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ISOLATION AND CHARACTERIZATION OF A BUTYRYLESTERASE FROM HUMAN ERYTHROCYTES

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Summary

Human erythrocytes contain a butyrylesterase which, judging from the ease with which it can be solubilized, is present in the cytoplasm of these cells. This enzyme has been isolated and a number of its properties characterized. The purified enzyme hydrolyzed butyryl esters with both a lower K_m and higher V than is seen with esters containing longer or shorter acyl groups. It has a molecular weight of 320 000 and an isoelectric point of 4.1. This low isoelectric point is apparently a result of the relatively high content of glutamic and aspartic acids. The stability of the isolated butyrylesterase has been examined under a number of different conditions. The enzyme is inhibited by low concentrations of Hg^{2+}, Cd^{2+}, Zn^{2+} and the organophosphorus compound Mipafox, but is insensitive to eserine. The properties of this butyrylesterase, including its ability to hydrolyze thiocholine esters at a relatively rapid rate (albeit with a high K_m), are a mixture of those expected for an arylesterase and a cholinesterase.

Introduction

The presence of various types of esterases in human erythrocytes has been described [1]. Preliminary experiments in our own laboratory [2] have also revealed that human erythrocytes contain an esterase activity which differs from the four main types of blood plasma esterase, i.e., arylesterases (EC 3.1.1.2), acetylesterases (EC 3.1.1.6), carboxylesterases (EC 3.1.1.1), and

† Deceased 27th October, 1978.
Abbreviation: ACD, acid citrate destrose.

cholinesterases (EC 3.1.1.8). In the present study we have isolated this enzyme and have investigated a number of its properties. On the basis of its substrate specificity, we have tentatively classified the enzyme as a butyrylesterase. During the course of our investigations, Højring and Svensmark [3] reported on an esterase from human brain which has properties similar to those of the enzyme described here.

Materials and Methods

Chemicals

Butyrylthiocholine, α-naphthylacetate, α-naphthylbutyrate, *p*-nitrophenyl esters, albumin, carbonic anhydrase, and neuraminidase (*Clostridium perfringens*, Type VI) were obtained from Sigma Chemical Company, St. Louis, MO. Phenylthioacetate and phenylthiobutyrate were purchased from Polysciences Inc., Warrington, PA. Phenylacetate came from Fluka AG and phenylbutyrate from the British Drug Houses Ltd., Poole (U.K.). Glycerokinase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, alkaline phosphatase, β-galactosidase, glutamate dehydrogenase, fumarase, and catalase were all obtained from Boehringer Mannheim GmbH, Mannheim (F.R.G.). Butyrylcholinesterase was purchased from Organon, Oss (The Netherlands). Mipafox (bis-monoisopropylamine fluorophosphate) was a gift from the Swedish National Defense Institute and bovine heart mitochondrial ATPase (F_1) was a kind gift from Prof. Lars Ernster. All other chemicals were purchased from common commercial sources and were of analytical purity.

Preparation of hemolysate

Outdated human ACD whole blood was obtained from a hospital blood bank. About 1 l blood was centrifuged at 1000 × *g* in a Beckman J-21B centrifuge for 25 min and the plasma and buffy coat were subsequently removed by suction and discarded. After being washed three times in cold isotonic NaCl solution, the erythrocytes were hemolysed by incubation for 30 min in 9 vols. of 10 mM Tris-HCl, pH 7.35. The hemolysate was then centrifuged at 30 000 × *g* for 25 min, the clear supernatant containing the butyrylesterase was carefully decanted, and the pellet containing the stroma was discarded.

Isolation of the butyrylesterase

Ion-exchange chromatography. About 2.5 l of the stroma-free hemolysate was applied to a DEAE-Sephadex A-25 (Pharmacia) column (4 × 16 cm) which had been previously equilibrated with 0.1 M Tris-HCl, pH 7.35. After washing with the same buffer, elution was carried out using a continuous gradient formed from 500 ml 0.1 M Tris-HCl, pH 7.35, and 500 ml of the same buffer containing 0.5 M NaCl. Fractions of about 20 ml were collected with a Fractomax FR5 drop counter fraction collector.

