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

the muscle immediately after washing out of the inhibitor, and P_t the response at time t, we obtain the monomolecular expression

$$k = \frac{1}{t} \log \frac{100 - P_0}{100 - P_t} \qquad \text{(I)}$$

By calculating k, in a series of experiments employing antihistaminics and atropine-like substances, we have found that k is by no means a constant. Its value increases continuously as the process of recovery goes on and we have obtained a linear relationship between k and successive values of P. Thus the velocity of recovery increases linearly with the responses to the testing dose of the agonist. Since k increases linearly with P, we have derived a constant $k' = 100\ k/P$ for different inhibitors and found that it behaves as a constant over a wide range of concentrations (Table I). For each value of P, *i.e.*, for each response of the muscle, we can calculate a value for k and from each value of k, we can derive a value for k'. The average of all values of k' will give the value of *constant* k' for each dose or concentration of the inhibitor.

If is therefore obvious that the process of recovery does not follow the equation of a monomolecular reaction. Since the velocity of recovery of "receptors" increases as the process progresses we have tentatively assumed that the process is an autocatalytic one. This view was further confirmed by fitting the logistic equation to the curve of recovery from inhibition, as will be shown in a later paragraph.

The index R_{50}. As a useful index of the intensity of the inhibition produced by a certain dose of the antagonist, we have selected the time (in seconds) necessary for a 50% recovery of the muscle, after washing out of the inhibitor. This index could be measured directly upon the recovery plots, when increasing concentrations of the antagonist were applied for exactly one minute contact and washed out. Every one and a half minutes the same dose of the agonist was applied and the percentage recovery measured directly in millimeters. The index R_{50} can be measured independently of any theoretical considerations by estimating graphically the abscissa of the point of intersection of the recovery curve on the line corresponding to 50% recovery. It can also be deduced from any point on the curve by calculating k' and using the equation:

$$R_{50} = 36.1/k' \qquad \text{(II)}$$

For each antagonist we have a family of curves and for each curve we can thus measure the respective "index R_{50}" (these values are indicated in Table I, as "R_{50} found"). It has been shown that this index is linearly related to the logarithm of the dose of antagonist and might be utilized as a measure of intensity of inhibition.

Characteristics of the recovery process as measured by R_{50}. It is interesting to stress a few points which have been extensively discussed in the original publications, as regards the intimate mechanism of the recovery process, as measured by the index R_{50}: (a) The process does not appear to depend upon the nature of the substances employed as antagonists; after inhibition by such unrelated substances as lysolecithin, benadryl, neoantergan, atropine and others, the muscle

TABLE I

Examples of calculation of constant k'

Lysocithin (1 mg) × (histamine)				Benadryl (5 μg) × (histamine)				Atropine (20 μg) × (acetylcholine)			
t (min)	P (%)	k	k'	t (min)	P (%)	k	k'	t (min)	P (%)	k	k'
0	3.0	—	—	0 to 3.0	0	—	—	0 to 9.0	0	—	—
1.5	4.4	0.003	0.060	4.5	8.5	0.008	(0.01)	10.5	2.5	0.001	0.040
3.0	6.6	0.003	0.046	6.0	28.5	0.024	0.085	12.0	5.0	0.002	0.040
4.5	8.9	0.006	0.067	7.5	46.0	0.036	0.078	13.5	7.5	0.002	0.033
6.0	11.1	0.007	0.063	9.0	60.0	0.044	0.061	15.0	10.0	0.003	0.030
7.5	17.7	0.009	0.051	10.5	72.0	0.053	0.073	16.5	20.0	0.006	0.030
9.0	26.8	0.013	0.049	12.0	72.0	0.046	0.061	18.0	40.0	0.012	0.030
10.5	42.5	0.021	0.050	13.5	85.0	0.061	0.072	19.5	45.0	0.013	0.029
12.0	55.0	0.028	0.052	15.0	100.0	—	—	21.0	75.0	0.028	0.037
13.5	64.0	0.032	0.050					23.5	75.0	0.027	0.036
15.0	77.5	0.041	0.053					25.0	100.0	—	—
k' (average)			0.054	k' (average)			0.071	k' (average)			0.034
±SE			±0.002	±SE			±0.004	±SE			±0.001
R_{50} (found) = 640″				R_{50} (found) = 480″				R_{50} (found) = 1176″			
R_{50} (calc.) = 660″				R_{50} (calc.) = 510″				R_{50} (calc.) = 1062″			

