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Receptor Mechanisms
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To link a biennial lecture such as this with the name of J. H. Gaddum was an apt choice, for so broad were Gaddum's interests and so important his contributions that there can be few pharmacologists working today who are not scientifically in his debt. In my own case, the debt is a direct and obvious one, for Gaddum was the first person to formulate the theory of competitive drug antagonism in a quantitative way. This he chose to do, remarkably enough, in a two-page communication to the Physiological Society (Gaddum, 1937) which contains not only an explicit formulation of the theory but also draws attention to the kinetic difficulties which have been much discussed since (Arunlakshana & Schild, 1959 ; Rang, 1966 ; Thron & Waud, 1968). The germination of this extremely valuable theory took a long time. Langley in his classic paper of 1905 clearly expressed the idea of specific receptors in skeletal muscle, and in 1914 analysed the interaction between nicotine and 'curari' in terms of what we should now call a dose-ratio. He obviously had in mind the idea of competitive antagonism between the two drugs but in spite of A. V. Hill's thorough quantitative treatment in 1909 of the reaction of nicotine with its postulated receptor substance, Langley took the idea no further. Simultaneous studies by Gaddum (1926), on the antagonism of adrenaline by ergotamine on the rabbit uterus (Fig. 1) and by Clark (1926a) on the antagonism of acetylcholine by atropine on the frog heart showed the now familiar pattern of

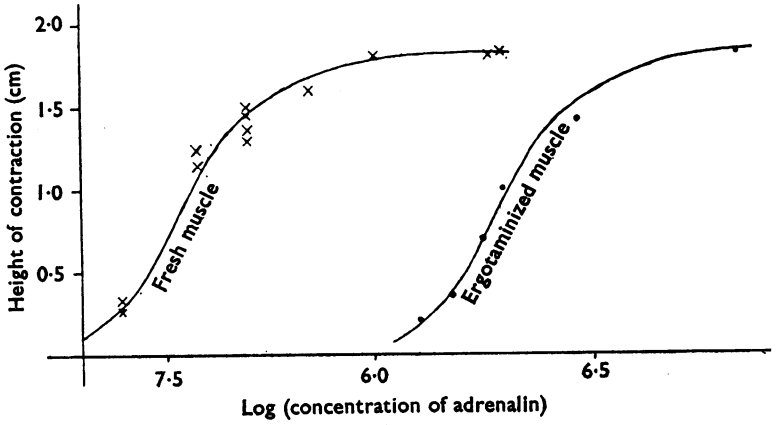

FIG. 1. The effect of ergotamine on the log-concentration effect curve of adrenaline acting on the rabbit uterus. The ergotamine concentration was 5 parts per million. (From Gaddum (1926), reproduced by permission of the Journal of Physiology.)

a parallel shift to the right of the agonist log concentration-effect curves, without any change in slope or maximum; both used the agonist dose-ratio as a quantitative measure of antagonism, and showed that dose-ratio was linearly related to the antagonist concentration (Fig. 2). Clark, in this study, considered the possibility of simple competition between atropine and acetylcholine, but was deterred by a single observation. This was that the slow recovery of the sensitivity of the heart to acetylcholine after the atropine was washed out of the bathing fluid was not accelerated in the presence of high concentrations of acetylcholine (in contrast, as he said, to the accelerated dissociation of carboxyhaemoglobin in the presence of oxygen). In retrospect it seems surprising that this should have been a stumbling block, for the simple theory of competitive antagonism that Gaddum (1937) put forward, which formed the basis for the more elaborate theories built up later (e.g. Schild, 1947; Ariens, van Rossum & Simonis, 1957; Stephenson, 1956; Paton, 1961), did not actually predict the acceleration effect that Clark failed to see. Indeed, had such an effect occurred, the simple competitive theory would have been in trouble.

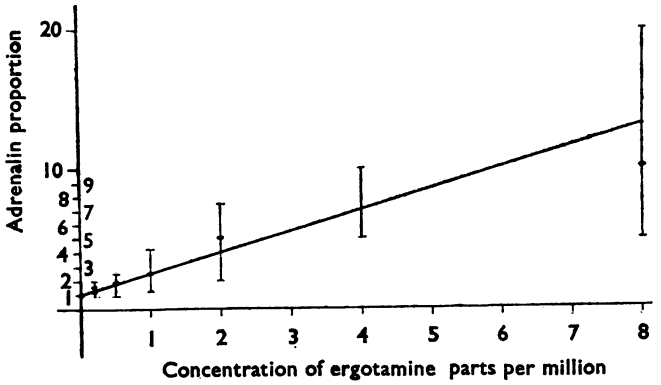

FIG. 2. The relationship between the 'adrenalin proportion' (the ratio by which the adrenaline concentration must be increased in order to produce a matching effect in the presence of ergotamine) and the concentration of ergotamine. (From Gaddum (1926), reproduced by permission of the Journal of Physiology.)

These early ideas about drug receptor interactions, both the attempts by Clark (1926b) and others to fit concentration-effect curves by means of the hyperbolic Langmuir adsorption isotherm, and the analysis of competitive antagonism, have obvious parallels in enzymology, and represent an almost exact equivalent of the Michaelis–Menten theory of enzyme kinetics. The point at which the two fields diverge is, of course, that the salient feature of an enzyme is that it brings about a change in the substrate molecule, whereas with drug-receptor interactions it is the macromolecule that is altered to bring about a physiological response, rather than the drug molecule. Developments in enzymology over the last ten years have, however, brought the two fields back into much closer correspondence, with the recognition that many enzymes are regulated allosterically by small molecules which bear no relation at all to their substrates, as well as being conformationally altered by the binding of substrate molecules (see review by Koshland & Neet, 1968). Thus for enzymes as well as receptors, the macromolecule can be greatly affected by association with a regulatory ligand. The main theme of this lecture

will be to discuss some experimental observations on cholinergic receptors, mainly those at the motor end-plate, and to attempt to relate them to mechanisms that have been proposed for regulatory enzymes.

Desensitization

It is well known that, in many tissues, application of a large concentration of an agonist drug will desensitize the tissue and that recovery of sensitivity after the drug is washed away may take many minutes (Barsoum & Gaddum, 1935; Cantoni & Eastman, 1946; Paton, 1961). This phenomenon has been studied in considerable detail at the neuromuscular junction (Fatt, 1950; Thesleff, 1955; Katz & Thesleff, 1957; Elmqvist & Thesleff, 1962; Manthey, 1966, 1970; Nastuk, 1967; Parsons, 1969; Magazanik & Vyskocil, 1970; Nastuk & Parsons, 1970).

