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STUDIES OF LYSOSOMAL SIALIC ACID METABOLISM: RETENTION OF SIALIC ACID BY SALLA DISEASE LYSOSOMES

Adam J. Jonas

Department of Pediatrics, University of Texas Medical School at Houston, Texas, 77030

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Purified rat liver lysosomes were incubated in 0.2 M sialic acid resulting in an increase in lysosomal free sialic acid of 3.8 ± 1.5 nmol /unit β hexosaminidase. Sialic acid loss by these lysosomes was stimulated 2-3 fold by 25 mM sodium phosphate. Loss of sialic acid by lysosomes from cultured human diploid fibroblasts was similar to that observed in rat liver lysosomes while loss of sialic acid by lysosomes from cultured fibroblasts from a patient with infantile Salla disease occurred much more slowly. Salla disease appears to be the consequence of defective lysosomal transport of sialic acid and is analagous to cystinosis, a disorder of lysosomal amino acid transport.

The lysosomal efflux of small molecular weight substances, the products of degradative processes, is an area of increasing interest. Studies of human cells have revealed a lysosomal transport system for cationic amino acids as well as a unique lysosomal transport system for the amino acid cystine (1-5). Similar systems have now been demonstrated in rat liver lysosomes (6). A defect in the lysosomal transport of cystine has been demonstrated to be the basis of the human disorder cystinosis, a disease which results in renal failure and which is marked by lysosomal cystine storage (1-4). Studies of the lysosomal transport of simple sugars have been performed in rat liver lysosomes and have implicated a system of facilitated diffusion in sugar transport (7-10). No disorder of lysosomal sugar transport has as yet been recognized in humans.

Salla disease is an autosomal recessive human disorder which is characterized biochemically by the lysosomal storage of free sialic acid (11, 12). Two forms of the disorder exist, an acute infantile form and a more slowly progressive adult form (13, 14). Affected individuals develop ataxia,

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coarse facial features, and psychomotor retardation. Investigation of the cells of these individuals has shown that lysosomal neuraminidase activities are normal (15), leaving no explanation for the accumulation of free sialic acid in this disorder. Recent studies have suggested disordered transport of lysosomal sialic acid in Salla disease (16). We have questioned whether this disorder is analagous to cystinosis, a disorder of lysosomal cystine transport. Sialic acid metabolism in lysosomes from rat liver and cultured human fibroblasts has been examined in order to determine whether Salla disease is the consequence of a defect in the lysosomal transport of sialic acid.

Materials and Methods

Materials: Unless otherwise noted, all chemicals and enzyme substrates were of the highest purity available and were obtained from the Sigma Chemical Company. Percoll density centrifugation media was obtained from Pharmacia. Coon's modified Ham's F-12 medium and fetal bovine serum were obtained from Irvine Scientific Company. N-acetyl [4,5,6,7,8,9 ^{14}C] neuraminic acid was obtained from the Amersham Corporation. Sprague-Dawley female rats (150 - 200 g) were obtained from the Timco Company.

Assays: Lysosomal sialic acid content was measured by fluorometric assay (17). β hexosaminidase was measured fluorometrically (18). One unit of β hexosaminidase activity was defined as the release of one umol of 4-methylumbelliferone per minute. Protein content was determined spectrophotometrically (19).

Fibroblast Culture: Fibroblasts were grown in Coon's modified Ham's F-12 medium with 10% fetal calf serum in roller bottles gassed with 10% CO_2 and 90% air (20). Infantile Salla disease fibroblasts were generously supplied by Dr. Roger Stevenson.

Lysosomal Purification: Lysosomes were purified approximately 89 fold from rat liver by density gradient centrifugation (6). Lysosomes were prepared from cultured fibroblasts by differential centrifugation and Percoll density gradient centrifugation (21, 22). Fibroblast gradients (1.2 ml crude granular fraction suspension: 0.8 ml buffered Percoll) were centrifuged in 3 ml centrifuge tubes for 30 minutes at 40,000 g at 4°C. The lower 1.5 ml was removed, diluted to 12 ml with 0.25 M sucrose, 20 mM Hepes, pH 7.0 and centrifuged for 10 minutes at 11,000 g. The lysosomal pellet thus obtained was used for further studies.

Lysosomal Loading: Lysosomes were incubated at pH 7.0 in 200 mM sialic acid, neutralized with either Tris base or sodium hydroxide, for 30 minutes at 25°C unless otherwise specified. Lysosomes were then diluted 10-fold with ice cold 0.25 M sucrose, 20 mM Hepes, pH 7.0 and collected by centrifugation. The pellet was resuspended and centrifuged an additional time.

