The Rate of Equilibration in a Competitive n Drug System and the Auto-Inhibitory Equations of Enzyme Kinetics: Some Properties of Simple Models for Passive Sensitization

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The rate of equilibration in a competitive n drug system and the auto-inhibitory equations of enzyme kinetics: some properties of simple models for passive sensitization

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The solution of the equation for the rate of equilibration of n drugs all competing for the same receptors is presented and the two-drug case is given explicitly. The case of two competing drugs with the same rate constants is discussed in detail as it is the simplest plausible model for the rate of passive sensitization. The predictions of this model are compared with present knowledge about the rate of onset and the persistence of sensitization. The model suggests that if the observed rate of sensitization depends on the rates of reaction with receptors then (1) equilibration will be too slow to be seen in the usual length of passive sensitization experiment *in vitro*, (2) there will be no fast initial phase of sensitization, and (3) sensitizing immunoglobulins which persist for a long time (e.g. those of man and rat) will have a slower rate of onset than those which persist for a shorter time (e.g. those of the guinea-pig). It is also emphasized that there are at present no firm grounds for supposing that the more persistent sensitizing antibodies have a higher affinity for cells than the less persistent since the association rate constants are unknown.

The model proposed by Mongar & Winne, which accounted for the auto-inhibition observed during passive sensitization by a mechanism involving two-point attachment of antibody to cells, is discussed in relation to uncompetitive and non-competitive models for auto-inhibition. These models all fit the available observations equally well but the last of them does not involve two-point attachment of antibody. The presence of non-specific immunoglobulin in the antibody preparation used is shown to have a potentially very serious effect on some of the parameter estimates in all three models, and corrections for errors from this source are discussed.

Introduction

It is now customary (see, for example, Mongar & Schild 1962; Austen & Humphrey 1963; Bloch 1967) to think of the process of sensitization as involving affinity of antibody immunoglobulin molecules for specific cellular sites, in competition with non-specific immunoglobulin molecules of the same type, which may be referred to as inhibitor. This interaction is usually assumed, implicitly or explicitly, to obey the law of mass action and it therefore seems desirable to formulate models so that quantitative experimental tests can be done, and so that inconsistent inferences are not made from experimental results.

The predictions of the simplest reasonable model for the rate of attainment of equilibrium, a competitive two drug system, will be considered first. Some alternative models based on equilibrium receptor occupancy in auto-inhibitory systems, all of which fit the available experimental sensitization results, will then be discussed.

THE RATE OF EQUILIBRATION IN A COMPETITIVE TWO-DRUG SYSTEM

The reaction of two drugs (A and B) with one sort of receptor (R) according the law of mass action is conventionally represented

$$\left.\begin{aligned} A+R &\underset{k_2}{\overset{k_1}{\rightleftharpoons}} AR, \\ B+R &\underset{k_4}{\overset{k_3}{\rightleftharpoons}} BR, \end{aligned}\right\} \tag{1}$$

and the corresponding rate equations are

$$\left.\begin{aligned} \frac{dp_A}{dt} &= k_1 A(1-p_A-p_B)-k_2 p_A, \\ \frac{dp_B}{dt} &= k_3 B(1-p_A-p_B)-k_4 p_B, \end{aligned}\right\} \tag{2}$$

where k_1, k_2, k_3 and k_4 are rate constants and p_A and p_B are the proportions of receptors occupied by drugs A and B. In almost all cases (see appendices) solution if of these equations gives the proportion of receptors occupied by drug A at time t, the concentrations of drugs A and B are changed at $t = 0$ from A_0 and B_0 ($t < 0$) to A and B ($t \geqslant 0$), as

$$p_A = c_{1A}\,e^{\lambda_1 t} + c_{2A}\,e^{\lambda_2 t} + p_A(\infty) \tag{3}$$

and the proportion occupied by drug B as

$$p_B = c_{1B}\,e^{\lambda_1 t} + c_{2B}\,e^{\lambda_2 t} + p_B(\infty), \tag{4}$$

where

$$\lambda_1 = -0{\cdot}5\{z + \sqrt{(z^2-w)}\}, \tag{5}$$

$$\lambda_2 = -0{\cdot}5\{z - \sqrt{(z^2-w)}\}, \tag{6}$$

$$z = k_2(D_A+1)+k_4(D_B+1), \tag{7}$$

$$w = 4k_2k_4(D_A+D_B+1). \tag{8}$$

The initial slopes of the occupancy-time curves are

$$p'_A(0) = k_2[D_A(1-p_A(0)-p_B(0))-p_A(0)], \tag{9}$$

$$p'_B(0) = k_4[D_B(1-p_A(0)-p_B(0))-p_B(0)], \tag{10}$$

where the concentration unit, D, is the ratio of concentration to dissociation constant (as used by Paton & Waud 1964). In full

$$\left.\begin{aligned} D_A &= (k_1/k_2)A, \quad D_{A_0} = (k_1/k_2)A_0, \\ D_B &= (k_3/k_4)B, \quad D_{B_0} = (k_3/k_4)B_0. \end{aligned}\right\} \tag{11}$$

If the receptors have been equilibrated with the drugs at $t < 0$ then the initial occupancies are

$$p_A(0) = \frac{D_{A_0}}{D_{A_0}+D_{B_0}+1}, \quad p_B(0) = \frac{D_{B_0}}{D_{A_0}+D_{B_0}+1}. \tag{12}$$

The final equilibrium occupancies will be

$$p_A(\infty) = \frac{D_A}{D_A+D_B+1}, \quad p_B(\infty) = \frac{D_B}{D_A+D_B+1}, \tag{13}$$

and if we define

$$\Delta p_A = p_A(0)-p_A(\infty), \quad \Delta p_B = p_B(0)-p_B(\infty),$$

the constants in equations (3) and (4) can be written

$$\left.\begin{aligned} c_{1A} &= (\Delta p_A - p'_A(0)/\lambda_2)/(1-\lambda_1/\lambda_2), \\ c_{2A} &= \Delta p_A - c_{1A}, \end{aligned}\right\} \tag{14}$$

$$\left.\begin{aligned} c_{1B} &= (\Delta p_B - p'_B(0)/\lambda_2)/(1-\lambda_1/\lambda_2), \\ c_{2B} &= \Delta p_B - c_{1B}. \end{aligned}\right\} \tag{15}$$

A method of deriving this solution is outlined in appendix 1. The general solution for the rate of equilibration of n drugs competing for the same receptors is given in appendix 2. Rang (1966) has studied the solution of equations (2) using an analogue computer.

Special cases of the above equations

A special case of this equation describing the rate of attainment of equilibrium when drug A equilibrates instantaneously has been given by Rang (1966), and equation (4) reduces to Rang's equation (10) if $(k_1A+k_2)\to\infty$. In the case of immunoglobulins the most reasonable *a priori* assumption is that the rate constants are the same for antibody immunoglobulin (drug A, say) and non-specific immunoglobulin of the same immunoglobulin type (drug B), although there is, so far, no direct evidence for this.

These equations do not take into account the possibility of auto-inhibition but if either the uncompetitive or non-competitive mechanisms (which are discussed later) were correct equations (3) and (4) would be applicable at low drug concentrations. It may be that, in the guinea-pig, the normal level of γ_1-globulin (about 1 mg/ml., Binaghi 1966; Colquhoun unpublished) is too high for the approximation to be good.

