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BIOCHEMICAL AND GENETIC EVIDENCE FOR DISTINCT MEMBRANE-BOUND AND CYTOSOLIC SIALIC ACID O-ACETYL-ESTERASES: SERINE-ACTIVE-SITE ENZYMES

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A cytosolic sialic acid-specific O-acetyl-esterase was previously described that can remove O-acetyl esters from the 9-position of sialic acids. We show that rat liver Golgi vesicles contain a distinct sialic acid-esterase located within the lumen of the same vesicles that add O-acetyl esters to sialic acids. Studies of a retinoblastoma cell line genetically deficient in the cytosolic enzyme also confirm the existence of distinct membrane-associated sialic acid esterase activity. We developed a sensitive, specific and facile assay, which measures release of [^{3}H]acetyl groups from [^{3}H-acetyl]9-O-acetyl-N-acetylneuraminic acid. Using this assay, we show that rat liver membranes may contain different sialic acid O-acetyl-esterases. The membrane-associated enzyme(s) bind to Concanavalin A Sepharose,whereas the cytosolic enzyme does not. Membrane-bound and cytosolic esterases are inactivated by di-isopropyl-fluorophosphate, showing they are serine-active-site enzymes. © 1987 Academic Press, Inc.

While the most common sialic acid is N-acetyl-neuraminic acid (Neu5Ac)[1], more than 25 different naturally-occurring derivatives have been described (1,2). One common modification is the addition of O-acetyl esters at positions C-4, C-7, C-8 and/or C-9. These can affect the physical properties of the molecule (2), and alter recognition by various enzymes (4-6), antibodies (7) and viruses (8-9). Furthermore, the expression of O-acetylation is developmentally regulated in various tissues (10). O-acetylated sialic acids are synthesized from the donor acetyl-CoA (2,11). However, very little is known about the turn-over and catabolism of the O-acetyl groups. Since O-acetyl esters retard or abolish neuraminidase release of sialic acids (2,5), it is reasonable to predict that specific O-acetyl-esterases might be involved in the catabolism of these molecules. Shukla and Schauer first reported an equine liver cytosolic esterase which could hydrolyze O-acetyl groups

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Abbreviations used: Sialic acids are designated as previously described (2). For example, N-acetyl-9-mono-O-acetylneuraminic acid is written as Neu5,9Ac2; HPLC, High pressure liquid chromatography; Con A, Concanavalin A; TX-100, Triton X-100; DFP, Di-isopropyl-fluorophosphate.

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from free sialic acids (12). We have described a human red cell cytosolic sialate:9-O-acetyl-esterase which showed similarity in many properties to the "non-specific" esterase D (13). However, all known oligosaccharide-bound sialic acids face away from the cytosolic leaflet of the plasma-membrane and membrane-bound organelles (1,2). Thus, the cytosolic enzyme is an unlikely candidate for involvement in the primary regulation, turn-over or catabolism of O-acetyl groups on sialic acids bound to macromolecules. We previously suggested the existence of membrane-associated sialic acid esterases (11). In this report, we describe a specific and simple assay and use it to demonstrate the existence and characteristics of membrane-associated sialic acid O-acetyl-esterase(s).

METHODS

Chemicals, Enzymes Radioisotopes and Biologicals The following materials were obtained from the sources indicated: [acetyl-^{3}H]acetyl-coenzyme A (AcCoA) (24 Ci/mmol), ICN; [^{3}H-acetyl] acetylcholine (3.7 Ci/mmol), Amersham; V. cholerae and A. ureafaciens neuraminidases, Calbiochem-Behring; red cell acetylcholinesterase, porcine liver butyryl-esterase, 4-methylumbelliferyl acetate, p-chloromercuribenzoate, phenylmethyl-sulfonyl fluoride, Sigma; di-isopropylfluorophosphate, Aldrich; Concanavalin A Sepharose, Pharmacia. Partially purified human red cell sialic acid esterase was prepared as previously described (13). Stock cultures of influenza C virus were purchased from the American Type Culture Collection (Bethesda, Maryland). Platelet activating factor acetyl-hydrolase was kindly provided by Dr. S. Prescott, University of Utah.

