# Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 18 No. 4
April 1966

# Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

17 Bloomsbury Square, London, W.C.1. HOLborn 8967




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*J. Pharm. Pharmac.*, 1966, **18**, 201–222

# Review Article

## The mathematics of drug-receptor interactions

D. MACKAY, B.Sc., Ph.D.

MOST pharmacological observations support the hypothesis that drugs produce their effects by interacting in a specific way with some component of the living cell. This component, which is likely to be either an enzyme or a site on a cell membrane, is called the receptor. Substances which act on receptors may be classified as agonists, which produce an observable response from a tissue, or as antagonists, which do not themselves produce an observable response but prevent the response to agonists. The concept of specific receptors is supported mainly by the ability of some antagonists to block selectively the response of tissues to certain agonists.

### EXPERIMENTAL EVIDENCE

Any detailed conclusions about drug-receptor interactions must rest on information derived from studies of the kinetics of drug action or of dose-response curves. The kinetics of drug action, however, when measured on isolated tissues, are likely to depend on the rate of diffusion of the drug to the receptors, on the rate of reaction of the drug with the receptors, and on the rate of response of the cells to the drug-receptor reaction. These problems, which were discussed fully by Clark (1933a), may arise even when responses are measured on single cells. Analyses of drug-receptor interactions therefore tend to be based on dose-response curves measured under equilibrium conditions, so that complicated kinetic factors are eliminated. The responses obtained are then assumed to correspond to an equilibrium, or steady state, occupation of the receptors to which the law of mass action may be applied.

### APPLICATION OF THE LAW OF MASS ACTION TO DRUG-RECEPTOR INTERACTIONS

If the drug is given the symbol A and the specific receptor with which it interacts the symbol R, then the reaction of the drug with the receptor may be written as

$$R + A \rightleftharpoons RA$$

The reaction is usually assumed to be bimolecular. Then by the law of mass action, the affinity constant, $K_A$, of the drug for the receptor is given by the equation

$$K_A = \frac{\{RA\}}{\{R\}\,(A)} \qquad \ldots \quad \ldots \quad \ldots \quad (1)$$

where (A) is the equilibrium molar concentration of the drug in the region about the receptors. The braces around the symbols R and RA

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indicate that the concentrations of receptor and of drug-receptor complex are in arbitrary units. Since the arbitrary units cancel, $K_A$ is in litres/mole.

The total concentration of receptors in or on a cell is $\{R\}_T$ where

$$\{R\}_T = \{R\} + \{RA\} \quad .. \quad .. \quad .. \quad (2)$$

Eliminating $\{R\}$ from equation (2), by use of equation (1), and re-arranging, gives

$$\{R\}_T = \{RA\}\left[1 + \frac{1}{K_A(A)}\right]$$

Then the fraction of the receptors occupied by the drug A, at equilibrium, is

$$y_A = \frac{\{RA\}}{\{R\}_T} = \frac{1}{\left[1 + \frac{1}{K_A(A)}\right]} \quad .. \quad .. \quad (3)$$

The fraction of the receptors occupied at any given value of (A), therefore, depends only on $K_A$, and $y_A$ tends to unity when the concentration of A is made sufficiently high.

It can be shown, in the same way, that when two drugs A and B compete for the same receptor R, then the fraction of the receptors occupied by drug A, at equilibrium, is

$$y_A = \frac{\{RA\}}{\{R\}_T} = \frac{1}{1 + \frac{1}{K_A(A)} + \frac{K_B(B)}{K_A(A)}} \quad .. \quad .. \quad (4)$$

If, on the other hand, the drug B is a non-competitive antagonist then

$$y_A = \frac{1}{1 + \frac{1}{K_A(A)} + \frac{K_B(B)}{K_A(A)} + K_{AB}(B)} \quad .. \quad .. \quad (5)$$

where $K_{AB}$ is the affinity constant of the agonist-receptor complex for the antagonist and $K_A$ and $K_B$ have their usual significance [see equation (1)]. If B is a non-competitive antagonist in the strictest sense then $K_B$ and $K_{AB}$ are equal.

## 2. THE RELATIONSHIP BETWEEN RECEPTOR-OCCUPATION AND THE OBSERVED RESPONSE

This problem may be considered both qualitatively and quantitatively.

*Qualitative considerations.* According to Clark's ideas, occupation of a receptor by an agonist causes a change in some property of the cell, and this change persists as long as the agonist occupies the receptor. This hypothesis is known as "occupation theory". Other possibilities have been suggested (Paton, 1961; Mackay, 1963), but fortunately, from a mathematical point of view, occupation theory and the alternative ideas all lead to the conclusion that the response, under equilibrium or steady-state conditions, is likely to be some function of the concentration of agonist-receptor complex.

*Quantitative considerations.* It must be emphasised that the relation between the fraction of receptors occupied by the agonist and the observed

response is not known. Indeed, it is interesting to consider the various types of response which might be measured. In the case of muscle contraction the response might be the isometric tension produced, or the change in length of the tissue under isotonic conditions. Alternatively, changes in the electrical properties or membrane permeabilities of the cells might be used as a measure of the response. It seems very unlikely that these various types of response would all be related in the same way to the fraction of receptors occupied by the agonist.

### 3. CLARK'S QUANTITATIVE TREATMENT OF RECEPTOR THEORY

The first quantitative treatment of receptor theory was that of Clark (1933c). He applied the law of mass action to the drug-receptor interaction and also assumed that the response of a tissue is directly proportional to the fraction of receptors occupied by the agonist, although he clearly realised (Clark, 1933a,b) that this assumption might not be valid. Clark's assumption may be written as

$$r_A = k y_A \quad .. \quad .. \quad .. \quad .. \quad (6)$$

where $y_A$ is the fraction of receptors occupied and is given by equation (3), and $r_A$ is the response to the agonist A. The constant k applies to all agonists interacting with these specific receptors. At sufficiently high concentrations all the receptors are occupied and $y_A$ is then equal to unity. Under such conditions equation (6) becomes

$$r_A = k = r_{max}$$

where $r_{max}$ is the maximal response of the tissue and is the same for all agonists.

Hence, on the basis of Clark's assumptions, the fraction of receptors occupied should be related to the response by the equation

$$y_A = \frac{r_A}{r_{max}}$$

When $r_A = \frac{1}{2} r_{max}$, then $y_A = \frac{1}{2}$

$$= \frac{1}{1 + \frac{1}{K_A(A)_{50}}}, \text{ from equation (3),}$$

where $(A)_{50}$ is the concentration of agonist which produces 50% of the maximal response. It follows that

$$K_A = \frac{1}{(A)_{50}} \quad .. \quad .. \quad .. \quad .. \quad (7)$$

and so, on the basis of Clark's assumptions, the affinity constant of an agonist for its receptor may be estimated from the dose-response curve.

Gaddum (1937) extended Clark's theory to account for the effects of specific antagonists. These compounds were assumed to adsorb onto the receptors without producing the changes necessary for a response. In this way antagonists could prevent the formation of agonist-receptor complexes. Gaddum therefore suggested that equation (6) might also

apply to the action of an agonist in the presence of a competitive antagonist, the value of $y_A$ being given by equation (4), where B would then be the competitive antagonist.

## 4. APPLICATION OF THE NULL METHOD TO STUDIES OF DRUG ANTAGONISM

A basic weakness of Clark's quantitative treatment of receptor theory was the assumption of direct proportionality between the response and the fraction of receptors occupied by the agonist.

However, both Clark and Gaddum sometimes compared the concentrations of an agonist required to produce a selected response from a tissue before and after it had been treated with an antagonist. Clark & Raventos (1937) suggested that "an alternative method of estimating antagonistic power is to determine the concentration of B (the antagonist) which alters by a selected proportion (e.g. tenfold) the concentration of A (the agonist) needed to produce a selected effect." This suggestion contains the basis of the null method which was applied by Schild (1947) to the study of drug antagonism.

Suppose that a given value of the response, r, is produced by a concentration $(A)_1$ of the agonist acting alone. The response is some function of the fraction of receptors occupied by the agonist, but this function is not necessarily a linear one. The fraction of receptors occupied, when the concentration of A is $(A)_1$, is

$$y_{A_1} = \frac{1}{1 + \dfrac{1}{K_A(A)_1}} \quad \text{[see equation (3)]} \quad . . \qquad (8a)$$

When a competitive antagonist is also present it reduces the concentration of agonist-receptor complex produced by $(A)_1$ by competing for the receptors. This in turn reduces the response produced by $(A)_1$, but this antagonism can be counteracted by increasing the concentration of the agonist from $(A)_1$ to some higher value $(A)_2$. The value of $y_A$, when the concentration of agonist $(A)_2$ acts on the tissue in the presence of a concentration (B) of antagonist, is

$$y_{A_2} = \frac{1}{1 + \dfrac{1}{K_A(A)_2} + \dfrac{K_B(B)}{K_A(A)_2}} \quad \text{[see equation (4)]} \quad . . \qquad (8b)$$

If the response is determined by the fraction of receptors occupied by the agonist then equal values of $y_A$ should produce equal responses, and vice versa. This conclusion does not depend on the form of the relationship between $y_A$ and the response, since only equal responses are compared. Suppose that a value of $(A)_2$ is chosen and (B) is adjusted until the response to $(A)_2$, in the presence of (B), is equal to the response to $(A)_1$ alone. Then

$$y_{A_1} = y_{A_2}$$

and so, from equations (8a) and (8b),

$$1 + \frac{1}{K_A(A)_1} = 1 + \frac{1}{K_A(A)_2} + \frac{K_A(B)}{K_A(A)_2}$$

It follows that

$$\frac{1}{(A)_1} = \frac{1}{(A)_2}[1 + K_B(B)] \quad .. \quad .. \quad (9)$$

and if $(A)_2 = x\,(A)_1$

then $$(B)_x = \frac{[x-1]}{K_B} \quad .. \quad .. \quad .. \quad .. \quad (10)$$

where $(B)_x$ is the corresponding concentration of the antagonist. Schild defined the $pA_x$ as

$$pA_x = -\log_{10}(B)_x$$
$$= \log_{10}K_B - \log_{10}[x-1], \text{ from equation (10)} \quad .. \quad .. \quad (11a)$$

From equation (11a), when x is equal to 2,

$$pA_2 = \log_{10}K_B, \quad .. \quad .. \quad .. \quad (11b)$$

and when x is equal to 10

$$pA_{10} = \log_{10}K_B - \log_{10}9$$
$$= pA_2 - \log_{10}9, \text{ from equation (11b).}$$

Therefore $$pA_2 - pA_{10} = \log_{10}9.$$

If this relationship between $pA_2$ and $pA_{10}$ is found to be valid then the antagonist is probably acting competitively and $K_B$ can be calculated from equation (11b).

However, a more general test for competitive antagonism can be applied by using equation (11a) (Arunlakshana & Schild, 1959). This equation can be rearranged to give

$$\log_{10}[x-1] = \log_{10}K_B - pA_x \quad .. \quad .. \quad (12)$$

Various values of x can be chosen and the corresponding values of $pA_x$ can be found experimentally. Then, for a competitive antagonist, a plot of $\log_{10}[x-1]$ against $pA_x$ should give a straight line with an intercept equal to $\log_{10}K_B$.

When applied to competitive antagonists the $pA_x$ method should give correct values of the affinity constants of the antagonist for the receptor, since this method involves no assumptions about the form of the relationship between the response and the fraction of receptors occupied by the antagonist.

## 5. INTRINSIC ACTIVITY

According to Clark's quantitative treatment of receptor theory, drugs which act on any particular type of receptor should be either agonists or antagonists. In sufficiently high concentrations all agonists should be able to produce a maximal response from a tissue.

However, it was later observed (Raventos, 1937; Ariëns, 1954) that the maximal responses produced by some agonists were less than those produced by others. Agonists which produce the maximal response of the tissue may be called full agonists, while those which produce maximal responses which are less than the maximal response of the tissue may be

called partial agonists. In order to account for such findings, Ariëns (1954) introduced the term *intrinsic activity* and described it as "a substance-constant determining the effect per unit of pharmacon-receptor complex." In other words, it was suggested that a complex of a receptor with one agonist might differ from the complex with another agonist, in its ability to contribute to a response. In order to obtain values of the intrinsic activity, Ariëns retained Clark's assumption that the response is directly proportional to the fraction of receptors occupied by the agonist. The constant k in equation (6) was therefore considered to vary from one agonist to another.

According to Ariëns' assumptions the response to any agonist A is $r_A$ where

$$r_A = k_A y_A \quad .. \quad .. \quad .. \quad .. \quad (13)$$

The term $k_A$ is the intrinsic activity of the agonist and $y_A$ is the fraction of receptors occupied. The value of $y_A$ is given by equation (3), and tends to a maximum of unity when the concentration of agonist is made sufficiently high. Then, from equation (13), the maximum response to the agonist A is

$$[r_A]_{max} = k_A \quad .. \quad .. \quad .. \quad .. \quad (14)$$

and the intrinsic activity of an agonist is proportional to the maximum response which it can produce. Then for two agonists A and B, the ratio of their intrinsic activities is

$$\frac{k_B}{k_A} = \frac{[r_B]_{max}}{[r_A]_{max}} \quad .. \quad .. \quad .. \quad .. \quad (15)$$

Suppose that the maximum response which can be elicited from the tissue is $r_{max}$. Then all agonists which produce this response will be observed to have the same intrinsic activity, which may be set equal to unity. If $[r_A]_{max}$ is equal to $r_{max}$ then $k_A = 1$, and equation (15) becomes

$$k_B = \frac{[r_B]_{max}}{r_{max}} \quad .. \quad .. \quad .. \quad .. \quad (16)$$

If the simplifying assumptions made by Ariëns are correct, then the (relative) intrinsic activity of a partial agonist can be obtained by comparing maximum responses (see Fig. 1).

From equations (13) and (14)

$$\frac{r_A}{[r_A]_{max}} = y_A$$

Suppose that the concentration of agonist, or partial agonist, which produces a response equal to one half of $[r_A]_{max}$, is written as $(A)_{50}$. Then the corresponding fraction of receptors occupied is

$$y_A = \frac{r_A}{[r_A]_{max}} = \tfrac{1}{2}$$

$$= \frac{1}{1 + \dfrac{1}{K_A(A)_{50}}} \text{ [from equation (3)]}$$

It follows that

$$[1 + \frac{1}{K_A(A)_{50}}] = 2$$

and so

$$K_A = \frac{1}{(A)_{50}} \quad .. \quad .. \quad .. \quad .. \quad (17)$$

The simplifying assumptions made by Ariëns lead to the conclusion that the affinity constant of an agonist or partial agonist for its receptor can be calculated directly from the simple dose-response curve, since $(A)_{50}$ is readily determined (see Fig. 1). Clearly equation (17) is similar to

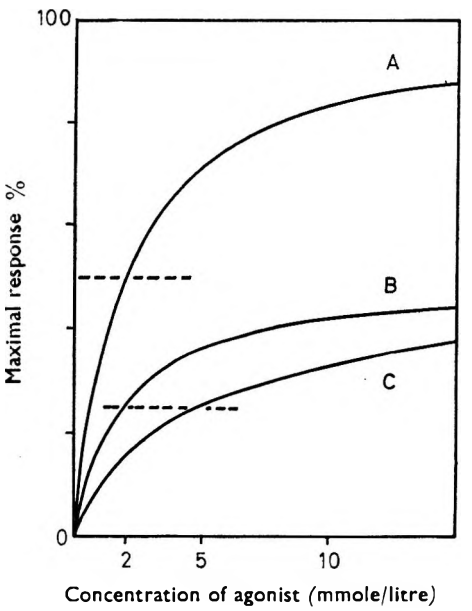


FIG. 1. The determination of affinity constants and intrinsic activities of agonists, on the basis of Ariëns' simplifying assumptions. Compound A has an intrinsic activity of unity and an affinity constant of 0·5 litre/mmole. Compounds B and C have intrinsic activities of 0·5. The affinity constants of compounds B and C are respectively 0·5 litre/mmole and 0·2 litre/mmole. The values of the affinity constants are equal to the reciprocal of the concentrations of the drugs which produce a response equal to one half of the maximal response which the drug can elicit from the tissue.

equation (7) except that $(A)_{50}$ now represents the concentration of drug which produces a response equal to one half of the maximal response which the drug can elicit from the tissue.

It may be noted that Ariëns' treatment separates agonists into two groups which are full agonists and partial agonists. However, as with Clark's treatment, the intrinsic activities of all fully active agonists are the same, since such compounds are assumed to occupy all the receptors when producing the maximal response of the tissue. If some fully active agonists could produce maximal responses from the tissue without occupying all the receptors, the intrinsic activities of these compounds

would appear to be the same, although actually different. Attempts have been made to differentiate between fully active agonists by using irreversible antagonists (van Rossum & Ariëns, 1962).

## 6. EFFICACY

The term efficacy, introduced by Stephenson (1956), is conceptually the same as the intrinsic activity. However, Stephenson did not retain Clark's assumption that the response is directly proportional to the fraction of receptors occupied by the agonist. Instead he assumed that some full agonists may produce a maximum response from a tissue when only a very small fraction of the receptors are occupied. If this assumption is valid then various full agonists may elicit the maximum response of which the tissue is capable, by occupying different fractions of the total number of available receptors.

Stephenson defined a quantity called the *stimulus* and distinguished clearly between this stimulus and the response. The stimulus is defined by the equation

$$s = ey \quad .. \quad .. \quad .. \quad .. \quad (18)$$

where e is a constant termed the efficacy, and y is the fraction of the receptors which the drug occupies when it produces the response corresponding to this stimulus. For any particular agonist, the stimulus is proportional to y, and y is given by equation (3). The response r is regarded as being a definite function of the stimulus, so that a given stimulus always produces the same response. It follows that any particular response might be produced by a very large number of values of e and y, provided that the product of e and y has the appropriate constant value.

By using the null method and by making certain simplifying assumptions, Stephenson was able to calculate the efficacies of partial agonists. The stimulus produced by an agonist A is

$$s_A = e_A y_A$$

$$= e_A \frac{K_A(A)}{1 + K_A(A)} \text{ [from equation (3)]} \quad .. \quad .. \quad (19a)$$

If the agonist A produces a maximal response from the tissue when $y_A$ is very much less than unity, which corresponds to the case when $K_A(A)$ is also very much less than unity (see equation 3), then equation (19a) reduces to

$$s_A = e_A K_A(A) \quad .. \quad .. \quad .. \quad (19b)$$

and this equation is then approximately valid for all values of (A) which produce responses between zero and the maximal response. In the case of a partial agonist P, which cannot produce the maximal response of the tissue, the stimulus is

$$s_P = e_P y_P \quad .. \quad .. \quad .. \quad .. \quad (20a)$$

where

$$y_P = \frac{K_P(P)}{1 + K_P(P)} \text{ [from equation (3)]} \quad .. \quad .. \quad (20b)$$

Suppose that a concentration $(A)_1$ of agonist, and a concentration (P) of partial agonist each produce the same response $r_1$, when applied separately to the same piece of tissue. Then provided that the stimulus-response relationship is constant, these concentrations of drugs also produce equal stimuli, so that from equations (19b) and (20a),

$$e_A K_A (A)_1 = e_P y_P \quad .. \quad .. \quad .. \quad (21)$$

Suppose also that a concentration $(A)_2$ of agonist produces a response $r_2$ which can also be produced by concentrations $(A)_3$ of agonist and (P) of partial agonist acting together. Then the corresponding stimuli are equal, so that

$$e_A K_A (A)_2 = e_A K_A (A)_3 [1 - y_P] + e_P y_P$$

[It was assumed at this stage that $y_A$ is negligible compared with $y_P$.] Then substituting equation (21) into the above equation,

$$e_A K_A (A)_2 = e_A K_A (A)_3 [1 - y_P] + e_A K_A (A)_1$$

It follows that

$$(A)_2 = (A)_3 [1 - y_P] + (A)_1$$

Then

$$y_P = \frac{(A)_3 - (A)_2 + (A)_1}{(A)_3} \quad .. \quad .. \quad .. \quad (22)$$

and so the value of $y_P$, which corresponds to (P), can be calculated from the experimentally observed values of $(A)_1$, $(A)_2$ and $(A)_3$. The value of $K_P$ is then estimated from the equation

$$K_P = \frac{y_P}{1 - y_P} \cdot \frac{1}{(P)} \quad .. \quad .. \quad .. \quad (23)$$

which is obtained by rearrangement of equation (20b).

Let the efficacy of a partial agonist which can produce a maximal response equal to one half of the maximal response of the tissue, be set equal to unity. When such a partial agonist elicits its maximal response then all of the receptors are occupied, and y is equal to unity. The stimulus which corresponds to this response is then

$$s = ey = 1.$$

Then for any other drug acting on the same receptors,

$$e = \frac{s}{y} = \frac{1}{y_{50}} \quad .. \quad .. \quad .. \quad .. \quad (24)$$

where $y_{50}$ is the fraction of the receptors occupied by the drug when it produces unit stimulus, which in turn produces a response equal to 50% of the maximal response of the tissue. In order to calculate $y_{50}$ directly from equation (3), it is necessary to know both the affinity constant of the agonist for the receptor and the concentration of the agonist which produces 50% of the maximal response of the tissue. The latter is readily obtained from the dose-response curve.

Stephenson was able to produce a solution to this problem by obtaining values of the affinity constants of partial agonists using equation (23).

