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Multiple Forms of a Cholinesterase from Body Muscle of Plaice (*Pleuronectes platessa*) and Possible Role of Sialic Acid in Cholinesterase Reaction Specificity

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Solubilized and partially purified cholinesterase from the body muscle of plaice (*Pleuronectes platessa*) exists as a mixture of multiple forms that could be separated by isoelectric focusing into 2 – 3 species with isoelectric points of pH 5.3, 6.9, and 7.5. These species did not differ, however, with respect to their catalytic properties when assayed with acetyl-, butyryl-, and benzoylcholine as substrates. Upon incubation of the enzyme with neuraminidase a new form with an isoelectric point of pH 8.0 was obtained. The catalytic properties of this desialylated enzyme was the same as that of the untreated one. When the new species was reincubated with neuraminidase an acetylcholinesterase with an isoelectric point of pH about 10 was obtained that had lost the ability to hydrolyse butyryl- and benzoylcholine. These data suggest that removing one or more sialic acids gives raise to an enzyme form specific for acetylcholine only.

As shown previously [1] a butyrylcholine splitting cholinesterase * was found in body muscle of plaice (*Pleuronectes platessa*) and a method of purifying the enzyme [2] has been described and also some of the enzyme properties.[3] The enzyme shows some characteristics typical for an acetylcholinesterase and some others typical for a butyrylcholinesterase. An acetylcholinesterase splits acetylcholine faster than propionyl- and butyrylcholine, the latter being hydrolyzed very slowly or not at all. A butyrylcholinesterase splits butyrylcholine faster than propionylcholine and acetylcholine. It is more sensitive toward certain inhibitors of the organophosphorus type; butyrylcholinesterases are inactivated at a much lower level of inhibitor than acetylcholinesterases. The two enzymes differ also in other respects. Acetylcholinesterase displays

* Enzymes: Acetylcholinesterase (EC 3.1.1.7), butyrylcholinesterase (EC 3.1.1.8), benzoylcholinesterase (EC 3.1.1.9). Acylcholine acylhydrolase (EC 3.1.1.8) is not called cholinesterase as recommended by IUPAC but butyrylcholinesterase in order to facilitate the representation. The cholinesterase investigated is called plaice cholinesterase. Neuraminidase (EC 3.2.1.18).

the phenomenon of substrate inhibition with the cholinesters split. This is not the case with butyrylcholinesterase.

For plaice cholinesterase the turnover number is highest for acetylcholine; thus this enzyme resembles an acetylcholinesterase.[3] Other substrates such as propionyl-, butyryl-, valeryl-, and benzoylcholine are hydrolyzed at a rate much higher than expected for an acetylcholinesterase. With these substrates the enzyme from plaice body muscle resembles more a butyrylcholinesterase. With respect to inhibition by organophosphorus compounds plaice cholinesterase differs from both acetylcholine- and butyrylcholinesterase showing even greater sensitivity towards this type of inhibitor than the other cholinesterases.[3] In addition, at high substrate levels an abnormal kinetic behaviour was observed [4] giving raise to the phenomenon of "bumpy curves" described by Teipel and Koshland [5] for a number of enzymes other than acetylcholinesterase.

Since such differences in catalytic properties could arise from a mixture of kinetically distinguishable multiple forms of an enzyme [6] plaice cholinesterase was subjected to isoelectric focusing on analytical and preparative scales. By these methods possible heterogeneities would be detected and it would be possible to isolate the forms obtained on a preparative scale. The present investigation describes the resolution of plaice cholinesterase into several species with different isoelectric points. It gives a characterization of the obtained forms with respect to their substrate specificity and their sensitivity towards diisopropylfluorophosphate. Evidence is presented that upon incubation of the enzyme with neuraminidase the isoelectric points are shifted towards higher pH values. A preliminary account of this work has been presented.[7]

MATERIALS AND METHODS

Enzymes. Lyophilized plaice cholinesterase, purified to a specific activity of 8 IU/mg of protein was obtained as described earlier.[2] The enzyme (10 mg of lyophilized powder) was dissolved in 5 ml of 5 mM Na-phosphate buffer, pH 7.0 and centrifuged at 4°C for 10 min at 2500 *g*. The clear supernatant containing 90 % or more of the total plaice cholinesterase activity was routinely used. Depending on the experiments the enzyme was stored in solution at 4°C or kept frozen at −10°C. Neuraminidase, type VI purified from *Cl. perfringens* was purchased from Sigma Chem. Comp., St. Louis, Mo., USA.

