The Kinetics of Inhibition of Erythrocyte Cholinesterase by Monomethylcarbamates

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1. The kinetics of the interaction of erythrocyte cholinesterase with 1-naphthyl *N*-methylcarbamate, 2-isopropoxyphenyl *N*-methylcarbamate and phenyl *N*-methylcarbamate were studied. Rate constants for inhibition and rate constants for spontaneous reactivation were determined. The calculated rate constants for spontaneous reactivation agreed well with those obtained experimentally. 2. The degree of inhibition obtained after preincubation of enzyme and inhibitor was found to be independent of both the substrate concentration and the dilution of the inhibited enzyme. 3. The reaction between the enzyme and the inhibitor was consistent with carbamates being regarded as poor substrates of cholinesterases. There was no evidence for the formation of a reversible complex between the enzyme and the carbamate.

Carbamates are known as inhibitors of cholinesterases and the mechanism of inhibition is considered analogous to the mechanism of inhibition of cholinesterase by organophosphorus compounds (Wilson, Hatch & Ginsburg, 1960). In distinction to the phosphorylated enzyme, carbamoylated cholinesterases reactivated more rapidly. The rapid spontaneous reactivation creates difficulties in determining the degree of inhibition of the enzyme in the presence of carbamates (Witter, 1963). Winteringham & Disney (1964) reported that the evaluation of cholinesterase inhibition by carbamates depends on the concentration of the substrate present during the measurement of enzyme activity, as well as on the degree of dilution of the inhibited enzyme. They calculated the dissociation constants between 2-isopropoxyphenyl *N*-methylcarbamate and housefly head or human whole blood cholinesterase. On the other hand, Wilson *et al.* (1960) obtained results which allowed them to omit the reversible complex in presenting the overall reaction scheme of the inhibitory process.

In this paper a kinetic study of the inhibition of erythrocyte cholinesterase by some monomethylcarbamates is presented. Both the inhibition and the rate of spontaneous reactivation of the inhibited enzyme in the presence of substrate have been determined. The effect of dilution of the inhibited enzyme preparation and the effect of substrate concentration on the enzyme inhibition have been studied.

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MATERIALS AND METHODS

The enzyme preparation used was purified bovine erythrocyte cholinesterase (Winthrop, New York, N.Y., U.S.A.). Stock solutions (5 mg./ml.) were prepared in 1% gelatin and kept at 4°.

The substrates, acetylcholine chloride, acetylcholine perchlorate and acetylthiocholine iodide, were obtained from Pliva, Zagreb, Yugoslavia, British Drug Houses Ltd., Poole, Dorset, and Calbiochem, Los Angeles, Calif., U.S.A., respectively. The 5,5'-dithiobis-(2-nitrobenzoic acid) reagent was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

Three monomethylcarbamates were used as inhibitors: 1-naphthyl *N*-methylcarbamate, 2-isopropoxyphenyl *N*-methylcarbamate and phenyl *N*-methylcarbamate. The compounds were provided as technical products (95–99% purity) from the World Health Organisation, Geneva, Switzerland, and were several times recrystallized from ethanol and water. Stock solutions (0.05 M) prepared in dimethylformamide were kept at 4°, and were suitably diluted with buffer or 0.9% NaCl soln. immediately before use.

Two methods were chosen for measuring cholinesterase activity in the presence of carbamates: the titrimetric method of Jørgenson (1959) and the spectrophotometric method of Ellman, Courtney, Andres & Featherstone (1961). These methods were used because the activities of enzyme could be obtained within a short time (several minutes) of assay, and the activities could be continually measured from 20 to 30 sec. after addition of substrate. At low substrate concentrations (below 1.0 mM) the spectrophotometric method has advantages because smaller enzyme concentrations are required than in the titrimetric procedure. To follow the rate of reactivation the standard manometric method was used (Aldridge, 1950).