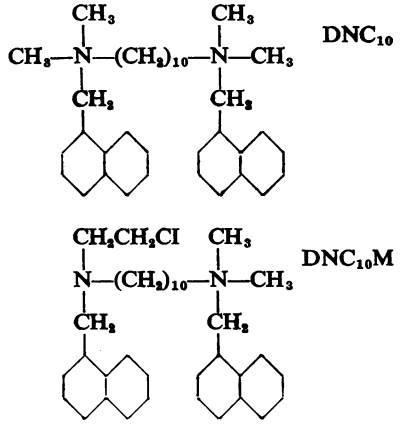

FIG. 3. Structures of decamethonium derivatives used in studies of desensitization in chick muscle. DNC_{10}=dinaphthyldecamethonium, $DNC_{10}M$=dinaphthyldecamethonium mustard.

We became interested in desensitization (as a phenomenon rather than a nuisance) in the course of some studies on aromatically-substituted decamethonium derivatives, diphenyldecamethonium (DPC_{10}) and dinaphthyldecamethonium (DNC_{10}: see Fig. 3) in chick muscle (Rang & Ritter, 1969). Having expected that these agents, which caused no depolarization themselves, would act simply as tubocurarine-like competitive blocking agents, we were surprised to find that they caused rather little direct blocking action against depolarizing drugs. Instead, they caused

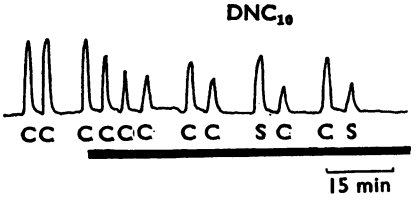

FIG. 4. Isometric contractions of a thin strip of chick biventer cervicis muscle in response to carbachol $1{\cdot}5\times10^{-5}$M (C) and suxamethonium $5{\cdot}6\times10^{-7}$M (S). Dinaphthyldecamethonium (DNC_{10}) $1{\cdot}9\times10^{-7}$M was present during the time shown by the black bar. The contractions on the left represent a tension of 200 mg. DNC_{10} causes only slight antagonism if the responses are preceded by a 10 min rest period, but much greater antagonism if the responses are closely spaced. (From Rang & Ritter (1969), reproduced by permission of Academic Press.)

a marked enhancement of desensitization, so that a series of equal test applications of carbachol caused a series of decreasing responses which recovered if the preparation was given a rest for a few minutes (Fig. 4). Studies with other substances that cause contraction of the muscle, such as potassium ions or caffeine, showed that the effect was specific to cholinergic receptors. Moreover, in leech muscle, where there are separate receptors for agents such as carbachol and acetylcholine on the one hand, and for suxamethonium and decamethonium on the other (Flacke & Yeoh, 1968a), the enhancement of desensitization by DPC_{10} affected only the suxamethonium receptors. Flacke & Yeoh (1968b) found that tubocurarine itself acted in the same way when tested against suxamethonium in leech muscle, but not when tested against carbachol. There were two plausible explanations of these results. One was that the antagonists exerted a direct effect on desensitization; the second was that the effect of an agonist was somehow to make the receptors more susceptible to the blocking effect of the antagonist, the declining response then being attributed to an increase in degree of block rather than to desensitization as such. The synthesis of an alkylating derivative of DNC_{10} (Fig. 3) enabled these possibilities to be distinguished. This compound, $DNC_{10}M$, caused an irreversible block of the receptors, and we found that the degree of block produced was much enhanced if an agonist such as carbachol or suxamethonium was added simultaneously with the blocking drug. On the other hand, once the block was established and the $DNC_{10}M$ washed away, successive responses to carbachol showed no greater tendency to desensitize than normally. The conclusion from these studies was that these blocking agents do not directly influence desensitization, but that agonists transform the receptors in such a way as to increase their affinity for DNC_{10} and $DNC_{10}M$. At first it seemed possible that the change in the receptors responsible for the enhanced binding of $DNC_{10}M$ might be the same change that caused the agonist response, i.e. the process of receptor activation. But one result made this extremely unlikely, and this was the finding that the enhanced binding could be detected even if the $DNC_{10}M$ was applied some minutes after the 'conditioning' dose of carbachol had been washed away. There was still a noticeable effect 15 min after the carbachol was washed away (Fig. 5), whereas the

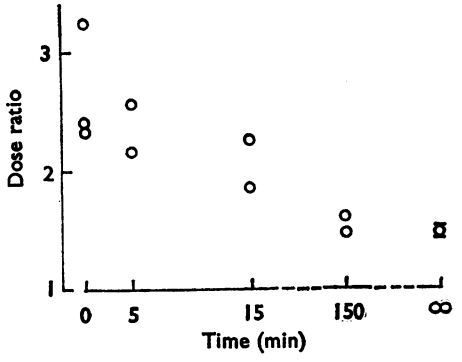

FIG. 5. Carbachol dose-ratio produced by diphenyldecamethonium mustard ($2{\cdot}2\times10^{-6}$M for 15 min) applied to a strip of chick biventer cervicis muscle at different times after a conditioning dose of carbachol ($1{\cdot}5\times10^{-4}$M for 1·5 min) was washed out of the organ bath. The point on the right hand side shows the mean (±standard error, $n=11$) dose-ratio produced when no large conditioning dose of carbachol was given. The enhanced block due to the conditioning dose declines slowly after the conditioning dose is washed out of the bath. (From Rang & Ritter (1969), reproduced by permission of Academic Press.)

contraction caused by carbachol disappeared within a few seconds. The enhancement of $DNC_{10}M$ block therefore outlasted the receptor activation when carbachol was washed away, and the two could not be ascribed to the same conformation change in the receptor. The time-course of the enhancement of $DNC_{10}M$ block was, on the other hand, very reminiscent of the slow recovery from desensitization following a large conditioning dose of agonist, so we next explored the possibility that the same conformational transition might be responsible for both phenomena.

The mechanism that seemed to give a reasonable account of these findings is essentially the same as that put forward by Katz & Thesleff (1957), and is closely related to the mechanism proposed for allosteric transitions of regulatory enzymes by Monod, Wyman & Changeux (1965). This had already been suggested by Karlin (1967) and by Changeux, Thiery, Tung & Kittel (1967), as a possible model for drug action, though as a mechanism for receptor activation rather than desensitization. The salient features of this model (Fig. 6) are that the receptor can exist in two conformations, R being the normal conformation and R′ the desensitized conformation. Either of these conformations can bind a drug molecule, though the dissociation constants, K and K′, differ. The conformational transitions $R \rightleftharpoons R'$ are assumed to be slow (corresponding to the time-course of desensitization and recovery) in relation to the rates of association and dissociation. This

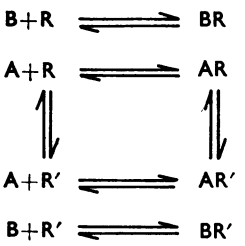

FIG. 6. Suggested mechanism of desensitization. R represents the normal conformation and R′ the desensitized conformation of the receptor. The interconversion of R and R′ occurs relatively slowly compared with the association and dissociation of drug molecules. Thus, if an agonist A has a higher affinity for R′ than for R it will shift the balance in favour of R′. When the agonist is washed away, vacant R′ receptors will remain, and can be blocked by an antagonist B, which also has higher affinity for R′ than for R.