Since the group of compounds which he studied comprised a homologous series, he was able to estimate the affinity constants of full agonists for the receptors by extrapolating the values which he obtained for those members of the series which were partial agonists. In this way he obtained estimates of the efficacies of both agonists and partial agonists, from equation (24).

## 7. THE STIMULUS-RESPONSE RELATIONSHIP

In applying the null method the aim is to eliminate assumptions about the relationship between stimulus and response. This is only partly achieved in the method described above, because the form of the stimulus-response relationship is partly determined by Stephenson's simplifying assumptions.

The concentration of any agonist A which produces the response r can be read from its dose-response curve and if $K_A$ is known then $y_A$ can be calculated from equation (3). If the value of $e_A$ is also available, then the stimulus which produces the response r can be obtained from equation (18), or from the related equation (19a). This calculation can be repeated for several values of r and so the stimulus-response relationship can be plotted. The type of stimulus-response curve obtained by Stephenson is shown in Fig. 2. Its validity depends on the validity of the estimated values of e and K.

## 8. COMPARISON OF INTRINSIC ACTIVITY AND EFFICACY

Although intrinsic activity and efficacy are conceptually the same they are quantitatively different, because of the different assumptions made in their calculation. What is even more important, from the point of view of structure-activity relationships, is the fact that these two approaches give different values of the affinity constants of agonists for the receptors. If the response is proportional to the fraction of receptors occupied by the agonist (as was assumed initially by Ariëns) then it follows that the response would be proportional to the stimulus. The differences in the mathematical treatments of Ariëns and Stephenson can therefore be summarised as differences in the assumed forms of the stimulus-response relationships, as shown in Fig. 2. According to Stephenson, an agonist which produces a maximal response equal to 50% of the maximal response of the tissue then produces unit stimulus and has an efficacy of unity. Since the assumed stimulus-response relationships are very similar below unit stimulus (see Fig. 2) it follows that an efficacy of 1·0 or less corresponds to an intrinsic activity approximately equal to one half of the efficacy. However, for compounds with intrinsic activities greater than 0·5, the discrepancy between the intrinsic activity (calculated in its simplest form) and the efficacy increases rapidly and is large for compounds which produce the maximal response of the tissue.

The assumption that the response is directly proportional to the fraction of receptors occupied by the agonist automatically means that all agonists producing the maximum response of the tissue should have the same intrinsic activity. On the other hand, Stephenson's assumptions would

allow drugs with different efficacies to produce the maximum response of the tissue, by occupying different fractions of receptors. The fraction of receptors occupied could be increased still further by increasing the concentration of agonist until all the receptors were saturated, but, because of the shape of the stimulus-response curve suggested by Stephenson (see Fig. 2), the increased stimulus would produce very little change in the response.

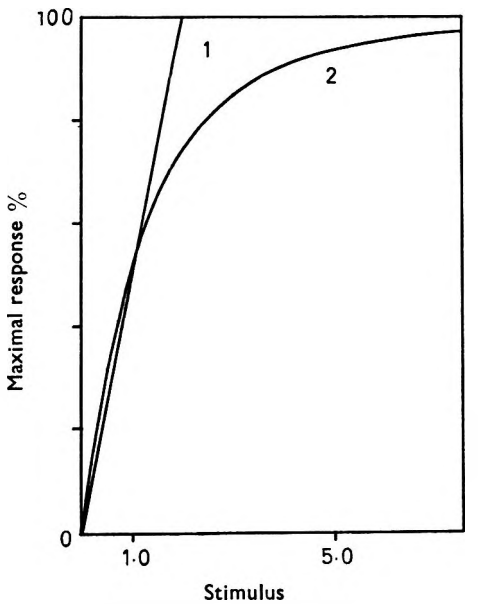


FIG. 2. The stimulus-response relationship. 1. As assumed by Ariëns. 2. As derived by Stephenson (1956) on the basis of his simplifying assumptions.

## 9. FURCHGOTT'S METHOD FOR THE DETERMINATION OF THE AFFINITY CONSTANTS OF AGONISTS FOR RECEPTORS

According to the ideas of Ariëns and Stephenson, agonist-receptor interactions are characterised by two parameters, the affinity constant and the intrinsic activity or efficacy. Furchgott (1965) suggested that the hybrid term *intrinsic efficacy* might be used for the second parameter. Since the terms intrinsic activity and efficacy are associated with certain assumptions which are not necessarily correct, or which may be correct only in certain cases, it seems advisable to use this hybrid term to describe the parameter itself, as distinct from any experimental estimate of the parameter.

If the affinity constants of agonists for their receptors could be calculated by some dependable method, relative values of their intrinsic efficacies could be obtained from a knowledge of equi-effective concentrations. The symbol f will be used here to denote intrinsic efficacy, although Furchgott uses the symbol $\epsilon$. Suppose that concentrations (A) of drug A and

(B) of drug B, each acting alone, produce the same response r. Then the respective stimuli are $s_A$ and $s_B$, where

$$s_A = f_A y_A \{R\}_T \quad .. \quad .. \quad .. \quad (25)$$

and

$$s_B = f_B y_B \{R\}_T \quad .. \quad .. \quad .. \quad (26)$$

(It may be noted that the stimulus as used in this and in subsequent sections differs slightly from Stephenson's earlier definition. The stimulus is now re-defined as the product of the intrinsic efficacy and the concentration of drug-receptor complex.) Since the stimuli $s_A$ and $s_B$ produce the same response they are assumed to be equal, so that equations (25) and (26) give

$$\frac{f_A}{f_B} = \frac{y_B}{y_A} \quad .. \quad .. \quad .. \quad .. \quad (27)$$

If $K_A$, $K_B$, (A) and (B) are known, then the values of $y_A$ and $y_B$ can be calculated from equation (3) and so the ratio of the intrinsic efficacies of the drugs A and B can be estimated from equation (27).

Furchgott (1965) suggested a method for calculating the affinity constants of agonists, based on the use of irreversible antagonists. These antagonists are assumed to react with the receptors in such a way as to inactivate them for a period of time which is long compared with the duration of the experiment. This is conducted in the following manner. First the log dose-response curve of an agonist A is determined for a piece of tissue. The tissue is then incubated with an irreversible antagonist for a period of time. Excess antagonist is then washed out of the tissue, and the log dose-response curve is re-determined for the treated tissue. The irreversible antagonist will have blocked some of the receptors, so that the log dose-response curve will be altered. Treatment with the irreversible antagonist is then repeated, log dose-response curves being determined after each such treatment. The type of results obtained is shown in Figs 3a and 3b.

These observations can be explained by the form of stimulus-response relationship suggested by Stephenson (1956). At very high concentrations of agonist, $y_A$ tends to unity. The maximum stimulus which the agonist can produce is therefore $[s_A]_{max}$ where

$$[s_A]_{max} = f_A \{R\}_T \text{ [from equation (25)]} \quad .. \quad .. \quad (28)$$

As the value of $\{R\}_T$ decreases, due to reaction of receptors with the irreversible antagonists, so the value of $[s_A]_{max}$ decreases. Ultimately it reaches a value such that it can no longer produce the maximal response of the tissue. The value of $\{R_T\}$ at which this occurs depends on the value of $f_A$, since it is the stimulus $[s_A]_{max}$ which then determines the response. The greater the value of $f_A$ the smaller must be $\{R\}_T$ and hence the longer must be the time of incubation of the tissue with the irreversible antagonist before the maximum response is reduced. It follows that with an agonist of high intrinsic efficacy, repeated treatment of the tissue with an irreversible antagonist causes the log dose-response curve to be shifted almost parallel to itself, before producing a reduction in the maximal response. On the other hand, if A is a partial agonist then the

maximal response is reduced immediately by treatment with an irreversible antagonist (see Figs. 3a and 3b).

Suppose that the stimulus response relationship can be written in the form

$$s = ar + br^2 + cr^3 + \ldots, \qquad \ldots \quad \ldots \quad (29a)$$

where s is the stimulus, r is the response and $a$, $b$, $c$, . . . are constants.

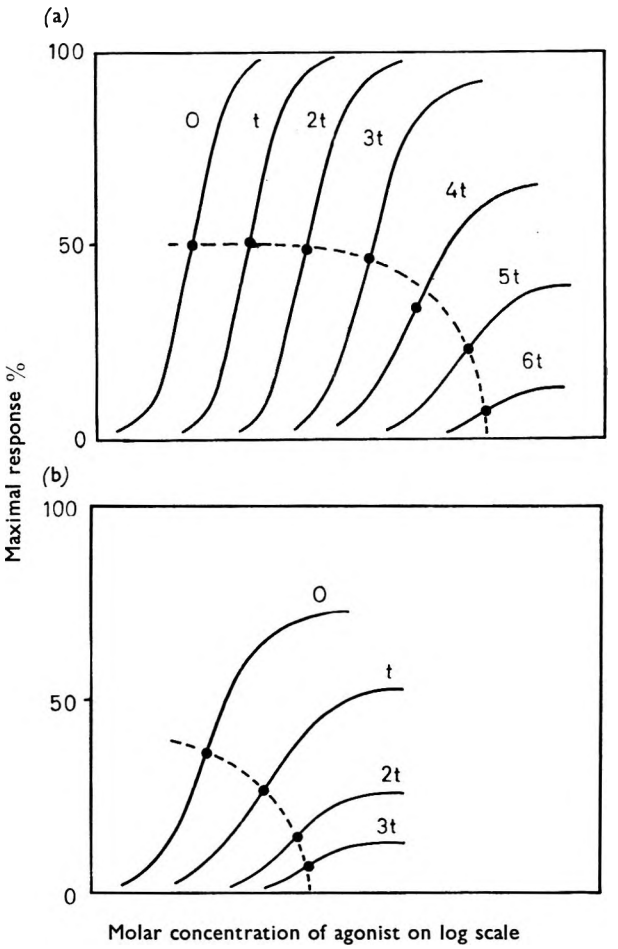


FIG. 3. Dose-response curves measured on a tissue which has been repeatedly treated with an irreversible antagonist. The standard time of incubation with the irreversible antagonist is taken as t. The total times of incubation, which apply to each dose-response curve, are indicated by the multiples of t. The results in Fig. 3a suggest that this agonist produces a maximal response when it occupies only a fraction of the total number of available receptors. The results in Fig. 3b suggest that in the case of a partial agonist a maximal response is produced only when all of the receptors are occupied. In both figures the filled circles indicate $(A)_{50}$ values. The dotted curve indicates the method of obtaining limiting values of $(A)_{50}$, at very small maximal responses.

Equation (29a) could be fitted to a large number of possible stimulus-response relationships. Provided that the constant $a$ is not equal to zero then the limiting form of equation (29a), at very small values of r, is

$$s = ar \quad .. \quad .. \quad .. \quad .. \quad (29b)$$

This indicates that at sufficiently small values of the response, its value is directly proportional to the stimulus. If the entire dose-response curve of the agonist falls within the range of r for which equation (29b) applies, then Ariëns' approximations are valid [see section 8, Fig. 2, and equation (17)] and the affinity constant of the agonist for its receptor can be calculated from the corresponding value of $(A)_{50}$ by use of equation (17). As the irreversible antagonist inactivates the receptors it reduces the maximum stimulus which the agonist A can produce, and hence reduces the maximal response until finally equation (29b) may become valid. The value of $(A)_{50}$ to be used in the calculation of $K_A$ is the limiting value as $[r_A]_{max}$ tends to zero, and is obtained by extrapolation as shown in Figs 3a and 3b. It is assumed that the irreversible antagonist merely inactivates the receptors and does not alter the stimulus-response relationship.

## 10. A GENERAL METHOD FOR THE ANALYSIS OF DRUG-RECEPTOR INTERACTIONS

### (i) *Application to Reversible Interactions*

As already pointed out, the main difficulty in analysing the interactions of drugs with receptors is the lack of knowledge of the stimulus-response relationship. This difficulty was overcome, in the case of drug antagonism, by applying the null method. Mackay (1965a,b) suggested that a simple mathematical transformation should be applied to dose-response curves to obtain useful information about the interaction of receptors with antagonists, partial agonists, and full agonists.

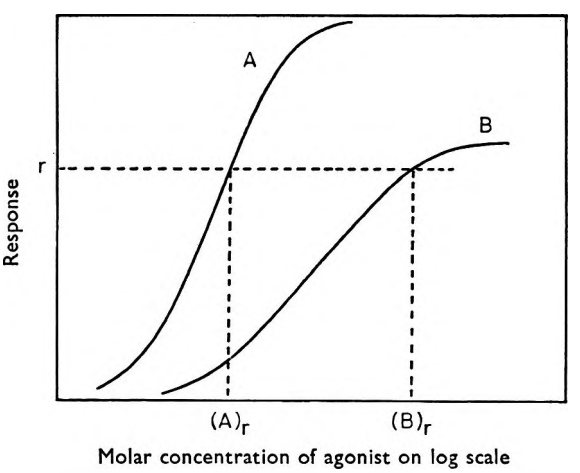


FIG. 4. Comparison of the dose-response curves of two agonists, A and B, measured on the same cell or tissue. $(A)_r$ and $(B)_r$ are the concentrations of the agonists which produce the chosen response r.

Suppose that the log dose-response curves shown in Fig. 4 have been obtained for two agonists A and B acting on the same cell or tissue. If a response r is chosen, then the corresponding pharmacological stimulus produced by the agonist A is given by equation (25). The stimulus $s_A$ is then that producing the response r, and may therefore be written as $[s_A]_r$. The term $y_A$ in equation (25) then also has a definite value, written as $[y_A]_r$, which in turn corresponds to a definite value of the concentration of agonist A, written as $(A)_r$. Thus equation (25) takes the general form

$$[s_A]_r = f_A[y_A]_r\{R\}_T \quad .. \quad .. \quad .. \quad (30)$$

which applies to any chosen response r. Similarly for the agonist B acting on the same cell or tissue,

$$[s_B]_r = f_B[y_B]_r\{R\}_T \quad .. \quad .. \quad .. \quad (31)$$

From equation (3),

$$[y_A]_r = \frac{1}{1 + \dfrac{1}{K_A(A)_r}} \quad .. \quad .. \quad .. \quad (32)$$

and

$$[y_B]_r = \frac{1}{1 - \dfrac{1}{K_B(B)_r}} \quad .. \quad .. \quad .. \quad (33)$$

If $(A)_r$ and $(B)_r$ are the concentrations of agonists A and B which produce the same response r, then they also produce equal stimuli. This is an application of the null method, and involves the assumption that the stimulus-response relationship has remained unchanged during the determination of the dose-response curves. Then from equations (30) and (31)

$$\beta_{AB} = \frac{f_A}{f_B} = \frac{[y_B]_r}{[y_A]_r} \quad .. \quad .. \quad .. \quad (34)$$

where $\beta_{AB}$ is the ratio of the intrinsic efficacy of drug A to that of drug B. Substituting equations (32) and (33) into equation (34) and rearranging gives

$$\frac{1}{(A)_r} = \frac{K_A}{K_B}\beta_{AB}\frac{1}{(B)_r} + K_A[\beta_{AB} - 1] \quad .. \quad .. \quad (35)$$

This equation indicates that if $1/(A)_r$ is plotted against $1/(B)_r$ then a straight line should be obtained of slope $\psi_{AB}$ and intercept $I_{AB}$

$$\text{where } \psi_{AB} = K_A\beta_{AB}/K_B \quad .. \quad .. \quad .. \quad (36)$$

$$\text{and } I_{AB} = K_A[\beta_{AB} - 1] \quad .. \quad .. \quad (37)$$

The values of $(A)_r$ and $(B)_r$ which produce the response r, are simply read from the log dose-response curves, as shown in Fig. 4. $1/(A)_r$ is then plotted against $1/(B)_r$ for a series of chosen values of r. This method therefore makes maximum use of the information available in the log dose-response curves.

If $I_{AB}$ is positive then $\beta_{AB}$ must be greater than one and so $f_A$ must be greater than $f_B$. The sign of $I_{AB}$ can therefore be used to decide which of the agonists A and B has the greater intrinsic efficacy. The experimental constants $\psi_{AB}$ and $I_{AB}$ are related to the fundamental parameters $K_A$, $K_B$ and $\beta_{AB}$. (Only relative values of the intrinsic efficacies can be determined.) However, there are only two equations, (36) and (37), containing these three unknowns. The individual parameters of the agonist-receptor interactions therefore cannot be determined from these equations. In the more general case of N agonists, all of which act on the same type of receptor, comparison of the log dose-response curves gives $(N - 1)$ independent values of $\psi$ and $(N - 1)$ independent values of I. The order of the intrinsic efficacies can be determined from the signs of the values of I. The N agonists will have N unknown values of K and $(N-1)$ unknown values of $\beta$. There will therefore be $(2N - 2)$ independent equations with $(2N - 1)$ unknown parameters. It follows that even if all the experimental constants can be accurately determined, the values of the fundamental parameters cannot be obtained from such data alone. In fact, an infinitely large number of sets of fundamental parameters can be made to fit any given group of dose-response curves (Mackay, 1965b). The fundamental parameters of the series of agonists could however, be estimated from the values of $\psi$ and I, provided that *one* of the following conditions is valid.

(1) The ratio of the intrinsic efficacy of one of the agonists to that of another (partial) agonist, is very much greater than unity. The values of $\beta$, obtained on the basis of this assumption, are then similar to Stephenson's efficacies (Mackay, 1965b).

(2) The affinity constant of one of the agonists is known.

(3) Another independent equation is available which relates the fundamental parameters.

Ideally, the values of the experimental constants $\psi_{AB}$ and $I_{AB}$ should be obtained from the log dose-response curves of the agonists, measured on the same piece of tissue with the same recording system. The values of the experimental constants should not depend on the method of recording the response (since the null method is employed) provided that $K_A$, $K_B$ and $\beta_{AB}$ do not themselves depend on the recording method.

The method of analysis described above can also be applied to other drug-receptor systems. If the log dose-response curve of an agonist, acting on a cell or tissue, is determined first in the absence and then in the presence of a competitive antagonist, it can be shown that the appropriate equation for the comparison of the dose-response curves is

$$\frac{1}{(A)_r} = \frac{1}{(A)'_r}[1 + K_B(B)] \quad .. \quad .. \quad .. \quad (38)$$

where $(A)_r$ is the concentration of the agonist which produces the response r in the absence of the antagonist, and $(A)'_r$ is the concentration which produces the response r in the presence of a constant concentration (B) of the competitive antagonist. [If this equation is compared

with equation (9) it will be seen that they are equivalent.] A plot of $1/(A)_r$ against $1/(A)'_r$ should give a straight line of slope $[1 + K_B(B)]$ passing through the origin, and so $K_B$ can be estimated from the slope.

It can also be shown, by application of this method, that if B is a non-competitive antagonist then the appropriate equation, assuming bimolecular drug-receptor interactions, is

$$\frac{1}{(A)_r} = \frac{1}{(A)'_r}[1 + K_B(B)] + K_A K_B(B) \quad .. \quad .. \quad (39)$$

Once again, a plot of $1/(A)_r$ against $1/(A)'_r$ should give a straight line. In this case the slope is $[1 + K_B(B)]$ and the intercept is $[K_A K_B(B)]$. The values of $K_B$ and of $K_A$ can therefore be calculated. The term *non-competitive* is used here in the enzymic sense. The antagonist is supposed to be adsorbed close to the adsorption site for the agonist, without interfering with the adsorption of the agonist. However, the presence of the adsorbed non-competitive antagonist is assumed to block the stimulus which normally results from the agonist-receptor interaction.

Mackay (1965d) also suggested a method for the determination of affinity constants and relative intrinsic efficacies of agonists, based on the kinetics of action of specific irreversible antagonists. However, this method has not been used, since it is more difficult to apply and theoretically less satisfactory than Stephenson's new method which is described in the next section.

(ii) *Application to Irreversible Antagonism*

Stephenson (1965) pointed out that dose-response curves obtained before and after treatment of a tissue with a specific irreversible antagonist, can be compared as described in the previous section, so as to obtain the affinity constant of an agonist for its receptors.

