the $pA_2 - pA_{10}$ differences of non-competitive antagonists approach those of competitive antagonists and the distinction between the two types of antagonism becomes altogether blurred. It can sometimes be assumed on the basis of the concentration-action curve of the agonist that the effect is proportional to y. In that case for a non-competitive antagonist $pA_h = \log K'_2$; this relation is obviously less likely to be experimentally realized than the corresponding competitive relation $pA_2 = \log K_2$.

The pA_h can be considered as primarily an empirical measurement. Many drugs which depress the maximum of the concentration-action curve are probably not true non-competitive antagonists but unspecific depressants. When the pA_h of these drugs is measured it is found to be closely correlated with depression of oxygen consumption (106).

Conclusion. The pA_x and pA_h are empirical measures of the activity of drug antagonists. In some special cases they have theoretical significance since they may correspond to the mass equation constants of competitive and non-competitive antagonists.

NONEQUILIBRIUM DRUG ANTAGONISM[1, 2]

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Pharmacological antagonists to endogenous or exogenous chemical stimuli classically have been categorized as competitive or noncompetitive. Competitive antagonists are believed to react with the same groupings or configurations on or in cells with which the agonist combines to produce its characteristic effect, the specific receptors[3]. Noncompetitive antagonists may act at any other point,

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[2] There is no general agreement on the appropriate term to be applied to the type of pharmacological action discussed herein, *i.e.*, the blockade by a drug which forms a stable bond with specific receptors, and as a result is not in mass-action equilibrium with the agonist. It is the opinion of the author that "nonequilibrium blockade" is the most suitable. This designation has been used to distinguish the action of members of the Dibenamine series from that of other adrenergic blocking agents (115), and appears to most closely describe the action in question. The terms "irreversible competitive blockade" (49) and "unsurmountable blockade" (55) also have been applied to this type of action. However, the blockade is not strictly irreversible, and the term unsurmountable is appropriate only when the antagonist is used in sufficiently large doses to prevent a maximal response even in the presence of massive doses of agonist.

[3] The concept that drugs produce their effects by combining with specific receptors (or receptive substances) in cells originated with Langley (82), and has been very fruitful in the development of pharmacology. It has been attacked from time to time, but it is difficult to deny that chemical agents must combine or react with something in order to produce an effect. It is also clear that receptors may be specific for certain compounds or groups of compounds because it is possible to block responses to one substance or group without

presumably somewhere along the chain of events between the receptor and the observed response. Thus it may be anticipated that competitive antagonists will exhibit greater specificity than noncompetitive. Indeed, the ultimate in nonspecific, noncompetitive antagonism is death of the responding cell. Inasmuch as the search for more specific agents is one of the keynotes of pharmacological investigation, it is not surprising that competitive antagonists have received major attention, and all of the important, commonly employed blocking agents appear to have this type of action.

Although most specific blocking agents are assigned, probably correctly, to the classification of competitive antagonists, the evidence upon which this classification is based may be open to question. The classical basis for distinguishing between competitive and noncompetitive antagonism has been the effect on the dose-response curve of the agonist in question. Mathematical formulation of the distinction between competitive and noncompetitive antagonism was presented by Lineweaver and Burk (84) in relation to enzyme activity, and similar principles have been applied by many investigators to the much more complex responses of cells, tissues and organ systems (30, 49, 51, 131, 134). The equations employed are derived on the assumption that the agonist and antagonist are in mass-action equilibrium with the receptors, and are equivalent to the adsorption equations of Langmuir (69, 83).

In simplest terms the formulations for competitive and noncompetitive drug antagonism predict that increasing concentrations of the former will shift the characteristic log dose-response curve of the agonist to the right along the dose axis without changing either its slope or its asymptote, whereas a noncompetitive inhibitor will decrease both the slope and the asymptote. These changes in the dose-response curves are distinctive because the noncompetitive inhibitor and agonist are not in mass-action equilibrium as they are in cases of competitive inhibition. Consequently, antagonists which form stable bonds with specific receptors, nonequilibrium blockade, should appear to be noncompetitive by this type of analysis.

The interpretation of experimental data in terms of competitive or noncompetitive inhibition is relatively simple when a single enzyme is involved. Here the active center (receptor) of the enzyme is directly involved in splitting the specific substrate (equivalent to agonist). Activity is proportional to enzyme-substrate complex [ES] formed and alteration of the substrate provides a direct measure of activity. However, the situation is much more complex when responses of cells, tissues or organs are considered. Here the combination of agonist and receptor is probably only the first of a series of events leading to the recorded response.

