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DIFFERENCES IN SUBUNIT ACTIVITIES IN ACETYLCHOLINESTERASE AS POSSIBLE CAUSE FOR APPARENT DEVIATION FROM NORMAL MICHAELIS-MENTEN KINETICS

RENÉ GENTINETTA and URS BRODBECK *

Medizinisch-chemisches Institut der Universität Bern, Bühlstrasse 28, CH-3000 Bern 9 (Switzerland)

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Summary

1. Form G_p of acetylcholinesterase (EC 3.1.1.7) from electric eel gave curved Lineweaver-Burk plots with acetylcholine. The Hill coefficients were 0.50—0.55 at low ionic strength and increased to 0.93 with increasing ionic strength. In presence of atropine or hexamethonium normal Michaelis-Menten kinetics were observed.

2. Inhibition of the enzyme by iPr_2P-F was biphasic. One half of the total enzyme activity decreased at a faster rate than the other half. With [^{3}H]iPr_2P-F, the extent of labelling was determined for the light and heavy subunits. At low [^{3}H]iPr_2P-F concentration only the light subunit was phosphorylated. Higher [^{3}H]iPr_2P-F concentrations and prolonged treatment increased the amount of label in the heavy subunit.

3. From these data it is concluded that the two subunits are labelled at different rates indicating different reactivity towards iPr_2P-F as well as towards acetylcholine. These data might account for the apparent non-Michaelis-Menten type kinetics obtained at low ionic strength.

Introduction

Acetylcholinesterase (EC 3.1.1.7) from the electric organ of *Electrophorus electricus* exists in a complex state of aggregation [1—3]. The multiple molecular forms of the enzyme can be isolated by the method of affinity chromatog-

* To whom reprint requests should be addressed.
Abbreviations: Nbs_2: 5,5'-Dithiobis (2-nitrobenzoic acid); iPr_2P-F: diisopropyl fluorophosphate; BBOT: 2,5-Bis[5'-tert-butylbenzoxazolyl(2')]-thiophene.

raphy [3—5] and can be separated by sucrose density-gradient centrifugation into the grape-like structures A, C and D as well as the more globular form G [5,6]. The oligomeric structure of acetylcholinesterase brings about apparent cooperative binding of substrate and other ligands. Since the early studies of Changeux [7,8] on the regulatory properties of a membrane-bound enzyme preparation, much additional evidence for the existence of a peripheral, non-catalytic binding site, that might exert regulatory properties, has accumulated [9—13]. In addition, non Michaelis-Menten type kinetics were observed in a number of cases [14—17].

Using acriflavine as an effector, Wermuth and Brodbeck [18] showed that form G of acetylcholinesterase exists in two catalytically different states. In State I, the enzyme has a higher affinity for the substrate than in State II.

Furthermore, in the latter state a substrate induced negative cooperativity was observed with acetylthiocholine. The production of thiocholine was assayed in presence of Nbs_2 following the procedure of Ellman et al. [19]. As discussed by Brownson and Watts [20], the presence of this thiol reagent might modify acetylcholinesterase by apparently activating the enzyme. The possibility thus existed that the negative cooperative effect obtained with acetylthiocholine was due to an Nbs_2 induced artefact.

As shown by a number of authors, the subunit molecular weight of the eel enzyme is estimated between 80 000 and 90 000 [21,22] and varies with the mode of solubilization [3]. Owing to autolysis during enzyme extraction and purification one subunit apparently breaks down to a peptide of 60 000—65 000 daltons. Both polypeptides of 80 000 and 65 000 contain active sites. In addition, a catalytically inactive peptide of approx. 25 000 molecular weight is formed [21,23]. According to Massoulié's notation [6] the resulting subunit composition for forms G_p, C and D then are h_2l_2, h_4l_4q and h_6l_6q [21]. In the present communication, the non Michaelis-Menten type kinetics of the enzyme are confirmed in absence of Nbs_2 with acetylcholine as the substrate. The deviation from normal Michaelis-Menten kinetics might be explained by differences in subunit reactivity towards acetylcholine. Since these differences might also exist in the reactivity towards iPr_2P-F, the reaction with this inhibitor was studied. A preliminary account of this work has been presented [24].