Buffers and Solutions PKM buffer: 10 mM KPi pH 6.5, 150 mM KCl and 1 mM $MgCl_2$; PS buffer: 0.25 M sucrose, 20 mM KPi, pH 7; stopping solution: 1 M chloroacetic acid, 0.5 M NaOH and 2 M NaCl.

Preparation of Membranes and Cytosol from Tissue Culture Cell Lines. The retinoblastoma cell lines LA-RB69A and LA-RB94 were kindly provided by Dr. William Benedict of the Children's Hospital of Los Angeles, and were cultured in alpha-MEM containing 15% normal human serum. All other cell lines were cultured in alpha-MEM or RPMI-1640 with 10% fetal bovine serum. Cells were harvested without trypsinisation, washed 3x in cold PBS, and incubated in PBS with 0.05% saponin for 30 min to allow complete permeabilization without solubilization (14), and release of cytosolic contents. The cellular membranes were then pelleted at 10,000g for 5 min at 0°C, and the supernate saved (cytosol). The membrane pellet was washed with 1.5 ml of PBS with 0.05% saponin.

Preparation of Rat Liver Fractions. A total rat liver homogenate and post-nuclear supernate were obtained as previously described (11,15). In some studies, the post-nuclear supernate was spun at 100,000g for 30 min to obtain a "total membrane" pellet and a "cytosol" fraction. The cytosol fraction was passed over a glass wool plug to remove lipids and particulates, and the membrane fraction was gently resuspended in PS buffer. For other studies the post-nuclear supernate was further fractionated to obtain Golgi vesicles that were highly purified (50-100-fold purification of galactosyltransferase) (11,15). Total membrane and cytosolic fractions were stored on ice and used within 18 hours. Golgi vesicles were washed and suspended in PS buffer containing 10% BSA, and stored under liquid nitrogen. Under these conditions, the Golgi vesicles were shown to be sealed and right-side out by previously described criteria (16).

Biosynthesis and Purification of [^{3}H]Neu5,9Ac2 A reaction mixture (1 ml) containing 2 uM [^{3}H]AcCoA (55 uCi), 1 mM ATP and 400 ul of Golgi vesicles (5-10 mg/ml) in PKM buffer, was incubated at room temperature for 30 min, quenched with 5 ml of ice cold PKM buffer, and centrifuged at 100,000g for 30 min at 4°C. The supernate was discarded, the pellet surface-washed three times with ice cold PKM buffer, and sonicated into 1 ml of 10 mM pyridinium formate pH 5.5. The sonicate was treated with 1 mM DFP (stock 100 mM in isopropanol) at 4°C for 15 min on ice, centrifuged at 100,000g for 30 min at 4°C, and the pellet briefly sonicated into 400 ul of 100 mM sodium acetate pH 6 with 4 mM calcium acetate. [^{3}H]mono-O-acetyl-sialic acids were released by incubation at 37°C for 3 h with 50 mU each of A. ureafaciens and V. cholerae neuraminidase and 0.1 % Triton X-100 (TX-100) (final). The reaction was quenched on ice and

centrifuged at 100,000g for 30 min at 4°C. The [^{3}H]O-acetyl-sialic acids in the supernate were purified by sequential ion exchange chromatography on Dowex 50 and Dowex 3x4A (formate form) as described previously (17). The mixture of [^{3}H]7-, 8- and 9-mono-O-acetyl N-acetyl-neuraminic acids eluted from Dowex 3x4A was dried, reconstituted in 1 ml of freshly-made 20 mM ammonium hydroxide, incubated at room temperature for 30 min, dried immediately on a Buchler Shaker evaporator, brought up in 10 mM acetic acid, and aliquots containing 10,000 cpm each dried into individual microfuge assay tubes using a Savant Centrifuge evaporator. The tubes were capped immediately and stored in a dessicator at 20°C until use.

