Turnover Number of Acetylcholinesterase*

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The turnover number of acetylcholinesterase is of special interest because the enzyme is thought to be one of the fastest enzymes and because a high speed is a prerequisite for the role of the enzyme in nervous function (1). The earliest value of the turnover number was given by Rothenberg and Nachmansohn (2) in terms of the molecular weight for enzyme obtained from electric eel. The molecular weight was estimated to be 3×10^6 from the rate of sedimentation in the ultracentrifuge and the preparation with the highest specific activity was assumed to be pure. The turnover number so obtained was 3×10^7 per minute per molecule of enzyme.

Michel and Krop (3) used the irreversible inhibitor, diisopropylfluorophosphate. This compound phosphorylates the active site of acetylcholinesterase (and also that of other enzymes). In that study an enzyme preparation from electric eel was treated with diisopropylfluorophosphate containing radioactive phosphorus. The protein was precipitated with trichloroacetic acid and washed free of unreacted diisopropylfluorophosphate. The radioactivity which remained with the protein gave the number of active sites. A possible error in this method, and one which can be very large, is that diisopropylfluorophosphate, which is a fairly active phosphorylating agent, may phosphorylate other proteins or other groups in addition to the active site. This possibility was discounted when it was found that little radioactivity was carried by the protein when the phosphorylation was carried out in the presence of acetylcholine. The value obtained in that study was 4.9×10^5 per minute at 0.14 ionic strength, pH 7.4, 38°, and acetylcholine 0.015 M. The value per active complex was calculated to be 7.2×10^5 per minute.

This method was modified by Cohen and Warringa (4) and used with enzyme from human red cells. During phosphorylation with diisopropylfluorophosphate the active site was protected with the poor substrate, butyrylcholine. Excess diisopropylfluorophosphate and butyrylcholine were removed by dialysis and the still active enzyme was finally treated with radioactive diisopropylfluorophosphate. In this way it was hoped that, although other groups might be phosphorylated, only the active site would be phosphorylated with radioactive phosphorus. With the simpler technique, several times as much radioactivity was carried by the protein so that, in this case, the simpler technique was not satisfactory. These studies gave a value of 3×10^5 per minute per active site.

A new method, which is described in this paper, uses dimethylcarbamyl fluoride, which is a very poor substrate and an in-

hibitor of acetylcholinesterase. This substance was shown to be an inhibitor by Myers and Kemp (5). These authors suggested that the behavior of this compound was similar to that of diisopropylfluorophosphate and other alkylphosphates (5, 6). This theory was substantiated by the fact that the inhibited enzyme is reactivated by hydroxylamine and also by other observations (7).

This inhibitor carbamylates the active site of the enzyme at a measurable rate and the resulting dimethylcarbamyl enzyme, in turn, hydrolyzes at a measurable rate. The same dimethylcarbamyl enzyme can be obtained from a number of other substrate-inhibitors such as dimethylcarbamyl choline and the dimethyl carbamates of 3-hydroxyphenyltrimethyl ammonium ion and of 3-hydroxy methylpyridinium ion (8).

The reaction of the enzyme with dimethylcarbamyl fluoride is:
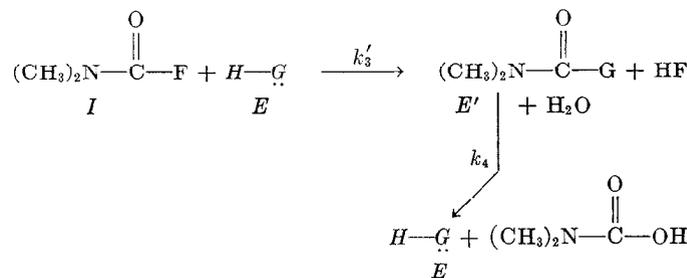
Here $H\text{-}G$ represents the esteratic site of the enzyme. This scheme is the same as for a substrate but the enzyme-inhibitor (substrate) complex is not explicitly indicated because the inhibitor (substrate) concentration is much smaller than the Michaelis constant. If there is only a small amount of enzyme present, the concentration of inhibitor remains sensibly constant and a steady state is reached in which the rate of formation of dimethylcarbamyl enzyme equals its rate of hydrolysis. With a suitable amount of inhibitor and a very low concentration of enzyme, the steady state is reached in about 1 hour and persists indefinitely. We have for the ratio (R) of the normality of the inhibited enzyme (E') to active enzyme (E) at the steady state (subscript ss)

$$\left(\frac{dE'}{dt}\right)_{ss} = k_3'(I)(E) - k_4(E') = 0$$

$$\therefore R_{ss} = \left(\frac{E'}{E}\right)_{ss} = \frac{k_3'}{k_4}(I) \tag{1}$$

in which (I) is the concentration of dimethylcarbamyl fluoride. The ratio (E'/E) is determined with a good substrate—acetyl-