Titrimetric determination of enzyme inhibition. The

enzyme was incubated with a given concentration of inhibitor at 25° in 0.9% NaCl soln. at an adjusted pH 7.4. At suitable time-intervals samples (10.0 ml.) were withdrawn for assay. After adding the substrate (2.0 ml. of a given concentration of acetylcholine perchlorate) the activity of enzyme was determined by an automatic titration assembly (Radiometer, Copenhagen, Denmark). The acetic acid liberated was titrated with 0.02N-NaOH for 2–5 min. During this period, for all concentrations of acetylcholine used the amount of alkali used was linear with time both for uninhibited and inhibited enzyme samples. The final concentration of enzyme preparation during assay was 0.15 mg./ml.

Spectrophotometric determination of enzyme inhibition. The enzyme was incubated with a given concentration of inhibitor at 20° in 0.1 M-phosphate buffer, pH 7.4. At suitable time-intervals samples (3.0 ml.) were withdrawn and added to 100 μ l. of dithiobisnitrobenzoate reagent plus 50 μ l. of substrate solution (acetylthiocholine iodide of a given concentration). The reaction mixture was immediately transferred to a 1.0 cm. photocell and the extinction was read, against a blank which did not contain the enzyme, on a Beckman spectrophotometer (model DU) at 412 m μ . Readings were done at 30 sec. intervals for 2–5 min., the first reading being taken 30 sec. after the enzyme had been added to the reagents. During the assay the extinction was linear with time for both inhibited and uninhibited enzyme samples. The final concentration of enzyme during assay was 0.015 mg./ml.

In experiments where the effect of dilution was studied samples (x ml.) were taken from the reaction mixture of enzyme and inhibitor, and added to 3–x ml. of phosphate buffer plus 100 μ l. of dithiobisnitrobenzoate, and 50 μ l. of substrate solution, giving a total volume of 3.150 ml.

Measurement of spontaneous reactivation. The enzyme was incubated with a given concentration of inhibitor in the main compartment of the Warburg flask in 1.8 ml. of bicarbonate buffer, in an atmosphere of CO₂+N₂ (5:95) at pH 7.4 and 25°. After a suitable time-interval 0.02 ml. of substrate (acetylcholine chloride of a given concentration) was added, and the CO₂ output was followed for 1–2 hr. Readings of the CO₂ output were stopped when the amount was reached which in the control flask was no longer linear with time (about 250 μ l.).

RESULTS

Degree of inhibition as measured at different substrate concentrations. After the enzyme had been incubated with 2-isopropoxyphenyl *N*-methylcarbamate for different times the activity of cholinesterase was measured either titrimetrically at 0.15 M and 1.5 mM substrate concentration, or by the spectrophotometric method at a substrate concentration of 1.0 mM and 0.1 mM. As shown in Fig. 1 the degree of inhibition was not influenced by the concentration of the substrate used.

Similar results were obtained when the inhibition of enzyme by 1-naphthyl *N*-methylcarbamate was estimated titrimetrically at 0.15 M and 1.5 mM substrate concentration.

Fig. 1. Degree of inhibition of erythrocyte cholinesterase by 2-isopropoxyphenyl *N*-methylcarbamate measured at different substrate concentrations by (a) the titrimetric and (b) the spectrophotometric method. (a) Substrate concn.: 1.5 mM (○) and 0.15 M (●); inhibitor concn.: 1.0 μ M. (b) Substrate concn.: 1.0 mM (Δ , ○, ∇ , □) and 0.1 mM ($\blacktriangle$, ●, $\blacktriangledown$, $\blacksquare$); inhibitor concn. (μ M): 0.5 (Δ , $\blacktriangle$), 1.0 (○, ●), 2.0 (∇ , $\blacktriangledown$) and 4.0 (□, $\blacksquare$).

Degree of inhibition as measured after dilution of the inhibited enzyme preparation. The enzyme was incubated with a given concentration of 2-isopropoxyphenyl *N*-methylcarbamate for different times, and the activity of cholinesterase was measured spectrophotometrically at a substrate concentration 1.0 mM. Immediately before assay different dilutions of the inhibited enzyme were prepared. The same degree of inhibition was obtained whether the inhibited enzyme was assayed undiluted or diluted three, ten or 30 times (Fig. 2).