model was analysed in some detail by Katz & Thesleff (1957) and by Rang & Ritter (1970a). The main features of it are: (1) The degree of desensitization produced by different agonists would be expected to vary according to the relative affinities (K′/K) of the drug for the two conformational states, so that drugs with a relatively high affinity for R′ should desensitize more than those with a low affinity. (2) Antagonists as well as agonists would be expected to show varying relative affinities for the two states. (3) The time-course of recovery from desensitization when the agonist is washed away should be exponential, and since it represents simply the first order $R' \rightarrow R$ transition, should occur at the same rate irrespective of the nature of the drug that caused it. (4) If enhancement of $DNC_{10}M$ block by agonists results from its relatively high affinity for receptors in the desensitized (R′) conformation, then it should be possible to demonstrate quantitatively the correlation between the two.

All of these predictions were borne out by experiments on desensitization in chick and frog muscles (Rang & Ritter, 1970a, b). The varying desensitization with

different agonists is shown (Fig. 7) by the greater rate of decline of the response with n-decyltrimethylammonium (C_{10}-TMA) than with carbachol in chick muscle, and a similar result with phenyl-trimethylammonium and carbachol in frog muscle.

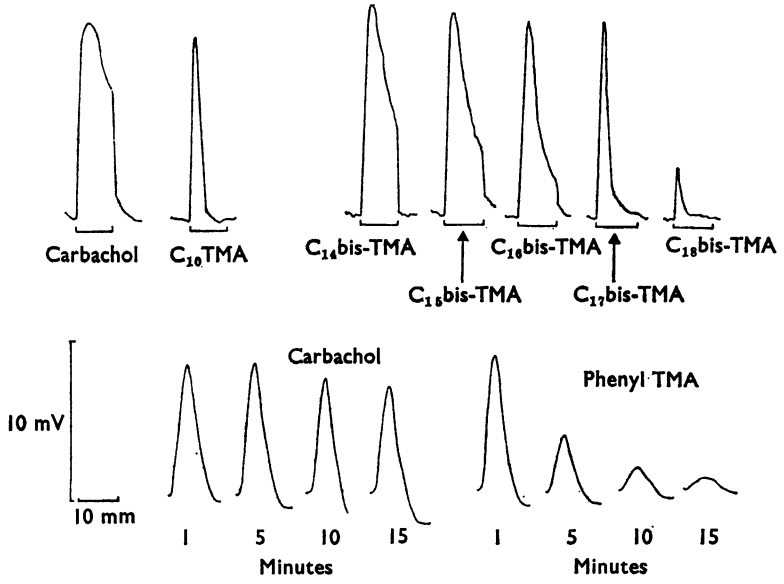

FIG. 7. Varying desensitization with different agonists in chick and frog muscle. Upper records: Contractions of strip of chick biventer cervicis muscle in response to different agonists applied for 10 minutes. The C_n-bis-TMA compounds are long-chain derivatives of decamethonium, where n is the number of carbon atoms in the polymethylene chain. C_{10}-TMA causes more rapid desensitization than carbachol, and a similar effect is seen with lengthening of the chain in the bisquaternary series. Lower records: Moving fluid recordings of endplate depolarization in frog toe muscle. Successive records were taken at the indicated times after adding the depolarizing drug to the bath. With carbachol the depolarization is quite well maintained, whereas with phenyl-TMA it subsides rapidly.

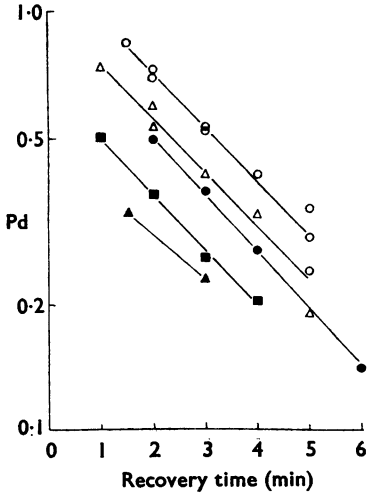

FIG. 8. Kinetics of recovery from desensitization in chick muscle. The fraction of receptors (Pd) in the desensitized state (estimated from the carbachol dose-ratio) is plotted semi-logarithmically against the time after washing out the conditioning agonist. The conditioning agonists were: ○ C_{10}-TMA $2{\cdot}2\times10^{-5}$M for 10 min; △ C_{10}-TMA $7{\cdot}4\times10^{-6}$M for 10 min; ● Carbachol $7{\cdot}4\times10^{-5}$M for 4 min; ■ C_{16} bis-TMA $2{\cdot}0\times10^{-7}$M for 5 min; ▲ C_{16} bis-TMA $1{\cdot}3\times10^{-7}$M for 5 min. (From Rang & Ritter (1970a), reproduced by permission of Academic Press.)

The exponential recovery from desensitization with carbachol or C_{10}-TMA in chick muscle is shown in Fig. 8. The fraction of desensitized receptors, estimated by a dose-ratio technique, decreased with the same time-course irrespective of the nature and concentration of the conditioning drug used to produce the desensitization. This finding constitutes strong evidence against various alternative models for desensitization (see Rang & Ritter, 1970a), in particular those in which recovery is associated with slow dissociation of drug molecules from the receptors (as in the rate theory of drug action; Paton, 1961) or in slow conformational transition of the agonist-receptor complex of the form

$$A + R \underset{\text{fast}}{\rightleftharpoons} AR \underset{\text{slow}}{\rightleftharpoons} AR'$$

Hammes, Porter & Stark (1971) have recently measured the rate of the conformational change in the isolated catalytic subunit of aspartate transcarbamylase, induced by the binding of succinate and malate respectively, which follows the kinetic behaviour of the model shown above. In contrast to the desensitization results shown in Fig. 8, they found a 9-fold difference in the rate constant of the AR'→AR transition with these two ligands, a difference which rules out in this case a mechanism of the type shown in Figure 6.