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choline. The value of (E) is determined in arbitrary units by diluting the solution of enzyme and inhibitor and measuring the rate at which an aliquot hydrolyzes acetylcholine. The difference between this rate and the rate found in the absence of dimethylcarbamyl fluoride yields the value of (E') in the same units. The ratio $(E')/(E)$ is, of course, dimensionless.

The kinetic constants k_4 and k_3' were previously measured; the best values are (7, 8):

$$k_4 = 2.6 \times 10^{-2} \text{ min}^{-1}$$

$$\frac{k_3'}{k_4} = 6.9 \times 10^4$$

$$k_3' = 1.8 \times 10^3 \text{ min}^{-1}$$

If there is a very large amount of enzyme present, the concentration of dimethylcarbamyl fluoride continually diminishes, *i.e.* it is hydrolyzed, and R slowly approaches zero. The rate of change of R is a measure of the rate of hydrolysis of inhibitor.

The differential equations are

$$\frac{d(E')}{dt} = k_3'(I) \times (E) - k_4(E') \tag{2}$$

$$\frac{d(I)}{dt} = -k_3'(I) \times (E) \tag{3}$$

and

$$(E^0) = (E) + (E') \tag{4}$$

in which (E^0) is the normality of the enzyme, and is, of course, constant throughout the experiment.

These equations have not been completely solved but a useful partial solution is obtained by substituting Equation 3 into Equation 2 and integrating between $t = 0$ and $t = t$.

$$(I) = (I^0) - (E') - k_4 \int_0^t (E') \, dt \tag{5}$$

(This equation can also be written down by inspection) where I^0 is the initial concentration of inhibitor (substrate). The initial value of (E') is zero. Because, as time progresses, (E') and (I) approach zero,

$$\frac{(I^0)}{(E^0)} = k_4 \int_0^\infty \frac{(E')}{(E^0)} \, dt \tag{6}$$

Thus, by plotting $(E')/(E^0)$ as a function of t and measuring the area under the curve, E^0 can be evaluated if k_4 is known. This constant was previously measured by greatly diluting an inhibited enzyme solution and measuring the return of enzyme activity.

A special solution of the differential equations which is useful for determining E^0 can be obtained for a limited time interval during which

$$|\alpha| = \left| \frac{1}{k_4(E^0)} \frac{d(E')}{dt} \right| \ll 1$$

Under these conditions we have

$$\ln R + R = -k_3 E^0 (1 + \bar{\alpha})t + \ln R^0 - R^0 \tag{7}*$$

where R^0 is a constant—it is the extrapolated value of R for $t = 0$. In two experiments to be described, $\bar{\alpha}$ (the average value of α) $= -0.02$ and -0.04 for a time interval starting

somewhat later than the time required to reach the steady state

$$\left(\frac{1}{(E^0)} \frac{d(E')}{dt} = 0 \right)$$

and extending until R declines to about 0.3 to 0.4. The above equation is valid for conditions which do not differ very much from the steady state, *i.e.* conditions under which Equation 1 is nearly satisfied.

The value of $\bar{\alpha}$ is obtained from a plot of $(E')/(E^0)$ against time (Fig. 3). The slope at any point is $1/(E^0) \, d(E')/dt$. The slope is fairly constant (the curve is nearly linear) in this interval and this "average" slope was used for evaluating $\bar{\alpha}$. The value of $\bar{\alpha}$ need be known only approximately inasmuch as neglecting the parameter completely would introduce an error of only 2 and 4% in the values of E^0. The value of R was determined (as shown previously) as a function of time and the data was plotted so as to give a straight line in accordance with

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* Equation 7 is derived as follows:

Substitution of Equation 4 in Equation 2 yields

$$\frac{d(E')}{dt} = (k_3'(I) + k_4)(E) - k_4(E^0)$$

$$(E) = \frac{k_4(E^0)}{k_3'(I) + k_4} + \frac{d(E')}{dt} \frac{1}{k_3'(I) + k_4} \tag{8}$$

$$(E) = \frac{k_4(E^0)(1 + \alpha)}{k_3'(I) + k_4}; \qquad \alpha = \frac{1}{k_4(E^0)} \frac{d(E')}{dt}$$

