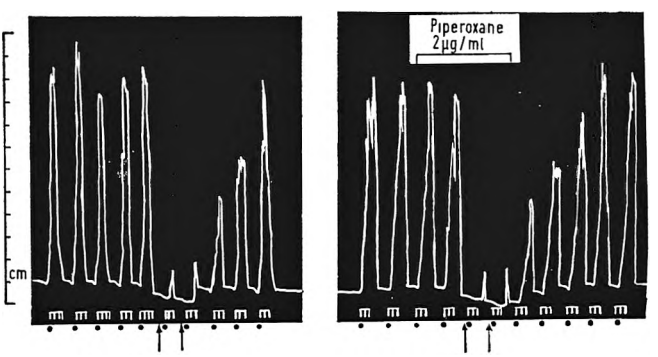

FIG. 3. The effect of piperoxane on the spasmolytic action of aminophylline. At ● methacholine added to the bath fluid to give a bath concentration of 1·0 μg/ml. At arrows, aminophylline added to bath fluid to give a bath concentration of 67·5 μg/ml. ⌐ shows presence of piperoxane (2·0 μg/ml) fluid. Time marker, 30 sec.

THE EFFECT OF DICHLOROISOPRENALINE ON THE SPASMOLYTIC ACTIVITY OF ADRENALINE

Dichloroisoprenaline (DCI), 1·0 μg/ml, did not have any spasmolytic activity on contractions of the circular muscle strip produced by methacholine, 2·0 μg/ml, and did not modify the inhibitory action of adrenaline (Fig. 4).

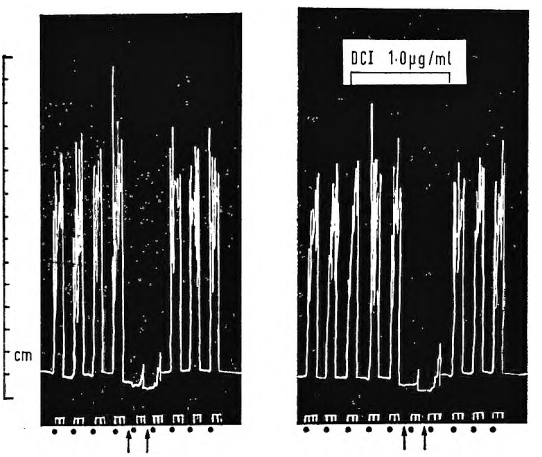

FIG. 4. The effect of dichloroisoprenaline (DCI) on the inhibitory action of adrenaline. At ● methacholine added to bath fluid to give a concentration of 2·0 μg/ml. At arrow, adrenaline added to bath fluid to give a concentration of 0·1 μg/ml ⌐ shows presence of DCI (1·0 μg/ml) in bath fluid. Time marker, 30 sec.

Discussion

The results of these experiments show that the circular muscle of the guinea-pig isolated ileum possesses adrenotropic receptors which are inhibitory. Further the receptors can be classified as α-receptors (Alquist, 1948). Three pieces of evidence are offered in support. Firstly, noradrenaline was more active than adrenaline and both of these substances were much more active than isoprenaline on the circular muscle strip; secondly the inhibitory action of adrenaline but not aminophylline was specifically antagonised by the α-receptor blocking agent piperoxane, and thirdly dichloroisoprenaline, which antagonises the β-receptor actions of adrenaline (Powell & Slater, 1958; Alquist & Levy, 1959), did not modify the inhibitory action of adrenaline on the circular muscle strip.

The observation that hexamethonium did not influence periarterial sympathetic stimulation of a segment of isolated guinea-pig ileum (Szerb, 1961) suggested that the efferent sympathetic chain is not functionally related to the enteric plexuses of the ileum but terminates in the intestinal musculature. Taken together with the results reported above this allows me to conclude that the site of action of noradrenaline, adrenaline and isoprenaline on the circular muscle of the guinea-pig ileum is located at post-ganglionic neuro-effector junctions in the smooth muscle. These

conclusions differ from those of McDougal & West (1954). But their interpretations of the action of sympathomimetic amines on peristalsis from observations on the longitudinal tubular preparation of the guinea-pig isolated ileum, led them to infer that the intramural nerve plexus was involved. However, Brownlee & Harry (1963) have shown a basic difference pharmacologically between the longitudinal and circular muscle layers of the guinea-pig ileum and thus the inferences of McDougal & West concerning the site of action of sympathomimetic amines on circular muscle activity from experiments on the longitudinal muscle must now be reconsidered.

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Translocation of alkaloids in *Datura* species

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The ditigloyl esters of the hydroxytropanes, normally found in the roots of *Datura* spp. occur also in the transpiration stream of *D. innoxia* and *D. cornigera* although they do not accumulate in the aerial parts. An alkaloid previously isolated from the leaves or roots of a number of species, and now obtained from the transpiration stream is probably an optical isomer of norhyoscine. Hyoscine is the principal alkaloid of the sap of both the species investigated and noratropine and hyoscyamine are also present.

HYOSCINE is usually the principal alkaloid of the aerial parts of *Datura innoxia* and *D. cornigera*. In addition, hyoscyamine and meteloidine have been isolated from *D. innoxia* and, noratropine and 3-hydroxy-6-tigloyloxytropane from *D. cornigera*. The roots of both species contain 3,6-ditigloyloxytropane, 7-hydroxy-3,6-ditigloyloxytropane, hyoscine and hyoscyamine or atropine. Noratropine and dihydroxytropane have been obtained from *D. cornigera* roots and tigloidine, tropine and pseudotropine from *D. innoxia* roots (see Evans & Wellendorf, 1959; Evans & Than, 1962; Evans & Griffin, 1963). The roots of both species contain more hyoscyamine or atropine than hyoscine.

As a preliminary step in the study of the metabolism of the ditigloyl esters we have investigated the upward translocation of alkaloids in *D. innoxia* and *D. cornigera* by the analysis of the bleeding sap drawn from the stocks of decapitated plants and by grafts. In previous work on *D. innoxia*, Romeike (1953) has detected hyoscine in the bleeding sap and a hyoscine: hyoscyamine ratio of about 3:1 in the leaves of *Cyphomandra betacea* (a non-alkaloid producing plant) grafted on to *D. innoxia* stocks.

Methods

ANALYSIS OF SAP

Datura innoxia. 50 flowering plants of *D. innoxia* growing in the field (August, 1963) were decapitated at soil level and the bleeding sap (3·7 kg) from the cut ends of the root stocks absorbed into weighed columns of cellulose (about 100 g). The lower ends of the columns were plugged with absorbent cotton wool and were pressed firmly onto the cut surfaces. After 48 hr the tubes were removed, reweighed and the sap displaced from the columns with acetone and evaporated to dryness under reduced pressure. The residues were treated with chloroform–ammonia† (3 ml), the solvent removed and then, the crude bases in ether, submitted to column chromatography [kieselguhr (15 g) with 0·5 M phosphate buffer solution, pH 6·4 (5 ml)]. Light petroleum (b.p. 40–60°), ether, chloroform and chloroform–ammonia were used successively as developing solvents and the eluted bases were titrated with 0·005 N sulphuric acid where

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† The lower layer produced by shaking chloroform (500 ml) with strong solution of ammonia B.P. (20 ml).

possible. Individual bases were characterised by thin-layer and paper chromatography and where feasible, by the preparation and infra-red examination of the picrates.

Another sample of bleeding sap (340 g) collected in September, 1962, was similarly analysed [kieselguhr (10 g) with 0·25 M phosphate buffer solution, pH 6·1 (3·2 ml)].

For comparison with the above, the dried, coarsely powdered leaves and stems of the 1963 plants were also analysed. A sample (50 g) was mixed with lime (5 g) and moistened with water (15 ml); after standing for 30 min, it was exhausted with ether. The solvent was removed and the residue transferred, in ether, to a chromatographic column [kieselguhr (15 g) with 0·5 M phosphate buffer solution, pH 6·8 (5·0 ml)]. Pigments were removed from the column with light petroleum (b.p. 40–60°) and the alkaloids were fractionally eluted by ether and chloroform.

D. cornigera. Two, four-year old trees of *D. cornigera* growing in a temperate greenhouse, were cut back to within a few feet of the ground and the bleeding sap (481 g) collected and treated as described above [kieselguhr (10 g) with 0·25 M phosphate buffer solution pH 6·1 (3·2 ml)].

ANALYSIS OF GRAFTED PLANTS

Tomato shoots were grafted on to *D. innoxia* stocks when both species were about 8 inches tall. The scions and roots were separately harvested at about 5 months and dried (15 g aerial parts; 3 g stocks). Chromatographic analysis was performed as for *D. cornigera.*

CHARACTERISATION OF NORHYOSCINE

One base, eluted from the chromatographic columns immediately after hyoscine, gave a picrate m.p. 230° and was obtained from the sap of both *D. innoxia* and *D. cornigera.* This base has previously been recorded as a constituent of the roots of *D. innoxia* (Evans & Wellendorf, 1959) and the aerial portions of *D. ferox*, Indian henbane (Evans & Partridge, 1949) and *D. cornigera* (Evans & Than, 1962). For its further study, material obtained from an investigation (Evans & Griffin, 1963) of *D. cornigera* was used. The details of the chemical examination are recorded below.