Values for constant k' and index R_{50} obtained with increasing doses of atropine × (acetylcholine)

Doses of atropine	$k' \pm$ SE	R_{50} (calc.)	R_{50} (found)
μg		(sec)	(sec)
0.5	0.270 ± 0.027	125	102
1.0	0.176 ± 0.045	212	144
5.0	0.056 ± 0.040	649	672
10.0	0.049 ± 0.004	740	780
20.0	0.034 ± 0.001	1062	1176
50.0	0.029 ± 0.0004	1250	1380
100.0	0.023 ± 0.001	1570	1512

Note: If the values for R_{50} (found) are plotted against R_{50} (calc.) a straight line is obtained.

follows the same law of recovery. This means that if a certain degree of inhibition is attained, the time for 50% recovery, and the shape of the curve relating recovery to time, are the same. (b) The value of R_{50} is independent of the number of washings of the preparation with new Tyrode solution: the degree of recovery is about the same if the muscle is washed once or repeatedly. (c) An excess of the active drug during the period of recovery *does not* accelerate the process; this condition has also been studied in detail by Bucher (24). (d) The process of recovery, and therefore the values of R_{50} are strongly dependent upon temperature; if the velocities of recovery are compared at 37° C., the ratio of velocities (Q_{10}) is 2–3. (e) Finally, certain cations, such as K^+, Li^+ and Sr^{++}, accelerate the process, while others, such as Mg^{++}, produce a definite retardation of the recovery process.

Such characteristics of the process of recovery would hardly be compatible with a simple diffusion process. They rather appear to depend upon the intimate potentialities of the muscle itself to regenerate some inherent structure that has been impaired by contact with the antagonist. It is even doubtful whether the antagonist is still present, since the whole process might depend upon correction or "healing" of a sequel or "lesion" left behind after washing out of the inhibitor. However, an irreversible combination of the inhibitor with the receptors and a process of slow elimination of the complex by some inherent activity of the muscle itself might also account for the observed facts.

The logistic treatment of the data. It has been shown (4) that the curve of recovery for atropine and trasentine (toward acetylcholine) can also be fitted by the logistic equation:

$$P = \frac{100}{1 + e^{-aKt}} \qquad \text{(III)}$$

where P represents the percentage of recovery attained at time t and the constant K is dependent upon the velocity of recovery. Details for the fitting of the recovery curve to this equation can be found elsewhere (125). It was also shown that the curves of recovery from atropine and trasentine can be rectified by using the *logit* transformation, according to Berkson (20).

Concluding remarks. Little can be added to what has been said in the original publications about the mechanism of the process of spontaneous recovery. The phenomenon appears to follow the course of an autocatalytic process, and can by no means be explained by the breakdown of a complex (IR) between inhibitor and receptors, according to the law of mass action. If one accepts the postulate that the intensity of the effect, as registered upon a smoked drum by a linear writing lever, measures the number of receptors occupied by the active drug, the increase in response during the process of recovery would measure the number of receptors set free from its blockade by the inhibitor, if all conditions of the assay are kept constant. Therefore, the facts presented above would indicate that the reappearance of receptors follows an autocatalytic course. We might formulate the above postulate in a more axiomatic language and say that "there is a function tentatively called *number of receptors*, which is linearly related to the intensity of the effect observed". It is, however, to be stressed that the analogy of the muscle surface with an adsorbing metal surface which has been basically utilized in the establishment of Clark's equation, could point to an identification of that linear function with the number of receptors actually occupied by the active drug. Other possibilities have been considered in order to explain the course of the recovery process. For instance, the sigmoid curve might represent an integrated frequency distribution of receptors with different affinities for antagonists. If this were the case it might be expected that variations of sensitivity at the beginning of the process would be as large as at the end, but the responses are much less variable at the beginning of recovery than at the end.

