THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 264, No. 32, Issue of November 15, pp. 19435-19442, 1989
Printed in U.S.A.

O-Acetylation and De-O-acetylation of Sialic Acids

PURIFICATION, CHARACTERIZATION, AND PROPERTIES OF A GLYCOSYLATED RAT LIVER ESTERASE SPECIFIC FOR 9-O-ACETYLATED SIALIC ACIDS*

(Received for publication, February 22, 1989)

Herman H. Higa‡, Adriana Manzi§, and Ajit Varki¶

From the Division of Hematology/Oncology, Department of Medicine, San Diego Veterans Medical Center and Cancer Biology Program, University of California at San Diego, La Jolla, California 92093

We have previously described the preparation and use of 9-O-[*acetyl*-^{3}H]acetyl-*N*-acetylneuraminic acid to identify sialic acid *O*-acetylesterases in tissues and cells (Higa, H. H., Diaz, S., and Varki, A. (1987) *Biochem. Biophys. Res. Commun.* 144, 1099–1108). All tissues of the adult rat showed these activities, with the exception of plasma. Rat liver contained two major sialic acid esterases: a cytosolic nonglycosylated enzyme and a membrane-associated glycosylated enzyme. The two enzymes were found in similar proportions and specific activities in a buffer extract of rat liver acetone powder. By using the latter as a source, the two enzymes were separated, and the glycosylated enzyme was purified to apparent homogeneity by multiple steps, including ConA-Sepharose affinity chromatography and Procion Red-agarose chromatography (yield, 13%; fold purification, ~3000). The homogeneous enzyme is a 61.5-kDa disulfide-linked heterodimeric protein, whose serine active site can be labeled with [^{3}H]diisopropyl fluorophosphate. Upon reduction, two subunits of 36 kDa and 30 kDa are generated, and the 30-kDa subunit carries the [^{3}H]diisopropyl fluorophosphate label. The protein has *N*-linked oligosaccharides that are cleaved by Peptide *N*-glycosidase F. These chains are cleaved to a much lesser extent by endo-β-*N*-acetylglycosaminidase H, indicating that they are mainly complex-type glycans.

The enzyme activity has a broad pH optimum range between 6 and 7.5, has no divalent cation requirements, is unaffected by reduction, and is inhibited by the serine active site inhibitors, diisopropyl fluorophosphate (DFP) and diethyl-*p*-nitrophenyl phosphate (Paraoxon). Kinetic studies with various substrates show that the enzyme is specific for sialic acids and selectively cleaves acetyl groups in the 9-position. It shows little activity against a variety of other natural compounds bearing *O*-acetyl esters. It appears to deacetylate di-*O*-acetyl- and tri-*O*-acetyl-*N*-acetylneuraminic acids by first cleaving the *O*-acetyl ester at the 9-position. The 7- and 8-*O*-acetyl esters then undergo spontaneous migration to the 9-position, where they can be cleaved, resulting in the production of *N*-acetylneuraminic acid. In view of its interesting substrate specificity, complex *N*-linked glycan structure, and neutral pH optimum, it is suggested that this enzyme is involved in the regulation of *O*-acetylation in membrane-bound sialic acids.

Recently, several esterases have been discovered that appear to be specific for removal of *O*-acetyl esters from the 9-position of naturally occurring sialic acids (1–4). A sialic acid esterase from equine liver described by Schauer (1) was found in the cytosolic fraction and cleaved acetyl groups from the 9- and 4-positions of naturally occurring sialic acids. We reported a human red cell cytosolic enzyme with properties very similar to "nonspecific" esterase D. This enzyme also cleaved 9-*O*-acetyl esters from sialic acids (3). A similar activity has been detected in the influenza C surface glycoprotein (2, 5) and recently in coronaviruses (6, 7). We have also detected such enzyme activities in several tissue culture cell lines, using as a substrate biosynthetically prepared 9-*O*-[*acetyl*-^{3}H]acetyl-*N*-acetylneuraminic acid ([^{3}H]Neu5,9Ac$_2$)[1] (4). Some of these esterases have been shown to be serine active site enzymes susceptible to inactivation with diisopropyl fluorophosphate (DFP)[2] and can be labeled with [^{3}H]DFP (1, 7, 8).

In the rat liver, the two major esterases detected with this substrate at neutral pH are a cytosolic nonglycosylated enzyme and a membrane-associated glycosylated enzyme, which are present in approximately equal amounts. A portion of the membrane-associated esterase activity was found within isolated rat liver Golgi vesicles, in the same location where the *O*-acetylation of sialic acids is known to occur (4, 9). This suggested that the membrane-associated esterase might be involved in cellular regulation of membrane sialic acid *O*-acetylation, in contrast to the cytosolic esterase, which might act as a scavenger of recycled free *O*-acetylsialic acids. We have now found that both of these enzyme activities are present in a buffer extract of commercially available rat liver acetone powder (at a specific activity similar to that found in fresh rat liver extract). In this report, we describe the purifi-

* This research was supported in part by United States Public Health Service Grant GM32373 and a Merit review grant from the Veterans Administration Research Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Postdoctoral trainee supported by Grant T-32CA09290.

§ Supported by a fellowship from the Consejo Nacional de Investigaciones Cientificas y Techicas, Argentina.

¶ Recipient of Senior Faculty Research Award FRA-295 from the American Cancer Society. To whom correspondence should be addressed: Dept. of Medicine, Q-063, University of California at San Diego, La Jolla, CA 92093.

[1] The various sialic acids are designated according to Schauer (42) using combinations of Neu (neuraminic), Ac (acetyl), and Gc (glycolyl), *e.g.* Neu5,7Ac$_2$ is 7-*O*-acetyl-*N*-acetylneuraminic acid.

[2] The abbreviations used are: DFP, diisopropyl fluorophosphate; Endo H; endo-β-*N*-acetylglucosaminidase H; PAF, platelet-activating factor; 4-MU, 4-methylumbelliferone; ConA, concanavalin A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

cation to apparent homogeneity, the characterization, and the properties of the glycosylated enzyme from this source.

EXPERIMENTAL PROCEDURES[3]

RESULTS

Distribution of Sialic Acid O-Acetylesterase in Rat Liver Tissues—Extracts from several tissues of adult male Sprague-Dawley rats were assayed for sialic acid 9-*O*-acetylesterase activity. In each case, the extracts were also fractionated into glycosylated and nonglycosylated forms using ConA-Sepharose. As shown in Fig. 1, enzyme activity could be detected in all tissues examined with the exception of plasma and showed a wide variability in specific activity. Notably, the testis, kidney, and colonic mucosa showed very high levels of activity of both forms, the brain and the heart contained almost exclusively the nonglycosylated form, and only the glycosylated form was found in skeletal muscle. Liver extracts contained nearly equal amounts of both enzymes. Both of these activities were also found in buffer extracts of commercially available rat liver acetone powder in similar proportions and specific activity to those found in the fresh tissue (data not shown). In view of this, rat liver acetone powder was chosen as a source for purification and further study of the enzymes.

Purification of a Glycosylated Sialate O-Acetylesterase from Rat Liver—The schematic diagram of the overall purification procedure is shown in Fig. 2 (see Miniprint section). The glycosylated enzyme does not bind to DEAE-cellulose but subsequently binds to ConA-Sepharose. On the other hand, the cytosolic (nonglycosylated) enzyme binds to the DEAE-column, but runs through the ConA-Sepharose column. Thus, these two steps ensure complete separation of the two activities. Further purification of the glycosylated enzyme to homogeneity involves gel filtration on Sephacryl S-200 followed by Procion Red-agarose affinity chromatography. The last step separates the glycosylated sialate 9-*O*-acetylesterase from another [^{3}H]DFP-binding esterase of apparent M_r = 64,800. As shown in Fig. 3 (see Miniprint section), the sialate 9-*O*-acetylesterase was eluted from Procion Red-agarose with buffer containing 370 mM NaCl, while the 64.8-kDa esterase was eluted with 900 mM NaCl. The latter esterase hydrolyzes 4-MU acetate, but does not hydrolyze Neu5,9Ac$_2$.

The glycosylated sialate 9-*O*-acetylesterase was purified 3068-fold to apparent homogeneity as described in the Miniprint section and summarized in Table I. Since the two sialate *O*-acetylesterases were separated at the DEAE-step, the fold purification and the overall yield from the crude homogenate do not accurately reflect that of the glycosylated enzyme alone. Assuming that all of the glycosylated enzyme is recovered in the DEAE run-through, the final preparation shows approximately a 6000-fold purification and a 40% recovery. Since the specific activity in the buffer extract of the acetone powder is similar to that found in fresh rat liver extracts, it is inferred that this enzyme is relatively abundant in this tissue.