Results

CONSTITUENTS OF THE SAP

D. innoxia. From the titration liquids of the light petroleum eluate a colourless base (2·5 mg) was obtained which, neutralised with sulphuric acid (0·02 N) and treated with sodium picrate solution gave 3,6-ditigloyloxytropane picrate, needles from aqueous ethanol, m.p. and mixed m.p. with authentic material 151°. The infra-red spectrum and Rf value by paper chromatography were identical with those of the reference compound. The first portion of the ether eluate (25 ml) contained a base (2·2 mg) affording an alkaloid picrate, plates from aqueous ethanol, m.p. and mixed m.p. with authentic 7-hydroxy-3,6-ditigloyloxytropane picrate,

183°. Comparison of the infra-red spectrum of the picrate and Rf value of the base with those of the authentic compound further substantiated the identification. From the second portion of the ether eluate (150 ml) another base (95 mg) was obtained; it was shown to be hyoscine by the characters of the picrate, needles from aqueous ethanol, m.p. and mixed m.p. with authentic hyoscine picrate, 188°. Found: C, 51·6; H, 4·5. Calc. for $C_{17}H_{21}NO_4$, $C_6H_3N_3O_7$: C, 51·9; H, 4·5%. Two peaks were evident on the titration curve of the chloroform eluate. The first (6·8 ml) gave a base, picrate m.p. 230° on one recrystallisation and the further investigation of this is recorded below; the second peak corresponded to hyoscyamine (30 mg), picrate m.p. and mixed m.p. with authentic hyoscyamine picrate 163°. Found: C, 53·1; H, 4·9. Calc. for $C_{17}H_{23}NO_3$,$C_6H_3N_3O_7$: C, 53·3; H, 5·0%. The chloroform–ammonia eluate contained a mixture of bases which was resolved by chromatography on alumina (50 g). Ether: ethanol (75:25) eluted hyoscyamine (3 mg), characterised as the picrate and ethanol eluted noratropine (7 mg), picrate, needles from ethanol m.p. and mixed m.p. with authentic material, 227°. The isolated noratropine base and the reference compound had the same Rf values on thin-layer chromatograms (alumina with chloroform–ethanol 1:9 as developing solvent).

From the *D. innoxia* bleeding sap collected in 1962, hyoscine (4·5 mg) and hyoscyamine (5·4 mg) were isolated as the corresponding picrates; 3,6-ditigloyloxytropane (0·5 mg) was indicated by paper chromatography.

The partition chromatographic separation of the basic mixture derived from the decapitated shoots yielded hyoscine (145 mg) and hyoscyamine (56 mg); both were identified by the preparation of picrates and by thin-layer chromatography (alumina with chloroform–ethanol 1 : 1 as developing solvent). No alkaloids were detected in the coloured light petroleum eluate.

D. cornigera. The light petroleum eluate contained a base (1·8 mg) having the same Rf value as 3,6-ditigloyloxytropane (alumina with ether as developing solvent) and which, on neutralisation and treatment with sodium picrate solution, gave 3,6-ditigloyloxytropane picrate, m.p. 150° undepressed on admixture with the authentic compound. The regenerated base, in dilute hydrochloric acid, gave with chloroplatinic acid solution 3,6-ditigloyloxytropane chloroplatinate, feathery needles, m.p. 228° undepressed by admixture with authentic material, m.p. 230°. Two bases were evident in the ether fraction. The first (initial 35 ml eluate) was equivalent to 0·5 ml 0·005 N acid and was tentatively identified by thin-layer chromatography (alumina with ether as developing solvent) as 7-hydroxy-3,6-ditigloyloxytropane although a derivative could not be prepared. The remainder of the ether eluate contained hyoscine (10·5 mg), which yielded hyoscine picrate m.p. 188°, undepressed by admixture with authentic hyoscine picrate. The titration curve obtained from the chloroform eluate showed a sharp, small peak followed by a larger one. The base (1·5 mg) corresponding to the first peak, on neutralisation and treatment with aqueous sodium picrate gave a picrate, serrated needles from aqueous ethanol m.p. 232°, the characterisation of which is described

below. The oily base (5 mg) from the second peak was shown by thin-layer chromatography (alumina, developing solvent chloroform : ethanol 1 : 1) to consist largely of hyoscyamine with traces of noratropine. Noratropine (1·5 mg) was eluted from the column by chloroform–ammonia and on neutralisation and treatment with aqueous sodium picrate afforded noratropine picrate, long needles from aqueous ethanol m.p. 226°, undepressed by admixture with authentic noratropine picrate.

GRAFTS OF TOMATO ON *D. innoxia*

The light petroleum eluate from the chromatographic analysis of the scions was shaken three times with 0·02 N acid (5 ml each) and the basic material (0·7 mg) recovered from the acid solution in chloroform. Paper chromatography of the base showed it to have an Rf value similar to that of 3,6-ditigloyloxytropane but insufficient material precluded the preparation of a derivative. Hyoscine (10·0 mg), picrate needles from ethanol m.p. 188° was obtained from the ether eluate and hyoscyamine (5·0 mg), picrate prisms from aqueous ethanol m.p. 160°, from the main chloroform eluate. The initial portion of the chloroform eluate contained a small quantity of base (about 0·4 mg) having an Rf value on paper chromatography intermediate between that of hyoscyamine and 7-hydroxy-3,6-ditigloyloxytropane and similar to that of tigloyloxytropane. Insufficient material prevented the preparation of a derivative.

The *D. innoxia* stocks of the graft combination contained 3,6-ditigloyloxytropane (1·5 mg), picrate m.p. and mixed m.p. 150°; 7-hydroxy-3,6-ditigloyloxytropane (1·5 mg), picrate m.p. and mixed m.p. 182°; hyoscine (0·6 mg), picrate m.p. 187° and a hyoscyamine fraction (3·0 mg) from which it was not possible to prepare a crystalline derivative.

CHARACTERISATION OF NORHYOSCINE

Analysis of the picrate m.p. 232° gave: C, 51·1, 50·7; H, 4·2, 4·2; N, 11·4, 10·6. Calc. for $C_{16}H_{19}NO_4$, $C_6H_3N_3O_7$: C, 51·0; H, 4·3; N, 10·8%; no *N*-methyl groups were present and the base gave a positive Vitali-Morin reaction. By many recrystallisations of the picrate it was possible to raise its m.p. to 238° and admixture with ±-norhyoscine picrate m.p. 245° resulted in no depression of the m.p. below 238°. The infra-red spectra of the two compounds were virtually identical. The base (25 mg), regenerated from the picrate, in methanol (0·5 ml) was treated with methyl iodide (0·02 ml). After standing at room temperature for 2 hr, the mixture was gently warmed to remove the solvent. Thin-layer chromatography (alumina with ether : ethanol 1 : 1 as developing solvent) of the residue indicated it to contain a mixture of unchanged alkaloid, hyoscine and hyoscine methiodide. The mixture was transferred to alumina (30 g) and with ether–ethanol (90 : 10) as eluant, a base (5 mg) was obtained which afforded a picrate, short needles from aqueous ethanol, m.p. 175–179°, undepressed by admixture with authentic (−)-hyoscine picrate. The infra-red spectra of the two compounds showed them to be the same. Unchanged alkaloid (9·0 mg), picrate m.p. 237°

was eluted by ether–ethanol (75:25). Insufficient base was obtained for the measurement of its optical rotation.

Discussion

The occurrence of 3,6-ditigloyloxytropane and 7-hydroxy-3,6-ditigloyloxytropane in the rising sap and in the roots of *D. innoxia* and *D. cornigera* suggests that further metabolism of these alkaloids takes place in the aerial parts of the plant since they do not accumulate in the leaves. This is further supported by the observation that 3,6-ditigloyloxytropane also appears in tomato scions grafted on to *D. innoxia* stocks. The hyoscine: hyoscyamine ratios found in the aerial portions of grafted plants and in the bleeding saps, more closely resemble those found in the normal leaves than in the roots of these species. This is in general agreement with previous studies on *D. innoxia* (Romeike 1953), although hyoscyamine was not reported as a component of the sap.

The alkaloid, picrate m.p. 230–232°, obtained from a number of *Datura* spp. appears to be norhyoscine. Norhyoscine was discovered as the ±-form in mother liquors remaining from the manufacture of hyoscine by Gmelin (1941). The infra-red spectra of the picrates of authentic material and the isolated alkaloid and of hyoscine and the *N*-methyl derivative afford good evidence for identification. The low m.p. of the picrate compared with ±-norhyoscine picrate (245°) and its gradual rise on recrystallisation without change in composition suggest that racemisation of the optically active salt may be taking place as also occurs with some samples of hyoscyamine and norhyoscyamine. In accordance with this is the somewhat low m.p. of the chemically pure *N*-methyl derivative (hyoscine). A larger quantity of the alkaloid is now required for an optical rotation measurement.

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Hydroxylation *in vitro* of pharmacologically active phenothiazine derivatives

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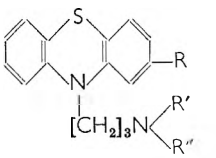
The hydroxylation of promazine, chlorpromazine, chlorproethazine, triflupromazine and promethazine by a rat liver preparation is described. It is concluded that promazine is converted to 3-hydroxypromazine by this system.

PREVIOUS studies have demonstrated that chlorpromazine (I, R = Cl, R′ = R″ = Me) and related phenothiazine derivatives are oxidised



(I)

to the sulphoxide and that the dialkylamino-group (if present) is dealkylated on incubation with certain liver homogenate systems (see reviews by Emmerson & Miya, 1963; Schenker & Herbst, 1963). The products resulting from the *in vivo* metabolism of these compounds indicate that hydroxylation of the phenothiazine moiety precedes the formation of a glucosiduronic acid derivative (Emmerson & Miya, 1963; Fishman & Goldenberg, 1963; Posner, Culpan & Levine, 1963; Schenker & Herbst, 1963; and references there cited). The present communication reports the *in vitro* hydroxylation of chlorpromazine and related compounds and demonstrates that promazine (I, R = H, R′ = R″ = Me) is probably hydroxylated in the 3-position.

Methods

The livers of adult male albino Wistar rats (200–300 g) were homogenised in the cold with two volumes of potassium chloride solution (1·15% w/v). The homogenate (2 ml) was incubated in a reaction mixture (total volume, 8 ml) which was modified from that described by Mueller & Miller (1953) by substituting glucose-6-phosphate for glucose-1,6-diphosphate and adding reduced nicotinamide adenine dinucleotide phosphate (100 μg) and the phenothiazine derivative (2·5 μmoles). The reaction vessels (50 ml conical flasks) were incubated at 37° with shaking for 1 hr. Trichloroacetic acid (to 5% w/v) was added to a volume of the incubation mixture, containing at least 5 μmoles of the phenothiazine derivative, and the precipitated protein was hydrolysed by heating with sodium hydroxide solution (30 ml of 3% w/v) on a boiling water-bath for 1 hr. The supernatant fluid (acid-soluble fraction) obtained after removal of the protein precipitate was made alkaline. This and the

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hydrolysis mixture were separately extracted with washed diethyl ether, and the ether extracts were evaporated almost to dryness. The concentrated extracts were chromatographed on paper using the solvent system of Eisdorfer & Ellenbogen (1960) and on thin-layer plates prepared from Aluminium oxide G (Merck) using chloroform containing absolute ethanol (10%, by volume) as the solvent. The chromatograms were examined as described previously (Beckett, Beaven & Robinson, 1963). Paper chromatographic separation resolves mixtures of the phenothiazine compounds and their sulphoxide analogues, while the thin-layer method satisfactorily separates the parent compounds from their dealkylated analogues (see Table 1).