Suppose that the log dose-response curve of an agonist A, acting on a single cell or tissue, is determined, and that the tissue is then incubated with an irreversible antagonist B. The incubation may be continued until the maximum response which the agonist can produce on the treated tissue, after washing out the excess antagonist, is definitely reduced. The log dose-response curve is then re-determined on the treated tissue. Let the concentration of receptors, before treatment of the tissue with the antagonist, be $\{R\}_T$, and the concentration after treatment $\{R\}'_T$. Then

$$\{R\}'_T = \{R\}_T [1 - y_B] \quad .. \quad .. \quad .. \quad (40)$$

where $y_B$ is the fraction of the receptors inactivated by the irreversible antagonist. If $(A)_r$ is the concentration of agonist which produces the response r from the untreated tissue then the corresponding stimulus is

$$[s_A]_r = f_A[y_A]_r\{R\}_T$$

$$= \frac{f_A\{R\}_T}{1 + \dfrac{1}{K_A(A)_r}} \quad \text{[from equation (3)]}$$

If $(A)'_r$ is the concentration of agonist which produces the same response r from the treated tissue then the corresponding stimulus is written as

$$[s_A]'_r = f_A[y_A]'_r\{R\}'_T$$
$$= \frac{f_A\{R\}_T[1 - y_B]}{1 + \dfrac{1}{K_A(A)'_r}} \text{ [from equations (3) and (40)].}$$

Provided that the stimulus-response relationship has not been altered by the action of the irreversible antagonist then the response r will have been produced by equal stimuli, so that

$$[s_A]_r = [s_A]'_r.$$

It follows that

$$\frac{1}{1 - \dfrac{1}{K_A(A)_r}} = \frac{[1 - y_B]}{1 + \dfrac{1}{K_A(A)'_r}}$$

Rearrangement of this equation gives

$$\frac{1}{(A)_r} = \frac{1}{[1 - y_B]} \cdot \frac{1}{(A)'_r} + K_A \frac{y_B}{[1 - y_B]} \quad \ldots \quad (41)$$

Therefore a plot of $1/(A)_r$ against $1/(A)'_r$ gives a straight line. In this case the slope is $1/[1 - y]_B$ and the intercept is $K_A y_B/[1 - y_B]$. Then $K_A$ can be calculated from the equation,

$$K_A = \frac{\text{intercept}}{[\text{slope} - 1]} \quad \ldots \quad \ldots \quad \ldots \quad (42)$$

This equation can also be applied to dose-response curves obtained in the presence of pseudo-irreversible and non-competitive antagonists, as discussed in detail elsewhere (Mackay, 1965c). Equations (41) and (42) have been derived on the assumption that the dose-response curves to be compared are those measured before and after treatment of the tissue with an irreversible antagonist. However, these equations apply equally well to comparisons of two dose-response curves obtained after two different periods of incubation of the tissue with the irreversible antagonist.

The validity of the equations derived in section 10 depends almost entirely on the assumption that the stimulus-response relationship does not change while the dose-response curves are being determined. This is a basic assumption of the null method. If this assumption is valid then the application of equations (41) and (42) may give good estimates of the affinity constants of agonists for their receptors. If the value of the affinity constant of each agonist is determined in this way, together with the equi-effective concentrations, then the relative intrinsic efficacies of the agonists can be estimated from equation (27) (see section 9).

However, it was pointed out in section 10 (i) that if accurate experimental estimates of $\psi_{AB}$ and $I_{AB}$ are available for a series of agonists then knowledge of the value of one of the affinity constants would enable all the other fundamental parameters to be calculated. Therefore, if all of the values of the affinity constants are determined by use of equations

(41) and (42), and the value of $\psi$ and I are also determined, there will be a surplus of experimental data and cross-checks become possible. Thus, the value of $\beta_{AB}$ can be estimated from equation (36), if $\psi_{AB}$, $K_A$ and $K_B$ are known. A theoretical value of $I_{AB}$ can then be calculated from equation (37), and this can be compared with the observed values. Any serious discrepancy between the calculated and observed values of $I_{AB}$ would throw doubt on the estimated values of the fundamental parameters, since the null method is more likely to be valid in the case of equation (35), than in the case of equation (41).

It must also be emphasised that the equations derived in section 10 ought to be taken as applying to graded responses measured on single cells, since there are the responding units. In certain circumstances these equations can also be applied to multicellular tissues (Mackay, 1965b).

### EXPERIMENTAL CONSIDERATIONS

The equations discussed in this review should strictly be applied only to measurements made on tissues which contain cells capable of producing graded responses. In applying these equations it is also assumed that the response which is measured is produced by the cell on which the drug-receptor interaction occurs and that the response is a result of the interaction of the drug with only one type of receptor.

The values of the concentrations of drugs which have to be inserted in the various equations are strictly the concentrations close to the receptors at equilibrium. However, these concentrations are usually assumed to be the same as those present in the bathing solution before it was applied to the tissue. This approximation is satisfactory only if the receptors are on the surface of the cells and if the amount of drug adsorbed by the tissue is not sufficient to produce any significant change in the concentration of the drug in the solution. This can usually be ensured, if necessary, by using a small piece of tissue and a large volume of bathing solution. Similar problems arise if the drug is metabolised or absorbed by the tissue, but these are not so readily solved. In some instances it may be possible to block the metabolism or uptake of the drug.

In section 10 it has been stated that the dose-response curves which are to be compared should be measured on a single piece of tissue, or on a single cell. This is because the validity of the equations derived in that section usually depends on the constancy of the stimulus-response relationship (and sometimes also of $\{R\}_T$) during the determination of the dose-response curves. However, *all* the dose-response curves of a group of agonists cannot be measured on a single piece of tissue, and in any case the stimulus-response relationship may vary with time. Such practical difficulties can be overcome to a large extent by carrying out experiments in such a way that one agonist is repeatedly used as a reference compound.

## General discussion and conclusion

It has already been emphasised that in analysing drug-receptor interactions no assumptions should be made about the form of the relationship

between the response and the fraction of receptors occupied by the agonist. This means that those methods of analysis discussed in sections 4 and 10, which are based only on the null method and the law of mass action, are the most likely to give dependable values for the fundamental parameters of drug-receptor interactions. However, such results must also be considered cautiously since these methods depend implicitly on the validity of two basic assumptions. The first assumption is that when drugs interact with receptors to produce an equilibrium or steady state concentration of drug-receptor complex then this corresponds to a steady response. The second assumption is that a definite stimulus-response relationship exists for any given piece of tissue. The exact validity of these assumptions can be questioned.

Experimentally, steady responses are seldom seen. Instead, the response to an agonist usually reaches a maximum and subsequently declines, sometimes to zero, even though the agonist is still present. Such observations were partly responsible for the introduction of alternative forms of receptor theory, such as the *rate theory* proposed by Paton (1961) and the *flux-carrier hypothesis* suggested by Mackay (1963). All of the mathematical treatments discussed here have been derived on the basis of *occupation theory*. The equations derived in sections 4 and 10 could also be applied to the alternative forms of receptor theory, provided that the appropriate responses are measured from the response-time curves. These appropriate responses are respectively the steady-state plateau response in the case of rate theory, and the maximal response in the case of the flux-carrier hypothesis.

Although the alternative forms of receptor theory require investigation and evaluation, there seems to be no strong reason for discarding occupation theory at the present time. The fact that the variation of a response with time does not follow the pattern predicted by occupation theory, does not necessarily mean that the response is not due to simple occupation of the receptors. The complicated forms of the response-time curves may be due to secondary effects. In such circumstances the methods of analysis discussed here can be applied, provided that the maximum response corresponds to equilibrium occupation of the receptors.

It seems likely that any increased permeability of the cell membrane would cause a greater influx of sodium ions. The ionic composition of the intracellular fluid is maintained by one or more "pump" systems. The properties of the sodium pump and of its adenosine-triphosphatase, have recently been reviewed by Skou (1965). An increased influx of sodium ions would be expected to stimulate the sodium pump and so tend to annul the effect of any increased permeability. The initial change in membrane permeability might be brought about by the interaction of an agonist with its receptors, and the sodium pump would provide a negative feedback. Many interesting pharmacological phenomena, which cannot be explained on the basis of simple occupation theory alone, could be explained by such a "feedback" model. If such a time-dependent feedback process does in fact occur, then the idea of a definite stimulus-response relationship can be only an approximation to the truth. The

feedback model is still highly speculative, but such biochemical considerations are likely to be of some importance, regardless of whether an agonist produces its primary action by simply occupying the receptors, or by the mechanisms suggested by the rate theory or the flux-carrier hypothesis.

It may therefore be concluded that, of the various methods which have been considered for analysing drug-receptor interactions, those which depend solely on the application of the null method and on the law of mass action, are probably the best available at the present time. Studies of response-time relationships may lead to new concepts which in turn may require further modification of receptor theory.

## Summary

The law of mass action can be applied to the interaction of a drug with a receptor, but the relationship between the response and the fraction of receptors, $y_A$, occupied by the agonist, is not known. In fact, it seems unlikely that the relationship between the response and $y_A$ would be the same for all the different types of response which might be measured.

The first quantitative treatment of receptor theory, set out by Clark (1933c), separated drugs into two groups which were the agonists and the antagonists. In the case of drug antagonism, techniques were developed (Gaddum, 1937; Clark & Raventos, 1937; Schild, 1947) which enabled the affinity constants of competitive antagonists to be estimated without making any assumptions about the relationship between $y_A$ and the response. This technique was based on the comparison of equal responses and was therefore called a null method.

The discovery of partial agonists led to the introduction of the terms *intrinsic activity* (Ariëns, 1954) and *efficacy* (Stephenson, 1956), which are conceptually the same but which are quantitatively different, especially in the case of agonists which elicit the maximal response of the tissue. The terms intrinsic activity and efficacy both imply that the complexes between the receptor and various agonists may differ in their ability to contribute to a response. Whereas Ariëns assumed that all of the receptors have to be occupied in order to produce a maximal response from the tissue, Stephenson assumed that some agonists can produce maximal responses when they occupy only a small fraction of the receptors. Stephenson also drew a clear-cut distinction between receptor occupation and the response, by introducing the term stimulus.

According to the ideas of Ariëns and of Stephenson, any agonist-receptor interaction can be characterised by two parameters, the affinity constant and the intrinsic activity or efficacy. Furchgott (1965) introduced the hybrid term *intrinsic efficacy* for the latter parameter. His method for the determination of the affinity constants of agonists depends on the use of irreversible antagonists. The assumption made by Furchgott, in order to estimate these affinity constants, is that when the maximal response of the tissue to a fully active agonist is made vanishingly small then the stimulus is proportional to the response. This assumption is in some way less restrictive than Stephenson's earlier assumptions. When

the affinity constants of the agonists for the receptors have been obtained, then their relative intrinsic efficacies can be estimated from their equieffective concentrations, assuming the null method to be valid.

Mackay (1965a,b) introduced a mathematical transformation of dose-response curves, which allowed the null method to be applied to the analysis of the interactions of receptors with competitive and non-competitive antagonists, partial agonists, and fully active agonists. He showed that in the case of partial agonists and fully active agonists, experimental constants could be obtained by comparing simple dose-response curves measured on a single piece of tissue. These experimental constants are related to the fundamental parameters of the agonist-receptor interactions, but can be broken down into the constituent parameters only under certain circumstances.

Stephenson (1965) pointed out that the same basic principles could be applied to obtain the affinity constants of agonists for their receptors, by comparing the dose-response curves of the agonist measured on a piece of tissue before and after it had been treated with an irreversible antagonist.

It is obvious that the most dependable techniques for analysing drug-receptor interactions are those which involve the smallest number of doubtful assumptions. The methods discussed in sections 4 and 10 are therefore recommended, since they depend only on the validity of the null method and the law of mass action. Application of these methods, assuming that the maximal response corresponds to equilibrium occupation of the receptors, has so far given satisfactory results. Nevertheless, the response-time relationship requires more detailed investigation and such studies may lead to further modification of receptor theory.

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*J. Pharm. Pharmac.*, 1966, **18**, 223–227 Received January 12, 1966

# The polarographic determination of chlorpromazine and chlorpromazine sulphoxide

G. S. PORTER AND JEAN BERESFORD

Cathodic polarography of solutions containing between 0·1 and 5·0 μg/ml of both chlorpromazine and its sulphoxide enables the sulphoxide to be determined directly. Reductive polarography of the solution after bromination gives an additive wave from which the chlorpromazine is determined by subtraction. The method has been applied to urine, interfering substances being removed by ion-exchange on Amberlite resins.

CHLORPROMAZINE, although not reduced at the dropping mercury cathode, can be determined by reductive polarography after bromination (Porter, 1964). This method has been adapted to the determination of chlorpromazine and its detoxication product chlorpromazine sulphoxide when present together in solution. The sulphoxide is directly reducible and neither bromination nor the presence of chlorpromazine quantitatively affects its calibration graph.

Chlorpromazine and chlorpromazine sulphoxide, on bromination under the conditions described, each give a reducible derivative. The two bromination derivatives are polarographically and chromatographically indistinguishable and have identical ultraviolet spectra. In mixtures, therefore, the sulphoxide is reducible before bromination; after bromination an additive wave is obtained from which the chlorpromazine concentration is obtained by subtraction of the sulphoxide wave.

Determinations were made on urine containing low concentrations of added chlorpromazine and sulphoxide, and on urine from volunteers who had taken chlorpromazine orally. Quantitative differentiation was possible between reducible and non-reducible excreted forms of the drug.

## Experimental and results

### APPARATUS

A Southern Analytical K 1000 polarograph was used. Determinations were made at 25° using a mercury pool anode, a start potential of −0·5 V and the derivative circuit. All peak current readings were expressed in graticule units taken at, or corrected to, an instrument sensitivity of 0·004.

### CALIBRATION GRAPHS

*Chlorpromazine sulphoxide.* Twenty-five ml quantities of solutions containing up to 5 μg/ml of base were prepared in 0·5N hydrochloric acid. One ml amounts of these solutions were reductively polarographed after flushing with nitrogen (3 min), and a peak current-concentration graph constructed.

From the Pharmaceutical Chemistry Laboratories, Department of Pharmacy, Liverpool Regional College of Technology, New Building, Byrom Street, Liverpool.

*Chlorpromazine.* Solutions of the base were prepared as for the sulphoxide. Each 25 ml quantity was treated with two drops of saturated bromine water, shaken, stood for 1 min and reductively polarographed after flushing with nitrogen (3 min). The peak current-concentration graph was then plotted.

*Brominated sulphoxide.* Solutions of sulphoxide when brominated as described for chlorpromazine gave a calibration graph identical with that of the unbrominated sulphoxide.

*Peak voltages.* Untreated sulphoxide, brominated sulphoxide and brominated chlorpromazine all gave cathodic waves whose peak voltages were about −0·75 V.

### ANALYSIS OF SOLUTIONS CONTAINING BOTH CHLORPROMAZINE AND CHLORPROMAZINE SULPHOXIDE

Twenty-five ml quantities of solutions containing 4 ml of deionised normal urine and up to 5 μg/ml of each base were prepared in 0·5N hydrochloric acid. Each solution was polarographed as described for the preparation of the sulphoxide calibration graph. The peak current was recorded and the sulphoxide content calculated. One drop of bromine water was added to the solution in the cell and the determination completed as for chlorpromazine. The additive peak current was recorded. Subtraction of the sulphoxide peak current from this, and reference to the chlorpromazine calibration graph, gave the chlorpromazine concentration. Results are summarised in Table 1.

TABLE 1. ANALYSIS OF SOLUTIONS CONTAINING CHLORPROMAZINE AND CHLORPROMAZINE SULPHOXIDE

| Substance | No. of determinations | Range of concentrations polarographed (μg/ml) | Standard deviation (μg/ml) |
|---|---|---|---|
| Chlorpromazine .. .. | 13 | 0·1 to 0·5 | 0·030 |
| | 25 | 0·5 to 1·0 | 0·041 |
| | 40 | 1·0 to 3·0 | 0·099 |
| | 8 | 3·0 to 5·0 | 0·115 |
| Chlorpromazine sulphoxide .. | 17 | 0·5 to 1·0 | 0·042 |
| | 33 | 1·0 to 3·0 | 0·105 |
| | 36 | 3·0 to 5·0 | 0·114 |

*Application to urine.* Varying amounts of chlorpromazine and its sulphoxide were added to normal urine samples to give concentrations of up to 125 μg/ml of each base. Urine (up to 4 ml) accurately measured, was applied to a 10 × 1 cm column of Amberlite resin IRA 400 (Cl) The column was washed with water and the water-white eluate and washings were collected in a 25 ml flask containing 2·5 ml of 5N hydrochloric acid and made up to the mark. A suitable quantity of solution was transferred to a polarographic cell and the determination completed as described.

Results from three determinations are in Table 2. Twelve analyses gave recoveries between 91 and 106% for chlorpromazine and between 90 and 102% for the sulphoxide.

TABLE 2. ANALYSIS OF NORMAL URINE SAMPLES CONTAINING ADDED CHLORPROMAZINE AND CHLORPROMAZINE SULPHOXIDE

| Sample | 1 | 2 | 3 |
|---|---|---|---|
| Volume analysed (ml) | 2·0 | 0·5 | 1·0 |
| Content (μg/ml) chlorpromazine sulphoxide | 35·0 | 65·1 | 21·0 |
| chlorpromazine | 12·8 | 65·1 | 10·5 |
| Peak current before bromination (graticule units) | 3·10 | 1·50 | 0·90 |
| Peak current after bromination (graticule its) | 4·35 | 3·18 | 1·45 |
| Difference | 1·25 | 1·68 | 0·55 |
| Recovery % chlorpromazine sulphoxide | 97 | 101 | 95 |
| chlorpromazine | 96 | 101 | 105 |

*Application to urine containing chlorpromazine metabolites.* 100 mg of chlorpromazine hydrochloride was taken orally by each of two volunteers before retiring. Urine was collected in fractions as voided over the next 24 hr and each fraction was analysed. Results are in Table 3.

TABLE 3. DETERMINATION OF REDUCIBLE AND NON-REDUCIBLE METABOLITES IN URINE AFTER A SINGLE 100 MG DOSE OF CHLORPROMAZINE HYDROCHLORIDE

| Sample | Fraction | Amount excreted (mg) | |
|---|---|---|---|
| | | Reducible metabolites calculated as sulphoxide | Non-reducible metabolites calculated as chlorpromazine |
| 1. | 1 | 2·6 | 3·0 |
| | 2 | 0·4 | 0·6 |
| | 3 | 0·4 | 0·9 |
| | 4 | 0·2 | 1·2 |
| | 5 | 0·2 | 1·0 |
| | 6 | 0·0 | 0·8 |
| | | 3·8 | 7·5 |
| 2. | 1 | 2·7 | 2·1 |
| | 2 | 1·2 | 1·2 |
| | 3 | 0·3 | 0·7 |
| | 4 | 0·2 | 0·5 |
| | 5 | 0·1 | 0·4 |
| | | 4·5 | 4·9 |

DETECTION AND REMOVAL OF INTERFERENCE

In the determinations on normal urine, samples from twenty-three adult male and seven adult female volunteers were examined. Two of the females were pregnant and two provided samples during menstruation.

Four ml quantities were diluted to 25 ml, the solution containing sufficient hydrochloric acid to render it 0·5N. On polarographing, a number of waves of varying amplitude were seen in the range −0·5 to −0·9 V. Bromination produced a further wave in this voltage range.

Deionisation [IRA 400 (Cl)] of urine samples from 29 of the volunteers before preparing the 25 ml of solution resulted in no polarographic wave being seen in the region −0·5 to −0·85 V before or after bromination.

Since after deionisation of the urine samples no interfering wave occurred within 0·1 V of the peak voltage of the phenothiazine compounds

(−0·75 V), interference with the determination of added chlorpromazine or sulphoxide, or both, was neither anticipated nor encountered in these 29 cases.

One sample (J.B.) on deionisation showed a wave at −0·79 V which interfered with the determination of added sulphoxide giving results up to 20% low, while the determination of chlorpromazine was unaffected. The interfering wave could be demonstrated consistently in successive urine samples, varying only in amplitude from time to time.

Using this sample (J.B.), containing added chlorpromazine and sulphoxide, a method adapted from an ion-exchange procedure of Forrest, Wechsler & Sperco (1963) was devised to detect and eliminate interference.

With an approximate knowledge of the total phenothiazine content obtained by the method already described, a definite weight of cation exchange resin was used to remove the phenothiazines from a suitable volume of the sample, these being subsequently eluted with N sodium hydroxide solution and determined.

The full procedure for a sample (J.B.) containing 4·0 μg/ml of chlorpromazine and 10·0 μg/ml of sulphoxide is described below.

Four ml of sample were analysed as described under *Application to urine*. The results gave 8 μg/ml for sulphoxide and 3·8 μg/ml for chlorpromazine. Concurrently with this determination a further 4 ml of sample was passed through two vertical ion-exchange columns in series, the upper one being a 5 × 1 cm column of Amberlite IRC 50 (H) analytical grade and the lower a 10 × 1 cm column of Amberlite IRA 400 (Cl). The eluate and washings were collected in a 25 ml flask containing 2·5 ml of 5N hydrochloric acid and made up to the mark. The polarogram of this solution showed only the interference wave at −0·79 V.

A fresh 4 ml portion of sample was shaken (5 min) with 0·5 g of Amberlite IRC 50 (H) resin in a stoppered cylinder, tapered at one end and having a sintered glass filter fused into the taper. The supernatant liquid was removed and the resin washed with water, liquid and washings being passed through a 10 × 1 cm column of IRA 400 (Cl) resin into a 25 ml flask containing 2·5 ml of 5N hydrochloric acid. No polarographic wave due to sulphoxide or chlorpromazine was seen in this solution either before or after bromination, indicating complete adsorption of the phenothiazines on the IRC 50 (H) resin. Although the interfering wave at −0·79 V was still apparent, the procedure was found to be a more rapid and reliable indication of complete phenothiazine adsorption than use of a colorimetric test.

The IRC 50 (H) resin remaining in the cylinder was shaken (30 min) with 5 ml of N sodium hydroxide solution to extract adsorbed phenothiazines. The residual liquid was filtered into a 25 ml flask containing 3 ml of 5N hydrochloric acid, the resin washed with water and the solution made up to the mark with washings. Polarography of this solution gave results for chlorpromazine and chlorpromazine sulphoxide content in precise agreement with theory.

Working with 0·5 g of resin in each case, and on volumes from 10 ml down to 4 ml, analyses of 12 portions of the urine sample (J.B.) containing

concentrations of added chlorpromazine and sulphoxide between 2·0 μg/ml and 10·0 μg/ml gave recoveries between 95 and 100%.