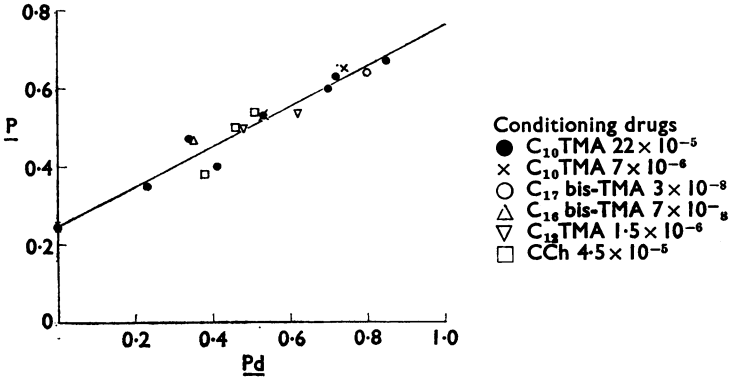

FIG. 9. Correlation between desensitization and blocking action of $DNC_{10}M$ in chick muscle. Desensitization was produced by various conditioning drugs, and the fraction of receptors in the desensitized state (Pd) measured by a dose-ratio method. In parallel experiments the same conditioning drug was used and the fraction of receptors blocked (P) by a standard application of $DNC_{10}M$ ($1{\cdot}5 \times 10^{-6}$M for 2 min) was measured. The correlation obtained supports the hypothesis that $DNC_{10}M$ combines preferentially with desensitized receptors, and that the desensitized conformation is the same irrespective of the nature of the conditioning drug. (From Rang & Ritter (1970b), reproduced by permission of Academic Press.)

Finally, the quantitative correlation between desensitization and the enhancement of the blocking action of $DNC_{10}M$ is shown in Figure 9. In these experiments (Rang & Ritter, 1970b) $DNC_{10}M$ was applied for 2 min at various times after washing out the various conditioning agonists. The degree of irreversible block produced by $DNC_{10}M$, expressed as a fraction of the total receptors inactivated, was then plotted against the fraction of receptors in the desensitized state at the time when $DNC_{10}M$ was applied, which was estimated in parallel experiments on the same strips of muscle. A linear relationship was predicted and this was borne out in a series of experiments with different conditioning agonists. The essential features of the mechanism shown in Fig. 6, which gives a better explanation of the experimental results than any of the other models considered (Rang & Ritter,

1970a), are (1) that the conformational transition $R \rightleftharpoons R'$ can occur in the absence of any ligand, and is not 'induced' directly by the binding of the ligand, and (2) that the species R′ is the same irrespective of the chemical nature of the desensitizing drug. The mechanism postulated for the conformation change is thus what Koshland & Neet (1968) have termed a *prior isomerization* pathway, rather than a *substrate-guided* pathway.

One of the striking features about the conformation change that appears to be responsible for desensitization is its slowness. The time constant for the transition $R' \rightarrow R$, from the results in Fig. 8, is 3·3 min, which may be compared with typical time constants for enzyme conformation changes that have been measured by various high speed relaxation techniques: 5·5 s and 0·18 s for the forward and backward isomerization reactions of glyceraldehyde 3-phosphate dehydrogenase (Kirschner, Eigen, Bittman & Voigt, 1966); 0·2 ms and 1·6 ms for the forward and backward isomerization reactions of the catalytic subunit of aspartate transcarbamylase induced by succinate (Hammes *et al.*, 1971); various other examples in the range 0·1–1 ms are given by Hammes (1968).

However, there are also examples of much slower ligand-induced conformation changes, such as the activation of D-amino acid oxidase by flavine adenine dinucleotide (FADN) which occurs with a time constant of about 5 min even though the binding of FADN is rapid (Massey & Curti, 1966), and also a NAD-oxidase activated by adenosine monophosphate (AMP) where the conformation change persists for many minutes after AMP dissociates (Worcel, Goldman & Cleland, 1965).

Receptor activation

The model (Fig. 6) discussed in relation to the phenomenon of desensitization falls into the general category of *2-state* models (see Colquhoun, 1973), there being only two conformational states represented, the ligand serving merely to shift the pre-existing equilibrium between them. The two states discussed so far represent only the 'resting' (R) and desensitized (R′) states, so it is necessary to ask where the process of activation (leading to the response) fits into the picture. Originally we discussed the possibility that the activated state might be a transitional state on the way from AR to AR′ (Rang & Ritter, 1970a), which would account for the fact that all agonists appear to cause some degree of desensitization. Such a mechanism would, however, imply that the cycle in Fig. 6 must contain thermodynamically irreversible steps, and consequently require a source of energy to keep it going. Since Kasai & Changeux (1971) have obtained permeability changes with cholinergic agonists acting on suspensions of fragments of cell membranes from eel electroplax, in which there is no obvious source of external energy, this hypothesis seems rather unlikely and it would be preferable to consider a mechanism not involving metabolic energy. One possibility, discussed by Karlin (1967) and by Changeux *et al.* (1967), is that a 2-state mechanism similar to that in Fig. 6 could account for receptor activation, if a different conformational transition $R \rightleftharpoons R^*$ is postulated. Since activation is much more rapid than desensitization, the rate constants governing this transition would be much higher than for the $R \rightleftharpoons R'$ reaction. The attractive feature of such a mechanism for receptor activation is that it gives a physical interpretation to the concept of 'efficacy' (Stephenson, 1956) or 'intrinsic activity' (Ariens *et al.*, 1957; van Rossum, 1966) which has for a

long time appeared as an empirical and rather mysterious parameter in the conventional occupation theory of drug action. Efficacy is used to denote the difference between pure antagonists which occupy receptors without producing any overt response, and agonists which, as well as occupying receptors, activate them so as to produce a biological response. In between the two lie partial agonists which, even when occupying 100 per cent of the receptors, produce only a submaximal response. The mechanistic idea behind efficacy, though it has never been clearly stated, appears to be that different drug molecules cause different degrees of distortion, and hence of 'activation' in the receptors that they occupy. Because of the unknown relationship between receptor occupancy and the response of the tissue, Stephenson (1956) introduced a new concept, the stimulus, S, which was by definition proportional to occupancy, the proportionality constant being equal to the efficacy of the drug. The formulation was thus,

Response=f(S), f being the same for all drugs
S =ep, where e=efficacy, p=fractional receptor occupancy.

The inherent assumption is that the stimulus has simple colligative properties so that, for example, halving the occupancy or halving the efficacy should affect the response in an *identical* manner. Since, however, efficacy refers to the *kind* of conformation change associated with occupation, we should not expect this assumption to be necessarily true. If there is really a large variety of ways in which the receptor can be distorted in order to give rise to a biological response, then it is unlikely that a single parameter will be enough to describe the variation between different drugs.

Experimental observations seem, however, to conform quite well with Stephenson's equations and equations based on them (see Stephenson, 1956; Furchgott, 1966; van Rossum, 1966), suggesting that the equations are probably valid even if the type of mechanism just discussed is not.

In terms of the two-state model, efficacy can be explained much more economically in terms of the relative affinity of a drug for the two states R and R*. Thus, a drug with a high affinity for R* relative to that for R will tend to shift the conformational equilibrium in favour of R* and will consequently be an agonist. If it has no affinity whatever for R, then it will bind only to receptors in the R* conformation; consequently, if it occupies all of the receptors then all of them must be in the R* conformation and efficacy is maximal. If the drug has equal affinity for R and R*, then its presence will make no difference to the pre-existing equilibrium, but it will competitively prevent the binding of any other drug and so will behave as an antagonist. A drug with a greater affinity for R and for R* will also behave as an antagonist, but by shifting the conformational equilibrium away from R* as well as by simple competition.