The simplest model for passive sensitization is thus the special case of equations (3) and (4) with $A_0 = 0$, $k_1 = k_3$ and $k_2 = k_4$. Some predictions of this model will be discussed.

In the case where the drugs have equal rate constants (as also in Rang's case) p_A and p_B can be expressed as functions of the dimensionless quantities D_{A_0}, D_{B_0}, D_A, D_B and k_2t only, and the shape of the calculated curves is therefore not dependent on the particular values of the rate constants chosen. When $k_2 = k_4$

$$\lambda_1 = -k_2(D_A+D_B+1), \tag{16}$$

$$\lambda_2 = -k_2. \tag{17}$$

It can also be shown that at any given time (at $t > 0$) p_A will be less than the value predicted by a simple exponential process with rate constant k_2, viz.

$$p_A = \Delta p_A\, e^{-k_2t} + p_A(\infty), \tag{18}$$

if the total drug concentration, $D_{A_0}+D_{B_0}$ (e.g. antibody plus non-specific immunoglobulin inhibitor), with which the tissue was initially in equilibrium is greater than the total drug concentration present at $t \geqslant 0$, i.e. if $(D_{A_0}+D_{B_0}) > (D_A+D_B)$, as long as $D_A \neq 0$. When this is the case c_{1A} will be positive. If $(D_{A_0}+D_{B_0}) = (D_A+D_B)$, or $D_A = 0$, then c_{1A} will be zero so that p_A will be given by equation (18) and if $(D_{A_0}+D_{B_0}) < (D_A+D_B)$, $D_A \neq 0$, then c_{1A} will be negative and p_A will be greater than the value given by equation (18). These equations have several interesting implications.

The rate of onset of passive sensitization

There are, of course, no reliable estimates of the rate constants for immunoglobulins. However, the dissociation rate constant k_2 is the rate constant for the desorption of drug A into a solution free of A (whether or not B is present). The observation that passive sensitization can be reversed little if at all by washing the tissue with physiological saline solution for a few hours suggests that *if* the rate of reversal is controlled by dissociation from receptors (as is commonly assumed) the dissociation rate constant k_2 must be rather small, and it is therefore of interest to discuss the implications of a slow dissociation rate constant. However, it should be emphasized that, although the slow rate of reversal cannot be accounted for by diffusion through the extracellular space (Brocklehurst & Colquhoun 1965), other explanations are possible. For example there could be a diffusion barrier separating the biophase (which contains the receptors) from the external solution, as proposed by Furchgott (1955, 1964) to account for the slowness of action of antagonist drugs. It has recently been shown by Rang (1966) that the rate of action of atropine is controlled by the rate at which it combines with and dissociates from receptors, but no comparable evidence exists for immunoglobulins.

Passive sensitization *in vitro* uses tissues recently removed from normal animals (i.e. $A_0 = 0$) with a substantial level (B_0) of non-specific immunoglobulin of the sensitizing type (7 S γ_1-globulin in the guinea-pig). Therefore when, for example, normal guinea-pig lung is sensitized with low concentrations of antibody (even if the antibody is not specifically purified) it is certainly true that $B_0 > (A+B)$ and thus (if k_2 is in fact small so that $p_B(0)$ is not reduced much during the washing of the tissue after removal from the animal) the rate of increase of p_A should be slower than given by equation (18), i.e. slower than the dissociation rate of antibody from receptors. Now the rate of sensitization observed experimentally is certainly *not* as slow as the rate of reversal of sensitization by washing the tissue. However, this does not necessarily mean that the latter cannot be dissociation controlled. If there were many more receptors than the number needed for maximal sensitization the observed rates could be consistent with the simple competitive model under discussion since a maximal response could be reached long before equilibrium. But the model *would* predict that the attainment of an equilibrium level of sensitization that was *submaximal* should have a rate constant of the order of k_2 and would be, therefore, probably too slow for equilibrium ever to be reached during the length of the usual experiment (for example if k_2^{-1} were, say, 12 h it would take 27·6 h for p_A to reach 90% of its equilibrium value according to equation (18)). The experimental evidence on this point is rather contradictory. Although it is often assumed

that equilibrium can be reached the observations of, for example, Mongar & Schild (1960), Brocklehurst & Colquhoun (1965) and Spuzic, Bloch & Austen (1966) all suggest that this remains to be demonstrated. We are at present investigating this point further using specifically purified guinea-pig antibodies.

The initial phase of sensitization

Another point of interest concerns the possible existence of a fast initial phase of passive sensitization (Mongar & Schild 1960, 1962). No evidence for such a phase was found by Brocklehurst & Colquhoun (1965). A fast phase is predicted by the model represented by equation (2) when λ_1 has a much larger negative value than λ_2 (i.e. if drugs A and B have the same dissociation rate constants, when $(D_A+D_B) \gg 1$) so that the term $c_{1A}e^{\lambda_1 t}$ rapidly disappears and p_A tends to jump towards $p_A^* = p_A(0)-c_{1A}$ (and similarly for p_B). When $p_A(0) = 0$ this can obviously only occur when c_{1A} is negative, i.e. if drugs A and B have the same rate constants, when

$$(A+B) > (A_0+B_0).$$

If c_{1A} is a large enough proportion of Δp_A such a jump is seen as a sharp bend or overshoot in the curve relating p_A and time. It is seen in figure 1 that this effect only becomes noticeable when $(A+B)$ is more than ten times B_0 and it is therefore