In complex systems, interpretation of dose-response curves in terms of mechanism of drug action must be approached with extreme caution. Although means are not currently available for rigorously disproving the theoretical significance of

affecting responses to others. It is only when "receptor" is taken out of its proper place as a descriptive term and is assumed to be an explanation of drug action that this concept becomes a hindrance to progress. Combination with receptors cannot be considered to be a useful explanation of drug action because all active agents must combine or react with something, and because we know nothing regarding the nature or location of most receptors or how the drug-receptor combination produces cellular or tissue responses.

hyperbolic dose-response or sigmoid log dose-response curves obtained from experiments on intact animals, it appears quite obvious that any relation of reactions such as the pressor response to adrenaline or noradrenaline to theoretical drug adsorption curves must be fortuitous. Such responses can be easily discounted because the response measured is obviously the resultant of many factors, *e.g.*, cardiac stimulation, and vasodilatation and vasoconstriction in many vascular beds, each receiving an unknown amount of drug, contributing a variable amount to the overall response and altering resistance in relation to the fourth power of changes in radius of the limiting vessels. The fact that such responses not infrequently conform to those expected from mass-action considerations should not tempt one to assume that they are a measure of this type of drug-receptor interaction. Rather it should suggest that this type of curve is common to a wide variety of biological responses and consequently, standing alone it may have very little theoretical significance, a situation comparable to that encountered in the evaluation of "autocatalytic" growth curves (40). Indeed, Clark (30) warned of this possibility in his early discussion of pharmacological responses in terms of adsorption isotherms.

Even in much simpler systems, such as isolated organs or organ segments with relatively parallel reacting elements, "mass action" dose-response curves and modification of these curves by antagonists can provide reliable critera of the nature of the drug-receptor combination and of the antagonism only if certain assumptions are valid. The most important of these for the present discussion are: 1) The reaction between the drug and its receptors is reversible and obeys the laws of mass action, 2) all receptors are equally accessible to the drug, and 3) the response is proportional to the number of receptors occupied, *i.e.*, the maximal response occurs when all receptors are occupied. These assumptions have in the past been tacitly accepted by many workers, although they only recently have become even partially amenable to experimental scrutiny. It should be noted that A. J. Clark, one of the pioneers in the study of dose-response relations, called attention not only to the unproved nature, but also to the improbability of some of these assumptions (30).

Reliance on the interpretation of graphic dose-response data in determining the competitive or nonequilibrium nature of a particular antagonism has led to apparent misinterpretations of the nature of the blockade produced by various β-haloalkylamines (27, 28, 62). Except at high concentrations of inhibitor, data for the blockade of responses to adrenaline or histamine by these agents are typical of classical competitive inhibition when plotted by the methods of either Lineweaver and Burk (84) or Gaddum (51). This observation conflicts with several lines of evidence which indicate that the blockade, although it is "competitive" in the sense that the agonist and antagonist react with the same receptors, is predominantly nonequilibrium because of stable bonding of the inhibitor to some tissue constituent in the area of the receptor (*vide infra*). This discrepancy appears to be due to the fact that the maximal tissue response does not require occupancy of all receptors as originally postulated (*vide infra*), a fact which makes the above formulations unsuitable as a basis for classifying types of blockade.

If the classical mass-action theory equations cannot be used as reliable tests of the competitive or nonequilibrium nature of a blockade, what tests are reliable? At the present time it appears that no one universally applicable test is completely reliable, and the results of a variety of studies must be evaluated before positive identification of nonequilibrium blockade can be made. In general, the nonequilibrium blocking agents tend to have a more prolonged action than reversible competitive agents. However, wide variations in duration of action are present within both groups, and there is sufficient overlap to preclude the use of duration of action as a definitive criterion.

The process of blockade by any agent which reacts with specific receptors to produce its effect may be represented by the equations

$$I_a \underset{k_b}{\overset{k_a}{\rightleftarrows}} I_b; \qquad I_b + R \underset{k_2}{\overset{k_1}{\rightleftarrows}} IR,$$

where I_a is inhibitor in the medium surrounding the tissues in question (organ bath fluid, blood and extracellular fluid, *etc.*), I_b is inhibitor in the immediate environment of the receptors, the biophase (42) and R the specific receptors. If the duration of action were limited by the equilibrium $I_b + R \underset{k_2}{\overset{k_1}{\rightleftarrows}} IR$, a prolonged action after removal of active drug from the surrounding medium would indicate a very low value of k_2, a reliable measure of nonequilibrium blockade. This condition appears to be satisfied in solutions of enzymes, and failure to regenerate activity after prolonged dialysis effectively distinguishes the nonequilibrium action of DFP (diisopropylfluorophosphate) from the classical competitive action of physostigmine (96, 102, 103). However, Gaddum suggested in 1937 (51) that other factors, such as diffusion, are involved in tissues. The response to an adequate dose of agonist may be rapid in tissues treated with a classical competitive blocking agent with relatively persistent action, and the presence of a large, effective dose of agonist does not speed the disappearance of the antagonist during washing (29). More recently Furchgott (49) has pointed out that penetration into and escape from the biophase effectively limit the rate of onset and duration of action of most or all competitive blocking agents (k_1 and $k_2 \gg k_a$ and k_b) (*cf.* 44).

