THE INHIBITION OF CHOLINESTERASE BY PHYSOSTIGMINE AND PROSTIGMINE

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It has been generally assumed that the inhibition of cholinesterase by physostigmine and prostigmine is non-competitive. This is implicit in the equation given by Matthes (1) relating the degree of inhibition to the concentration of physostigmine, for it contains no term for substrate concentration, an omission justified only if the inhibition is non-competitive. However, it should be noted, as Matthes himself points out, that his equation fits only a certain range of the data, and that the discrepancies with low physostigmine concentration are probably not to be accounted for by experimental error. In any case, as Lineweaver and Burk (2) have made clear, the type of inhibition cannot be determined from this relationship.

Some years later Easson and Stedman (3) reached the tentative conclusion that inhibition by prostigmine is non-competitive on the indirect evidence that, if enzyme and inhibitor are allowed to reach equilibrium before the substrate is added, hydrolysis of the latter, provided a sufficient excess is present, proceeds at a constant rate. Their theoretical relationship between prostigmine concentration and the degree of inhibition is essentially the same as that of Matthes: there is assumed to be a reversible but non-competitive combination between 1 molecule of inhibitor and one active center of enzyme to form an inactive compound. They state that it is necessary to leave prostigmine in contact with the enzyme for at least 9 hours before measuring the inhibition in order to insure equilibrium, and in their experiments they apparently left the mixture for about 24 hours. Similarly Roepke (4) states that approximately 8 hours are required for physostigmine and prostigmine to reach an equilibrium with the enzyme. Using an indicator method, he measured the rate of hydrolysis of varying concentrations of acetylcholine by a cholinesterase preparation which had been mixed with the drug and left overnight in the ice box. When the reciprocals of the velocities thus obtained were plotted against the reciprocals of the substrate concentrations (cf. Lineweaver and Burk (2)), it was found that the points lay upon two straight lines which did not cut the 1/v axis at the same point, typical of non-competitive inhibition. On the other hand he failed to confirm Easson and Stedman, finding that the rate
of hydrolysis very slowly decreased on addition of substrate, and concluded that these drugs are probably also competitive inhibitors.

An equilibrium reached only after 8 or 9 hours would seem to be, in the case of these drugs, of only slight physiological importance, for the action in the body begins within a few minutes and is usually over within a couple of hours. Effects obtained immediately after the drug was mixed with the enzyme were accordingly investigated with an electrometric titration method slightly modified from that of Alles and Hawes (5) and described elsewhere (Eadie (6)). Serum was kept in a stoppered glass tube, through which bubbled nitrogen. This precaution was taken because otherwise blanks with serum alone were found to become alkaline at a measurable rate, presumably from loss of carbon dioxide. Dog serum was used as the source of enzyme, 1 cc. being added to the titration mixture, the total volume of which was 25 cc. The alkali used was 0.01 N NaOH, and the temperature was 36.5°.

Existence of Early Equilibria—The usual criterion for the existence of an equilibrium is taken to be the constancy of the rate of hydrolysis of acetylcholine. Three sets of conditions, however, must be distinguished. (1) When the initial concentration of acetylcholine is sufficiently high so that it remains practically constant during the course of the experiment (20 minutes), the amount hydrolyzed per minute will remain constant. (2) With lower acetylcholine and moderately low inhibitor concentrations, the rate will follow the usual logarithmic law for monomolecular reactions. (3) It will be shown later that the inhibition is competitive; because of this the degree of inhibition will be greater, the lower the substrate concentration. Hence with low acetylcholine and relatively high inhibitor concentrations, the rate will fall off more quickly than predicted by the monomolecular equation. This departure, however, will not occur until an appreciable amount of substrate has been hydrolyzed.

When the experiments were analyzed, it was found that with high acetylcholine concentrations plotting the NaOH used against time yielded a straight line; plotting \( \log a/(a - x) \) against time also yielded a straight line not only here but with lower acetylcholine concentrations, the only exceptions being when the inhibitor concentrations were very high, and even in these cases the divergence did not occur before 15 minutes or so. Fig. 1 illustrates the first of these cases, and also shows that the new equilibrium became established within 5 minutes. In most cases examined it took about half this time.

Velocities, accordingly, were calculated by fitting the data to the logarithmic equation by the method of least squares. The velocity constant thus obtained was multiplied by the initial acetylcholine concentration to get the actual initial velocity, a correction being made for hydrolysis due to OH ion.
All experiments were rejected in which the variance of estimate (i.e. the sum of the squares of deviation from a straight line divided by the degrees of freedom) exceeded an arbitrary value which was fixed at $1.0 \times 10^{-6}$. In the case of the experiments in which a departure from linearity occurred towards the end, only the earlier points were used, provided, of course, that they met the preceding criterion.