The Homogeneous Glycosylated Rat Liver Sialic Acid O-Acetylesterase Is Composed of Two Disulfide-linked Heterodimeric Subunits—The final preparation showed a single band on an SDS-PAGE gel. The purity of the enzyme was confirmed by gel filtration on a Bio-Gel P-100 column (a matrix *not* used in the purification protocol) and by SDS-PAGE. As shown in Fig. 4 (see Miniprint section), a single symmetrical elution peak of the enzyme from the P-100 column (determined by protein absorbance at 220 nm) coincided almost exactly with activity determined with both [^{3}H]Neu5,9Ac$_2$ and 4-MU acetate assays. The enzyme activities and protein also corresponded to the intensity of the labeled bands obtained in a nonreducing 10% SDS-PAGE fluorogram of [^{3}H]DFP-labeled proteins from each fraction. The purified enzyme was also analyzed by nonreducing 10% SDS-polyacrylamide gel electrophoresis and stained with silver nitrate. As shown in Fig. 5 (see Miniprint section), the enzyme corresponds to the major band at 61.5 kDa with a slight residual contami-

[3] Portions of this paper (including "Experimental Procedures" and Figs. 2–9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

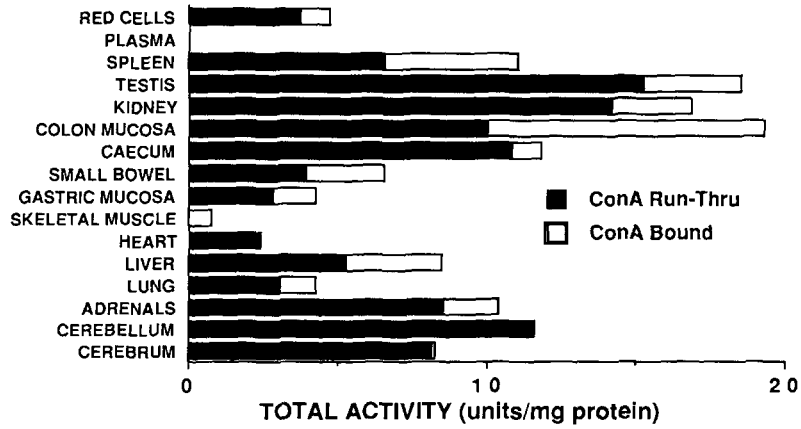

FIG. 1. **Distribution of sialic acid *O*-acetylesterase activity in rat tissues.** Adult male Sprague-Dawley rats were killed by anesthesia and decapitation and permitted to exsanguinate into 1/10 volume of 3.8% citrate. Red cells (washed free of the buffy coat) and plasma were obtained as previously described (3). Organs and tissues were removed immediately, chilled, and dissected free of connective tissue. Cells and tissues were washed in ice-cold phosphate-buffered saline, pH 7.5, and homogenized in phosphate-buffered saline (0.25 g/ml) containing 0.5% Triton X-100. The detergent extracts were centrifuged at 100,000 × *g* for 30 min at 4 °C, and aliquots (250 μl) of supernatants were applied to 0.5-ml ConA-Sepharose columns equilibrated in phosphate-buffered saline, pH 7.5. The samples were chased into the columns with 150 μl of buffer, the flow was stopped for 15 min, and the elution continued with 4 × 200 μl of buffer. The starting extracts and the ConA run-through fractions were assayed with the [*acetyl*-^{3}H]Neu5,9Ac$_2$ esterase assay as described under "Experimental Procedures." *Open bars*, ConA run-through activity; *closed bars*, ConA-bound activity. Protein was determined as described (43).

TABLE I

Purification of rat liver glycoprotein sialate 9-O-acetylesterase

The sialate 9-*O*-acetylesterase activity was determined by the radiometric assay described under "Experimental Procedures." Protein was determined by the Lowry method with bovine serum albumin as a standard.

Step	Volume	Protein	Total protein	Specific activity	Total activity	Purification	Yield
	ml	*mg/ml*	*mg*	*units*		*-fold*	*%*
Total homogenate	700	99	69,272	6.6	458,500	1	100
Supernatant	780	17.4	13,541	26.8	362,895	4.0	79.2
DEAE-cellulose[a]	904	5.2	4,710	30.9	145,544	4.7	31.7
ConA-Sepharose	338	.3	102	973	99,597	147	21.7
Ammonium sulfate/Sephacryl S-200	21	2.8	59	1,638	97,020	248	21.2
Procion Red-agarose and concentration	0.33	9.0	3	20,311	60,706	3,068[a]	13.2[a]

[a] At the DEAE-step, the glycoprotein enzyme is separated from the cytosolic enzyme, which represents about 60% of the total activity in the supernatant. Thus, the actual fold purification and yield of the glycoprotein enzyme are a little more than twice those listed.

nation (<5%) of the 64.8-kDa esterase.

Upon reduction of the enzyme, two subunits with molecular weights of 36,400 and 30,300, respectively, are generated (see *right panel* of Fig. 5 and *lower panel* of Fig. 6). To determine if this heterodimer represents the native form of the enzyme, the elution profile of activity and protein were monitored on a Sephacryl S-200 column, calibrated with appropriate molecular weight standards. As shown in Fig. 6 (see Miniprint section), the calculated apparent molecular weight of the native enzyme by gel filtration was 62,800, corresponding well to that determined by SDS-PAGE. Of the two subunits, the smaller one contains the serine active site which can be inactivated and labeled with [^{3}H]DFP (Fig. 7) (see Miniprint section).

The Protein Contains Complex-type N-Linked Oligosaccharides—The diffuse nature of the band on SDS-polyacrylamide gel electrophoresis and the ability of the enzyme to bind to ConA-Sepharose suggested the presence of *N*-linked oligosaccharides. The nature of the glycans was analyzed by treatments with Peptide *N*-glycosidase F, endoglycosidase H, and *Arthrobacter ureafaciens* neuraminidase (Fig. 7) (see Miniprint section). Peptide *N*-glycosidase F, which cleaves all known types of *N*-linked oligosaccharides (10), caused a significant shift of both the native enzyme and the M_r 30,300 subunit to lower molecular weights (*lanes 1, 2* and *6, 7*). The Peptide *N*-glycosidase F caused a decrease of ~10,000 in the apparent molecular weight of the unreduced protein. Endo H, which cleaves high mannose-type and certain hybrid-type *N*-linked chains (23), caused a much smaller molecular weight shift of the enzyme (*lanes 3, 4* and *8, 9*). Neuraminidase treatment caused no noticeable shift in migration (*lanes 3, 5* and *8, 10*). However, direct analysis of the protein by a picomole scale adaptation of the thiobarbituric acid assay (21) showed the presence of sialic acid in the protein (1–2 mol/mol of protein). Thus, the enzyme contains complex-type *N*-linked oligosaccharides which probably carry sialic acids.

Properties of the Purified Esterase—The enzymatic activity had no divalent cation requirements and was unaffected by EDTA. It was irreversibly inactivated with DFP and diethyl-*p*-nitrophenyl phosphate (Paraoxon), indicating a trypsin-like serine active site mechanism. However, unlike other serine esterases, this enzyme was also irreversibly inhibited by the arginine-modifying reagents phenylglyoxal and butanedione (see Ref. 24 for details). Fluoride, bis-*p*-nitrophenyl phosphate, Hg^{2+} ions, and *p*-chloromercuribenzoate had little effect on the activity. The nonhydrolyzable analogue of Neu5,9Ac$_2$, 9-acetamido-Neu5Ac, also had no effect at concentrations up to 5 mM. The pH activity profile of the enzyme is shown in Fig. 8 (see Miniprint section). It has a broad neutral pH optimum range with the highest apparent activity at pH 7. Detergents do not activate the enzyme. At 0.5–2.0% concentration, Triton X-100, Triton CF-54, and saponin have no significant effect, while CHAPS lowered the activity by 40%. Deoxycholate resulted in significant loss of activity (by 75–80% at 2% concentration).

The activity of the concentrated purified enzyme is stable at 4 °C for at least 1 month; at 37 °C, 40% activity is lost in 20 h. It is stable for prolonged periods (>6 months) in the frozen state. A single cycle of freeze-thaw results in little loss of activity. However, repeated freeze-thaw results in gradual loss of activity. Lyophilization and reconstitution results in loss of 30% of activity. The activity also becomes unstable upon excessive dilution (>100-fold).

