Studies on Cholinesterase

8. DETERMINATION OF REACTION VELOCITY CONSTANTS WITH A REVERSIBLE INHIBITOR OF PSEUDO-CHOLINESTERASE

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According to the classical theory of enzyme-substrate kinetics as proposed by Michaelis & Menten (1913), the activity of an enzyme is dependent on, and directly proportional to, the concentration of an intermediary enzyme-substrate complex. The concentration of this complex (ES) is determined by the mass law principles governing a reversible reaction between free enzyme (E_f) and free substrate (S_f), this reaction being of the form

Similarly, it is postulated that the degree of inhibition of enzyme activity by a reversible competitive inhibitor (I_f) is proportional to the concentration of an intermediary enzyme-inhibitor complex which is formed by the reaction

Straus & Goldstein (1943) and Goldstein (1944) have analysed the kinetics of enzyme-substrate-inhibitor reactions from this theoretical viewpoint, and predicted that the relative enzyme concentration could play an important role in determining the mechanism of these reactions. These theoretical predictions have been confirmed experimentally (Myers, 1952), and to this extent the theory not only describes the known facts but can also be used to make a successful prediction.

However, it has been pointed out by Briggs & Haldane (1925) that the overall reaction is actually of the form

and that this complete equation must be considered in theoretical treatments of enzyme kinetics. This formulation has been applied to the kinetics of peroxidase activity by Chance (1943), and in this case it was shown that the velocity constant for breakdown of the substrate in the enzyme-substrate complex is much larger than the velocity constant for dissociation of this complex. However, the results with this enzyme system were of a somewhat different nature than is usual with esterases in that the rate of enzymic breakdown of substrate was proportional to the concentration of a third reactant in the system, the oxygen acceptor. It was of interest, therefore, to determine the individual

velocity constants involved in a reaction with the cholinesterase system.

Direct measurements of these velocity constants is not possible with an enzyme such as cholinesterase, but in the treatment of enzyme kinetics given by Goldstein (1944), methods were introduced for the indirect determinations of the various velocity constants of each of the individual reactions involved in the above equations. However, it was impossible to arrive at any definite value for enzyme concentration in the cholinesterase system with which the theoretical data were illustrated. Consequently the reaction constants themselves could not be accurately evaluated at that time, and, moreover, the equations given by Goldstein (1944) require some modifications in view of the theoretical considerations of Briggs & Haldane (1925). It has recently been possible to obtain a reliable and relatively accurate estimation of pseudo-cholinesterase concentration by the use of potent reversible competitive inhibitors (Myers, 1952). Knowing the enzyme concentration, definite values can be assigned to all of the determinable reactions constants.

THEORETICAL

The theoretical formulations given below represent a modification and extension of Goldstein's treatment and therefore the mathematical symbols used by Goldstein have been retained throughout. One exception is made to distinguish the velocity constants for the destruction of inhibitor and of substrate, these being designated as k_5 and k_6 respectively. For a comprehensive background to this work, see Goldstein (1944).

In the treatment presented by Goldstein, it has been assumed that the enzyme reactions can be adequately represented by the equations

where enzyme activity is dependent upon the concentration of ES and inhibition of enzyme activity is dependent upon the concentration of EI. The symbols E, I and S are used to designate the molar concentrations of enzyme, inhibitor, and substrate, respectively. Thus the theoretical equation for the progress of the reaction between enzyme and substrate can be written as

$$\frac{d(ES)}{dt} = k_3 E_f S_f - k_4 ES. \quad (1)$$

At equilibrium, when the concentration of the enzyme-substrate complex is constant,

$$\frac{d(ES)}{dt} = 0, \quad \text{and} \quad \frac{E_t S_t}{ES} = \frac{k_4}{k_3} = K_s.$$

In this case, K_s could be termed a 'dissociation constant'.

The overall reaction between enzyme and substrate is actually of the form

when the initial constant velocity of enzyme activity is being measured (Briggs & Haldane, 1925). Thus the correct equation for the progress of the reaction between enzyme and substrate will be

$$\begin{aligned} \frac{d(ES)}{dt} &= k_3 E_t S_t - k_4 ES - k_5 ES \\ &= k_3 E_t S_t - (k_4 + k_5) ES. \end{aligned} \quad (2)$$

In this case $K_s = \frac{(k_4 + k_5)}{k_3}$, where K_s is now the experimental 'Michaelis constant'. Only if k_5 were very small in comparison to k_4 could these two values of K_s be taken as identical. However, the work of Chance (1943) on peroxidase has shown that, in this case at least, the Michaelis constant $(k_4 + k_5)/k_3$ is quite different from the dissociation constant k_4/k_3 .