Gel filtration. Fractions in the region of 31—36 from the ion exchange column containing butyrylesterase activity were pooled and concentrated 5—10-fold with a 50 ml Amicon Diaflo cell model 52 using an XM-50 filter. 6 ml of this concentrate were applied to an Agarose A-0.5 m (Bio-Rad) column (2 × 83 cm) preequilibrated with 10 mM sodium phosphate, pH 6.8, containing

1 mM EDTA. Elution was performed with this same phosphate/EDTA buffer and 5—10 ml fractions were collected.

Hydroxyapatite chromatography. Fractions in the region of 52—58 from the Agarose column contained butyrylesterase activity and were pooled and put onto a Bio-Gel HTP column (2 × 6 cm) preequilibrated with 10 mM sodium phosphate, pH 6.8, containing 1 mM EDTA. A continuous gradient formed from 30 ml of the same phosphate/EDTA buffer and 30 ml 0.5 M potassium phosphate, pH 6.8, containing 1 mM EDTA was used for elution. The volume of the fractions collected was 1—3 ml.

Determination of molecular weight

Thin-layer gel filtration was carried out on a 0.6 mm layer of Sephadex G-200 Superfine in 0.1 M Tris (pH 7.35)/0.1 M NaCl. Protein was stained with bromthymol blue according to the Pharmacia instructions and esterase activity was detected using α-naphthylacetate and α-naphthylbutyrate as substrates, followed by an azo coupling technique using Echtblausalz B (Merck) in 10 mM Tris-HCl, pH 8.0 [4].

Gradient centrifugation was performed using a modification of the method described by Martin and Ames [5]. We used an isokinetic gradient instead of a linear one [6].

Gradient gel electrophoresis was performed with commercially obtained Pharmacia gradient gels PAA 4/30. Electrophoresis was carried out in 90 mM Tris/80 mM boric acid/2.5 mM Na_2EDTA, pH 8.35, at 14—15°C using 125 V for 15 h. Protein was stained with Coomassie Brilliant Blue and esterase activity detected by incubating the gel in 1 mM phenylthiobutyrate containing 1 mg/ml nitro blue tetrazolium.

SDS-gel electrophoresis. The samples contained 0.1% SDS, 10% sucrose, 10 mM Tris-HCl, pH 7.35, 40 mM dithiothreitol, and about 50 μg protein and were heated for 1 h at 50°C. SDS gel electrophoresis was performed by the procedure of Weber and Osborne [7], and protein and glycoprotein were stained according to Fairbanks [8].

Polyacrylamide gel electrophoresis. Electrophoresis was performed in 0.5 × 6 cm gel rods according to Maurer's system No. 6 [9] in a Pharmacia GE-4 apparatus. During the 17-h run the apparatus was cooled with tap water. Protein was stained with Coomassie Brilliant Blue and esterase with α-naphthylacetate and α-naphthylbutyrate as described for thin-layer gel filtration.

Isoelectric focusing. Plates for focusing were occasionally made from 5.5% acrylamide, 0.0165% bisacrylamide and 12% sucrose. The gel contained a total of 2% ampholine, including equal amounts of pH 4—6, pH 5—7 and pH 5—8. In most cases ampholine polyacrylamide gel plates pH 3.5—9.5, purchased from Pharmacia, Uppsala, Sweden, were used. 10 μl of the pooled dialyzed fractions from peak A (hydroxyapatite column) was applied to paper squares and laid on the plate. Focusing was performed at 50 mA and 200 V for 60 min with continuous cooling to 14°C using tap water. Protein was stained with Coomassie Brilliant Blue. Staining for esterase with α-naphthylacetate and α-naphthylbutyrate was performed as described for thin-layer gel filtration.

Amino acid composition. Protein was hydrolyzed for 24 h in 6 N HCl10% phenol at 120°C in vacuo. The amino acid composition of the isolated butyryl-

esterase was determined in a Stein-Moore amino acid analyzer at the Central Amino Acid Analysis Laboratory, Uppsala, Sweden.

Assay of esterase activity. When thiolesters were used as substrates, assay of esterase activity was based on a technique described by Ellman et al. [10] and recently applied to the quantitative assay of arylesterase activity [11]. When *p*-nitrophenyl esters were used as substrates, appearance of the *p*-nitrophenolate ion was followed spectrophotometrically at 400 nm. In some cases the assay was conducted using the Warburg procedure [12]. Unless otherwise stated, incubation was performed at 30°C (25°C for the Warburg procedure); 50 mM Tris-HCl, pH 8.0, was present; and the concentration of substrate was 1 mM.