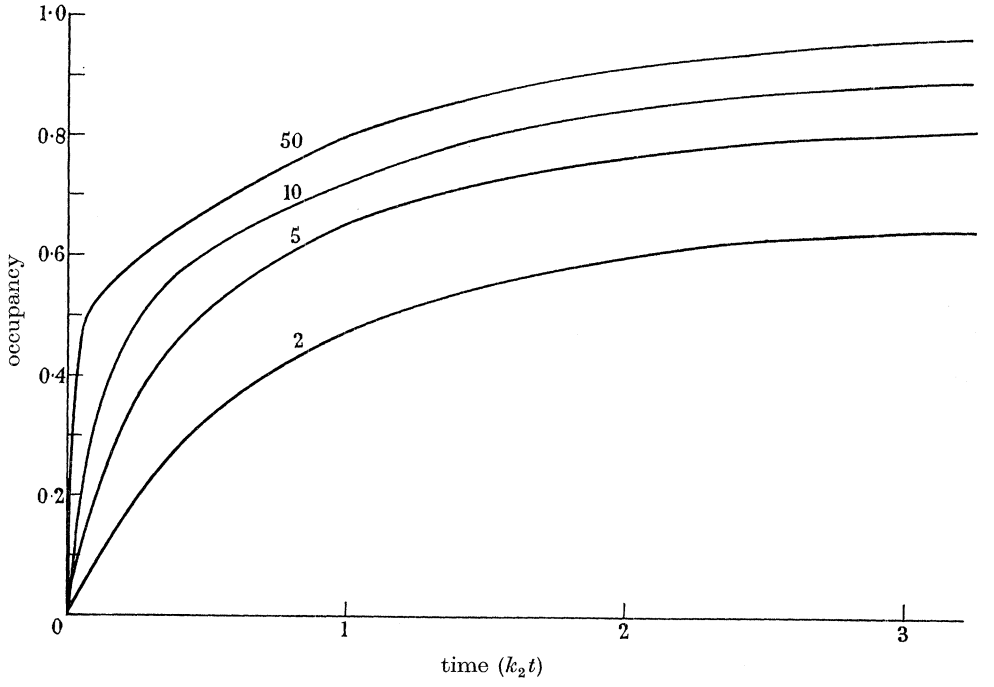


FIGURE 1. Calculated time course of receptor occupancy by drug A (representing antibody) using equation (3). Drug A is added at zero time in concentrations corresponding to $D_A = 2$, 5, 10 and 50, and inhibitor is removed so $D_B = 0$. The receptors have been previously equilibrated with drug B (representing non-specific immunoglobulin, supposed to have the same rate constants as A), at $D_{B_0} = 1$ so that 50 % of receptors are initially occupied by B. The abscissa is time expressed in units of the time constant for dissociation $(1/k_2)$.

a prediction of the model that an initial fast phase will only be seen when using a total concentration of immunoglobulin (antibody or inhibitor or both) far greater than that usually used for passive sensitization.

If $k_2 = k_4$ there cannot be an overshoot (i.e. a stationary point) in the curve for total occupancy, $p_A + p_B$, but there can be an overshoot in the p_A against time curve as long as $p_A(0) \neq 0$. Rang (1966) has observed experimentally the predicted overshoot in total occupancy using two inhibitors with different rate constants.

The latent period

The predictions of the simple competitive model may also be relevant to the frequently discussed 'latent period' for the onset of passive sensitization *in vivo*. The term is usually only vaguely defined, but the fact that the rate of onset of passive sensitization of skin *in vivo* appears to be lower for those immunoglobulins which persist for the longest time, those of man, rat, rabbit and dog (see reviews by Benacerraf (1967) and Bloch (1967)), is at least qualitatively consistent with the prediction of the simple mass action mechanism that the rate constants for onset as well as for offset should be of the order of k_2 when a small injected concentration of antibody (A) sensitizes submaximally a tissue which is in contact with much larger concentrations of autologous inhibitor immunoglobulin before (B_0) and during (B) sensitization, so $B_0 \simeq A + B$.

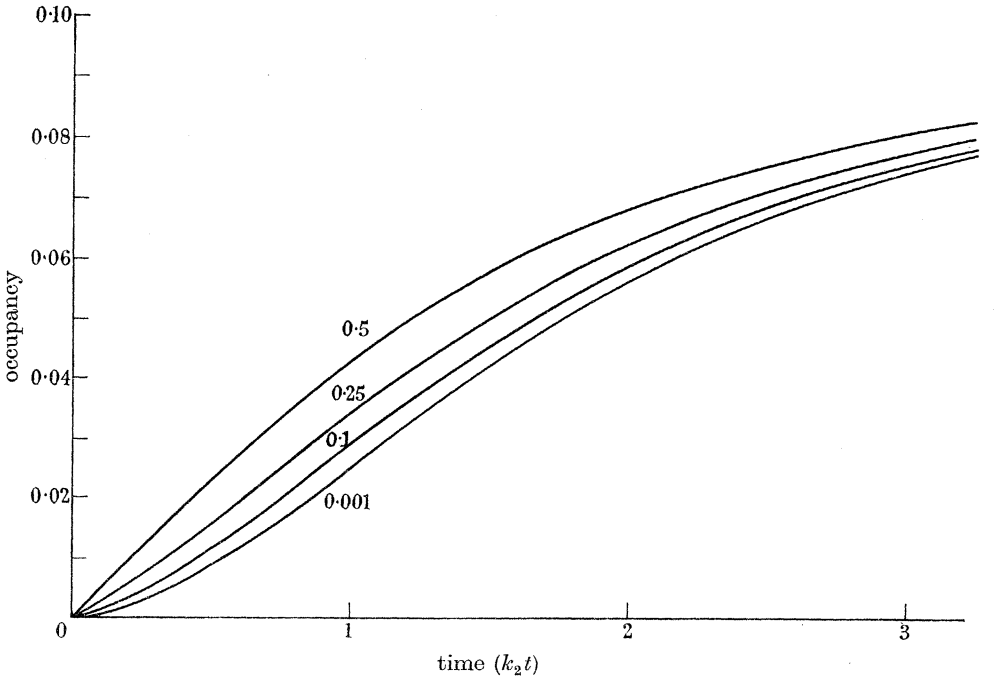


FIGURE 2. Calculated time course of receptor occupancy by drug A. Drug B (with the same rate constants as A) is initially present at concentrations ($D_{B_0} = 1$, 3, 9 and 999) such that the proportion of receptors free at zero time, $1 - p_B(0)$, is 0·5, 0·25, 0·1 and 0·001. Drug B is removed at zero time and A added in a concentration corresponding to $D_A = 0{\cdot}1$. The abscissa is the same as in figure 1.

Figure 2 shows the calculated rates when the inhibitor initially present in relatively high concentration (B_0) is removed at the same time that a low concentration of $A(D_A = 0{\cdot}1)$ is added. If $B = 2A$ (rather than $B = 0$), as might be the case if antibody was not specifically purified, the rate is decreased and the rate constant increased but the shape is not drastically altered. Lower concentrations of A give similarly shaped curves.

It has been suggested (Binaghi & Benacerraf 1964; Benacerraf 1967) that the long latent period for sensitization of skin by the reaginic type of anaphylactic antibody might be needed for exchange with non-specific immunoglobulin already bound and the above arguments give some sort of quantitative basis for this mechanism (though the antibody concentration at the site of injection, D_A, must be very far from constant during the latent period).

It is, perhaps, worth pointing out that if the antibody concentration needed for sensitization is low enough ($D_A \ll 1$) the rate constant for onset of submaximal sensitization (as well as for offset) would be expected to be of the order of k_2 whether or not there was non-specific immunoglobulin already bound to the tissue, so it may be quite unnecessary to suppose that the slowness is caused by an exchange process. This follows from the fact that the onset of antibody occupancy in the absence of inhibitor would have the rate constant $k_2(D_A + 1)$.

The minimum rate of onset

It can be shown that when drugs A and B have the same rate constants, and when $D_{B_0} \to \infty$ so that the receptors are all initially occupied, and when the agonist concentration is very low ($D_A \to 0$, $D_B \to 0$) the onset rate approaches a curve similar in shape to the lowest one in figure 2. In this limiting case p_A takes a time $t = 2{\cdot}146/k_2$ to reach $(1 - 1/\mathrm{e}) \times 100 = 63{\cdot}2\,\%$ of its equilibrium value, and $t = 3{\cdot}890/k_2$ to reach 90 % of its equilibrium value.

The rate of reversal of sensitization

The faster rate of reversal of sensitization in the presence of high concentrations of immunoglobulins reported by Halpern, Liacopoulos, Liacopoulos-Briot, Binaghi & van Neer (1959) and by Mongar & Schild (1960) could only be accounted for by the simple rate equations described if diffusive delay maintained a substantial antibody concentration near the receptors during the washing process. However, the auto-inhibition mechanisms to be discussed probably *can* account for this phenomenon. Further investigation of these possibilities is unlikely to be worth while until more is known about the rate constants.