The Enzyme Has Strict Substrate Specificity for O-Acetylsialic Acids and a Broad Specificity for Small Synthetic Esters—The specificity of the esterase was determined with various natural and synthetic substrates. The enzyme had little or no activity against a variety of natural acetylated compounds other than sialic acids (see Table II). However, it showed significant activity with small synthetic acetyl ester

TABLE II

Comparison of activity of purified rat liver glycoprotein sialate 9-O-acetylesterase against various substrates

Details regarding all assays are described under "Experimental Procedures."

Substrate	% activity[a] compared against:		
	Neu5,9Ac$_2$	[^{3}H]Neu5,9Ac$_2$	4-MU-OAc[b]
4-MU-butyrate			<5
p-Nitrophenyl acetate			72
α-Naphthyl acetate			65
[^{3}H]Neu5,9Ac$_2$-α-linked to *N*-linked oligosaccharides		46	
[^{3}H]Neu2en5,9Ac$_2$		46	
O-[^{3}H]Acetyl colominic acid		<5	
[^{3}H]Acetylcholine		<5	
[^{3}H]Acetyl-PAF		<5	
[4-^{14}C]Neu5,9Ac$_2$β2Me		<5	
Neu2-*O*-benzyl,5,9Ac$_2$	26		
Triacetin	26		
Neu2-*O*-benzyl,4,9Ac$_2$	8		
3′-*O*-Acetylthymidine	8		
O-Acetylserine	<5		

[a] Activity against Neu5,9Ac$_2$ is nonsaturating at concentrations greater than 10 mM (see text for discussion). The individual substrates were therefore compared against the indicated primary substrate for the percent hydrolysis of acetate/min/mg of protein.

[b] The enzyme has activity against 4-MU-OAc. However, this substrate is unstable at the pH (8.0) used for all the other substrates and therefore cannot be compared directly with them. For this reason, the three synthetic esters were compared at pH 5.5.

substrates, such as *p*-nitrophenyl acetate, α-naphthyl acetate, and 4-MU acetate. Hydrolysis of 4-MU butyl esters was present at a much lower level, but too low for accurate quantitation. Hydrolysis of *O*-acetyl esters from triacetin (tri-*O*-acetyl glycerol) occurred at a significant rate. This is of interest because of its similarity to the glycerol-like side chains of the sialic acids.

Free Neu5,9Ac_2 is the best substrate. However, the hydrolysis rate with this compound failed to reach saturation at 1 mM concentration. Further study of the saturation kinetics showed an apparent K_m value of 8.8 mM for Neu5,9Ac_2, with a V_{max} of 48 nmol/min/mg of protein. When compared with the free sugar, Neu5,9Ac_2 α-glycosidically linked to *N*-linked oligosaccharides was hydrolyzed at about one-half the rate (Table II). On the other hand, labeled *O*-acetyl groups on polysialic acid (colominic acid) were not removed. This implies that the sialic acid must be in a terminal α-glycosidic linkage or in a free state to be a substrate for the enzyme. When both 4-^{14}C-labeled Neu5,9Ac_2β20Me and *O*-[*acetyl*-^{3}H] Neu5,9Ac_2 were present in the same reaction with the enzyme, HPLC analysis showed that no significant hydrolysis of the ^{14}C-labeled compound occurred under conditions where the ^{3}H-compound was almost completely cleaved (data not shown). Thus, addition of a β-linked methyl group at the 2-position of Neu5,9Ac_2 also abolished activity. On the other hand, the α-benzyl derivative of Neu5,9Ac_2 was hydrolyzed at a significant rate, as was Neu2en5,9Ac_2 (Table II). These data indicate that the enzyme recognizes certain features of the sialic acid molecule other than the ester group itself including the anomeric carbon and the glycerol side chain. In the following paper (24), we show that the enzyme also may recognize the negatively charged carboxyl group of the substrate. Taken together, the data indicate that sialic acids may be the natural substrate for this enzyme.

Enzymatic Activity on O-Acetyl Esters from Mono-, Di-, and Tri-O-acetylsialic Acids Shows Specific Hydrolysis of 9-O-Acetyl Groups—To determine the positional specificity of the enzyme for *O*-acetyl groups on the 7-, 8-, and 9-exocyclic hydroxyl group of sialic acid, hydrolysis of mono-*O*-acetyl, di-*O*-acetyl, and tri-*O*-acetylsialic acids were examined by HPLC analysis (Figs. 9 and 10). The position of the acetyl group had a significant effect upon the activity. As shown in Fig. 9 (see Miniprint section), when a mixture of Neu5,7Ac_2 and Neu5,9Ac_2 were exposed to the enzyme in the same reaction, little hydrolysis of the 7-*O*-acetyl ester was seen, even when the 9-*O*-acetyl groups were completely cleaved. Acetyl esters at the 4-position were hydrolyzed at a very slow rate (Table II). Thus, among the mono-*O*-acetylsialic acids, the 9-*O*-acetyl isomer is selectively cleaved by the enzyme.

Di-*O*-acetylsialic acids with ester groups located at the 7- and 8- or the 7- and 9- positions were studied next. Neu5,7,8Ac_3 and Neu5,7,9Ac_3 are normally present in a 1:1 equilibrium mixture (25). Esterase treatment of this mixture yielded approximately equal amounts of Neu5Ac and Neu5,7Ac_2 after short incubations (Fig. 10). After 20 h, a small drop in the level of Neu5,7Ac_2 and a proportional increase in Neu5Ac was seen. Since Neu5,7Ac_2 is a poor substrate for this enzyme (Fig. 9), this slight drop in the level of Neu5,7Ac_2 after 20 h of incubation is likely due to the slow rate of spontaneous migration of the acetyl group under these conditions (pH 7.2) from the 7- to the 9-position of sialic acid. The resulting Neu5,9Ac_2 would then be rapidly cleaved by the enzyme. The results obtained with tri-*O*-acetylated sialic acids were very similar, with Neu5Ac and Neu5,7Ac_2 being the initial products of short incubations (data not shown). As summarized in Fig. 11, the esterase activity on di- and tri-*O*-acetylsialic acids thus occurs by first rapidly cleaving 9-*O*-acetyl groups. Any 8-*O*-acetyl esters that are adjacent to free 9-hydroxyl groups would now undergo very rapid migration to the 9-position (25), where they can be cleaved. On the other hand, acetyl esters at the 7-position would undergo slow migration (at neutral pH) to the 9-position and then be cleaved by the enzyme. Thus, given sufficient time, the enzyme can ultimately remove all *O*-acetyl esters on the glycerol side chain in this sequential manner (see Fig. 11 for a summary).

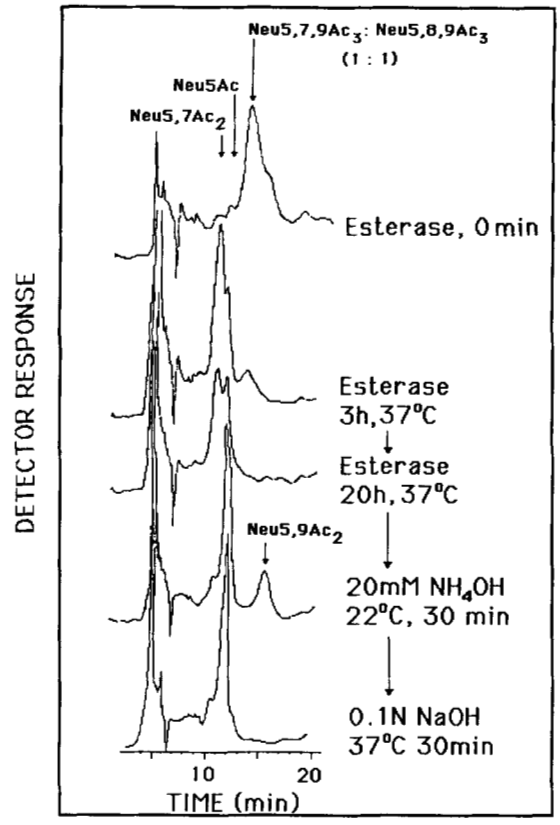

FIG. 10. **HPLC analysis of products of esterase activity on di-Q-acetylated Neu5Ac.** Unlabeled di-*O*-acetylated *N*-acetylneuraminic acids (an equilibrium mixture of the 7,9- and 8,9-di-*O*-acetylated molecules (25) were treated with 43 units of the purified esterase, and the products were examined by HPLC in System A at various times. The positions of elution of the standards are shown. To confirm the identity of some of the intermediate products (particularly Neu5,7Ac_2), a portion of the reaction mixture after 20 h was subjected to induced migration of *O*-acetyl groups from the 7- to 9-position, and then to de-*O*-acetylation by NaOH (see "Experimental Procedures" for details). The relevant areas of the HPLC profiles are superimposed for comparison.