Lysosomal Efflux: Lysosomes were suspended in Hepes sucrose buffer at 4°C. Incubations were initiated by adding 20 μl of lysosomal suspension to 1 ml of 0.25 M sucrose, 20 mM Hepes with 0.4 mg/ml of human serum albumin at 25°C (6). Lysosomes were collected by centrifugation for 2 minutes in a microcentrifuge and the pellets were broken by sonication in 150 μl of

distilled H_2O. The sonicate was assayed for both β hexosaminidase activity and sialic acid content. Lysosomes loaded with [^{14}C] sialic acid were collected on glass fiber filters by vacuum filtration and were washed with 5 ml of buffer. Sialic acid content was determined by liquid scintillation counting.

Results

Sialic acid uptake by purified rat liver lysosomes was both temperature and time dependent (Figure 1). Results were similar whether uptake was measured using fluorometric assay or using radiolabeled sialic. Loading, measured by both methods, was enhanced by as much as 50% at 25°C in the presence of the protonophore CCCP. Lysosomes loaded for 30 minutes at 25°C accumulated 3.8 $\pm$ 1.5 nmol sialic acid/unit β hexosaminidase (mean $\pm$ standard deviation, n = 14) as measured by fluorescent assay (corrected for background reactive material). This represented a three-fold rise ($P < 0.001$ by Student's t test) over background assay values of unloaded lysosomes (2.0 nmol sialic acid $\pm$ 0.5, mean $\pm$ s.d., n = 14).

Loaded rat liver lysosomes which were frozen and thawed 5 times before collection and assay lost both 97% of their β hexosaminidase activity and 95% of their sialic acid content when compared to unfrozen samples. Lysosomal sialic acid thus appeared to be soluble and internalized within the lysosome and not merely bound to the lysosomal surface.

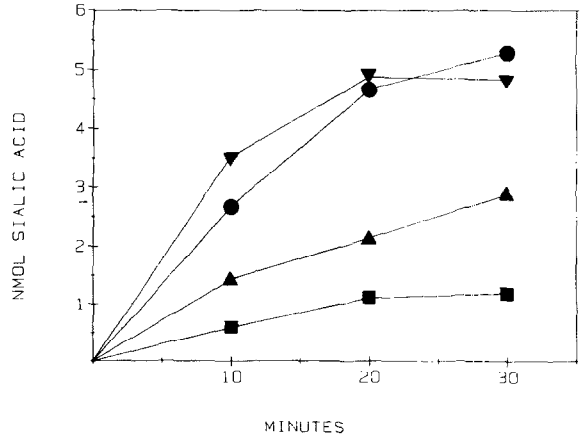

Figure 1: Uptake of [^{14}C] sialic acid by rat liver lysosomes.
Lysosomes were incubated in 0.2 M sialic acids as described in methods. Uptake was determined at 10°C ■, at 25°C ▲, at 37°C ●, and at 25°C in the presence of 10 uM CCCP▼.

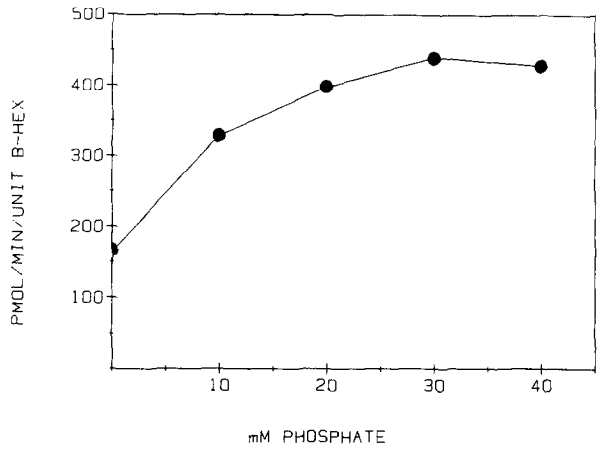

Figure 2: Sodium phosphate stimulation of sialic acid loss by rat liver lysosomes.
Sialic acid loaded lysosomes were incubated in 0.25 M sucrose, 20 mM Hepes, pH 7.0 for 5 minutes at 25°C. Values represent the decrease in lysosomal sialic acid content. Points represent duplicate values.