Assay of Sialic Acid 9-O-acetyl-esterase Activity The tubes containing 10,000 cpm of [^{3}H]Neu5,9Ac$_2$ were incubated with enzyme in 100ul of the buffers at the various pH values and conditions indicated. The reactions were quenched by the addition of 100 ul of "stopping mixture" (see above), chilled on ice for 15 min, and spun at 10,000g for 5 min. Aliquots (170-190 ul) of the supernatant were transferred to 10 ml of scintillation cocktail containing 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 released radioactivity determined. The blank values ranged from 100-200 cpm, depending upon the particular batch of substrate, and the pH and time of incubation. One unit of activity is defined as release of 1% of the radioactivity per hour of incubation. All assays were performed under conditions where the release was linear with time and added enzyme, and less than 20% of the substrate was consumed. A similar assay was used to directly monitor the release of free [^{3}H] acetate from pre-labelled Golgi vesicles.

RESULTS AND DISCUSSION

A Sialic Acid O-acetyl-esterase Appears to be Localized Within the Lumen of Golgi Vesicles. We previously reported that when Golgi vesicles are labelled with [^{3}H]acetyl-CoA, a major portion (70-80%) of the macromolecule-bound label is in [^{3}H-O-acetyl]-sialic acids (11). However, during incubation with neuraminidases in the presence of detergents, a variable but significant portion of this label was converted to free [^{3}H]acetate. This suggested that a sialic acid O-acetyl-esterase activity was present either within the Golgi vesicles, or in some other fraction contaminating the Golgi preparation. To study this, vesicles were incubated with [^{3}H]AcCoA, reisolated, washed, and gently resuspended (11). Under these conditions, the labelled vesicles remained greater than 90% intact, by previously described criteria (16). The labelled vesicles were then incubated at 37°C; aliquots were removed at various times and the increase in free [^{3}H]acetate determined. As shown in Figure 1, >60% of the bound [^{3}H]acetate was released in 3 h. Since at least 80 % of the bound label is in O-acetylated sialic acids, much of the release must represent the action of an O-acetyl-esterase capable of cleaving such molecules. This esterase activity could be either in the same vesicles as the labelled sialic acids, or in a different population of vesicles. In the latter case, the contact between the labelled sialic acids and the esterase must occur by spontaneous breakage of the two populations of vesicles, and should be enhanced by the addition of a detergent. In fact, as shown in Figure 1, the presence of 0.2% TX-100 actually markedly decreased the release of [^{3}H]acetate. This indicates that the esterase is likely to be in the lumen of the same Golgi vesicles that contain the [^{3}H-O-acetyl] sialic acids. The alternate possibility that the decrease in activity is due to inhibition by TX-100 is ruled out by direct assay of the enzyme in the presence of the

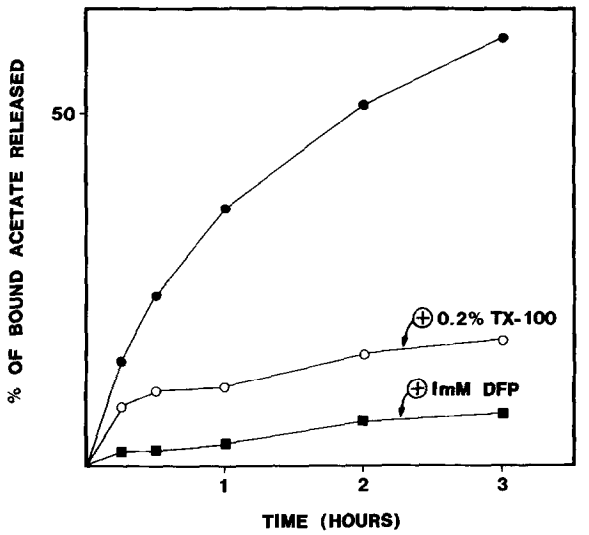

Figure 1. Release of [^{3}H]Acetate from Rat Liver Golgi Vesicles pre-labelled with [^{3}H]acetyl-CoA. Rat liver Golgi vesicles were labelled with [^{3}H]acetyl-CoA for 20 min at 22°C (11), re-isolated, washed and resuspended in PS buffer. Aliquots were incubated at 37°C in PKM buffer, in the absence (●) or presence of either 0.2 % TX-100 (○) or 1 mM DFP (■). At each time point, aliquots were quenched with 100ul of "stopping mixture" and quantitated for the amount of [^{3}H]acetate released as described under "Methods". The 100% value for total amount of bound [^{3}H]acetate was determined by complete de-O-acetylation in 0.1 N NaOH at 37°C for 30 min.

detergent (see below). Addition of di-isopropyl-fluorophosphate (DFP) (which can penetrate lipid bilayers) almost completely abolishes the release of free acetate. This indicates an esterase enzyme with a serine active site.