The values $k_4 = 0.32 \text{ min.}^{-1}$ and $k_5 > 3500 \text{ min.}^{-1}$ given by Goldstein (1944) indicate very definitely that k_5 , the destruction constant, must also be taken into consideration when working with pseudo-cholinesterase. But the interpretation of these results is further complicated by the fact that one of these values must be erroneous. A consideration of the Eqns. (1) and (2) above will show that the mathematical treatments proposed by Goldstein are still valid if the correct constant $(k_4 + k_5)$ is substituted for k_4 throughout these equations and that the experimental value given for ' k_4 ' must, therefore, be the value of $(k_4 + k_5)$. This leaves us with the problem of reconciling the value $(k_4 + k_5) = 0.32 \text{ min.}^{-1}$ with the value $k_5 > 3500 \text{ min.}^{-1}$.

The value of $(k_4 + k_5)$ was determined simultaneously with the value of ' k_3 ' (or more correctly $k_3 + k_5$) from experimental data for the displacement of acetylcholine from pseudo-cholinesterase by added eserine. The equation representing the change in fractional activity a with time t will be

$$\frac{da}{dt} = \frac{S'(1-a) - I'a}{\frac{I'}{(k_4 + k_5)} + \frac{S'}{(k_3 + k_5)}}. \quad (3)$$

(See Goldstein's equation 15A.) From the data given by Goldstein, $I' = I/K_I$ had the values 35.5 and 355, while $S' = S/K_s$ was constant at 64.4. Thus, using the value $(k_3 + k_5) = 0.026$, $S'/(k_3 + k_5) = 2500$. For $(k_4 + k_5) = 0.32$, $I'/(k_4 + k_5) = 111$ and 1110 which, in comparison to the value of $S'/(k_3 + k_5)$, would be large enough to affect the value of da/dt appreciably, especially for the higher concentration of inhibitor. But if $(k_4 + k_5)$ had a very high value, let us say > 3500 , then the value of $I'/(k_4 + k_5)$ would be negligible in comparison to $S'/(k_3 + k_5)$, and thus $(k_4 + k_5)$ could not be determined from this type of experiment.

Integration of Eqn. (3), setting $a = 1$ when $t = 0$, gives

$$a = \frac{1}{S' + I'} \left\{ S' + I' \exp \left[- \frac{(S' + I') t}{I'/(k_4 + k_5) + S'/(k_3 + k_5)} \right] \right\}. \quad (4)$$

(See Goldstein's equation 16A.) On plotting the theoretical curves calculated from this equation, it is found, in fact, that the scattered experimental points can be fitted just as well by an equation where the term $I'/(k_4 + k_5)$ is neglected as by an equation where this term has the finite values given by Goldstein. This is illustrated in Fig. 1.

Fig. 1. Displacement of substrate by inhibitor. Eserine added at zero time to an equilibrated mixture of dog serum and acetylcholine. Data of fractional activity of enzyme (a) taken from table IV of Goldstein (1944). Points near A, with $1.1 \times 10^{-6} \text{ M}$ - and points near B, with $1.1 \times 10^{-5} \text{ M}$ - eserine. Theoretical curves calculated from Eqn. (4), see text. ---, $(k_3 + k_5) = 0.026$, $(k_4 + k_5) = 0.32 \text{ min.}^{-1}$ (identical with curves of fig. 8 of Goldstein, 1944); —, $(k_3 + k_5) = 0.026$, $(k_4 + k_5) > 3500$; —, $(k_3 + k_5) = 0.020$, $(k_4 + k_5) > 3500$.

A similar criticism also applies to the simultaneous determination of $(k_4 + k_5)$ and $(k_3 + k_5)$ from the experimental data for displacement of eserine by acetylcholine (Goldstein, 1944). Actually, Goldstein has noted in this case that the 'theoretical treatment does not correctly describe the reaction at low inhibitor concentrations'; but then the assumption that 'free enzyme is negligible' does not apply to the theoretical curves of Fig. 9 in Goldstein's paper. A more correct treatment of the kinetics of displacement of inhibitor by substrate is evident from consideration of the experimental fact that cholinesterase activity reaches a maximum within a very short time after addition of acetylcholine.