Substitution of Equation 8 in Equation 3 yields

$$\frac{d(I)}{dt} = -k_3'(I) \frac{k_4 E^0 (1 + \alpha)}{k_3'(I) + k_4}$$

$$\frac{k_3'}{k_4} d(I) + \frac{d(I)}{(I)} = -k_3'(E^0)(1 + \alpha) \, dt$$

Integration (if α is constant or small) gives

$$\frac{k_3'}{k_4} [(I) - (I^0)] + \ln \frac{(I)}{(I^0)} = -k_3'(E^0)(1 + \alpha)t$$

but from Equation 8 and Equation 4

$$\frac{k_3'(I)}{k_4} = (1 + \alpha) \left[\frac{(E) + (E')}{(E)} \right] - 1 = \alpha + (1 + \alpha)R \tag{9}$$

$$\therefore R(1 + \alpha) + \ln [R(1 + \alpha) + \alpha] - R^0(1 + \alpha)$$

$$- \ln [R^0(1 + \alpha) + \alpha] = -k_3(E^0)(1 + \alpha)t$$

(Because α can be evaluated, this equation can be used, but because α is small, further simplification is possible.)

$$\ln [R(1 + \alpha) + \alpha] = \ln R + \ln \left(1 + \alpha + \frac{\alpha}{R} \right) \therefore R + \ln R$$

$$= -k_3(E^0)(1 + \alpha)t + R^0 + \ln R^0 + f(\alpha, R, R^0)$$

where

$$f(\alpha, R, R^0) = \alpha R + \ln \left(1 + \alpha + \frac{\alpha}{R} \right) - \alpha R^0 - \ln \left(1 + \alpha + \frac{\alpha}{R^0} \right)$$

The value of $f(\alpha, R, R^0)$ is less than α for $0.3 < R < 3$ and can be omitted without significantly affecting the slope or the intercept.

Equation 7 ($2.3 \log R + R$ was plotted against t). With the known value of k'_3, (E^0) was obtained from the slope. The value of R^0 agreed with previously measured values of k'_3/k_4 (see Equation 9).

In this paper we use these two methods of evaluating E^0, one based on Equation 6 and the other on Equation 7.

EXPERIMENTAL PROCEDURE

The acetylcholinesterase preparation was obtained from the electric organ of *Electrophorus electricus* (9). The solution was 0.050 M sodium phosphate buffer, pH 7.0, contained 2.4 mg of protein per ml, and had an activity of 5.3 mmoles of acetylcholine hydrolyzed per minute per ml.

Activity was measured by the colorimetric method of Hestrin (10) with acetylcholine, 2.5×10^{-3} M, in buffer of the composition, 0.1 M NaCl, 0.01 M MgCl$_2$, and 0.02 M sodium phosphate at pH 7.0, 25.0°. The assay time was 1 minute and the dilution of enzyme was such that with uninhibited enzyme about 1.5 μmoles of acetylcholine were hydrolyzed.

In this work, the integrated kinetic equation which contains terms for substrate inhibition and for product inhibition was used to calculate the enzyme activity. For hydrolysis of about 1.5 μmoles per ml, the calculated initial velocity is 18 % higher and agrees precisely with experimental initial velocities measured by the method of continuous automatic titration.

In one experiment, 0.300 ml of enzyme solution was mixed with 0.300 ml of saline buffer containing 8×10^{-5} M dimethyl-
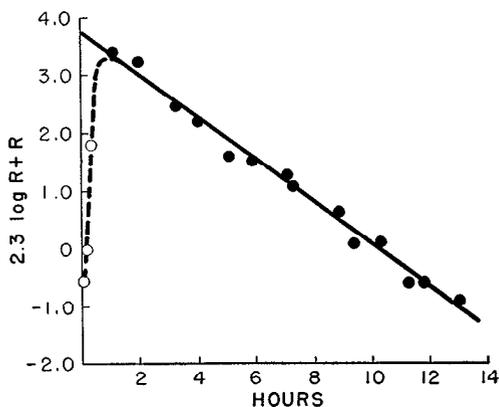