Models for auto-inhibition

Mongar & Schild (1960) suggested that antibody was attached to cellular receptors by more than one bond. Recently Mongar & Winne (1966) have found that when histamine release is plotted against the antibody concentration used for passive sensitization in the presence of various concentrations of non-specific immuno-

globulin, a series of bell-shaped curves is observed. These results could be quantitatively accounted for by a theory involving double attachment of antibody to cellular receptors. As Mongar & Winne pointed out, their model is not unique. Two other models which fit the available results equally well will be described. It is also possible that the shape of the curves is affected by the antigen–antibody ratio. The consequences of the presence of non-specific immunoglobulin in the antibody preparation, and methods of parameter estimation will be discussed.

All the models to be discussed assume that the observed effect, E (while it is submaximal) is directly proportional to the proportion of receptors effectively occupied by antibody p_A. The proportionality constant, k_e, is related to the efficacy of antibody in the sense of Stephenson (1956). Its value is not restricted and a value much greater than the maximum possible effect would suggest the existence of spare receptors. The assumption of proportionality is unlikely to be exactly true. If there were spare receptors it would imply a sharp bend in the dose-response curve (i.e. a discontinuity in its first derivative) when the maximum response ($E_{\max}$) was reached. This will formally be the case for any sort of occupancy-effect relationship such that the maximum response of the tissue is attained before the product $k_e p_A$ has reached its maximum value (k_e). However, the assumption could be roughly correct over a sufficient range to account for the fact that the models fit the available experimental results.

1. *Model of Mongar & Winne*

The model proposed by Mongar & Winne (1966) was as follows:

$$\left.\begin{aligned} A + R &\rightleftharpoons AR \quad \text{affinity constant } K_1, \\ AR + R &\rightleftharpoons AR_2 \quad \text{affinity constant } K_2, \end{aligned}\right\} \tag{19}$$

where R represents a cellular receptor and A represents an antibody molecule. The inhibitor molecule, represented by B, is supposed to react in a similar way with the receptors but with affinity constants K_1' and K_2'. If the second reaction, the combination of a singly bound antibody molecule with a second surface receptor to give the anaphylactically effective complex AR_2, conformed to the simple law of mass action, the result would be the equation derived by Mongar & Winne, viz.

$$E = 2k_e K_1 K_2 R_0 A R^2, \tag{20}$$

where E is the percentage of histamine released from the lung tissue, k_e is the proportionality constant mentioned above, A is the antibody concentration and R is the concentration of free receptors as a fraction of the total concentration, R_0, of receptors. R is given by

$$R = \{-n + \sqrt{(n^2 + 8d)}\}/4d, \tag{21}$$

where

$$n = 1 + K_1 A + K_1' B, \tag{22}$$

$$d = K_1 K_2 R_0 A + K_1' K_2' R_0 B, \tag{23}$$

and B is the concentration of non-specific immunoglobulin inhibitor present. It is a consequence of the model that the absolute values of K_2 and K_2' cannot be estimated, but only $K_2 R_0$ and $K_2' R_0$.

2. *Non-competitive auto-inhibition in the presence of a competitive inhibitor*

The only mechanism of substrate inhibition for which evidence exists is the non-competitive mechanism which Hofstee (1955) found could account for the inhibition of xanthine oxidase by its substrate, xanthine. This mechanism involves single point attachment of antibody to cells but fits the results of Mongar & Winne as well as the models which postulate two point attachment (see figure 3).

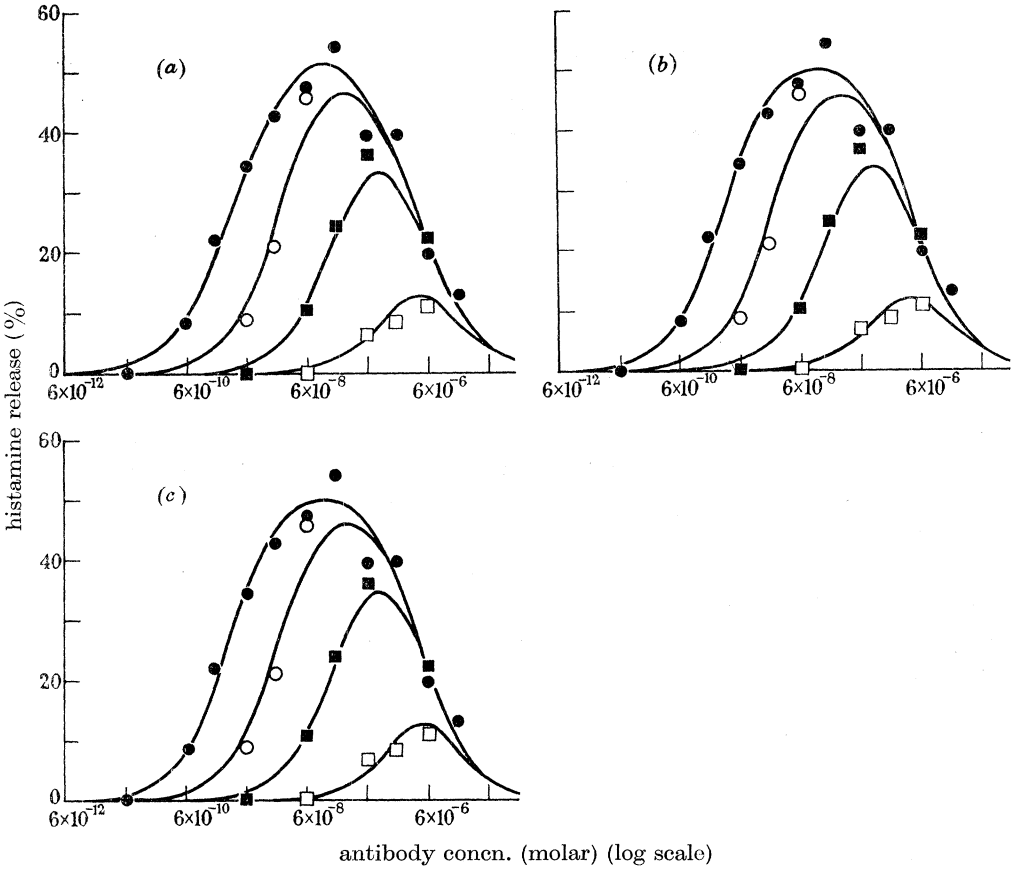
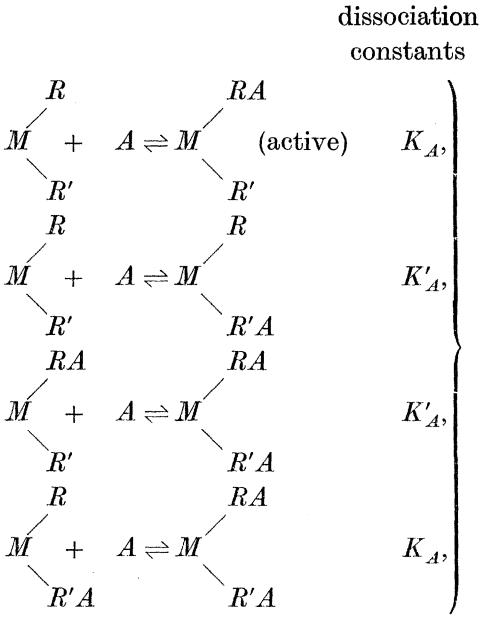