The times required for penetration of and escape from the biophase appear to be essentially equal for most classical competitive blocking agents, *i.e.*, the rate of development and dissipation of the blockade are equal. The duration of the blockade produced by nonequilibrium agents under most experimental conditions appears to be much greater than the time required for onset, probably because the reaction $I_b + R \underset{k_2}{\overset{k_1}{\rightleftarrows}} IR$ is limiting and $k_1 \gg k_2$. Under suitable conditions this difference in the rates of onset and dissipation may be taken as presumptive evidence of nonequilibrium blockade. However, the fact that $k_1 \gg k_2$ provides the condition necessary for prolonged accumulation of IR, which can be demonstrated to continue well beyond the time usually required for development of the blockade when tissues are exposed to very low concentrations of a β-haloalkyl-

amine (112), and it is thus theoretically possible for a nonequilibrium blockade to have equal onset and disappearance times.

Classical competitive antagonism can be distinguished from nonequilibrium antagonism also by other tests which take advantage of the fact that different factors control the temporal development and disappearance of these two types of blockade. The time-course appears to be limited primarily by penetration into and escape from the biophase (k_a and k_b) in the case of competitive agents (49), and by the very low value of k_2 in the case of nonequilibrium agents. Development of the blockade produced by a classical competitive agent ceases and is reversed as soon as active drug is removed from the surrounding medium, whereas formation of stable IR complex from active nonequilibrium inhibitor present in the biophase may continue for some time after washing. Consequently, the cumulative effect of several short exposures to a competitive blocking agent should be less than that produced by a single exposure of equal total duration, whereas the cumulative effect of short exposures to a nonequilibrium agent should be greater than the effect of the single longer treatment. Although experimental verification of this assumption has been limited to a relatively small number of compounds, comparison of the effects of single and repeated exposures has provided a clear differentiation of blockade produced by Dibenzyline from that due to several competitive inhibitors (112).

The fact that the reactions of all known nonequilibrium inhibitors (organophosphorus anticholinesterases and β-haloalkylamines) with receptors appears to occur in two steps, the first reversible "adsorption" and the second a more stable chemical reaction (107, 115, 116), makes it possible to draw reasonably reliable conclusions regarding the nature of the blockade from its rate of dissipation during dialysis of enzymes or washing of isolated tissues. Classical competitive agents wash out of tissues or dialyze from enzyme solutions as a continuous function of the concentration gradient, whereas the dissipation of a nonequilibrium blocking agent reveals a discontinuity. A portion of the blockade disappears relatively rapidly, a measure of the escape of inhibitor from the biophase (k_b), whereas the remainder is removed very slowly, limited by the k_2 of the drug-receptor complex. The second component increases with increased time of drug-enzyme or drug-tissue incubation (107, 112).

Under appropriate conditions classical competitive and nonequilibrium antagonism can be distinguished by a simple plot of a series of the ratios $\frac{\text{agonist}}{\text{antagonist}}$ required to produce a standard response (the "isobol"). The position of these points is determined primarily by $I + R \rightleftarrows IR$ if the effects of rate of penetration and escape of drug are controlled by the use of standard conditions. However, because both competitive and nonequilibrium antagonists can shift the dose-response curve of the agonist over a limited range without changing its shape (*vide infra*), differentiation of types of antagonism by this method requires that studies be made over a considerable range of doses of antagonist, and this increases the danger of the appearance of nonspecific, noncompetitive effects which may complicate interpretation. If this plot is a straight line, the agent in question may be considered to be a classical competitive antagonist. If the ratio

increases with increasing concentration of antagonist, and if complicating side reactions can be ruled out, the agent is probably a nonequilibrium or noncompetitive inhibitor. Even in intact animals this method clearly differentiates the competitive adrenergic blockade produced by Hydergine, phentolamine (Priscoline) and azapetine (Ilidar) from the nonequilibrium blockade produced by Dibenzyline (112).

