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Defective lysosomal release of glycoprotein-derived sialic acid in fibroblasts from patients with sialic acid storage disease

Klaus MENDLA,* Joachim BAUMKÖTTER,† Charlotte ROSENAU, Barbara ULRICH-BOTT and Michael CANTZ‡

Institute of Pathochemistry and General Neurochemistry, University of Heidelberg, Im Neuenheimer Feld 220, D-6900 Heidelberg, Federal Republic of Germany

Fibroblasts from patients with sialic acid storage disease (SASD), sialidosis, mucolipidosis II, and from normal controls, were incubated in the presence of the glycoprotein fetuin that was tritium-labelled in its sialic acid residues by the periodate/[^{3}H]borohydride reduction method, and the fate of the intracellular radioactive sialic acid (C_7-sialic acid) followed in pulse–chase experiments. The model glycoprotein was readily endocytosed and degraded, more than 90% of the radioactivity being trichloroacetic acid (TCA)-soluble after 4 days of incubation. In all of the patients' fibroblasts, there was an increased accumulation of TCA-soluble radioactivity and, upon chase, a much lower rate of elimination than in normal controls. Gel chromatography of the material from the chase experiment showed that, in normal cells, most of the radioactivity at zero time behaved as free C_7-sialic acid. This, as well as material of larger size (sialyloligosaccharides), was very much diminished by 48 h. In cells from two patients with SASD, there were large peaks both in the sialic acid and oligosaccharide positions; whereas the oligosaccharides were somewhat decreased by the end of the chase period, the sialic acid was essentially unchanged. In sialidosis fibroblasts, the radioactive material consisted of oligosaccharides, but very little C_7-sialic acid; the elimination of the oligosaccharides was retarded. In normal cells, about 80% of the radioactivity released into the medium after 48 h chase behaved as free C_7-sialic acid upon gel chromatography and t.l.c. Subcellular fractionation in Percoll gradients showed that the radioactive C_7-sialic acid remaining in normal cells after 48 h of chase was mainly localized in the cytosol. In SASD cells, on the other hand, it was associated with lysosomal fractions which, unexpectedly, exhibited an abnormally low density. Our findings demonstrate that SASD fibroblasts degrade the sialoglycoprotein but, unlike normal cells, accumulate the liberated C_7-sialic acid along with sialyloligosaccharides in their lysosomes. The results therefore support the concept of a defective transport system for sialic acid in the lysosomal membrane of patients with SASD.

INTRODUCTION

Sialic acids are constituents of many glycoproteins and glycolipids. Genetic defects in the catabolism of these glycoconjugates due to deficient activity of an oligosaccharide (glycoprotein)-specific sialidase cause the lysosomal accumulation of sialic acid-containing oligosaccharides and lead to clinical disorders which have been termed sialidoses (Lowden & O'Brien, 1979; Cantz, 1982). Intralysosomal accumulation and increased concentration in body fluids of free sialic acid is associated with another group of genetic disorders, appropriately called sialic acid storage disease (SASD), or 'Salla' disease after the Finnish district where it was first discovered (Aula *et al.*, 1979; Hancock *et al.*, 1982; Tondeur *et al.*, 1982; Stevenson *et al.*, 1983). The clinical symptoms include mental and motor retardation, coarse facial features, small stature, and neurological degeneration, with considerable heterogeneity of disease (Baumkötter *et al.*, 1985).

As sialidase and other enzymes involved in sialic acid metabolism were found to function normally in SASD cells, it was suggested that the lysosomal release of free sialic acid was impaired because of a defective transport system in the lysosomal membrane (Hancock *et al.*, 1983; Renlund *et al.*, 1983). As the accumulated sialic acid should arise from the catabolism of sialoglycoproteins and gangliosides, we reasoned that the fate of the liberated sialic acid could conveniently be followed by allowing cultured fibroblasts to endocytose the glycoprotein fetuin which was radioactively labelled in its sialic acid residues. For the labelling of fetuin, we used the periodate oxidation/[^{3}H]borohydride reduction method (Van Lenten & Ashwell, 1971), which converts sialic acid residues into a labelled, C_7, amino sugar that is still susceptible to sialidase but is otherwise not metabolized. The metabolism of the model glycoprotein was then studied in pulse–chase and subcellular fractionation experiments.