FIGURE 3. Calculated curves fitted by the method of least squares to the observations of Mongar & Winne (1966). (*a*) The model of Mongar & Winne (equation (20)); (*b*) non-competitive auto-inhibition (equation (25)); and (*c*) uncompetitive auto-inhibition of the Haldane type (equations (27) and (28)). These curves have been fitted with $r = 0$, but curve (*a*) would be identical and the others would not be noticeably changed (the parameter estimates being altered appropriately) if there were non-specific immunoglobulin in the antibody preparation. Concentration of non-specific immunoglobulin added: ●, none, ○, $4{\cdot}29 \times 10^{-8}$ M; ■, $4{\cdot}29 \times 10^{-7}$ M; and □, $4{\cdot}29 \times 10^{-6}$ M.

The mechanism requires that two sorts of cellular receptor exist, say R and R'. One of these (R) when combined with antibody results in sensitization of the cell so that histamine is released on subsequent contact with antigen, but only as long as the other receptor (R') is *not* combined with immunoglobulin (antibody or

non-specific). If M represents the cell membrane then dissociation constants are defined as follows:



(24)

where A represents an antibody molecule. Eight more analogous equations represent the combination of non-specific (inhibitor) immunoglobulin with the same receptors (with dissociation constants K_B and K'_B), and the formation of mixed complexes.

Hofstee (1955) considers only the cases in which the inhibitor combines with *either R or R'*. Obviously when both agonist and antagonist are immunoglobulins the approach must be extended to the case when the inhibitor can combine with *both* receptors. When this is done the histamine release (percentage) is predicted to be

$$E = \frac{k_e}{\left(1+\frac{K_A}{A}+\frac{K_A B}{A K_B}\right)\left(1+\frac{A}{K'_A}+\frac{B}{K'_B}\right)}. \tag{25}$$

Hofstee's equations (9) and (13) are special cases of this equation.

3. *Uncompetitive auto-inhibition in the presence of a competitive inhibitor*

The shape of the curve obtained by Mongar & Winne (1966) suggests the bell-shaped plots of reaction velocity against log substrate concentration which Haldane (1930) attempted to explain by means of a reaction mechanism which involved a two-point attachment of substrate to enzyme. This mechanism has been used to explain the substrate inhibition shown by acetylcholinesterase, and van Rossum (1964) has obtained curves showing the competitive inhibition of acetylcholinesterase which look very much like those in figure 3.

In terms of passive sensitization Haldane's mechanism suggests that only two-point attachment of the antibody molecule to the cell results in passive sensitization,

whereas the single point attachment predominating at high antibody concentrations is ineffective.

If a single divalent receptor, as conceived by Haldane, is symbolized R, then the reactions to be considered are as follows:

$$\begin{array}{lll} & & \text{dissociation constants} \\ R\diagup\!\diagdown + A \rightleftharpoons R\frown A \quad \text{(active)} & & K_A, \\ R\frown A + A \rightleftharpoons R\begin{array}{l}\diagup A\\ \diagdown A\end{array} & & K'_A, \\ R\diagup\!\diagdown + B \rightleftharpoons R\frown B & & K_B, \\ R\frown B + B \rightleftharpoons R\begin{array}{l}\diagup B\\ \diagdown B\end{array} & & K'_B, \\ R\frown A + B \rightleftharpoons R\begin{array}{l}\diagup A\\ \diagdown B\end{array} & & K'_{AB}, \\ R\frown B + A \rightleftharpoons R\begin{array}{l}\diagup A\\ \diagdown B\end{array} & & K'_{BA}, \end{array} \tag{26}$$

Again Hofstee, and other authors who have considered this mechanism, treat only special cases for the operation of the inhibitor. The full set of reactions shown above, which are required when both agonist and antagonist are immunoglobulins, implies the following expression for the percentage of histamine released:

$$E = \frac{k_e}{1 + \frac{K_A}{A} + \frac{K_A B}{A K_B} + \frac{A}{K'_A} + \frac{B^2 K_A}{A K_B K'_B} + \frac{B}{K'_{AB}}} \tag{27}$$

Hofstee's equation (3) is a special case of this relationship. This uncompetitive mechanism is obviously closely related to that proposed by Mongar & Winne, but because of the way the reactions are symbolized the relationship is not immediately obvious. The reaction written by Haldane as

$$R\frown A + A \rightleftharpoons R\begin{array}{l}\diagup A\\ \diagdown A\end{array}$$

might actually take place in two stages, thus:

$$R\frown A \rightleftharpoons R\diagup\!\diagdown A \quad \text{and} \quad R\diagup\!\diagdown A + A \rightleftharpoons R\begin{array}{l}\diagup A\\ \diagdown A\end{array}.$$

This would show one difference between Haldane's mechanism and that of Mongar & Winne. Haldane implicitly assumed the rate of cyclization of $\overset{A}{\diagdown R\diagup}$ to be proportional to the concentration of $\overset{A}{\diagdown R\diagup}$ whereas Mongar & Winne assumed it to be proportional to the product of this concentration with the concentration of free receptors.

At low concentrations of A and B both the non-competitive and uncompetitive models reduce to the usual competitive inhibition equations and the rising parts of the curves in figures 3*b* and 3*c* are parallel. A similar analogue of the Mongar & Winne model with a negligible concentration of singly bound molecules is obtained