Materials and methods

Enzymes

Form G of acetylcholinesterase was extracted after limited proteolysis under toluene from electric organs of *Electrophorus electricus* (Paramount Research Supply Co. Ardsley, New York) and purified by affinity chromatography [4] to a minimal specific activity of 9000 I.U./mg of protein (assay 2). According to Massoulié's notation [6], this form of acetylcholinesterase is called G_p. Forms C and D were extracted from frozen tissue as described previously [25] and purified by affinity chromatography to a specific activity of 8000 I.U/mg of protein (assay 2). The forms were separated by density gradient centrifugation in a linear 5—30% (w/v) sucrose gradient according to the method of Martin and Ames [6]. Catalase was purchased from Sigma Chemical Company, Ltd. St. Louis/Mo. USA.

Chemicals

[^{3}H]-iPr$_2$-P-F was from New England Nuclear (Dreieichenhain), and Bio Gel P-10 from Bio Rad Laboratories (Richmond). Urea, ultra pure grade, was purchased from Mann Research Laboratories (New York). BBOT was from Ciba-Geigy (Basel). All other chemicals were standard commercial products obtained from Fluka (Buchs) or from Merck (Darmstadt).

Enzyme assay

Acetylcholinesterase activity was determined at 30° C either by measuring the amount of acetic acid produced (assay 1) or by following the production of thiocholine (assay 2) according to the method of Ellman et al. [19]. Enzyme activity is expressed in μmol substrate hydrolyzed per min. Results of kinetic experiments are presented as Lineweaver-Burk plots [27] or in semi-logarithmic fashion [28]. As acetylcholinesterase shows the phenomenon of substrate inhibition [29] the maximum reaction velocity was estimated by the method of Cleland [30]. Hill plots [31] are plotted as log $[v/(V-v)]$ vs. log [S].

Assay 1. In a total volume of 20 ml water, the standard mixture contained varying amounts of the chloride salts of mono- and divalent cations, acetylcholine chloride and acetylcholinesterase. The reaction was started by the addition of enzyme. The pH was kept constant at 7.4 by addition of 0.01 M NaOH using a Metrohm pH-meter E 300 B equipped with Impulsomat E 473 and Dosimat E 425. The reaction mixture was kept under CO_2-free nitrogen. For kinetic experiments at substrate concentrations below 0.2 mM acetylcholine the assay was modified as follows: employing the double-syringe technique described by Heilbronn [32] the Dosimat was filled with $2 \cdot 10^{-3}$ M NaOH (made freshly and titrated against standard acid every day prior to its use). The second syringe (Metrohm E 457 manual burette) was filled with an aqueous solution of $2 \cdot 10^{-3}$ M acetylcholine chloride. During the enzymic reaction the second syringe was advanced at the same rate as the one of Dosimat E 425. By this method, it was possible to keep the substrate concentration constant during the course of hydrolysis thus effectively changing the reaction kinetics from pseudo first-order to pseudo zero-order, allowing the determination of acetylcholinesterase activity at substrate levels as low as 5 μM.

Assay 2. In a total volume of 3.0 ml the standard assay mixture contained 100 mM sodium phosphate buffer pH 7.4, 0.125 mM Nbs_2 and 1 mM acetylthiocholine. The reaction was started by addition of acetylcholinesterase and followed spectrophotometrically at 412 nm (on a Beckman DB-G spectrophotometer equipped with a W+W recorder 3002).