Parts of this work have been published in abstract form (Baumkötter *et al.*, 1984, 1986).

MATERIALS AND METHODS

Fetuin (purified from fetal calf serum), *Vibrio cholerae* neuraminidase and bovine serum albumin were pur-

Abbreviations used: SASD, sialic acid storage disease; TCA, trichloroacetic acid; sialidase, neuraminidase, *N*-acetylneuraminosylglycohydrolase (EC 3.2.1.18).

* Present address: Department of Biochemistry, Boehringer Ingelheim Co., D-6507 Ingelheim, Federal Republic of Germany.

† Present address: Department of Pediatrics, Technical University of Munich, D-8000 München 40, Federal Republic of Germany.

‡ To whom correspondence and reprint requests should be addressed.

chased from Serva, Heidelberg, Federal Republic of Germany. Sodium boro[^{3}H]hydride with a specific activity of 7.6 Ci/mmol was obtained from NEN, Dreieich, Germany. Instagel was obtained from Packard. Cellulose high performance t.l.c. plates and Fractogel TSK HW-40 S, were purchased from Merck, Darmstadt, Germany. Sephadex G-25 and Percoll were obtained from Pharmacia, Freiburg, Germany. AG 1 × 8 resin (acetate form, 200–400 mesh) was from Bio-Rad, München, Germany. All other chemicals were of analytical grade.

Cell culture

Human diploid fibroblasts were cultured from skin biopsies of patients with genetic metabolic disorders and from normal subjects as controls, and were maintained in Eagle's minimum essential medium (Serva, Heidelberg, Germany) supplemented with 10% fetal calf serum (Boehringer, Mannheim, Germany) and antibiotics as described (Cantz *et al.*, 1972). The fibroblast lines were in their 7th to 11th passage.

Diagnosis of the patients was based on appropriate biochemical tests including determination of lysosomal hydrolases; the SASD cell lines were from recently reported patients with the infantile form (Baumkötter *et al.*, 1985; Wolburg-Buchholz *et al.*, 1985).

Preparation of [^{3}H]sialic acid-labelled fetuin and ^{3}H-labelled sialic acid

The basic methodology was that of Van Lenten & Ashwell (1971). To 409 mg of fetuin containing 94 μmol of sialic acid [determined according to the method of Skoza & Mohos (1976)], and dissolved in 20 ml of 0.1 M-sodium acetate buffer (pH 5.5), 1 ml of 0.141 M-$NaIO_4$ was added on ice. After 20 min stirring in the dark, 3 ml of ethylene glycol was added by stirring for another 20 min. The reaction mixture was dialysed exhaustively, first against distilled water, and then against 0.1 M-$NaHCO_3$/0.1 M-NaCl. To the dialysed fetuin solution, 100 mCi of sodium boro[^{3}H]hydride dissolved in 1 ml of 0.01 M-NaOH was slowly added by stirring at ambient temperature in a ventilated isotope cabinet. After 3 h, 3.76 mmol of 'cold' sodium borohydride dissolved in 1 ml of 0.01 M-NaOH was very slowly added (to avoid excessive foaming) by stirring, and the mixture was stirred for another 2 h. Reduction was stopped by adjusting the pH to 4.5 with 10 M-acetic acid. The reaction mixture was dialysed at 4 °C for 20 h against several changes of 0.15 M-NaCl, and the labelled fetuin further purified by chromatography on a column of Sephadex G-25 (2.6 cm × 65 cm, equilibrated and eluted with 0.15 M-NaCl). The peak fractions eluting with the void volume were pooled. The specific radioactivity of the final product was 53.6 μCi/mg of protein.

A portion of the radioactive fetuin was subjected to enzymic hydrolysis by *N*-acetylneuraminosylglycohydrolase (neuraminidase) to obtain labelled C_7-sialic acid for use as a chromatography standard. [^{3}H]Fetuin (0.11 mg) was incubated for 4 h at 37 °C in the presence of 50 μmol of sodium acetate buffer, pH 5.5, 10 μmol of $CaCl_2$, 15 μmol of NaCl and 0.2 unit of *Vibrio cholerae* neuraminidase in a total volume of 1 ml. The reaction mixture was dialysed against 400 ml of water and the dialysate containing the free sialic acid was lyophilized and redissolved in 30 ml of 0.15 M-NaCl. Nearly 100% of the label after such neuraminidase treatment was recovered in the dialysate fraction, indicating specific labelling of sialic acid residues in the fetuin preparation.