None of the tests mentioned above determines whether or not a nonequilibrium antagonist is reacting with specific receptors. Fortunately, the initial competitive nature of a nonequilibrium blockade can be identified by direct methods. If the agonist and antagonist both react with the same receptors, a reciprocal inhibition should be demonstrable. Interference with inhibitor-receptor combination is not easy to demonstrate directly with classical competitive antagonists, probably because their action is limited by diffusion factors rather than by combination with receptors. However, if a tissue is exposed to a nonequilibrium antagonist for a short period of time in the presence of and in the absence of agonist, and the degree of "irreversible" blockade produced subsequently determined, it can be demonstrated that the reduction of the blockade is linearly related to the logarithm of the concentration of agonist (109). The specificity of this test is indicated by the fact that, where an antagonist is effective against responses to several types of stimulants (adrenergic, cholinergic, histamine, *etc.*), the presence of one agonist during exposure to the blocking agent prevents blockade of responses to only that specific type of agonist (48). This effect represents a specific protection of receptors.

Specific protection experiments with enzyme inhibitors are complicated by the fact that rapid splitting of the normal substrate limits the extent to which it can interfere with attachment of the antagonist. It was first reported that acetylcholine does not protect cholinesterase against the action of DFP (80), although subsequent studies using high concentrations of substrate have demonstrated protection against both diisopropylfluorophosphate (DFP) and tetraethylpyrophosphate (TEPP) (16). Strong indirect support for the assumption that DFP produces a nonequilibrium inhibition of cholinesterase by combining with the active sites on the enzyme is supplied by the observation that known competitive inhibitors of this enzyme, such as physostigmine (59), effectively protect cholinesterase against irreversible inactivation by DFP (16, 80).

Only two groups of agents have been shown to conform sufficiently closely to the characteristics outlined above to be classified as nonequilibrium antagonists—the organophosphorus anticholinesterases and the β-haloalkylamine adrenergic blocking agents and antihistaminics (Dibenamine and congeners). The following discussion of the mechanisms by which this type of blockade is produced will be limited largely to the latter, which more closely resemble classical pharmacological antagonists.

It was early noted that blockade by Dibenamine of responses to sympathetic nerve activity, adrenaline and other sympathomimetics was prolonged and effective against very large doses of agonist (113). These characteristics, particularly the latter, provided presumptive, but not conclusive evidence that the blockade produced was of the nonequilibrium type. Coupled with the known in-

stability of compounds of this type at body pH, these observations were taken to indicate that the prolonged blockade was due to some effect of the drug which occurred shortly after administration and was only slowly reversed (108, 113). However, this concept was questioned by Brodie and coworkers (17, 22), who found that following the administration of massive doses of Dibenamine or Dibenzyline (necessary to allow quantitative analytical procedures), a considerable amount of drug accumulated in neutral fat depots and this material disappeared slowly as the blockade wore off. They suggested that slow release of active compound from fat depots rather than stable combination with the blocked tissue was responsible for the prolonged action. Possible accumulation of active material in fat was suggested earlier by Nickerson and Goodman to explain the appearance of blockade when thiosulfate blood levels were allowed to decline some hours after the administration of Dibenamine (114). However, it was pointed out that the blockade produced by this agent far outlasts the presence of active drug.

Although persistence of active drug in the body might explain the prolonged action of Dibenamine and related agents, it fails to explain the completeness of the established blockade, and this interpretation is strongly opposed also by more direct evidence. Studies with Dibenzyline-C^{14}, employing doses closer to the range usually used to produce adrenergic blockade, failed to confirm any appreciable accumulation of active or inactive drug in neutral fat depots (72). The difference between these results and those of Brodie and coworkers can probably be explained simply on the basis of the doses employed.

The results of cross-circulation experiments indicate that the blood stream of blocked animals is free of effective concentrations of active drug long before the blockade disappears (1, 63), and local blockade produced by intra-arterial administration of Dibenzyline has been shown to persist for twenty-four hours or longer without extension to other areas (1). Perhaps the most conclusive evidence that the β-haloalkylamine blockade is due to some persistent effect directly on the blocked tissue is the persistence of the blockade *in vitro*. Exposure of rabbit aorta strips to Dibenamine or Dibenzyline for periods as short as five minutes produces a blockade which persists for several days in the absence of any possible reservoir of active drug (48, 110).

Thiosulfate, which chemically inactivates β-haloalkylamines by reacting with the intermediate ethyleneiminium, can prevent the development of blockade *in vivo* when administered prior to the blocking agent, but has little effect when administered after the development of the blockade (41, 63, 73, 108, 114). Carefully timed experiments on rats injected with Dibenzyline intramuscularly indicate that blood levels of thiosulfate (1.0 mg/ml), which essentially completely prevent the development of blockade, do not significantly alter an established blockade over a period of at least ten hours, the maximum time that the experimental animals could be kept in good condition with this blood level of thiosulfate (110). That some active drug may enter the circulation long after intramuscular administration was indicated by the fact that if the thiosulfate blood level was allowed to decline as late as ten hours after injection, some blockade developed. However, this cannot be taken as evidence that active drug

was entering the blood stream from fat depots. It is more probable that in these experiments a local depot was established by precipitation of drug at the site of injection.