TABLE 1. RESULTS OF THIN-LAYER CHROMATOGRAPHY ON ALUMINA PLATES

Solvent: chloroform containing 10% v/v ethanol. The solvent moved 100 mm. All spots showed a blue fluorescence when irradiated with ultra-violet light

Compound	Distance from base line in mm*	50% v/v H_2SO_4	Reaction with $HCl/NaNO_2$
Chlorpromazine	87–92	pink	pink
Desmonomethylchlorpromazine	40–44	pink	pink
Desdimethylchlorpromazine	10–15	pink	pink
Promazine	90–95	orange-pink	pink
2-Hydroxypromazine	42–48	blue	pink
Extracts of chlorpromazine incubation mixture:			
acid-soluble fraction	85–90	pink	pink
	55–60	blue-purple	pink
	40–45	pink	pink
	7–10	pink	pink
Alkali treated fraction	85–90	pink	pink
	40–45	pink	pink

* The distances given are for the rear and advanced edges of the spots.

Spectroscopic measurements were made under optimum conditions using a Beckman DK2 recording spectrophotometer.

Results

CHLORPROMAZINE

Paper chromatography of the acid-soluble fraction showed the presence of desmonomethylchlorpromazine, (I, R = Cl, R′ = Me and R″ = H) and to a lesser extent desdimethylchlorpromazine (I, R = Cl and R′ = R″ = H) and unchanged chlorpromazine. Chlorpromazine sulphoxide was not detected. Thin-layer chromatograms confirmed the presence of desmonomethyl- and desdimethyl-chlorpromazine and chlorpromazine itself (Table 1). In addition, a fourth component which gave a purple colour with 50% v/v sulphuric acid was not present in sufficient quantity to permit a satisfactory test for the amine group. The ultra-violet absorption curves of the acid solutions of the extracts of the acid-soluble fraction showed peaks at 260, 290 and 340 mμ approximately; chlorpromazine sulphoxide (λ_{max} 237, 273, 298 and 340 mμ) was not present. Differential measurements using chlorpromazine solutions in the reference beam indicated the presence of oxidised and reduced nicotinamide adenine dinucleotide phosphate in the extracts. After treating the acid-soluble fraction extract with 50% v/v sulphuric acid,

a purple colour (λ_{max} 555–60 mμ) was obtained. In contrast, chlorpromazine treated similarly gives a red colour (λ_{max} 525–7 mμ). The alteration in the position of the absorption peak was considered to result from aromatic hydroxylation of the compound by the liver homogenate. Since this could not be confirmed because the appropriate reference compounds were not available, the *in vitro* hydroxylation of promazine was studied. The extracts of the alkaline hydrolysate gave two spots on thin-layer chromatograms which corresponded with chlorpromazine (which predominated) and desmonomethylchlorpromazine. The ultraviolet absorption curve in dilute acid solution and the visible absorption curve after sulphuric acid treatment were similar to those for chlorpromazine. This fraction was normally devoid of the hydroxylated component.

PROMAZINE (I, R = H, R′ = R″ = Me)

Thin-layer chromatograms of the extracts of the acid-soluble fraction from incubation mixtures containing promazine yielded at least two phenothiazine spots; of these, one corresponded with unchanged promazine and the other moved approximately the same distance as 2-hydroxypromazine and gave a purple spot with the sulphuric acid reagent (promazine gives a pink-orange colour). The demethylated analogues were not available, but, by analogy with chlorpromazine and by comparison with 2-hydroxypromazine, the hydroxylated compound probably retains the tertiary amine group. The ultra-violet absorption curves of the dilute acid solutions of the extracts again indicated the presence of cofactors in the acid-soluble fraction and the absence of sulphoxide in either fraction. After treatment of the acid-soluble fraction extract with sulphuric acid, the main absorption peak was at 555 mμ with minor peaks at 345 and 375 mμ whereas promazine gives a peak at 510 mμ under these conditions. In some instances, when the extract contained an appreciable proportion of "unchanged" promazine the visible absorption peaks were not well-defined, however, differential spectrophotometry, using sulphuric acid-treated promazine solutions in the reference cuvette, clearly confirmed the presence of the hydroxylated component. The latter possessed a main absorption peak at 555 mμ, minor peaks at 345 and 375 mμ and a shoulder at 450–470 mμ. This absorption spectrum is similar to those of analogous compounds in ethanolic sulphuric acid, viz 3-methoxypromazine (342, 372, 565 mμ), 3-hydroxyphenothiazine (342, 369 (443), 549 mμ) and differs from that of 2-hydroxypromazine (343, 558 mμ) by the presence of the additional peak at 375 mμ (Beckett & Curry, 1963). There was no similarity with the spectra of 1-hydroxypromazine or 4-hydroxypromazine. The presence of ethanol in the sulphuric acid does not alter the positions of the absorption peaks significantly.

OTHER COMPOUNDS

The relevant absorption data for chlorproethazine (I, R = Cl, R′ = R″ = Et) triflupromazine (I, R = CF_3, R′ = R″ = Me) and

promethazine (10-(2-dimethylamino-2-methyl ethyl) phenothiazine) and their metabolites are given in Table 2.

TABLE 2. VISIBLE ABSORPTION PEAKS OF PHENOTHIAZINE DERIVATIVES IN 50% v/v SULPHURIC ACID BEFORE INCUBATION AND AFTER RECOVERY FROM THE ACID-SOLUBLE FRACTION OF THE INCUBATION MIXTURE

Compound	λ_{max} in mμ	
	Before incubation	Recovered compound(s)
Promazine	510	345, 375, 555
Chlorpromazine	525–8	555–60
Chlorproethazine	525	565
Trifluprom azine	498	533
Promethazine	514	564

Discussion

The lack of suitable analytical methods for the detection of hydroxyl groups attached to the phenothiazine ring sytem has made it difficult to determine the biological fate of these compounds in animals and in isolated tissue systems. However, the 30–40 mμ bathochromic shift in the absorption peak obtained on treatment with sulphuric acid affords a distinction between the parent compounds and their hydroxylated analogues (Beckett & Curry, 1963). Incubation of promazine, chlorpromazine, chlorproethazine, trifluprom azine and promethazine in the liver homogenate system yielded metabolites which, in 50% v/v sulphuric acid, showed similar shifts in the locations of the absorption peaks when compared with those of the starting compounds. It is probable therefore, that all these drugs are hydroxylated *in vitro*.

In addition, experiments with thin-layer chromatography also indicate that promazine is hydroxylated, since one of the promazine metabolites moved approximately the same distance as 2-hydroxypromazine. We believe that promazine is hydroxylated in the 3-position because this metabolite yields an absorption peak at 375 mμ in 50% v/v sulphuric acid, which is not found with either 1-, 2- or 4-hydroxypromazine.

The hydroxylated chlorpromazine metabolite was detected on thin-layer chromatograms, but lack of reference compounds hindered adequate identification of the product. The most likely sites for biochemical hydroxylation appear to be the 3- and 7-positions.

The *in vitro* hydroxylation of chlorpromazine is in accord with the finding that these drugs are excreted in human urine as *O*-glucuronides.

A large proportion of the phenothiazine-positive material remains bound to the liver tissue after incubation, but could be recovered after alkaline hydrolysis. The hydroxylated products were found in the acid-soluble fraction (before hydrolysis) or in the supernatant solution after centrifuging the incubation mixture at 140,000 rcf for 30 min. If hydroxylation is effected by enzymes associated with liver microsomes, then release of the hydroxylated compound into the medium after the reaction is in accord with the more polar nature of the product.

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Determination of *o*-methoxycinnamaldehyde in cassia oil by infra-red spectrophotometry

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The determination of *o*-methoxycinnamaldehyde is described, based upon an absorption measurement at 1266 cm^{-1} in the infra-red region of the spectrum together with the normal assay for total aldehydes. Comparison of the absorption at 1266 and 1312 cm^{-1} may be used qualitatively to distinguish between oils derived from the bark of *Cinnamomum cassia* Blume and those from the leaves and twigs.

OIL of cassia is the volatile oil distilled from the leaves and twigs of *Cinnamomum cassia* Blume, and it consists mainly of cinnamaldehyde together with *o*-methoxycinnamaldehyde, cinnamic acid and esters (Guenther, 1950). The composition of the bark oil is similar but Darlington & Christensen (1944) have noted that the percentage of *o*-methoxycinnamaldehyde is much less than that of the leaf and twig oil. The determination was made using a modification of Zeisel's method for the methoxyl group and special precautions were taken by the authors to ensure reliable results.

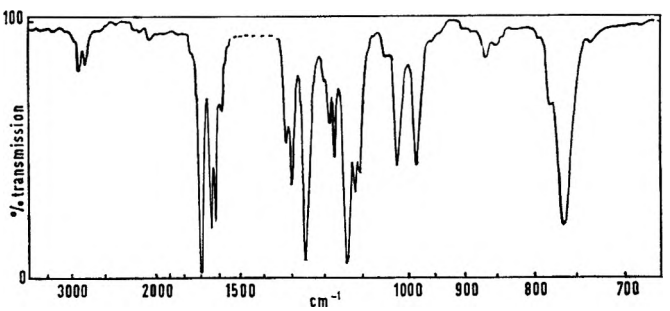

FIG. 1. 5 % w/v *o*-methoxycinnamaldehyde in acetonitrile (0·1 mm cell)

Infra-red spectrophotometry has not been used for the determination of *o*-methoxycinnamaldehyde and this investigation followed the preparation of a small sample of the aldehyde for photochemical study. Comparison of the infra-red absorption curve (Fig. 1) with that of cinnamaldehyde (Fig. 2) showed that only one absorption band, *viz.* that at about 1260 cm^{-1}, was likely to be of value. Cinnamaldehyde showed absorption in this region but the intensity was very much less than that due to *o*-methoxycinnamaldehyde. The difference was deemed sufficient to justify the use of a correction for the cinnamaldehyde.