Incorporation studies with [^{3}H]sialic acid-labelled fetuin

Labelled fetuin was added to maintenance medium (Eagles minimum essential medium containing 10% fetal calf serum) at a final radioactivity of 3.75 μCi/ml and was sterilized by filtration (Schleicher and Schüll, disposable filter holder FP 030/2). In some experiments, serum-free Waymouth MAB 87/3 medium (Seromed Biochrom, Berlin, Germany) containing 1.6 g of $NaHCO_3$/l, 192 i.u. of insulin/l, 10000 units of penicillin/l and 100 mg of streptomycin/l, and the same amount of radioactive fetuin as above, was used. Fibroblasts grown to confluency in 35 mm Petri dishes (Falcon) were incubated at 37 °C in the presence of 2 ml of the radioactive medium in an atmosphere of 19:1 air/CO_2. Cells were harvested by trypsinization (Cantz *et al.*, 1972), washed three times with 5 ml of 0.15 M-NaCl, and resuspended in 800 μl of 0.15 M-NaCl. After ten freeze–thaw cycles, portions of the well-mixed homogenate were taken for protein determination (Lowry *et al.*, 1951). To another portion, bovine serum albumin at a final concentration of 2 mg/ml (as a carrier) and trichloroacetic acid (TCA) at a final concentration of 10% (w/v), were added. After 20 min standing on ice, the suspension was centrifuged. Supernatant was removed and counted in a liquid-scintillation counter (Searle Mark III) using Instagel. Precipitate was washed three times (500 μl of 10% TCA) and redissolved by heating at 95 °C for 10 min in 500 μl of 0.5 M-NaOH/10% (w/v) SDS.

Gel chromatography of [^{3}H]sialic acid-labelled material

Products generated by the intracellular degradation of labelled fetuin were analysed on a column (2.6 cm × 90 cm) of Fractogel TSK HW-40S, equilibrated, and eluted with 0.15 M-NaCl/0.02% (w/v) NaN_3. TCA supernatant (300 μl) was applied to the column which was eluted at a rate of 37 ml/h, fractions of 2.67 ml being collected. The column was operated at 4 °C and calibrated with labelled fetuin (V_0) and labelled C_7-sialic acid.

T.l.c.

Radioactive fractions obtained upon gel chromatography eluting in the position of free sialic acid were desalted on a column (2.6 cm × 60 cm) of Fractogel TSK HW-40S using water as eluent. The radioactive fractions were pooled, lyophilized, and reconstituted with 1 ml of water. Samples were subjected to chromatography on cellulose high performance t.l.c. plates using the solvent system butan-1-ol/propan-1-ol/0.1 M-HCl (1:2:1, by vol.) according to Schauer & Corfield (1982). ^{3}H-labelled sialic acids were detected using a radioactivity scanner (Berthold automatic TLC linear analyser, Berthold, Wildbad, Federal Republic of Germany); unlabelled *N*-acetylneuraminic acid was visualized by spraying with Ehrlich's reagent. Labelled C_7-sialic acid, prepared as described above, served as a standard and showed an R_F of 0.58, the same as cited in the literature (Schauer & Corfield, 1982).

Subcellular fractionation studies

Fibroblasts grown to confluency in 75 cm^2 Falcon plastic flasks (2 weeks after subculture) were incubated

for 4 days in 20 ml of medium containing 76.3 μCi of radioactive fetuin and 'chased' for 2 days, using unlabelled medium. Subcellular fractionation was performed on gradients of Percoll and the marker enzymes β-*N*-acetylhexosaminidase (lysosomes), lactate dehydrogenase (cytosol), succinate dehydrogenase (mitochondria), galactosyltransferase (Golgi) and 5'-nucleotidase (plasma membrane) of the fractions determined using the methodology of Rome *et al.* (1979). Centrifugation was for 2.5 h (18000 rev./min, 4 °C) in a Sorvall OTD 65 centrifuge using a Sorvall TV 850 rotor and 1 × 3.5 in polyallomer tubes. Fractions (1 ml) were collected from the bottoms of the tubes.