On the basis of the varied experiments mentioned above, it appears probable that the prolonged blockade produced by β-haloalkylamines is due to some relatively irreversible action on the blocked tissues, and that accumulation of active drug in neutral fat depots plays no more than a minor role, if, indeed, such accumulation does occur to any significant extent when the usual blocking doses of these agents are employed.

If the duration of action of the β-haloalkylamines is due to some relatively irreversible reaction with the blocked tissues, the variable duration of the blockade produced by different members of the series, and differences between the duration of blockade of responses to adrenaline and to histamine require explanation. It is well established that the blockade produced *in vivo* by SKF 638A (N,N-dimethyl-β-phenyl-β-chloroethylamine) is much shorter than that produced by Dibenamine and most other members of the series (41, 112). It also has been reported that the duration of the adrenergic blocking action of SY-28 (J11; N-1-naphthylmethyl-N-ethyl-β-bromoethylamine) is only about one half that of Dibenamine, and that the duration of the antihistaminic action of SY-28 is much shorter than that of the adrenergic blockade produced (62). However, the rate of disappearance of the persistent, nonequilibrium portion of the blockade produced by SKF 638A in rabbit aorta strips is essentially the same as that of Dibenzyline (112). In addition, disappearance of the blockade produced by Dibenamine or Dibenzyline appears to be somewhat slower in isolated strips than *in vivo* (48, 112).

In the first discussion of the prolonged action of the β-haloalkylamine adrenergic blocking agents, it was suggested that the slow disappearance of the blockade was due either to slow removal of drug from the blocked receptors or to formation of new receptors (113). Evidence accumulated since that time indicates that the first alternative is more probably correct. A relatively slow enzymatic splitting of the drug-receptor bond would explain all of the currently available facts relating to the duration of action of these agents. Inasmuch as all active members of the β-haloalkylamine series appear to act through similar iminium intermediates (*vide infra*), the drug-receptor bonds should be comparable. However, differences in duration of action could be explained on the reasonable assumption that the configurations of both the drug and the receptor (adrenergic or histamine) affect the rate of splitting of this bond. It is also not unreasonable to assume that enzymatic splitting might be impaired in tissues maintained for a considerable period of time *in vitro*, and at lower temperatures. This mechanism may be considered analogous to the reactivation by "phosphatase" (15) or by nucleophilic reagents (70, 146) of cholinesterase "irreversibly" inactivated by the organophosphorus inhibitors, although the action of the nucleophilic reagents is nonenzymatic.

The concept of enzymatic splitting of the drug-receptor complex might also help to explain the fact that the homologous secondary amines are the only degradation products of the β-haloalkylamines definitely identified, although they

have been shown to account for only a small percentage of the injected drug (17, 22). One would not expect the free drugs or their hydrolysis products to be dealkylated in this manner, but it is quite possible that drug firmly bound to some tissue constituent might be split at this locus.

Although the established blockade produced by β-haloalkylamines has typical nonequilibrium characteristics, the development of the blockade appears to occur in at least two stages, one of which is competitive and reversible. A competitive component of the blockade was first suggested by observations that the degree of blockade produced in intact animals was decreased by administration of adrenaline (108, 115), other sympathomimetic amines (117), or piperoxan (933F) (135) prior to the β-haloalkylamine or during the course of development of the blockade.

Direct evidence for two stages in the development of the blockade of both adrenaline and histamine by β-haloalkylamines has been obtained in experiments in which isolated tissues were exposed to the blocking agents for short periods of time, and the degree of blockade then tested at intervals as the drug was washed out (112, 116). If the blockade has not gone to completion, a variable percentage disappears during the early stages of washing, with a half life of ten to twenty-five minutes. After this component has been removed, the residual blockade is essentially unaffected by continued washing. In very prolonged *in vitro* experiments the relatively irreversible component of the β-haloalkylamine adrenergic blockade has been shown to decrease at a rate of 10% to 30% per 24 hours (48, 112). The relative magnitude of the reversible component can be varied from over 50% to essentially zero with different agents and periods of incubation. With all agents studied, the ratio reversible/irreversible decreases as the incubation time is increased (112).