When the inhibitory effects of metal ions were tested, special problems were encountered in the assay of esterase activity. In the first place, the solution of purified enzyme contained EDTA (see above) and had to be dialyzed before incubation with metal ions. In addition, heavy metal ions react with sulfhydryl groups, so that thiol esters could not be used as substrates in experiments involving such ions.

Treatment with neuraminidase. To approximately 0.2 mg purified enzyme in 0.4 ml 10 mM Tris-HCl, pH 7.3, were added 50 μg of lyophilized neuraminidase (*Clostridium perfringens*, Type VI, Sigma). After incubation for 3 h at room temperature samples were taken for isoelectric focusing.

Determination of protein. Protein concentration was determined either according to Lowry et al. [13] or by direct measurement of absorbance at 260 and 280 nm or at 215 and 225 nm.

Results

Purification

After hemolysis of erythrocytes at least three different soluble enzymes capable of hydrolyzing phenylthioacetate could be discerned by ion-exchange chromatography (Fig. 1). Little activity was found in the stroma. These enzymes are apparently localized in the cytoplasm. Groups II and III were

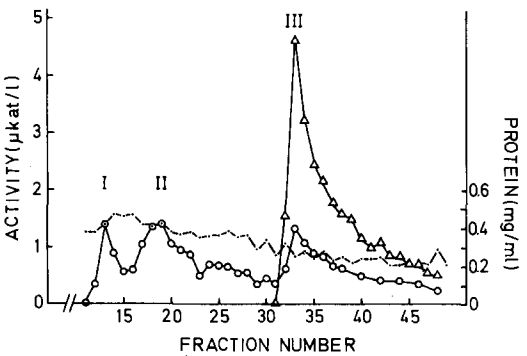

Fig. 1. Separation of esterase activities by DEAE-Sephadex ion exchange chromatography of the hemolysate from human erythrocytes. Enzyme activity was subsequently measured using phenylthiobutyrate (△———△) and phenylthioacetate (○———○) as substrates, both at a final concentration of 1 mM. The Roman numerals indicate three different peaks of esterase activity. The line · — · — · indicates the protein concentration in the fractions collected.

TABLE I

ACTIVITY OF THE GROUP II AND III ESTERASES TOWARDS DIFFERENT ESTERS

The most active fractions from groups II and III indicated in Fig. 1 were tested with four substrates using the Warburg procedure. Each substrate was used at a final concentration of 2 mM. Enzyme activities are expressed as μkat/l.

Group	Substrate			
	Phenylacetate	Phenylthioacetate	Phenylbutyrate	Phenylthiobutyrate
II	3.3	6.0	0.3	0
III	1.8	3.5	3.6	7.8

assayed using the Warburg procedure with phenylbutyrate, phenylacetate, phenylthiobutyrate, and phenylthioacetate as substrates. As can be seen in Table I, the enzymes in group II readily hydrolyze phenylacetate and phenylthioacetate, while phenylbutyrate and phenylthiobutyrate are poor substrates for these esterases. In contrast the enzymes in group III hydrolyze all four of the esters tested. In general thiolesters are more susceptible to hydrolysis by the easily-solubilized esterases of human erythrocytes than are their hydroxyl analogues.

The polyacrylamide gel electrophoretic patterns shown in Fig. 2 reveal a number of interesting characteristics of these three groups of esterases. In the first place α-naphthylacetate is substrate for all three groups, whereas α-naphthylbutyrate is hydrolyzed only by group III. In addition, even though each esterase fraction contained a number of different proteins, only a limited number of these proteins stained for esterase activity. Thus, only one esterase band is seen for group I and another esterase band, migrating more rapidly, is seen for group II. There is only one region of the electrophoretogram for group III which stains for esterase activity with both α-naphthylacetate and α-naphthylbutyrate as substrates.