When the measurement of the normal activity of a cholinesterase is begun 1-3 min. after the addition of substrate to the enzyme, it is commonly found that the activity is linear in relation to time from the beginning of the determination (reference to this linearity will be found in, for example, Easson & Stedman, 1936; Goldstein, 1944;

Myers, 1951, 1952; Berry, 1951). In other words, the cholinesterase activity has increased from zero and has become constant and maximal within 1-3 min. after the enzyme and substrate were mixed. It is not feasible to measure the rate of increase of activity during the first minute by the techniques commonly used, but we can calculate on a theoretical basis how quickly this must occur. The theoretical equation for the rate of combination of enzyme with substrate, as derived from Eqn. (2), will be

$$\frac{da}{dt} = k_3[S - a(S + K_s)].$$

Integrating and setting $a=0$ when $t=0$,

$$a = \frac{S'}{S' + 1} \{1 - \exp[-(k_4 + k_5)(S' + 1)t]\}. \quad (5)$$

Even using the value $(k_4 + k_5) = 0.32 \text{ min.}^{-1}$ it is evident that the combination of enzyme with substrate will proceed very rapidly; if $S' = 64$, the enzyme activity is 98% of the maximum it will reach at this specific concentration of substrate within 0.2 min. after the enzyme and substrate are mixed.

Thus we can consider the combination of substrate with free enzyme to be instantaneous under these conditions, especially in view of the fact that the correct value of $(k_4 + k_5)$ is probably greater than 3500 min.^{-1} . The relative concentration, a , of free enzyme in an equilibrated enzyme-inhibitor system will be given by the equation $I' = (1 - a)/a$, or $a = 1/(I' + 1)$. Thus a more correct solution for Goldstein's equation 17A will be obtained by setting $a = 1/(I' + 1)$ when $t=0$ in the integration of Eqn. (3). It follows that

$$a = \frac{1}{(S' + I')} \left\{ S' + \left[\frac{I'(1 - S')}{(I' + 1)} \right] \exp \left[- \frac{(S' + I')t}{\frac{I'}{k_4 + k_5} + \frac{S'}{k_2 + k_5}} \right] \right\}. \quad (6)$$

On plotting the theoretical curves calculated from the equation (see Fig. 2), it is again found that the experimental data obtained by Goldstein (1944) can be fitted just as well by an equation where the value of $(k_4 + k_5)$ is high enough so that $I'/(k_4 + k_5)$ can be neglected, as by an equation in which $(k_4 + k_5) = 0.32 \text{ min.}^{-1}$.

Using these corrected equations, it would seem that the best fit to all the data of both figures is given by a value of 0.020 min.^{-1} for $(k_2 + k_3)$ (see Figs. 1 and 2). The experimental data do not allow any definite value to be assigned to $(k_4 + k_5)$, however, nor do they contradict the fact that $(k_4 + k_5)$ must be $> 3500 \text{ min.}^{-1}$.

Although this discussion has dealt with the reaction between enzyme and substrate, it is obvious that a similar consideration will apply in the case of an inhibitor which itself is susceptible to slow hydrolysis by the enzyme. Thus the reaction between pseudo-cholinesterase and eserine, for example, should be written in the form

Then, as before in the case of the substrate, the experimental value of K_I will represent $(k_2 + k_3)/k_1$ rather than k_2/k_1 , and the value 0.020 min.^{-1} will represent $(k_2 + k_3)$ rather than ' k_2 '.

