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A sialic acid-specific *O*-acetylesterase in human erythrocytes: Possible identity with esterase D, the genetic marker of retinoblastomas and Wilson disease

(*O*-acetyl esters/chromosome 13)

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ABSTRACT **The "nonspecific" esterases are a family of enzymes that were originally identified because of their reaction with synthetic *O*-acetyl ester substrates. While the electrophoretic polymorphisms of these enzymes have been extremely useful for genetic studies, their biological functions have remained completely unknown. Esterase D is characterized by its reactivity with 4-methylumbelliferyl acetate. This enzyme has recently been of particular interest because of its tight linkage to the putative recessive gene causing retinoblastomas, and to the recessive gene causing Wilson disease. We describe here the partial purification of a human erythrocyte esterase that appears to be highly specific for *O*-acetylated sialic acids. We next present evidence that suggests that esterase D is identical to this sialic acid-specific *O*-acetylesterase. First, both activities copurify from human erythrocyte lysates through several different purification steps, each of which use different principles of separation. Second, both activities show a remarkably similar profile of inhibition with a variety of different agents. Third, they both show a nearly identical heat-inactivation profile. This cytosolic sialic acid-specific *O*-acetylesterase appears to be involved in the "recycling" of *O*-acetylated sialic acid molecules. Thus, esterase D may be the first nonspecific esterase for which a specific biological role can be predicted.**

The sialic acids are a family of *N*- and *O*-substituted derivatives of *N*-acetylneuraminic acid (1, 2).† While a great deal has been written about the sialic acids and their probable biological functions, many studies fail to take into account the extent of diversity in these molecules. One common type of substitution responsible for such diversity is that of an *O*-acetyl ester on the exocyclic (7/8/9) side chain of the molecule. These substitutions could affect the conformation of the parent molecule and clearly have significant effects on enzyme function (3–6), complement activation (7) and virus binding (8), and on the antigenicity of gangliosides (9–11) and bacterial polysaccharides (12). There is also evidence for developmental regulation of these substitutions in tissues as diverse as embryonic brain (11) and neonatal colonic mucosa (13). However, the exact biological roles of these substitutions are currently unknown.

We have been studying the biosynthesis and reutilization of such *O*-acetylated sialic acids in various systems (13–15). Several proteins appear to be involved in these processes, including one or more *O*-acetyltransferases (1), a Golgi acetylcoenzyme A transporter (14), and one or more *O*-acetylesterases (16). A sialic *O*-acetylesterase was first described by Shukla and Schauer in equine liver (16). In the course of studying a similar activity in human erythrocytes, we have discovered that it may be identical to the "nonspecific" esterase D. This enzyme is already known to be localized to human chromosome 13 (band 13q14.11), in very tight linkage to a putative recessive "retinoblastoma gene" (17–23), and to the recessive gene causing Wilson disease (24).

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MATERIALS AND METHODS

Materials. Most of the chemical and biological reagents used here were obtained from Sigma. The following materials were obtained from the sources indicated: Dowex 50 AG (1 × 2) (H^+ form) and Dowex 3 × 4A (100–200 mesh, chloride form) (Bio-Rad); chemically synthesized *N*-acetylneuraminic acid (Neu5Ac) (>99% purity) (Kantoishi Pharmaceutical, Tokyo, Japan); and *N*-[4-^{14}C]acetylneuraminic acid (56.8 mCi/mmol; 1 Ci = 37 GBq), New England Nuclear. The Dowex 3 × 4A resin was converted to the formate form, as recommended by the manufacturer. All other chemicals were of reagent grade, and were purchased from commercial sources. 9-*O*-[*acetyl*-^{3}H]acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$) was released and purified from [6-^{3}H]glucosamine-labeled Friend erythroleukemia cells as described (15). Authentic Neu5,9Ac$_2$ was kindly provided by Roland Schauer (Kiel, F.R.G.). A mixture of several *O*-[*acetyl*-H]-acetylsialic acids were released and purified from rat liver Golgi vesicles incubated with [^{3}H]acetyl CoA, exactly as described in ref. 14. The major peak in this mixture is *O*-[*acetyl*-^{3}H]Neu5,9Ac$_2$.