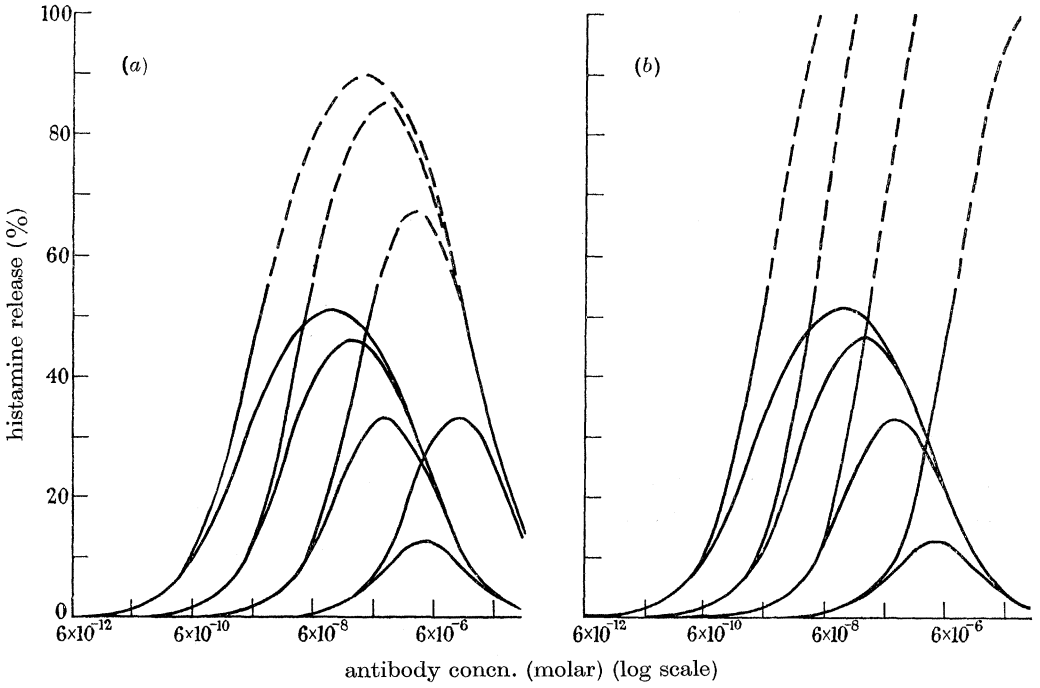


FIGURE 4. An example of the potential effects of incorrect assumptions about the proportion of non-specific immunoglobulin in the antibody. The lower sets of curves are the same as those in figure 3(*a*). The upper sets of curves are calculated for pure antibody ($r = 0$) using the model of Mongar & Winne with the parameters estimated from their observations assuming that their antibody preparation in fact had (*a*) 50 % specific antibody ($r = 1{\cdot}0$), and (*b*) 41·7 % specific antibody ($r = 1{\cdot}4$). The sets of four curves correspond to the inhibitor concentrations specified in figure 3. The curves should be cut off at the unknown maximum histamine release (not necessarily 100 % release) and are therefore broken at values greater than the largest observed release.

if K_2 and K_2' are increased and K_1 and K_1' are decreased such that K_1K_2 and $K_1'K_2'$ remain constant. This, however, does not lead to the usual competitive inhibition equations, and the rising curves in figures 3*a*, 4*a* and 4*b* are not parallel.

These points suggest that it might be better to allow for the concentrations of the species $R\begin{smallmatrix}/^{A}\\ \backslash\end{smallmatrix}$ and $R\begin{smallmatrix}/^{B}\\ \backslash\end{smallmatrix}$ which do not appear in Haldane's formal reaction scheme. One way of doing this results in the addition of $[\sqrt{(K_A/K'_A)}+(BK_A)/\{A\sqrt{(K_BK'_B)}\}]$ to the denominator of equation (27) but this has little effect in the present case.

This approach to Haldane's mechanism allows K'_{AB} to be expressed in terms of the other equilibrium constants making no more drastic assumptions about the independence and equivalence of receptors than have already been made. The result,

$$K'_{AB}=\sqrt{\left(\frac{K_BK'_BK'_A}{K_A}\right)}, \tag{28}$$

allows one arbitrary parameter to be eliminated from equation (27), leaving five arbitrary parameters as in equations (20), (22) and (23) together, and as in equation (25).

Curves calculated from equation (27) with equation (28) are almost indistinguishable from those calculated using the other models, as shown in figure 3.

The consequences of not using pure antibody

If sensitization is performed with a whole antiserum, or with whole (not specifically purified) immunoglobulin, the presence of non-specific immunoglobulin in the antibody preparation needs to be allowed for in the equations. If the ratio of non-specific to antibody immunoglobulin in the antibody preparation is denoted by r then the inhibitor concentration, B, in all the equations given should be replaced by

$$B = \textit{added } B+(A\times r), \tag{29}$$

where *added* B is the concentration of inhibitor added deliberately and A is the concentration of antibody (not total protein) added. For most hyperimmune rabbit antisera r would be expected to have a value between 1 and 3.

When equation (29) is substituted in equations (20), (25) and (27) it is found that the form of the equations for the percentage histamine release is exactly (Mongar & Winne, and uncompetitive models) or approximately (non-competitive model) the same whether $r = 0$ or not. In other words, if one of these models fits the observations when r is given its correct value, it can be made to fit for any other value of r merely by adjusting the parameter estimates. The parameter estimates obtained when r is erroneously taken to be zero will be referred to as the apparent parameters.

There is an exact relationship between the true and apparent parameters for Mongar & Winne's model. The relationship is also exact for the uncompetitive model if K'_{AB} is separately estimated but only a good approximation if equation (28) is used. The non-competitive model also gives rise to a relationship which is a good approximation.

It is found that the estimates of the two inhibitor equilibrium constants are unaffected by the presence of non-specific immunoglobulin in the antibody but the estimates of the other three parameters depend on the value of r.

The Mongar & Winne model gives rise to the following relationships:

$$K_1 = K_1(app) - rK_1', \tag{30}$$

$$K_1' = K_1'(app), \tag{31}$$

$$K_2R_0 = \frac{K_1(app)\,K_2R_0(app) - rK_1'K_2'R_0}{K_1(app) - rK_1'}, \tag{32}$$

$$K_2'R_0 = K_2'R_0(app), \tag{33}$$

$$k_e = \frac{K_1(app)\,K_2R_0(app)\,k_e(app)}{K_1K_2R_0}. \tag{34}$$

For the uncompetitive model the analogous relationships, if K'_{AB} is independently estimated, are

$$K_A = \frac{1}{1/K_A(app) - r/K_B}, \tag{35}$$

$$K_B = K_B(app), \tag{36}$$

$$K'_A = \frac{1}{\dfrac{(1+rK_A/K_B)}{K'_A(app)} - \dfrac{r^2K_A}{K_BK'_B} - \dfrac{r}{K'_{AB}}}, \tag{37}$$

$$K'_B = K'_B(app), \tag{38}$$

$$k_e = \frac{k_e(app)}{1 - rK_A(app)/K_B}, \tag{39}$$

$$K'_{AB} = \frac{1}{\dfrac{(1+rK_A/K_B)}{K'_{AB}(app)} - \dfrac{2rK_A}{K_BK'_B}}. \tag{40}$$

Similar relationships can be found for the non-competitive model which, though not exact, are a good approximation in the present case.

In principle the apparent values of the parameters could be corrected to allow for the presence of inhibitor, if the value of r were known. However, the correction is found to be impossible in practice unless the proportion of inhibitor is small because the correction becomes exceedingly sensitive to the value of r (and also to experimental errors in the estimation of the apparent parameters) as the proportion of inhibitor increases. Thus, if equation (30) is applied to the results of Mongar & Winne (using either their parameter estimates or the least squares estimates discussed below) the corrected value of K_1 appears to be negative even if a value as small as 1·0 is assumed for r. Because of this finding the concentration of γ-globulin in the normal rabbit serum used by Mongar & Winne as an inhibitor has been estimated immunochemically. Using a small-scale modification of the method of Darcy (1960) similar to that described by Darcy (1965), with an unabsorbed goat anti-rabbit-γ-globulin serum (kindly given by Dr J. H. Humphrey), the γ-globulin

content was estimated to be 6·4 mg/ml., considerably bigger than the electrophoretic estimate of 1·9 mg/ml. When the estimate of the inhibitor concentration is changed the estimate of K_1' only is altered. When the new estimate of K_1' is inserted in equation (30) Mongar & Winne's results give non-negative parameter estimates with any value of r up to 1·41 (i.e. the antibody preparation must have contained at least 41 % of specific antibody if the results are to be compatible with the theory). It follows from equations (30) to (34) that if the presence of non-specific immunoglobulin in the antibody is ignored the value of k_e found is only a minimum value, and the value of the affinity constant K_1 is a maximum value.