By the methods outlined by Goldstein (1944) k_3 itself can be determined, but a definite value can be assigned only if

the enzyme concentration is known. Using a zone A equation despite the assumption of $E' = 12.8$, Goldstein has calculated that $k_3 = 0.00182 \text{ min.}^{-1}$ for the destruction of eserine in horse serum as given by the data of Ellis, Plachte & Straus (1943). From the data given in a previous article (Myers, 1952), it can be calculated that the concentration of pseudo-cholinesterase in the full horse serum used by Ellis *et al.* (1943) was probably $6.2 \times 10^{-8} \text{ M}$. Using $K_I = 3 \times 10^{-8} \text{ M}$ -eserine, then $E' = E/K_I = 2.1$ and the value of k_3 would become 0.011 min.^{-1} . It is interesting that this value of k_3

Fig. 2. Displacement of inhibitor by substrate. Acetylcholine added at zero time to an equilibrated mixture of dog serum and eserine. Data of fractional activity of enzyme (a) taken from table V of Goldstein (1944). Points near A with $5.5 \times 10^{-9} \text{ M}$ -, B with $5.5 \times 10^{-8} \text{ M}$ -, C with $5.5 \times 10^{-7} \text{ M}$ -, and E with $5.5 \times 10^{-6} \text{ M}$ -eserine. Theoretical curves calculated from equation 6, see text. ---, $(k_2 + k_3) = 0.026$, $(k_4 + k_5) = 0.32 \text{ min.}^{-1}$; - · - · -, $(k_2 + k_3) = 0.026$, $(k_4 + k_5) > 3500$; these two lines are practically identical near points A and B; —, $(k_2 + k_3) = 0.020$, $(k_4 + k_5) > 3500$.

is of a similar order of magnitude to the value of $(k_2 + k_3)$ calculated previously, but this is only a very rough approximation since it is a value dependent on the results of three separate investigations. Further, the value of k_3 obtained with eserine and the pseudo-cholinesterase of horse serum cannot be strictly compared with the previous value of $(k_2 + k_3)$ obtained with eserine and the pseudo-cholinesterase of dog serum, even if the experimental conditions were not somewhat different in the three experiments.

EXPERIMENTAL AND RESULTS

The data given by Goldstein (1944) suffer from the disadvantage of being determined with dog serum using acetylcholine as substrate for pseudo-cholinesterase and eserine as cholinesterase inhibitor. Dog serum also contains appreciable

amounts of true cholinesterase (Mendel, Mundell & Rudney, 1943) which would also hydrolyse acetylcholine and would also be inhibited by the concentrations of eserine used to inhibit the pseudo-cholinesterase. In human serum, on the other hand, the amount of true cholinesterase is very small and would, in any case, have no effect upon the results when benzoylcholine is used as a specific substrate (Mendel *et al.* 1943) and the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)trimethylammonium bromide (Hoffman-La Roche, Nu 683) as a specific inhibitor (Hawkins & Gunter, 1946) for the pseudo-cholinesterase.

Table 1. *Destruction of Nu 683 by the pseudo-cholinesterase of human serum on incubation at 37.5° and pH 7.4*

(The solution contained 0.025 M-bicarbonate, a volume of dialysed human serum equivalent to 10% (v/v) native serum, and 2×10^{-6} M-Nu 683. After incubation for various periods of time, the activity of 5 ml. was measured using 0.006 M-benzoylcholine as substrate for the pseudo-cholinesterase. Three control serum solutions with no added inhibitor were treated similarly.)

Time of incubation (min.)	Activity in the first 10 min. after addition of substrate	Activity in the first 20 min. after addition of substrate
	Activity of the control solutions (μ l. CO ₂ /5 ml. solution)	
15	163	330
340	157	323
510	159	325
	av. 160	av. 326
	Activity of the serum solutions with Nu 683 calculated as a fraction (a) of the corresponding average control value	
15	0.054	0.072
30	0.041	0.052
60	0.043	0.059
120	0.029	0.051
200	0.060	0.075
260	0.094	0.101
320	0.157	0.168
380	0.321	0.324
410	0.421	0.419
430	0.502	0.509
450	0.533	0.549
480	0.679	0.674
510	0.748	0.753

To obtain comparable values for k_5 and $(k_2 + k_5)$, these reaction velocity constants were determined on the same enzyme-inhibitor system as was used previously for the determination of enzyme concentration (Myers, 1952), that is to say, with Nu 683 as inhibitor of the pseudo-cholinesterase activity of dialysed human serum.

The experimental methods used were essentially the same as those described in a previous article (Myers, 1952). A 0.025 M-bicarbonate buffer saturated with 5% CO₂ in

95% (v/v) N₂ gave a pH of 7.4 at 37.5°. The substrate in the first experiment below was 0.006 M-benzoylcholine; in the second experiment 0.06 M-acetylcholine was employed.