Chemicals. Acetylthiocholine iodide, butyrylthiocholine iodide, diisopropylfluorophosphate, and 5,5′-dithiobis-2-nitrobenzoic acid were obtained from Fluka AG, Buchs, Switzerland. Benzoylcholine chloride was from F. Hoffmann-La Roche AG, Basel, Switzerland, and carrier ampholine (pH 3 − 10) was purchased from LKB, Stockholm, Sweden.

All other chemicals employed were standard commercial products. Deionized water was used for the preparative work and double distilled water for the analytical procedures.

Enzyme assays. Acetylcholinesterase activity was determined at 30°C either by following the production of thiocholine (assay 1) according to the method of Ellman *et al.*[8] or by measuring the amount of benzoate produced [9] (assay 2). Enzyme activity was expressed in international units (IU = μmol of product produced per minute).

Assay 1. In a total volume of 3.0 ml the standard assay mixture contained 0.1 M Na-phosphate, pH 7.4, 0.125 mM 5,5′-dithiobis-2-nitrobenzoic acid and 1 mM acetylthiocholine or butyrylthiocholine. The reaction was started by adding varying amounts of plaice cholinesterase and was followed spectrophotometrically by measuring the increase in absorbance at 412 nm (on a Beckman DB-G Spectrophotometer equipped with a W+W Recorder 3002).

Assay 2. The incubation mixture contained the same components described in assay 1 except that 80 μM benzoylcholine was used instead of acetylthiocholine. The reaction was followed spectrophotometrically by measuring the decrease in absorbance at 240 nm.

Treatment of plaice cholinesterase with neuraminidase. Sialic acid residues were removed from plaice cholinesterase essentially according to the procedure of Carlsen and Svensmark.[10] Cholinesterase (20 IU in 2.5 ml of buffer) was diluted with an equal volume of 0.1 M Na-acetate buffer, pH 5.5, containing 0.2 M NaCl and 20 mM $CaCl_2$. To this mixture 10 μg of purified neuraminidase were added. The solution was incubated at 30°C for different amounts of time. Before isoelectric focusing the mixture was dialyzed against 3 × 1 l of a solution of 1 % glycine, pH 7.4.

Treatment of plaice cholinesterase with purified solubilizing factor from Cytophaga sp. Cholinesterase was treated with the purified cholinesterase solubilizing factor isolated from *Cytophaga sp.* as described earlier.[11] Two ml of cholinesterase (5.6 IU/ml) in 5 mM Na-phosphate buffer, pH 7.4, were added to 2 ml of a solution of 0.1 M Na-acetate buffer, pH 5.5, containing 0.1 M NaCl and 20 mM $CaCl_2$. To this mixture 80 μl of a solution of 1 mg of purified Cytophaga factor in 10 ml of 5 mM Na-phosphate buffer, pH 7.4, were added. The incubation and dialysis were carried out as desrcibed above.

Analytical isoelectric focusing. Analytical isoelectric focusing was carried out essentially as described by Godson [12] except that J-type tubes were used, filled with 36 – 40 ml of a gradient (0 – 45 % sucrose) containing 1 % ampholine solution. The tubes were filled to the top and the upper part was sealed by a dialysis membrane. To prevent the formation of Joule's heat the voltage was increased gradually over a period of 12 – 18 h keeping the total current to 1.5 mA or lower per tube. A maximum of four samples were focused together at one time. After reaching 1000 V the electrofocusing was continued for 3 days. The removal of the gradients from the J-type tubes was carried out according to the procedure of Godson. Fractions, 0.8 – 1.0 ml each were collected.

Preparative isoelectric focusing. The multiple forms of plaice cholinesterase were separated on a preparative scale using a 150 ml electrofocusing column (LKB 8001). Conditions similar to the one described above were employed. Fractions, 1.2 – 1.5 ml each, were collected.