Preparation of β-[^{14}C]methylglycoside of 9-*O*-Acetyl-*N*-acetylneuraminic Acid. The β-methylglycoside of [4-^{14}C]Neu5,9Ac$_2$ ([4-^{14}C]Neu5,9Ac$_2$,2OMe) was prepared in a manner similar to that described by Haverkamp *et al.* for the nonradioactive compound (25). [4-^{14}C]Neu5Ac (0.45 Ci/mmol) was converted to its methyl ester β-methylglycoside by the method of Karkas and Chargaff (26). The Dowex-50 beads were removed by passing over a glass wool filter and the methanolic acid was removed by evaporation. The residue was fractionated by preparative paper chromatography on Whatman 3MM paper in *n*-butanol/acetic acid/water (4:1:5, upper phase). Strips (1 cm) were cut, soaked in 50% methanol, and aliquots were counted. The major peak with an *Rf* of 0.58 was pooled, dried, saponified with 3 ml of 2 M NH_4OH for 2 hr at 100°C, dried again, and subjected to paper chromatography in the same system. The major peak was again pooled (β-methylglycoside) and the sialic acid content was determined by the 2-thiobarbituric

Abbreviations: Neu, neuraminic acid; Ac, acetyl; Gc, glycolyl; OMe, *O*-methyl.

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†The various sialic acids are designed by combinations of Neu (neuraminic acid), Ac (acetyl), Gc (glycolyl), and OMe (*O*-methyl). The amino group at the 5 position is always substituted with an Ac or a Gc group. Other substitution positions are indicated by numerals. For example, *N*-acetyl-9-mono-*O*-acetylneuraminic acid is written as Neu5,9Ac$_2$ (after R. Schauer and others; ref. 2).

acid method after acid hydrolysis. The pooled material was clarified by passage through a 0.22-μm filter in 10 mM formic acid, taken to dryness in a glass reactivial, and dissolved in 25 μl of dry pyridine by heating at 37°C for 24 hr. A 1.2-fold molar excess of *N*-acetylimidazole in dry pyridine was added, and the mixture was incubated at 65°C for 2 hr. The reaction mixture was taken to dryness in a rotary evaporator, brought up in 300 μl of 50% methanol, and passed over 1 ml of Dowex-50 (H^+ form) in 50% methanol. The column was washed with 4 ml of 50% methanol, and the pooled washings were taken to dryness. Final preparative purification of the product was achieved by HPLC, using the system described below. Fig. 1 shows a comparison of the structure of this compound with that of the native compound Neu5,9Ac_2. The identity of the compound was confirmed by its elution position on HPLC before and after de-*O*-acetylation (see below).

High-Pressure Liquid Chromatography. HPLC was used to separate the various types of sialic acids. A Varian Micropak AX-5 column eluted in the isocratic mode with acetonitrile/water/0.5 M NaH_2PO_4 (64:26:10) (see refs. 14 and 15 for other details). Neu5Ac and its β-methylglycoside eluted very close together but could be differentiated if they were labeled with ^3H and ^{14}C, respectively (Fig. 2). The chemically synthesized [^{14}C]Neu5,9Ac_2,2OMe eluted very close to [^3H]Neu5,9Ac_2. Upon de-*O*-acetylation, both compounds shifted to the elution position of the parent compound (not shown).

Assay of Sialic Acid *O*-Acetylesterase. Three different types of assays have been developed.

Assay 1. [^3H]Neu5,9Ac_2 (3000–5000 cpm) was incubated with the enzyme in 50 μl of 50 mM Tris·HCl, pH 7.5/1 mM EDTA. At the end of 30–60 min, the reaction mixture was chilled, transferred into a chilled prewashed Amicon Centrifree micropartition unit, and spun at 3000 × *g* for 30 min. The ultrafiltrate was injected onto a 50-μl loop and was studied by HPLC as described above. Fractions (0.6 ml) were collected, and the radioactivity was monitored. Appearance of the product ([^3H]Neu5Ac) is expressed as a percentage of the total radioactivity. One unit represents conversion of 1% per hr.

Assay 2. Unlabeled Neu5,9Ac_2 (10 nmol; final concentration, 0.2 mM) was incubated with enzyme in 50 μl of 50 mM potassium phosphate buffer (pH 7.5) at 37°C for 30 min. The reaction was quenched by chilling and then immediately injected directly onto the HPLC column. In this case, the substrate and product were monitored by UV detection at 200 nm. This assay could only be used for more-purified preparations of the esterase because of the presence of interfering substances in cruder samples. In this case, 1 milliunit represented conversion of 1 nmol/min.