By following the change in the fractional activity a caused by enzymic destruction of inhibitor over a period of several hours, k_5 can be determined directly (see Goldstein, 1944; Ellis *et al.* 1943; Easson & Stedman, 1936). Under the conditions used, the most accurate estimate of k_5 could be obtained by using a concentration of inhibitor which caused almost complete inhibition initially (Fig. 3). However, it might be expected that the small activity initially observed during the first 2 hr. of the incubation would be more susceptible to errors caused by the displacement of combined inhibitor after the addition of substrate.

Theoretical curves for this type of displacement were shown in Fig. 2 above. But the results given in Table 1 show that it does not make much difference to these particular results whether the fractional activity is calculated for the first 10 min. or for the first 20 min. after addition of substrate. This does not mean that combined Nu 683 was not displaced by added benzoylcholine, but rather, that the displacement was very slow. Over longer periods of time after the addition of substrate, a marked displacement of combined inhibitor was evident at all stages of the experiment represented by Fig. 3. This confirms the previous conclusion that Nu 683 is a reversible competitive inhibitor of the pseudo-cholinesterase.

The values calculated from the activity observed in the first 10 min. after the addition of substrate were used as a somewhat better approximation to the 'inhibition' existing before substrate was added. On plotting these results and applying the correct theoretical equation

$$\left[\frac{1}{a_0} + \ln \frac{(1-a_0)}{a_0} + E' \ln(1-a_0) \right] - \left[\frac{1}{a} + \ln \frac{(1-a)}{a} + E' \ln(1-a) \right] = k_5 E' t \quad (7)$$

in place of Goldstein's equation 25, the experimental results are found to correspond fairly well with these theoretical curves (Fig. 3).

$$K_I = 8.8 \times 10^{-10} \text{ M-Nu 683}$$

for this enzyme-inhibitor system and

$$E = 2.3 \times 10^{-9} \text{ M-pseudo-cholinesterase}$$

in a solution of which 5 ml. give an activity of 100 μ l. CO₂/20 min. with 0.006 M-benzoylcholine as substrate under the standard experimental conditions (Myers, 1952). For the above experiment, thus $E = 7.5 \times 10^{-9}$ M-pseudo-cholinesterase and

$$E' = E/K_I = 8.5.$$

Using this value for E' , the best average value of k_5 was 0.0119 min.⁻¹.

By measuring the rate of displacement of substrate by inhibitor, $(k_2 + k_5)$ was then determined. Under the conditions used, $E'/S' = 0.04$ so that zone

A equations can be applied. With other kinetic data from which $(k_2 + k_5)$ could be estimated (displacement of inhibitor by substrate, effect of dilution, combination of inhibitor with enzyme), the enzyme-inhibitor system would be in zone B, rendering the mathematical solution for $(k_2 + k_5)$ much more difficult.

In this second experiment, 0.06 M-acetylcholine was used as substrate for the pseudo-cholinesterase. The affinity of benzoylcholine for the pseudo-

equivalent to $(a \cdot k_6 \cdot E)$ (Goldstein, 1944). From the equation $S' = a/(1-a)$, $a = 0.978$ when $S' = 45$; from the data given previously (Myers, 1952), $E = 1.72 \times 10^{-9}$ M-pseudo-cholinesterase at this particular concentration of serum. Substituting these values for a and E in the equation

$$(a \cdot k_6 \cdot E) = 9.76 \times 10^{-5} \text{ mole l.}^{-1} \text{ min.}^{-1},$$

then $k_6 = 5.80 \times 10^4 \text{ min.}^{-1}$

and $(k_4 + k_5) \geq 5.80 \times 10^4 \text{ min.}^{-1}$.

Fig. 3. Enzymic destruction of Nu 683 by the pseudo-cholinesterase of human serum. Experimental conditions as in Table 1. Ordinate fractional activity of enzyme (a). Theoretical curves (—) calculated from Eqn. (7) where $k_4 = 0.0119 \text{ min.}^{-1}$, $E = 7.5 \times 10^{-9}$ M-pseudo-cholinesterase. The experimental curve (— · —) deviates from the theoretical curve initially because of the fact that the inhibitor is added at zero time and requires 50–60 min. to come to equilibrium with the enzyme. Curve A using 1×10^{-8} M-Nu 683, curve B using 2×10^{-8} M-Nu 683.

cholinesterase of dialysed human serum is so high that an accurate determination of pS (0.5) (see Fig. 4) was impossible with the Warburg technique. Since an accurate value for S' is necessary in determining $(k_2 + k_5)$, the use of benzoylcholine as substrate was not practicable for this experiment.