Similar results are obtained using the uncompetitive and non-competitive models. The apparent value of k_e is again only a minimum value, and the apparent value of the dissociation constant K_A is also a minimum value.

It can be seen that the presence of non-specific immunoglobulin in the antibody preparation can lead to very great uncertainty in the parameter estimates (except those for non-specific immunoglobulin which are unaffected), as illustrated in figure 4. It cannot therefore be stated with any certainty that the affinities of antibody and non-specific immunoglobulins for cellular receptors are the same, although *a priori* it seems likely that they will be.

A further point of interest is that it is possible for auto-inhibition to arise solely as a result of the presence of non-specific immunoglobulin in the antibody preparation, though this could happen only if the affinity constants for specific and non-specific immunoglobulin were very different. This situation would arise if $K_A' \gg K_B'$ in the case of both the non-competitive and the uncompetitive models. In the case of Mongar & Winne's model it is found that if K_2 is increased and K_1 is decreased so that the product K_1K_2 remains constant the curves predicted for pure antibody show little or no auto-inhibition. The parameter estimates necessary to produce the results just described are not incompatible with the experimental results available at the moment, but there is no decisive evidence for or against them.

Estimation of the parameters

Linearizing transformations allow estimation of the parameters (the affinity constants and k_e) from experimental results in some simple cases (Dixon & Webb 1964; Hofstee 1955). These methods cannot be used to estimate all the parameters in any of the models described, and furthermore Dowd & Riggs (1965) have shown that in the case of the simple Michaelis–Menten equation they may, unless properly weighted, give very poor estimates of the parameters. Simultaneous least squares estimation of all parameters might be expected on theoretical grounds to give better estimates of the parameters (if the scatter of observations were roughly constant). It has been shown that this is actually so in the case of the Michaelis–Menten equation (Colquhoun, unpublished). Curve fitting programmes for each of the models have therefore been written in Algol for the University of London Atlas computer. The parameter estimates which minimize the sum of squared deviations between the observed and calculated histamine releases were found using a minimization procedure, *patternsearch* written by M. Bell of the University of London

Institute of Computer Science. (The algorithm of Bell & Pike (1966) is very similar.)

The least squares parameter estimates for various models obtained using the observations of Mongar & Winne (assuming various values for r) are shown in table 1. These values have been used to calculate the curves shown in figures 3 and 4. The value of the maximum possible histamine release is not known (though given suitable data the methods used could probably be modified to estimate it) but it is not necessarily 100 % release. The upper sets of curves in figure 4 would, according to the model used, become flat topped at $E = E_{max}$ when E calculated from equation (20) became greater than E_{max}.

TABLE 1. LEAST SQUARES ESTIMATES OF THE PARAMETERS OF THE MODELS DESCRIBED CALCULATED USING THE OBSERVATIONS OF MONGAR & WINNE WITH THE REVISED INHIBITOR CONCENTRATION OF 6·4 mg/ml.

Concentrations were molar assuming a molecular weight of 150000. The sum of squared deviations (S.S.D.) is given as an indication of the relative badness of fit of the calculated curves.

model	r	K_1	K_1'	K_2R_0	$K_2'R_0$	k_e	S.S.D.
Mongar & Winne	0	$8{\cdot}5 \times 10^6$	$6{\cdot}0 \times 10^6$	17·6	9·1	71·7	195·5
	1	$2{\cdot}5 \times 10^6$	$6{\cdot}0 \times 10^6$	38·5	9·1	113	195·5
	1·4	$0{\cdot}15 \times 10^6$	$6{\cdot}0 \times 10^6$	486	9·2	147	195·5
		K_A	K_B	K_A'	K_B'	k_e	
non-competitive auto-inhibition	0	$3{\cdot}0 \times 10^{-9}$	$7{\cdot}8 \times 10^{-9}$	$4{\cdot}5 \times 10^{-6}$	$3{\cdot}9 \times 10^{-6}$	52·6	206·2
	1	$5{\cdot}1 \times 10^{-9}$	$7{\cdot}6 \times 10^{-9}$	infinite	$4{\cdot}4 \times 10^{-6}$	87·9	207·0
uncompetitive auto-inhibition	0	$3{\cdot}0 \times 10^{-9}$	$8{\cdot}0 \times 10^{-9}$	$4{\cdot}4 \times 10^{-6}$	$1{\cdot}9 \times 10^{-6}$	52·5	199·6
	1	$4{\cdot}9 \times 10^{-9}$	$7{\cdot}9 \times 10^{-9}$	19×10^{-6}	$3{\cdot}1 \times 10^{-6}$	85·3	203·9
	1·4	$6{\cdot}6 \times 10^{-9}$	$7{\cdot}8 \times 10^{-9}$	$1{\cdot}2 \times 10^{7}$	$3{\cdot}4 \times 10^{-6}$	115	204·6

It is well known that K_B can be estimated from the horizontal separation of the rising part of the response–log dose curves and that this estimate is independent of assumptions about the occupancy–response relationship. The fact that estimates obtained in this way are much the same as the values estimated assuming direct proportionality (given in table 1) is merely a reflexion of the fact that the curves calculated on the basis of this assumption fit the observations reasonably well.

DISCUSSION

It is not proposed that any of the models presented represent reality, but merely that they are the simplest quantitative consequences of the receptor mechanisms now generally (but qualitatively) considered (see Mongar & Schild 1962; Austen & Humphrey 1963; Bloch 1967) to be involved in the process of sensitization. Therefore, by the application of Occam's razor, it is these models which must be shown to be wrong before further progress can be made. Their consequences for the conduct of future experiments and the interpretation of past ones are worth brief discussion.

First, it remains to be shown that equilibrium submaximal sensitization can be reached *in vitro* with low antibody concentrations in the time usually available. If

the simplest rate theory were true it probably could not and this would make difficult the testing of models based on the assumption of equilibrium.

Secondly, it has been shown that because the onset of action of long-persisting sensitizing antibodies is slow it cannot necessarily be inferred that the slowness depends on an exchange process of antibody for inhibitor already occupying receptors (although this may nevertheless be what occurs) even if the observed rates depend on the rate of association with and dissociation from receptors.

Thirdly, no initial fast phase of passive sensitization is predicted except at very high immunoglobulin concentrations.

Furthermore, no inference can be made about the relative affinities of different sorts of sensitizing antibodies for receptors from qualitative observations of the onset rate or of the persistence of sensitization. In the simplest case of dissociation of antibody into an antibody-free solution the decrease in receptor occupancy would have a rate constant k_2, and since k_1 is completely unknown it cannot be assumed that because an antibody is very persistent (k_2 small) that it must necessarily have a high affinity (k_1/k_2) for receptors. In reality the situation is even more difficult to interpret as it will also involve diffusion, efficacy, spare receptors and the relation between p_A and histamine release. It is therefore perhaps premature to speak of reaginic antibodies as being 'cytophilic' or 'high affinity' antibodies.