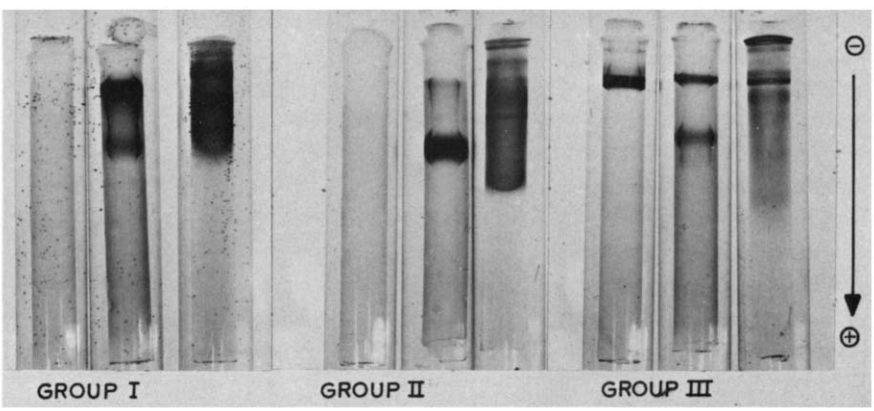

Fig. 2. Polyacrylamide gel electrophoresis of the groups of esterases obtained by DEAE-Sephadex ion exchange chromatography. The most active fractions from groups I, II, and III indicated in Fig. 1 were subjected to polyacrylamide electrophoresis. The gels on the left in each group were stained for esterase activity with α-naphthylbutyrate as substrate, those in the middle for esterase activity with α-naphthylacetate as substrate, and the gels on the right were stained with Coomassie Brilliant Blue.

TABLE II

PURIFICATION OF BUTYRYLESTERASE FROM THE ERYTHROCYTE HEMOLYSATE

Chromatographic step	Activity							
	Total activity * (μkat)	Total protein (mg)	Specific activity * (mkat/kg) (μkat/l)	With phenyl-thiobutyrate (μkat/l)	With phenyl-thioacetate (μkat/l)	Phenylthio-butyrate/ phenylthio-acetate ratio	Purifi-cation	Yield (%)
DEAE-Sephadex A-25	0.218	12.8	17.0	7.5	3.5	2.1	1	100
Agarose A-0.5	0.173	1.9	91.2	20.3	6.6	3.1	5.4	79
Bio-gel HTP, Peak A	0.132	0.78	169	19.8	6.5	3.0	9.9	60

* The substrate used in determining total and specific activity was phenylthiobutyrate. The group III fractions from DEAE-Sephadex ion-exchange chromatography (see Fig. 1) were assigned a purification factor of 1 and a yield of 100%. It would be misleading to use the original hemolysate as a starting point, since so much of the protein in this preparation is hemoglobin.

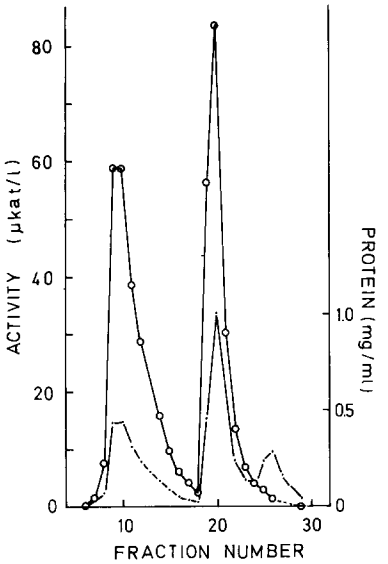

Fig. 3. Elution of esterase activity from the hydroxyapatite column. Enzyme activity (solid line) was measured using 1 mM phenylthiobutyrate as substrate. The broken line represents protein.

However, isoelectric focusing (not shown) revealed that each of these esterase fractions contained more than one protein with esterase activity. The esterases of group I focused in the region of pH 5.8, those of group II around pH 5.3, and those of group III near pH 4.

As can be seen from the purification scheme diagrammed in Table II, gel filtration and hydroxyapatite chromatography of the group III esterase obtained from ion-exchange chromatography resulted in a further 10-fold purification of the butyrylesterase. The maximum yield obtained was 60%. The gel filtration step increased the rate of phenylthiobutyrate hydrolyses relative to phenylthioacetate hydrolysis, but hydroxylapatite chromatography did not further affect this ratio. This finding would suggest that contaminating esterases were removed by gel filtration. However, two peaks of esterase activity were obtained from the hydroxyapatite column (Fig. 3).

Peak A, which was eluted first, had the higher specific activity and was used in subsequent experiments. The specific esterase activity of the different fractions of this peak was essentially constant. Both polyacrylamide gel electrophoresis and isoelectric focusing of this enzyme revealed only a single band staining for protein and esterase activity. In addition, gradient gel electrophoresis, which was used to determine the molecular weight of the esterase in peak A (see below), did not reveal any contaminating protein or esterase activity. All of these criteria speak for the homogeneity of the isolated enzyme.