Inhibition of erythrocyte cholinesterase and reactivation of the inhibited enzyme. The titrimetric method was used for studying the kinetics of inhibition of erythrocyte cholinesterase by 1-naphthyl

N-methylcarbamate, 2-isopropoxyphenyl *N*-methylcarbamate and phenyl *N*-methylcarbamate. The activity of cholinesterase was measured at 3.0mM substrate concentration. Similar results were obtained with all the three inhibitors, the results obtained with 2-isopropoxyphenyl *N*-methylcarbamate being presented in Fig. 3.

When the concentration of inhibitor was small cholinesterase inhibition followed first-order kinetics up to approx. 1hr., whereas at high inhibitor concentrations deviations from first-order kinetics were observed already after 10min. inhibition. Rate constants for inhibition (k_i) were calculated from the first part of the inhibition curves according to the equation (1) for a bimolecular reaction with one component in excess (Aldridge, 1950; Aldridge & Davison, 1952), where c_i stands for the molar

$$k_i c_i t_i = \ln \frac{100}{a}$$

(1)

concentration of inhibitor, t_i for the time of inhibi-

tion in minutes, and a for the activity of cholinesterase expressed as percentage of the control. The calculated k_i values are listed in Table 1.

After the inhibition deviated from first-order kinetics the reaction gradually approached a state of apparent equilibrium. Equilibrium constants (K) were calculated according to equation (2) from

$$K = \frac{a_e c_i}{100 - a_e}$$

(2)

the activities of enzyme at equilibrium (a_e) expressed as percentage of the control. The mean of three or four enzyme activities determined at equilibrium was used for calculating the equilibrium constant at each concentration of inhibitor; K values given in Table 1 represent the means obtained at several inhibitor concentrations.

Since the equilibrium constant represents the ratio between the rate constant for spontaneous reactivation (k_r) and the rate constant for inhibition (k_i), the k_r value can be calculated from

Fig. 2. Degree of inhibition of erythrocyte cholinesterase by 2-isopropoxyphenyl *N*-methylcarbamate measured by the spectrophotometric method after different dilutions of the inhibited enzyme preparation. Substrate concn.: 1.0mM; inhibitor concn.: 1.0μM. Enzyme dilution: ○, nil; △, threefold; □, tenfold; ▽, 30-fold.

Fig. 3. Inhibition of erythrocyte cholinesterase by 2-isopropoxyphenyl *N*-methylcarbamate measured by the titrimetric method. Inhibitor concn. (μM): 0.5 (○), 1.0 (●), 2.0 (△), 5.0 (▲). Substrate concn.: 3.0mM.

Table 1. *Kinetic constants for inhibition of erythrocyte cholinesterase by monomethylcarbamates and for reactivation of the inhibited enzyme*

k_i (mol.⁻¹. min.⁻¹) is the rate constant for inhibition calculated from the regression lines according to equation (1); constants are given ± the standard error of the slope of the line. K is the mean of the equilibrium constant calculated according to equation (2). k_r (min.⁻¹) calculated is the rate constant for spontaneous reactivation according to equation (3). k_r (min.⁻¹) obtained is the mean of the rate constants for spontaneous reactivation obtained experimentally; values are given ± the standard error of the mean. Numbers in parentheses indicate the number of individual estimates.

Inhibitor	10 ⁻³ k_i	10 ⁶ K	10 ² k_r	
			Calculated	Observed
1-Naphthyl <i>N</i> -methylcarbamate	17.9 ± 0.95 (18)	0.794 (19)	1.42	0.90 ± 0.48 (33)
2-Isopropoxyphenyl <i>N</i> -methylcarbamate	40 ± 13 (17)	0.384 (18)	1.54	1.23 ± 0.21 (6)
Phenyl <i>N</i> -methylcarbamate	0.2681 ± 0.0069 (12)	54.3 (12)	1.46	1.30 ± 0.98 (7)

equation (3), if K and k are known. For all the

$$K = \frac{k_r}{k_i} \quad (3)$$

three inhibitors studied the calculated k_r values were practically the same (Table 1).