The equations governing the two-state model are, as would be expected, different from the simple Langmuir binding equation that is usually used in discussions of drug action. It is therefore important to know whether or not the experimental results that have been analysed in terms of the Langmuir equation are also compatible with the two-state model. Colquhoun (1973) has analysed the properties of the two-state model in some detail with a view to discovering what kind of experimental evidence would be needed to distinguish it from the conventional theory, and how far the available evidence allows the distinction to be made.

Several types of experimental analysis have been based on the conventional Langmuir binding equation, the most important being the following four:

1. *Analysis of competitive antagonism* (Gaddum, 1937; Schild, 1947; Arunlakshana & Schild, 1959). The basic assumptions made are that the agonist and the antagonist interact competitively, and that the response is a function only of the fractional occupancy achieved by the agonist. The predictions are that the log-concentration-effect curve for the agonist should be shifted to the right without change in slope, and that a plot of (dose ratio−1) against antagonist concentration should be linear, the slope giving a measure of the dissociation constant of the antagonist. There are many examples where these predictions are borne out with considerable precision (see Rang, 1971).

The two-state model involves different assumptions and different equations: the binding curve does not agree with the Langmuir equation, and the effect is assumed to be a function, not of occupancy, but of the fraction of receptors in the activated conformation (given by the 'state function', rather than the 'binding function' defined by Monod *et al.*, 1965). Both Karlin (1967) and Colquhoun (1973) have shown, however, that the predicted form of competitive antagonism according to this model is almost exactly the same as for the conventional model. The parallelism of the log-concentration-effect curves is not exact, but the deviation is very unlikely to be detectable experimentally. Notwithstanding this, for any given level of response, the relationship between dose-ratio and antagonist concentration is exactly as predicted by the conventional theory.

2. *Comparisons of full and partial agonists.* If concentration-effect curves are obtained on the same tissue for a full agonist and a partial agonist, then the equi-effective concentrations of the two drugs should be related in a fairly simple way, if the only difference between the two drugs is in their efficacy as defined by Stephenson (1956). Experiments of this kind have been described by various authors (Mackay, 1966; Barlow, Scott & Stephenson, 1967; Waud, 1969) and shown, generally by means of a reciprocal plot analysis, to agree quite well with the theory. An example of this type of experiment is shown in Figure 10.

Again, however, it turns out (see Colquhoun, 1973 for details) that the two-state

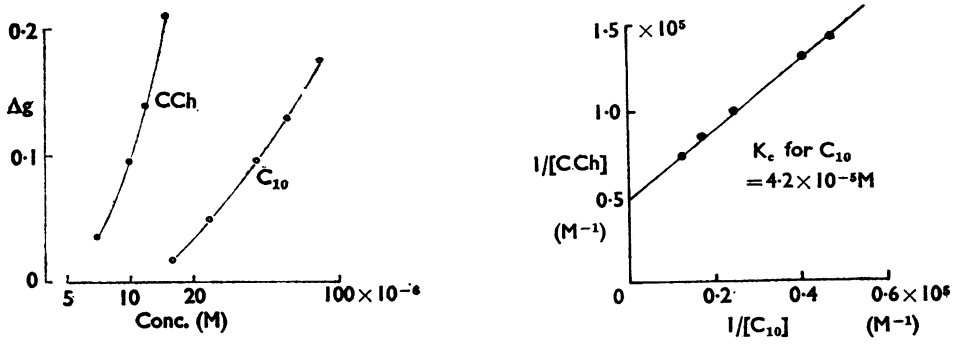

FIG. 10. Action of carbachol (CCh) and decamethonium (C_{10}) on the end-plate region of frog semitendinosus muscle fibres at 4° C. The depolarization produced by bath application of the drug was measured and converted to a relative conductance change (Δg) assuming a reversal potential of—15 mV. Concentration-effect curves are shown on the left, and the double reciprocal plot of equieffective concentrations on the right. The equilibrium constant for decamethonium estimated from this experiment was $4{\cdot}2\times10^{-5}$M.

model leads to virtually the same predictions ; agreement is not exact, but the extent of the discrepancy is almost certainly too small to detect experimentally.

3. *Interaction between full and partial agonists.* Comparison of the concentration-effect curve for a full agonist on its own with that obtained in the presence of a fixed concentration of a partial agonist, effectively combines the previous two sorts of experiment, for the partial agonist simultaneously exerts an effect of its own and competitively inhibits the full agonist. Stephenson (1956) estimated the dissociation constants of partial agonists by this method, and experimental results obtained in frog muscle are shown in Figure 11. Once again, however, Colquhoun (1973) has found that the two-state model leads to virtually identical predictions.

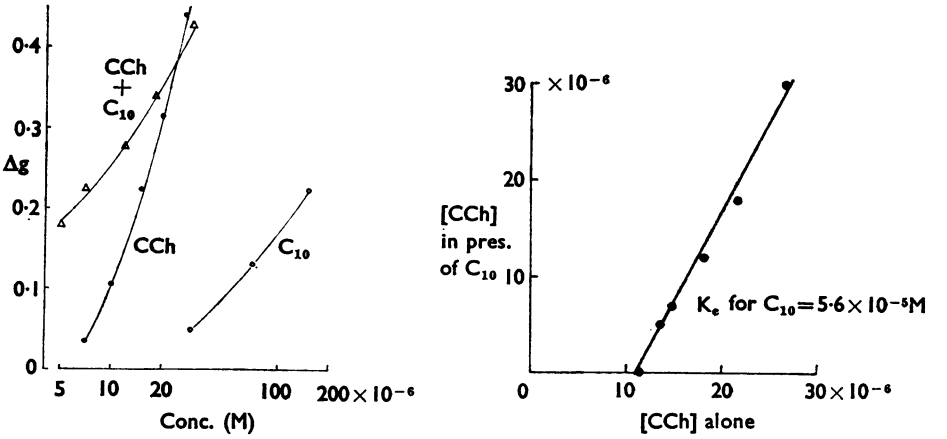

FIG. 11. Interaction of carbachol and decamethonium on the frog motor end-plate, recorded as in Fig. 10. Concentration-effect curves are shown on the left for carbachol (CCh) and decamethonium (C_{10}) separately, and for carbachol in the presence of 7×10^{-6}M decamethonium. The plot on the right is of equieffective concentrations of carbachol on its own (abscissae) and in the presence of decamethonium (ordinates). The predicted linear relationship is observed, and the calculated equilibrium constant for decamethonium was $5{\cdot}6\times10^{-5}$M.