Labelling of acetylcholinesterase with [^{3}H]iPr$_2$P-F. The enzyme was inactivated at varying concentrations of [^{3}H]iPr$_2$P-F (specific radioactivity 1 Ci/mmol) essentially as described by Berman [25]. To 200—500 units of pure enzyme (assay 2) in 50 μl of 50 mM sodium phosphate buffer, pH 7.4, [^{3}H]-iPr$_2$P-F in acetonitrile was added to varying final concentrations of inhibitor. The concentration of acetonitrile in all experiments was 0.5% (v/v). After incubation for the desired length of time, inhibition by [^{3}H]iPr$_2$P-F was stopped by adding butyrylcholine to a final concentration of 200 mM. Unreacted [^{3}H]-iPr$_2$P-F was separated from the labelled enzyme by chromatography through columns of BioGel P-10 made of Pasteur-pipettes (0.5 × 7.0 cm). The fractions

corresponding to the void volume contained the labelled enzyme. They were pooled, lyophilized and prepared for sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Disc electrophoresis on 5% polyacrylamide gels in presence of 0.1% sodium dodecyl sulfate and 8 M urea in 0.1 M sodium phosphate buffer, pH 7.1 was carried out according to Berman [33]. Samples were incubated at 100° C for 3 min in 3% sodium dodecyl sulfate containing 10% 2-mercaptoethanol. After addition of 7 M urea, the samples were layered directly onto the gels. The tracking dye was bromophenol blue. Electrophoresis was carried out for 6.5 h using the Ortec 4100 pulsed power supply. After staining with Coomassie brillant blue the gels were sliced and prepared for liquid scintillation counting according to the procedure of Tishler and Epstein [34].

Radioactivity. Radioactivity was detected on a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3320. Aqueous solutions of 0.4 ml containing solubilized gel material were counted after addition of 4 ml methanol and 10 ml of a solution of 8 g BBOT per l toluene.

Results

Evidence for non Michaelis-Menten kinetics: as shown previously [18], non Michaelis-Menten kinetics were obtained for the hydrolysis of acetylthiocholine by acetylcholinesterase when the assay mixture contained 10 μM acriflavine. This apparent negative cooperative effect was seen between substrate concentrations of 0.2 and 5 mM. A similar effect could be shown in absence of acriflavine when the substrate concentration was extended to 5 μM [3]. According to the arguments of Brownson and Watts [20] this effect might be due to alterations of the enzymic properties by the thiol reagent Nbs_2 present in the assay mixture.

Using the double syringe technique of Heilbronn [32], it was possible to assay acetylcholinesterase activity in absence of Nbs_2 with acetylcholine at substrate concentrations as low as 5 μM. It could be demonstrated (Fig. 1) that with form G_P, the hydrolysis of acetylcholine yields similar non Michaelis-Menten type kinetics, as shown previously using the Ellman assay procedure [19]. As summarized in Table I, increases in ionic strength decreased the apparent negative cooperative effect of acetylcholine on the enzyme. Furthermore, addition of atropine also changed the apparent negative cooperative effect to yield normal Michaelis-Menten kinetics (Fig. 2A). These results were compared to those obtained with the thio-substrate analogue (Fig. 2B). To avoid the use of the Ellman reagent, Nbs_2 was replaced in the assay mixture by dichlorophenolindophenol. The effect differed from the one shown in Figure 2A in as much as an apparent activation of the enzyme by atropine could be seen.

Working with purified acetylcholinesterase from head ganglia of squid, Kato and coworkers [15,16] showed that in presence of atropine the kinetics of acetylthiocholine hydrolysis were non Michaelis-Menten and resembled the bumpy curves described by Koshland for a number of enzymes [35]. Kato and coworkers also showed that atropine could lower to some extent the inhibition observed at high substrate concentration. In the present study, when form G_P