Two determinations are therefore necessary; infra-red absorption for total aldehyde calculated as *o*-methoxycinnamaldehyde and a chemical (or physical) method for total aldehyde calculated as cinnamaldehyde. The latter assay is normally carried out in assessing the quality of an oil

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and the final expression for *o*-methoxycinnamaldehyde will vary slightly according to the method adopted. The formula for calculation of the true percentage of *o*-methoxycinnamaldehyde is readily derived, bearing in mind that with hydroxylamine hydrochloride for example 162·2 g *o*-methoxycinnamaldehyde = 132·2 g cinnamaldehyde and from infra-red analysis 100% cinnamaldehyde = 6·8% *o*-methoxycinnamaldehyde. Using ultra-violet absorption at the wavelength (286 mμ) for *cinnamaldehyde*, E (1%, 1 cm) for *o*-methoxycinnamaldehyde was found to be 1110.

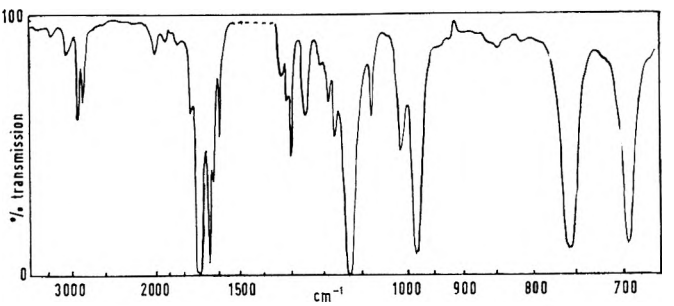

FIG. 2. 10·4 % w/v cinnamaldehyde in acetonitrile (0·1 mm cell)

Preliminary experiments indicated that the presence of terpenes and esters in the form of an aldehyde-free oil (see below) caused a decrease in the extinctions of solutions of *o*-methoxycinnamaldehyde and of cinnamaldehyde although the relationship between them remained the same. It is desirable therefore to prepare the calibration curve with mixtures closely akin to natural oils. In the absence of a suitable quantity of cassia oil, the non-aldehyde fraction of cinnamon oil was isolated for this purpose; it contained a small quantity of esters (absorption at 1740 cm^{-1}) which were probably formed during its isolation. It was used to prepare the standard oil for admixture with varying quantities of *o*-methoxycinnamaldehyde. Beer's law applied over the concentration range used but the curve is not reproduced here as it may be obtained from Table 1 (columns 6 and 7).

Experimental

Infra-red absorption spectra were measured by means of a Perkin-Elmer Infracord, model 137, fitted with sodium chloride optics; the accurate figures 1312 and 1266 cm^{-1} were obtained on a Perkin-Elmer model 237 instrument; ultra-violet absorption spectra were recorded on an Optica CF4D.R. instrument. Melting-points are uncorrected.

o-*Methoxycinnamaldehyde.* The aldehyde was prepared in low yield by the method of Vorländer & Gieseler (1929) for *p*-methoxycinnamaldehyde as pale yellow glistening flakes, m.p. 44–46°; (phenylhydrazone, m.p. 116–118°) (Bertram & Kürsten, 1895, give m.p. 45–46° and 116–117° respectively); (OMe) 19·0%, Calc. for C_9H_7O (OMe), 19·1%; E (1%, 1 cm) at 281 mμ, 1160 (max.); E (1%, 1 cm) at 286 mμ = 1110.

Aldehyde-free oil. Cinnamon oil (50 ml) in methanol (500 ml) was treated with Girard's reagent T (50 g) and boiled under reflux for 1 hr. The mixture was cooled, water (1000 ml) was added and the oil was extracted with light petroleum (b.p. 40–60°). The ether extract was washed once with dilute solution of sodium hydroxide (1%, 50 ml) and with brine (3 × 50 ml). The ether extract was dried (Na_2SO_4) and evaporated to yield a brown aldehyde-free oil (9 g, weak ester absorption at 1740 cm^{-1}).

TABLE 1. DATA USED IN CONSTRUCTION OF CALIBRATION CURVE

M* (mg)	Standard o.l (mg)	Finale volume (ml)	Cinnamalde-hyde (mg/ml)	*E*	*E* for M* only	M* mg/ml of final solution
0	517·9	5·00	88·3	0·127	—	—
17·6	482·3	5·00	82·3	0·192	0·074	3·52
17·3	185·2	2·00	77·9	0·301	0·189	8·65
24·8	172·3	2·00	74·5	0·370	0·263	12·4
84·9	414·1	5·00	70·7	0·450	0·348	17·0
37·8	170·6	2·00	69·7	0·507	0·407	18·9

* M = *o*-Methoxycinnamaldehyde.

Standard oil free from o-methoxycinnamaldehyde. The aldehyde-free oil was mixed with redistilled cinnamaldehyde so that the mixture contained 85·2% w/w cinnamaldehyde.

Calibration curve. Accurately weighed quantities of *o*-methoxycinnamaldehyde were added to weighed amounts of standard oil and dissolved in acetonitrile at a standard temperature. Table 1 incorporates the quantities and volumes. The absorption curve of each solution was recorded over the region 2000 to 1000 cm^{-1} using a 0·1 mm cell. A reference cell with acetonitrile was used to compensate for solvent absorption.

The extinction of each solution was calculated for the peak absorption at about 1260 cm^{-1} using the base line technique (cf. Cross, Gunn & Stevens, 1957). The calculated extinction for the cinnamaldehyde was subtracted from the observed extinction and the difference was plotted against the concentration of *o*-methoxycinnamaldehyde expressed in mg/ml of final solution. All the results are recorded in Table 1.

Correction for cinnamaldehyde. The extinctions of solutions of cinnamaldehyde in acetonitrile were compared with those for *o*-methoxycinnamaldehyde. 100% cinnamaldehyde was equivalent to 6·8% *o*-methoxycinnamaldehyde.

Method. Prepare, at a standard temperature, 2·00 ml of a 10% w/v solution of the oil in acetonitrile and record the absorption spectrum over the region 2000–1000 cm^{-1}. Calculate the extinction using the base line technique and read off from the calibration curve the apparent content of *o*-methoxycinnamaldehyde in mg/ml of final solution. Calculate the apparent percentage of the aldehyde (B).

Determine the percentage of total aldehyde, calculated as cinnamaldehyde (A) by a suitable method. The true percentage of *o*-methoxycinnamaldehyde is given by the expression % *o*-methoxycinnamaldehyde

= 1·06 B—0·072 A when hydroxylamine hydrochloride is used for the determination of A. When ultra-violet spectrophotometry is used the percentage is given by 1·04 B—0·071 A.

The results obtained on various samples are recorded in Table 2, which also includes the results of chemical determinations of the methoxyl group by a modified Zeisel method.

TABLE 2. RESULTS

Sample	% *o*-Methoxycinnamaldehyde		% Total aldehyde calculated as cinnamaldehyde	
	By infra-red	By methoxyl determination	By hydroxylamine	By ultra-violet spectrophotometry
Bark oil A	3·7; 3·7; 3·2	3·60; 3·74; 3·80	86·5	84·5
B	3·2	3·20; 3·27; 3·12	85·7	85·0
C	1·5; 1·45	1·59; 1·49; 1·50	81·5	79·1
D	3·7; 3·7	3·40; 3·0; 3·40	—	82·8
Leaf and twig oil ..	11·6; 11·4	11·24; 11·16; 11·20	85·7	—
Mixture I	10·0; 10·3	10·0 } Theory	—	—
Mixture II	4·8; 4·8	5·0 }		
Cinnamon oil ..	not detected	—	61·1	—

Results and discussion

The results for cassia oils (Table 2) fall into the two categories observed by Darlington & Christensen (1944) *viz.* a low percentage of *o*-methoxycinnamaldehyde in the bark oils and a much higher percentage in the leaf and twig oil. It would appear that genuine cassia oil contains high percentages of *o*-methoxycinnamaldehyde as observed by Dodge (1939). It is unfortunate that we were able to obtain only one sample of the leaf and twig oil so that some caution must be observed in drawing comparisons between the two oils. The value of infra-red absorption in this respect lies in the fact that a simple *qualitative* run on a liquid film may distinguish the bark from the leaf and twig oil because the band at about 1312 cm^{-1} acts as an internal standard. In bark oils the band is more intense than that at about 1260 cm^{-1}, whereas in the leaf and twig oil the converse is true.

It was hoped that examination of the infra-red absorption curves over the region 800 to 700 cm^{-1} might also provide information on the cinnamaldehyde content, hence the interest in selecting a solvent which was sufficiently transparent in that region. On re-appraisal of the many possible variables, *viz.* the number and proportion of mono-substituted benzene compounds other than cinnamaldehyde in cassia oil (Guenther, 1950), it was decided to abandon this approach, attractive though it seemed. Total aldehyde was therefore determined by hydroxylamine hydrochloride and by direct ultra-violet spectrophotometry. These results are included in Table 2.

The methoxyl content of the oils was checked by a modified Zeisel method and results in agreement with those found by infra-red spectrophotometry (Table 2) were obtained.

It appeared of interest to examine cinnamon oil by the method for

cassia oil, but, unfortunately, the presence of eugenol interferes in the region 1300 to 1200 cm^{-1}. Addition of 1·25% of *o*-methoxycinnamaldehyde to the oil did, however, introduce a detectable absorption at about 1260 cm^{-1} and it therefore appears unlikely that the methoxy-compound occurs in cinnamon oil.

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Note on the *in vitro* assay of corticotrophin

M. C. CANN, W. F. DEVLIN AND N. R. STEPHENSON

Factors affecting the precision of the *in vitro* assay of corticotrophin using quartered adrenals of rats have been examined. Satisfactory results were obtained only by using tissue from healthy adult male Wistar rats which had been kept quiet and at a constant temperature for several days before the assay. Greater precision was obtained from rats first anaesthetised with pentobarbitone and then decapitated. The assay is not considered to be an adequate procedure for checking commercial injections of corticotrophin.