Other Purified O-Acetylesterases from Rat Liver Do Not Cleave 9-O-Acetylsialic Acids—Two other purified esterases from rat liver were obtained for comparison. Although they have somewhat similar molecular weights, neither Egasyn (26, 27) nor a previously described 60-kDa glycoprotein esterase from rabbit liver (14) showed any detectable activity with the 9-*O*-acetylated sialic acid substrate (less than 5% of the activity of the esterase purified in this study; data not shown).

DISCUSSION

In this study, we have described the purification and characterization of an esterase from rat liver that can cleave 9-*O*-acetyl esters from mono-, di-, and tri-*O*-acetylated sialic acids. In contrast to the previously described cytosolic 9-*O*-acetylesterase(s) (1, 3), this enzyme is a glycoprotein with complex-type *N*-linked oligosaccharides. In addition, we have recently

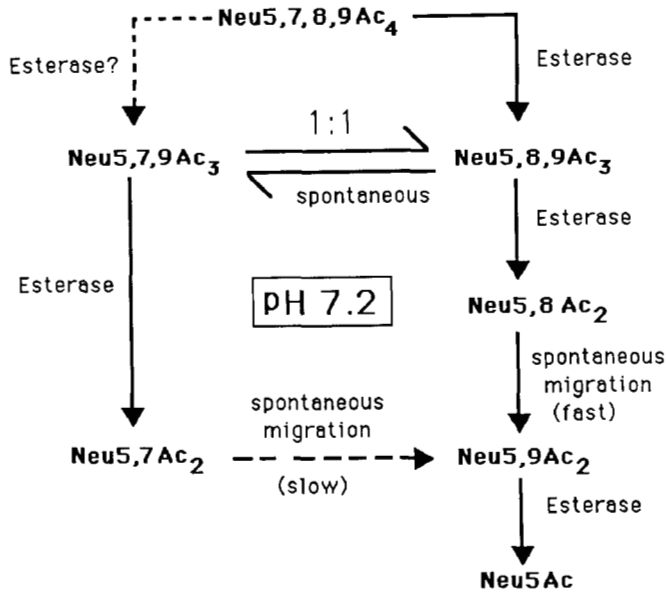

FIG. 11. **Proposed scheme for sequential cleavage of di- and tri-*O*-acetylated sialic acids by the rat liver glycoprotein esterase.** The pathways shown in this figure are based upon the data shown in Figs. 10 and 11 and the results obtained by treatment of tri-*O*-acetylated Neu5Ac (data not shown). See text for further discussion.

found that polyclonal monospecific antibodies raised against this glycoprotein esterase do not cross-react significantly with the cytosolic enzyme.[4]

A large number of *O*-acetylesterases have been discovered, described, and purified on the basis of their reactivity with synthetic chromogenic or fluorogenic substrates (14, 28–32). While they have served as extremely useful markers in genetic linkage studies (33–35), there are very few instances where the natural biological substrates of these enzymes have been identified. The enzyme described in this study was purified on the basis of its activity against 9-*O*-acetylated sialic acids. Although the K_m of the enzyme for this substrate is rather high (8.8 mM), several lines of evidence indicate that it does indeed have specificity for sialic acid substrates. Firstly, it does not cleave *O*-acetyl esters from a variety of other natural substrates bearing *O*-acetyl groups. Secondly, it appears to recognize several features of the sialic acid molecule including the anomeric carbon and the glycerol side chain. Thirdly, as shown in the following paper (24), it appears to recognize the carboxyl group of the sialic acid substrate, likely via a specific arginine residue. It is, of course, possible that in the intact cell there are cofactors that improve the affinity of the enzyme for the substrate. So far, we have been unable to detect such cofactors. However, there are other enzymes with relatively high K_m values for their natural substrate that are still functionally important in the intact cell. There are also well known examples of enzymes that are active against synthetic substrates, but are nevertheless highly specific for their natural substrates (36, 37). The ultimate proof of the biological specificity of this enzyme must come from study of the consequences of its absence. The availability of a rapid, simple, and specific assay for the enzyme will facilitate the identification of such a mutant.

Of the large number of esterases that have been characterized, only a few have been shown to be glycoproteins (28, 38). The presence of *N*-linked glycosylation suggests that a protein is located at some point along the endoplasmic reticulum-Golgi secretory pathway. One such family of glycoprotein esterases have high mannose-type oligosaccharides and appear to reside in the lumen of the rough endoplasmic reticulum (28, 38). The enzyme described in this study is different from all others discovered to date, in that it has complex-type *N*-linked oligosaccharides and sialic acid residues. This indicates that this enzyme has passed through the various stacks of the Golgi apparatus, where *N*-linked oligosaccharide processing is known to occur (39). The exact subcellular location of this enzyme remains unknown. In fresh homogenates of rat liver, a substantial proportion of the ConA binding 9-*O*-acetylesterase is found to be associated with crude smooth membranes and is cryptic. However, almost all of this activity can be released from the membranes by repeated freeze-thaw, or by permeabilization with low concentrations of saponin without a requirement for nonionic detergents.[4] In a buffer extract of acetone powder, all of the activity is found in the soluble fraction. We do not at present know whether the enzyme is initially membrane-associated via a hydrophobic anchor which is cleaved during homogenization or if the protein is in fact a soluble molecule that is confined within the lumen of the vesicular pathway.

Since we have shown that *O*-acetylation of sialic acids in the rat liver takes place after their transfer to macromolecules (9, 18), it follows that the enzyme cannot encounter its natural substrate until it passes through the *trans*-Golgi apparatus, where the sialyltransferases are located (40, 41). However, only a relatively small proportion of the activity from crude smooth membranes is recovered in intact Golgi vesicles.[4] On the other hand, the activity is not found in plasma, the ultimate end point of the secretory pathway of hepatocytes. Thus, it appears that the enzyme might be retained in subcellular compartments of the exocytic/endocytic pathways or in the plasma membrane by as yet unknown mechanisms. Since the enzyme has a neutral pH optimum and appears to pass through the Golgi apparatus, we hypothesize that its function might be to regulate the turnover of *O*-acetyl groups on membrane-bound sialic acids. It remains to be seen whether this is a simple degradative reaction or is part of a more complex acetylation-deacetylation cycle.

Acknowledgments—We thank Drs. Reinhard Brossmer, Richard Swank, Stephen Prescott, and Juris Ozols for providing materials, Sandra Diaz and Bruce Gill for help with many of the experiments, and Bradley Hayes for helpful discussions.

REFERENCES

1. Schauer, R. (1987) *Methods Enzymol.* **138,** 611–626
2. Herrler, G., Rott, R., Klenk, H. D., Muller, H. P., Shukla, A. K., and Schauer, R. (1985) *EMBO J.* **4,** 1503–1506
3. Varki, A., Muchmore, E., and Diaz, S. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 882–886
4. Higa, H. H., Diaz, S., and Varki, A. (1987) *Biochem. Biophys. Res. Commun.* **144,** 1099–1108
5. Rogers, G. N., Herrler, G., Paulson, J. C., and Klenk, H. D. (1986) *J. Biol. Chem.* **261,** 5947–5951
6. Vlasak, R., Luytjes, W., Spaan, W., and Palese, P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 4526–4529
7. Vlasak, R., Luytjes, W., Leider, J., Spaan, W., and Palese, P. (1988) *J. Virol.* **62,** 4686–4690
8. Muchmore, E., and Varki, A. (1987) *Science* **236,** 1293–1295
9. Varki, A., and Diaz, S. (1985) *J. Biol. Chem.* **260,** 6600–6608
10. Tarentino, A. L., and Plummer, T. H., Jr. (1987) *Methods Enzymol.* **138,** 770–778
11. Stafforini, D. M., Prescott, S. M., and McIntyre, T. M. (1987) *J. Biol. Chem.* **262,** 4223–4230
12. Stafforini, D. M., McIntyre, T. M., Carter, M. E., and Prescott, S. M. (1987) *J. Biol. Chem.* **262,** 4215–4222
13. von Deimling, O., Ronai, A., and de Looze, S. (1985) *Histochemistry* **82,** 547–555

[4] H. H. Higa, S. Diaz, and A. Varki, unpublished observations.