Determination on the urine (J.B.) after the donor had taken a single oral dose of 50 mg of chlorpromazine hydrochloride gave a total phenothiazine recovery of about 10% in 24 hr.

## Discussion

Each five point calibration graph can be prepared in about 30 min; a single determination on urine in the absence of interference takes 15 min. The longer procedure requires an hour for a single determination and about 2 hr for four concurrent determinations.

Calibrations were unaffected by up to 4 ml of deionised [IRA 400(Cl)] normal urine and by such quantities of sodium chloride as were introduced after eluting the IRC 50 (H) resin with sodium hydroxide.

Both resins were used as supplied but whereas the IRA 400 (Cl) was regenerated with saturated sodium chloride solution it was found advisable to use fresh IRC 50 (H) for each determination. Blank determinations on eluates from both resins showed no polarographic waves from either resin, before or after bromination.

Fels, Kaufman & Karczmar (1958) reported instability of chlorpromazine in N sodium hydroxide solution as evidenced by spectrophotometric determinations. This was not found in trial experiments where chlorpromazine was adsorbed on IRC 50 (H) resin, eluted with N sodium hydroxide solution and polarographed in acid medium.

Since ion-exchange would be expected to account for all soluble chlorpromazine metabolites (Forrest & others, 1963), ether extraction (Flanagan, Lin, Novick, Rondish, Bocher & Van Loon, 1952) followed by polarography will differentiate not only between oxidised and non-oxidised derivatives but also polar and non-polar excreted forms of the drug.

The slope and linearity of the calibration graphs depend on the electrolyte, capillary characteristics and instrument parameters. Variation of the start potential caused much variation in peak height for a given concentration. This effect was noted by R. C. Rooney & D. L. Jones (private communication) and is thought to be caused by some of the current being due to catalytic hydrogen discharge. Determinations throughout, therefore, were made with the start potential constant at −0·5 V.

*Acknowledgments.* We wish to thank Dr. Birchall, Sefton General Hospital, Liverpool, and Dr. E. T. Stok, The Surgery, Greasby, Wirral for obtaining urine samples, also Messrs. S. G. E. Stevens and R. M. J. Ratcliffe of Smith, Kline and French Laboratories for helpful suggestions. Chlorpromazine and chlorpromazine sulphoxide samples were kindly supplied by Messrs. May and Baker Ltd., Dagenham.

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*J. Pharm. Pharmac.*, 1966, **18**, 228–235 Received December 6, 1965

# Inhibition of intestinal absorption by different samples of cetrimide and the homologous alkyl series C10-C20

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The effects of different samples of cetrimide on the intestinal absorption of D-glucose, sodium butyrate and DL-methionine were compared with those of homologous compounds $C_{(10)}$–$C_{(20)}$ of the same alkyl series, by *in vivo* intraluminal perfusion in the mouse, rat and guinea-pig. Two samples of "cetrimide" had different inhibitory activities in the mouse and rat but were equiactive in the guinea-pig in which both samples caused glucose reversal. None of the homologues, $C_{(10)}$–$C_{(20)}$, paralleled the activity of either of the cetrimide samples exactly. It is concluded that cetrimide should be assayed in the rat before its use in studies of absorption and protein binding.

ABOUT five years ago cetrimide (cetyltrimethylammonium bromide) was shown to have a striking inhibitory effect on the intestinal absorption of nutrients in several species (Nissim, 1960a,b; 1961; 1963). Experiments for the elucidation of the mode of action of cetrimide and related compounds and of their interactions with other drugs such as phloridzin have resulted in a new theory of intestinal absorption (Nissim, 1964).

During these investigations the original sample of cetrimide was used up, and it was found that new batches of cetrimide were less active. The change in activity coincided with a change in the strain of mice from C3H to CBA. It was thus necessary to suspend the studies of the mechanism of action of cetrimide and investigate the activities of the various batches of cetrimide in different species. Two of the new batches of cetrimide were found to have activity similar to that of the original cetrimide and were designated sample A. The other batches were all less active and are referred to as sample B. This paper describes the comparative activities of the two samples A and B, thus defined, on the intestinal absorption of glucose, sodium butyrate and methionine in mice, rats and guinea-pigs. A preliminary report of these findings has been published (Hart & Nissim, 1963). It appeared likely that differences in the activities of the samples were caused by admixtures of homologues of cetrimide (then largely $C_{(16)}$) of different chain length. Accordingly, homologues $C_{(10)}$–$C_{(20)}$, of a high degree of purity, were examined for their potency on the absorption of the three nutrients and for their physical properties. The activities of the two samples A and B of cetrimide were compared with those of the pure alkyl homologues.

## Materials and methods

### CETRIMIDE SAMPLES

Sample A. The original cetrimide was obtained from the British Drug Houses Ltd., and the new batches which proved active were obtained from Kodak Ltd. and Judex (from B.D.H.).

From the Department of Pharmacology, Guy's Hospital Medical School, London, S.E.1.

Sample B. The batches found to have a low activity were obtained from the British Drug Houses Ltd. and from Hopkin & Williams Ltd.

All batches were supplied as cetrimide, i.e. cetyltrimethylammonium bromide $C_{(16)}$, except those from Hopkin & Williams which were supplied as 'cetrimide B.P.'

The $C_{(12)}$, $C_{(14)}$ and $C_{(16)}$ homologues were obtained through the special courtesy of Printar Industries Ltd., and the $C_{(10)}$, $C_{(18)}$ and $C_{(20)}$ compounds from Glovers Chemicals Ltd.

### PERFUSION TECHNIQUE

Except for certain details, the same *in vivo* perfusion method (Nissim, 1965) was used in the mouse, rat and guinea-pig. A known volume of 0·9% saline, containing 0·2% D-glucose, sodium butyrate and DL-methionine, was perfused through the lumen of the small intestine for a known time and the final volume measured. The concentration of each of the three nutrients was then determined and the percentage absorption calculated. The activity of the drug was investigated by dissolving it in the perfusion fluid and comparing the percentage absorption of the nutrients with that obtained in control animals. Some control experiments were always made concurrently with those in which drugs were tested. All concentrations are expressed as w/v.

*Mice.* Male C3H and CBA mice of either sex bred at Guy's Hospital Medical School and weighing 18–24 g were separated from their food overnight and anaesthetised with sodium pentobarbitone (Veterinary Nembutal, Abbott Laboratories), 60 mg/kg subcutaneously. The whole small intestine was perfused for $\frac{1}{2}$ hr with 25 ml of the nutrient solution.

*Rats.* Male albino rats (obtained from A. Tuck & Son Ltd. or bred at Guy's), weighing 200–400 g, were separated from their food overnight and anaesthetised with sodium pentobarbitone, 60 mg/kg subcutaneously. The proximal 60 cm of small intestine was perfused for $\frac{1}{2}$ hr with 50 ml of the nutrient solution. In 1 hr perfusion experiments, 50 ml of 0·2% D-glucose alone in 0·9% saline was used.

*Guinea-pigs.* Male and female albino guinea-pigs weighing about 500 g were anaesthetised with sodium pentobarbitone, 45 mg/kg subcutaneously, after a subcutaneous dose of atropine, 1 mg/kg. The whole small intestine was perfused with 100 ml of the required perfusion fluid for $\frac{1}{2}$, 1 or 2 hr. Samples were taken for analysis during the longer experiments, usually at $\frac{1}{2}$, 1 and 2 hr. In some experiments the drug under investigation was dissolved in 0·9% saline and perfused without nutrient to test for leakage of glucose from the blood or, in other words, reversal of glucose absorption. In these experiments the animals were not separated from their food overnight as this procedure lowered the blood sugar in guinea-pigs excessively.

### HISTOLOGY

Segments of rat intestine perfused with control or drug-containing solution were fixed in 10% formol saline. Sections were stained with haematoxylin and eosin.

CHEMICAL ESTIMATIONS

Glucose was estimated either by the method of Haslewood & Strookman (1939), or by a modification of the method of Hoffman (1937), using an autoanalyser. Butyrate was estimated by the method of Smyth & Taylor (1958). Methionine was estimated by a modification of the method of McCarthy & Sullivan (1941). The addition of one drop of 0·1% sodium lauryl sulphate gave readings which were more consistent owing to the elimination of unduly low readings apparently caused by some interfering substance from the intestine. Thus previously published figures for the absorption of methionine should be divided by 1·4 to correspond to recent values. Values quoted from previous papers in the series have been modified in this manner.

## Results

SAMPLES A AND B OF CETRIMIDE

*Mice.* In Table 1 the results obtained with the new batches of cetrimide in CBA mice are compared with those obtained with the original sample of cetrimide, sample A, in C3H mice (Nissim, 1962). The inhibitory effect of the drug on the absorption of glucose was significantly smaller. The change in the strain of mice being studied necessitated the examination of sample B in C3H mice. The results in Table 1 show that the difference

TABLE 1. THE EFFECTS OF THE ORIGINAL CETRIMIDE, SAMPLE A, AND THE NEW BATCHES OF CETRIMIDE, SAMPLE B, ON THE ABSORPTION OF GLUCOSE, BUTYRATE AND METHIONINE IN ½-HR INTRALUMINAL PERFUSION EXPERIMENTS IN CBA AND C3H MICE

| Treatment | n | Percentage absorption | | | P |
|---|---|---|---|---|---|
| | | Glucose | Butyrate | Methionine | |
| *Sample A, male C3H mice* | | | | | |
| Controls | 27 | 35·4 ± 1·1 | 34·0 ± 1·5 | 33·3 ± 1·1 | |
| Cetrimide, $10^{-3}$ | 4 | 3·7 ± 1·5 | 24·9 ± 1·5 | 22·9 ± 1·6 | |
| Cetrimide, $10^{-4}$ | 10 | 50·9 ± 2·2 | 32·9 ± 1·8 | 30·1 ± 1·8 | |
| *Sample B, male and female CBA mice* | | | | | |
| Controls | 20 | 41·3 ± 2·9 | 38·5 ± 2·1 | 28·1 ± 1·4 | <0·1 |
| Cetrimide, $10^{-3}$ | 7 | 23·6 ± 2·0 | 22·3 ± 2·6 | 17·3 ± 2·0 | <0·001 |
| Cetrimide, $10^{-5}$ | 4 | 40·9 ± 4·1 | — | 25·1 ± 2·4 | |
| *Sample B, male C3H mice* | | | | | |
| Controls | 15 | 34·2 ± 1·2 | — | — | |
| Cetrimide, $10^{-3}$ | 4 | 10·2 ± 0·7 | 21·4 ± 3·4 | 18·6 ± 3·1 | <0·01 |

Values are means with standard errors. n = number of experiments. P refers to comparison with glucose values obtained with sample A and C3H male mice.

in activity between the two samples was now far less marked, though still statistically significant with $P < 0{\cdot}01$. Sample variation and strain variation were therefore both involved.

*Rats.* The examination of the new batches of cetrimide, which had shown low activity in mice, was continued in the rat, but before this was done the reliability of the assay was given a further check. Cetrimonium stearate had been examined originally in albino rats bred at Guy's (Nissim, 1960b), and at a concentration of $5 \times 10^{-3}$ had given a reduction of glucose absorption of 37·3%. When the same sample of cetrimonium

stearate was re-examined on the same stock of rats, at a concentration of $5 \times 10^{-3}$, 35·8% inhibition of glucose absorption was obtained, giving $P < 0{\cdot}8 > 0{\cdot}7$ between the two sets of experiments. With this confirmation of the reliability of the assay procedure, the new batches of cetrimide were examined in the rat. In two sets of experiments, with four rats in each set, the absorption of glucose in the presence of the new cetrimide, at a concentration of $10^{-3}$, was 28·8 ± 2·2% and 27·5 ± 5·2% as compared with 41·5 ± 1·0% in the control. These results indicated that the new batches of cetrimide were comparatively inactive in inhibiting the glucose absorption, for the original cetrimide had reduced this to 5·4 ± 2·4%. These batches of cetrimide showing low activity in both mice and rats were designated sample B. Eventually two new batches of cetrimide were obtained which, in initial experiments in rats, showed activity similar to that of the original cetrimide. One batch, from Judex, was obtained through the courtesy of C. B. Taylor who had confirmed our early results by examining cetrimide in an *in vitro* preparation (Taylor, 1963). The second batch was obtained from Kodak Ltd. The inhibitory activity of these two new batches, designated sample A, was compared with the activity of the other batches, grouped together under sample B, at three concentrations in the rat. The results are summarised in Table 2, where

TABLE 2. THE EFFECTS OF THE TWO SAMPLES OF CETRIMIDE ON THE ABSORPTION OF GLUCOSE, BUTYRATE AND METHIONINE IN ½-HR INTRALUMINAL INTESTINAL PERFUSION EXPERIMENTS IN MALE ALBINO RATS

| | Absorption % | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Glucose | | | Butyrate | | | Methionine | | |
| Treatment | Sample A | Sample B | P | Sample A | Sample B | P | Sample A | Sample B | P |
| Controls | 41·5 ± 1·0 (28) | | | 43·0 ± 1·5 (20) | | | 29·0 ± 1·3 (20) | | |
| Cetrimide $10^{-3}$ | 6·4 ± 1·0 (16) | 22·8 ± 1·7 (19) | <0·0001 | 22·3 ± 1·6 (16) | 31·8 ± 1·4 (19) | <0·001 | 11·4 ± 1·1 (16) | 26·0 ± 2·0 (19) | <0·001 |
| Cetrimide $10^{-4}$ | 33·3 ± 2·4 (8) | 38·1 ± 3·5 (4) | <0·3>0·2 | 38·4 ± 1·9 (8) | 44·0 ± 3·1 (4) | <0·2>0·1 | 25·2 ± 1·9 (8) | 25·3 ± 3·1 (4) | 0·99 |
| Cetrimide $10^{-5}$ | 43·0 ± 2·9 (4) | 45·9 ± 1·7 (8) | <0·4>0·3 | 43·7 ± 1·9 (4) | 45·7 ± 1·9 (6) | <0·2>0·1 | 29·9 ± 2·7 (4) | 38·0 ± 1·8 (8) | <0·05 >0·025 |

Values are means with standard errors; the number of animals is shown in parentheses. P refers to comparison between sample A and sample B.

it is seen that the two samples had significantly different effects on the absorption of all three nutrients. This was in contrast to the results in the mouse, in which the differences with respect to methionine and butyrate were negligible. The difference in inhibitory activity was also observed in 1-hr experiments on two groups of four rats. Sample A, at $10^{-3}$, reduced the absorption of glucose by 62·3%, whilst the same concentration of sample B reduced it by only 23·8%.

*Guinea-pigs.* Two types of experiments were made in guinea-pigs, the perfusion fluid being either the usual nutrient-containing solution for the study of absorption, or the nutrient-free 0·9% saline for the study of glucose reversal.

When the original cetrimide was previously examined for its effect on the absorption of glucose in guinea-pigs it gave a steep dose-response curve and, at a concentration of $10^{-3}$, a negative value for glucose absorption. This latter observation meant that glucose passed from the blood vessels into the lumen of the intestine, a finding which was most striking when cetrimide was perfused in 0·9% saline alone.

2-hr perfusion experiments with the three nutrients were performed on one control animal, and two animals which had in addition sample B at a concentration of $10^{-3}$ (Table 3). The glucose reversal obtained with

TABLE 3. THE EFFECTS OF THE TWO SAMPLES OF CETRIMIDE ON THE CONCENTRATION OF GLUCOSE, BUTYRATE AND METHIONINE IN THE LUMEN OF THE GUINEA-PIG SMALL INTESTINE DURING PERFUSION EXPERIMENTS

| Treatment | Nutrient | n | Nutrient concentration in lumen | | | |
|---|---|---|---|---|---|---|
| | | | Initially | ½ hr | 1 hr | 2 hr |
| Control nutrient | Glucose | 1 | 200 | — | 60 | 10 |
| | Butyrate | | 200 | — | 50 | 50 |
| | Methionine | | 200 | — | 80 | 40 |
| Nutrient solution sample B, $10^{-3}$ | Glucose | 2 | 200 | | 211<br>255 | 222<br>26 |
| | Butyrate | | 200 | | 107 | 84<br>44 |
| | Methionine | | 200 | | 123<br>99 | 100<br>108 |
| Saline solution, 0·9% | Glucose | 1 | 0 | — | — | 0 |
| | Butyrate | | 0 | — | — | 0 |
| | Methionine | | 0 | — | — | 0 |
| Saline solution, 0·9% sample B, $10^{-3}$ | Glucose | 2 | 0 | — | 79<br>81 | 112<br>100 |
| | Butyrate | | 0 | — | — | 0 |
| | Methionine | | 0 | — | — | 0 |
| Saline solution, 0·9% sample A, $10^{-3}$ | Glucose | 3 | 0 | 72·3 ± 8·7 | 102·5 ± 22·5 | |
| | Butyrate | | 0 | — | 0 | |
| | Methionine | | 0 | — | 0 | |
| Saline solution, 0·9% sample B, $10^{-3}$ | Glucose | 3 | 0 | 62·0 ± 4·1 | 116·8 ± 7·1 | 168·4 ± 14·7 |
| | Butyrate | | 0 | — | 0 | |
| | Methionine | | 0 | — | 0 | |

Values are concentrations in mg/100 ml of either individual experiments or means and standard errors; n = number of animals. P between glucose values for two samples at ½ hr $<0{\cdot}4 >0{\cdot}3$ and at 1 hr $>0{\cdot}7 >0{\cdot}6$ in last two experiments.

sample B indicated clearly that in the guinea-pig it was no less active than the original cetrimide, for the dose-response curve obtained with the original cetrimide showed that no reversal would have occurred at a concentration of sample A as high as $0{\cdot}9 \times 10^{-3}$.

Confirmation of the glucose reversal was obtained by perfusing sample B, at a concentration of $10^{-3}$, in 0·9% saline containing no nutrient. Preliminary experiments had shown distinctly that the degree of glucose reversal depended on the blood sugar level. Animals perfused with nutrient-free saline were not therefore separated from their food overnight. In this way a blood sugar level between 200 and 300 mg 100 ml was assured. In two 2-hr experiments a final concentration of 100 mg% or more was observed (Table 3) with, of course, no trace of either methionine or butyrate. Finally, the potency of the two samples of cetrimide in causing glucose reversal was compared in two groups of three guinea-pigs in which the cetrimide was perfused at a concentration of $10^{-3}$ in

nutrient-free 0·9% saline. The results in Table 3 show that both samples produced a similar degree of glucose reversal and thus had equivalent activity in the guinea-pig, in marked contrast to their distinctly different activities in the mouse and rat.

### PHYSICAL AND HISTOLOGICAL OBSERVATIONS

During the present experiments, the surface-activities of the two cetrimide samples, as assessed by the degree of frothing of the perfusate in the apparatus, appeared to be similar. On the other hand, differences in the solubilities of the two samples were quite marked. Sample A dissolved easily at $10^{-3}$, in the nutrient solution, and did not precipitate when stored at a low temperature (0·5°). Sample B had to be warmed to about 35–40° before it dissolved completely to give a concentration of $10^{-3}$, and it reprecipitated when stored in the cold. Both samples remained in solution at room temperature.

The perfusion fluid collected at the end of the experiment was always inspected for cloudiness and mucosal debris. At concentrations of $10^{-3}$, both samples produced some damage to the intestinal mucosa, no difference in the amount of debris being noted. Sections of the small intestine from such experiments were examined histologically, but again it was impossible to distinguish between sections perfused with sample A and those perfused with sample B. At concentrations of $10^{-3}$, both caused some breakdown at the tips of villi with comparable accumulation of cellular debris in the lumen.

### HOMOLOGOUS COMPOUNDS

Pure members of the homologous series of chain length $C_{(10)}$–$C_{(20)}$ were examined for their activities at a concentration of $10^{-3}$ in $\frac{1}{2}$-hr perfusion experiments in rats. The results are shown in Table 4, where

TABLE 4. THE EFFECTS OF THE TWO SAMPLES OF CETRIMIDE COMPARED WITH THOSE OF HOMOLOGOUS COMPOUNDS AT A CONCENTRATION OF $10^{-3}$, ON THE ABSORPTION OF GLUCOSE, BUTYRATE AND METHIONINE IN $\frac{1}{2}$-HR INTRALUMINAL INTESTINAL PERFUSION EXPERIMENTS IN MALE ALBINO RATS

| Treatment | n | Absorption, % | | |
|---|---|---|---|---|
| | | Glucose | Butyrate | Methionine |
| Controls | 20 | 41·5 ± 1·0 | 43·0 ± 1·5 | 29·0 ± 1·3 |
| Cetrimide | | | | |
| Sample A | 16 | 6·4 ± 1·0 | 22·3 ± 1·6 | 11·4 ± 1·1 |
| Sample B | 19 | 22·8 ± 1·7 | 31·8 ± 1·4 | 26·0 ± 2·0 |
| $C_{(10)}$ | 4 | 33·6 ± 1·3 | 45·3 ± 0·7 | 19·3 ± 1·2 |
| $C_{(12)}$ | 4 | 7·3 ± 2·2 | 31·5 ± 1·1 | 14·6 ± 0·9 |
| $C_{(14)}$ | 4 | 10·7 ± 1·5 | 30·7 ± 3·1 | 14·8 ± 3·2 |
| $C_{(16)}$ | 4 | 14·3 ± 2·8 | 29·2 ± 0·7 | 8·2 ± 2·2 |
| $C_{(18)}$ | 4 | 23·9 ± 1·7 | 29·6 ± 1·5 | 25·1 ± 2·0 |
| $C_{(20)}$ | 4 | 26·0 ± 2·1 | 30·9 ± 0·8 | 17·9 ± 1·9 |

Values are means with standard errors; n = number of animals.

they are compared with those obtained at the same concentration of the two samples of cetrimide. Compounds $C_{(10)}$–$C_{(16)}$ dissolved easily in the nutrient solution, whilst $C_{(18)}$ and $C_{(20)}$ had to be warmed to 50° before they dissolved, though once dissolved they remained in solution at

room temperature. The inhibitory effect on glucose absorption was maximal with $C_{(12)}$, and decreased thereafter with increasing chain length. There were no significant differences in the effects of the five compounds, $C_{(12)}$–$C_{(20)}$, on butyrate absorption, all producing about 30% inhibition, while $C_{(10)}$ had no significant effect. A peak in the inhibitory activity on methionine absorption occurred at $C_{(16)}$.