Preparation of [^{3}H-O-acetyl]Neu5,9Ac$_2$ Substrate for Sialic Acid O-acetyl-esterase Assay The cytosolic sialic acid O-acetyl-esterase (12) and the influenza C O-acetyl-esterase (18) have previously been shown to work on Neu5,9Ac$_2$, but not on Neu5,7Ac$_2$. We developed an assay that measures the release of [^{3}H]acetate from [^{3}H-O-acetyl]Neu5,9Ac$_2$ (see "Methods" for details). The substrate was synthesized by labelling intact Golgi vesicles with [^{3}H]AcCoA, and releasing and purifying the [^{3}H-mono-O-acetyl]sialic acids from the labelled vesicles. As shown in Figure 2 (Panel A), the resulting material is primarily a mixture of Neu5,7Ac$_2$ and Neu5,9Ac$_2$. However, we have defined conditions (Panels B & C) under which such mixtures can be almost completely converted into [^{3}H]Neu5,9Ac$_2$ by induced migration of the O-acetyl groups from C-7 to C-9, without loss of free acetate. The resulting substrate is radiochemically pure and was used to assay for the sialic acid O-acetyl-esterase. The principle of the assay is similar to that previously described for acetylcholinesterase (19). 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 ([^{3}H]Neu5,9Ac$_2$) cannot enter the toluene phase and hence cannot be counted, whereas

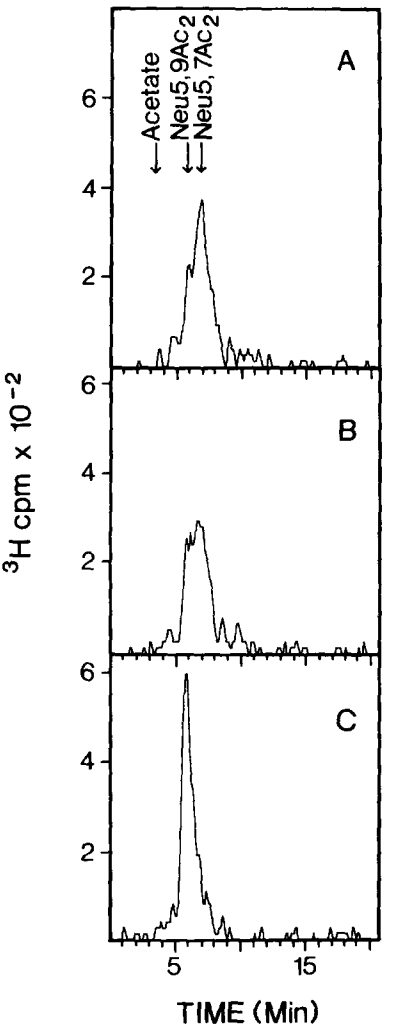

Figure 2. High Performance Liquid Chromatography Analysis of [^{3}H] O-acetylated Sialic Acids. [^{3}H-O-acetyl]mono-acetylated sialic acids were released and purified from [^{3}H]acetyl-CoA-labelled Golgi vesicles as described under "Methods". An aliquot (10,000 cpm) was fractionated on a Micro-Pak AX-5 HPLC column (4 x 300 mm) eluted at 1 ml/min under isocratic conditions with 64% acetonitrile, 20% water, 16% 0.25M NaH_2PO_4 (27). Similar aliquots were treated with freshly-made 20mM NH_4OH for 15 min (panel B) or 30 min (panel C) at room temperature, taken to dryness with a Buchler Shaker evaporator, brought up in water, and applied to the HPLC column.