**Effect of Varying Substrate Concentration**—The difference between competitive and non-competitive inhibition can be determined only by experiments with varying substrate concentration (2, 7). The relationship between velocity of hydrolysis and substrate concentration is given by the equation, modified from Michaelis and Menten (8)

$$v = \frac{Vx}{x + K_p}$$

where $v$ is the initial velocity, $x$ the molar substrate concentration, and $V$ the value of $v$ when $1/x$ is 0. $K_p$ stands for $K_M (1 + f^n/K_f)$ where $K_M$ and $K_f$ are the enzyme-substrate and enzyme-inhibitor dissociation constants respectively, $f$ the molar concentration of inhibitor, and $n$ the number of molecules of inhibitor combining with 1 molecule (or active center) of enzyme. In the absence of inhibitor $K_p$ reduces to $K_M$. In non-competitive inhibition the value of $V$ is decreased, and in competitive inhibition that of $K_p$ is increased.

The equation in the form given is not very easy to deal with, and Lineweaver and Burk (2) suggested using $1/x$ and $1/v$ as the variables; so that it becomes

$$\frac{1}{v} = \frac{1}{V} + \frac{K_p}{V} \frac{1}{x}$$
When plotted, this gives a straight line of slope $K_p/V$ cutting the $1/v$ axis at $1/V$. Competitive inhibition is now characterized by a change in slope due to a change in $K_p$ (but not in $V$), and no change in the point of interception on the $1/v$ axis. Fig. 2 shows the data from a typical experiment with physostigmine plotted in this way, and Fig. 3 one with prostigmine. It is clear that in each case the data indicate competitive inhibition.

**Fig. 2.** The effect of substrate concentration on the velocity of hydrolysis of acetylcholine by dog serum in the presence (upper curve) and absence (lower curve) of physostigmine, $1 \times 10^{-6}$ M.

**Fig. 3.** The effect of substrate concentration on the velocity of hydrolysis of acetylcholine by dog serum in the presence (upper curve) and absence (lower curve) of prostigmine, $1.5 \times 10^{-3}$ M.

The data show enough variation to make a statistical analysis advisable. For this purpose the equation as just given is not very satisfactory, particularly if we are interested in the value of $K_p$. We may, however, rewrite it in the form

$$v = V - K_p c$$

where $c$ is the velocity constant; i.e., $v/x$. This form has the advantage that the slope, $K_p$, is now the regression coefficient, and the two constants
have been placed in separate terms. \( V \) can be shown to be equal to \( \bar{v} - K_p\bar{c} \), where \( \bar{v} \) and \( \bar{c} \) are the mean values of these variables.

The question whether the variation in the values obtained for \( K_p \) in different experiments may be attributed to the experimental error of the individual determinations can be answered by calculating \( F \), the ratio of the two variances involved. In each of the three cases (acetylcholine alone, and with physostigmine and prostigmine) \( F \) was found to be below the level of significance; experimental error is therefore sufficient to account for the differences. The test also indicates that pooling of data is permissible. Table I shows the values of the constants obtained by successive poolings, and illustrates the more or less rapid approach to a fairly stable value. The most probable value is the final one in each series. The
standard error of the mean is also given. The difference in $K_p$ produced by the addition of physostigmine and prostigmine was found to be highly significant by the $t$ test. This is characteristic of competitive inhibition.

$V$ is a value obtained by extrapolation, and therefore subject to greater errors than $K_p$. Calculation of $F$, however, gave values well beyond the 1 per cent level of significance, indicating that the differences between individual estimations are greater than might be expected from the errors involved. This is not surprising, since $V$ depends not only on the characteristics of the enzyme but also on the amount of it per cc. of serum. Significant alterations in $V$ were also found to occur on storage. We are therefore not justified in pooling the data to arrive at the most probable value of $V$, but must compare averages. For each batch of serum there was usually one determination of $V$ without inhibitor, and several with. Each determination was made from several titrations, usually three to five. For the sera used for the physostigmine experiments the values of $V$ without and with inhibitor were averaged, each being weighted according to the number of titrations involved in its determination; the standard deviations were also calculated. In the absence of physostigmine the average $V$ was 0.207 ± 0.47 (six experiments), and in the presence of $2.4 \times 10^{-7}$ M inhibitor 0.233 ± 0.032 (seven experiments). There is no significant difference between these values, and in any case the second is higher than the first rather than lower. For the sera used in the prostigmine experiments the values were without prostigmine 0.178 ± 0.028 (six experiments) and with prostigmine ($1.5 \times 10^{-7}$ M) 0.176 ± 0.013 (fifteen experiments). Here again there is no significant difference. There is thus no evidence of any change in $V$ as would have occurred with non-competitive inhibition. Statistical analysis thus confirms the conclusion that the inhibition is competitive.

The best value for the enzyme-substrate dissociation constant is 0.0017 ± 0.0001. The calculation of the inhibitor-enzyme dissociation constants must be postponed until we know the value of $n$, the number of molecules of inhibitor combining with 1 of enzyme.