Selected characteristics of the esterase in peak B were also examined.

Substrate specificity

The rate of hydrolysis of a series of different esters of *p*-nitrophenol by the purified enzyme was determined using a 1 mM substrate concentration. As can

TABLE III

ACTIVITY OF THE PURIFIED BUTYRYLESTERASE WITH DIFFERENT *p*-NITROPHENYL ESTERS

The activity of the purified butyrylesterase was measured with *p*-nitrophenyl esters in which the length of the acyl moiety varied. These esters were dissolved in acetone and 50 µl of the substrate solution added to a 3.1 ml assay volume buffered at pH 8.0 to give a final concentration of 1 mM. The appearance of *p*-nitrophenolate upon incubation at 30°C was followed spectrophotometrically at 400 nm.

Number of carbon atoms in the acyl chain	Activity (%)
2	12.2
3	58.5
4	100
5	42.7
6	25.6
8	2.2
10	0.7

be seen from Table III, the butyryl ester is hydrolyzed most rapidly; the hexyl ester is hydrolyzed at a rate which is about twice that observed with the acetyl ester; and esters with eight or more carbon atoms in the alkyl chain no longer serve as effective substrates for the enzyme.

Table IV further illustrates that of the esters tested butyryl esters appear to be the best substrates for our enzyme. In the case of aromatic esters the butyryl esters have K_m values which are about 20 times lower and V values which are 3—5 times higher than those obtained with the acetyl and propionyl esters.

Furthermore, our purified enzyme can hydrolyze butyrylthiocholine relatively rapidly, albeit with a very high K_m.

These findings on substrate specificity have led us to designate the enzyme as a butyrylesterase.

Molecular weight

A number of different methods were employed to estimate the molecular weight of the isolated butyrylesterase. Gel filtration on a column indicated a

TABLE IV

KINETIC PARAMETERS OF THE PURIFIED BUTYRYLESTERASE WITH DIFFERENT SUBSTRATES

The activity with each substrate was determined in 50 mM Tris-HCl, pH 8.0, at 30°C using five different substrate concentrations and in duplicate. The same amount of enzyme was used in all measurements. The apparent kinetic constants were subsequently obtained using a plot of S/v vs. S.

Substrate	K_m (mM)	V (mkat/kg)
Phenylthioacetate	0.165	28.2
Phenylthioburyrate	0.009	87.6
p-Nitrophenylacetate	0.175	13.4
p-Nitrophenylpropionate	0.131	13.4
p-Nitrophenylbutyrate	0.007	63.1
Butyrylthiocholine	4.9	72.8

molecular weight of about 360 000. Upon sucrose gradient centrifugation the purified butyrylesterase behaved in a manner identical to that of horse plasma butyrylcholinesterase, which was used as a reference and which has a reported molecular weight of 320 000. Finally, gradient gel electrophoresis gave a result which agrees with that obtained by gradient centrifugation. In addition, thin-layer gel filtration revealed that one enzyme from group I and another from group II (Fig. 1), which both stain for esterase activity with α-naphthylacetate as substrate, both have a molecular weight of 60 000.

The quarternary structure of the butyrylesterase was investigated using SDS gel electrophoresis. Several weak protein bands were visible (see Discussion) and one thick band migrated at a position corresponding to a molecular weight of 78 000. All of the peptides could be stained with the Schiff reagent. These findings together suggest that the butyrylesterase is a glycoprotein containing four subunits.

Isoelectric point

The butyrylesterase was shown by isoelectric focusing to have no net charge at a pH of 4.1. The isoelectric point is not changed by more than 0.1 pH upon treating the enzyme with neuraminidase, which indicates that bound sialic acid residues are not influencing this property of the protein. The relatively low isoelectric point causes the enzyme to be strongly bound to a DEAE ion-exchange column at pH 7.35; while its relatively high molecular weight results in its early elution from an agarose A-0.5m column. These two somewhat extreme properties of the butyrylesterase make it easy to purify.