A plot of the activity at various substrate concentrations against the log of substrate concentration indicated that half-maximal activity is obtained with a concentration of 1.34×10^{-3} M-acetylcholine (Fig. 4). Thus $K_s = 1.34 \times 10^{-3}$ M and, for $S = 0.06$ M-acetylcholine, $S' = S/K_s = 45$.

At this particular concentration of acetylcholine, the activity observed with the 2% (v/v) serum solution used was 218 μ l. CO_2 /5 ml. solution/20 min. or 0.0976 m-mole/l./min. This rate of hydrolysis is

In the theoretical equation for change in a as the substrate is displaced by added inhibitor, these reaction constants occur in the term

$$\left[\frac{I'}{(k_4 + k_5)} + \frac{S'}{(k_2 + k_5)} \right].$$

When $I' = 228$, as in the present experiment, $I'/(k_4 + k_5) = 0.004 \text{ min.}$ For $S' = 45$ and

$$(k_2 + k_5) = 0.0119 \text{ min.}^{-1},$$

on the other hand, $S'/(k_2 + k_5) = 3800 \text{ min.}$ Therefore, under these experimental conditions, $I'/(k_4 + k_5)$ will be quite negligible in comparison to $S'/(k_2 + k_5)$. Thus Eqn. (4) can be rewritten in the simplified form

$$a = \frac{1}{(S' + I')} \{S' + I' \cdot \exp[-t(k_2 + k_5)(S' + I')/S']\}. \quad (8)$$

Using this equation, the best average value of $(k_2 + k_3)$ can be calculated for the experimental points of Fig. 5. Satisfactory agreement with the experimental points is obtained by giving $(k_2 + k_3)$

Fig. 4. Pseudo-cholinesterase activity at various concentrations of acetylcholine. Ordinate: fractional activity of enzyme (a). Abscissa: negative log. of the molar concentration of acetylcholine. Theoretical curve calculated from the equation $S = K_s a / (1 - a)$ using the value $K_s = 1.34 \times 10^{-3} \text{ M}$.

a value of 0.0119 min.^{-1} . The experimental error precludes the determination of a more precise value for these velocity constants; but it can be seen from Fig. 5 that the rate of displacement of substrate by inhibitor is, if anything, slightly slower than the rate expected if $(k_2 + k_3) = 0.0119 \text{ min.}^{-1}$. This

would mean that $(k_2 + k_3)$, as calculated from this experiment, might be slightly less than 0.0119 min.^{-1} . Since this work was completed, a method has been described for the determination of the limiting concentration of pseudo-cholinesterase in serum by the use of an irreversible fluorophosphonate inhibitor (Berry, 1951). Therefore the pseudo-cholinesterase concentration in human serum was estimated by a similar technique using diisopropyl fluorophosphonate as enzyme inhibitor in the present investigation. The results indicated that the enzyme concentration was $2.20 \times 10^{-9} \text{ M}$ -pseudo-cholinesterase (range 2.12 – $2.34 \times 10^{-9} \text{ M}$)/(100 $\mu\text{l. CO}_2/20 \text{ min.}$) activity under the standard conditions used above. This would mean that the value of k_3 is about 0.0115 min.^{-1} , which gives an even better fit to the experimental points of Fig. 5.

From these results it can be concluded that $(k_2 + k_3)$ is not significantly greater than k_3 alone, in other words, that k_2 must be of negligible value in comparison to k_3 . This would mean that in this particular case the 'reversible reaction' between enzyme and inhibitor can, for practical purposes, be written as

and that the Michaelis constant K_1 could be equated to k_3/k_2 .

Fig. 5. Displacement of substrate by inhibitor. Nu 683 was added at zero time to an equilibrated mixture containing 2% (v/v) dialysed human serum and 0.06 M -acetylcholine. Ordinate: fractional activity of enzyme (a). Theoretical curves were calculated from Eqn. (8) (see text) for $S' = 45$ and $(k_2 + k_3) = 0.0119 \text{ min.}^{-1}$; $I' = 28.5$ for curve A, 56.9 for curve B, 114 for curve C, and 228 for curve D.