SAFFRAN, Grad & Bayliss (1952) and Saffran & Bayliss (1953) have shown that incubation of rat adrenal tissue *in vitro* with corticotrophin resulted in the release of corticosteroids into the medium. This response of the isolated adrenal gland has been used as the basis for an assay procedure for corticotrophin. To obtain reproducible results it was necessary to use quartered adrenals equally among the dose groups and to introduce a pre-incubation period which reduced the initial output of corticosteroids to a more or less constant base level (Saffran & Schally, 1955; McKerns & Nordstrand, 1955b). van der Vies (1957) pointed out that the test animals in this assay method had to be kept under carefully controlled conditions in order to improve the precision of the assay.

We have examined various factors which might increase the precision of the assay.

Experimental methods

The *in vitro* method used was essentially that of Saffran & Schally (1955) as modified by van der Vies (1957). Male albino rats, 150–200 g, born and raised in the animal colony of the Food and Drug Directorate and derived from an inbred Wistar strain, were acclimatised for two weeks at a constant temperature of 76° ± 1° F. After killing the animals by decapitation or pentobarbitone anaesthesia or decapitation after pentobarbitone anaesthesia, the adrenals were carefully removed and weighed. The glands were then cut into quarters and subjected to the preincubation period in the Warburg apparatus as described by Saffran & Schally (1955). In our hands, the quartering of the adrenals was more uniform when a razor blade was used instead of fine scissors which is in agreement with Kitay, Holub & Jailer (1958).

Results and discussion

Exposure of the donor rats to continuous darkness for periods up to one week did not change the response of the isolated adrenal tissue to corticotrophin *in vitro*. However, a change of more than 2° F in the temperature of the animal room usually produced a large variation in the

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production of corticoids by the adrenal tissue. This sensitivity to environmental temperature confirmed the observations of McKerns & Nordstrand (1955a) and Kitay & others (1958).

Although quartered adrenal tissue obtained from Sprague-Dawley and Wistar rats from our colony responded equally well to corticotrophin, the Wistar rats were employed routinely in this study because they were usually the easier to handle. The age of the donor rat had no significant effect on the response of the adrenal cortex to stimulation with corticotrophin. However, like Tanaka (1957), we found adrenal tissue from male rats to be more responsive than that from female rats. A typical set of data shown in Table 1 indicates that the glands from donor male rats usually gave a steeper slope and a larger mean response than those from females.

TABLE 1. EFFECT OF THE SEX OF THE DONOR RAT ON THE *In Vitro* ADRENAL RESPONSE TO CORTICOTROPHIN

Sex of the donor rat	Dose of corticotrophin (mU)	Response (μg/100 mg tissue)	Slope log dose response line
Female	10·0	32·6 ± 1·0*	19·7
	30·0	42·0 ± 1·1	
Male	10·0	47·4 ± 2·4	28·3
	30·0	60·9 ± 3·7	

* Average of 4 values ± standard error.

Adrenal glands from rats with an upper respiratory infection were unable to distinguish between the low and high dose levels. The fact that the output of corticoids was in the lower part of the normal range suggested that the adrenal tissue from these infected rats could not be further stimulated by exogenous corticotrophin.

The results in Table 2 demonstrate that adrenal glands from rats that were anaesthetised with pentobarbitone sodium and then decapitated provided a steeper log dose response line than adrenal tissue from rats that were killed by either decapitation or pentobarbitone sodium anaesthesia alone.

TABLE 2. INFLUENCE OF THE METHOD OF KILLING OF THE DONOR RATS ON THE RESPONSE OF THE ADRENAL TISSUE TO CORTICOTROPHIN

Treatment	Dose of corticotrophin (mU)	Response (μg/100 mg tissue)	Slope log dose response line
Pentobarbitone sodium	10·0	43·3 ± 3·2*	25·2
	30·0	55·3 ± 1·3	
Decapitation	10·0	46·3 ± 0·8	27·7
	30·0	59·5 ± 1·0	
Pentobarbitone sodium and decapitation ..	10·0	44·1 ± 1·6	42·5
	30·0	64·4 ± 2·9	

* Average of 4 values ± standard error.

When halved adrenal glands were used in each flask, and the number of rats in each assay was increased from 8 to 16, the variation within dose groups was as large as that between dose groups. However, when

quartered adrenals were employed and tissue from each donor rat was included in each dose group, valid assays were obtained. This confirms the work of Saffran & Schally (1955); and McKerns & Nordstrand (1955b). This finding limits the number of dose groups for each assay to two, unless the adrenal glands were divided into sixths or eighths. However, it was very difficult to divide the adrenal tissue evenly when fragments were smaller than quarters. Since each of the eight flasks in the assay contains tissues from each rat, the variation within dose groups should be a minimum, and could be attributed to errors in sectioning of the glands, weighing, extraction and estimation of the corticosteroids produced, as well as variation in the viability of the individual quarters. Consequently, the standard error of such an assay was probably not dependent on biological variation, but rather could be attributed to mechanical and chemical manipulations. According to Bangham, Mussett & Stack-Dunne (1962) the estimates of error obtained from the collaborative assay of the Third International Standard for corticotrophin by the *in vitro* method were unreliable and led to weights which showed large variations between assays. In their opinion the tests of statistical validity in this assay procedure may have very little meaning. The data in Table 3 show that the potency of commercial preparations assayed by

TABLE 3. THE DETERMINATION OF THE POTENCY OF COMMERCIAL PREPARATIONS OF CORTICOTROPHIN BY THE *In Vitro* METHOD

Preparation	Labelled route of administration	Labelled potency (I.U.)	Found potency *in vitro* (I.U.)
A	Intramuscular	25·0	17·3 (10·2–29·6)
		25·0	14·0 (4·6–42·8)
		25·0	19·9 (11·6–33·9)
B	,,	40·0	22·4 (8·4–60·0)
		40·0	14·4 (10·3–43·9)
C	,,	40·0	27·6 (17·6–43·2)
		40·0	19·6 (9·3–41·2)
D	Intramuscular and intravenous	25·0	12·7 (5·3–30·6)
	,,	25·0	7·6 (3·5–16·7)

the *in vitro* procedure varied from approximately 30 to 80 % of the labelled value. However, the wide fiducial limits for each assay usually included the labelled potency. In assays of this type, an unweighted mean potency may provide a more reliable estimate than one in which weights for individual potency ratios are taken into consideration. The finding that the *in vitro* assay gave a lower potency than that stated on the label was not unexpected since the commercial preparations were assayed by the manufacturer by a method involving subcutaneous administration of the corticotrophin.

CONCLUSION

Satisfactory results with the *in vitro* method of assay of corticotrophin were obtained in our laboratory only by employing quartered adrenal tissue from healthy adult male Wistar rats which were kept as quiet as possible and at a constant temperature for several days before the assay.

In addition, the use of adrenal glands from rats which were anaesthetised with pentobarbitone sodium before decapitation, usually resulted in an assay with increased precision since the log dose response lines tended to be steeper.

According to our data the *in vitro* assay does not provide an adequate procedure for checking the potency of commercial samples of corticotrophin in pharmaceutical form which have been labelled for intramuscular or subcutaneous use.

Acknowledgments. The authors wish to express their appreciation to Mr. E. R. W. Gregory, Animal Colony, for the supply and care of rats and Mr. A. Bayne for technical assistance.

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Note on the cardiac effects of nystatin

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Nystatin, a polyene antifungal antibiotic, induces systolic arrest of the isolated hearts of the frog, rat or rabbit. In the frog, heart failure induced by raising venous pressure was antagonised and the cardiac outflow was increased. Coronary flow in the perfused heart of the rat or rabbit was decreased.

ARORA & Baghchi (1963) reported the cardiotonic activity of nystatin in the perfused frog heart. The present report communicates the results of experiments on the effects of nystatin on the hearts of the frog, rat and rabbit.

Methods and results

A 0·5% solution of nystatin in propylene glycol at 85° was diluted as necessary. Since it lost cardiotonic activity completely in 2 to 3 hr, it was freshly prepared before each experiment.

Frog hearts were perfused as described by Burn (1952). In 6 experiments, failure was induced by raising perfusion pressure in steps of 1 cm before perfusing with drug. In another 6 experiments, failure was not induced before perfusion. Cardiac outflow was measured through a cannula in the aorta. Comparisons were made with ouabain and with control preparations without any drug.

Langendorff's preparation of the albino rat and the rabbit heart was set up as described earlier (Arora & Arora, 1963) and the effects of perfusion with nystatin were compared with those produced by ouabain and propylene glycol.

In both kinds of preparation, the Ringer-Locke solution used before beginning drug perfusion contained the same amount of propylene glycol as the Ringer-Locke solution containing nystatin.

In the non-failing perfused frog heart, nystatin, 2×10^{-5} g/ml, induced systolic arrest in 20 to 30 min. There was an initial slight decrease in the amplitude of contraction and cardiac outflow followed by an increase in the amplitude of contraction and cardiac outflow and then a gradual increase in the diastolic tone and a decrease in cardiac outflow terminating gradually in systolic arrest (Fig. 1a). Changes in the heart rate were variable but generally a slight initial increase was followed by a gradual reduction. In the perfused frog hearts where failure had been induced, perfusion with nystatin 2×10^{-5} g/ml, produced effects similar to those seen in the non-failing heart (Fig. 1b). In 6 experiments with ouabain, 2×10^{-5} g/ml, effects were essentially similar to those seen with nystatin although the systolic arrest was not as complete as with nystatin, i.e., the ventricles did not become as contracted, white and button-like as with nystatin. In 6 experiments with propylene glycol, a progressive decrease in the amplitude of contraction and cardiac outflow followed the induction of failure.