RESULTS

Multiple forms of plaice cholinesterase. As shown in Fig. 1 freshly dissolved lyophilized powder of plaice cholinesterase could be resolved by preparative isoelectric focusing into two fractions with isoelectric points at pH 5.5 and 6.9. Similar results were obtained when the same enzyme preparation was subjected to analytical isoelectric focusing (Fig. 2). The total amount of cholinesterase used in the former experiment was approximately 40 – 100 times that required for an analytical isoelectric focusing.

When the solution containing plaice cholinesterase was kept at 4°C for several weeks a pronounced shift in the elution profile was observed. Generally the total amount of enzyme focusing at pH 5.5 was decreased and a third species focusing at pH 7.6 was gradually obtained (Fig. 3). In extreme cases practically all of the enzyme had converted to forms with isoelectric points higher than pH 6. The enzyme obtained from the preparative separation was pooled into three fractions with isoelectric points at pH 5.5, 7.0, and 7.6. The three enzyme forms were analyzed for possible differences in substrate specificity. As shown in Table 1 the enzyme forms of all three pools could not be distinguished from one another on kinetic basis and did not differ from the enzyme before its separation into the three species. Furthermore no differences among the three forms were found when the inhibition of the three enzyme species by diisopropylfluorophosphate was compared (Fig. 4). The common pI_{50} value of 8.7 is similar to the one reported for the unresolved enzyme.[3]

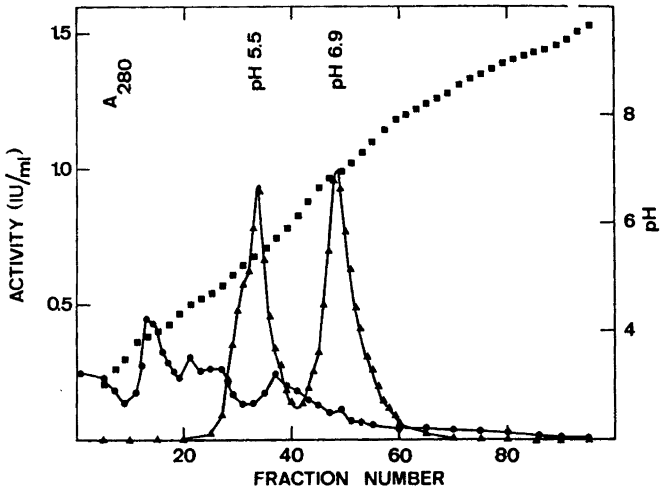

Fig. 1. Preparative isoelectric focusing of freshly dissolved plaice cholinesterase. The enzyme was dissolved and subjected to isoelectric focusing as described in METHODS. Enzyme activity: ▲, acetylthiocholine as substrate; ●, A_{280}, and ■, pH.

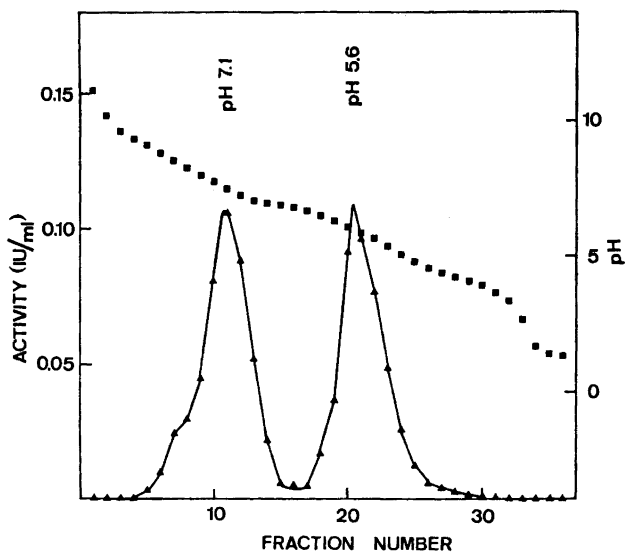

Fig. 2. Analytical isoelectric focusing of same sample as in Fig. 1. Symbols similar to Fig. 1.