ADDENDUM*

Considerable discussion has been aroused concerning the statement that the response is linearly related to the number of receptors occupied by the active drug. This postulate is implied in the deduction of Clark's equation (see 30) and also in the deduction of Gaddum's equation (51) for antagonism of the competitive type. The validity of this postulate was recently challenged by Stephenson (141) on the basis that the concentration ratios for producing 80%/20% or 50%/20% or 80%/50% of the maximum response deviate very strongly from the theoretical ratios 16:4:4. However, Stephenson's method of obtaining the parameters of Clark's equation, by application of massive doses of the agonist in order to obtain the maximum effect ($y = 1$), is open to strong criticism. The correct way to verify the validity of Clark's equation is to take the reciprocal of both sides (27):

$$\frac{1}{Kx} = \frac{1}{y} - 1$$

and to test for the linearity of the relationship between $1/x$ and $1/y$, using the actual measurements obtained in the assay. The maximum response will be given by the intercept of the line with the ordinate axis, and from this value, a sort of "normalized" curve can be obtained, with intercept 1 and the reciprocals of the percentages linearly related to the reciprocal of the doses. Deviation from linearity has been found always non-significant when compared with the error of the assay. There is no reason, therefore, to reject Clark's equation and the assumption that there is a linear relationship between the number of receptors occupied by the active drug and the effect observed in the smoked drum still holds, as far as histamine is concerned.

If a concentration (I) of a competitive inhibitor, such as benadryl for histamine, is present, the line will turn a certain angle around the intercept ($1/y_{max}$) in such a way that the constant K (reciprocal of the slope) will be altered as follows:

$$\beta/K = k' \qquad \text{and} \qquad \beta = 1 + \frac{(I)}{K_i}$$

where k' is the slope of the new line (in presence of the antagonist) and K_i is a constant depending upon the nature of the antagonist. The significance of β is easy to grasp, since it is the ratio of the slopes "with" antagonist and "without" antagonist. It is also easy to find that the new constant K_i can be calculated as a measure of the concentration of the inhibitor which makes $\beta = 2$. Therefore, K_i represents the concentration of the inhibitor which reduces the effect produced by a double dose to that produced by a single one. The relationship between K_i and the index pA_2 introduced by Schild (131, 133) is obvious and one can write

$$\beta = 1 + \frac{(I)}{10^{-pA_2}}$$

From these considerations we have derived a rational method for determining

* During proof corrections.

the value of pA_2 by simply measuring the ratio of slopes (β) of the lines of the reciprocals of (responses-doses) and deducing, from the above equation, by the following formula:

$$-pA_2 = \log(I) - \log(\beta - 1)$$

By using this method we have calculated the pA_2 for the pair benadryl-histamine, and the values obtained agree with those of previous investigators, using more direct methods of determination.

Since such deductions depend upon the validity of the main arguments involved in Clark's and Gaddum's equations, their agreement with the experimental findings have, as a consequence, the rejection of Stephenson's hypothesis and a confirmation of the postulate invoking linearity between the number of receptors occupied by the drug and the effect as measured upon the smoked drum. Also the idea that a maximum contraction can be produced by a small number of receptors occupied by the drug should be rejected. There is no reason to suppose that the interrelationship between agonist and antagonist, when competition is established, will not follow Clark's and Gaddum's equations.

METABOLITE ANTAGONISMS IN BACTERIA

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Perhaps by historical accident this symposium on drug antagonism has been based largely on experiments on vertebrate tissues. In order to bring antagonisms in bacteria into the same picture, I would like to describe recent work (35) on the relations of growth inhibitors to purines in a strain of *Escherichia coli* (NCTC 8242) that requires purine. In this strain, the curve of growth plotted against log concentration of adenine follows a sigmoid course, but after reaching a peak turns downwards because excess adenine inhibits. The general antibacterial compound Dequadin (decamethylene-bis-4-aminoquinaldinium) (18) vertically depresses this curve, except where lack of adenine limits growth. On the other hand, the antipurine 6-mercaptopurine (39) shifts the log adenine-growth curve along the horizontal axis of the graph without changing its height or its eventual downward turn, so that the ascending portions of curves obtained in the presence and absence of 6-mercaptopurine run roughly parallel. If the extent of the horizontal shift, which characterizes competitive inhibition, is plotted against the negative log molar concentration of 6-mercaptopurine, a straight line is obtained. From this line the pA values of Schild (131) may conveniently be read off. For example, the pA_{10} of 6-mercaptopurine against adenine for *Esch. coli* (8242) is 3.12. It thus seems that in bacteria metabolite antagonisms may be analysed on similar lines to those described for vertebrate tissues.