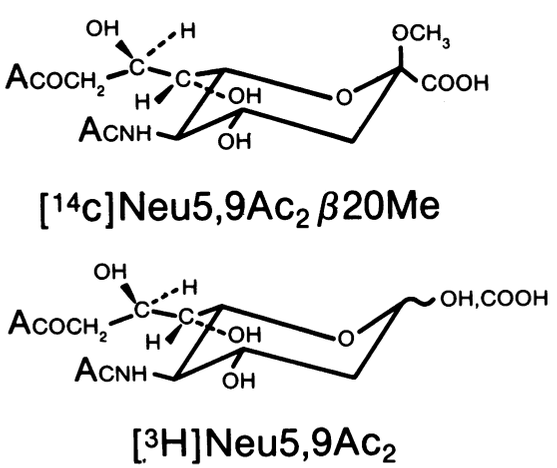

FIG. 1. Comparison of the structures of 9-*O*-acetyl-*N*-acetylneuraminic acid and its β-methylglycoside. The two labeled compounds were prepared as described. The structures are shown in chair conformation.

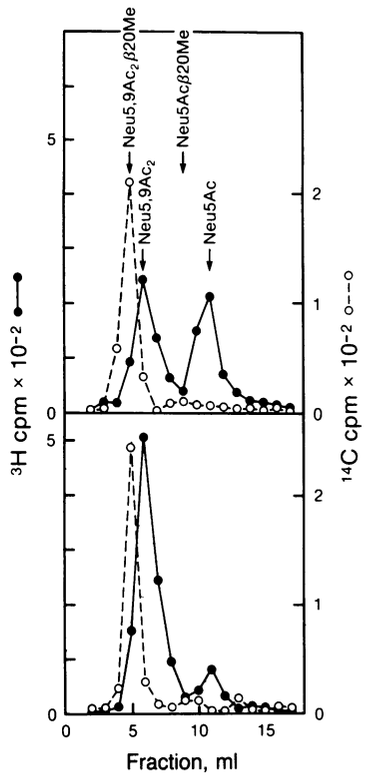

FIG. 2. Demonstration of the substrate specificity of erythrocyte sialic acid *O*-acetylesterase. [^3H]Neu5,9Ac_2 (from metabolically labeled erythroleukemia cells, contains 10% [^3H]Neu5Ac) and [^{14}C]Neu5,9Ac_2,2OMe (chemically synthesized) were incubated in 50 mM Tris·HCl (pH 7.4) for 30 min at 37°C, in the presence (*Upper*) or absence (*Lower*) of the partially purified erythrocyte enzyme. The reaction mixtures (50 μl) were filtered through Amicon Centrifree micropartition cartridges and examined by HPLC using the system described in ref. 5. Fractions (0.6 ml) were collected and the radioactivity was determined. The elution positions of the de-*O*-acetylated products were confirmed in a separate run after complete base hydrolysis (not shown).

Assay 3. The incubation conditions were identical to those in assay 1, except that 5000 cpm of a mixture of *O*-[*acetyl*-^3H]acetylsialic acids prepared from [^3H]acetyl CoA-labeled rat liver Golgi (see above) was used as substrate. The reactions were quenched with 10 μl of 10 M acetic acid, taken to dryness on a rotary evaporator and the residual radioactivity was determined. Since hydrolysis of the *O*-[^3H]acetyl groups converted them into volatile [^3H]acetic acid, the loss of radioactivity could be used to measure the enzyme activity. As for assay 1, 1 unit was taken as a conversion of 1% per hr. Because of the ease of performance, this assay was used to monitor the purification described below. However, at each step, the more specific and accurate assay 1 was used to define the -fold purification and yield.

Purification of Human Erythrocyte Sialic Acid *O*-Acetylesterase. *Lysis.* Human erythrocytes were collected from fresh defibrinated blood, washed three times in phosphate-buffered saline, and stored frozen at −20°C until used. The frozen pellets were lysed into 2 vol of 5 mM potassium phosphate (pH 7.0) with 0.1% 2-mercaptoethanol and 1 mM EDTA, spun at 100,000 × *g* for 30 min, and the supernatant fluid (lysate) was used for further purification.

Ion-exchange chromatography. The lysate (6.5 liters) was applied to a 1.7-liter DE-52 (DEAE Cellulose) column equilibrated in 10 mM Tris·HCl/1 mM EDTA, pH 7.5, washed

with 2 column vol of the same buffer, and then eluted with a linear gradient from 0 to 200 mM NaCl. Fractions (25 ml) were collected and 15-μl aliquots were monitored for activity by assay 3.

Ammonium sulfate precipitation. The peak fractions from the DE-52 step were pooled, gradually adjusted to 40% ammonium sulfate, and the precipitate was removed by centrifugation. The supernate was then adjusted to 60% saturation, and the resulting precipitate was collected.