Incubation of sialic acid loaded rat liver lysosomes in 0.25 M sucrose, 20 mM Hepes, pH 7.0 with 0.4 mg/ml human serum albumin resulted in the loss of sialic acid (Figure 2). Unloaded lysosomes showed no change in their background values by fluorometric assay over a 1 hour period at 25°C. Thus, the changes observed in the sialic acid content of loaded lysosomes were unrelated to background variability. The β hexosaminidase content of samples did not change significantly over the incubation period reflecting lysosomal integrity. Loss of lysosomal sialic acid was stimulated approximately two-threefold by the presence of 25 mM phosphate (Figure 2) and was unaffected by either 20 mM NaCl or 2 mM $MgCl_2$. Sialic acid loss was low when buffer pH was 6.2 (19 pmol/min/ β hex) but increased when buffer was at pH 7.2 (271 pmol/min/ β hex) or pH 8.2 (350 pmol/min/β hex).

Lysosomes were prepared from 9 roller bottles of normal cultured human diploid fibroblasts and from 4 roller bottles of fibroblasts from a patient with infantile Salla disease. Salla disease lysosomes were distributed widely throughout the density gradient and were not concentrated at a particular density. Lysosomes from both cell types were purified approximately 9-fold as measured by the specific activity of β hexosaminidase. Loaded normal fibroblast lysosomes contained 5.0 nmol sialic acid/unit β hexosaminidase

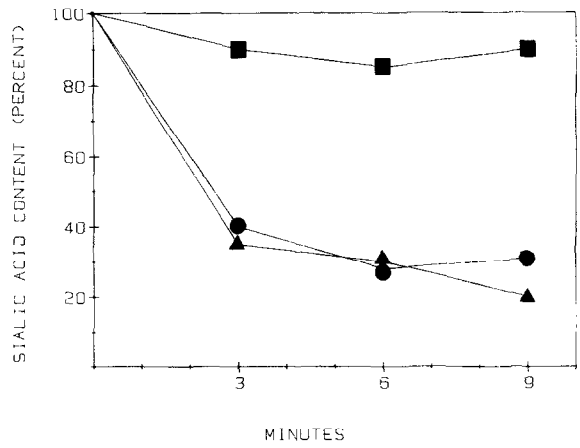

Figure 3: Sialic acid loss by Salla disease lysosomes.
Lysosomes from sialic acid loaded rat liver and cultured normal human diploid fibroblasts were incubated in buffer with 25 mM phosphate. Lysosomes from Salla disease cultured fibroblasts were isolated and similarly incubated. Sialic acid content at zero time was 1.9 nmol/unit β hex for rat liver lysosomes▲, 5.0 nmol/unit β hex for normal fibroblasts lysosomes ●, and 229 nmol/unit β hex for Salla disease fibroblast lysosomes■.

while lysosomes from Salla fibroblasts contained 229 nmol sialic acid/unit β hexosaminidase. When loaded, normal fibroblast lysosomes were frozen and thawed, they lost 93% of their sialic acid content and 95% of their β hexosaminidase activity. When incubated in buffer containing sodium phosphate, normal fibroblast lysosomes, similar to rat liver lysosomes, rapidly lost their sialic acid content (Figure 3). Experiments using Salla fibroblast crude granular fractions showed virtually no loss of sialic acid content over a 30 minute period. Experiments with gradient purified Salla fibroblast lysosomes showed little, if any loss of sialic acid content (Figure 3).

Discussion

Sialic acid loading and loss in rat liver and cultured human fibroblast lysosomes appears to be similar. The finding that the protonophore CCCP improves the loading of lysosomes with sialic acid is particularly intriguing. CCCP has been demonstrated to enhance the rate of lysosomal alkalinization in the presence of permeable monovalent cation (23). It may be that conditions are more favorable for the movement of sialic acid into lysosomes when the lysosomal interior is alkaline. This would be consistent

with the observation that sialic acid appears to be more readily lost by lysosomes when the external pH is alkaline. Alternatively, movement of sialic acid across the lysosomal membrane may involve the symport or counter transport of monovalent cations. The role of sodium phosphate in sialic acid loss by lysosomes is currently unclear and will require further study.

The slow loss of sialic aicd by Salla cell fibroblast lysosomes is compatible with a defect in the transport of sialic acid. This finding is analagous to findings in lysosomes from patients with cystinosis where the loss of lysosomal cystine is markedly impaired. Thus, Salla disease appears to be caused by an inherited defect in lysosomal sugar transport while cystinosis is the result of the inherited defect in lysosomal amino acid transport. The transport system for cystine is quite specific and is dependent upon pH gradients as well as divalent cations. Investigations are under way to examine the transport of sugars other than sialic acid in Salla disease lysosomes.

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