4. *Effects of irreversible antagonists* By comparing the concentration-effect curve for an agonist before and after irreversible occlusion of a fraction of the receptors by an alkylating agent such as dibenamine, it is possible, according to the conventional model, to estimate the dissociation constant for the agonist drug (Furchgott, 1966 ; Furchgott & Bursztyn, 1967 ; Parker & Waud, 1971 ; Sastry & Cheng, 1972). The usual analytical method is (as in (2)) to plot the results in double reciprocal form, when a straight line relationship is predicted. This is the only method so far available for determining the dissociation constants of full agonists.

Predictions based on the two-state model (Colquhoun, 1973) are more uncertain than in the other examples (especially for agonists of very high efficacy), because arbitrary assumptions must be made about the binding of the alkylating agent to the two states. In general, however, it is very unlikely that experimental results woud succeed in distinguishing these two models.

Overall, it therefore appears that the available experimental evidence is just as compatible with the two-state model as it is with the conventional occupation theory on which the analyses were originally based, and there appears to be no evidence at present which strongly favours one view or the other. An interesting point to emerge from Colquhoun's analysis is the significance of the estimates of agonist dissociation constants which come from the type of experiments just discussed. The

two-state model implies that any drug must have two different dissociation constants, one for each of the two conformations of the receptor, so the question arises what the single estimated value actually relates to. The answer is that the value lies somewhere between the two actual dissociation constants, but if one assumes (as seems reasonable) that the $R \rightleftharpoons R^*$ equilibrium in the absence of any drug lies well over to the left, so that the degree of activation in the absence of the drug is very small, then all of the methods discussed give an estimate that approximates to the dissociation constant for the *inactive* (R) conformation. One hitherto puzzling feature of the estimates of dissociation constants that have been published (Table 1) is that the most potent agonists, at least those that act on muscarinic receptors in smooth muscle, have an affinity for the receptors that is roughly one thousand times lower than the affinity of potent antagonists for the receptors. If the two-state model is correct, and these estimates actually relate to the inactive receptors, this is exactly what we would expect, for potent agonists are characterized by a much higher affinity for active than for inactive receptors, whereas this is not so for antagonists. For agonists of very high efficacy, exact interpretation of the affinity constant measured by the use of irreversible antagonists, in the framework of two-state models, is difficult (see above), but it would certainly be expected that in all cases it would be much less than the affinity for the active state.

TABLE 1. *Dissociation constants of drugs acting on intestinal smooth muscle*

Potent agonists	
(Rabbit stomach; Furchgott & Bursztyn, 1967)	
Acetylcholine	$2{\cdot}08 \pm 0{\cdot}24 \times 10^{-6}$ M
Carbachol	$1{\cdot}59 \pm 0{\cdot}24 \times 10^{-5}$ M
Methacholine	$2{\cdot}48 \pm 0{\cdot}20 \times 10^{-6}$ M
(Guinea-pig ileum; Sastry and Cheng, 1972)	
Acetylcholine	$1{\cdot}08 \pm 0{\cdot}21 \times 10^{-6}$ M
Propionylcholine	$1{\cdot}21 \pm 0{\cdot}25 \times 10^{-5}$ M
Potent antagonists	
(Guinea-pig ileum; Paton & Rang, 1965)	
Atropine	$1{\cdot}11 \pm 0{\cdot}03 \times 10^{-9}$ M
Methylatropine	$4{\cdot}7 \pm 0{\cdot}1 \times 10^{-10}$ M
Lachesine	$1{\cdot}46 \pm 0{\cdot}04 \times 10^{-9}$ M
Hyoscine	3×10^{-10} M

Is there any other experimental evidence that helps to distinguish between the conventional occupation theory and the two-state model? At present the evidence is fragmentary and indecisive. One prediction of the two-state model is that all agonists should produce qualitatively identical effects, since all are acting by shifting the conformational equilibrium $R \rightleftharpoons R^*$, and only one conformational species R^* is postulated. The change produced at the neuromuscular junction by cholinergic agonists involves an increase in permeability to both Na^+ and K^+, and the relative contributions of these two ions determines the reversal potential for the effect (Fatt & Katz, 1951; Takeuchi & Takeuchi, 1960; see review by Ginsborg, 1967). If the activated conformation is identical for different agonists, then the reversal potential must also be the same, whereas this would not necessarily be expected for the conventional model. Various authors have reported that reversal potentials for different agonists applied ionophoretically to the frog neuromuscular junction are indeed identical (Manalis & Werman, 1969; Koester & Nastuk, 1970; Feltz & Mallart, 1971a, b) and a similar inference has been drawn from rather less direct results obtained on eel electroplax cells by Changeux & Podleski (1968). The

interesting finding by Takeuchi & Takeuchi (1971) that the reversal potential for gamma-aminobutyric acid (GABA) acting on the crayfish muscle varies with the concentration of GABA applied is not inconsistent with the two-state model, but clearly introduces a complication in the determination of reversal potentials. Strictly speaking, it would appear necessary to compare different substances at equieffective concentrations to obtain valid comparisons, but in practice the small effect detected by Takeuchi & Takeuchi may not be a serious source of error. It obviously would be interesting to know whether or not analogues of GABA produce effects with different reversal potentials.

In studies of the properties of muscarinic receptors in smooth muscle, Burgen & Spero (1968) found that cholinomimetic agonists produced two distinct effects: contraction and an increased efflux of radioactive potassium or rubidium; the relative potencies in producing these effects varied very greatly from one drug to another. Later they found (Burgen & Spero, 1970) that these relative potencies were markedly affected by the calcium concentration. One interpretation, favoured in the first paper, was that there are two receptor types, one leading to an increase in ionic permeability, the other to contraction. Although it would introduce complications, such a mechanism is not basically at variance with the two-state model. In 1970, however, they suggested that a single type of receptor was independently 'coupled' to the two responses, the coupling varying from one drug to another, and this clearly *is* at variance with the two-state model, which requires that an activated receptor should produce identical effects irrespective of what drug is involved. At present it is hard to reconcile all of their results with any single hypothesis, so one cannot say whether or not they effectively refute the two-state model.

Studies in other systems

Although not directly relevant to the drug-receptor problem, studies of other systems, notably of enzymes and of excitable membranes, are of interest in connexion with the two-state model, partly because they suggest what kind of experiments might be usefully carried out on receptor-mediated responses.

In the field of regulatory enzyme mechanisms, one of the central points of discussion is the distinction between the 'induced fit' mechanism for conformation changes (Koshland, Nemethy & Filmer, 1966) and the 'symmetry' mechanism (Monod *et al.*, 1965), which is discussed in detail by Koshland & Neet (1968). In relating these ideas to drug-receptor mechanisms, we need to distinguish two important points of difference between these two theories. The first concerns the nature of the conformation change. The induced fit theory regards the protein molecule as being highly flexible, and able to assume a wide range of conformations in response to the binding of different ligands (substrates or regulatory molecules). The symmetry theory, however, assumes that only two conformational states are possible, and is, in this respect, exactly equivalent to the two-state model discussed above (being, in fact, the father of it; see Karlin, 1967; Changeux *et al.*, 1967).