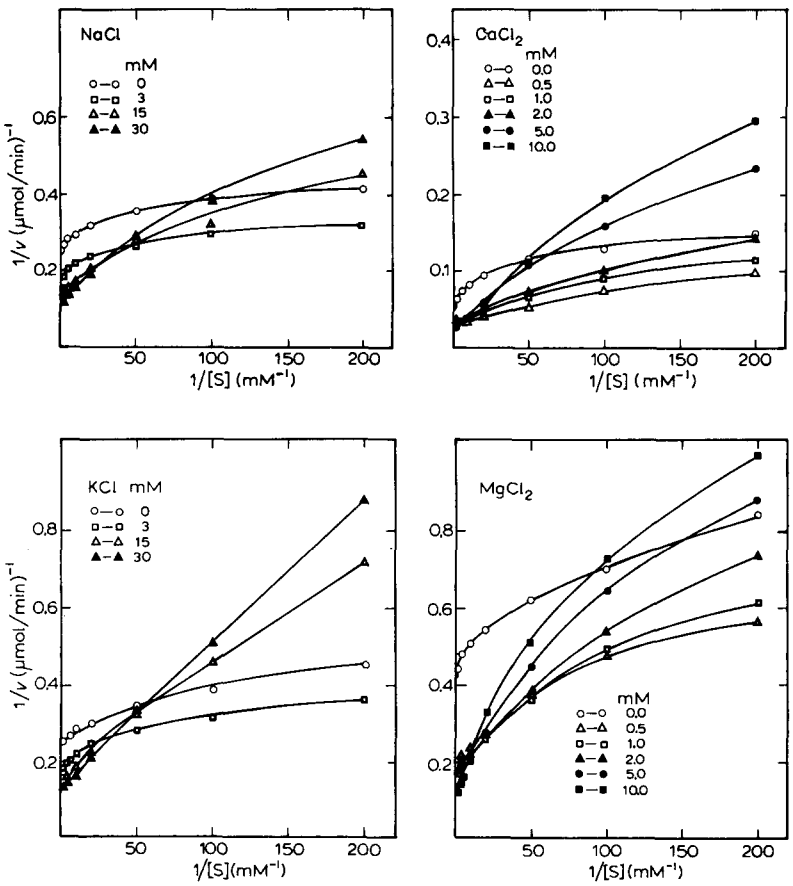

Fig. 1. Effect of increasing ionic strength on the hydrolysis of acetylcholine by form Gp of acetylcholinesterase shown in a Lineweaver-Burk plot. Substrate hydrolysis was determined using assay 1. Prior to its use, the enzyme was dialyzed against 1 mM of the respective salt solution and diluted 1000-fold into the assay medium.

of the eel enzyme was assayed using the conditions described by Kato and coworkers (100 mM phosphate buffer, pH 8.0 containing 10 mM Nbs_2, sodium bicarbonate (1.5 mg/ml) and increasing amounts of acetylthiocholine), the enzyme saturation curves did not show intermediary plateau regions at atropine

TABLE I

HILL COEFFICIENTS IN DEPENDENCE OF SALT CONCENTRATION

Salt concentration (mM)	Hill coefficient (n)		Salt concentration (mM)	Hill coefficient (n)	
	$CaCl_2$	$MgCl_2$		NaCl	KCl
0.0	0.50	0.55	0.0	0.54	0.55
0.5	0.72	0.74	3.0	0.50	0.55
1.0	0.73	0.77	15.0	0.74	0.84
2.0	0.77	0.80	30.0	0.72	0.90
5.0	0.79	0.78			
10.0	0.93	0.75			