FIG. 1. The data of Experiment I are plotted in accordance with Equation 7. $R = (E')/(E)$
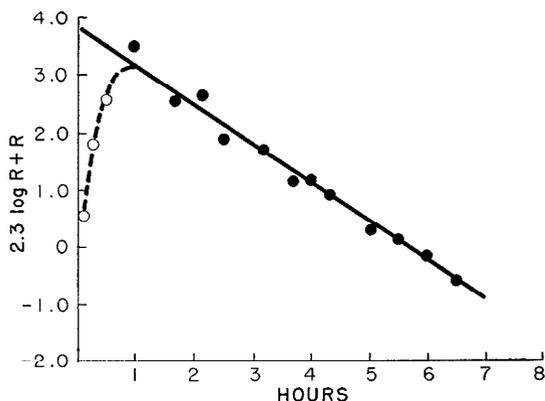

FIG. 2. The data of Experiment II are plotted in accordance with Equation 7. $R = (E')/(E)$

TABLE I

Method based on Equation 7

Experiment	$(1 + \bar{\alpha}) \dfrac{R^0}{I^0} = \dfrac{k_3'^*}{k_4}$	$E^0 = \dfrac{\text{slope}}{k_3' \ (1 + \bar{\alpha})}$	$\dfrac{v\dagger}{E}$
I, $\alpha = -0.02$	6.7×10^5	3.6×10^{-6}	7.4×10^5
II, $\alpha = -0.04$	6.8×10^5	6.7×10^{-6}	7.4×10^5

Method based on Equation 6

	Area$\ddagger$ — hrs	$E^0 = \dfrac{I^0}{k_4 \times \text{area}}$	
I	7.20	3.6×10^{-6}	7.4×10^5
II	3.86	6.7×10^{-6}	7.5×10^5

* Equation 9.

† v is the initial velocity of hydrolysis of acetylcholine in mmoles per ml per minute multiplied by the extent to which the enzyme was diluted in order to assay it.

‡ Area under the curve of (E'/E^0) *versus* time from $t = 0$ to $t = \infty$.

carbamyl fluoride and kept at 25.0°. At various times an aliquot was withdrawn, diluted 300 times with buffer, and assayed 1 minute later by adding 0.200 ml to 1.00 ml of acetylcholine buffer. A small correction was made for the recovery during this minute. Uninhibited enzyme hydrolyzed 1.50 μmoles in 1 minute. The initial velocity was, therefore, $(1.5 \times 10^{-3} \times 1.18)/1.20 = 1.48 \times 10^{-3}$ mmoles per minute per ml. The enzyme was diluted 1.8×10^3 times in the assay.

In a second experiment, 0.300 ml of enzyme solution was mixed with 0.0100 ml of saline buffer containing 1.24×10^{-3} M dimethylcarbamyl fluoride. The final concentration of inhibitor-substrate was the same in both experiments, 4×10^{-5} M, but in the second the enzyme concentration was almost twice as great. For assay an aliquot was diluted 600 times and 0.200 ml added to 1.00 ml of an acetylcholine buffer. Uninhibited enzyme hydrolyzed 1.41 μmoles in 1 minute. The initial velocity was $(1.41 \times 10^{-3} \times 1.18)/1.20 = 1.39 \times 10^{-3}$ mmoles per minute per ml. The enzyme was diluted 3.6×10^3 times in the assay.

RESULTS

The data were plotted in accordance with Equation 7 and the expected straight lines were realized (Figs. 1 and 2). The steady states were reached in about 1 hour. The values of the parameters calculated from these lines and from the enzyme activities with acetylcholine as substrate are given in Table I and it will be noted that the two experiments are in good agreement. The values of k'_3/k_4 agree with previous measurements.

The values of $(E')/(E^0)$ as a function of time are shown in Fig. 3 for Experiment II. The data for Experiment I are very similar but the time scale extends twice as long. The extrapolated area is less than 2 % of the total area. The pertinent data are presented in Table I. Both experiments agree and the two methods yield about the same result.

The kinetics of the hydrolysis of acetylcholine follows the Michaelis equation modified for substrate inhibition:

$$v = \frac{kE^0}{1 + \dfrac{K_m}{S} + \dfrac{S}{K_2}} \tag{10}$$

in which $K_m = 9.2 \times 10^{-5}$ (10) and $K_2 = 3 \times 10^{-2}$. For $S = 2.5 \times 10^{-3}$ M, $v/E^0 = 7.4 \times 10^5$ min^{-1} (Table I), and, therefore, $k = 8.3 \times 10^5$ min^{-1}. At the optimal substrate concentration the turnover number is 7.4×10^5 per minute per active site and the average time required to hydrolyze 1 molecule of acetylcholine is 81 μseconds. The turnover number per occupied site is 8.3×10^5 per minute. This value is somewhat higher than the value of Michel and Krop (7.2×10^5), especially because their value is for a higher temperature, 38°, and a more favorable pH (7.4). Lawler, in this *Journal*, reports measurements of the order of 6×10^5 for the turnover number at 30°, pH 7, acetylcholine 3×10^{-3} M.*