The rate of spontaneous reactivation was measured directly in the presence of 0.02M-substrate after the enzyme was treated with various concentrations of inhibitor. The rate constant for spontaneous reactivation (k_r) was calculated according to equation (4), where a_0 represents the activity

$$k_r = \frac{\ln(a_0 - a_1) - \ln(a_0 - a_2)}{t_2 - t_1} \quad (4)$$

of the uninhibited sample, and a_1 and a_2 represent the activity of the inhibited sample at t_1 and t_2 min. after addition of substrate. As a_1 and a_2 were usually calculated for enzyme activities at 15 and 75 min. after addition of substrate, the difference $t_2 - t_1$ corresponded to 60 min. In Table 1, the mean k_r values obtained from several runs are given for each inhibitor studied. The experimentally obtained k_r constants were about 10–40% smaller than the calculated values.

DISCUSSION

Many authors studying cholinesterase inhibition have regarded carbamates as poor substrates of cholinesterases (Myers, 1952; Myers & Kemp, 1954; Wilson *et al.* 1960; Wilson, Harrison & Ginsburg, 1961; Brick, Brestkin & Yakovljev, 1964). Wilson *et al.* (1960) postulated a reaction scheme for the inhibition of cholinesterases by carbamates and the reactivation of the inhibited enzyme (equation 5),

where E, I and EI' stand for the free enzyme, inhibitor and carbamoylated enzyme respectively.

The results we have obtained for the kinetics of inhibition and spontaneous reactivation of erythrocyte cholinesterase by monomethylcarbamates can be represented by the same reaction scheme (equation 5), but the way in which k_i and k_r were obtained differed from that of Wilson *et al.* (1960). Whereas these authors obtained k_i and k_r from the derived differential equations comprising both the time-course of inhibition and the steady-state conditions, we calculated these constants separately: k_i from the first part of the inhibition curve and k_r from the equilibrium constant obtained from the inhibition curve at steady state (equations 2 and 3). This method of determination proved satis-

factory, as the results obtained fit the mechanism proposed in equation (5).

According to equation (5) the inhibition of cholinesterase should follow first-order kinetics at the start of the reaction, as only a small amount of inhibited enzyme is present and the spontaneous reactivation is consequently small. This proved to be the case with all the three inhibitors studied. From the activity of enzyme as determined at steady state the equilibrium constant K can be evaluated, and from K and k_i the rate constant for spontaneous reactivation can be calculated (equation 3). As predicted from equation (5), k_r should be equal for all the three inhibitors used, since all yield monomethylcarbamoylated enzyme. The k_r values, both calculated and obtained experimentally, were in reasonably good agreement for all the three carbamates studied. However, values of k_r when obtained experimentally were somewhat lower than those calculated; this may be attributed to the presence of inhibitor during the measurement.

Winteringham & Disney (1964) studied the effect of substrate concentration, and the effect of dilution of the inhibited enzyme upon the measured degree of inhibition, and interpreted the interaction of cholinesterase with carbamates as a competitive reversible reaction. However, the authors assumed that under their experimental conditions their results could be interpreted from a hypothesis involving carbamoylation of the enzyme. In our studies 'initial' rate measurements have been made, as the time of contact of substrate and inhibited enzyme did not exceed 5 min., and the dilution of enzyme was accomplished by adding an appropriately diluted substrate to the inhibited enzyme. Under these conditions the inhibition of enzyme by carbamates did not depend upon the concentration of substrate over a wide range (0.15M–0.1mM) and a dilution of inhibited enzyme up to 30 times did not affect the degree of inhibition. If the recovery of the enzyme activity is a result of dissociation of a reversible complex between the enzyme and the inhibitor, the k_r values should differ from one monomethylcarbamate to the other. Contrary to that, calculated and experimentally obtained k_r values in our studies suggest that the reversal of the enzyme-inhibitor complex plays only a minor part in the whole reactivation process, if any.

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