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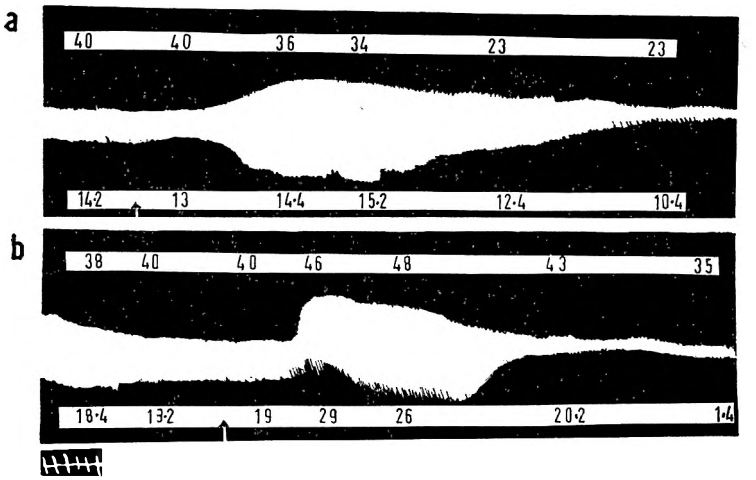

FIG. 1. Effects of nystatin, 2×10^{-5} g/ml, on the perfused frog heart. a, effects on the normal heart; b, effects after inducing failure by increasing perfusion pressure. Drug perfusion was started at arrow. The records from above downwards are: heart rate/min, contractions of the heart, cardiac outflow ml/min. Time tracing in 30 sec intervals.

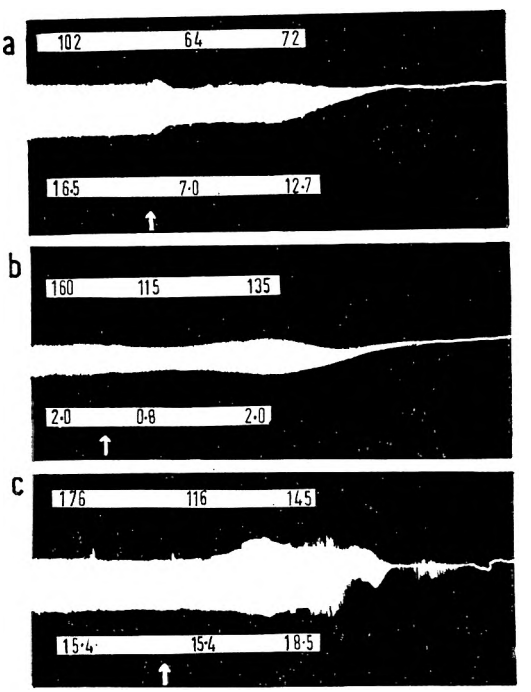

FIG. 2. Effects of nystatin on the Langendorff's preparation of (a) rabbit heart and (b) rat heart. Concentrations used were (a) 4×10^{-5} g/ml and (b) 3×10^{-5} g/ml. (c) shows the effects of ouabain, $2{\cdot}5 \times 10^{-6}$ g/ml, on the perfused rabbit heart. Drug perfusion was started at arrow. The records from above downwards are: heart rate/min, tracings of contractions of the heart, coronary flow ml/min.

In 7 experiments on the rat heart, using nystatin, 3×10^{-5} g/ml, and 6 experiments on the rabbit heart using nystatin, 4×10^{-5} g/ml, changes were essentially similar. An initial decrease in the amplitude of contraction was followed by a partial or complete recovery terminating in systolic arrest in 10 to 15 min (Fig. 2). Coronary flow decreased markedly immediately after starting nystatin perfusion followed by a partial recovery towards normal. In one experiment each on the rat and the rabbit heart, recovery in coronary flow was complete. In all but one experiment on the rat and the rabbit heart, where no change in the heart rate could be seen, there was an initial decrease in the heart rate which was followed either by a partial recovery and then again a decrease and finally cessation of ventricular activity, or by a progressive decrease terminating in ventricular arrest. In all cases, the auricles continued to beat after the ventricles had stopped.

In 6 experiments on the rat heart, ouabain, 10^{-5} g/ml, failed to have any action on the heart rate, coronary flow or the amplitude of contraction. However, in 5 experiments on the rabbit heart, $2{\cdot}5 \times 10^{-6}$ g/ml, induced systolic arrest in 10 to 15 min. Unlike nystatin, there was an increase in the amplitude of contraction above normal level from the start without any initial decrease (Fig. 2). The coronary flow also increased above control values in 3 out of 5 experiments. In one experiment on the rabbit heart auriculo-ventricular block was seen.

Discussion

Nystatin thus resembles hamycin and trichomycin in its ability to induce systolic arrest of the heart in various species. Certain differences with ouabain, however, exist such as those on the amplitude of contraction and coronary flow. It is interesting to note that nystatin, a polyene antifungal antibiotic, is a tetraene (Vining, 1960) while hamycin and trichomycin are heptaenes and yet they all share the cardiotonic activity. Unlike hamycin and trichomycin, nystatin solution is highly unstable in its cardiotonic activity. The significance of the cardiac effects of nystatin remains to be established.

Acknowledgements. The author thanks M/s Squibb Institute for Medical Research, New Brunswick, New Jersey, U.S.A. for nystatin; Dr. K. K. Chen, Eli Lilly and Co., Indianapolis, U.S.A., for ouabain and Sh. S. L. Kapoor for technical assistance.

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Letters to the Editor

Purification of glycyrrhizates through the lead salt

SIR,—In a reported method for the preparation of glycyrrhizic acid (Lythgoe & Trippett, 1950; Kuboki & Hoshizaki, 1954; Ito, Kirita & Kuroda, 1955) the precipitate produced by adding lead acetate to an aqueous solution of crude ammonium glycyrrhizate is suspended in aqueous ethanol and decomposed with hydrogen sulphide; the precipitate is removed, and the solid obtained on evaporating the filtrate recrystallised. The reaction sequence would be expected to produce lead glycyrrhizate, which is subsequently decomposed to give lead sulphide and glycyrrhizic acid, but Marsh & Levvy (1956) using the same process, claimed that mono-ammonium glycyrrhizate was the end product. Thus the lead derivative could be a mixed lead-ammonium salt, and to investigate this possibility we prepared the compound and examined it. Mono-ammonium glycyrrhizate, prepared as described previously (Gilbert & James, 1964) was dissolved in 30% ethanol and made alkaline to litmus with ammonia. Excess lead acetate solution was added giving a white precipitate which was removed, washed six times with water, and dried *in vacuo* at 50°.

Gravimetric determination as chromate gave Pb 20·00%; $(C_{42}H_{59}O_{16})_2Pb_3$ requires Pb 27·41%; $C_{42}H_{59}O_{16}NH_4Pb$ requires Pb 19·83%. Ammonia distillation gave NH_4 1·56%; $C_{42}H_{59}O_{16}NH_4Pb$ requires NH_4 1·63%.

Thus mono-ammonium glycyrrhizate is the logical product, providing the first pK_a value is lower than that of hydrogen sulphide (pK_a 7·04), for if hydrogen sulphite were the stronger acid it would replace the ammonium in the lead-ammonium salt with hydrogen. Potentiometric titration of glycyrrhizic acid with sodium hydroxide gave only one inflection, which showed that the pK_a values for all three carboxyl groups lay between 3·5 and 5·0, while pH measurements of mixtures of mono-ammonium glycyrrhizate and hydrochloric acid gave a mean first pK_a value of 3·74 at 25°. Decomposition of the lead ammonium glycyrrhizate with hydrogen sulphide gave a product identical with mono-ammonium glycyrrhizate.

Examination of the papers which described the products as glycyrrhizic acid revealed some support for our conclusions. Both Kuboki & Hoshizaki (1954) and Ito & others (1955) quoted the melting-point of their product as 170°, mono-ammonium glycyrrhizate turns yellow at this temperature, but the acid does not. It would therefore appear that the material these workers described as glycyrrhizic acid was the mono-ammonium salt.

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Resistance of *Pseudomonas aeruginosa* to chemical inactivation

SIR,—The resistance of *Pseudomonas aeruginosa* to chemical antibacterial agents causes serious difficulties in ophthalmology (Brown, Foster, Norton & Richards, 1964) and in the control of cross infection (Rogers, 1960). We have found that the antibacterial activity of polymyxin B sulphate, benzalkonium chloride and chlorhexidine against this organism is substantially increased in the presence of the non-ionic surface-active agent polyoxyethylene sorbitan mono-oleate (Tween 80).

Cultures of *Ps. aeruginosa* NCTC 8203 in nutrient broth were measured in the log phase of growth spectrophotometrically at 420 mμ. Difficulties were met in establishing a satisfactory growth curve because of the short initial log phase, and it was also observed microscopically that the initial rate slowed at the same time as cell clumping increased substantially. *Ps. aeruginosa* produces much slime (Rhodes, 1959) and it seemed likely that this contributed to the clumping. Tween 80 added to the broth in an attempt to disperse the slime and prolong the initial phase. Concentrations of Tween 80 greater than 0·01% satisfactorily increased the length of the initial log phase in nutrient broth, and microscopic examination made concurrently showed that clumping was eliminated.

Antibacterial activity was measured by adding a small volume of a pre-warmed solution of the antibacterial agent to cultures of logarithmically dividing cells and measuring subsequent changes in growth rate (Brown & Garrett, 1964). There was no appreciable effect upon the growth rate in nutrient broth when benzalkonium chloride 35 μg/ml was added to log phase *Ps. aeruginosa* cells. The same concentration immediately reduced to zero the rate in broth

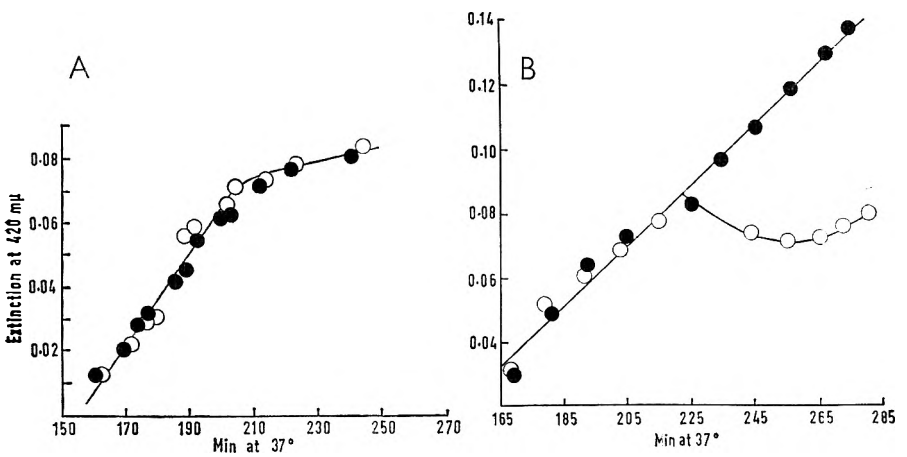

FIG. 1. Effect of Tween 80 on the action of benzalkonium against log phase cultures of *Ps. aeruginosa*. ● Control culture. ○ Benzalkonium added A, to nutrient broth after 18 min; B, to nutrient broth + 0·02% Tween 80 after 223 min.

containing 0·02% Tween 80 (Fig. 1). This effect is particularly remarkable in view of the fact that Tween 80 is an antagonist of benzalkonium (Kohn, Gershenfeld & Barr, 1963). We found that the growth rate in broth with 0·5% Tween 80 was unaffected by the addition of benzalkonium 35 μg/ml. A similar phenomenon was observed with chlorhexidine, when 0·02% Tween 80 significantly enhanced its activity but 0·5% Tween 80 eliminated any observable effect.