The rate constant k is composed of two constants related by

$$\frac{1}{k} = \frac{1}{k_3} + \frac{1}{k_4}$$

where k_3 is the specific rate of formation of the acetyl enzyme from the enzyme substrate complex and k_4 is the pseudo first order rate constant for the hydrolysis of the acetyl enzyme. For acetylcholine at 25° the ratio k_3/k_4 seems to be of the order of 6 (10). Assuming this value to be correct, we have $k_3 = 6 \times 10^6$ min^{-1}, $k_4 = 1.0 \times 10^6$ min^{-1}. The half-time for the formation of the acetyl enzyme is 7 μseconds and the half-time of the acetyl enzyme is 42 μseconds.

The purest enzyme prepared by Nachmansohn and Rothenberg had an optimal activity of 6.9 mmoles of acetylcholine hydrolyzed per minute per mg of protein at 25° and pH 7.2. Recent values by Lawler are 7.2 mmoles per minute per mg of protein at 30° and pH 7.0. Probably 6.8 is a reasonable estimate for our conditions. This value gives an equivalent weight of 1.07×10^5.

This general method for determining the turnover number would seem to be of general applicability.

It is pertinent to inquire into what consequences this turnover number has for the theories that involve acetylcholinesterase in neurological events. In these phenomena it is assumed that acetylcholine is released and acts on a specific receptor, and that this action is terminated by hydrolysis. The time is limited to about 200 μseconds for conduction and 2 msec for transmission. The concentration of acetylcholine is evidently quite low, certainly far below the K_m for acetylcholinesterase, 10^{-4} M. The turnover number depends, of course, on the substrate concentration and is quite low at low substrate concentrations, but the fractional rate of hydrolysis is greater at lower concentrations and is essentially independent of the concentration for values well below the Michaelis constant. We can, therefore, answer the questions, "What normality of enzyme is required to hydrolyze 80% of the acetylcholine present in 200 μseconds; in 2 msec"? The answer obtained from the integration of Equation 10 is about 5×10^{-5} N and 5×10^{-6} N. This is, of course, quite a high concentration but it does not seem unreasonably high for localized portions of the cells. It is interesting that this concentration of enzyme is greater than biologically active con-

* The application of carbamyl enzyme studies to the determination of turnover number described in this paper was started as Lawler was engaged in her work.
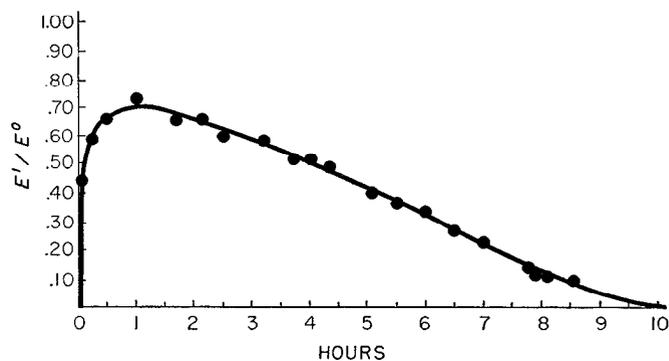

FIG. 3. The data of Experiment II are plotted in accordance with Equation 6.

centrations of acetylcholine. (Equation 10 is not accurate under these conditions and gives a low but roughly correct answer.)

SUMMARY

A new method for determining the normality of an enzyme solution and its turnover number is described. The basis of the method lies in the fact that certain compounds which are generally regarded as inhibitors are, in reality, extremely poor substrates. The reactions of these substances, in particular, dimethylcarbamyl fluoride with acetylcholinesterase, are so slow that the rate constants for the individual steps can be evaluated and these values, in combination with information concerning the over-all rate of hydrolysis, are sufficient to calculate the normality of the enzyme solution. Because dimethylcarbamyl fluoride inhibits the enzyme with regard to the hydrolysis of acetylcholine, the hydrolysis of dimethylcarbamyl fluoride is readily indicated by the declining inhibition.

Equations are derived which are convenient for determining the normality of the enzyme solution from the time course of the inhibition of the hydrolysis of acetylcholine.

The turnover number at 25°, pH 7.0, acetylcholine 2.5×10^{-3} M, was found to be 7.4×10^5 min^{-1}.

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