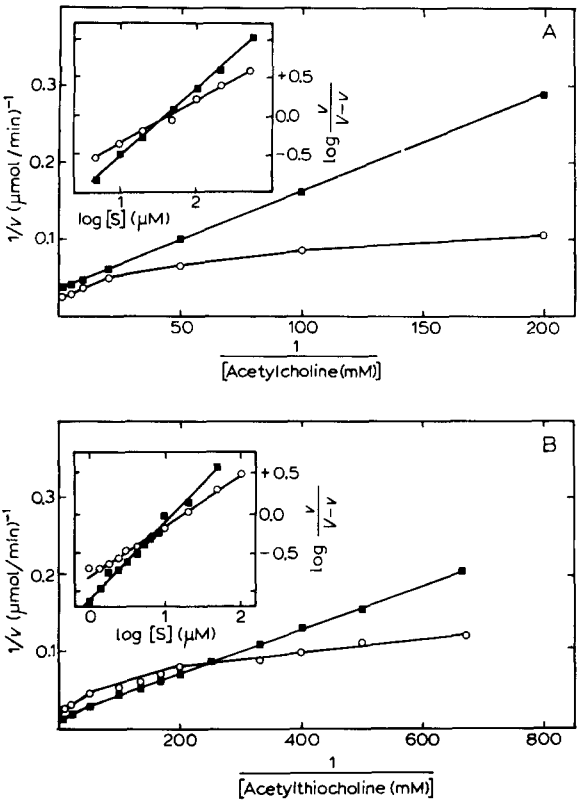

Fig. 2. Effect of atropine on the kinetics of substrate hydrolysis by acetylcholinesterase shown in a Lineweaver-Burk plot. (A) Acetylcholine hydrolysis in presence of 1 mM NaCl was monitored by using assay 1. (B) Acetylthiocholine hydrolysis was determined using the spectrophotometric assay with 2,4-dichlorophenol indophenol (50 μM) as redox indicator instead of Nbs_2 and 1 mM Na-phosphate buffer, pH 7.4. Substrate hydrolysis in presence of 0.5 mM atropine (■———■) and in absence thereof (○———○). Inserts show the same data plotted according to the Hill equation. In (A), the Hill coefficients are 0.91 in presence and 0.53 in absence of atropine. In (B), the values are 1.02 and 0.66 respectively.

concentrations of 1, 5 and 10 mM respectively. However, when the inhibition by substrate was measured at different ionic strengths the curves were unsymmetrical and at low ionic strength showed intermediary plateau regions (Figs. 3 and 4). Applying the Hill equation to those portions of the curves showing substrate inhibition [16,36], a biphasic Hill plot was obtained at low ionic strength (inserts, Figs. 3 and 4) with Hill coefficients below 1.

From these results it was concluded that the heavy and light subunit might show different reactivity towards acetylcholine. To investigate this possibility further, the effect of iPr_2P-F on the subunits of the enzyme was investigated.

Effects of iPr_2P-F on acetylcholinesterase

Forms G_P, C and D were separated by sucrose density gradient centrifugation (Fig. 5, top). Acetylcholinesterase from the peak fractions was reacted with 0.05 mM iPr_2P-F. At that concentration, the organophosphorous compound was in large excess over enzyme concentration, effectively yielding pseudo first-order kinetics of the reaction between enzyme and inhibitor. When