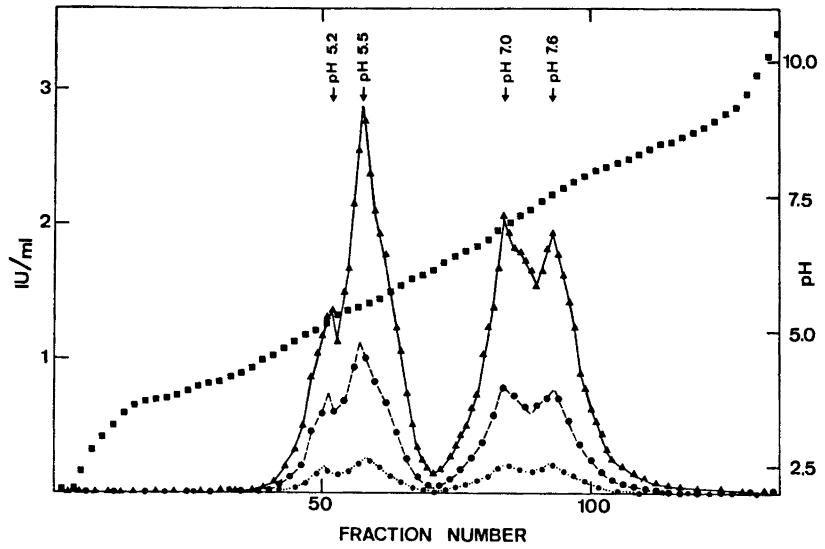

Fig. 3. Preparative isoelectric focusing of plaice cholinesterase stored at 4°C in solution for one week. Enzyme activity was assayed with acetylthiocholine (▲), butyrylthiocholine (●) and benzoylcholine (*); ■, pH.

Incubation of plaice cholinesterase with neuraminidase. The increase in the isoelectric points of plaice cholinesterase could possibly have arisen from a release of an acidic component such as sialic acid residues. Accordingly the enzyme was incubated with neuraminidase at 30°C for different periods of

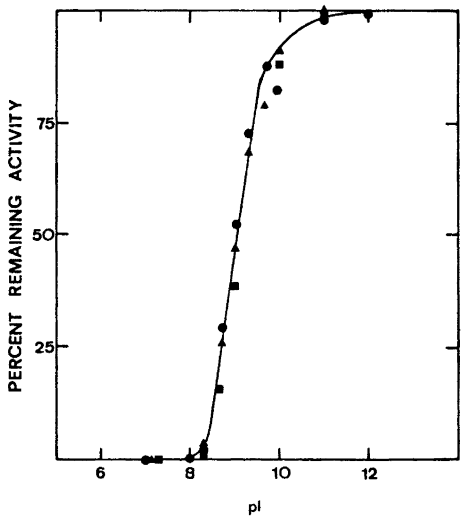

Fig. 4. Inhibition of the different forms of plaice cholinesterase by diisopropylfluorophosphate. The enzyme eluted from the preparative isoelectric focusing column (Fig. 3) was pooled as follows: pool I, fractions 55–65; pool II, fractions 80–85; pool III, fractions 92–100. The pooled fractions were incubated in 50 mM K-phosphate buffer, pH 8.2, at room temperature with increasing amounts of diisopropylfluorophosphate. After 30 min the remaining activity was determined spectrophotometrically with acetylthiocholine as substrate. pI curve of the enzyme from pool I (●); pool II (▲) and pool III (■).

Table 1. Comparison of kinetic parameters of plaice cholinesterase fractions.

Fraction	Isoelectric point	Acetylthiocholine			Butyrylthiocholine			Benzoylcholine		
	pH	K_m mM	V_{max} rel. units	Optimum activity pS	K_m mM	V_{max} rel. units	Optimum activity pS	K_m mM	V_{max} rel. units	Optimum activity pS
Enzyme before separation	–	0.19	0.33	2.47	.038	0.13	3.52	.018	.026	4.17
Pool I	5.5	0.18	0.31	2.55	.037	0.11	3.50	.018	.025	4.16
Pool II	7.0	0.18	0.36	2.55	.039	0.13	3.55	.021	.028	4.17
Pool III	7.6	0.19	0.34	2.60	.036	0.12	3.50	.020	.024	4.18

The fractions were pooled as indicated in the legend to Fig. 3 and assayed spectrophotometrically under standard conditions. The values of V_{max} and K_m were obtained from Lineweaver-Burk plots, optimal activity from plots of activity *vs.* pS. Relative units of enzyme activity are given since the exact amounts of enzyme in the different pools are unknown.