The inhibitory effect of polymyxin B sulphate on *Ps. aeruginosa* was enhanced in the presence of all concentrations of Tween 80 tested (0·004–0·5%); the effect increasing with increasing concentrations of Tween 80.

Preliminary experiments using end-point methods have confirmed these results and shown that the activity of our test agents is substantially increased by the presence of Tween 80.

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Pharmacologically active constituents of *Girardinia heterophylla* (Dcne)

SIR,—*Girardinia heterophylla* (Dcne), a stinging nettle obtained from the Kumaoun hills (India) was tested for the active constituents.

An acetone extract (100% w/v) from the leaves of the plant was prepared. The acetone was immediately evaporated at room temperature and the volume of the extract was made up to the original by adding normal saline. The presence of 5-hydroxytryptamine (5-HT) in the acetone extracted material from *G. heterophylla* was indicated by a spasmogenic response on atropinised oestrus rat uterus, which was completely blocked by brom-lysergic acid diethylamide. Identification of histamine was done after boiling the acetone extracted material with strong hydrochloric acid and removing the acid by distillation (Gaddum, 1953). This acid-treated extract contracted the atropinised guinea-pig ileum and elicited a fall in the cat blood pressure, the two responses were blocked after mepyramine.

Chromatographically, 5-HT was detected by the method of Jepson & Stevens (1953) and histamine by that of Pratt & Auclair (1948). The Rf values obtained by using the solvents (*a*) n-butanol : acetic acid : water (4 : 1 : 5 v/v) were for 5-HT and the extract each 0·25; for histamine and the extract 0·19 and (*b*) n-butanol : ethanol : acetic acid : water (8 : 2 : 1 : 3 v/v) were for 5-HT and the extract each 0·60; for histamine and the extract 0·20.

Thus, the presence of 5-HT and histamine in *G. heterophylla* (Dcne) has been demonstrated both biologically and chromatographically.

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Sensitivity of isolated atria from reserpine-treated rats to noradrenaline

SIR,—Chronic sympathetic postganglionic denervation reduces the noradrenaline content of smooth or cardiac muscle (Goodall, 1951; von Euler & Purkhol, 1951; Burn & Rand, 1959) increases its sensitivity to injected noradrenaline and decreases its reactivity to tyramine (Bulbring & Burn, 1938). Pretreatment with reserpine causes similar changes (Burn & Rand, 1958a). Burn & Rand (1959) therefore suggested the possibility of an inverse relationship between tissue stores of noradrenaline and sensitivity to exogenous noradrenaline. Recent observations failed to support such a view, since pretreatment of short duration (24 to 48 hr) with large doses of reserpine failed to cause supersensitivity (Fleming & Trendenburg, 1961). These authors pointed out that the time factor must also be considered, although this factor seemed to be more important for the appearance of supersensitivity of the nictitating membrane than of the cardiovascular system. In the present study, sensitivity to exogenous noradrenaline was reinvestigated in isolated atrial preparations made from rats treated with reserpine at various times.

Male albino rats of the Holtzman strain, weighing 225 to 250 g, were used in all experiments. Animals were killed by a blow at the base of the neck, decapitated and the heart rapidly removed. Atria were freed of ventricular muscle, connective tissue, fat and blood vessels, then placed in a modified Tyrode's solution (Bhagat & Shideman, 1963b) maintained at 28° and containing $2{\cdot}9 \times 10^{-8}$M atropine sulphate. A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the bathing fluid via a sintered glass plate at the bottom of the bath. Isometric contractile amplitude (resting tension of approximately 0·5 g) and rate of spontaneous beat were recorded. Drugs were added to the bath after the preparation had attained a constant amplitude of contraction and responses were calculated as percentage changes relative to the amplitude existing just before the addition of the drug.

The concentrations of catecholamines in the ventricular myocardium were determined by the trihydroxyindole fluorimetric procedure of Shore & Olin (1958) and are expressed as μg of noradrenaline per g of fresh tissue.

Noradrenaline bitartrate monohydrate and reserpine (Serpasil, Ciba) are expressed as base and cocaine hydrochloride as the salt. In one group, rats were given 1·5 mg/kg of reserpine intramuscularly 24 hr before the experiment, while in another group, 0·5 mg/kg of reserpine was administered every 18 hr and the animals were used 72 hr after the first injection.

TABLE 1. POSITIVE INOTROPIC EFFECT OF NORADRENALINE ON ISOLATED RAT ATRIA

Treatment	Catecholamine concentration (μg/g of fresh tissue) mean ± s.e.	Sensitivity to noradrenaline (0·5 μg/ml) Increase in size of contraction mean % change ± s.e.	
			in presence of cocaine 5 μg/ml
Control	1·06 ± 0·04 (8)	98 ± 11·4 (12)	132 ± 12·6 (6)
Reserpine 1·5 mg/kg 24 hr before experiment	0·06 ± 0·03 (10)	89 ± 6·8 (10)	128 ± 9·5 (5)
Reserpine 0·5 mg every 18 hr (3 doses) rats killed at 72 hr ..	0·08 ± 0·04 (10)	105 ± 9·8 (10)	136 ± 8·7 (6)

Results in Table 1 show that the sensitivity to noradrenaline of isolated atria obtained from rats pretreated with reserpine was normal. This confirms the

findings of Bhagat & Shideman (1962a, b) who did not find any supersensitivity to noradrenaline in atria from rats depleted of their catecholamines with reserpine or guanethidine. Perhaps organ and species differences may be of importance, since the hearts of spinal cats (Fleming & Trendelenburg, 1961) and isolated atria from reserpinised rabbits are supersensitive (MacMillan, 1959), whereas those from reserpine-pretreated rats are not. Crout, Muskus & Trendelenburg (1962) also reported that isolated atria from reserpine-pretreated guinea-pigs are normal in their sensitivity to noradrenaline. They attributed this difference to the schedule of treatment with reserpine, but this could not be so in our experiment, since reserpine was injected in small doses and 72 hr were allowed for the development of supersensitivity.

Reserpinised atria not only behaved like normal tissues in their sensitivity to noradrenaline but also to the sensitising effect of cocaine. Similar findings with cocaine have been reported by Trendelenburg, Muskus, Fleming, & Gomez (1962) with the nictitating membrane of the cat. The ability of cocaine to potentiate the effects of adrenaline on blood pressure and on sympathetically innervated organs was first reported by Frohlich & Loewi (1910). Since then, several studies have been made to explain this phenomenon. The most generally accepted hypothesis is that cocaine impairs the rapid uptake of noradrenaline by storage sites in tissues thereby increasing the amount of exogenous noradrenaline available for reaction with sympathetic receptors (Whitby, Hertting & Axelrod, 1960; Hertting, Axelrod, Kopin & Whitby, 1961; Bhagat, 1964). Similarly, reserpine is known to block the uptake of noradrenaline. But recent chemical investigation by Kopin & Gordon (1963) suggest that reserpine-treated animals can take up noradrenaline but they disposed of it primarily by enzymatic inactivation rather than by storage. With such a system, the potentiating action of cocaine could then be attributed to an impairment of uptake of catecholamines into the structures rather than to blockade of either subsequent storage (predominant in normal tissues) or subsequent enzymatic degradation (predominant in reserpinised tissues).

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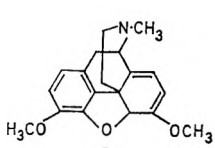
Structure-activity requirements in some novel thebaine-derived analgesics

SIR,—Thebaine (I) an alkaloid present in opium (0·2–0·8%) has no therapeutic activity and little commercial value. Unlike the chemically related central nervous system depressants morphine and codeine, thebaine is devoid of analgesic activity and acts as a central nervous system stimulant. Bentley & Hardy (1963) have recently described the synthesis of a series of dienophil derivatives of thebaine showing high analgesic potencies.

A number of the ketonic adducts, II (Table 1), were found to have analgesic actions similar to that of morphine.

Primary and secondary alcohols, III, produced by the reaction of appropriate Grignard reagents on these and other related ketones were found to possess greater analgesic activity (Table 2).

O-Demethylation of the thebaine derivatives to the corresponding oripavines increased the activity in a manner similar to that seen on the conversion of codeine to morphine.



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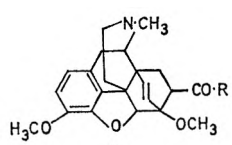
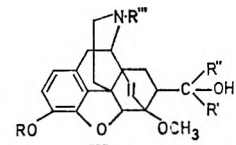


II



III

When tested in rats by a modification of the tail-pressure method of Green & Young (1951), as described by Lister (1960) or in mice by the hot-plate method (Eddy & Leimbach, 1953) or by the phenylquinone writhing test in mice (Hendershot & Forsaith, 1959) these compounds showed analgesic potencies ranging from 0·1 to approximately 8,000 times that of morphine. The relative molar analgesic potencies and intravenous toxicity of typical members of these two series are shown in Tables 1 and 2. Although the analgesic activities of members of these series are so high when compared with morphine, their toxicities are not increased to the same degree.

TABLE 1. ANALGESIC ACTIVITIES AND TOXICITIES OF SOME KETONIC DERIVATIVES OF TETRAHYDRO-6,14-*ENDO*ETHENOTHEBAINE

Code No.	Structure II, R =	Molar Potency Ratios: Morphine = 1	
		Analgesia in rats s.c.	Toxicity in mice i.v.
M.61	H	0·6	2·0
M.39	Me	1·2	3·8
M.70	Bu^t	9·0	5·0
M.85	C_6H_5	0·7	2·7
M.88	$[CH_2]_7Me$	0·03	2·3

Further modifications of the molecule have been studied; in some instances acetylation of the phenolic group increased analgesic activity and in others reduced it. *N*-Demethylation to the secondary base reduced activity in all compounds but the analgesic potency retained was still from 0·5 to 6 times that of morphine.

