On the Kinetics of Inhibition of rat-brain Acetylcholine esterase by Sevin

A. HASSAN, F. M. ABDEL-HAMID and M. R. E. BAHIG
Dept. of Biology, Atomic Energy Establishment, Cairo, U.A.R.

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The inhibition of rat-brain acetylcholinesterase with Sevin was shown to be partly reversible. The rate of progression of the irreversible inhibition is extremely slow and follows the kinetics of a uni-molecular reaction, with $K=6.62 \times 10^{-5} \text{ min}^{-1}$ and $t_{0.5}=190 \text{ minutes}$. The reversible inhibition of the enzyme was found to be of competitive nature. The dissociation constant of $E-I$ ($K_I$) was determined—by two different methods—to be $5.7$ and $7.6 \times 10^{-4} \text{ M}$. The presence of acetylcholine protects the enzyme against the irreversible attack of Sevin. It is believed that acetylcholine esterase does not participate in the biochemical degradation of Sevin in the nervous tissue.

Many organophosphorus esters used as insecticides are potent cholinesterase inhibitors. In many cases, the effect is produced as a result of the irreversible combination of the inhibitor with the enzyme. The carbamate insecticides are also becoming increasingly important, in the field of crop protection. A well known member of this group is 1-naphthyl-N-methyl carbamate (Sevin), which manifests its action by inhibiting cholinesterase strongly. However, the mechanism of this inhibition is not yet fully understood; and this raises the question whether the nature of inhibition simulates that of some organophosphorus esters. In this investigation, a kinetic study of the reaction between rat-brain acetylcholine esterase and Sevin is presented.

Experimental

Rat brain homogenates were used as the source of acetylcholine esterase. Rats were killed by a blow on the neck and the brain was dissected out and immediately homogenized in ice-cold isotonic potassium chloride solution. Unless otherwise stated, 10 per cent homogenate was used.

Sevin was prepared according to the procedure of LAMBR~CH~5 and the insecticide was purified by twice crystallization from carbon tetrachloride (m. p. 142°). An aqueous solution of Sevin was obtained by shaking the crystals with distilled water for two hours at room temperature. The solution was stored at $2^\circ$ (stable for two weeks). For quantitative determination of Sevin, a part of the solution was subjected to alkaline hydrolysis, and the liberated 1-naphthol was determined colourimetrically, using 4-amino antipyrine reagent.

For the assay of acetylcholine esterase, the hydroxylamine-Ferrie chloride method of HESTRIN was adopted. The period of assay was 30 minutes. In experiments where Sevin was incubated with the rat-brain homogenates, the $E-I$ reaction was stopped by the addition of 8 mM acetylcholine chloride solution (final concentration), and the enzyme assayed for 30 minutes.

In general the reaction mixture had the following composition:

- 0.9 ml 0.2 M phosphate buffer, pH 7.2;
- 0.3 ml 0.4 M magnesium chloride solution;
- 0.3 ml 1.0 M sodium chloride solution;
- 0.5 ml rat brain homogenate in isotonic KCl;
- 0.5 ml distilled water or variable concentrations of the insecticide in distilled water;
- 0.5 ml 48 mM acetylcholine chloride in 0.001 M sodium acetate (final concentration 8 mM, unless otherwise stated).

Results and discussion

Nature of the $E-I$ reaction

The reaction between the inhibitor and the enzyme was studied by incubating different concentrations of Sevin with crude rat-brain homogenates, for different periods. The $E-I$ reaction was stopped by the addition of 8 mM acetylcholine chloride solution. When log-remaining activity was plotted against time, the curves in Fig. 1 were obtained. An important feature of these curves is that the lines

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K = 3.65 \times 10^{-3} \text{ mol} \text{ min}^{-1}$
$$

$K$ being the dissociation constant of $E-I$.

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6 M. E. MARTIN, Analytic. Chem. 21, 1419 [1949].
do not pass through the origin, and for different inhibitor concentrations, the lines are parallel. According to Aldridge, the lines give a measure of the rate of the enzyme becoming irreversibly inhibited, and the amount below the origin, where the lines produced back cross the ordinate, is a measure of the reversible inhibition. Since the rate of the irreversible inhibition is independent on the concentration of I, the reaction is therefore unimolecular. The velocity constant (K) was determined from the slope in Fig. 1 to be \(3.65 \times 10^{-3}\) min.\(^{-1}\), and the \(i_{0.5}\) was calculated to be 190 minutes.

**Reversibility**

An examination of the rate of increase of the irreversible inhibition shows that it is extremely slow; and therefore the contribution of the reaction leading to irreversibility may be assumed negligible during the first few minutes (Fig. 1). The kinetics of the reversible reaction were studied by incubating S and I with E (20\% homogenate) for seven minutes (arbitrarily chosen) to eliminate the possible contribution of the irreversible reaction. This is again ensured by the finding that the presence of substrate protected the enzyme against the irreversible attack of Sevin (see below).