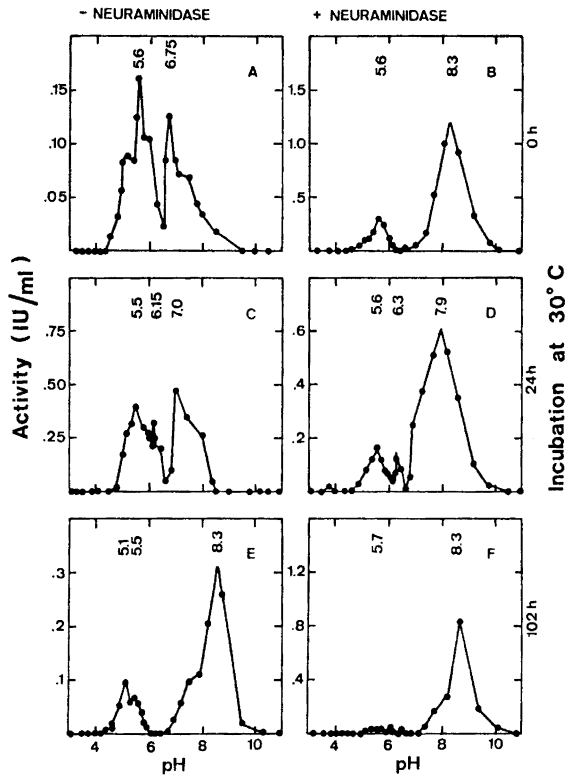

Fig. 5. Effect of neuraminidase on the isoelectric points of plaice cholinesterase. The enzyme was incubated at 30°C in presence and absence of neuraminidase for different lengths of time. Analytical isoelectric focusing was carried out on each incubation mixture after neuraminidase action. Elution patterns were drawn to a constant pH scale. Enzymatic activity assayed with acetylthiocholine as substrate (●). Enclosed numbers denominate the isoelectric points of the fractions obtained.

time. A summary of these results is shown in Fig. 5. In order to directly compare the elution patterns they were redrawn to a constant pH scale rather than to constant elution volume. As can be seen prolonged incubation with neuraminidase changes the isoelectric points of all species to a new single isoelectric point at pH 8.2 – 8.3 (Fig. 5F). In absence of neuraminidase no complete conversion to the pH 8.3 species was obtained even after a 102 h incubation period at 30°C (Fig. 5E).

To analyze each fraction for its activity towards the three substrates acetylthiocholine, butyrylthiocholine, and benzoylcholine, plaice cholinesterase was incubated with neuraminidase on a preparative scale. The elution profile obtained after isoelectric focusing of such an experiment was similar to the one in Fig. 3, and again no differences in catalytic properties could be found among the different forms of the enzyme.

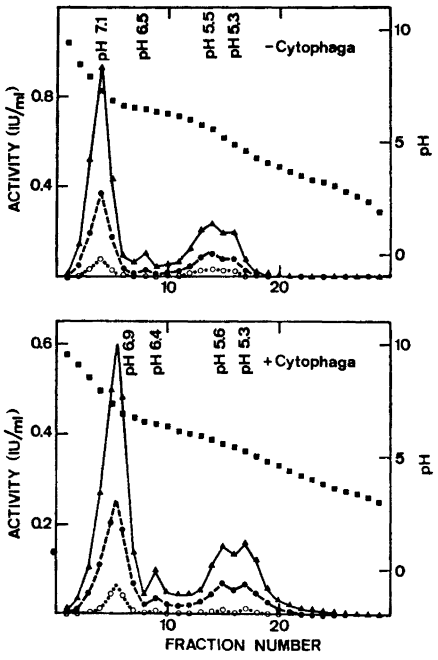

Fig. 6. Effect of enzyme solubilizing factor from Cytophaga. The enzyme was incubated at 30°C for 100 h with the purified cholinesterase solubilizing factor from Cytophaga. Analytical isoelectric focusing was carried out after completion of the incubation with the Cytophaga factor. Enzyme activities: ▲, acetylthiocholine; ●, butyrylthiocholine, and ○, benzoylcholine. ■, pH.