14. Ozols, J. (1987) *J. Biol. Chem.* **262,** 15316–15321
15. Varki, A., and Diaz, S. (1984) *Anal. Biochem.* **137,** 236–247
16. Schauer, R. (1978) *Methods Enzymol.* **50,** 64–89
17. Higa, H. H., and Varki, A. (1988) *J. Biol. Chem.* **263,** 8872–8878
18. Diaz, S., Higa, H. H., Hayes, B. K., and Varki, A. (1989) *J. Biol. Chem.* **264,** 19416–19426
19. Roux, L., Holoyda, S., Sunblad, G., Freeze, H. H., and Varki, A. (1988) *J. Biol. Chem.* **236,** 8879–8889
20. Johnson, C. D., and Russell, R. L. (1975) *Anal. Biochem.* **64,** 229–238
21. Powell, L. D., and Hort, G. W. (1986) *Anal. Biochem.* **157,** 179
22. Laemmli, U. (1970) *Nature* **227,** 680–682
23. Varki, A., and Kornfeld, S. (1983) *J. Biol. Chem.* **258,** 2808–2818
24. Hayes, B. K., and Varki, A. (1989) *J. Biol. Chem.* **264,** 19443–19448
25. Kamerling, J. P., Schauer, R., Shukla, A. K., Stoll, S., van Halbeek, H., and Vliegenthart, J. F. (1987) *Eur. J. Biochem.* **162,** 601–607
26. Medda, S., Stevens, A. M., and Swank, R. T. (1987) *Cell* **50,** 301–310
27. Medda, S., Takeuchi, K., Devore Carter, D., von Deimling, O., Heymann, E., and Swank, R. T. (1987) *J. Biol. Chem.* **262,** 7248–7253
28. Korza, G., and Ozols, J. (1988) *J. Biol. Chem.* **263,** 3486–3495
29. Mentlein, R., Schumann, M., and Heymann, E. (1984) *Arch. Biochem. Biophys.* **234,** 612–621
30. Mentlein, R., Heiland, S., and Heymann, E. (1980) *Arch. Biochem. Biophys.* **200,** 547–559
31. Torres, J. L., Rush, R. S., and Main, A. R. (1988) *Arch. Biochem. Biophys.* **267,** 271–279
32. Brenner, S. (1988) *Nature* **334,** 528–530
33. Mentlein, R., Ronai, A., Robbi, M., Heymann, E., and von Deimling, O. (1987) *Biochim. Biophys. Acta* **913,** 27–38
34. Murphree, A. L., and Benedict, W. F. (1984) *Science* **223,** 1028–1033
35. Peters, J. (1982) *Biochem. Genet.* **20,** 585–606
36. Tabas, I., and Kornfeld, S. (1978) *J. Biol. Chem.* **253,** 7779–7786
37. Tulsiani, D. R., Hubbard, S. C., Robbins, P. W., and Touster, O. (1982) *J. Biol. Chem.* **257,** 3660–3668
38. Takagi, Y., Morohashi, K.-I., Kawabata, S.-I., Go, M., and Omura, T. (1988) *J. Biochem. (Tokyo)* **104,** 801–806
39. Kornfeld, R., and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54,** 631–664
40. Taatjes, D. J., Roth, J., Weinstein, J., and Paulson, J. C. (1988) *J. Biol. Chem.* **263,** 6302–6309
41. Griffiths, G., and Simons, K. (1986) *Science* **234,** 438–443
42. Schauer, R. (1982) *Sialic Acids: Chemistry, Metabolism and Function, Cell Biology Monographs*, Vol. 10, Springer-Verlag New York Inc., New York
43. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85

SUPPLEMENTAL MATERIAL TO:

ACETYLATION AND DE-ACETYLATION OF SIALIC ACIDS: PURIFICATION, CHARACTERIZATION AND PROPERTIES OF A GLYCOSYLATED RAT LIVER ESTERASE SPECIFIC FOR 9-O-ACETYLATED SIALIC ACIDS

by
Herman H. Higa, Adriana Manzi, and Ajit Varki

Materials

The following materials were obtained from the sources indicated: [3H-acetyl]acetyl-coenzyme A (AcCoA)[1] (13-15 Ci/mmol), ICN; [3H-isopropyl]diisopropyl fluorophosphate (DFP), (56.8 Ci/mmol), NEN; [4-14C]Neu5Ac, (56.8Ci/mmol), NEN; rat liver acetone powder, *Clostridium perfringens* neuraminidase (Type X), diethyl-p-nitrophenyl phosphate (Paraoxan), acetyl-serine, acetyl-thymidine, triacetin, p-chloromercurobenzoate, bis-pnp-phosphate, acetyl-choline, p-nitro-phenyl acetate, alpha-naphthyl acetate, 4-methyl-umbelliferyl acetate, 4 methyl-umbelliferyl butyrate, alpha-methyl mannopyranoside, saponin, CHAPS, Triton X-100, Triton CF-54 and deoxycholate, Sigma Chemical Company; *Arthrobacter ureafaciens* neuraminidase, Calbiochem; Procion Red Agarose, Amicon; Molecular weight standards for SDS polyacrylamide gel electrophoresis, and for gel filtration, Bio-Rad; DEAE Cellulose (DE-52), Whatman; Concanavalin A Sepharose, Pharmacia; and endo-ß-N-acetylglucosaminidase H (endo H); Miles. Diisopropyl fluorophosphate (Aldrich) was prepared as 1M and 100mM stocks in isopropanol, and stored in a desiccator at -20ºC: Dowex-50 AG 1x8 (H+form, BioRad) was washed extensively in water prior to use. Dowex AG 3x4A (BioRad) was converted to the formate form, and equilibrated in 10mM sodium formate, pH 5.5. Sprague-Dawley rats (male, 3 month old) were from Charles River.

Peptide:N-glycosidase F (PNGase F) was prepared as described (10). Acetyl platelet activating factor (PAF) and PAF acetyl-hydrolase were kind gifts of Dr. Stephen Prescott, University of Utah (11,12); Egasyn was kindly provided by Dr. Richard Swank, Roswell Park Memorial Institute (13), and the 60 Kd glycoprotein esterase was a gift from Dr. Juris Ozols, University of Connecticut at Farmington (14). Alpha-benzyl $Neu5,9Ac_2$, alpha-benzyl $Neu4,5Ac_2$, and 9-acetamido-Neu5Ac were generous gifts of Dr. Reinhard Brossmer, University of Heidelberg, FRG. Mono-, di- and tri-O-acetylated sialic acids were prepared from bovine submaxillary mucin as previously described (15) with the addition of cellulose chromatography (16), and preparative HPLC in System A. The 9-O-acetylated ß-methylglycoside of [4-14C]Neu5Ac ($Neu5,9Ac_2$ß2Ome), was prepared as previously described (3). [3H-O-acetyl]colominic acid (polysialic acid) was prepared using an *E.coli* O-acetyltransferase and commercially obtained colominic acid (Sigma), as previously described (17). Alpha-glycosidically bound [3H-acetyl]-labelled sialic acids on N-linked oligosaccharides were prepared by Peptide:N-glycosidase F treatment of Golgi vesicles labelled with [3H-acetyl]AcCoA (18). The released oligosaccharides were collected by gel filtration on Sephacryl S-200 in a manner similar to that recently described for sulfated N-linked oligosaccharides (19).

Methods

Biosynthetic Preparation of [3H-acetyl]9-O-acetyl-N-acetyl-Neuraminic Acid

Isolated, intact rat liver Golgi vesicles (18) were pretreated with DFP (final 1mM DFP, 1% isopropanol, on ice for 15 min) to inactivate endogenous esterase activity. The reaction mixture (1 ml) containing 1-2 uM [3H]AcCoA (undiluted stock) and 400 ul of Golgi vesicles (5-10 mg/ml) in PK buffer (10mM Potassium phosphate, 150mM potassium chloride pH 6.5) was incubated at 22ºC for 20 min. The reaction was quenched by filling the tube with ice cold PK buffer, and centrifuging at 100,000 x g for 30 min at 4ºC. The supernate was discarded, the pellet surface-washed three times with ice cold PK buffer, and sonicated into 1 ml of 10 mM sodium acetate pH 5.5. The sonicated membranes were recovered by centrifugation at 100,000 x g for 30 min at 4ºC, and the pellet briefly sonicated into 400 ul of 100 mM sodium acetate pH 5.5. [3H]mono-O-acetylsialic acids were released by incubation at 37ºC for 3 h with 100 mU *Arthrobacter ureafaciens* neuraminidase and 0.1% Triton X-100 (final). The mixture was centrifuged at 100,000 x g for 30 min at 4ºC, and the [3H]O-acetyl-sialic acids in the supernate purified by sequential ion exchange chromatography, at room temperature. The supernate was loaded onto a 1ml column of Dowex 50 AG1x8(H+form, in water) and eluted with 4ml of water. The pooled washings were loaded directly onto a 1ml column of Dowex AG 3x4A (formate form) in 10mM sodium formate, pH 5.5. The column was washed with 7ml of 10mM formic acid, and the mixture of [3H]mono-O-acetyl-N-acetyl-neuraminic acids eluted with 10ml of 1M formic acid. The eluate was dried on a Buchler shaker-evaporator, reconstituted in 1 ml of *freshly*-made 20 mM ammonium hydroxide and incubated at room temperature for 30 min, to induce migration of all the [3H]O-acetyl groups to the C-9 position. The sample was dried immediately on the shaker evaporator, brought up in 10 mM acetic acid, and aliquots containing 10,000-50,000 cpm dried into individual microfuge assay tubes using a Savant Centrifuge evaporator. The tubes were dried further for 30 min on a lyophyllizer, capped immediately, and stored in a desiccator at -20ºC until use. Recovery of radioactivity was monitored at each step by counting 0.5% aliquots. Final yield of radioactivity is somewhat variable, depending upon the batches of Golgi and [3H-acetyl]AcCoA. In a typical preparation, starting with 5uCi of [3H-acetyl]AcCoA (specific activity 5Ci/mmol) and 400ul of Golgi vesicles, about 1uCi is found in the labelled membranes, and about 0.5uCi is recovered in the final purified substrate. Note that [3H-acetyl]AcCoA breaks down upon prolonged storage and that the resulting unlabelled CoA is inhibitory for labelling of the Golgi vesicles.