Histological examination of sections from intestine perfused with the pure and active $C_{(12)}$ and $C_{(14)}$ homologues showed greater damage to villi than was obtained either with sample A or B of cetrimide. This difference was reflected in the greater amount of debris observed in the naked-eye inspection of the perfusion fluid.

## Discussion

Commercial samples of cetrimide, even from the same manufacturer, were not equivalent in effect. This finding is of importance on account of the value of this drug as a tool for research in the study of the mechanisms involved in intestinal absorption. Previous evidence suggests that cetrimide inhibits absorption by binding to the mobile intracellular proteins which constitute the normal carriers for active nutrient absorption (Nissim, 1964). The results obtained with the two samples of cetrimide may indicate that species and strain differences exist in the protein carriers themselves or in the factors controlling the association-dissociation constants between nutrients and proteins.

Both sample A and sample B, at a concentration of $10^{-3}$, caused the same degree of damage to the mucosa, while they exerted significantly different effects on the absorption of nutrients. This fact constituted yet another reason for concluding that the inhibitory effects of cetrimide were not simply due to some non-specific action on absorption, an action closely related to the mucosal damage it produced, as believed by Taylor (1963). It also suggested the interesting possibility that these two properties of cetrimide could be separated in some compound which might in future be synthesised. Such a compound, which would be devoid of any damaging effect on the intestinal mucosa and which would reduce the absorption of carbohydrates, fats and proteins, might prove of value in the treatment of obesity or of atherosclerosis.

The results obtained with the quaternary ammonium homologues indicate the importance of the length of the alkyl chain in determining activity. Other changes in the molecule, however, must also be considered (Nissim, 1960c). In the present experiments, not all the differences between the two samples of cetrimide could be explained on the basis of differences in chain length. A comparison of the activities of the two cetrimide samples with those of the pure homologous series would suggest that sample A may be composed largely of the shorter chain compounds, and sample B of chain lengths $C_{(10)}$–$C_{(20)}$. At least one significant anomaly would remain unexplained, however, according to this hypothesis. None of the pure homologous compounds were as active in inhibiting butyrate absorption as sample A of the so-called 'cetrimide', and the

difference between the percentage absorption of 22·3 ± 1·6 for 16 animals and the percentage absorption of any of the homologues, $C_{(10)}$–$C_{(20)}$, gave $P < 0{\cdot}05$. Furthermore, the damage to intestinal villi produced by the pure $C_{(12)}$ and $C_{(14)}$ homologues was appreciably greater than that produced by the no less active sample A of cetrimide. It is quite probable that differences exist in the molecule, such as the number of methyl groups, which could also partly contribute to the differences in activity. Further, the long chain quaternary ammonium compounds would be expected to form micelles in solution, and this may also be associated with reduction in activity.

According to the specification of the British Pharmacopoeia 1953, cetrimide consisted largely of the $C_{(16)}$ compound with only small amounts of the other homologues and was soluble in 10 parts of water. In the 1958 specification, cetrimide B.P. contained a mixture of the $C_{(12)}$, $C_{(14)}$ and $C_{(16)}$ compounds, which on drying gave 94–100% of bromide calculated as the $C_{(14)}$ homologue, and this was soluble in only two parts of water. Lastly, according to the British Pharmacopoeia 1963, cetrimide contains mainly the $C_{(14)}$ compound with only small amounts of the $C_{(12)}$ and $C_{(16)}$ homologues. This gradual replacement of the $C_{(16)}$ by the $C_{(14)}$ compound in cetrimide B.P. and the resulting increase in solubility was noted by Jones (1963), for these changes affected the formation of the cetrimide salts of nucleic acids during their isolation. The present investigation shows that preparations supplied as $C_{(16)}$ have differed, and indicates that differences beside chain length are involved.

Finally, since the difference in the chemical nature between samples A and B has not yet been elucidated, it seems essential that the identity of samples of cetrimide, intended for absorption and other biological studies involving protein binding, should be determined by prior biological assay in the rat.

*Acknowledgement.* We are grateful to the Medical Research Council for a personal grant (to S.L.H.) and for a grant for expenses.

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*J. Pharm. Pharmac.*, 1966, **18**, 236–238 Received October 29, 1965

# A note on the assay of the contribution of the pentose phosphate pathway to glucose metabolism in human red cells

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When human red cell suspensions were incubated with [$^{14}C_1$] glucose a substantial fraction of the incorporated radioactivity was found in phosphate compounds. If some of these phosphates are precursors of $^{14}CO_2$ then an error is introduced into the conventional calculation of the proportion of glucose metabolised by the pentose phosphate pathway. The existence of this error was demonstrated and its magnitude was assessed by studying the effects of the subsequent addition of unlabelled glucose to the reaction mixtures in either the absence or the presence of methylene blue.

THE human red cell metabolises glucose by the glycolytic and pentose phosphate pathways. Mature erythrocytes lack certain enzymes of the tricarboxylic acid cycle (Prankerd, 1955; Murphy, 1960) and the oxidative reactions of the pentose phosphate pathway appear to be the sole mechanism for the conversion of glucose carbons to $CO_2$. The relative contribution of this pathway to glucose metabolism has been assessed by measuring the evolution of $^{14}CO_2$ from red cells incubated with [$^{14}C_1$] glucose (De Loecker & Prankerd, 1961). The use of radioactive glucose, labelled in the 1 position only, avoids difficulties arising from the possible recycling of hexose phosphates (Murphy, 1960). The ratio:

$$\frac{\text{Radioactivity in evolved } ^{14}CO_2}{\text{Radioactivity lost from } [^{14}C_1] \text{ glucose}}$$

is the basis of calculations performed to derive the proportion of glucose metabolised by the pentose phosphate pathway (De Loecker, 1964). It is assumed that the radioactivity lost from the [$^{14}C_1$] glucose and metabolised by the pentose phosphate pathway has all been recovered as $^{14}CO_2$. The validity of this assumption is questionable since under the experimental conditions used by many investigators (De Loecker & Prankerd, 1961; Bonsignore, Fornaini, Segni, Leoncini & Chieffi, 1963) variable amounts of radioactivity may remain in certain intermediary compounds such as 6-phosphogluconate and glucose-6-phosphate, which are precursors of $^{14}CO_2$. Retention of radiocarbon in these intermediates at the end of the incubation would be expected to yield low values for the percentages of glucose metabolised by the pentose phosphate pathway. The following experiments were performed to assess the magnitude of this error under defined conditions.

## Experimental

White cells were removed from defibrinated human blood by filtration through cotton wool and washing three times with a solution consisting of seven parts of 0·15 M sodium chloride and three parts of 0·1 M phosphate

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buffer, pH 7·4 (Buchanan, 1960). The remaining red cells were re-suspended in an equal volume of the above medium, which was also used to prepare all other solutions. The incubations were at 37° in stoppered Warburg flasks with two side arms, one of which contained 0·5 ml of 50% w/v trichloroacetic acid and the other contained quantities of unlabelled glucose, ranging from 0–70 $\mu$moles, dissolved in 0·5 ml of medium; the centre well contained 0·2 ml of 20% w/v potassium hydroxide. Red cell suspension (2 ml) was added to the main compartment of each flask followed by 1 ml of medium containing [$^{14}C_1$] glucose (1 $\mu$c, 0·03 $\mu$mole) and sufficient methylene blue, when present, to give a final concentration of 1 mM. After an initial incubation period of 2 hr the unlabelled glucose was added from one side arm and the incubation continued for a further 2 hr. The trichloroacetic acid was then added from the other side arm and the incubation continued for an additional hour to ensure that all the liberated $^{14}CO_2$ was absorbed in the centre well. Glucose, lactic acid and phosphate compounds were separated by chromatography on Whatman No. 4 paper using n-butanol: propionic acid: water (3:2:2) and radioactive compounds were located by a Nuclear-Chicago Actigraph scanner. The amounts of [$^{14}C_1$] glucose initially present were measured in corresponding mixtures to which the trichloracetic acid was added at zero time. No radioactive glucose was detected in the incubation mixtures at the end of the experiments. The $^{14}CO_2$ liberated from the cell suspensions was absorbed in potassium hydroxide and portions of the resulting solution dried on Whatman No. 4 paper. All radioactive counting was performed directly on paper with a Packard Tri-Carb liquid scintillation counter, using as phosphor 15 ml of 0·4% 2,5-diphenyloxazole and 0·01% 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene in toluene.

## Results and discussion

The results (Table 1) show that when the red cells were incubated with [$^{14}C_1$] glucose for 4 hr, the incorporated radioactivity was distributed

TABLE 1. EFFECT OF THE SUBSEQUENT ADDITION OF UNLABELLED GLUCOSE ON THE DISTRIBUTION OF $^{14}C$ FROM [$^{14}C_1$] GLUCOSE INTO PHOSPHATES, LACTIC ACID AND CARBON DIOXIDE OF HUMAN RED CELL SUSPENSIONS
The results represent the means of four experiments ± standard deviations and are expressed as the radioactivity in each fraction calculated as a percentage of that lost from the [$^{14}C_1$] glucose.

| Unlabelled glucose added ($\mu$moles) | Phosphates | Lactic acid | Carbon dioxide |
|---|---|---|---|
| 0 | 61·6 ± 0·9 | 13·1 ± 0·7 | 14·3 ± 0·2 |
| 0·7 | 36·8 ± 0·8 | 27·4 ± 0·6 | 14·9 ± 0·3 |
| 7 | 24·3 ± 1·0 | 40·2 ± 0·9 | 16·6 ± 0·3 |
| 35 | 16·6 ± 0·9 | 49·5 ± 0·5 | 17·8 ± 0·2 |
| 70 | 16·4 ± 0·9 | 50·7 ± 0·6 | 18·1 ± 0·3 |
| In presence of methylene blue | | | |
| 0 | 16·5 ± 0·8 | 3·3 ± 0·1 | 63·4 ± 0·3 |
| 0·7 | 10·2 ± 0·5 | 4·8 ± 0·1 | 65·9 ± 0·4 |
| 7 | 3·1 ± 0·2 | 5·5 ± 0·4 | 73·3 ± 0·7 |
| 35 | 2·5 ± 0·2 | 6·8 ± 0·3 | 76·8 ± 0·7 |
| 70 | 2·3 ± 0·2 | 6·8 ± 0·3 | 76·6 ± 0·3 |

between intermediate phosphate compounds and metabolic end products, lactic acid and carbon dioxide. If some of the labelled phosphates were precursors of $^{14}CO_2$ then the conventional method (cf. Murphy, 1960) for the calculation of the proportion of glucose metabolised by the pentose phosphate pathway must be in error. This hypothesis was tested by adding increasing amounts of unlabelled glucose to the incubation mixtures after 2 hr and continuing the incubation for a further 2 hr. Marked changes in the distribution of the radiocarbon from the [$^{14}C_1$] glucose between the various fractions were observed. More isotope appeared in lactic acid and carbon dioxide and less in the phosphates. Thus the proportion of [$^{14}C_1$] glucose converted to $^{14}CO_2$ was increased up to 25% by the addition of the unlabelled glucose. The amount of $^{14}CO_2$ liberated from red cells incubated with concentrations of [$^{14}C_1$] glucose such that all the labelled glucose is utilised during the experiments (Bonsignore & others, 1963) is, therefore, not a reliable measure of the quantity of sugar metabolised by the pentose phosphate pathway. The present results show that the magnitude of this error may be diminished by adding unlabelled glucose to the incubation mixtures, but it will be affected by variation in experimental conditions such as the time of incubation and the glucose concentrations present at the beginning of the experiments.

In the presence of methylene blue, added as an electron acceptor to facilitate the oxidation of $NADPH_2$ by molecular oxygen (Brin & Yonemoto, 1958), the distribution of radioactivity from the [$^{14}C_1$] glucose was found to alter in a manner consistent with a higher proportion of the radioactive glucose being metabolised by the pentose phosphate pathway (Table 1). Nevertheless, the addition of unlabelled glucose again increased the evolution of $^{14}CO_2$ and decreased the retention of radiocarbon in phosphates. The magnitude of this effect was similar to that found in the absence of the dye.

*Acknowledgement.* We wish to thank the King's College Hospital and Medical School Research Committee for financial support.

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*J. Pharm. Pharmac.* 1966, **18**, 239–245 Received November 25, 1965

# The analgesic and related properties of 6-deoxy-6,6-ethylenedioxy-7,8-dihydro-14-hydroxycodeine hydrochloride

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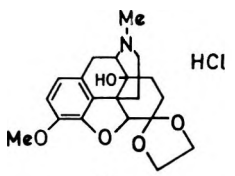
The compound 6-deoxy-6,6-ethylenedioxy-7,8-dihydro-14-hydroxycodeine hydrochloride possesses analgesic potency greater than either morphine or codeine when examined by a variety of methods in mice and rats. Compared with these two analgesics the compound showed greater separation between effective analgesic doses and doses producing inhibition of gastrointestinal motility or death, but not for mydriasis or respiratory depression. Some evidence for a lower addictive liability than morphine is discussed.

A NUMBER of cyclic ketals derived from 14-hydroxydihydrocodeinone have been synthesised as potential analgesic drugs by Lester, Petrow & Stephenson (1965). Routine biological screening indicated that the most active of these was the 6-ethylene ketal. The pharmacology of this compound (I; 6-deoxy-6,6-ethylenedioxy-7,8-dihydro-14-hydroxycodeine hydrochloride, BDH 5499) is now reported.




I

## Methods

*General.* Female albino mice, about 20 g, from the BDH colony, male Sprague-Dawley rats, about 160 g, from Animal Supplies Ltd., cats of either sex, 2·6–4·3 kg, and adult male rabbits, 2·4–3·4 kg, of various breeds, were used. Unless otherwise stated, the compounds were administered at varying dose levels with a dose ratio of 2·0 to groups of twenty animals. Drugs were administered in 5% (w/v) acacia or physiological saline; dose volumes were 25 ml/kg orally or subcutaneously and 10 ml/kg intravenously or intraperitoneally for mice and rats, and 5 ml/kg orally for rabbits. ED50 or LD50 values and their confidence limits ($P = 0.95$) were estimated by the method of Litchfield & Wilcoxon (1949) where appropriate.

*Acute toxicity.* The acute oral and subcutaneous toxicities of BDH 5499, morphine hydrochloride and codeine phosphate were compared in mice using a dose ratio of 1·5. The number of animals dying within seven days was recorded.

*Analgesic activity.* The oral and subcutaneous analgesic potencies of BDH 5499 and morphine hydrochloride and the oral potency of codeine

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phosphate were compared using the tail pinch, hot plate and phenylquinone-induced writhing methods in mice. Oral potencies were also assessed by a radiant heat method in rats.

The criterion of analgesia in the tail pinch method (Bianchi & Franceschini, 1954) was the absence of any attempt to remove an artery clip applied for 30 sec 1 cm from the base of the tail 30 min after administration of the compound. Only animals which previously made repeated attempts to remove the clip within 15 sec were used.

The criterion of analgesia in the hot plate method of Eddy & Leimbach (1953) was the absence of any sign of discomfort within 30 sec when the animal was placed 30 min after dosing on a hot plate maintained at 55–56°.

The writhing test was essentially as described by Siegmund, Cadmus & Lu (1957). The criterion of analgesia was the total absence of writhing during the 15 min after an intraperitoneal injection of phenylquinone (2 mg/kg) given 30 min after the drug.

The radiant heat method was based on that described by D'Amour & Smith (1941). Ten rats were used at each dose level. The criterion of analgesia was the absence of the typical tail flick response within 10 sec of the application of radiant heat from a 6 V, 45 W lamp 1 hr after dosing.

The development of tolerance was assessed by giving a single oral dose on five consecutive days in each week for three weeks. The presence of analgesia was determined daily by the hot plate method 30 min after dosing.

The possibility of antagonism by nalorphine was examined by adminstering varying doses of nalorphine hydrobromide subcutaneously to groups of 10 mice. BDH 5499, morphine hydrochloride or codeine phosphate were given at the same time at dose levels producing a near-maximal effect in animals not treated with nalorphine. Analgesia was determined by the hot plate method.

*Straub index.* The method was essentially as described by Shemano & Wendel (1964). The numbers of mice with the tail raised through at least 90° (Straub effect) within 2 min of intravenous administration of the drugs were determined as also were the numbers dying within 24 hr. The Straub index is given by the ratio LD50 : ED50.

*Mydriatic activity.* Pupil diameters of mice were determined under constant illumination and magnification with a binocular dissecting microscope before, and 30 min after giving BDH 5499, morphine hydrochloride or codeine phosphate orally. An increase in pupil diameter of 50% was recorded as a positive response.

*Antitussive activity* (Domenjoz, 1952). Square wave stimuli (width 10 msec, frequency 5/sec, intensity 2–6 V) were applied at 5 min intervals to the cut central end of the superior laryngeal nerve of cats anaesthetised with pentobarbitone sodium (37–58 mg/kg intraperitoneally). Movements of the diaphragm were recorded with an isometric lever attached by a thread to the abdominal wall. BDH 5499 was given by way of the femoral vein.

*Respiratory depression.* Thirty min after oral dosing, groups of 10

mice immobilised individually in paper cones were placed inside a desiccator containing self-indicating soda lime ("Carbosorb," BDH). The desiccator was connected to a float recorder writing on a smoked drum and then allowed to equilibrate to a constant temperature by complete immersion in a water-bath at 27°. The time required by each group to take up a constant amount of oxygen was determined, an increase in time being a measure of the degree of respiratory depression. Five groups of ten mice were used at each dose of BDH 5499 or morphine; five groups were given the vehicle only. The dose of each drug producing a 20% increase compared with the control in the time taken to consume the given volume of oxygen was estimated graphically.

The effect of BDH 5499, morphine hydrochloride and codeine phosphate on the respiratory minute volume of restrained conscious rabbits was determined using Gaddum's (1941) apparatus. Ten animals were used at each dose. When the animals had become accustomed to the apparatus, measurements were made before and 60 min after the drugs were given orally. The dose of drug producing a 30% decrease in respiratory volume compared with a group of animals given the vehicle alone was estimated graphically.

*Effects on the gastrointestinal tract.* The effect of BDH 5499, morphine hydrochloride and codeine phosphate on defaecation by mice was examined using a method based on that of Lou (1949). The number of faecal pellets eliminated by each group of mice during the 24 hr following oral administration was determined and compared with that of a group receiving the vehicle alone. The dose of each compound required to produce a 50% decrease in the number of faecal pellets was estimated graphically.

The effect on gastrointestinal propulsion was also determined using the method described by Macht & Barba-Gose (1931). Twenty-five min after oral administration of the above compounds or the vehicle alone, 0·5 ml of a 10% w/v suspension of charcoal in 5% w/v acacia was administered by stomach tube. Ten min later the animals were killed, the gastrointestinal tract dissected out and the distance which had been traversed by the charcoal measured. Ten animals were used for each dose with a dose ratio of 3·0. The amount of each compound producing a 50% decrease in the distance travelled was estimated graphically.

The effects of the three compounds on smooth muscle *in vitro* were examined using rabbit duodenum and guinea-pig ileum preparations. Short segments of rabbit duodenum were suspended in oxygenated Tyrode solution at 37° and the contractions recorded with a frontal writing lever. The compounds were added to the bath in saline, and were washed out after 90 sec contact. Short segments of guinea-pig ileum were suspended in oxygenated Tyrode solution at 32°. The compounds were given 30 sec before the various agonists. In some experiments, the peristaltic reflex was recorded (Trendelenburg, 1917) and the compounds were introduced 150 sec before eliciting the reflex.

*Cardiovascular effects.* Cats were anaesthetised with chloralose (80 mg/kg intravenously) following induction with ether. Mean arterial

pressure was recorded from the carotid artery with a mercury manometer. BDH 5499 was administered via the femoral vein.

Isolated rabbit hearts were perfused with Ringer-Locke solution at 37°, the amplitude and rate of ventricular beat being recorded on a smoked drum. BDH 5499 was dissolved in saline and added to the perfusing fluid just before its entry into the coronary vessels.

## Results

*Acute toxicity.* There is little difference in acute toxicity between BDH 5499 and morphine hydrochloride after oral or subcutaneous administration. Codeine phosphate is 2·5 times as toxic as BDH 5499 by both routes (Table 1).