Amino acid composition

The amino acid composition of the purified butyrylesterase is illustrated in Table V, column A. As might be expected from the enzymes low isoelectric point, the combined content of the acidic amino acids (and their amides) is considerably greater than the combined content of the basic amino acids. Glutamic acid (plus glutamine) is the single amino acid present in the largest amount. The protein also contains relatively much serine and much of the hydrophobic amino acids leucine, valine, glycine, proline, and alanine; while it contains only small amounts of the aromatic amino acids (tryptophan was not determined). It can be calculated that 50.3% of the total amino acid residues of the butyrylesterase are non-polar. A minimal molecular weight of 81 000 could be determined, a finding in good agreement with the results from SDS gel electrophoresis (see above).

It is interesting to note that the second peak of esterase activity obtained from the hydroxyapatite column (Fig. 3, peak B) has an amino acid composition which is very similar to that of the butyrylesterase of primary interest (Table V, column B).

Stability

The stability of the purified enzyme under different conditions and the effects of various substances on this stability were examined. When frozen at −86°C and then immediately thawed, 77% of the enzyme activity is lost (Table VI). In the presence of Ca^{2+} this denaturation is even greater, while EDTA has a

TABLE V

AMINO ACID COMPOSITION OF THE PURIFIED BUTYRYLESTERASE

All values are given in mol%. Column A gives the amino acid composition of the first peak (peak A), while column B documents the amino acid composition of the second peak (peak B) obtained by hydroxyapatite chromatography (Fig. 3). Tryptophan was not determined.

Substance	A	B
Aspartic acid	7.62	8.17
Threonine	4.04	4.28
Serine	8.97	8.95
Glutamic acid	12.11	12.45
Proline	7.17	6.61
Glycine	8.52	8.17
Alanine	6.28	6.61
Half-cystine	2.69	2.72
Valine	8.97	8.56
Methionine	2.24	1.95
Isoleucine	3.14	3.50
Leucine	10.31	10.12
Tyrosine	2.69	2.33
Phenylalanine	3.59	3.50
Histidine	2.69	2.72
Lysine	4.04	4.28
Arginine	4.93	5.06

slight protective effect. When 50% glycerol (v/v) is included in the medium, freeze-thawing with or without Ca^{2+} or EDTA does not affect the activity of the enzyme.

When the enzyme is left standing at room temperature or at 4°C in buffer, essentially all of the activity is lost within 26 days. Neither Ca^{2+} nor EDTA has any effect on this loss. In 50% glycerol with or without Ca^{2+} or EDTA at 0°C the enzyme maintains full activity for at least 3 months. At room temperature

TABLE VI

THE EFFECT OF FREEZE-THAWING IN THE PRESENCE OR ABSENCE OF GLYCEROL, Ca^{2+}, AND/OR EDTA ON ENZYME ACTIVITY

The samples containing the butyrylesterase in 0.1 M Tris-HCl, pH 7.35, were frozen at −86°C (acetone-solid CO_2). They were then immediately thawed and examined for esterase activity towards 1 mM phenylthiobutyrate. All values shown are the average of duplicates.

Sample containing			Remaining activity (%)
50% glycerol	1 mM Ca^{2+}	1 mM EDTA	
—	—	—	23
+	—	—	94
—	+	—	6
—	—	+	35
+	+	—	101
+	—	+	105

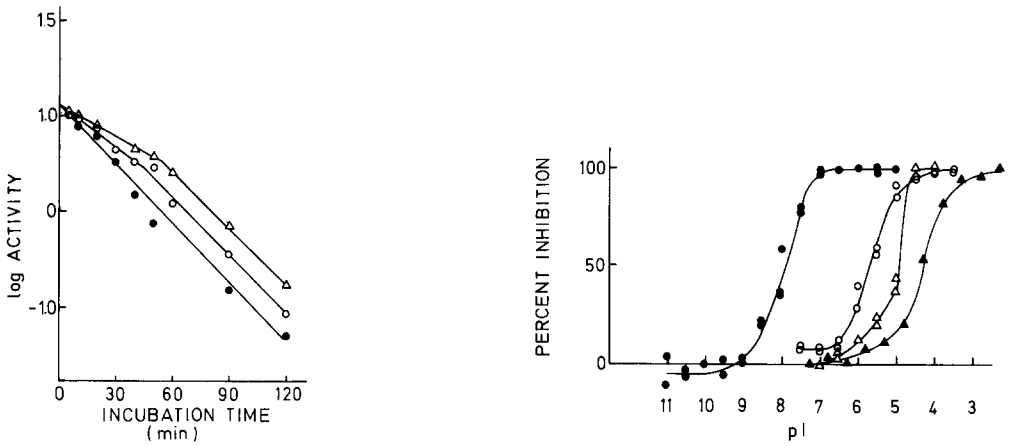

Fig. 4. Heat inactivation of the purified butyrylesterase. The butyrylesterase was incubated at 45°C in 50 mM Tris, pH 8.0, in the absence (△———△) and presence of 1 mM $MgCl_2$ (○———○) or $CaCl_2$ (●———●). Activity was measured with 1 mM phenylthiobutyrate and expressed in μkat.