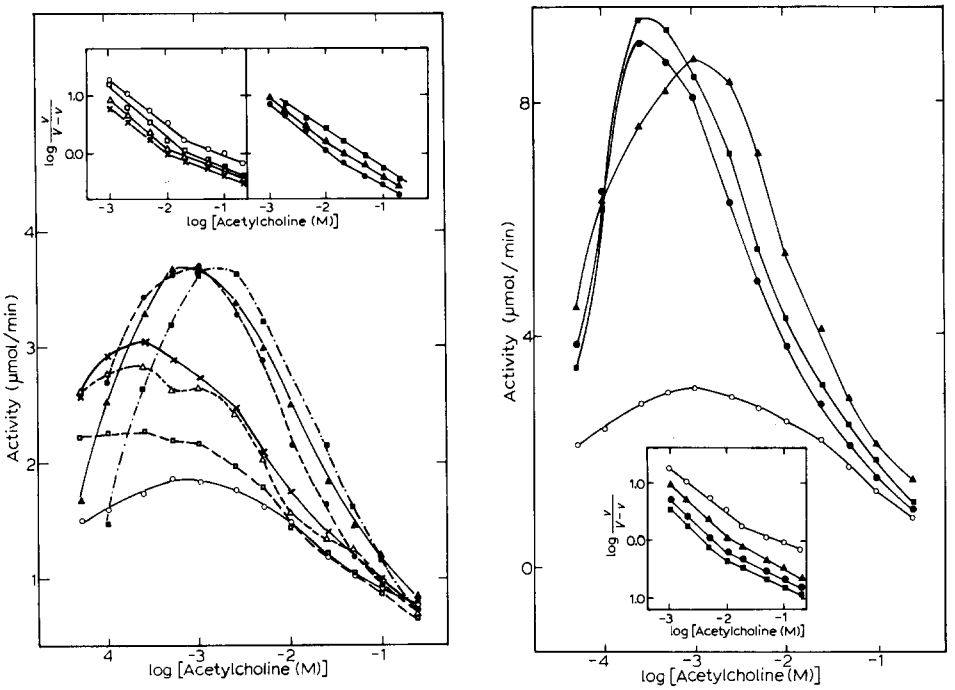

Fig. 3. Effect of increasing KCl concentrations on the substrate inhibition of acetylcholinesterase. Enzyme activity was determined using assay 1. The enzyme was dialyzed against 1 mM KCl and diluted 100-fold into the reaction mixture. Curves obtained at 0 mM (○———○), 1 mM (□———□), 5 mM (△———△), 10 mM (X———X), 50 mM (●———●), 100 mM (▲———▲) and 250 mM (■———■) KCl. Insets show data plotted according to the Hill equation.

Fig. 4. Effect of increasing $MgCl_2$ concentrations on the substrate inhibition of acetylcholinesterase. Assay conditions similar to those described in Fig. 3. Curves were obtained at 0 mM (○———○), 1 mM (●———●), 2 mM (■———■) and 10 mM (▲———▲) $MgCl_2$. Inset shows data plotted according to the Hill equation.

the decrease in enzyme activity was plotted in semilogarithmic fashion as a function of time, curved lines were obtained (Fig. 5, bottom, filled circles). The experimental results were fitted to the equation [44]:

$$\frac{X}{X_0} = f_1 \cdot e^{-k_1 \cdot t} + f_2\, e^{-k_2 \cdot t} \qquad (1)$$

in which X/X_0 is the fraction of the total activity remaining after time t; f_1 and f_2 are the fractions of the total activity of the uninhibited enzyme and k_1 and k_2 are the first-order rate constants for enzyme inhibition by iPr_2P-F. Graphical analysis of the data resulted in $f_1 = f_2 = 0.5$. Under the conditions of this experiment, where iPr_2P-F was present in such excess that its concentration did not change effectively during the course of the reaction, the bimolecular inhibition constant k_1 could be calculated from the slopes (Table II).

The results shown in Fig. 5 suggested that the heavy and light subunit possibly were inhibited by iPr_2P-F at different rates. To check this hypothesis, the extent of labelling in the two subunits was determined. Form G_P was reacted with [^{3}H]iPr_2P-F at different concentrations for varying lengths of time. After denaturation and reduction, the subunits were separated by sodium dodecyl