The nonequilibrium, "irreversible" stage of the blockade produced by the β-haloalkylamines is "competitive" in the sense that the antagonist reacts with the same area or tissue constituent as the agonist. This was first clearly shown for the antihistaminic action of members of this series by exposing strips of guinea-pig ileum to a β-haloalkylamine in the presence of histamine or of a reversible antihistaminic such as diphenhydramine (Benadryl) or tripelennamine (Pyribenzamine), and washing out the agents simultaneously. Both histamine and the reversible competitive antihistaminics prevented development of the "irreversible" blockade of responses to histamine (116). Dibenamine and other β-haloalkylamines can block responses to 5-hydroxytryptamine and acetylcholine as well as those to histamine and adrenergic agents *in vitro* where high concentrations of antagonist can be employed. If a high concentration of any one of these agents is present during exposure to Dibenamine, blockade of responses to the specific stimulant present is inhibited without significant alteration of the blockade of responses to the other types of stimulants (48). These experiments demonstrate 1) that in each case the Dibenamine is reacting with the same locus as the specific stimulant, and 2) that the receptors for these different stimulants are distinct. Subsequent studies have shown that the degree of inhibition of a threshold blocking dose of Dibenzyline is linearly related to the logarithm of the concentration of agonist, or of a classical competitive antagonist present over a

considerable range, and, consequently, it may be assumed that this inhibition of the blockade is a measure of the relative number of receptors occupied by the agonist (109, 112).

In summary, current evidence indicates that the blockade produced by the β-haloalkylamines develops in two stages. 1) The inhibitor is attached to the receptor by the same relatively weak forces (hydrogen bond, ionic, *etc.*) which are involved in binding most agonists and classical competitive antagonists. During this stage the antagonist is in mass-action equilibrium with the agonist and may be removed relatively easily by washing the tissue. 2) The blocking agent then reacts with the receptor or some adjacent grouping to form a much more stable bond and is no longer in mass-action equilibrium with the agonist. During the first stage, the persistence of the blockade appears to be limited by diffusion of the drug out of the biophase; during the second stage it is determined by the very low dissociation constant of the inhibitor-receptor complex.

Although the occurrence of a competitive reversible phase during the development of the β-haloalkylamine blockade appears to be established, the presence of this component does not provide an adequate basis for the conclusion that the β-haloalkylamines produce a classical competitive blockade except when administered in large doses (27, 28, 62). This inadequacy is emphasized by the fact that a "competitive" blockade has been noted during the wearing off of a previously nonequilibrium blockade (62). Classical competitive blocking properties were ascribed to the β-haloalkylamines on the basis of the form of the dose-response curves, plotted according to the formulae of Lineweaver and Burk (84) and of Gaddum (51), for adrenaline and histamine in the presence of inhibitor. As mentioned above, such plots provide reliable information regarding the nature of the inhibition only if certain assumptions are valid. In particular, they assume that the maximal tissue response occurs only when all receptors are occupied by agonist. This assumption has been shown to be incorrect. Dose-response curves for histamine, determined on isolated segments of guinea-pig ileum treated with low concentrations of GD 121 (N-1-naphthylmethyl-N-ethyl-β-chloroethylamine, the chloro analogue of SY-28 and J11) and washed until only the nonequilibrium, "irreversible" component of the blockade remained, may be shifted at least two log units along the dose axis without significantly changing either the slope or asymptote (111).[4] A similar but smaller shift of the response to adrenaline of aorta strips treated with Dibenamine also has been reported (49). When the degree of blockade is further increased in either of these preparations, both the slope and asymptote are decreased. These observations can best be explained by assuming that receptor occupancy is not the limiting factor in the tissue activation and that occupancy of only about 1 % of the histamine receptors (perhaps up to ⅓ of the adrenergic receptors) is adequate to produce a maximal response. As the total number of free receptors is progressively decreased by "irreversible" combination with blocking agent, larger concentrations of agonist are required to provide the receptor occupancy necessary for any given response. Ultimately the total number of free receptors is reduced below the number required for a

[4] See (49) for a report of a personal communication from R. P. Stephenson indicating similar results.

maximal response even when they are saturated by a very high concentration of agonist.

The above observations provide an explanation for the fact that classical dose-response plots indicate "competitive" antagonism at low and "irreversible" or noncompetitive antagonism at high concentrations of a β-haloalkylamine. In addition, the difference between the relative numbers of histamine and adrenergic receptors required for a maximal response provides an explanation for the apparently more competitive nature of the blockade of histamine (62).

In contrast to most other pharmacological agents, the β-haloalkylamines possess high chemical reactivity, which provides a satisfactory basis for the nonequilibrium characteristics of the blockade produced. In common with that of the nitrogen mustards, the β-halogen of these compounds is readily lost in neutral or alkaline solution with formation of a highly reactive and unstable ethyleneiminium ring (26, 67, 121). This may react with water to form the corresponding primary alcohol, or it may react with a variety of chemical groupings, including a number found in tissues. It has been claimed that failure to find the primary alcohols of Dibenamine and Dibenzyline in the urine of animals administered the parent compounds indicates that the ethyleneiminium intermediates are not formed *in vivo* (17, 22). However, these agents have been shown to react predominantly with sulfhydryl even when this is present in only equimolar amounts (68). Consequently, it would not be surprising if very little of the active intermediate reacted with water in the presence of the large excess of sulfhydryl and other reactive groupings *in vivo*.