Gel filtration chromatography. A Sephacryl S-200 column (2.5 × 110 cm) was equilibrated in 50 mM Tris·HCl/1 mM EDTA/100 mM NaCl, pH 7.5. The ammonium sulfate precipitate was dissolved in 10 ml of this buffer, applied to this column, and eluted at 1 ml/min. Fractions (8.5 ml) were collected and 10-μl aliquots were assayed for activity.

Amicon dye-matrex agarose chromatography. The peak fractions from the previous step were pooled and passed over a 50-ml column of Amicon Dye-Matrex red agarose in the same buffer. The activity ran through the column. Similarly, the activity ran through a 10-ml column of blue agarose and a 5-ml column of green agarose. With each of these steps, other proteins were removed from the preparation.

Assay of esterase D. By using 4-methylumbelliferyl acetate as a substrate, esterase D was assayed exactly as described by Sparkes *et al.* (27). Activity against other synthetic substrates was measured under similar conditions and the products were monitored by spectrophotometry or fluorescence as appropriate.

RESULTS AND DISCUSSION

Sialic Acid *O*-Acetylesterase Activity Is Present in Many Tissues. Radioactive 9-*O*-acetyl-*N*-acetylneuraminic acid was obtained from metabolically labeled mouse erythroleukemia cells (15). An *O*-acetylesterase activity against this substrate was demonstrated by HPLC separation of the substrate from the product *N*-[^{3}H]acetylneuraminic acid (Neu5Ac) (see Fig. 2). Such an activity was found in the high-speed supernate of homogenates from several tissues such as rat liver, human placenta, and human erythrocytes. As shown below, the possible identity of this enzyme with esterase D would predict that it is present in all tissues except plasma.

Partial Purification of the Enzyme from Human Erythrocytes. Assays 1 and 3 were used to monitor the purification of the enzyme from human erythrocytes. A partial purification of 891-fold was achieved by using a combination of several different steps, including ion-exchange and gel-filtration chromatography (see Table 1, column 1).

The Enzyme Is Specific for Unbound Native Sialic Acids. It was possible that the enzyme that we had partially purified was simply a true nonspecific esterase that happened to work on *O*-acetylated sialic acids. We reasoned that if this enzyme were indeed specific for native sialic acid molecules, its activity might be affected by some slight modifications of the native substrate. To address this question we chemically synthesized the β-methylglycoside of [^{14}C]Neu5,9Ac$_2$ by using modifications of previously established methods. The structure of the native *O*-acetylated molecule and the methylglycoside are presented in Fig. 1. As shown in Fig. 2, the partially purified enzyme had no activity against the closely related nonbiological analogue under conditions where extensive hydrolysis of the biological substrate occurred. Thus, a minor structural modification of the substrate in a region distant from the *O*-acetylester itself completely abolished the activity, suggesting that the enzyme recognizes the entire sialic acid molecule. Our preliminary studies also suggest a lack of activity against glycosidically bound *O*-acetylated sialic acids in biological substrates. *O*-acetylated sialic acids bound to both submaxillary mucins and to gangliosides were resistant to the partially purified enzyme (E.M., A.V., and D. Cheresh, unpublished observations). Furthermore, recent evidence from other workers suggests that a very similar enzyme from equine liver (16) will not hydrolyze 7-*O*-acetyl groups from Neu5,7AC$_2$. Taken together, all of these findings suggest that this enzyme has a high degree of specificity for free 9-*O*-acetylated sialic acids.

Activity Against Synthetic Substrates: Copurification with 4-Methylumbelliferyl Acetate Esterase. There are previously known examples of highly specific enzymes that are nevertheless active against certain synthetic substrates. For example, the highly specific Golgi α-mannosidase II involved in the processing of *N*-linked oligosaccharides will hydrolyze *p*-nitrophenol α-D-mannoside (28, 29). We therefore asked if any of the commercially available synthetic substrates containing *O*-acetyl ester groups would be hydrolyzed by the sialic acid-specific enzyme. As expected, the crude erythrocyte lysate contained many "nonspecific esterase" activities directed against a number of substrates. However, as shown in Table 1, the activity against 4-methylumbelliferyl acetate copurified through several different steps with the sialic acid-specific esterase. The yield and -fold purification of the two activities were remarkably similar through several different steps that used different principles of separation. Previous studies have shown that the human esterases A and C do not have significant activity against 4-methylumbelliferyl acetate (30). Although human esterase B has some limited activity against 4-methylumbelliferyl acetate, it is primarily a butyryl esterase (31). Furthermore, as shown in Table 1, the esterase B activity against 4-methylumbelliferyl butyrate did not copurify with the sialic acid-specific enzyme. In contrast to esterases A, B, and C, esterase D is characterized primarily by its specificity for 4-methylumbelliferyl