TABLE 1. ACUTE TOXICITY OF BDH 5499, MORPHINE HYDROCHLORIDE AND CODEINE PHOSPHATE IN MICE

| Compound | Oral route | | Subcutaneous route | |
|---|---|---|---|---|
| | LD50 and confidence limits (P = 0·95), mg/kg | Relative toxicity | LD50 and confidence limits (P = 0·95), mg/kg | Relative toxicity |
| BDH 5499 .. .. .. .. | 1470 (1246–1734) | 1·3 | 590 (454–767) | 1·1 |
| Morphine hydrochloride .. .. | 1900 (1552–2325) | 1·0 | 620 (504–763) | 1·0 |
| Codeine phosphate .. .. | 580 (491–684) | 3·3 | 260 (218–309) | 2·4 |

*Analgesic activity.* The results are summarised in Table 2. By mouth BDH 5499 is 10–17 times as potent as codeine phosphate and 3–4 times as active as morphine hydrochloride. It is 0·6–2·3 times as potent as morphine hydrochloride after subcutaneous administration. In contrast to morphine, which is more active by injection, BDH 5499 appears to be

TABLE 2. ANALGESIC ACTIVITY OF BDH 5499, MORPHINE HYDROCHLORIDE AND CODEINE PHOSPHATE

| Test | Compound | Oral route | | Subcutaneous route | |
|---|---|---|---|---|---|
| | | ED50 and confidence limits (P = 0·95), mg/kg | Relative activity | ED50 and confidence limits (P = 0·95), mg/kg | Relative activity |
| Hot plate (mice) | BDH 5499 | 10·0 (7·0–14·3) | 3·4 | 7·0 (4·5–10·8) | 0·6 |
| | Morphine hydrochloride | 34·0 (24·3–47·6) | 1·0 | 4·0 (3·0–5·4) | 1·0 |
| | Codeine phosphate | 100·0 (69·9–143·0) | 0·3 | | |
| Tail pinch (mice) | BDH 5499 | 2·9 (2·1–4·1) | 4·1 | 2·6 (2·0–3·4) | 2·3 |
| | Morphine hydrochloride | 11·8 (8·3–16·8) | 1·0 | 6·1 (4·3–8·6) | 1·0 |
| | Codeine phosphate | 38·0 (28·1–51·3) | 0·3 | | |
| Writhing (mice) | BDH 5499 | 0·34 (0·24–0·49) | 3·4 | 0·52 (0·39–0·69) | 0·9 |
| | Morphine hydrochloride | 1·15 (0·78–1·70) | 1·0 | 0·58 (0·36–0·65) | 1·0 |
| | Codeine phosphate | 5·8 (3·9–8·7) | 0·2 | | |
| Radiant heat (rats) | BDH 5499 | 6·2 (4·3–9·0) | 2·7 | | |
| | Morphine hydrochloride | 17·0 (11·0–26·3) | 1·0 | | |
| | Codeine phosphate | 82·5 (47·2–144·5) | 0·2 | | |

as active orally as subcutaneously. After repeated administration there is essentially no difference between the three compounds either in the extent or rate at which tolerance develops (Fig. 1). The analgesic action of all three compounds is readily prevented by simultaneous administration of nalorphine hydrobromide; 2 mg/kg antagonised the analgesia produced by 30 mg/kg BDH 5499 or 80 mg/kg morphine hydrochloride and 4 mg/kg antagonised 200 mg/kg codeine phosphate.

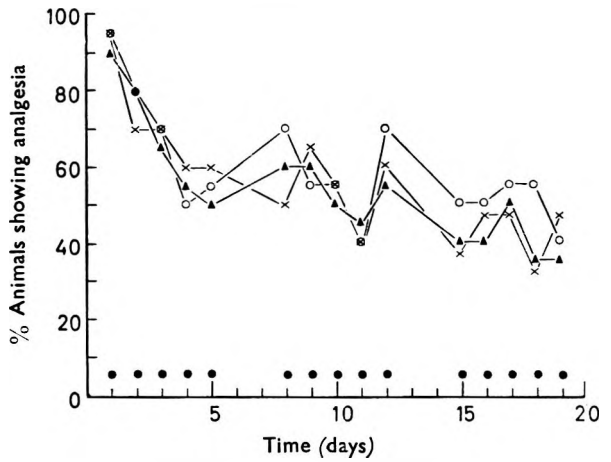


FIG. 1. The development of tolerance to the analgesic action of BDH 5499, 30 mg/kg/day (○—○), morphine hydrochloride, 80 mg/kg/day (▲—▲) and codeine phosphate, 200 mg/kg/day (X—X) administered orally on the days marked ●.

*Straub index.* The Straub tail effect is only apparent at near-toxic dose levels of BDH 5499, Straub index 2·1, and codeine phosphate, Straub index, 1·2, whereas with morphine hydrochloride the effect is evident at comparatively low dose levels, Straub index 19·6. The indices for codeine and morphine are in reasonable agreement with those obtained by Shemano & Wendel (1964).

*Mydriatic activity.* BDH 5499 is 9·8 and 2·4 times as active as codeine phosphate and morphine hydrochloride respectively. [ED50 (with confidence limits, P = 0·95) mg/kg: BDH 5499, 7·7 (5·2–11·3); morphine hydrochloride, 18·5 (13·7–25·0); codeine phosphate, 75·0 (52·8–106·4)]. Lenticular opacity was not observed, in contrast to the observations of Weinstock, Stewart & Butterworth (1958).

*Antitussive activity.* BDH 5499 appeared to have well marked antitussive activity at dose levels of 0·5–1·0 mg/kg intravenously. Although direct comparisons with morphine and codeine were not made, it is likely that BDH 5499 has a similar order of activity to codeine phosphate (David, Leith-Ross & Vallance, 1957).

*Respiratory depression.* BDH 5499 is approximately four times as potent as morphine hydrochloride in causing respiratory depression in mice (7·5 : 34·0 mg/kg) and rabbits (7·3 : 28·2 mg/kg). In rabbits, it is approximately 36 times as potent as codeine phosphate (7·3 : 263 mg/kg).

*Effect on the gastrointestinal tract.* BDH 5499 has similar potency to morphine hydrochloride in decreasing the number of faecal pellets by 50% over 24 hr in mice (48 : 52 mg/kg) and is 3·1 times as potent as codeine phosphate (149 mg/kg). In rats, however, BDH 5499 has only 0·4 times the potency of morphine hydrochloride (19·1 : 8·3 mg/kg) and 2·5 times that of codeine phosphate (46·8 mg/kg) in decreasing movement of a charcoal meal by 50%.

BDH 5499 caused a slight decrease in tone of the isolated rabbit duodenum at a concentration of 60 μg/ml. Similar effects were seen with codeine phosphate (6 μg/ml). Morphine hydrochloride (240 μg/ml) was without effect. BDH 5499 (0·05–50 μg/ml) produced varying degrees of inhibition of contractions of the isolated guinea-pig ileum due to histamine (0·05 μg/ml) and barium chloride (30 μg/ml) but never complete suppression. Contractions due to acetylcholine (0·25 μg/ml) were unaffected. When the peristaltic reflex was elicited by an increase in intraluminal pressure, BDH 5499 (1·3 μg/ml) produced almost complete inhibition. A similar degree of inhibition was produced by morphine hydrochloride (0·15 μg/ml) and codeine phosphate (3·3 μg/ml).

*Cardiovascular effects.* BDH 5499 produced a slight fall (15 mm Hg) in mean arterial pressure of the anaesthetised cat at a dose level of 6 mg/kg i.v. This effect was prevented by prior administration of mepyramine maleate (0·3 mg/kg i.v.). BDH 5499 (8 mg) caused a slight reduction in amplitude of the isolated rabbit heart but no change in rate.

## Discussion

The analgesic properties of BDH 5499 have been demonstrated in the mouse and the rat using a variety of stimuli (chemical, mechanical, thermal conduction, and thermal radiation). Although a structural analogue of codeine, it possesses greater potency than morphine. The compound also possesses other properties associated with narcotic analgesics, namely, the Straub tail effect, rapid development of tolerance to its analgesic action, respiratory depression, cough suppression and reduced gastrointestinal motility.

In the evaluation of a narcotic analgesic, particular attention must be paid to the degree of separation between analgesic activity and the associated side-effects since the severity of the latter will be an important factor in determining the clinical utility of the compound. The results indicate that BDH 5499 possesses a greater separation between effective analgesic doses and those inhibiting gastrointestinal function than either codeine or morphine, and the ratio of the lethal to the analgesic dose is also greater. BDH 5499 does not, however, show a markedly greater separation between analgesic and respiratory depressant or mydriatic doses.

The Straub index for BDH 5499 is particularly interesting since Shemano & Wendel have suggested that compounds showing little or no separation between lethal doses and doses producing the Straub effect (i.e. possessing a low Straub index) are likely to be only minimally or mildly addicting.

In contrast, BDH 5499 has been shown to be capable of suppressing withdrawal symptoms in morphine-dependent monkeys and would thus appear to have high physical dependence capacity (personal communication from Dr. G. A. Deneau). Nevertheless, since the dose required to produce suppression of withdrawal symptoms was eleven times that of an equivalent dose of morphine, it seems unlikely that addiction would develop as rapidly as with morphine, particularly in view of the greater analgesic potency of BDH 5499.

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*J. Pharm. Pharmac.*, 1966, **18**, 246–251 Received October 5, 1965

# The passage of drugs across the rat intestine *in vitro*

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The *in vitro* methods of Smyth & Taylor (1957) and everted sac technique of Crane & Wilson (1958) have been evaluated for studying the passage of ferrioxamine-B hydrochloride and its derivatives, glutethimide, thalidomide, a polypeptide, salicylic acid, aniline and phenol red from the mucosal to serosal side of the rat intestinal barrier *in vitro*. The ferrioxamines were not significantly absorbed, glutethimide was more rapidly absorbed than thalidomide, the polypeptide was broken down rapidly and appeared as amino-acids in the serosal fluid, salicylic acid and aniline were found in the serosal fluid but phenol red was little absorbed. Although allowance must be made for differences in the transit pathways of *in vivo* and *in vitro* systems because of the non-functional nature of the capillary network in *in vitro* studies, our results suggest that these methods can supply useful information on the permeability characteristics of newly developed drugs through the intestinal barrier.

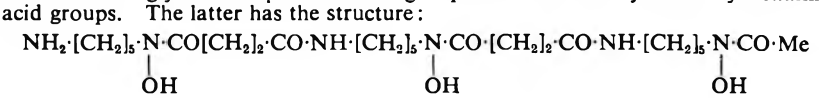
MUCH evidence (Schanker, 1960, 1961; Brodie, 1964) has been found to support the role of pH gradients, partition coefficients and lipid solubility in the gastrointestinal absorption of drugs. The concept of a lipid-intestinal barrier preferentially permeable to lipid-soluble molecules in undissociated form does not, however, satisfy all the requirements for the absorption of a drug. The passage of some drugs in their dissociated forms must undoubtedly be governed by other special transport mechanisms. Hogben (1960a, b) and Smyth (1960, 1964) have emphasised the complex nature of this intestinal barrier and Wilson (1962) has pointed out the difficulties which the membrane thickness and available surface area impose on the application of Fick's diffusion equation for passage of drugs across intestinal epithelium.

As the diffusion involved movement across three permeability-barriers (two cell membranes and a basement membrane), Wilson (1962) conceives one membrane to be the effective permeability-barrier for water-soluble substances and another for lipid-soluble substances. In spite of an incomplete understanding of the intestinal barrier, there has been a growing interest in intestinal absorption in recent years and Nelson (1961), Wagner (1961) and Nogami & Matsuzawa (1961, 1962, 1963) have given excellent treatments of the kinetic and physico-chemical factors underlying absorption processes.

In the present report two recent methods, those of Smyth & Taylor (1957) and Crane & Wilson (1958), have been evaluated for studying the passage of drugs from the mucosal to serosal side of rat intestinal preparations *in vitro* using compounds of current pharmaceutical interest. These included ferrioxamine-B hydrochloride* (Bickel, Hall, Keller-Schierlein, Prelog, Vischer & Wettstein, 1960) and its derivatives, deferrioxamine

From CIBA Ltd., Basle, Switzerland.

* Ferrioxamine-B is the $Fe^{3+}$ complex of deferrioxamine, an amphoteric compound with one strongly basic aliphatic amino-group and three weakly acidic hydroxamic acid groups. The latter has the structure:



(Tripod & Keberle, 1962), a metabolite of a species of *Streptomyces* which specifically binds $Fe^{3+}$ to give ferrioxamine-B, glutethimide (Tagmann, Sury & Hoffman, 1952), thalidomide and $Val^5$-angiotensin-Asp-$\beta$-amide. For the sake of comparison salicylic acid, aniline and phenol red have also been investigated.

## Experimental

### METHODS

Male and female rats weighing between 200–240 g had their food withheld 24 hr before use and were given plain water and 5% glucose solution (100 ml) to clear the intestine of solid contents.

*The method of Smith & Taylor* (1957). The experiment was set up according to Smyth & Taylor (1957). The intestine was removed as described by Wiseman (1953) except for the use of light anaesthesia with chloroform. The compounds were dissolved in Krebs Ringer bicarbonate saline (pH 7·4) containing glucose (5 mg/ml) in concentrations given in Table 1. Before circulating the drug solution, Ringer saline containing glucose was circulated for about 3 min through the intestinal segments and then taken out and replaced by 25 ml of a solution of drug. The head of pressure at the upper end of the intestinal segment was 7 cm and at the lower end was 21 cm of water. All experiments lasted 75 min. At the end of each experiment, volumes of mucosal and serosal fluids were noted and suitable aliquots analysed for content of drugs.

*Everted sac method of Crane & Wilson* (1958). These authors used a simple everted sac technique incorporating features of several earlier methods, for the study of intestinal absorption of sugars. Because of its simplicity, we have examined its suitability for studying the permeability characteristics of the drug.

The experiment was similar to that of Crane & Wilson (1958). The sac was everted according to Wilson & Wiseman (1954). 10 ml of Krebs Ringer bicarbonate saline with a glucose concentration of 5 mg/ml was placed in the outer glass jacket and 1 ml of Ringer solution in the everted sac. After 5–10 min of oxygenation in a thermostat at 37°, the sac relaxed and was filled with Ringer. The volume of fluid in the cannula was sufficient to give about 1–2 cm of fluid above that in the outer jacket. The sac was then transferred to another glass jacket containing a known concentration of drug dissolved in 10 ml of Krebs Ringer bicarbonate saline containing glucose. The gas mixture was allowed to bubble at a moderate rate. The duration of the experiment was 40 min. With glutethimide-$^{14}C$ and thalidomide-$^{14}C$, aliquots of 0·1 ml fluid from serosal side were withdrawn at various time intervals for counting the radioactivity. In other instances, the serosal and mucosal fluids were removed, their volumes recorded and suitable aliquots analysed for drug content.

### ESTIMATIONS

Phenol red and salicylic acid were estimated by the method of Schanker, Shore, Brodie & Hogben (1957), aniline by Bratton & Marshall's method

(1939), ferrioxamines and deferrioxamine by Tripod & Keberle's method (1962), non-labelled glutethimide by the method of Goldbaum & Williams (1960) and labelled glutethimide and thalidomide by liquid scintillation counting. Due to low solubility of thalidomide in Ringer solution, only a small concentration was used. For the estimation of Val$^5$-angiotensin-Asp-$\beta$-amide, the mucosal and serosal fluids from the experiments were immediately immersed in hot water at 95° for about 3 min to check enzymatic cleavage; suitable aliquots of the fluid were then freeze-dried and the residue extracted repeatedly with n-butanol saturated with water. The residue from butanol extracts was dissolved in a known volume of distilled water for paper chromatography. Aliquots of angiotensin amide solution containing known concentrations were extracted concurrently and worked up by the above procedure to serve as standard references for paper chromatography.

## Results and discussion

The results of investigations using Smyth & Taylor's method are presented in Tables 1 and 2. The data on diffusion of phenol red, salicylic acid and aniline show a parallel with *in vivo* absorption data of Schanker & others (1957). Phenol red is known not to be absorbed

TABLE 1. TRANSPORT OF DRUGS BY *in vitro* RAT INTESTINAL PREPARATION (SMITH & TAYLOR, 1957)

| Compound | Conc. used (μg/ml) | Vol. of serosal fluid (ml) | Vol. of mucosal fluid (ml) | Ratio of serosal to mucosal conc. per ml |
|---|---|---|---|---|
| Phenol red | 200 | 4·2 | 19·6 | 0·164 |
| Salicylic acid | 400 | 3·0 | 19·4 | 1·086 |
| | | 2·2 | 21·2 | 1·081 |
| Aniline | 408·8 | 2·2 | 19·8 | 1·070 |
| | | 1·6 | 21·0 | 0·980 |
| Ferrioxamine-B hydrochloride[1] | 1600 | 2·4 | 20·0 | 0·200 |
| | | 3·5 | 19·5 | 0·100 |
| | | 2·3 | 19·0 | 0·310 |
| *N*-Acetylferrioxamine-B[2] | 1600 | 3·6 | 20·0 | 0·177 |
| | | 2·5 | 20·5 | 0·242 |
| *N*-Valerylferrioxamine-B[3] | 1600 | 2·7 | 20·6 | 0·200 |
| | | 5·1 | 17·2 | 0·070 |
| *N*-Benzoylferrioxamine-B[4] | 1600 | 3·3 | 20·8 | 0·157 |
| | | 2·3 | 19·6 | 0·384 |
| | | 2·9 | 20·6 | 0·150 |
| *N*-[2-(*p*-Ethoxyphenyl)-acetyl] ferrioxamine-B[5] | 1600 | 3·3 | 19·8 | 0·184 |
| | | 3·2 | 20·2 | 0·256 |
| Deferrioxamine | 1600 | 2·3 | 20·2 | 0·180 |
| | | 1·7 | 20·0 | 0·460 |
| | | 3·8 | 19·5 | 0·110 |
| Glutethimide | 133·3 | 2·5 | 19·0 | 0·830 |
| | 95 | 2·5 | 17·4 | 0·846 |
| Val$^5$-angiotensin-Asp-$\beta$-amide | 2000 | 3·0 | 20·0 | — |
| | | 2·5 | 17·5 | |

Water/chloroform ratios: [1] >99 : <1; [2] 98 : 2; [3] 58 : 42; [4] 36 : 64; [5] 7 : 93.

TABLE 2. DIFFUSION OF GLUTETHIMIDE-$^{14}$C AND THALIDOMIDE-$^{14}$C THROUGH RAT *in vitro* INTESTINAL PREPARATION (SMITH & TAYLOR, 1957)

| Compound | Time of sampling of serosal fluid (min) | Vol. of serosal fluid (ml) | Vol. of mucosal fluid at end of experiment (ml) | Ratio of radioactivity in serosal to that in mucosal fluid |
|---|---|---|---|---|
| Glutethimide-$^{14}$C .. | 20 | 0·40 | 18·5 | 0·62 |
| | 40 | 0·75 | | 0·83 |
| | 60 | 0·90 | | 0·70 |
| | 75 | 0·80 | | 0·99 |
| Thalidomide-$^{14}$C .. | 25 | 0·65 | 18·5 | 0·14 |
| | 40 | 0·75 | | 0·64 |
| | 60 | 0·90 | | 0·58 |
| | 75 | 0·75 | | 0·64 |

through the intestine *in vivo*. Ferrioxamines of increasing lipid solubility and deferrioxamine showed a ratio of serosal to mucosal concentration in the range 0·1–0·3 and would not therefore be taken to be significantly absorbed. The pharmacological and clinical *in vivo* data with ferrioxamine-B and deferrioxamine bear out this point. A striking contrast is noticeable with salicylic acid and aniline, both of which are known to be well absorbed through rat intestine *in vivo*. In these cases the ratio of concentration in serosal to that in mucosal fluid approached 1·0 to 1·1. These values are comparable with the ratio of 0·85 obtained with non-labelled glutethimide which is also known to be well absorbed *in vivo*. From the comparative data on rate of diffusion of glutethimide-$^{14}$C and thalidomide-$^{14}$C across the intestinal barrier, it is evident that glutethimide-$^{14}$C crosses the barrier faster than thalidomide-$^{14}$C for the duration of the experiment. The greater lipid-solubility of glutethimide compared to thalidomide may be a factor contributing to this effect. The effect of probable degradation of thalidomide to other products by intestinal mucosal cells has not been assessed in this study. The increasing lipid solubility with ferrioxamine derivatives did not seem to have any significant effect in promoting their passage through intestinal barrier *in vitro*. With Val$^5$-angiotensin-Asp-$\beta$-amide after 2 min circulation of Ringer saline containing drug, a marked degradation could be seen from the paper chromatography of an aliquot of mucosal fluid worked up according to the stated procedure, but angiotensin amide could still be detected, though in a much reduced concentration. However, the circulation of drug solution through intestinal segments for 1 hr led to disappearance of all angiotensin amide from the mucosal fluid, only degraded peptides or amino-acids being detected by paper chromatography. This was also the case with serosal fluid. No attempt was made to identify the degradation products. Newey & Smyth (1959a) found that when dipeptides are present in the lumen of intestine, it was the constituent amino-acids which appeared in the blood stream. Newey & Smyth (1959b) further concluded that dipeptides entered the mucosal cells as peptides, were hydrolysed intracellularly and emerged as amino acids. According to Wiggans & Johnston (1959) tri- and tetra-glycyl peptides, when present on the mucosal side of the intestine, also emerged as amino-acids on the serosal side.