Studies on enzymes tend, in general, to favour the induced fit model, good examples being the variety of conformational changes observed in creatine kinase by O'Sullivan & Cohn (1966) in the presence of different substrates, and the study of Hammes *et al.* (1971) on the catalytic subunit of aspartate transcarbamylase. By the use of a temperature-jump method, these authors showed that the rate con-

stant for the conformational isomerization of the enzyme in the presence of malate was nearly ten times as great as that measured in the presence of succinate. The two-state model predicts that the rate constant in question should be independent of the nature of the ligand (a situation exactly analogous to the kinetics of recovery from desensitization shown in Figure 8).

Thus there is no experimental support for the two-state model being a general mechanism for conformational changes in proteins. It is interesting, nevertheless, that regulation of the catalytic activity of enzymes by regulatory molecules unrelated to the substrate very often takes the form of a change in the apparent K_m of the enzyme for its substrate without any alteration of V_{max}, from which it has been argued (as was argued earlier in relation to the constancy of the reversal potentials for different agonists on the motor endplate) that the 'active' conformation of the enzyme is the same, irrespective of the nature of the regulatory ligand. In general, it seems that the two-state model is supported far more strongly by studies of enzymes consisting of associated sub-units, than on monomeric enzymes or on systems (such as that studied by Hammes *et al.* (1971), mentioned above) where the subunits have been dissociated.

Cooperativity

The second important point of difference between Koshland's induced fit theory, and the symmetry theory of Monod, Wyman & Changeux for enzyme regulation, concerns the assumptions that they make about subunit interactions. The experimental finding around which the discussion has been centred is that, for many enzymes (in particular those which consist of associated subunits, so that each oligomer possesses more than one catalytic and/or regulatory site), the relationship between reaction rate and substrate concentration is sigmoid instead of being a simple rectangular hyperbola as predicted by the Michaelis-Menten equation. The implication of this result is that the subunits interact with one another in such a way that binding of a substrate molecule at one site favours the association of substrate molecules at other sites within the oligomer, and this type of effect is therefore loosely referred to as 'cooperativity'.

The explanations for cooperativity offered by the induced fit and symmetry theories are contrasted in Figure 12. The induced fit theory suggests that binding of the substrate molecule to one subunit induces a conformational change in both it and its neighbours, so that its neighbours then show a higher affinity for substrate molecules. The overall conformation change thus takes place sequentially as successive substrate molecules are bound. The symmetry model, on the other hand, suggests that all of the subunits are constrained by their association to adopt the same conformation, so that the overall conformation change is concerted rather then sequential. The steady-state characteristics of these two models are very similar (see Koshland *et al.*, 1966) though with accurate measurements it appears that they are distinguishable (Cornish-Bowden & Koshland, 1970). The kinetic properties of the two mechanisms are, however, different, since the induced fit model postulates a sequence of conformational changes which would be expected to occur at intrinsically different rates, whereas the symmetry model entails only a single, concerted transition which should be characterized by a single rate constant. Relaxation methods, notably the temperature jump technique (Eigen & Hammes, 1963; Hammes, 1968) on various enzymes, including glyceraldehyde-3-phosphate

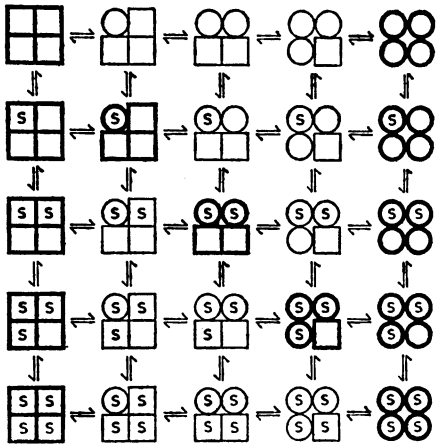

FIG. 12. General mechanism for cooperative binding of a ligand (S) to a tetrameric protein. The concerted transition model (Monod *et al.*, 1965) postulated that the monomers must all adopt the same conformation, and the permitted species are only those at the right and left of the diagram. The induced fit hypothesis (see Koshland & Neet, 1968) postulates that the monomers change conformation one by one as ligand molecules are bound (diagonal sequence).

dehydrogenase (Kirschner *et al.*, 1966) and aspartate transcarbamylase (Eckfeldt, Hammes, Mohr & Wu, 1970; Hammes & Wu, 1971) have been used to test this point, and have given results that seem to favour the symmetry model. However, the finding of Conway & Koshland (1968) and Cook & Koshland (1970) that glyceraldehyde-3-phosphate dehydrogenase shows *negative* as well as *positive* cooperativity (implying that the conformational change occurring in adjacent subunits when a ligand molecule is bound may be such as to *diminish* rather than *increase* their affinity for substrate molecules) appears to be incompatible with the symmetry model. The experimental evidence is therefore confusing in distinguishing between the two postulated mechanisms for enzyme cooperativity. The most extensively studied co-operative mechanism is that of haemoglobin, where there is general agreement that although small changes in tertiary structure of the subunits occur sequentially as oxygen molecules are bound, the change actually responsible for increasing the affinity of vacant sites for oxygen is a concerted transition involving all four subunits (Perutz, 1970; Ogata & McConnell, 1972).

There is considerable evidence that cooperativity is a feature also of cholinergic receptor activation. Sigmoid, rather than hyperbolic, concentration-effect curves were observed by Katz & Thesleff (1957) and by Jenkinson (1960) in studies of end-plate depolarization by acetylcholine. Changeux & Podleski (1968) showed the same for the depolarization of eel electroplax cells by cholinergic agonists. Though the highly non-linear electrical properties of this membrane (Nakamura, Nakajima & Grundfest, 1965) make this result rather difficult to interpret, the conclusion is strengthened by the finding of Kasai & Changeux (1971) that the increased sodium efflux from membrane fragments of electroplax cells shows similar cooperativity. Voltage-clamp studies have also been used to confirm the earlier findings of cooperativity at the frog motor end-plate (see Rang, 1971, 1973a). The Hill coefficients for these effects are generally quite low, in the range of 1·5–2·0, so that the degree of cooperativity is less than, for example, that of the binding of oxygen by haemoglobin, where the Hill coefficient is 2·6.

The conductance change at the crayfish neuromuscular junction produced by

gamma-aminobutyric acid (GABA) shows cooperativity with a Hill coefficient equal to 2·0 (Takeuchi & Takeuchi, 1969). However, their later finding (Takeuchi & Takeuchi, 1971) mentioned above, that the ionic specificity of these channels, as indicated by the reversal potential, changes with GABA concentration, makes this finding rather difficult to interpret; furthermore, Feltz (1971) has suggested that the marked cooperativity could be an artefact resulting from desensitization. In contrast, the effects of muscarinic agonists in causing a permeability increase in smooth muscle show no apparent cooperativity, the concentration-effect curve being hyperbolic in form (Burgen & Spero, 1968).