the product ([^{3}H]acetate), which is protonated under these conditions, can. To demonstrate the specificity of the assay we compared the hydrolysis of [^{3}H]Neu5,9Ac$_2$ by several different O-acetyl-esterases. As predicted, [^{3}H]Neu5,9Ac$_2$ was a substrate for partially-purified red cell sialic acid esterase (13) and influenza C virus (18). In contrast, it was not a substrate for porcine liver butyryl-esterase (20), red cell acetyl-cholinesterase (21), nor platelet-activating-factor acetyl-hydrolase (22) (data not shown). These results suggest that the [^{3}H]Neu5,9Ac$_2$ could be used as a relatively specific substrate for sialic acid O-acetyl-esterases in crude tissue samples. The Golgi-

Table 1. Distribution of membrane-bound and cytosolic sialic acid O-acetyl-esterases in Tissue Culture Cell lines and Rat Liver

CELL LINE/TISSUE (ref.)	Sialic Acid O-acetyl-esterase activity			
	Specific Activity (/mg)		% of Total Activity	
	Cytosol	Membranes	Cytosol	Membranes
1. LA-RB69A Retinoblastoma (23)	<2	1.3	<10	>90
2. LA-RB94 Retinoblastoma (23)	17	1.1	88	12
3. DS-19 Murine Erythroleukemia (28)	73	1.5	97	3
4. K-562 Human Erythroleukemia (29)	56	1.8	98	2
5. M-21 Human Melanoma (30)	44	3.4	71	29
6. CHO-K1 Chinese Hamster Ovary (31)	181	5.8	93	7
7. Rat Liver Homogenate	11.8	18.8	46	54

Cytosol and membrane fractions were prepared from various tissue culture cell lines (Nos.1-6) and from rat liver homogenate (No.7) as described under "Methods". Assays for sialic acid O-acetyl-esterase were carried out in 100 mM Tris HCl pH 8.0 with (membranes) or without (cytosol) 1% TX-100. Protein concentrations were determined by the Amido-Schwartz method (32). The results are expressed as specific activity or as percentage of total activity (taking into account the total volume of each fraction).

associated esterase activity was studied by this assay. In the presence of TX-100 (which activates the enzyme by 67%) the release of [^{3}H]acetate is linear up to 90 minutes of incubation at 37°C, and directly proportional to the amount of Golgi vesicles added to the assay.

Genetic evidence for distinct Membrane-bound Sialic Acid O-acetyl-esterase Acitivty We had earlier obtained indirect evidence that cytosolic sialic acid O-acetyl-esterase might be identical to the "non-specific" esterase D (13). A retinoblastoma cell line has been previously described that is deficient in esterase D on the basis of a sub-microscopic deletion in the band 13q14.1, combined with a loss of the second 13th chromosome (23). We examined the expression of the sialic acid-O-acetyl-esterases in this cell line(LA-RB69A), in comparison with another retinoblastoma(LA-RB94), that has normal levels of esterase D. As shown in Table 1, LA-RB69A is also deficient in cytosolic sialic acid O-acetyl-esterase. However, both retinoblastoma cell lines showed a small but significant portion of the sialic acid O-acetyl-esterase activity associated with the membrane-bound fraction. The similarity in the specific activity of the membrane bound activity in the two cell lines indicates that it does not represent a simple contaminant from the cytosolic activity. Similar studies of several other tissue culture cell lines and of rat liver homogenates show that a highly variable portion (2-54%) of the total sialic acid-O-acetyl-esterase activity is membrane-associated (Table 1).