Barry, Mathews & Smyth (1961) have shown that energy for glucose-dependent transfer of water through the intestines came from glycolysis or the hexose monophosphate shunt and energy for glucose-independent transfer of water came from the citric acid cycle. Since the drugs passed through the intestinal barrier into water in varying proportions, there may be some kind of coupled absorption of water and drug molecules as a basis of absorption of most drugs (Fisher, 1955). In such a process the solubility of drug in the lipid material of which the membrane is composed, partition coefficients and pH gradients, would all seem to play a dominant role.

The results with the everted sac technique are in Tables 3 and 4. In this preparation phenol red and ferrioxamine-B and its progressively more

TABLE 3. PASSAGE OF SUBSTANCES FROM MUCOSAL TO SEROSAL SIDE BY EVERTED SAC TECHNIQUE

| Compound | Conc. (μg/ml) | Initial and final vol. of mucosal fluid (ml) | Initial and final vol. of serosal fluid (ml) | Ratio of serosal to mucosal conc. |
|---|---|---|---|---|
| Phenol red | 375 | 10, 9·75 | 1, 0·85 | 0·015 |
| | 250 | 10, 10 | 1·5, 1·5 | 0·050 |
| Salicylic acid | 138 | 10, 9·75 | 1, 1 | 0·28 |
| | 138 | 10, 9·75 | 1, 0·90 | 0·29 |
| | 1000 | 10, 9·5 | 1·5, 1·5 | 0·49 |
| | | | | 0·38 |
| | | | | 0·45 |
| | | | | 0·30 |
| Aniline | 102·2 | 10, 9·8 | 1, 0·8 | 0·77 |
| | 670·0 | 10, 9·8 | 1·5, — | 0·77 |
| Ferrioxamine-B | 650 | 10, 9·9 | 1, 0·75 | 0·011 |
| *N*-[2-(*p*-Ethoxyphenyl)-acetyl]ferrioxamine-B | 812 | 10, 10 | 1, 0·9 | 0·06 |
| | 2960 | 10, 10 | 1·5, 1·4 | 0·025 |
| Glutethimide | 217 | 10, 9·3 | 1, 1 | 0·60 |
| | 217 | 10, 9·3 | | 0·56 |

TABLE 4. DIFFUSION OF GLUTETHIMIDE-$^{14}$C AND THALIDOMIDE-$^{14}$C THROUGH EVERTED INTESTINAL SAC PREPARATION

| Compound | Time of sampling of serosal fluid (min) | Vol. of serosal fluid taken (ml) | Vol. of mucosal fluid after expt. (ml) | Ratio of radioactivity in serosal to that in mucosal fluid | |
|---|---|---|---|---|---|
| Glutethimide-$^{14}$C | 10 | 0·1 | 9·85 | 0·26 | |
| | 20 | 0·1 | | 0·31 | |
| | 30 | 0·1 | | 0·72 | |
| | 40 | 0·1 | | 0·58 | |
| Thalidomide-$^{14}$C | 10 | 0·1 | 9·0 | 0·11 | 0·31 |
| | 20 | 0·1 | | 0·24 | 0·20 |
| | 30 | 0·1 | | 0·31 | 0·38 |
| | 40 | 0·1 | | 0·67 | 0·53 |

lipid-soluble derivatives did not pass from mucosal to serosal side of intestinal barrier and therefore would not be expected to be significantly absorbed. Salicylic acid permeated in amounts much less than those found using Smyth & Taylor's method (1957). Aniline, however, was transported well, giving a ratio of 0·77 of serosal to mucosal concentration.

Glutethimide crossed the barrier easily giving a ratio of 0·6. This observation was further strengthened by studies on the comparative rates of transfer of glutethimide-$^{14}$C and thalidomide-$^{14}$C, the results of which are given in Table 4. Glutethimide crossed the barrier faster than thalidomide. The mechanism of transfer of fluids and solutes in the everted sac preparations has been investigated by Barry & Smyth (1960) who pointed out that what appeared in serosal fluid *in vitro* using everted sac preparations was only a fraction of that transported by epithelial cells. This concept should be taken into account in the interpretation of results by such a technique. The parallelism in results, however, between this method and that of Smyth & Taylor (1957), suggests that these methods can supply useful information on the permeability characteristics of newly-developed drugs.

*Acknowledgements.* The authors thank Dr. K. Hoffmann for his continued interest in this investigation, and Dr. K. Schmid for the supply of labelled glutethimide and thalidomide and the measurement of radioactive samples.

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**Effects of amphetamine, tyramine, and protriptyline on reserpine-resistant amine-concentrating mechanisms of adrenergic nerves**

SIR,—The ability of adrenergic nerves to take up and concentrate amines even after blockade of the amine-storing granules by reserpine has been demonstrated, and evidence supporting the existence of a reserpine-resistant uptake mechanism at the level of the neuronal cell membrane ("the membrane pump") has been presented (see Malmfors, 1965; Carlsson & Waldeck, 1965a). Whether the amines that accumulate in the adrenergic nerves under these conditions exist entirely free in the cytoplasm or are partly bound extragranularly, has not been elucidated. An observation of Malmfors (1965) may indicate that the latter alternative is true. He found that even the virtually complete blockade of the "membrane pump" by desipramine was relatively inefficient in releasing extragranular noradrenaline or α-methylnoradrenaline. Nevertheless, tyramine caused rapid release of these amines. The present data lend additional support to this view. Tyramine, and particularly amphetamine, have been found to release extragranular $^3$H-noradrenaline under conditions where a large dose of an efficient "membrane pump"-blocking agent was found to be more or less inactive.

Mice were pretreated with reserpine and nialamide and then received $^3$H-noradrenaline in certain experiments together with carrier noradrenaline. The drugs to be investigated were given 15 min after the $^3$H-noradrenaline, and the animals were killed after another 15 min. For further experimental details, see Tables 1 and 2 and Carlsson & Waldeck (1963).

TABLE 1. RELEASE BY AMPHETAMINE OF $^3$H-NORADRENALINE FROM THE HEARTS OF MICE PRETREATED WITH RESERPINE AND NIALAMIDE. Reserpine (10 mg/kg) and nialamide (10 mg/kg) were given intraperitoneally 6 and 2 hr, respectively, before the intravenous injection of $^3$H-noradrenaline (1 μg/kg; approximately 6 c/mM). The amphetamine was given 15 min after this injection, and the animals killed after another 15 min. There were 6 animals/group.

| Drug | Dose mg/kg i.v. | $^3$H-noradrenaline, ng/g | | |
|---|---|---|---|---|
| | | Mean | Range | No. of groups |
| Control | — | 0·50 | 0·35–0·67 | 4 |
| (±)-Amphetamine | 0·15 | 0·15 | 0·07–0·23 | 2 |
| " | 0·4 | 0·11 | | 1 |
| " | 1·5 | 0·08 | | 1 |
| " | 4 | 0·11 | 0·09–0·12 | 2 |
| (+)-Amphetamine | 0·1 | 0·15 | 0·09–0·21 | 2 |

TABLE 2. COMPARISON OF $^3$H-NORADRENALINE RELEASING ACTIVITY OF TYRAMINE AND PROTRIPTYLINE FROM THE HEARTS OF MICE PRETREATED WITH RESERPINE AND NIALAMIDE. Reserpine (10 mg/kg) and nialamide (100 mg/kg) were given intraperitoneally 15–21 and 2 hr, respectively, before the intravenous injection of $^3$H-noradrenaline (1 μg/kg; approximately 6 c/mM). The drugs were given 15 min after this injection, and the animals killed after another 15 min. There were 6 animals/group.

| | Dose of drug mg/kg i.v. | Dose of carrier (−)-noradrenaline mg/kg | $^3$H-noradrenaline, ng/g | | |
|---|---|---|---|---|---|
| | | | Mean | Range | No. of groups |
| Control | — | — | 0·36 | 0·23–0·49 | 3 |
| Tyramine HCl | 10 | — | 0·17 | 0·16–0·18 | 3 |
| Protriptyline HCl | 10 | — | 0·36 | 0·23–0·49 | 2 |
| Control | — | 0·1–0·2 | 0·33 | 0·30–0·39 | 4 |
| Tyramine HCl | 10 | 0·1–0·2 | 0·10 | 0·09–0·12 | 3 |
| Protriptyline HCl | 10 | 0·1–0·2 | 0·26 | 0·24–0·31 | 3 |

Under the conditions described above, amphetamine in doses down to 0·10–0·15 mg/kg caused a marked release of $^3$H-noradrenaline accumulated in heart (Table 1). In contrast, amphetamine given to non-reserpine-treated mice in a dose of 1·5 mg/kg causes little or no release of $^3$H-noradrenaline previously taken up in heart (data not shown).

Under the present conditions tyramine also caused release of $^3$H-noradrenaline, whereas protriptyline was inactive in a dose of 10 mg/kg (Table 2). We have discovered that this drug blocks $^3$H-noradrenaline uptake in doses down to 1 mg/kg.

In the present experiments the animals had been pretreated with a large dose of reserpine, and thus the noradrenaline uptake by the amine-storing particles was blocked. The $^3$H-noradrenaline taken up by the adrenergic nerves thus accumulated outside the granules and was protected from destruction by the monoamine oxidase inhibitor nialamide. Blockade of the "membrane pump" by a large dose of protriptyline did not result in detectable release of this $^3$H-noradrenaline. This is in contrast to $^3$H-metaraminol which is rapidly lost after blockade of the "membrane pump" (Carlsson & Waldeck, 1965b; Carlsson, 1965). The explanation of this difference may be that the more lipid soluble metaraminol leaks out through the cell membrane more rapidly and its intraneuronal retention is thus more dependent on the "membrane pump". The question then arises how tyramine, and particularly amphetamine in doses which do not block the "membrane pump" efficiently (Carlsson & Waldeck, 1965c), are capable of releasing extragranular $^3$H-noradrenaline. The most likely explanation appears to be that the $^3$H-noradrenaline under the present conditions is largely bound to extragranular sites and that these drugs are capable of displacing the $^3$H-noradrenaline from these binding sites.

The question also arises whether the effects of tyramine and amphetamine described may be involved in their actions as indirect sympathomimetics. This question is difficult to answer, since the binding sites proposed above must be assumed to be occupied by noradrenaline to a much smaller degree normally than under the present conditions, where both monoamine oxidase and storage granules have been blocked. Further work is necessary to settle this point.

*Acknowledgements.* This work was supported by grants from the Swedish State Medical Research Council (14X-155-02), the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service (NB 04359-03) and Knut and Alice Wallenbergs Foundation. For generous gifts of drugs we thank The Swedish Ciba Ltd., Stockholm: reserpine (Serpasil) and the Swedish Pfizer Ltd., Stockholm, Sweden: nialamide (Niamid). For technical assistance we thank Miss Ingrid Weigner and Mrs. Evelin Öst.

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**Some central nervous properties of diethyldithiocarbamate**

SIR,—Diethyldithiocarbamate has been shown to inhibit dopamine β-oxidase in the ileum of rats and rabbits, so that 200–500 mg/kg of the compound decreases the noradrenaline content and increases the dopamine level (Collins, 1961). Carlsson, Lindquist, Fuxe & Hökfelt (1966) confirmed the noradrenaline-decreasing effect in rat ileum, heart and brain and demonstrated an increase of dopamine level in the brain stem and in the hemispheres; but no difference was found in the striatum or in the whole brain. We find that diethyldithiocarbamate has certain depressing effects on the central nervous system.

After the intravenous administration of 50 mg/kg of hexobarbitone the sleeping time in control mice was 429 sec ±129 (s.d.) When the animals were given diethyldithiocarbamate, 400 mg/kg, 2 hr before the experiment the sleeping time increased to 2478 sec ±746 (s.d.). The compound had no effect on tremorine-induced tremor in mice and it was also ineffective towards amphetamine toxicity in aggregated mice.

TABLE 1. THE EFFECT OF DIETHYLDITHIOCARBAMATE (400 mg/kg s.c.) ON THE HYPERMOTILITY PRODUCED BY AMPHETAMINE (5 mg/kg s.c.) AND COCAINE (20 mg/kg s.c.)

| Treatment | No. of animals | Motimeter counts (means ± s.d.) | |
|---|---|---|---|
| | | without diethyldithiocarbamate | with diethyldithiocarbamate |
| Amphetamine | 4 | 1436 ± 713 | 261 ± 318 |
| Cocaine | 4 | 967 ± 626 | 534 ± 204 |
| Amphetamine + nialamide | 3 | 1030 ± 485 | 1698 ± 962 |

The recording was made 2 hr after the administration of diethyldithiocarbamate and 1 hr after amphetamine or cocaine. Nialamide was administered 20 hr before the experiment.

In rats, diethyldithiocarbamate diminished the hypermotility caused by amphetamine or cocaine. The motility was measured with the motimeter Knoll (1960). The inhibiting effect of the compound in hypermotility may be related to its effect in decreasing noradrenaline in the brain, since in the presence of the monoamine oxidase inhibitor, nialamide, the inhibiting effect is not seen (Table 1).

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**An adrenergic neurone blocking action of emetine**

SIR,—In treating amoebiasis with emetine hydrochloride, some untoward effects are encountered which have not hitherto been explained fully. Prominent among these are diarrhoea and hypotension, both of which appear to reflect diminution in sympathetic tone. The possibility that emetine may exert this effect by adrenergic neurone blockade has been tested by the combined method of transmural and periarterial nerve stimulation described by Wilson (1962).

Segments of guinea-pig ileum, prepared according to the method of Finkleman (1930), are mounted for transmural stimulation (Paton, 1955) in 50 ml of McEwen's solution at 35° and bubbled with 95% oxygen and 5% carbon dioxide. With two sets of platinum electrodes and different stimulation parameters, it is possible to stimulate the parasympathetic cholinergic nerves in the intestinal wall and the periarterial sympathetic adrenergic fibres either independently or simultaneously. Continuous transmural twitches are first obtained with supramaximal stimuli (20 V; 0·5 msec; 6/min), and these are inhibited at 4-min intervals by the simultaneous stimulation of the perivascular nerves with supramaximal stimuli (20 V; 0·5 msec; 50 pulses/sec for 20 or 30 sec). These effects persist in the presence of hexamethonium $1 \times 10^{-4}$ g/ml, confirming the finding of Wilson (1962).

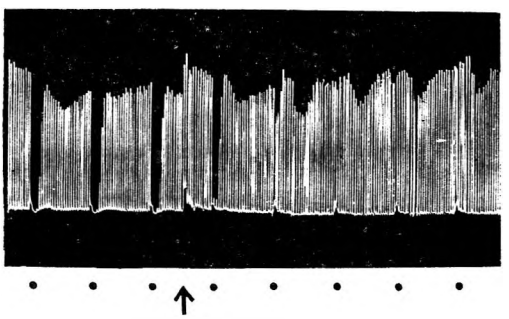

FIG. 1. Guinea-pig ileum. Continuous transmural stimulation (20V; 0·5 msec; 6/min) interrupted at 4-min intervals by simultaneous periarterial nerve stimulation at the dots (20 V; 0·5 sec; 50/sec for 20 sec). Emetine $2 \times 10^{-6}$ g/ml was added at the arrow indicated. The inhibitory response to periarterial nerve stimulation was abolished and replaced by augmented twitches.

Fig. 1 shows the effect of emetine, $2 \times 10^{-6}$ g/ml, on the response to cholinergic and simultaneous adrenergic nerve stimulation. The contractions to transmural stimulation are unaffected but the inhibition of these responses by sympathetic nerve stimulation are reduced and finally abolished. In some experiments there is a potentiation of the transmural twitches, and this is not abolished by hexamethonium, $1 \times 10^{-4}$ g/ml, but is abolished by hyoscine $1 \times 10^{-7}$ g/ml. When the inhibitory effect of sympathetic nerve stimulation is blocked by emetine, the inhibitory action of added noradrenaline on the transmural contraction is now potentiated. This is evidence that the action of emetine is located elsewhere than at the adrenergic receptor, possibly on the adrenergic fibres.

The evidence also indicates that emetine has no blocking action on the response mediated by cholinergic nerve in the guinea-pig ileum, but that it appears to act on the postganglionic adrenergic neurone interfering with the release of

the sympathetic transmitter substance. This observation may explain the nature of the diarrhoea and hypotension seen in clinical practice.

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**Gentisate and guinea-pig testis metabolism**

SIR,—Gentisic acid (2,5-dihydroxybenzoic acid) is devoid of uncoupling activity in mitochondrial suspensions (Brody, 1956; Whitehouse, 1964) and does not produce toxic symptoms in large doses in man (Smith, 1952). Claims that gentisate is a therapeutically active antirheumatic drug have been challenged in print (cited in Whitehouse, 1964), but the absence of any well controlled clinical trial still leaves gentisate as one salicylate congener of potential therapeutic value. Recent studies on a liver succinate oxidase preparation (Hines, Bryant & Smith, 1963) and on testis mitochondria (Hines & Bryant, 1966a), both from the guinea-pig, have demonstrated several effects of gentisate on biochemical parameters often greater than those found for the parent molecule, salicylate. These experiments compare salicylate and gentisate effects on the metabolism of radioactive substrates by preparations of guinea-pig testis; and of gentisate on several isolated dehydrogenase enzymes. The tissue was isolated, the fractions prepared and the incubation techniques performed as described previously (Hines & Bryant, 1966b), using 1 $\mu$c of each carbon labelled substrate. The radioactively labelled intermediates were extracted with ethanol, separated by two-dimensional paper chromatography, visualised by radioautography and the $^{14}C$ measured by established techniques.

The results (Table 1) show that gentisate closely parallels salicylate in its effects on preparations of isolated guinea-pig testis. Both drugs decrease the utilisation of (2-$^{14}C$) - acetate by an homogenate preparation. The qualitative pattern of incorporation of the radiocarbon by each preparation was not altered by either drug. Quantitative relationships were altered, and these are shown as changes in the pool sizes of the amino-acids (alanine, aspartate and glutamate) acids of the tricarboxylic acid cycle (succinate, fumarate, malate and citrate), and those intermediates associated with glycolysis (phosphates and lactate). The inhibitory effect of salicylate on many isolated dehydrogenase enzymes is well established, and the mechanism of the inhibition involves competition with the appropriate coenzyme (Hines & Smith, 1964). The inhibitory action of sodium gentisate (5mM) on several dehydrogenases was also investigated. The inhibitions % (calculated from initial rates) for those dehydrogenases studied are: malate 46, isocitrate 31, lactate (NAD→NADH) 16, (NADH→NAD) 13, glyceraldehyde-3-phosphate 17, $\alpha$-glycerophosphate 13, glucose-6-phosphate 21. It was possible to reduce the inhibition, in each instance, by the further addition of the respective coenzyme. The interference with transaminase enzyme activity is reflected in the reduced formation of radioactive amino-acids (Table 1) and conforms with established actions of salicylate on both glutamic - pyruvic and glutamic - oxaloacetic transaminase

TABLE 1. THE EFFECTS OF SODIUM SALICYLATE (5mM) AND SODIUM GENTISATE (5mM) ON THE METABOLISM OF RADIOACTIVE SUBSTRATES BY PREPARATIONS OF GUINEA-PIG TESTIS

(Results, the mean of four separate estimations, are expressed as the total % $^{14}C$ incorporated from the labelled substrates into the sum of all the separated soluble intermediates; the $^{14}C$ in each residual substrate being excluded)

| Soluble intermediate | (2-$^{14}C$) Acetate homogenate | | | (U-$^{14}C$) Glucose soluble fraction | | | (1:4-$^{14}C_2$) Succinate mitochondria | | | (5-$^{14}C$)α-Ketoglutarate mitochondria | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | None | Salicylate | Gentisate | None | Salicylate | Gentisate | None | Salicylate | Gentisate | None | Salicylate | Gentisate |
| Alanine | 0 | 0 | 0 | 8 | 7 | 6 | 0·7 | 0·2 | 0·5 | 0·3 | 0·6 | 0·4 |
| Aspartic acid | 3 | 2 | 2 | 20 | 24 | 24 | 30 | 18 | 14 | 4 | 3 | 3 |
| Asparagine | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0·8 | 0·7 | 0·4 | 0·6 |
| Glutamic acid | 64 | 59 | 56 | 0·5 | 0·3 | 0·2 | 4 | 2 | 2 | 12 | 10 | 11 |
| Citric acid | 7 | 11 | 10 | 0 | 0 | 0 | 12 | 16 | 15 | 50 | 42 | 39 |
| Fumaric acid | 2 | 1 | 2 | 0 | 0 | 0 | 15 | 19 | 22 | 6 | 8 | 6 |
| Lactic acid | 18 | 17 | 16 | 39 | 32 | 30 | 0·8 | 0·2 | 1 | 4 | 1 | 2 |
| Malic acid | 2 | 8 | 7 | 0·5 | 0·7 | 0·8 | 35 | 43 | 44 | 17 | 21 | 19 |
| α-Ketoglutaric acid | 0·5 | 1 | 2 | 0 | 0 | 0 | 0·5 | 0·6 | 0·7 | — | — | — |
| Succinic acid | 2 | 1 | 5 | 0 | 0 | 0 | — | — | — | 6 | 14 | 19 |
| Phosphates | 0·5 | 0 | 0 | 21 | 26 | 27 | 0 | 0 | 0 | 0 | 0 | 0 |
| Oligosaccharides | 0 | 0 | 0 | 11 | 10 | 12 | 0 | 0 | 0 | 0 | 0 | 0 |

activities (Smith, 1963). The diminished conversion of labelled glucose to lactate (probably a lactic dehydrogenase inhibition) may have been a causative factor in the unexpected increase in incorporation of radiocarbon into aspartate in the glucose experiments. There was no observable effect of either drug on the level of incorporation of radio-carbon from glucose in the oligosaccharide fraction, although γ-resorcylic acid (2,6-dihydroxybenzoic acid) is reported to double the incorporation of labelled glucose into an oligosaccharide fraction (Smith, 1963).