During these large scale incubation experiments a heavy protein precipitate was formed. After centrifugation the specific activity of plaice cholinesterase was increased 10 fold and the recovery of the enzyme was 92 – 95 %. However neuraminidase treatment on large amounts of plaice cholinesterase was less effective although the addition of neuraminidase was increased to the same extent as the total amount of cholinesterase units.

Incubation of soluble plaice cholinesterase with the enzyme solubilizing factor from Cytophaga. A unique feature in the preparation of the plaice enzyme is its solubilization from muscle tissue by a cholinesterase releasing factor obtained from *Cytophaga sp.*[11] Although the factor behaves like an enzyme its exact action on the muscle tissue remains obscure.[13] Thus it was of interest to obtain information on the possible effects of the Cytophaga enzyme on the soluble plaice cholinesterase. Plaice enzyme was incubated in presence and absence of the Cytophaga factor. After isoelectric focusing the fractions were analyzed for acetylthiocholine, butyrylthiocholine, and benzoylcholine activities. As shown in Fig. 6 the patterns obtained in presence and absence of the Cytophaga enzyme were identical.

Reincubation of plaice cholinesterase with neuraminidase. The forms of plaice cholinesterase with an isoelectric point of pH 7.0 and 7.6 isolated by isoelectric focusing were treated anew with neuraminidase and subjected to isoelectric focusing. The results of one of these experiments is shown in Fig. 7. After reincubation most of the enzyme was present in forms with isoelectric points

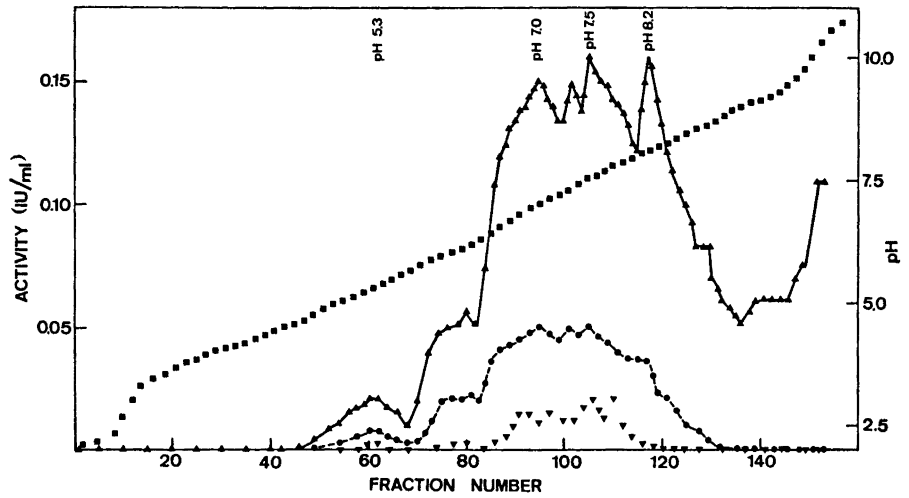

Fig. 7. Preparative isoelectric focusing pattern after reincubation of plaice cholinesterase with neuraminidase. Enzyme activities: ▲, acetylthiocholine; ●, butyrylthiocholine, and ▼, benzoylcholine. ■, pH.

at pH 7 or greater with a pronounced peak at pH 8.2. In fractions of high pH a new peak of enzyme activity was found that hydrolysed acetylthiocholine only and had no activity towards butyrylcholine and benzoylcholine. The activities of the peak fractions of this experiment together with the ratios between acetylthiocholine, butyrylthiocholine, and benzoylcholine are compared in Table 2.

Table 2. Comparison of activites of the different forms of plaice cholinesterase with acetylthiocholine, butyrylthiocholine, and benzoylcholine as substrates.

Enzyme form Isoelectric point	Activites			Ratio of activities		
	Acetyl-thio-choline	Butyryl-thio-choline	Benzoyl-choline	Acetyl-thio-choline	Butyryl-thio-choline	Benzoyl-choline
pH	IU/ml	IU/ml	IU/ml			
7.0	0.15	0.05	0.012	1.00	: 0.33	: 0.08
7.5	0.16	0.05	0.020	1.00	: 0.31	: 0.12
8.2	0.16	0.03	0.002	1.00	: 0.19	: 0.01
10.1	0.11	0.00	0.000	1.00	: 0.00	: 0.00

The peak fractions in Fig. 7 with isoelectric points at pH 7.0, 7.5, 8.2, and 10.1 were assayed spectrophotometrically for acetyl-, butyrylthiocholine, and benzoylcholine activities.