The effect of varying independently both the substrate concentration (S) and inhibitor concentration (i) was determined in order to obtain the dissociation constant of E-I (\(K_i\)). As Lineweaver and Burk pointed out, the plot of reciprocals of velocity and substrate concentration gives a straight line according to:

\[
\frac{1}{v} = \frac{K_s}{V_{max}} S + \frac{1}{V_{max}} \quad \text{(in absence of inhibitor)}
\]

and

\[
\frac{1}{v_i} = \frac{K_s}{V_{max}} \left(1 + \frac{i}{K_i} \right) \frac{1}{S} + \frac{1}{V_{max}} \quad \text{(in presence of a competitive inhibitor)}
\]

where \(v\) = reaction velocity, \(S\) = substrate concentration, \(K_s\) = dissociation constant of E-S, \(V_{max}\) = maximum reaction velocity, \(v_i\) = reaction velocity in presence of \(i\), \(i\) = inhibitor concentration, \(K_i\) = dissociation constant of E-I.

By graphical representation of these equations (Fig. 2), it was possible to determine \(K_i\) where:

- Slope of the upper line = \(\left(1 + \frac{i}{K_i}\right) \frac{K_s}{V_{max}}\) and
- slope of the lower line = \(\frac{K_s}{V_{max}}\)

\(K_i\) was calculated to be \(5.7 \times 10^{-6}\) M. The competitive nature of inhibition is indicated by the fact that the intercept does not increase in presence of the inhibitor.

A second value for \(K_i\) was obtained from the equation

\[
\frac{v}{v_i} = 1 + \frac{K_s}{K_i} \left(\frac{i}{K_s+i}\right).
\]

\(^8\) H. Lineweaver and D. Burk, J. Amer. chem. Soc. 56, 658 [1934].

According to Lineweaver and Burk, the plot of $v/v_i$ against different values of $i$, gives a straight line with unit intercept and slope dependent on $S$ in case of competitive inhibitors.

Fig. 3 illustrates the plot of $v/v_i$ versus $i$. Using the value of $1.6 \times 10^{-3}$ M for $K_s$ (determined from Fig. 2), $K_i$ was determined to be $7.6 \times 10^{-6}$ M, which is in good agreement with the first value. It was also found that the slope in Fig. 3 depends on $S$ which gives additional evidence for the competitive nature of inhibition. This seems to be in agreement with the work done by Winteringham and Disney, who interpreted the interaction of cholinesterase with carbamates as a competitive reversible reaction.

The washing technique was adopted to demonstrate the reversibility of inhibition. Using an inhibitor concentration of $3 \cdot 10^{-6}$ M (incubation period = 30 minutes), the remaining enzyme activity was only 32 per cent. After washing the free inhibitor and resuspending in buffer, the remaining activity increased to 68%, thus demonstrating partial recovery of the enzyme activity. Twice washings did not result in a much better recovery (remaining activity = 70%). In case of irreversible inhibitors, similar treatment does not result in recovery of the enzyme activity.

The effect of substrate on the enzyme inhibition was studied by determining the inhibition after simultaneous addition of acetylcholine and Sevin to the brain homogenate. Using an inhibitor concentration of $6 \cdot 10^{-6}$ M, and a substrate concentration of $8 \cdot 10^{-3}$ M, the inhibition was almost constant (18 – 24%) over the period 5 – 120 minutes. This suggests that only the reversible reaction (independent of time) is operating, while the progressive inhibition observed in absence of substrate (Fig. 1) could not be traced; even when the substrate concentration was lowered to the half. It is, therefore, believed that acetylcholine protects the enzyme against the irreversible attack of Sevin. This conclusion, together with the competitive nature of the reversible reaction, suggests that Sevin attaches itself to the substrate-binding group of the enzyme.

Though the reversible combination of E and I may refer to the participation of acetylcholine esterase in the hydrolysis of Sevin, it is believed that this enzyme is not involved in the detoxification of the insecticide in the nervous tissue to any significant extent. Direct evidence for this belief has been gained from experiments with $^{14}$C-Sevin (Carbonyl-labelled) and purified bovine erythrocyte cholinesterase (Sigma, U.S.A.). After 150 minutes, the system was found to be incapable of producing enzymatically-liberated $^{14}$CO$_2$ (cf. ref. 11).

The low value of $K_i$ clearly indicates the potency of Sevin as a cholinesterase inhibitor. This potency is further augmented by the contribution of the irreversible inhibition (though proceeding at a very slow rate). In fact, it is possible that, in the course of time, a complete block of cholinesterase results, and for all practical purposes the enzyme will remain permanently inhibited. This may be explained on the basis that since reversible inhibition is characterized by a state of equilibrium, there will be always a small amount of free enzyme in equilibrium, which becomes attacked irreversibly.

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