Fig. 5. Inhibition of the purified butyrylesterase by Mipafox, Hg^{2+}, Cd^{2+}, and Zn^{2+}. The enzyme was incubated in 50 mM Tris, pH 8.0, for 20 min at 0°C with different concentrations of Mipafox (▲———▲) or for 60 min at 0°C with different concentrations of $HgCl_2$ (●———●), $CdCl_2$ (○———○), or $ZnCl_2$ (△———△). The reaction was started by addition of 50 μl substrate (63 mM phenylthiobutyrate in the case of Mipafox and 63 mM *p*-nitrophenylbutyrate in the case of the heavy metals) and 3 ml Tris-HCl buffer to the 0.1 ml incubation medium. p*I* is the negative $\log_{10}$ of the inhibitor concentration.

denaturation still occurs and EDTA and especially Ca^{2+} protect the enzyme against inactivation under these conditions.

Finally, investigation of the heat inactivation of human erythrocyte butyrylesterase at 45°C revealed that in contrast to the findings with human serum arylesterase, Ca^{2+} does not protect the butyrylesterase (Fig. 4). Nor does Mg^{2+} afford any protection. On the contrary, both of these divalent cations promote inactivation, Ca^{2+} to a higher degree than Mg^{2+}. Heat inactivation proceeds in two phases; the rate of the first phase is affected by the presence of these cations while the second is not. The presence of 50% glycerol protects the enzyme partially at 45°C but Ca^{2+} and Mg^{2+} still promote the inactivation.

Inhibitors

Mipafox was found to inhibit the enzyme to 50% at a concentration of 50 μM and totally at a concentration of about 0.5 mM (Fig. 5). Approximately 70% of the inhibition obtained with 0.32 mM Mipafox occurs more rapidly at 0°C than an assay can be performed; while the remaining 30% of the activity is abolished during the 10 min of incubation.

The purified enzyme is not sensitive to eserine in concentrations as high as 1 mM.

Of the metal ions tested, only Zn^{2+}, Cd^{2+}, and Hg^{2+} inhibit the butyrylesterase. Al^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Sr^{2+}, Cu^{2+}, and La^{3+} are all without effect at a 1 mM concentration, as are Na^+ and K^+. The enzyme is sensitive to Hg^{2+} at concentrations as low as 1 nM (Fig. 5) and at 10 nM the inhibition is 50%. As also shown in Fig. 5, Cd^{2+} and Zn^{2+} are also effective inhibitors, although less effective than Hg^{2+}. Although the shapes of the p*I*-curves are the

same for all three of these divalent cations, 3.16 μM Cd^{2+} and 10 μM Zn^{2+} are required to achieve 50% inhibition. The inhibition by Hg^{2+} appears to proceed in two phases, an initial phase during which 70% of the activity is lost rapidly and a second phase during which the remaining activity is abolished more slowly. Inhibition by Cd^{2+} occurs more slowly.

Since Hg^{2+} is such an extremely effective inhibitor of the enzyme, the nature of this inhibition was examined more closely. As expected, this inhibition is non-competitive. In addition, the substrates phenylbutyrate and, to a lesser degree, phenylacetate protect the enzyme to a certain extent from inactivation by Hg^{2+}.

The esterase in peak B obtained from the hydroxyapatite column (see Fig. 1) is inhibited by Hg^{2+} and Cd^{2+} in a manner similar to that seen with the purified butyrylesterase (peak A).

Discussion

Human erythrocytes have long been known to contain at least six different types of enzymes capable of hydrolyzing carboxyl ester linkages, i.e., cholinesterase, carbonic anhydrase, three non-specific carboxylesterases [1], and at least one D-esterase [14]. The carboxylesterases have been classified according to their substrate specificities and sensitivities to various effectors. According to this classification the butyrylesterase studied here is a B-type esterase.