Further Studies of Rat Liver Sialic Acid O-acetyl-esterases When assayed at pH 8.0, more than 50% of the total sialic acid-O-acetyl-esterase activity remained associated with the membrane fraction of rat liver (Table 1). A more detailed study of the pH activity profile in the presence and absence of non-ionic detergent is presented in Figure 3 (left panel). The results suggest the

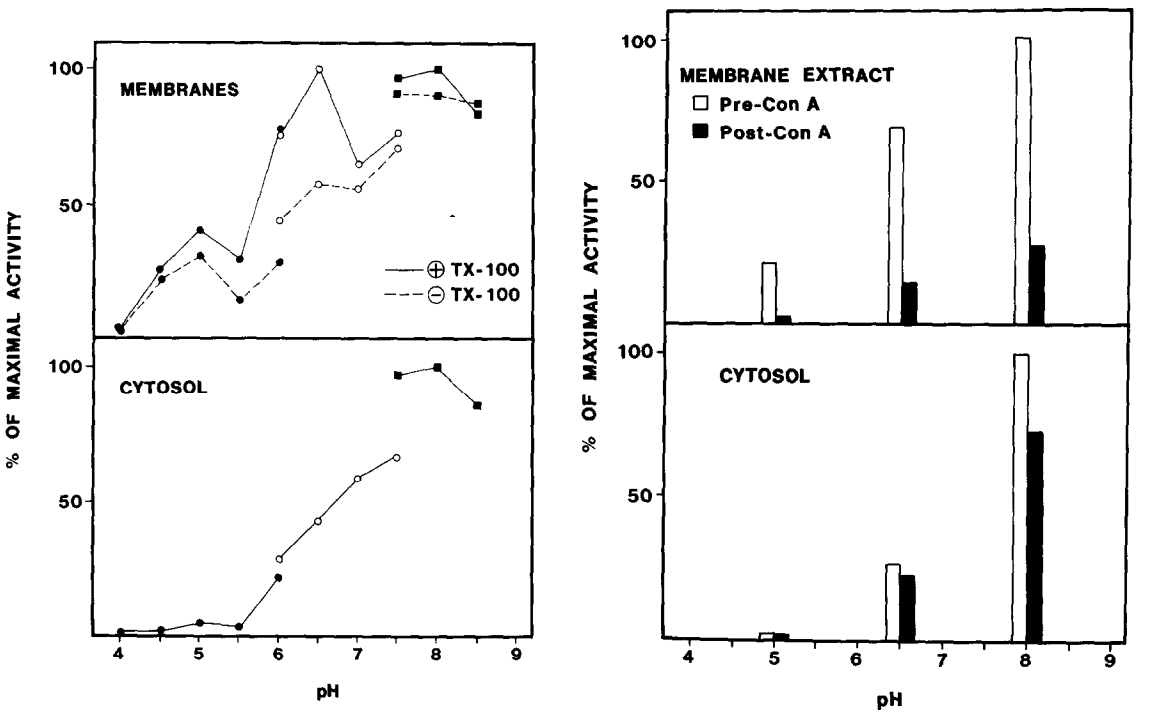

Figure 3. pH/Activity and Con A Sepharose Binding of Rat Liver Sialic Acid O-acetyl-esterases. Left Panel: Aliquots of freshly prepared rat liver membrane fraction (1.7mg protein, panel A), or cytosol fraction (0.58 mg protein, panel B) were assayed for sialic acid-O-acetyl-esterase in 100 mM of either sodium acetate pH 4-6.1 (●), KPi pH 6-7.5 (○) or Tris/HCl pH 7.5-8.5 (■) in the presence (solid line) or absence (broken line) of 0.5 % TX-100, as described under "Methods". Values were corrected for controls at each pH value. Right Panel: Rat liver membranes from the same preparation were extracted with 1% TX-100 in 20 mM KPi pH 6.5, and centrifuged at 100,000g for 30 min at 4°C. One ml of the supernatant was applied onto a 1.5 ml Con A Sepharose column equilibrated in 20 mM KPi pH 6.5 containing 1 % TX-100, and washed with the same buffer; fractions of 250 ul were collected. Rat liver cytosolic fraction was applied directly onto a 1.5 ml bed Con A Sepharose column, washed and collected as described above. Aliquots (70 ul) of the peak fractions of the eluates were assayed against [^{3}H]Neu5,9Ac$_2$ in either 100 mM sodium acetate pH 5, 100 mM KPi pH 6.5 or 100 mM TrisHCl pH 8, in comparison with 70 ul aliquots of the starting material.