Induced Migration and Removal of O-acetyl Esters

Migration of O-acetyl esters from the 7- to the 9-position, and de-O-acetylation were carried out exactly as described earlier (4,18).

Acetylesterase Assays

[3H]$Neu5,9Ac_2$ Assay

Reaction mixtures (100ul) containing [3H]$Neu5,9Ac_2$ (10,000 cpm), 100 mM Tris/HCl pH 8, and enzyme were incubated at 37ºC for 15-60 min. The amount of enzyme and time of incubation were varied such that less than 20% of the substrate is consumed. The reaction was quenched by addition of 100ul of "stopping solution" (1 M chloroacetic acid, 0.5 M NaOH, 2 M NaCl), chilled on ice for 15 min, and spun at 10,000 x g for 5 min at room temperature. Fixed aliquots (170-190 ul, depending upon the pellet size) of the supernatant are transferred to 10 ml of a toluene-based scintillation cocktail (0.5% PPO and 0.03% POPOP in toluene and 20% isoamyl alcohol). The mixture was shaken well, allowed to stand for 15 min, and the radioactivity released was determined by counting. Comparably incubated and processed blanks were always included in each assay. The blank values ranged from 100-300 cpm, depending upon the particular batch of substrate, the pH and time of incubation. The 100% value was determined by complete de-O-acetylation using 0.1N NaOH at 37ºC for 30 min, followed by "stopping solution", and scintillation cocktail. All assays were performed under conditions where the release was linear with time and added enzyme. After prolonged storage of the substrate, some breakdown occurs, and background values increase. This can be corrected by adding a small amount of 10mM acetic acid and evaporating the substrate once.

The principle of the assay is very similar to that previously described for acetyl-cholinesterase (20). The aqueous reaction mixture is quenched with a solution that acidifies it, and it is then mixed with a toluene-based scintillation counting cocktail. The substrate ([3H]$Neu5,9Ac_2$) cannot enter the toluene phase and hence cannot be counted, whereas the product ([3H]acetate), which is protonated under these conditions, can. Thus, any increase in radioactivity over the background represents release of [3H]acetate from the substrate. Note that because this esterase has a high Km value (8.8mM) for its natural substrate $Neu5,9Ac_2$, saturating assays are not practical. Thus, the first order kinetics (%/hour released from non-saturating radioactive substrate) were used to establish the activity. One unit of activity is defined as release of 1% of the radioactivity per hour of incubation at 37ºC. This assay is accurate and reliable even in crude extracts of tissues.

Other Assay methods

The same principle as above was applied to monitoring activity against other substrates with [3H]-O-acetyl groups except for [3H-acetyl]PAF (see below). The release of unlabelled free acetate from substrates was monitored using a commercially available kit (catalog #148-261 from Boehringer Mannheim). The components for this acetate assay can also be purchased individually. In the case of synthetic chromogenic substrates (e.g. 4-methyl-umbelliferyl acetate), hydrolysis was followed by monitoring the reaction fluorometrically or spectrophotometrically. Assays for acetylcholinesterase (20) and PAF-acetylhydrolase (11,12) were exactly as previously described. In all cases, assays were performed under conditions where product formation was linear with time and added enzyme.

High Pressure Liquid Chromatography (HPLC)

System A: The different sialic acids were separated using a BioRad HPX-72S column (300 X 7.8mm) eluted in the isocratic mode at 1 ml/min with 100mM Na_2SO_4 (18). For unlabelled sialic acids, the eluant was monitored by absorbance at 210nm. Labelled compounds were either detected with an on-line Flo-One beta Radiomatic Flow Detector, or by collecting fractions.

System B: Sialic acids were quantitated on a pmol scale, exactly as described by Powell and Hart (21).

[3H]DFP-labelling of Esterases

The purified esterase (4 ug) in 2 ml of 20 mM KPi pH7.0 was treated with 62.5 uCi of [3H]DFP at 4ºC for 1 h. After adding an additional 62.5 uCi of [3H]DFP, the mixture was incubated at 37ºC for 1 h. The reaction was quenched with 12.5 ul of 100 mM DFP (unlabelled), incubated at 4ºC for 1-2 days, applied to a Sephadex G-50-80 column (1.4 x 13 cm), and fractions (1 ml) were collected. Aliquots (1ul) were counted and peak radioactive fractions in the void volume were pooled. The pooled sample was aliquoted and stored at -20ºC. Samples for analysis by SDS-PAGE (65ul) were incubated with 1uCi of [3H]DFP at 25ºC for 30 min, before addition of sample buffer.

Glycosidase Treatments

Peptide N-Glycosidase F (PNGase F): [3H]DFP-labelled esterase (15,000 cpm) prepared as described above, was boiled in 0.2 % SDS for 3 minutes to denature the protein. Reaction mixtures (65ul) containing the denatured esterase, 50 mM EDTA, 1% NP-40, 2 units of PNGase F (10), in 50 mM Tris/HCl pH 8 with (reduced) or without 50mM 2-mercaptoethanol (nonreduced), were incubated at 37ºC for 4 hours.

Endo-ß-N-acetylglucosaminidase H (Endo H): [3H]DFP-labelled esterase (15,000 cpm) was denatured in 0.2 % SDS as described above. The denatured esterase was treated with Endo H (7 mU) in 200 mM citrate-phosphate pH 6 (65 ul) with or without 50 mM 2-mercaptoethanol, and incubated at 37ºC for 4 hours.

Arthrobacter ureafaciens Neuraminidase: Reaction mixtures contained neuraminidase (2mU) in 200 mM citrate phosphate pH 6, with or without 50 mM 2-mercaptoethanol, and were incubated at 37ºC for 4 hours.

SDS Polyacrylamide Gel Electrophoresis/Detection of Proteins

Samples were heated for 5 min in sample buffer (4% SDS, 65mM Tris HCl pH 6.8) with (reducing) or without (% 2-mercaptoethanol (non-reducing). The solubilized proteins were electrophoresed according to the method described by Laemmli (22). Unlabelled protein bands were visualized either by staining with Coomassie Brilliant Blue R-250, or with silver nitrate. [3H]DFP-labelled protein bands were visualized by fluorography after treatment with Enhance (NEN) according to the manufacturer's recommended procedure.

Purification of the Glycoprotein Esterase

All steps were performed at 4ºC. The following buffers were used: Buffer A, 20 mM KPi pH 8; Buffer B, 20 mM KPi pH 8 with 100mM NaCl; Buffer C, 20 mM KPi pH 5.5 with 50mM NaCl; Buffer D, 20 mM KPi pH 5.5; Buffer E, 20 mM KPi pH 5.5 with 370mM NaCl; Buffer F. 20 mM KPi pH 5.5 with 900mM NaCl; Buffer G, 30mM K_2HPO_4, 2mM EDTA.

STEP 1. Extraction. Rat liver acetone powder (100 g) was homogenized gently into 500 ml of Buffer A with a polytron homogenizer for approximately 1 min, until all clumps were dispersed. The homogenate was stirred for 1 hr, and the mixture centrifuged at 100,000 x g for 30 min (33,000 rpm, 50.2 Ti Beckman rotor). The supernatant was passed through a plug of glass wool and saved. The pellet was resuspended, and re-extracted as above.