The existence of spare receptors is quite compatible with available experimental results and also with the models which have been discussed. They are thought to exist for conventional drugs (see, for example, Paton & Rang 1966). If there are spare receptors it might have interesting implications for the reversibility of sensitization. It has been shown that the reversal of passive sensitization by non-specific immunoglobulin becomes more difficult with time (Mongar & Schild 1962) and that active sensitization is very difficult to reverse. It has been proposed that this is because additional bonds between antibody and receptor are formed with time. Until it is definitely shown that a steady level of *submaximal* sensitization becomes more difficult to reverse with time an alternative explanation is that maximal sensitization is reached after a fairly short time but antibody continues to occupy spare receptors until, after a longer time, equilibrium is reached. Thus a certain amount of antibody (increasing with time until equilibrium occupancy was reached) would have to be removed, or made ineffective, before any reversal of sensitization was seen.

It is evident that the experimental results available at the moment are compatible with mechanisms involving two-point attachment of antibody, but that, as Mongar & Winne (1966) thought likely, they are also compatible with a mechanism which does not involve two-point attachment, apart from the possibility that the shape of the observed curves may depend on the antigen concentration. Furthermore, the presence of non-specific immunoglobulin in the antibody preparation can have a very large effect on the parameter estimates so that it is not at present possible to be sure that the affinities of antibody and of non-specific immunoglobulins are the same or even that the observed auto-inhibition was not caused entirely by the presence of such non-specific immunoglobulin, unlikely as this seems.

The method of least squares estimation of parameters, apart from its simplicity

and probable greater precision, may in general have a considerable advantage over trial and error fitting of complex models since it is much more likely to be noticed if the fit of the calculated curves to the results is insensitive to the values of some parameters. Under some conditions the value of a parameter was in fact found to be indeterminate, but if the fitting had been done by trial and error the first guess at value of this parameter would have been found to give an adequate fit and would probably have been accepted.

The only way to remedy the present uncertainties is to use purified antibodies which contain a minimum (but known) proportion of non-specific immunoglobulin, and a range of antigen concentrations. Such experiments are now in progress.

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Appendix 1

If we define

$$\Delta p_A(t) = p_A(t) - p_A(\infty),$$

then the equations to be solved can be written in the form

$$\frac{d\Delta p_A}{dt} = a_{11}\Delta p_A + a_{12}\Delta p_B, \tag{A 1}$$

$$\frac{d\Delta p_B}{dt} = a_{21}\Delta p_A + a_{22}\Delta p_B, \tag{A 2}$$

where $a_{11} = -k_2(D_A+1)$, $a_{12} = -k_2 D_A$, $a_{21} = -k_4 D_B$ and $a_{22} = -k_4(D_B+1)$. If equation (A 1) is rearranged to give Δp_B and substituted in equation (A 2) the resulting second-order differential equation can be solved for p_A as described, for example, by Kaplan (1958, p. 123). The values of λ_1 and λ_2 are found by solving the quadratic characteristic equation

$$(a_{11}-\lambda)(a_{22}-\lambda) - a_{12}a_{21} = 0.$$

The constants c_{1A}, c_{2A}, c_{1B} and c_{2B} can be found from the initial values of Δp and of the slope p'. Equivalently the method described, for example, by Kaplan (1958, p. 224) can be used.

If $\lambda_1 = \lambda_2$ equations (14) and (15) cannot be used to find the constants and equations (3) and (4) may not be the correct solution. If $k_2 = k_4$ the two roots are equal only when $D_A = D_B = 0$, and in this case equations (A 1) and (A 2) reduce to two independent equations.

Appendix 2

The rate of equilibration of n drugs competing for the same receptors

The equations to be solved can be written

$$\mathrm{d}\mathbf{p}/\mathrm{d}t = \mathbf{A}\mathbf{p}, \tag{A 3}$$

where $\mathbf{p}$ is the $(n \times 1)$ vector of the quantities $\Delta p_i(t) = p_i(t) - p_i(\infty)$, and $\mathbf{A}$ is an $(n \times n)$ matrix of constants. The elements of $\mathbf{A}$ are

$$a_{ij} = -k_{-i}(D_i + \delta_{ij}) \tag{A 4}$$

where k_{-i} is the dissociation rate constant and D_i the ratio of concentration to dissociation equilibrium constant for the ith drug, and $\delta_{ij} = 1$ if $i = j$, $\delta_{ij} = 0$ otherwise. The equilibrium occupancies are

$$p_i(\infty) = D_i \Big/ \left(1 + \sum_{j=1}^{n} D_j\right) \qquad (i = 1, 2, \ldots, n). \tag{A 5}$$

The solutions, giving the receptor occupancy p_i by the ith drug at time t, are

$$p_i = \sum_{j=1}^{n} c_{ij} \mathrm{e}^{\lambda_j t} + p_i(\infty) \qquad (i = 1, 2, \ldots, n), \tag{A 6}$$

if the values of λ, which are the n roots of the characteristic equation,

$$|\mathbf{A} - \lambda \mathbf{I}| = 0, \tag{A 7}$$

are distinct. If the latent roots are not distinct the solution will not have this form unless $\mathbf{A}$ can, nevertheless, be reduced to diagonal form (e.g. unless $\mathbf{A}$ is symmetric). The matrix, $\mathbf{C}$, of the coefficients c_{ij}, has columns which are proportional to the latent vectors of $\mathbf{A}$, the proportionality constants being found from the n initial occupancies, $\Delta p_i(0)$. Thus if $c_{ij} = h_j b_{ij}$ then $\mathbf{h} = \mathbf{B}^{-1}\mathbf{p}(0)$ where $\mathbf{h}$ is the vector of proportionality constants (h_j) and $\mathbf{B}$ is a matrix (b_{ij}) the columns of which are a set of latent vectors of $\mathbf{A}$. Equivalently $\mathbf{C}$ can be found by defining the matrix of the initial values of the first $n-1$ derivatives for each drug

$$\mathbf{P} = \begin{bmatrix} \Delta p_1(0) & p_1'(0) & \ldots & p_1^{(n-1)}(0) \\ \Delta p_2(0) & p_2'(0) & \ldots & p_2^{(n-1)}(0) \\ \vdots & \vdots & \vdots & \vdots \\ \Delta p_n(0) & p_n'(0) & \ldots & p_n^{(n-1)}(0) \end{bmatrix} \tag{A 8}$$

(each column is $\mathbf{A}$ times the previous one so only n initial conditions are needed) and defining

$$\mathbf{\Lambda} = \begin{bmatrix} 1 & \lambda_1 & \ldots & \lambda_1^{n-1} \\ 1 & \lambda_2 & \ldots & \lambda_2^{n-1} \\ \vdots & \vdots & \vdots & \vdots \\ 1 & \lambda_n & \ldots & \lambda_n^{n-1} \end{bmatrix}. \tag{A 9}$$

The initial conditions can now be written $\mathbf{P} = \mathbf{C}\mathbf{\Lambda}$ and the coefficients are therefore given by

$$\mathbf{C} = \mathbf{P}\mathbf{\Lambda}^{-1} \tag{A 10}$$

if the latent roots are distinct (if they are not $\mathbf{\Lambda}$ will be singular).

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