The demonstration of salicylate as an inhibitor of certain vital groups of cellular enzymes (those concerned with oxidative phosphorylation, the transaminases, and the dehydrogenases) can now, with the exception of uncoupling, be extended to its congener, gentisic acid.

*Acknowledgements.* I should like to thank Professor M. J. H. Smith for continuous encouragement during this work and the Medical Research Council for generous financial assistance.

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**The effect of reserpine on amphetamine toxicity in aggregated mice**

SIR,—The enhancing effect of aggregation on the toxicity of amphetamine (Chance, 1946; Hohn & Lasagna, 1960; Mennear, 1965), can be prevented by prior administration of chlorpromazine (Lasagna & McCann, 1957), reserpine (Burn & Hobbs, 1958) and α- or β- adrenergic blocking agents (Mennear & Rudzik, 1965).

We have found the protection given by reserpine against the toxicity of amphetamine to be dependent on the dose of reserpine. Thus, doses of reserpine greater than 0·8 mg/kg intraperitoneally did not protect aggregated animals against amphetamine toxicity.

Male albino mice (Harlan Industries), 18–26 g, were given doses of reserpine 18–24 hr before (+)-amphetamine sulphate, 20 mg/kg i.p. Immediately after the administration of the amphetamine the mice were aggregated in groups of ten, in wire mesh cages measuring 15 cm on each side. The adrenergic blocking agents, MJ-1999 [4-(2-isopropylamino-1-hydroxyethyl)methane sulphonanilide], and phenoxybenzamine were administered 30 min before amphetamine was given to resperpine-pretreated animals.

In Table 1 it is seen that only doses of reserpine ranging from 0·1 to 0·3 mg/kg were effective in reducing the toxicity of amphetamine in aggregated mice. Doses of reserpine greater than 0·8 mg/kg did not reduce the % mortality produced by the 20 mg/kg dose of amphetamine.

TABLE 1. EFFECT OF VARYING DOSES OF RESERPINE ON THE TOXICITY OF AMPHETAMINE IN AGGREGATED MICE

| Reserpine dose (mg/kg i.p.) | No. dead / No. tested | % Mortality |
|---|---|---|
| — | 35/40 | 88 |
| 0·05 | 9/10 | 90 |
| 0·1 | 9/30 | 30 |
| 0·2 | 6/20 | 30 |
| 0·4 | 4/20 | 20 |
| 0·8 | 11/20 | 55 |
| 1·0 | 8/10 | 80 |
| 1·6 | 16/20 | 80 |
| 3·0 | 8/10 | 80 |
| 6·4 | 16/20 | 80 |

The β-adrenergic receptor blocking drug MJ-1999 (30 mg/kg i.p.) and the α-adrenergic receptor blocking agent phenoxybenzamine (10 mg/kg i.p.) antagonized the toxicity of amphetamine in groups of ten animals pretreated with doses of reserpine (1·0, 1·6, 3·0 and 6·4 mg/kg), only one death was recorded of the 100 animals tested; this was at a dose of 1·6 mg/kg i.p. reserpine + phenoxybenzamine.

The protective effect of low doses of reserpine on the toxicity of amphetamine in aggregated mice has been previously reported by Moore (1964). The lack of protection after large doses of reserpine was unexpected and may involve a toxicity of the reserpine itself. Since MJ-1999 and phenoxybenzamine are capable of blocking the toxicity of (+)-amphetamine after large doses of reserpine, the mechanism of action of reserpine in these tests remains obscure.

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## Desipramine and potentiation of noradrenaline in the isolated perfused renal artery

SIR,—Recently it was reported that desipramine potentiates the pressor effect of directly acting sympathomimetic amines in cats or rabbits (Sigg, Soffer & Gyermek, 1963) and in rats (Bonaccorsi, 1966). This action was thought to be the result of an inhibition of the uptake of noradrenaline by nerve endings (Iversen, 1965). We have now examined the interaction of desipramine and noradrenaline in an isolated sympathetically innervated peripheral tissue.

The isolated renal artery of the rat was found to show a constriction with a single dose of 0·1 $\mu$g of noradrenaline. The artery, removed from 200 $\pm$ 20 g Sprague-Dawley male rats, was cannulated and perfused by means of a peristaltic pump under constant flow with Krebs-bicarbonate solution (6–7 ml/min.) gassed with 95% oxygen and 5% carbon dioxide. There was about 5 mm of artery between the tip of the cannula and the open end through which perfusion fluid emerged. Under resting conditions the pressure was 40–50 mm Hg.

The constrictor response of the vessel was measured by raising the perfusion pressure recorded by a mercury manometer on a kymograph. The renal artery was immersed in a 50 ml bath with overflow and containing its own perfusion fluid at 37°.

TABLE 1. EFFECT OF COCAINE AND DESIPRAMINE ON PRESSOR RESPONSE OF ISOLATED RENAL ARTERY OF RAT TO NORADRENALINE

Perfusing pressure values (mm Hg) and resistance (R)[1] in presence of noradrenaline (bitartrate salt) before and after treatment with cocaine or desipramine

| 0·25 $\mu$g | | 1·0 $\mu$g | | 4·0 $\mu$g | |
|---|---|---|---|---|---|
| mm Hg $\pm$s.e. | R | mm Hg $\pm$s.e. | R | mm Hg $\pm$s.e. | R |
| 7 experiments. Drug: cocaine, 2·9 × $10^{-5}$M | | | | | |
| Before drug 61·2 $\pm$ 3·3 | 9·4 $\pm$ 0·5 | 81·9 $\pm$ 5·0 | 12·6 $\pm$ 0·8 | 104·4 $\pm$ 7·6 | 16·0 $\pm$ 1·1 |
| After drug 71·2 $\pm$ 3·3** | 10·9 $\pm$ 0·4** | 98·1 $\pm$ 4·6* | 15·1 $\pm$ 0·6* | 123·2 $\pm$ 7·7† | 18·9 $\pm$ 1·1* |
| 8 experiments. Drug: desipramine, 6·6 × $10^{-6}$M | | | | | |
| Before drug 64·7 $\pm$ 1·5 | 9·9 $\pm$ 0·3 | 86·5 $\pm$ 2·8 | 13·1 $\pm$ 0·5 | 112·3 $\pm$ 4·3 | 17·1 $\pm$ 0·8 |
| After drug 73·0 $\pm$ 2·6§ | 11·2 $\pm$ 0·6§ | 106·4 $\pm$ 4·4** | 16·2 $\pm$ 0·9§ | 142·6 $\pm$ 6·5* | 21·8 $\pm$ 1·3** |

The basal pressure was 45 $\pm$ 5 mm Hg. Optimal potentiation was obtained after 60 min of perfusion with desipramine and 10 min after perfusion with cocaine.
* $P < 0.001$. ** $P < 0.002$. † $P < 0.005$. § $P < 0.01$.
[1] Resistance = pressure (mm Hg)/flow (ml/min)

(–)-Noradrenaline (as bitartrate salt) was always injected in a volume of 0·1 ml through rubber tubing interposed just upstream from the cannula. For each preparation, the dose-response curve for noradrenaline was first established and the artery was then perfused with a solution containing desipramine, as hydrochloride, or cocaine, as hydrochloride. The dose-response curve of noradrenaline was then determined again. The sensitivity of preparations to

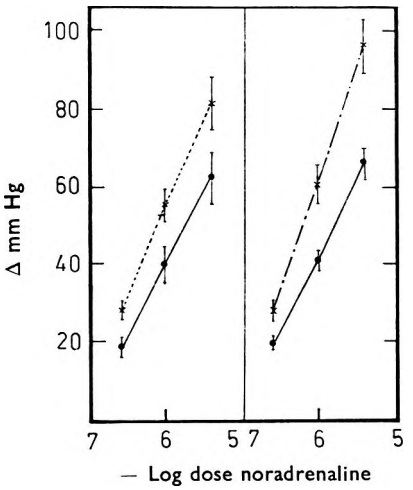


FIG. 1. Effect of cocaine and desipramine on the dose-response curve of noradrenaline in isolated perfused renal artery of rat; before ● — ● and after perfusion of artery with cocaine $2{\cdot}9 \times 10^{-5}$M (× - - - - ×) and desipramine $6{\cdot}6 \times 10^{-8}$M (× · — · — · ×).

noradrenaline in control experiments did not change significantly for several hours. Cocaine and desipramine did not show any effect on the baseline perfusion pressure. The results obtained are reported in Table 1 and in Fig. 1. Both cocaine ($2{\cdot}9 \times 10^{-5}$M) and desipramine ($6{\cdot}6 \times 10^{-8}$M) shift the dose-response curve of noradrenaline to the left.

The onset of the potentiating effect of desipramine required a longer time (60 min) than did that of cocaine (10 min).

These results are consistent with those obtained in rats in which the blood pressure response was measured (Bonaccorsi, 1966). They are also in agreement with the facts reported by Iversen (1965), who established that the ED50 of desipramine needed to inhibit the uptake of noradrenaline in the isolated rat heart was $1{\cdot}3 \times 10^{-8}$M, a concentration close to ours ($6{\cdot}6 \times 10^{-8}$M) shown here to potentiate noradrenaline contraction in the renal isolated artery.

The present results support the hypothesis that desipramine increases the pharmacological effect of noradrenaline by preventing its uptake by the arterial wall.

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**Indirect sympathomimetic actions of dopamine**

SIR,—Dopamine, the immediate precursor of noradrenaline, has direct sympathomimetic actions although there are reports which suggest that dopamine acts partly indirectly (Bülbring & Burn, 1938; Bejrablaya, Burn & Walker, 1958; Strömblad, 1960; Farmer, 1965). The acute administration of cocaine, or pretreatment with reserpine, enhances the response of smooth muscle structures to directly acting amines and decreases the response to indirectly acting amines (Tainter & Chang, 1927; Burn & Rand, 1958). Thus the response of the nictitating membrane, heart rate and blood pressure of spinal cats to graded doses of dopamine and their modification by cocaine or reserpine has been determined.

Cats weighing 2–3·5 kg were anaesthetised with halothane and made spinal by the method of Burn (1952). The movements of the right nictitating membrane were recorded with an isotonic, frontal writing point, lever (tension 7 g 15× magnification). Blood pressure was recorded from the right femoral artery with a mercury manometer. The heart rate was measured by a Devices Tachograph unit. Reserpine, 3 mg/kg, was administered intraperitoneally in 20% ascorbic acid 24 hr before the experiment. Cocaine hydrochloride, 5 mg/kg, was given as a slow intravenous injection 30 min before starting the dose-effect curve for dopamine.

The results are shown in Fig. 1. Table 1 compares the effects of cocaine and reserpine on the response of nictitating membrane, heart rate and blood pressure to dopamine, with the effect on response of these tissues to tyramine and noradrenaline taken from Fleming & Trendelenburg (1960).

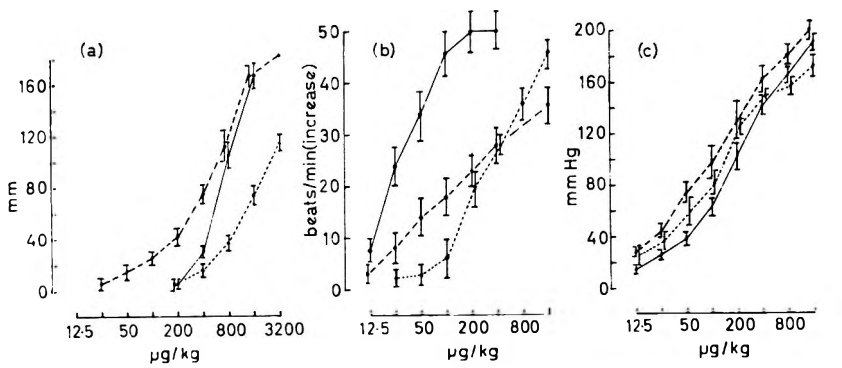


FIG. 1. Dose effect curves for dopamine on (a) nictitating membrane, (b) heart rate, (c) blood pressure of spinal cats. Each response is mean ± s.e. for 4 cats. ●——● control. ●- - - - - -●, resperpine pretreated (3 mg/kg/24 hr). ●— — —●, after cocaine 5 mg/kg i.v.

It is clear from the results in the Table that dopamine must be classified as a sympathomimetic amine with both direct and indirect actions. The extent to which the sympathomimetic response in the intact animal may be attributed to direct or indirect action depends upon the tissue studied. For instance the action of dopamine on the heart and nictitating membrane is mainly indirect whilst the vasopressor action is partly direct and partly indirect.

It has been suggested that dopamine might accumulate in sympathetic nerves and be released as a false transmitter after prolonged inhibition of the enzyme

TABLE 1. EFFECTS OF COCAINE AND RESERPINE ON THE RESPONSE OF CAT NICTITATING MEMBRANE, HEART RATE AND BLOOD PRESSURE TO DOPAMINE COMPARED WITH THE EFFECT OF THE RESPONSE OF THESE TISSUES TO TYRAMINE AND NORADRENALINE (FLEMING & TRENDELENBURG, 1960)

| Catechol-amine | Nictitating membrane | | Heart rate | | Blood pressure | |
|---|---|---|---|---|---|---|
| | Cocaine | Reserpine | Cocaine | Reserpine | Cocaine | Reserpine |
| Dopamine | Small doses (25–400 µg/kg) Increase. Maximum 4 fold | Decrease<br>Maximum 4 fold | Decrease<br>8 × | Decrease<br>8 × | Increase<br>2 × | Increase<br>1½ |
| Nor-adrenaline | Increase 30–60 × | No change | Increase | Increase | Increase 5 × | Increase 5 × |
| Tyramine | Decrease<br>2 × | Very marked decrease | Decrease<br>10 × | Abolished | Decrease<br>3 × | Very marked decrease |

monoamine oxidase (Farmer, 1965). Support for the hypothesis that dopamine can act as a false transmitter has since been demonstrated by Thoenen, Haefely, Grey & Hurlimann (1965). These authors observed that dopamine was released from the cat spleen in response to electrical stimulation of sympathetic nerves after treatment with the β-oxidase inhibitor disulphiram. The ability of dopamine to displace noradrenaline, i.e. to act indirectly, may partly account for the fall in noradrenaline content of certain tissues of cats treated with the monoamine oxidase inhibitor nialamide (Davey, Farmer & Reinert, 1963) or tissues of rats treated with the β-oxidase inhibitor disulphiram (Musacchio, Kopin & Snyder, 1964).

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# Book Reviews

*RECENT DEVELOPMENTS IN THE CHEMISTRY OF NATURAL CARBON COMPOUNDS, VOLUME* 1. By G. Fodor, K. Nador and I. V. Torgov. Pp. 319 (no Index). In English. Akademiai Kiado, Publishing House of the Hungarian Academy of Sciences, Budapest, 1965. 105s.

This book is divided into three sections. The first of these (160 pages) is a monograph by Professor G. Fodor about recent developments in the stereochemistry of the ephedrine, pyrrolizidine, granatoline and tropane alkaloids. He begins with some comments on methods which have been of particular value in elucidating the structures of these compounds. His subjects are those in which he is directly interested, much of the work having been done in his own department, consequently this account is authoritative and most useful. The chapter on tropane alkaloids is particularly extensive (78 pages) and must command the attention of all chemists interested in these compounds. Professor Fodor seems able to project his enthusiasm for his subject into his writing and this makes for stimulating reading.

The author of the second monograph (70 pages) is K. Nádor, who describes the results of the extensive biological tests which have been made on derivatives of tropine and pseudotropine. As in the first section, much of the work described was done in the author's own department. It is of particular value to English-speaking readers because many of the results have been published in Hungarian journals and have not been easily accessible until this account appeared. The section includes chapters on ability to block "muscarinic" receptors, receptors in ganglia and receptors at the neuromuscular junction, a short sub-section on local anaesthetic properties and a brief note of antihistamine activity and central effects. Although many questions remain unanswered, the review is of great importance, because work with rigid compounds like these tropeine derivatives is the most likely to lead to information about the relative positions of binding groups within the receptors.

In the last section (85 pages), I. V. Torgov reviews work on the total synthesis of natural steroids, equilenin, oestrone, androstane, derivatives of 11-desoxypregnane and of 11-hydroxypregnane, sapogenins, tomatidine, solasodine, vitamin $D_2$, conessine, lanosterol and digitoxigenin. It is clearly written and easy to follow and the diagrams of the reaction sequences are exceptionally well-produced. This review, like the other two sections, is well provided with references and is a very suitable introduction to the synthesis of this type of compound.

In the preface, Professor Fodor states that ". . . the individual feature of this series is that it collects for the first time the relevant work of Hungarian chemists . . ." and that ". . . it is hoped that the English speaking reader will find here material of interest that has been heretofore accessible to him only with difficulty". Considerable trouble has apparently been taken to see that the book lives up to these hopes. It is a pity that there is no index, but the book is arranged in a systematic way and the list of contents is extensive. The price, 5 guineas, is low enough to ensure it being readily accessible.

R. B. BARLOW

*HORIZONS IN NEURO-PSYCHOPHARMACOLOGY.* Edited by William A. Himwich and J. P. Schadé. (Progress in Brain Research, Volume 16). Pp. xii + 347 (including index). Elsevier Publishing Company, Barking, Essex, 1965. 100s.

This volume is dedicated to Dr. Harold E. Himwich, Director of the Thudichum Psychiatric Research Laboratory at Galesburg, Illinois, U.S.A., on the

occasion of his 70th birthday. It consists of contributions from past and present members of his laboratories and includes a list of Dr. Himwich's publications. As a timely acknowledgment of the signal achievements and industry of Dr. Himwich and his colleagues this volume is worthwhile; but it is difficult to recommend this book to the general reader because of the uneven nature of the contents, although several good discursive reviews are included. The specialised reader, however, may find something of interest in this volume.

Many of the contributions are concerned with the field of biological psychiatry. Smythies gives an account of the Osmond-Smythies' hypothesis of a disorder in methylation as the biochemical lesion in schizophrenia. He then describes his experiments to determine hallucinogenic properties in a wide variety of drugs on the basis of animal behaviour experiments. The role of amines in schizophrenia is developed by other contributors; Brune has studied the effect of psychotropic drugs on biogenic amine metabolism in schizophrenia, while Valcourt describes experiments showing that reserpine increases the urinary excretion of 5-hydroxyindoleacetic acid in mental patients.

Other contributors reflect a more basic neuropharmacological interest. Berlet gives a very interesting review of amino-acid metabolism in a wide range of amino acidopathies, including phenylketonuria, with and without cerebral dysfunction. A detailed account of his experiments on aceytcholine-acetylcholinesterase and 5-hydroxytryptamine-monoamine oxidase systems on whole animal behaviour is given by Aprison. Kobayashi describes the behavioural effects of various neurotropic drugs administered through the arachnoid space overlying the cortex. The effects on evoked potentials in the mid-brain reticular formation and the electroencephalogram of rabbits of a wide series of drugs active on the central nervous system are described by White, while Rinaldi discusses the effects of atropine applied topically and by infusion through a cortical artery on the electrical activity of the rabbit cortex.

D. W. STRAUGHAN

*PHARMACOGNOSY OF AYURVEDIC DRUGS.* (Kerala). By K. N. Aiyer and M. Kolammal. Pp. 4 + 129. Department of Pharmacognosy, University of Kerala, Trivandrum, India. Series 1, No. 7, 1963. Rs. 10.

Seventeen Indian plants yielding drugs used in Ayurvedic medicine are described in this number of the series dealing with indigenous drugs of Kerala. Each plant is considered under sub-headings, namely (1) notes in sanskrit text with transliterations and translations, (2) detailed description of the macroscopical structure and of the parts used medicinally, each illustrated by a good full-page drawing of the plant, (3) an account of the anatomy of the part or parts used medicinally, accompanied by careful drawings of their microscopical structure. One plant *Erythrina indica* is illustrated by a well-executed coloured drawing of the flowering plant, which forms a frontispiece to the book. Three of the plants are familiar in Britain; these are *Tinospora cordifolia* of which the dried stem is used and contains the alkaloid berberine; *Adhatoda vasica* the leaves of which contain the alkaloid vasicine, and *Terminalia chebula* which yields fruits that in the unripe and dried condition are known as *Chebulic Myrobalans*; these were in 1900 included in the Indian and Colonial Addendum to the British Pharmacopoeia 1898 and the Myrobalans were described also in the B.P. 1914. Throughout the book the drawings are labelled with the same lettering and an explanatory list is given in the introduction, thus avoiding printing a long legend for each drawing, of which there are 31 in the book. The whole forms a valuable contribution to the study of Indian drugs used in ayurvedic medicine.

T. E. WALLIS

Journal of Pharmacy and Pharmacology

APRIL 1966 VOL. 18 No. 4

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## Letters to the Editor



PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.