Step 2. DEAE-Cellulose Chromatography. The supernates are pooled, and loaded onto a column of DE-52(Whatman) (1130 ml packed volume 7.8 x 23.8 cm, in Buffer A). The column was washed with 600 ml of the same buffer and 20ml fractions collected. The glycoprotein enzyme ran through the column, whereas the cytosolic enzyme bound. If desired, a partially purified preparation of the latter can be obtained by elution with a 1700ml linear gradient of 0-300mM NaCl in Buffer A.

Step 3. Concanavalin A Sepharose Chromatography. The pooled run-through from the DE-52 column was adjusted to 0.1M NaCl with a stock 4M NaCl and loaded at about 1ml/min onto a 1.5 x 31 cm (55ml) ConA-Sepharose column equilibrated in Buffer B. The column was washed with 200-250ml of Buffer B. One column volume of 100mM alpha-methyl mannoside in Buffer B(55ml) was loaded onto the column, and the flow stopped for 8-15 h. The enzyme binds to the column and requires prolonged exposure to the glycoside for elution. The column flow was resumed and elution with alpha-methyl mannoside continued for 4 additional column volumes. Fractions (40 ml) were collected and monitored for activity. A small portion of the activity (5-20%) may run through the column. If optimal yield is desired, a second smaller column of ConA Sepharose (25ml) can be run to bind and elute this remaining activity.

Step 4. Ammonium Sulfate Precipitation/Sephacryl S-200 Chromatography. The active fractions eluted from the ConA-Sepharose column were pooled and adjusted to 80% saturation with ammonium sulfate (56.1G/100ml) by gradual addition of the solid powder with continuous stirring. After stirring for 3-6 h, the precipitate was collected by centrifugation at 30,000 x g (SS34 Sorvall rotor, 16,000 rpm) for 30 min. The ammonium sulfate precipitate was dissolved in 3ml of Buffer C, and loaded onto a Sephacryl S-200 column (1.5 x 47cm, 83 ml), equilibrated in the same buffer, and eluted at 20ml/h. Fractions (1ml) were collected, and monitored for activity. A single, symmetrical peak of activity was found between 40-60ml of the elution volume.

Step 5. Procion Red-Agarose Chromatography. The active Sephacryl S-200 fractions were pooled and applied to a column of Procion Red-Agarose (1.5 x 17 cm, 30ml) equilibrated in Buffer D, at a flow of 20ml/h. The column was washed with 300ml of the same buffer, and eluted with 400ml of Buffer E. Fractions (8.5ml) were collected and monitored for activity. The fractions containing sialic acid esterase activity, eluted with Buffer E, were pooled and diluted with an equal volume of Buffer G, to give a final concentration of 25mM KPi, 185mM NaCl, pH 7.5. The volume was then reduced to <0.5ml (5-10mg/ml), using several Centricon concentrators (Amicon, catalog #4301). The purified, concentrated enzyme was divided into aliquots and stored at -20ºC. Under these conditions, the enzyme is stable for at least 6 months. This final step separates the sialic acid esterase from another glycoprotein acetyl-esterase that can subsequently be eluted from the column with 300ml of Buffer F. The latter enzyme hydrolyzes 4-MU-OAc, but not the sialic acid substrate. Its natural substrate remains unknown.

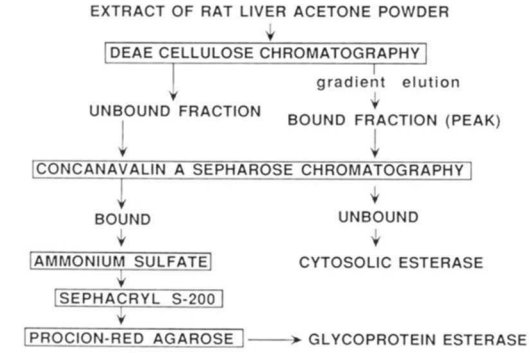

Figure 2. Purification Scheme of Rat-Liver Sialic Acid 9-O-acetylesterases: Two distinct sialic acid O-acetylesterases can be separated from a buffer extract of rat liver acetone powder. The individual separation steps are indicated in the scheme. The cytosolic (non-glycoprotein) esterase is only partially purified in this protocol. The glycoprotein esterase is purified to homogeneity by this protocol, and is the subject of the remainder of this paper.

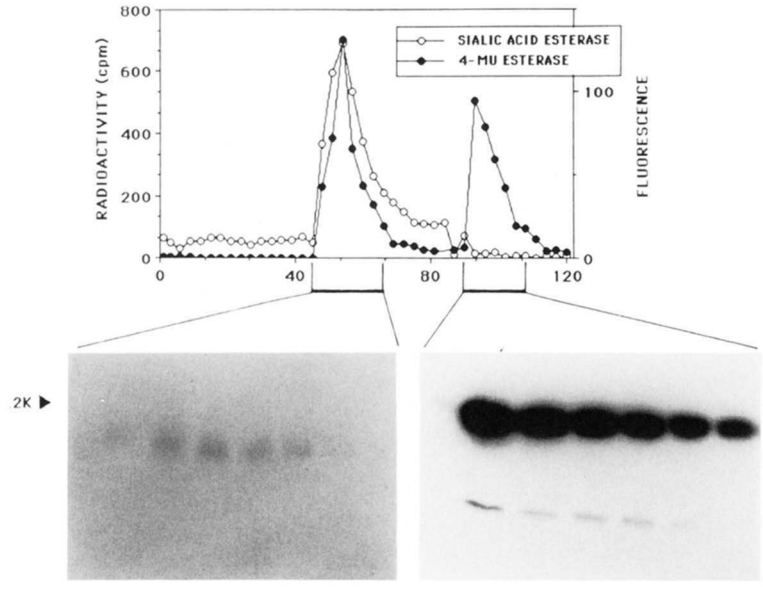

Figure 3. Elution Profile of Rat Liver Glycosylated Esterases on Procion Red Agarose. The active fractions from the Sephacryl S-200 column (step 4) were pooled and applied to a Procion Red Agarose column (1.4 x 13 cm). From fraction 1 through 41, the column was washed with 300 ml of 20 mM KPi pH 5.5 (Buffer D). At fraction 42 the sialic acid esterase was eluted with 300 ml of Buffer E, and at fraction 87, the 64.8Kd esterase was eluted with Buffer F. Fractions (8.5ml) were collected and monitored for sialate: 9-O-acetylesterase, and 4-MU acetate esterase. Aliquots of every third fraction were labelled with [^{3}H]DFP, and analyzed by nonreducing 10% SDS-PAGE, Coomassie blue staining and fluorography as described under "Experimental Procedures". The lower panels show the relevant regions of the fluorograms. The sialic acid esterase is present in smaller amounts, is a more diffuse band, and is also labelled less well than the 64.8Kd protein. The flourogram on the left is therefore exposed longer, and has a higher background.

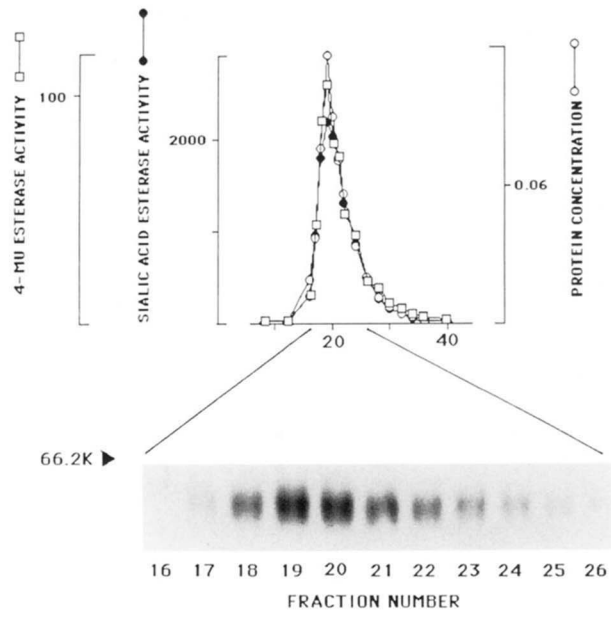

Figure 4. Proof of Purity of Rat Liver Glycosylated Sialate: 9-O-acetylesterase by Gel Filtration Analysis: Purified rat liver glycosylated sialate: 9-O-acetylesterase (270ug) was loaded onto a column of Bio-Gel P-100 (50-100 mesh, 0.7 cm x 40 cm) equilibrated in 20mM KPi pH 7.0 containing 100mM NaCl, and eluted with the same buffer. Fractions (200ul) were collected and assayed for sialate: 9-O-acetylesterase and 4-MU esterase activities as described under "Experimental Procedures". Protein was determined by absorbance at 220nm. Aliquots of the peak fractions were labelled with [^{3}H]DFP, and studied by SDS-PAGE (10%, non-reduced) and fluorography.