DISCUSSION

Plaice cholinesterase not only hydrolyzes acetylcholine but also choline esters of butyric, propionic, valeric, and benzoic acid. Thus this enzyme takes up a unique position between an acetylcholinesterase generally located in neuromuscular junctions and the soluble butyrylcholinesterases found in plasma and brain. Cholinesterases from a variety of sources exist as mixtures of multiple molecular forms. Differences in molecular and catalytic properties of butyrylcholinesterases were described on the basis of net charge, substrate specificity and sensitivity towards inhibition by fluorophosphate derivatives.[14,15] Multiple forms of human erythrocyte [16] and human brain acetylcholinesterase [17] have also been described. Acetylcholinesterase from the electric organ of the electric eel (*Electrophorus electricus*) exists in different oligomeric forms with apparently similar catalytic properties.[18]

The resolution of plaice cholinesterase into several enzymic species parallels the earlier observations made for acetyl- and butyrylcholinesterases. Contrary to the findings for the plasma enzyme,[14,15] no difference in catalytic properties were found for the various forms of plaice cholinesterase isolated by isoelectric focusing. Thus the unique ability of the plaice enzyme to hydrolyze acetylcholine and choline esters of aromatic and other aliphatic acids could not be explained on the basis of the presence of a mixture of kinetically distinguishable isoenzymes.

After prolonged standing an increase in the isoelectric point of plaice cholinesterase was observed which could be attributed to the spontaneous release of an acidic component from the enzyme. Treatment of this protein with neuraminidase yielded forms of the enzyme with isoelectric points up to pH 8.3 without any apparent change in catalytic reaction specificity. Similar observations were made by Svensmark and coworkers for human serum,[19,20] horse serum,[21] and human brain [10] butyrylcholinesterases. The results of Heilbronn [22] showed that there are 34 molecules of *N*-acetylneuraminic acid associated with each molecule of horse serum cholinesterase. Haupt *et al.*[23] reported figures for sialic acid content of human serum cholinesterase that would suggest that there are 80 molecules of this aminosugar in each molecule of the enzyme.

Besides cholinesterase a number of other enzymes are known to be glycoproteins in nature. In some cases it is possible to remove most of the carbohydrate from these glycoenzymes without impairment of enzymic function. Examples of this type of glycoprotein are thrombin,[24] glycoamylase,[25] and chloroperoxidase.[26] On the other hand there is a group of carbohydrate containing hormones of the anterior pituitary where even small changes in the carbohydrate moiety abolishes the specific interaction of the hormone with the target organ.[27] Similar observations were made with a number of blood group specific glycoproteins.[28]

According to a suggestion by Eylar [29] the attachement of sugars to a specific protein serves as the chemical passport for export from the synthesizing cell into the extracellular space. This hypothesis was rejected by Winterburn and Phelps [30] in favour of the idea that the attached carbohydrate determines the extracellular fate of a protein molecule in the sense that sugars serve as code

for the topographical location of the protein within an organism. Furthermore in some proteins the oligosaccharide structure attached may serve as signal for destruction of these proteins by catabolic enzymes. This effect is neutralized by the terminal sialic acid residues.[29]

Prolonged incubation of plaice cholinesterase with neuraminidase resulted in an enzyme preparation specific for acetylcholine only. Butyrylthiocholine and benzoylcholine were not hydrolyzed by this new form of the enzyme. The isoelectric point of the new species was at or around pH 10 and could not be determined accurately because the enzyme focused at the end of the ampholine gradient. In view of these results a new role for sialic acid residues might be described. In plaice cholinesterase, sialic acid residues could possibly be responsible for maintaining a specific ternary structure in this enzyme allowing the accomodation not only of acetylcholine but also of choline esters of aliphatic acids such as propionic, butyric, and valeric acid together with esters of aromatic carboxylic acids such as benzoylcholine. Upon removal of one or more sialic acid residues possibly near or at the active site the structure around the active site might become more closed up thus accomodating the smallest substrate molecule, acetylcholine, only. More detailed investigations on a pure enzyme preparation are, however, necessary to warrant this hypothesis.

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