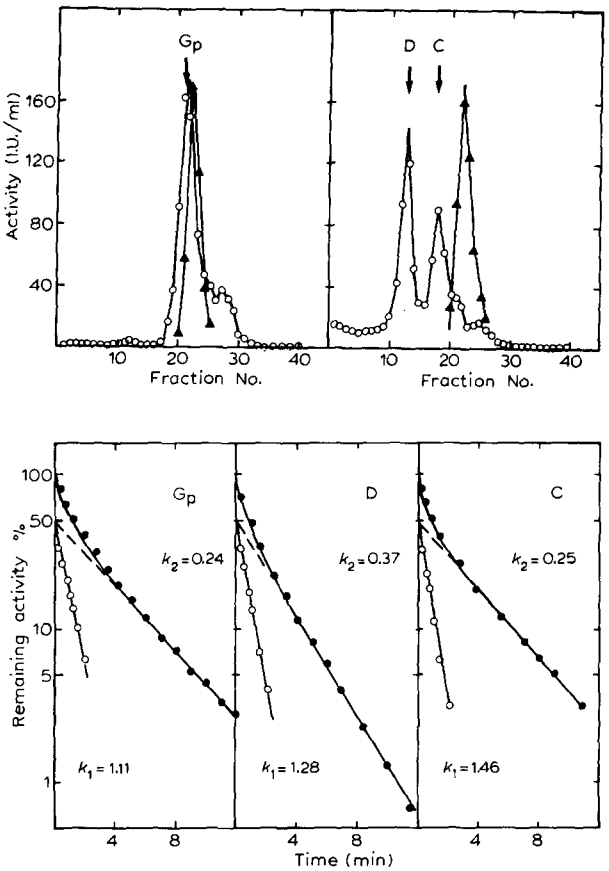

Fig. 5. Separation of oligomeric forms of acetylcholinesterase by sucrose density gradient centrifugation and inactivation by iPr_2P-F. Top: forms C and D were purified by affinity chromatography from frozen electric tissue and separated by sucrose density gradient centrifugation (right). Similarly form G_P was obtained from toluene treated, autolyzed tissue (left). Acetylcholinesterase activity (○———○) was determined by assay 2. Catalase served as marker protein (▲———▲). From the peak fractions indicated by arrow, 200 µl of enzyme was incubated with iPr_2P-F at a final concentration of 0.05 mM in a solution of 100 mM sodium phosphate buffer, pH 7.4, containing 0.5% acetonitrile. At the times indicated, 3 µl samples were withdrawn, diluted to 3 ml and assayed for remaining acetylcholinesterase activity using assay 2. Bottom: decrease in enzyme activity, corrected for the slight inactivation observed in presence of 0.5% acetonitrile alone (●). Upper curves (solid line) represents the decrease in activity calculated according to equation 1. Lower curves (○) were obtained by subtracting the values of the extrapolated, dashed lines from the corresponding values of the first portion of the upper curves. Left to right: forms G_P, D and C respectively. k-Values are first order rate constants in min^{-1}.

TABLE II

BIMOLECULAR RATE CONSTANTS FOR THE REACTION OF iPr_2P-F WITH FORMS D, C and G_P OF ACETYLCHOLINESTERASE

Enzyme form	Bimolecular rate constant k_i ($1 \cdot 10^{-4}$ $Mol^{-1} \cdot min^{-1}$)	
	fast reaction	slow reaction
D	2.56	0.73
C	2.92	0.51
G_P	2.21	0.49

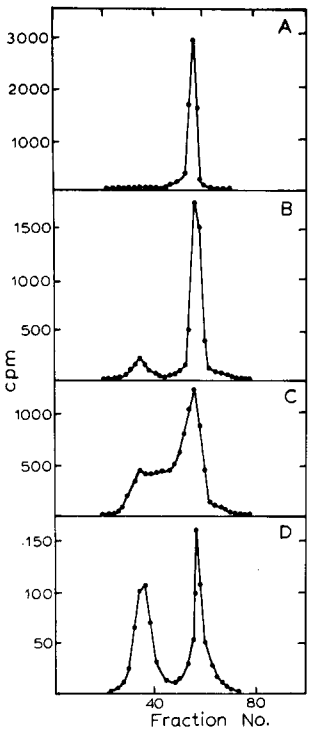

Fig. 6. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of [^{3}H]iPr$_2$P-F labelled reduced form Gp of acetylcholinesterase. The enzyme was incubated at different [^{3}H]iPr$_2$P-F concentrations for varying lengths of time. (A), 5 μM for 30 min, (B), 0.1 mM for 30 min, (C), 0.1 mM for 150 min and (D), 2 mM for 150 min. The specific radioactivity of [^{3}H]iPr$_2$P-F in experiments A to C was lCi/mmol; in experiment D it was 0.1 Ci/mmol. After the intervals indicated inhibition by [^{3}H]iPr$_2$P-F was stopped by addition of butyrylcholine and the samples were treated as indicated in methods.

sulfate gel electrophoresis. The gels were then fractioned and the radioactivity in the individual subunits was determined. As shown in Fig. 6A only the light, faster moving subunit was phosphorylated at low [^{3}H]iPr$_2$P-F concentrations. Increasing concentrations of [^{3}H]iPr$_2$P-F and prolonged reaction time resulted in labelling of the heavy subunit as well (Fig. 6, B-D). It should be noted that slightly different amounts of enzyme were subjected to sodium dodecyl sulfate gel electrophoresis giving different numbers of total counts (Fig. 6, A-C). In experiment D, the specific radioactivity was one tenth of that used in experiments A-C reducing the number of total counts by a factor of 10. Similar results were obtained when the subunits were separated by isoelectric focusing in presence of 6M urea after denaturation in sodium dodecyl sulfate and reduction by 2-mercaptoethanol.