possibility of more than one esterase activity. The membrane fraction shows three apparent pH optima at 5, 6.5 and 8. Assays at each pH also showed differing degrees of latency to TX-100. In contrast, the cytosolic sialic acid O-acetyl-esterase exhibited a single pH optimum of approximately 8, and no latency to detergent (not shown). It could still be argued that a major portion of the esterase activity seen in the membrane fraction actually represents trapped cytosolic enzyme. However, as shown in Figure 3 (right panel), a major portion of the detergent-solubilized activity from the membrane fraction bound to Con A Sepharose at all of the pH values studied. In contrast, the cytosolic enzyme showed only a minor component that bound to Con A. This indicates that the membrane-bound enzymes are distinct, and that cross-contamination is minimal.

Comparison of Properties and Inhibition Profiles of Rat Liver Sialic Acid O-acetyl-esterases When assayed at pH5.0, the membrane-associated enzyme had a similar latency (25%) to TX-100 as beta-hexosaminidase (32%), suggesting that it could represent a lysosomal sialic acid-O-acetyl-esterase. The membrane activity with a pH 8 optimum shows a latency of less than 10%, but is distinguished from the cytosolic enzyme because it is a glycoprotein, binding to Con A. The effect of various compounds including p-chloromercuribenzoate (2mM), Mercuric chloride (2mM), EDTA(2mM), 2-Mercaptoethanol(2mM), Sodium Fluoride(10mM), PMSF(2mM), and DFP(2mM) on these activities was studied at various pH values in the presence or absence of TX-100. The patterns of inhibition were similar for the membrane-bound pH 8 (non-latent) and the pH 6.5 (non-latent) activities, suggesting that the latter represents the residual activity of the former. Thus, the activity at pH 6.5 that is latent to TX-100 could be a distinct enzyme, or could be in a different subcellular compartment. For each of the activities the effects of the various compounds appeared to be significantly different (not shown). However, no single inhibitor was capable of distinguishing conclusively between them. All of the cytosolic and membrane-associated activities were abolished by DFP, which is a known inhibitor of serine active-site enzymes.

CONCLUSIONS

In this study we have provided biochemical and genetic evidence for multiple sialic acid-O-acetyl-esterase activities that are serine active-site enzymes. We have deliberately limited this survey to enzymes that can cleave 9-O-acetyl groups. Further studies with other O-acetylated substrates, purification of the individual enzymes, and analysis of kinetic properties are required before the specificity, number and relationships of these enzymes can be clearly established. The exact role of each of activities in the metabolism of O-acetylated sialic acids also remains to be defined. Biological roles for lysosomal and cytosolic enzymes are easiest to predict. Since oligosaccharide-bound sialic acids are relatively resistant to neuraminidases, a lysosomal esterase could facilitate primary degradation of glycoconjugates. On the other hand, if some O-acetylated sialic acids are released intact by lysosomal neuraminidases, they would presumably be transported to the cytosol by the recently recognized sialic acid exporter (24,25). However, such molecules would be relatively or absolutely resistant to further degradation by sialic acid lyase (2), and also to re-utilization by CMP-sialic acid synthase (6). Thus, the cytosolic esterase could function to facilitate "recycling" of such free O-acetylated sialic acids in the cytosol.

It is surprising that the major membrane-bound activity (pH optimum 8) is essentially non-latent to non-ionic detergent, in preparations where vesicle integrity appears to generally good. One possible explanation is that the active site of this enzyme is primarily on the outer face of the plasma membrane, which would be exposed in the plasma membrane-derived vesicles. The presence of sialic acid O-acetyl-esterase activity in the same vesicles where O-acetylation of sialic acids takes place is also surprising. Our studies on the O-acetylation mechanism suggest that this event should occur in the latter part of the Golgi apparatus, perhaps in the recently described trans-Golgi tubular network (26). This region represents a possible point of intersection between endocytic and exocytic pathways, and indirectly between the plasma membrane and the Golgi apparatus. Thus, it is possible that these O-acetyl-esterases and O-acetyl-transferases are involved in a de-O-acetylation/re-O-acetylation cycle that is responsible for maintaining a steady state level of O-acetylation. We are currently searching for evidence for such a pathway.

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