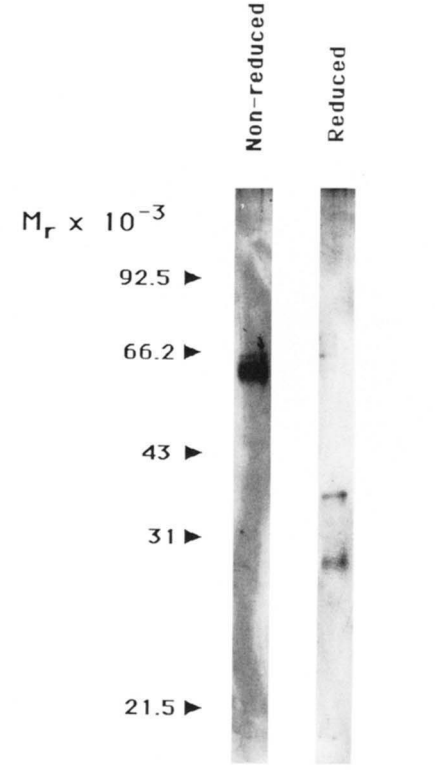

Figure 5. SDS Polyacrylamide Gel Electrophoresis of Purified Native and Reduced Glycosylated Rat Liver Sialate: 9-O-acetylesterase: Purified rat liver glycosylated sialate: 9-O-acetylesterase (2 ug) is shown stained with silver nitrate in its native form (left) or in its reduced form (right). Molecular weight (Mr) standards are phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). Conditions for reduction of the enzyme, SDS-PAGE (10%) and silver nitrate staining are described under "Experimental Procedures".

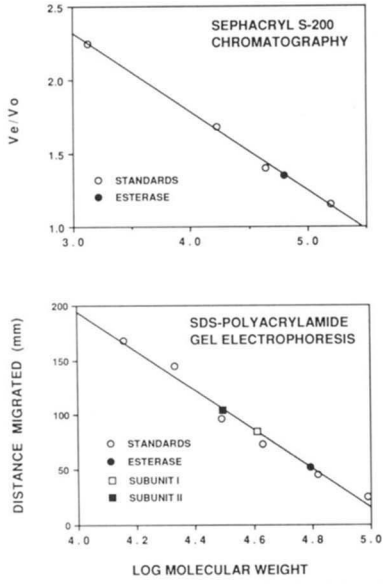

Figure 6: Molecular Weight Determination of Purified Rat Liver Glycosylated Sialate: 9-O-acetylesterase by Gel Filtration and SDS-Polyacrylamide Gel Electrophoresis.

Upper Panel: A mixture containing [^{14}C]-N-acetylmannosamine (10,000 cpm, for total volume), blue dextran (for excluded volume) and the purified esterase labelled with [^{3}H]DFP (10,000 cpm), was loaded onto a precalibrated Sephacryl S-200 column (0.7 x 40 cm) equilibrated in 20 mM KPi pH 7.0 containing 100 mM NaCl, and eluted with the same buffer. Aliquots (100ul) from fractions (230ul) were counted and peak fraction containing blue dextran was determined by absorbance at 550nm. The column had been calibrated immediately before the analysis of the enzyme with a mixture of thyroglobulin (670,000), gamma globulin (158,000), chicken ovalbumin (44,000), horse myoglobulin (17,000) and vitamin B12 (1,350). The standards were monitored by absorbance at 220nm. The closed circle represents Ve/Vo of the purified enzyme (apparent molecular weight 62,800).

Lower Panel: Nonreducing or reducing 10 % SDS polyacrylamide gel electrophoresis of the purified esterase were stained with silver nitrate and the distances migrated determined. Molecular weight standards are as described in Figure 5. The calculated molecular weights are: 61,500 daltons for the unreduced esterase; 36,400 daltons for subunit I, and 30,300 daltons for subunit II.

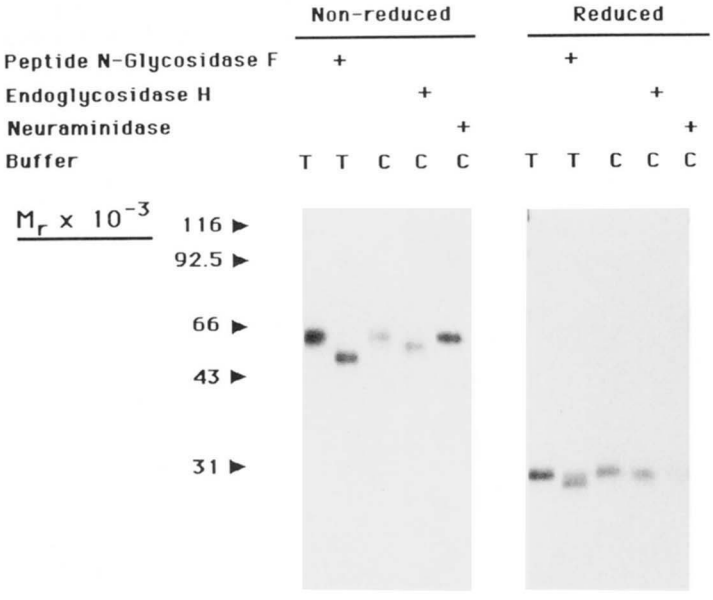

Figure 7: Effects of Glycosidases on the Purified Rat Liver Glycosylated Sialate: 9-O-acetylesterase: The native and reduced forms of [^{3}H]DFP-labelled rat liver glycosylated sialate: 9-O-acetylesterase (12,000 cpm each) were treated with either Peptide N-Glycosidase F, Endo-ß-N-acetylglucosaminidase H, or *Arthrobacter ureafaciens* Neuraminidase and visualized by nonreducing 10% SDS-PAGE and fluorography. Molecular weight standards are as described for Figure 5. Conditions for reduction of the enzyme, [^{3}H]DFP labelling, glycosidase treatments, SDS polyacrylamide gel electrophoresis, and fluorography are described under "Experimental Procedures". The buffers are indicated as T: 50mM Tris HCl pH 8.0; and C: 200mM citrate phosphate, pH 6.0. The individual lanes are referred to in the text by number (1-10) counting from left to right.

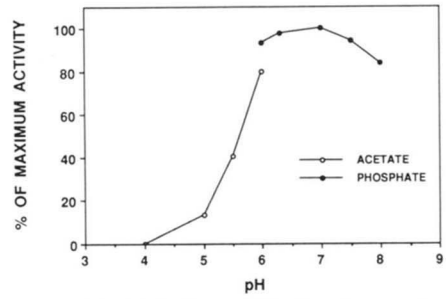

Figure 8: pH Activity Profile of Rat Liver Glycosylated Sialate: 9-O-acetylesterase: Purified rat liver glycosylated sialate: 9-O-acetylesterase was assayed at various pH values, as described under "Experimental Procedures", in 100mM concentrations of the buffers indicated.

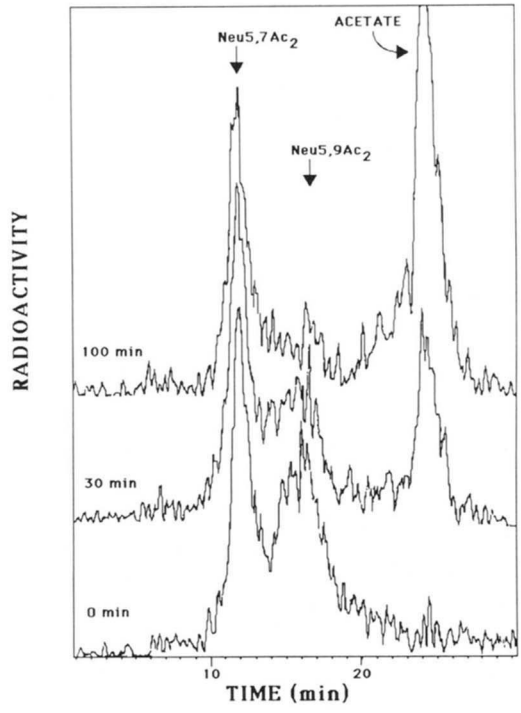

Figure 9: HPLC Analysis of Products of Esterase Activity on 7- and 9-O-acetylated Neu5Ac: A mixture of [^{3}H-O-acetyl] Neu5,9Ac$_2$ and [^{3}H-O-acetyl]Neu5,7Ac$_2$ obtained as described earlier (18), was treated with 62units of the purified glycosylated esterase for varying periods of time and then studied by HPLC in System A. The column eluant was monitored for radioactivity using a Radiomatic Flo-one beta radioactivity detector. The positions of elution of Neu5,9Ac$_2$, Neu5,7Ac$_2$ and acetate are indicated.