DISCUSSION

The determination of the individual rate constants for the reactions involved in the inhibition of pseudo-cholinesterase by Nu 683 showed that the rate of dissociation of combined enzyme-inhibitor (k_2) was negligible in comparison to the rate of hydrolysis of combined inhibitor (k_3). The rate of hydrolysis of a substrate such as acetylcholine is much too high to allow the direct determination of the corresponding velocity constants k_4 and k_5 . However, it seems quite possible that k_4 may also have a negligible value in comparison to the very high value of k_3 . No proof of this assumption can be given as yet, but for practical purposes we could consider that the Michaelis constant $K_s = (k_4 + k_5)/k_3 \geq k_5/k_3$.

In the case of the hydrolysis of acetylcholine by pseudo-cholinesterase, k_5 was found to have a value of $5.8 \times 10^4 \text{ min.}^{-1}$, while $K_s = 1.34 \times 10^{-3} \text{ moles/l.}$ under the experimental conditions. Thus

$$k_3 \geq k_5/K_s = 4.3 \times 10^7 \text{ l. mole}^{-1} \text{ min.}^{-1}.$$

The corresponding constant for Nu 683,

$$k_1 = k_5/K_1 = 1.35 \times 10^7 \text{ l. mole}^{-1} \text{ min.}^{-1}.$$

It appears that the rate of combination with pseudo-cholinesterase differs only by about three times for acetylcholine and for Nu 683. On the other hand, the rate of hydrolysis of acetylcholine

$$(k_6 = 5.8 \times 10^4 \text{ min.}^{-1})$$

would be nearly 5×10^6 times greater than the rate of hydrolysis of Nu 683 ($k_5 = 0.0119 \text{ min.}^{-1}$) by pseudo-cholinesterase. Apparently the chief difference between the reactions of acetylcholine and of Nu 683 with pseudo-cholinesterase lies in the rate of hydrolysis of these two compounds by the pseudo-cholinesterase.

For comparative purposes, approximate values of the corresponding constants (k_3 and k_5) were calculated from data on the true cholinesterase concentration which have been obtained with the *N-p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250) as inhibitor (Myers, 1952). These indicated that an enzyme concentration of 6.3×10^{-10} moles true cholinesterase/l. will give an activity of $100 \mu\text{l. CO}_2/20 \text{ min.}/5 \text{ ml. solution}$, or $4.46 \times 10^{-5} \text{ moles/l./min.}$, at a substrate concentration of $0.03 \text{ M-acetyl-}\beta\text{-methylcholine}$. From results obtained previously (Myers, 1951), we can calculate that S' has an average value of about 37 with $0.02 \text{ M-acetyl-}\beta\text{-methylcholine}$ and therefore $K_s = 5.4 \times 10^{-4} \text{ M}$. At a concentration of $0.03 \text{ M-acetyl-}\beta\text{-methylcholine}$, $S' = 56$ and $\alpha = 0.982$. Then, as before,

$$k_5 = (dS/dt)/\alpha E = 7.2 \times 10^4 \text{ min.}^{-1}$$

$$\text{and } k_3 \geq k_5/K_s = 1.3 \times 10^8 \text{ l. mole}^{-1} \text{ min.}^{-1}.$$

These values are slightly higher (1.2 and 3.0 times respectively) than the values of the same reaction

constants for the hydrolysis of acetylcholine by the pseudo-cholinesterase of human serum.

The most striking thing about all of these rate constants is the large order of magnitude, indicating a very high activity of the cholinesterases towards acetylcholine and related substrates. In the hydrogen peroxide-peroxidase system used by Chance (1943), k_3 also had a very high value which was, in fact, of the same order of magnitude as those obtained above with the cholinesterases. The value given for k_3 was $1.2 \times 10^7 \text{ l. mole}^{-1} \text{ sec.}^{-1}$ or $7.2 \times 10^8 \text{ l. mole}^{-1} \text{ min.}^{-1}$, as compared with the values 1.35×10^7 to $1.3 \times 10^8 \text{ l. mole}^{-1} \text{ min.}^{-1}$ calculated above. On the other hand, the destruction constant, k_6 , varied from nearly zero to higher than 300 min.^{-1} , depending upon the concentration of a third reactant which reacted with the activated enzyme-substrate complex (Chance, 1943). In the case of the cholinesterases, the third reactant is probably water, the concentration of which is very high and constant under the experimental conditions.