Table 1. Stepwise purification of sialic acid *O*-acetylesterase: Comparison with activity against synthetic substrates

	[^{3}H]Neu5,9Ac$_2$		4-Methylumbelliferyl acetate		4-Methylumbelliferyl butyrate	
	Purification, -fold	Yield, %	Purification -fold	Yield, %	Purification, -fold	Yield, %
1. Lysate	1	100	1	100	1	100
2. DEAE-cellulose	59	49	99	82	22	18
3. Ammonium sulfate/ Sephacryl S-200	372	27	319	23	67	5
4. Amicon Dye-Matrex agarose	861	23	891	23	34	1

Washed human erythroyctes were lysed in hypotonic buffer and the 100,000 × *g* supernate (called lysate) was used for the purification of the sialic acid-specific *O*-acetylesterase. The purification and recovery of this enzyme were monitored through several purification steps by assay 3 and was confirmed by assay 1 at each step. The data presented are those from assay 1. Aliquots at each step were also assayed with the synthetic substrates as indicated.

acetate, and indeed was originally identified with this substrate (32, 33). Furthermore, we found that plasma, the only biological material known to be devoid of esterase D, was also deficient in the sialic acid-specific *O*-acetylesterase (not shown). These facts suggested that the sialic acid-specific *O*-acetylesterase and esterase D might be identical.

Both Enzymatic Activities Have Similar Inhibition Profiles. To further explore this possibility, we compared the effects of several chemicals and inhibitors on the partially purified enzyme activities. As shown in Table 2, the effects of a wide variety of tested compounds on both the sialic acid-specific esterase and the esterase D activities were nearly identical. Of particular note was the marked inhibition of both activities by diisopropylfluorophosphate, *p*-chloromercuribenzoate, and mercuric chloride, and the lack of major effect of iodoacetamide. Previous studies have shown that human esterase B is activated by *p*-chloromercuribenzoate, esterase C is unaffected by diisopropylfluorophosphate and that the A esterases are completely inhibited by iodoacetamide (17). Thus, the behavior of the sialic acid-specific esterase was again remarkably similar to that of esterase D, but quite unlike that of the esterases A, B, and C.

Electrophoretic Studies of the Enzymes. Starch gel electrophoresis of the initial lysate and the partially purified enzyme was carried out with the kind help of E. Beutler and Carol West (Scripps Clinic and Research Foundation). The esterase D bands were visualized as described (31). Unfortunately, the thickness of the gel made it impossible to elute sufficient enzyme activity to assay for the sialic acid *O*-acetylesterase. Since overloading of the gels caused a loss of resolution, we were unable to compare the electrophoretic mobilities of the enzymes.

Both Enzymes Show Very Similar Heat-Inactivation Kinetics. To obtain alternative evidence for the identity of the two enzymes, we therefore studied their heat-inactivation profiles. Aliquots of the partially purified preparation were heated at different temperatures for 15 min, chilled on ice, and the residual activity of esterase D and the sialic acid esterase (assay 2) was determined. As shown in Fig. 3, the extent of inactivation of the two enzymatic activities very closely paralleled each other over a wide temperature range. Taken together, all of these results suggest that the two activities under study represent a single enzyme.