There is at present very little evidence to suggest the mechanism by which cooperativity in drug effects may be produced, but three general possibilities can be put forward:

1. Complex events intervene between the primary process of receptor activation and the measured effect, so that the concentration-effect relationship gives no direct information about receptor activation.

2. The mechanism is analogous to the type of mechanism discussed above in relation to cooperative effects in enzyme regulation, involving subunit interactions of the type postulated by Koshland or by Monod, Wyman & Changeux.

3. The mechanism resembles that by which membrane potential controls the sodium and potassium permeabilities of excitable membranes (Hodgkin & Huxley, 1952; see review by Noble, 1966).

The first of these possibilities is almost certainly correct in many situations and is forcefully argued by Waud (1968). The most commonly measured pharmacological response is contraction of a piece of isolated muscle, and physiological considerations alone make it very unlikely that the complex chain of events by which activation of a membrane receptor leads to contraction will show simple linear proportionality at each step. When the response consists of an ionic conductance or permeability change, however, there is no evidence about the type of linkage between receptor activation and response, and no reason to assume that it shows complex, non-linear properties.

The possibility that subunit interactions are responsible for cooperativity was explored by Karlin (1967) and by Changeux *et al.* (1967), and an experimental study was presented by Changeux & Podleski (1968). One interesting finding was that the cooperativity in the concentration-effect curve of carbachol on the electroplax disappeared when a low concentration of decamethonium (which by itself caused only very slight depolarization) was present. This result resembles effects seen with a number of regulatory enzymes, for example, deoxythymidine kinase (Okazaki & Kornberg, 1964) where the relation between substrate (ATP) concentration and reaction rate is normally sigmoid, but is converted to a hyperbola in the presence of the regulatory substance cytidine diphosphate, which is not a substrate for the enzyme. Interactions of this sort (see Monod *et al.*, 1965) require, in general, that the interacting ligands should combine with different sites on the enzyme. Whether this finding implies that carbachol and decamethonium also react with separate sites on the receptor is not clear, and the result remains a puzzling one.

The third possibility, that mechanisms similar to those postulated in the Hodgkin–Huxley theory of excitation are responsible for cooperativity in drug effects is interesting for several reasons, and has been analysed by Colquhoun (1973).

The complex changes in ionic conductance of the squid axon membrane in response to sudden changes in membrane potential have been very successfully analysed in terms of a two-state model showing properties of cooperativity, and therefore provide an important analogy with drug-induced conductance changes. The successful application of the two-state model is emphasized in a discussion by Tsien & Noble (1969) of the membrane properties of heart muscle. Heart muscle, unlike nerve, shows highly non-linear electrical properties (Noble, 1965 ; Noble & Tsien, 1968) but it is nevertheless found that the current-voltage characteristics of the potassium channels are independent of the state of activation of the channels, a powerful argument, as Tsien & Noble (1969) point out, for the all-or-nothing properties of individual channels.

The cooperative nature of both sodium and potassium conductances in the nerve membrane is implicit in the Hodgkin–Huxley equations. The sodium conductance is described in terms of a first-order variable, m, which can be regarded as the fraction of the ' gates ' which are in the open state and which changes exponentially when the membrane potential is suddenly altered. The sodium conductance, g_{Na}, is proportional to m^3 rather than to m, one interpretation being that each sodium channel is controlled by three independent ' gates ', all of which have to be in the open conformation for the channel to conduct. For potassium conductance, the first-order variable, n, is raised to the fourth power, so the potassium channels are assumed to be controlled by four ' gates '. The possibility that a mechanism based on a concerted transition of interacting subunits of the Monod–Wyman–Changeux type might equally well account for the kinetic properties of the conductance mechanisms of the axon membrane was considered by Hill & Chen (1971). They showed, however, that the kinetic properties of the potassium channels in nerve could *not* be fitted by such a model nearly so well as by the model that Hodgkin & Huxley used. It is worth emphasizing that in the Hodgkin–Huxley model the ' gates ' are assumed to function completely independently of each other, in contrast to the mechanisms postulated for enzyme regulation, which are based on interactions between subunits. Which (if either) of these two general types of mechanism is likely to be the correct explanation of receptor cooperativity is an open question. Colquhoun (1973) discusses both mechanisms and concludes that either is capable of accounting for most of the available data on cholinergic receptors.

Conclusions

In this lecture I have discussed some of the experimental evidence relating to the mechanisms by which agonists cause two types of change in cholinergic receptors, desensitization and activation, and have attempted to relate drug-receptor mechanisms to comparable processes of enzyme regulation and of the control of ionic permeability of excitable membranes. As soon as one attempts comparisons of this sort it becomes clear that very little is at present known about receptor-mediated permeability changes by contrast, say, with our knowledge of voltage-sensitive permeability mechanisms, which has been derived almost entirely from voltage-clamp studies.

What techniques are likely to reveal more about the mechanism of drug-induced permeability changes? The direct counterpart of voltage-clamping would require rapid, controlled changes of drug concentration in the neighbourhood of the receptors and this does not appear to be technically feasible at present. There have been

recent reports, however, suggesting that the drug-induced permeability changes at the motor end-plate are affected by the membrane potential to some extent (Kordas, 1969; Magleby & Stevens, 1972a, b). Thus it may be possible to apply abrupt changes in potential to the end-plate membrane by a voltage-clamp technique, and then to follow the kinetics of the relaxation process by which the drug-sensitive channels adjust to this perturbation, in a manner analogous to the temperature-jump technique.

A second very promising approach can be expected to come from the recent finding by Katz & Miledi (1970, 1971, 1972) that the electrical 'noise' of the end-plate membrane is increased when the membrane is depolarized by acetylcholine or carbachol. They have argued that these electrical fluctuations result from the switching on and off of individual receptor-operated channels, and from a spectral analysis of the noise they were able to calculate the number of channels opened by the acetylcholine released in a single transmitter quantum as well as the average duration for which the channels remain open (approximately 1 ms for acetylcholine, 0·4 ms for carbachol). In principle, analysis of this phenomenon could yield the same sort of conclusions as a relaxation method if, for example, the concentration dependence of the noise spectrum, or the effects of antagonists upon it can be measured with precision; it may therefore provide a means of distinguishing between the two-state model and the conventional model for receptor activation, and also of determining what kind of interaction is responsible for cooperativity.

Finally, no discussion of receptors would be complete without a mention of the recent rapid progress that has been made in isolating receptor macromolecules (see review by O'Brien, Eldefrawi & Eldefrawi, 1972 and papers in Symposium on Drug Receptors, Rang (ed.) 1973b). It is from these studies that we shall eventually expect to learn exactly how receptors respond to drug molecules.

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