Several lines of evidence indicate that the reactive iminium intermediate is the molecular species directly responsible for the blockade. All structure-activity data on the several hundred members of the series are compatible with this hypothesis. Derivatives which are incapable of ethyleneiminium formation are uniformly inactive. Fluoro, cyano, hydroxyl and other β-substitutions which are not readily displaced to allow cyclization abolish activity, whereas comparable β-chloro, bromo, iodo and sulfonic acid derivatives are active except when a substituent such as phenyl, which prevents displacement of the halogen by decreasing electron availability at the nitrogen, is added (26, 56, 62, 115).

All active members of this series are tertiary amines. Some quaternary derivatives were originally reported to be active (115, 119). However, this conclusion was apparently due to misidentification of the compounds studied (the members of this series do not form quaternary derivatives readily by the usual methods), and several authentic quaternaries have now been found to be uniformly inactive (78). Removal of an active halogen to the γ-position also abolishes activity. Although such γ-halogen compounds will cyclize, the four-membered rings formed lack the high reactivity of ethyleneiminium. In addition to the complete elimination of activity by structural changes which preclude formation of reactive ethyleneiminium intermediates, many quantitative differences in activity appear to be induced by more subtle effects of various substituents on the reactivity of the intermediate (108, 115).

The activity of phenoxyethyl substituted members of this series, including Dibenzyline, requires special comment. Although all highly active members of

this subgroup meet the requirements of intermediate ethyleneiminium formation, nonhalogenated primary, secondary and tertiary phenoxyethylamines with adrenergic blocking activity are known (76, 118). However, this is only a superficial discrepancy. More detailed analysis has revealed that this blockade is readily reversible, and that it differs from that characteristic of the β-haloalkylamines in several other respects (118). The phenoxyethyl grouping apparently is sterically suited to produce a competitive blockade. Indeed, combination of this sterically favorable group with portions of the Dibenamine molecule led to the production of some of the most active adrenergic blocking agents known (118).

Other evidence of the role of the iminium intermediate in the production of the blockade has been obtained from studies of the effect of prior administration of thiosulfate. This nucleophilic reagent reacts rapidly with the ethyleneiminium intermediate of nitrogen mustards or β-haloalkylamine blocking agents to produce an inactive product. Although a minor direct displacement of halogen may occur (67), the fact that prior thiosulfate administration effectively prevents blockade is strong evidence for the essential role of the intermediate. More direct evidence confirming this conclusion has been obtained from tests of the blocking activity of solutions of various β-haloalkylamines in which most or all of the parent compound has undergone cyclization. Such solutions produce a biological effect which closely corresponds to the amount of calculated or directly determined iminium intermediate present (26, 41, 63, 67). Although iminium ion formation is essential for blocking activity, several completely inactive compounds have been found to undergo comparable internal reactions (67), and it is obvious that several other rather specific structural features are required for activity (115).

The nature of the chemical groupings with which the β-haloalkylamines react *in vivo* to produce a stable blockade are unknown, but it may be assumed that most of the administered drug reacts with something other than water. Studies of excreted degradation products of Dibenamine and Dibenzyline have not demonstrated the corresponding alcohols although the methods were adequate to detect these materials in the urine when they were administered as such (17, 22). Suggestive indirect information regarding the groupings involved in blockade has been obtained from *in vitro* experiments. Dibenamine and Dibenzyline have been shown to react with sulfhydryl, amino and carboxyl groups both of relatively simple organic molecules and of intact proteins (68). Sulfhydryl is probably the best candidate for a role in the blocking reaction because this grouping reacts more completely with the blocking agents and is capable of competing successfully with the others mentioned to react with a major portion of a limited amount of Dibenamine. Evaluation of these *in vitro* results is complicated by the fact that they distinguish β-haloalkylamines devoid of blocking activity from active compounds only by minor quantitative differences. Among the compounds studied, active blocking agents combined somewhat more selectively than inactive compounds with sulfhydryl when the reaction occurred in the presence of amino groups, and the reaction of the former with sulfhydryl was less inhibited by the falling pH of unbuffered solutions (68).