Discussion

It is generally accepted that eel acetylcholinesterase assumes a complex oligomeric structure, the multiple molecular forms of which are, in absence of prolonged autolysis, readily purified by affinity chromatography (for recent reviews, see refs. 37 and 45). Numerous studies have established the existence of a

binding site spatially apart from the active site [9—12]. From fluorescent ligand displacing experiments, Mooser and Sigman [13] concluded that the enzyme probably exists in two conformationally distinct forms, thus confirming earlier observations made by Changeux on a partially purified torpedo acetylcholinesterase [7] and by Wermuth and Brodbeck on pure form G_P of electric eel acetylcholinesterase [18].

The present study shows that at low substrate concentration, the kinetics of acetylcholine hydrolysis differ from normal Michaelis-Menten type behavior, the degree of which varies with the ionic strength. An increase in salt concentration seems to decrease the non Michaelis-Menten behavior. A similar effect has been observed on crude bovine red cell acetylcholinesterase [38].

Atropine, which preferentially binds at the peripheral site according to the studies of Kato and coworkers [9,10], was shown in the present study to reduce the apparent non Michaelis-Menten behavior also. At substrate concentrations where inhibition of acetylcholine hydrolysis is seen, atropine did not, however, protect the eel enzyme against this inhibition. This observation is in contrast to the one made by Kato and coworkers on the enzyme from squid head ganglia [16]. On the other hand, when the kinetics of substrate inhibition were fitted to the Hill equation, the slopes of the straight lines always were below 1. The results again contrast with those of Kato who found for the squid enzyme a Hill coefficient of 2.0 for the part of the curve showing substrate inhibition [15].

The results of the present study together with the data from the literature suggest that either there are subunit interactions in eel acetylcholinesterase or the enzyme contains non-identical subunits that function independently and give different kinetics. As shown by different groups, the enzyme, following solubilization, contains subunits that differ with respect to their molecular weight but contain both catalytically active sites [3,21—23]. The possibility thus existed that the heavy and light subunit behave differently not only with respect to their catalytic efficiency but also with respect to their affinity towards organophosphorous compounds. To obtain additional information on the latter possibility, the covalent phosphorylation of the enzyme by iPr_2P-F was investigated.

The decrease in enzyme activity as a consequence of enzyme inactivation by iPr_2P-F was, at a given inhibitor concentration, faster during the first 2—3 min than thereafter. Similar inactivation kinetics have been observed by Massoulié and coworkers (personal communication). From extrapolation to zero time it was concluded that one half of the total enzyme activity decreased faster than the remaining 50%. These data suggested that the light and heavy subunits react differently towards iPr_2P-F. In addition, the results obtained after sodium dodecyl sulfate gel electrophoresis of [^{3}H]iPr_2P-F labelled acetylcholinesterase showed that at low inhibitor concentrations the light subunit was predominantly phosphorylated and only at a relatively high iPr_2P-F concentration did both subunits become labelled. Thus the light subunit seems to be more reactive than the heavy one. These data then suggest that the light and heavy subunit of the enzyme have different kinetic properties. The labelling pattern does not unequivocally support the hypothesis that there are indeed subunit interactions in acetylcholinesterase. On the other hand atropine was shown to reduce the

apparent negative cooperativity in acetylcholinesterase. This effect might be caused either by a decrease in subunit interactions or equally by rendering the heavy and light subunit kinetically indistinguishable without imposing subunit interactions.

Although it is generally accepted that each subunit of acetylcholinesterase carries an active site, other ratios have been reported. As reviewed recently [3] the enzyme purified by Leuzinger from toluene-treated tissue [39,40] had a tetrameric structure with a subunit composition of $\alpha_2\beta_2$ [41]. By using iPr_2P-F and *o*-nitrophenyl-dimethylcarbamate as active-site titrants, Leuzinger found only two active sites per tetramer [42]. Furthermore, Rosenberry and coworkers, studying a pure enzyme obtained from autolyzed eel tissue, reported at one time three active sites per tetramer [43]. The results presented in this paper might explain the discrepancies in the number of active sites estimated by different investigators. Depending on the relative concentration of the active site titrant to the amount of enzyme, two to four active sites per tetramer might be found.

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