All the compounds described have a pharmacological profile similar to that of morphine. When injected they produce analgesia, depression of the respiratory and the cough centres, inhibition of gastrointestinal motility, lowering of body temperature and other signs characteristic of morphine-like drugs. These pharmacological effects have been observed in the mouse, rat, pig, rabbit, cat, dog, guinea-pig, monkey and man. When the dose of these highly active compounds is increased beyond that necessary to produce analgesia, a tranquillising effect is seen and normally aggressive animals become tractable. Further increase in dosage produces a state of 'total analgesia' or areflexia without apparent loss of consciousness. All these depressant effects are antagonised by nalorphine or levallorphan. Despite the vast increase in potency of many of the compounds in these series, there appears to be only a minor degree of dissociation between their therapeutically desirable and undesirable properties when compared with equianalgesic doses of morphine. The duration of action is found to be less than that of morphine. Unlike morphine and pethidine these potent analgesics do not liberate histamine even in doses many times higher than that required to produce analgesia.

TABLE 2. ANALGESIC ACTIVITIES AND TOXICITIES OF SOME ALCOHOLIC DERIVATIVES OF TETRAHYDRO-6,14-*ENDO*ETHENOTHEBAINE

Code No.	Structure III				Molar Potency Ratios Morphine = 1	
	R =	R′=	R″=	R‴=	Analgesia in rats s.c.	Toxicity in mice i.v.
M.50	Me	Me	Me	Me	2·5	3·1
M.53	Me	Me	Pr^n	Me	89	4·2
M.53A	Me	Pr^n	Me	Me	0·7	3·2
M.99	H	Me	Pr^n	Me	2,060	10·8
M.183	COMe	Me	Pr^n	Me	5,330	16·7
M.74	H	Me	Me	Me	49	2·5
M.150	H	H	Me	Me	35	1·5
M.217	H	H	H	Me	13	1·2
M.89	Me	Pr^n	Pr^n	Me	3·8	6·3
M.79	Me	Me	Pr^n	Me	1·2	2·6
M.140	H	Me	$CH_3CH_2CH(Me)$	Me	7,800	27·5
M.191	H	Me	Pr^n	H	40	6·1

The introduction of the bridged system and the creation of a further centre at position 7 capable of chemical modification has made possible the synthesis of a large number of variants of the basic molecule.

Examination of the structure-activity relationships in the two series discussed, reveals a need to revive even further the classical ideas of these relationships in the morphine series first proposed by Braenden, Eddy & Halbach (1955).

In the ketonic series, Table 1, maximum activity was found in compound II with the substituent R = isobutyl (M. 70).

Examination of the analgesic activities of the alcoholic derivatives shown in Table 2 shows that optimal activity tends to occur under the following conditions.

1. The oripavine derivatives, R = H, or their acetylated derivatives, R = COMe, are more active analgesics than the corresponding thebaine derivatives R = Me, e.g. M.99 and M.183 > M.53.
2. The nature of the alcohol grouping attached to C7 is important; tertiary alcohols show a higher potency than secondary alcohols, e.g. M.74, > M.150, > M.217.
3. One of the substituents R′ or R″ on the carbon atom attached to C7 should be small, preferably methyl, e.g. M.53 > M.89.
4. Peak analgesic activity is found when the second of these substituents is an alkyl group with an overall chain length of 3–6 carbon atoms, e.g. M.99 and M.140.
5. The unsymmetrical tertiary alcohols exhibit optical isomerism with the two diastereoisomers showing marked differences in analgesic potencies, e.g. M.53 and M.53A. Difficulty has been encountered in assigning the configurations of the isomers but in all instances the isomer with the higher melting-point is found to be more active.
6. The piperidino-nitrogen atom is tertiary. The corresponding secondary amines are found to be less active, M.191 > M.99.

The preliminary results indicate that the simplified concept of the nature of the morphine receptor site proposed by Beckett (1959) must be revised to account for these new facts. These compounds have a more rigid molecular structure than morphine which may fit the receptor surface more closely and this may be a factor in explaining the greatly increased activity found in this series. A second binding site for the group at position 7 must be postulated and this appears to highly selective, judging by the differences in analgesic activity found in diastereoisomers.

Despite the vast increase in analgesic potency found in members of these series, no marked differentiation between analgesic activity and the other centrally mediated properties has been demonstrated. This suggests that these drugs may be acting on areas in the central nervous system through which pass afferent impulses to the specific controlling centres; these areas are likely to be in the mid-brain and reticular system.

Further work is in progress to evaluate the structure activity relationships in these series and to determine their site and mode of action.

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Wheatfield Road,
Edinburgh, 11.
March 17, 1964

ROBERT E. LISTER

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Book Reviews

GRUNDLAGEN DER ARZNEIMITTELFORSCHUNG UND DER SYNTHETISCHEN ARZNEIMITTEL. By J. Büchi, Pp. 744 (including 95 illustrations and Index). Birkhäuser Verlag, Basle, 1963. Sw. fr. 96.

In an era of increasing specialisation, it is a pleasant change to find a book which deals with research into medicinal substances as a unified whole. The scope of this book ranges through organic and physical chemistry, biochemistry, pharmacology and pharmacy; it is difficult to think of any scientific discipline involved in the discovery and development of a new drug which has been omitted. This diversity is the more remarkable when one considers that the entire book is the work of one author. Despite the complexity of his task, Professor Büchi has organised the widely varied material into a logical and lucid form, using the chemistry of medicinal substances as a broad framework but diverging widely as the occasion arises.

The text is divided into six chapters. After a brief introduction, including an outline of the history of research into drugs, there is a long but very well organised chapter on the practical aspects of developing a new medicinal substance. This deals with the broad principles governing the synthesis and isolation from natural sources of potentially active materials, and the possibilities of modifying these materials chemically or physically to increase their pharmacological activities, lower their toxicity or make their formulation more effective. It also covers chemical and biological testing, and some aspects of formulation.

The physical properties of drugs in relation to their pharmacological actions, the biological transformations and excretion of medicinal substances, and the vexed and thorny problems of structure-action relationship are treated with great clarity and reasonable detail in succeeding chapters. The inevitable compression required to deal with such a range of material in some 300 pages is offset by the wealth of references to original literature (up to 1960 and in some cases 1962). The inclusion of lists of reference in the middle of chapters is confusing, since the same reference numbers may be repeated five times in a single chapter. This is, however, offset by the provision of a complete author-index at the end of the volume.

The final chapter deals in some detail with pharmacology at the cellular and molecular level, particularly the nature of the bonds between molecules of the drugs and their tissue receptors, and their steric relationships.

All the chapters are divided systematically into sections, subsections and sub-subsections each classified numerically for ease of reference. This is typical of the admirable tidiness of the author's approach to a subject which is not in itself particularly orderly. The printing and general presentation are uniformly good, with clear explanatory diagrams and graphs.

The only adverse criticism which may possibly be made is that the author has attempted to put a quart into a pint pot, and the specialist may feel that some aspects have been treated too briefly. This problem is, however, insoluble in any book which attempts, within a reasonable number of pages, to span a wide range of research, and the author has done an excellent job of arrangement and condensation, including the wide use of tabulated information. The danger of producing a catalogue has on the whole been averted and the book is consistently readable. It fills most ably a gap between the undergraduate textbook and the specialised research review, and is to be recommended particularly to the specialist in any aspect of drug research who wishes to extend his knowledge of related fields of investigation.

J. CHILTON.

STATISTICS FOR MEDICAL STUDENTS. By R. G. Hoffman. Pp. xvi + 197 (including Index). Charles C. Thomas, Springfield, Ill., U.S.A., 1963. $7.50.

Some knowledge of statistical methods must soon become an essential part of a doctor's training, since he must as a practitioner or research worker be able to assess critically the merits of papers he reads in the journals.

The average medical student possesses only a limited ability to think mathematically, and how best to introduce and teach statistics in medical schools presents a problem. It is clear that the position could be eased if classes in mathematical statistics were to be included in the school syllabuses for those pupils who intend to take a University course in biology or medicine. In the medical schools statistics should go hand in hand with the practical work; biochemical, physiological and pharmacological experiments provide ideal material for statistical analysis.

Any book which helps to overcome the problem of teaching statistics to medical students is to be welcomed. Dr. Hoffmann's book was written to help the student overcome real problems requiring "decisions where uncertainty exists". There is a brief introduction, dealing with measurement, accuracy and precision, leading up to Section I which contains the information on statistical methods proper. This section contains seven chapters, each of which deals with a specific topic such as "the comparison of averages" or the "statistics of the straight line". The theory of each chapter is neatly summarised and then applied to real problems such as those encountered in laboratory experiments.

Part of Section I will be of little or no use to the medical student. I doubt very much, for example, whether he will ever need to know about the statistical design of experiments, which is dealt with at some length, but those students who later become involved in postgraduate work will perhaps find this aspect to be of use.

There is an odd error in the mathematical example of p. 12 ($\bar{x}$ (coded) = $\frac{1{\cdot}1}{4}$ not $\frac{0{\cdot}11}{4}$ and I disagree with the use of L.D.50 instead of E.D.50 in the chapter on dosage-response problems. But these are details, and must not be allowed to distract a would-be buyer.

Dr. Hoffmann has had several years' experience working alongside medical practitioners, and in the second and last section of this book he outlines some of the uses of statistical methods in private practice. These include chapters on storing and retrieving information and on the construction of hospital record abstracting systems. Most of the examples and information in this section are taken from the Teaching Hospital of the University of Florida.

As both a postgraduate research worker in pharmacology and a medical student I have found this book useful in evaluating my own experimental results. I think it is a good book, and I recommend it to all those who require a fundamental knowledge of statistical methods.

E. S. JOHNSON

ABSTRACTING AND CODING OF DRUG LITERATURE

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Applications close in London and Brisbane on 15th May, 1964.

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Review Article

Research Papers

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