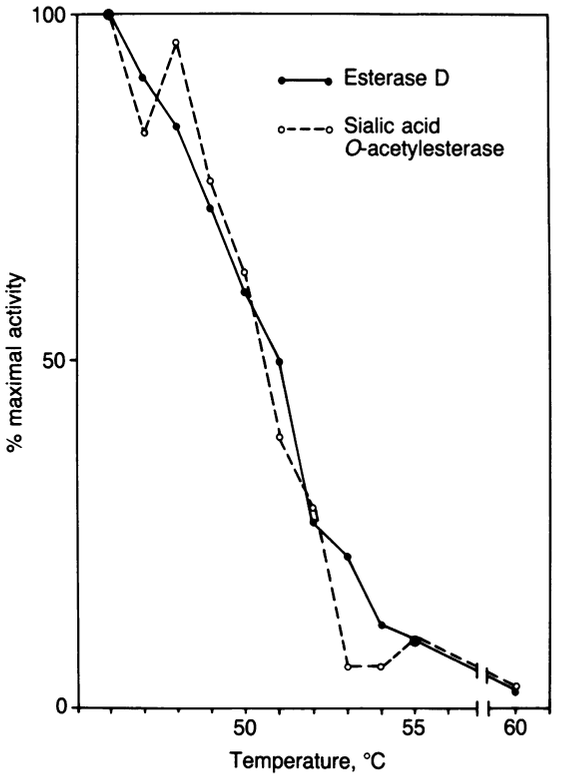

FIG. 3. Heat inactivation profile of enzymes. Separate aliquots of the partially purified erythrocyte enzyme were heated for 15 min at the various temperatures indicated in a Haake D1 controlled temperature circulating water bath. At the end of each heating period, the sample was placed on ice and immediately assayed for esterase D or for the sialic acid-specific esterase (assay 2).

CONCLUSIONS

A substantial body of literature now suggests that the homozygous deletion or inactivation of an unknown gene on chromosome 13 (band 13q14.11) may be the cause of both hereditary and sporadic human retinoblastomas (17–23). Although the nature of the gene is unknown, its chromosomal localization was possible only because of a very tight linkage to esterase D. We have presented evidence here that this enzyme may be highly specific sialic acid *O*-acetylesterase. However, since not all retinoblastomas are completely deficient in esterase D, the relevance of this finding to the biology of this tumor is uncertain. Very recently, the esterase D gene has also been found to be linked to the unknown recessive gene causing retinoblastomas in humans (24).

Table 2. Effect of various compounds on sialic acid *O*-acetylesterase and 4-methylumbelliferyl acetate esterase (esterase D) activity

Compound	Concentration	% of control activity against	
		[^{3}H]Neu5,9Ac$_2$	4-Methylumbelliferyl acetate
1. None	—	100	100
2. EDTA	1 mM	118	94
3. 2-Mercaptoethanol	0.1%	113	110
4. Iodoacetamide	1 mM	67	85
5. *p*-Chloromercuribenzoate	1 mM	10	10
6. Diisopropylfluoro phosphate	1 mM	11	13
7. Phenylmethylsulfonyl fluoride	1 mM	37	52
8. Dansylglutamylglycyl-arginyl-chloromethyl ketone	14 μM	88	85
9. Fluoride	10 mM	43	43
10. Mercuric chloride	0.1 mM	10	12

The partially purified preparation of the sialic acid *O*-acetylesterase was studied for its activity against [^{3}H]Neu5,9Ac$_2$ (assay 1) or against 4-methylumbelliferyl acetate for esterase D (29). The various agents were either preincubated with the enzyme (nos. 2–8, 15 min at 37°C) or were present in the incubation mixture (nos. 9 and 10).

To our knowledge, this is the first demonstration of a specific biological substrate for a nonspecific esterase. Since others have already shown that esterase D is widely distributed in all tissues examined (32), it appears that this sialic acid *O*-acetylesterase may be expressed in many cell types. The exact role of this *O*-acetylesterase in the metabolism of *O*-acetylated sialic acids is currently under active investigation. Our pulse–chase studies of sialic acid *O*-acetylation in Friend erythroleukemia cells suggest that *O*-acetylated sialic acids may be released intact from glycoconjugates and "recycled" into the cytosol (A.V. and S.D., unpublished observations). These *O*-acetylated molecules appear to be poor substrates for the CMP sialic acid synthetase (8), for at least some sialyltransferases (8), and also for degradation by acylneuraminate pyruvate lyase (1, 2, 6). Thus, in order for these recycled molecules to be efficiently reutilized in the cytosol, they would need to be de-*O*-acetylated back to the parent compound, *N*-acetylneuraminic acid. If this is indeed the case, the cytosolic location of this specific *O*-acetylesterase would make it important in the reutilization of sialic acids.

Our data suggest that this enzyme may be identical to esterase D. If so, the identification of a simple synthetic substrate assay for this enzyme, and the availability of both human retinoblastoma and fibroblast cell lines with genetically determined variations in this activity (17–23, 34, 35) should greatly aid in studying this enzyme. Furthermore, the existence of a closely homologous enzyme (esterase 10) in the mouse (36) and the known existence of a null allele for esterase D in the human population (27, 37) implies that the search for a homozygous deficiency state in intact organisms might be rewarding. Ultimately such studies could lead to a better understanding of the precise biological functions of this enzyme and of *O*-acetylation of sialic acids in general.

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