In the particular case investigated, the reaction between the reversible inhibitor and enzyme could be represented best by an equation of the form

Obviously this conclusion cannot be extended to all reversible competitive inhibitors of cholinesterases. A simple cationic inhibitor such as the sodium ion or choline is not susceptible to hydrolysis by cholinesterase and in such a case the reversible reaction will be strictly of the classical form

In still other cases, the reaction between enzyme and a reversible competitive inhibitor which is susceptible to hydrolysis may be represented best by the full equation

but no good examples of this third type of reaction have been described in the literature as yet.

The assumption, that the enzymic reaction with either substrate or inhibitor may follow the first type of equation, will not invalidate the basic theory of enzyme kinetics as developed for reactions of the second and third type (Michaelis & Menten, 1913; Briggs & Haldane, 1925; Goldstein, 1944). The main difference will be in the velocity constants which are represented in the Michaelis constants K_1 and K_s . It does of course lay more emphasis on the destruction of inhibitor or substrate by the enzyme, but this is a factor which must always be taken into account when measuring the initial constant velocity of the enzyme activity.

As noted above, the main difference between acetylcholine and Nu 683 in their reactions with pseudo-cholinesterase would appear to be the large

difference in the relative rates of hydrolysis. This forms an interesting comparison with the data of Chance (1943) on peroxidase, in which case the Michaelis constant K_s , with the same substrate throughout, was directly proportional to the velocity constant k_s for the rate of destruction of the substrate. The apparent dependence of K_s upon k_s would suggest that the values of k_1 and k_2 may be a measure of physical factors limiting the rate of combination of enzyme with substrate or inhibitor. However, it is rather improbable that this comparison between the values of k_s and K_s could be extended to all enzymes substrates under all experimental conditions.

The effect of the experimental conditions upon the values obtained for these velocity constants remains to be investigated. It has been demonstrated with cholinesterase that the Michaelis constants for both substrate and inhibitor may be considerably affected by the presence of salts in the medium (Mendel & Rudney, 1945; Myers, 1950). Thus the salt concentration must have a significant effect upon the observed values of the individual reaction constants. However, it would seem logical that these rate constants should be determined in a medium containing as few interfering factors as possible; the inherent velocity constants of the enzyme-inhibitor or enzyme-substrate system are probably those which would be obtained in the complete absence of salts or other interfering factors in the medium. Since high concentrations of salts are not necessary for the activity of the cholinesterases, the best practical solution to this problem is probably obtained by using only the low concentration of buffer solution required to maintain pH.

SUMMARY

1. The theoretical formulations of Goldstein (1944) were based on the assumption that the

reaction between an enzyme (E) and its substrate (S) could be adequately represented by an equation of the form

These formulations have been modified to comply with the correct equation for the overall reaction

2. The velocity constants for the reactions between the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)trimethylammonium bromide (Nu 683) and the pseudo-cholinesterase of human serum have been determined by the use of these modified theoretical equations. The results obtained were as follows: Michaelis constant $K_1 = (k_2 + k_3)/k_1 = 8.8 \times 10^{-10}$ mole l.⁻¹; $k_3 = 0.0119$ min.⁻¹ (rate of hydrolysis of inhibitor I); $(k_2 + k_3) = 0.0119$ min.⁻¹; k_2 = negligible value (rate of dissociation of EI); $k_1 = 1.35 \times 10^7$ l. mole⁻¹ min.⁻¹ (rate of combination of E with I).

3. The corresponding velocity constants between acetylcholine and the pseudo-cholinesterase were: Michaelis constant $K_s = (k_4 + k_5)/k_3 = 1.34 \times 10^{-3}$ mole l.⁻¹; $k_5 = 5.8 \times 10^4$ min.⁻¹ (rate of hydrolysis of S); $k_3 \geq 4.3 \times 10^7$ l. mole⁻¹ min.⁻¹ (rate of combination of E with S).

4. The significance of these results has been discussed. In the particular case of Nu 683 as inhibitor of the pseudo-cholinesterase activity of dialysed human serum, it would appear that the 'reversible reaction' between the enzyme and inhibitor could for practical purposes be represented by an equation of the form

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