Effect of Inhibitor Concentration—If our interest is in the relationship of the velocity to the inhibitor concentration with constant substrate concentration, it is convenient to rearrange the terms of the Michaelis equation and simplify by substituting new constants to get

$$\frac{1}{v} = a + b^f^n$$

If $n = 1$, which is usual, then the reciprocals of the velocities plotted against the inhibitor concentrations should form a straight line. Data for physostigmine are plotted in Fig. 4 and for prostigmine in Fig. 5. Only with lower concentrations of the inhibitor do the points approach a straight line.
If, however, \( n = 2 \), the points should fall on the curves shown in Figs. 4 and 5, and it is obvious that they fit them fairly closely.

**Fig. 4.** The effect of physostigmine concentration on the hydrolysis of acetylcholine (0.008 M) by dog serum. If 1 molecule of inhibitor combines with 1 of enzyme, the points should fall on the straight line; if 2 molecules of inhibitor, on the curved line.

**Fig. 5.** The effect of prostigmine concentration on the hydrolysis of acetylcholine (0.008 M) by dog serum. If 1 molecule of inhibitor combines with 1 of enzyme, the points should fall on the straight line; if 2 molecules of inhibitor, on the curved line.

The data have been analyzed statistically by fitting them, by the methods of multiple regression, to the equation

\[
\frac{1}{v} = A + Bf + Cf^2
\]

The standard partial regression coefficient for the first power of \( f \) was found to be negative and not significant; that for the second power, positive and significant. Deviations from linear regression were also found to be signif-
icantly greater than from curved regression. From this it may be concluded that $1/v$ varies as the second power of $f$, or that 2 molecules of these inhibitors combine with 1 of enzyme.

Since Easson and Stedman believe that their experiments indicate a combination of 1 molecule of prostigmine with 1 of enzyme, it is of interest to examine their data from this point of view. The results given in their Table VII have accordingly been plotted in Fig. 6, the (approximately) 24 hour figures in the column headed "Titration" being used as a measure of the velocity (two figures missing from this table can be supplied from the data in Table VIII). No correction was made for the difference in "time after mixing," since, as they point out, its magnitude is exceedingly small. The straight line and curve have the same significance as in Figs. 4 and 5. Here also the experimental points fit the curve very closely throughout the whole range, and do not fit the straight line except at lower concentrations, again indicating that the inhibiting combination is between 2 molecules of drug and 1 of enzyme.

**Inhibitor-Enzyme Dissociation Constants**—The enzyme-inhibitor dissociation constants may now be calculated from the data in Table I. They are $3.3 \times 10^{-14}$ for physostigmine and $3.6 \times 10^{-14}$ for prostigmine.

The standard deviation for these values cannot be calculated exactly, because $\rho$, the correlation coefficient between $K_M$ and $(K_p - K_M)$, is unknown. Maximum possible values for the standard deviations will be obtained if $\rho = -1$; these are $3.14 \times 10^{-14}$ and $4.33 \times 10^{-14}$ respectively; the true values are unquestionably less than this. Another estimate of the range may be obtained by using the ranges of $K_M$ and $K_p$ given by their standard deviations. This leads to a range of $K_f$ for physostigmine of $4.54 \times 10^{-14}$ to $2.47 \times 10^{-14}$, and for prostigmine of $5.40 \times 10^{-14}$ to $2.61 \times 10^{-14}$.
Comment

The type of inhibition has a bearing on the question whether the physiological effects are due to enzyme inhibition. In general, concentrations used in the test-tubes are likely to be greater than those reached at the point of action in the body, and, if inhibition is non-competitive, to produce the same degree of it would require the same concentration. On the other hand, if it is competitive, the extent of inhibition will depend also on the substrate concentration. Putting it in another way, we may say that the affinity of the enzyme for physostigmine or prostigmine is $10^{14}$ times its affinity for its substrate. These experiments therefore make it possible to explain the in vivo action on the basis of that in vitro.

The combination of 2 molecules of inhibitor with 1 of enzyme means that the number of enzyme molecules blocked increases with the square of the inhibitor concentration, rather than with the first power. It is thus possible to attain practically complete inhibition much more readily.

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SUMMARY

1. Mixtures of cholinesterase with physostigmine or prostigmine reach an equilibrium within a few minutes after being mixed.

2. The inhibition of the enzyme by these drugs is competitive.

3. 2 molecules of inhibitor combine with 1 molecule (or active center) of enzyme.

4. The inhibitor-enzyme dissociation constants are approximately $3 \times 10^{-14}$ for physostigmine and $2 \times 10^{-14}$ for prostigmine, the cholinesterase being that of dog serum at 36.5°. Under the same conditions the enzyme-substrate dissociation constant was found to be $1.7 \times 10^{-3} \pm 0.1$.

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