Even though several electrophoretic studies have been performed (e.g. Refs. 1 and 14), this is the first time that a B-type soluble esterase has been isolated from human erythrocytes and the molecular properties of the purified enzyme determined. The purified enzyme hydrolyzes butyryl esters with both a lower K_m and higher V than is seen with esters containing longer or shorter acyl chains. It has a molecular weight of about 320 000 and SDS gel electrophoresis suggests that the enzyme consists of four subunits. In addition, since all of the protein bands seen on SDS gel electrophoresis stain with the Schiff reagent, this butyrylesterase may be a glycoprotein. The enzymes isoelectric point of 4.1 is not due to the presence of terminal sialic acid residues, but is apparently the result of its relatively high content of glutamic and aspartic acids. Finally, the enzyme is inhibited by very low concentrations of Hg^{2+} and somewhat higher concentrations of Cd^{2+}, Zn^{2+} and Mipafox; but it is not sensitive to eserine in concentrations as high as 1 mM.

We originally chose to isolate this butyrylesterase because preliminary experiments indicated that is has characteristics which are a mixture of those expected of an arylesterase and of a cholinesterase. Arylesterases are characteristically very sensitive to heavy metal ions such as Hg^{2+}; are insensitive to eserine and to organophosphorus compounds such as Mipafox (on the contrary, such compounds may serve as substrates for arylesterases); and do not hydrolyze choline esters (for reviews, see Refs. 15 and 16). On the other hand, most cholinesterases are relatively insensitive to Hg^{2+} but are inhibited by low concentrations of eserine and organophosphorus compounds such as Mipafox (for review, see Ref. 15). A comparison of these properties to those of our enzyme, together with the observation that the purified butyrylesterase can hydrozyle butyryl thiocholine at a relatively rapid rate (albeit with a high K_m),

suggest that the distinction between arylesterases and cholinesterases as two different groups of enzymes may not be as clear as previously stated [12,16].

B-Esterases from a number of different tissues have been studied electrophoretically (e.g., Refs. 1, 14), but the only other tissue from which such an enzyme has been isolated is the human brain [3]. It is of interest to compare the butyrylesterases from human erythrocytes and brain tissue. The brain enzyme has a molecular weight of 340 000, very similar to that of the erythrocyte protein. Isoelectric focusing of the brain enzyme reveals several protein bands in the pH region of 4.0—4.7 and treatment with neuraminidase does not affect this relatively low isoelectric point. In addition to the fact that the erythrocyte enzyme is similarly acidic, it is of interest to note that both enzymes seem to be somewhat unstable. The erythrocyte protein appears homogenous upon polyacrylamide gel electrophoresis, isoelectric focusing, and gradient gel electrophoresis; while the brain enzyme gives a single band upon electrophoresis. However, the erythrocyte enzyme gives several weak and one strong band when subjected to SDS gel electrophoresis; and the brain butyrylesterase gives a number of bans on isoelectric focusing. We have observed the same behavior with commercially obtained (Organon, Oss, Holland) purified butyrylcholinesterase from horse plasma as with the erythrocyte butyrylesterase. This phenomenon has yet to be explained for butyrylesterase, but appears to result from the oxidation and reduction of sulfhydryl groups in the case of albumin [17]. The brain enzyme is, like the red cell esterase, insensitive to eserine; but unlike the red cell enzyme, it is also insensitive to heavy metal ions such as Zn^{2+}. Finally, the brain enzyme has been reported to have very little activity with thiocholine esters as substrates. However, this was tested at a relatively low substrate concentration (0.5 mM), so it may be a question of high K_m rather than low activity.

In conclusion, the butyrylesterase investigated here has a number of interesting properties and we are presently characterizing it further. In addition we plan to study the other esterases solubilized from human erythrocytes by hemolysis. The physiological function of these erythrocyte esterases remain a mystery. The report by Sidhu and Blair [18] that human liver aldehyde dehydrogenase can hydrolyze *p*-nitrophenyl esters suggests that the converse may also be true, ie., that enzymes detected by their esterase activity may actually be dehydrogenases. In contrast to the findings reported for aldehyde dehydrogenase we have so far been unable to observe any effect of $NADP^+$ or NADPH on the esterase activity of the erythrocyte butyrylesterase. However, we continue to examine the possibility that erythrocyte esterases may function as dehydrogenases in vivo.

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