Dibenamine effectively combines with liver sulfhydryl *in vitro* and Dibenzyline

causes a detectable decrease in total body sulfhydryl *in vivo*, but only when administered in near-lethal doses (68). The latter observation suggests a striking specificity for certain functional groupings. An average blocking dose of Dibenzyline could react with <0.01% of the total body sulfhydryl, and the fraction would be even smaller if amino or carboxyl groups were involved. Studies of reactions of an antimitotic β-haloalkylamine with thiol compounds have indicated that sulfhydryl groupings in a local alkaline medium would provide the nucleophilic characteristics necessary for a selective reaction (127). This presents an interesting possibility, but it must be concluded that present data are inadequate to warrant further speculation regarding the nature of the reactive groupings of the specific receptors with which β-haloalkylamines react to produce a nonequilibrium blockade.

SUMMARY

Numerous studies during the past decade have demonstrated that certain pharmacological antagonists act through a relatively stable combination with specific receptors. This action cannot be considered to be truly irreversible. However, it is qualitatively different from classical competitive antagonism in that the agonist and antagonist are not in mass-action equilibrium with the receptors. The blockade produced may therefore be referred to as nonequilibrium. It is clearly different from noncompetitive antagonism in which the antagonist acts at some point other than the site of action of the agonist in question. Nonequilibrium blockade cannot be adequately distinguished from competitive or noncompetitive blockade on the basis of effects on the agonist dose-response curves, or on the basis of duration of action. However, satisfactory differentiation can be made by a combination of tests, several of which depend upon the fact that the persistence of a competitive blockade appears to be limited by diffusion of antagonist into and out of the immediate environment of the receptors (biophase), whereas that of a nonequilibrium blockade is dependent upon the stability of the inhibitor-receptor complex.

The action of the two established groups of nonequilibrium inhibitors, the β-haloalkylamine antihistaminics and adrenergic blocking agents, and the organophosphorus anticholinesterases, appears to develop in two stages. 1) The inhibitor is attached to the receptor by the same relatively weak forces (hydrogen bond, ionic, *etc.*) which are involved in binding most agonists and classical competitive antagonists. During this stage the antagonist is in mass-action equilibrium with the agonist and may be removed relatively easily by washing the tissue. 2) The blocking agent then reacts with the receptor or some adjacent grouping to form a much more stable bond and is no longer in competitive equilibrium with the agonist. Because of the competitive phase early in the development of blockade, prior occupancy of the receptors by agonist inhibits the development of blockade and provides direct evidence that the reaction is within the specific receptors.

In the case of β-haloalkylamines, the development of a stable drug-receptor bond can be related directly to the chemical reactivity of the ethyleneiminium

intermediates formed at physiological pH. The nature of the chemical structures with which these drugs react in tissues has not been established, but *in vitro* studies suggest that sulfhydryl groups may be involved.

The availability of agents which form stable bonds with a variety of specific receptors provides an important tool for the analysis of drug effects. Use of these agents has made it possible to demonstrate that the receptors for adrenaline, histamine, acetylcholine and 5-hydroxytryptamine are distinct (48), that only a fraction of the total adrenergic or histamine receptors is necessary for the production of a maximal tissue response (48, 111), and that activation of receptors is not an all-or-none process (109). Many other applications of these agents to the analysis of mechanisms of drug action will undoubtedly be developed.

KINETICS OF RECOVERY FROM INHIBITION BY ANTIHISTAMINICS, ATROPINE AND ANTISPASMODICS

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The problem to be discussed here deals with the spontaneous recovery of the sensitivity of plain muscle after the antagonist has been washed out from the external fluid. Since the inhibition produced by antihistaminics, atropine-like substances and antispasmodics persists for a while after the antagonist has been washed out, there is a common feeling that the wash out phenomenon is due to a slow diffusion of the antagonist out of the biological structure, but the finding that the phenomenon is greatly influenced by temperature and the ionic composition of the bathing fluid suggests that it cannot be entirely accounted for by diffusion. A more fundamental process of recovery appears to be involved (19, 126).

I shall employ the expression "receptors" as indicative of patches at the surface of the muscle cells, suitable for the fixation of certain chemical configurations belonging either to the active or to the "false" drug and shall assume that the "number of receptors occupied by the active drug is linearly related to the intensity of the effect as measured upon the smoked drum". I shall also assume that if an effect (y) is reduced, for example, to 10% of its previous measure by contact of the muscle with the antagonist, 90% of receptors are blocked by the antagonist, provided the testing dose of the agonist and the conditions of the assay have not changed.

The inhibitor-receptor complex (IR). If we assume that the process of recovery depends upon the breakdown of a hypothetical complex (IR) of the inhibitor with the receptors and that the percentage of reduction of response at any moment measures the actual concentration of the complex still present, we can test in the first instance whether the breakdown of the complex (IR) follows the course of a monomolecular reaction. If P_0 indicates the percentage response of