Latency of the lysosomal marker β-hexosaminidase in postnuclear supernatants was 85% for the normal control and 75% for SASD cells.

Radioactivity of the subcellular fractions was measured in 100 μl portions. To determine the amounts of free and bound [^{3}H]sialic acid, the fractions corresponding to the 'heavy' and 'light' lysosomal and cytosolic compartments, respectively, were pooled and applied to AG 1 × 8 columns (0.5 cm × 5 cm), which were eluted with 20 ml of a linear gradient of 0–200 mM-sodium acetate; oligosaccharide-bound sialic acid was eluted at acetate concentrations below 70 mM, whereas free C_7-sialic acid was eluted at a concentration of 130 mM.

RESULTS

Uptake and degradation of labelled fetuin

Fibroblasts cultured from patients with SASD and normal controls were incubated for various time intervals in medium containing the glycoprotein fetuin which was ^{3}H-labelled in its sialic acid residues. For comparison, we also included fibroblasts from patients with sialidosis, which are deficient in a lysosomal sialidase specific for oligosaccharides and glycoproteins (Mendla & Cantz, 1984), and fibroblasts from patients with mucolipidosis II, which have a deficiency of this sialidase as well as other lysosomal hydrolases because of a general defect in their transport to lysosomes (Neufeld & McKusick, 1983).

Normal control cells incorporated about 0.6% of the radioactivity added to the medium per mg of cell protein during a 48 h incubation. This value was not much different when the labelling was performed in serum-free medium, indicating the uptake to be independent of the concentration of unlabelled fetuin in the fetal calf serum and thus presumably due to bulk pinocytosis. When the rate of endocytosis of the labelled fetuin was estimated from uptake and elimination rates, there was some variation between different cell lines, values ranging from 0.02 to 0.06%/h per mg of cell protein.

Cell-associated radioactivity was separated into a high molecular mass, a TCA-precipitable, and a low molecular mass, TCA-soluble, fraction. After 4 days of incubation, more than 90% of the radioactivity appeared in the TCA-soluble fraction in each of the cell lines, indicating an efficient degradation of the glycoprotein (Table 1).

Kinetics of the low molecular mass material is shown in Fig. 1. Whereas normal cell radioactivity reached a plateau after about 4 days, the patients' cells continued to accumulate radioactive material (Fig. 1*a*). In SASD cells, this accumulation amounted to nearly three times that of the normal control after 7 days. In sialidosis and mucolipidosis II fibroblasts, the level of accumulation was only slightly higher than in the control by the end of the experiment; in these cells, the rate of uptake of the glycoprotein was generally lower, however. When the fate of the TCA-soluble radioactivity was followed in chase experiments, normal control cells lost half of their radioactivity in about 22 h, whereas this rate was markedly decreased in the SASD and the other patients' lines (Fig. 1*b*).

Table 1. Intracellular radioactivity of TCA-soluble and TCA-precipitable fractions after 4 days of incubation in medium containing [^{3}H]sialic acid-labelled fetuin

Cell line	Radioactivity (c.p.m./mg of protein)	
	TCA-soluble	TCA-precipitable
Normal control	282130	13470
SASD patient	548870	13180
Sialidosis	270670	10970
Mucolipidosis II	167810	11060

TCA-soluble material from the chase experiment was fractionated according to size using gel chromatography (Fig. 2). In normal cells, most of the radioactivity at zero time was eluted in a position identical to authentic, free sialic acid. This peak, as well as other material of larger size (sialyloligosaccharides), was very much diminished by 48 h. In cells from two patients with SASD (Fig. 2 shows only one), there were large peaks both in the oligosaccharide and the sialic acid positions. The 'oligosaccharide' peaks were somewhat decreased by the end of the chase period, whereas the 'sialic acid' peak was still very prominent. In the fibroblasts from the sialidosis patient, the bulk of the radioactive material was in one major and several minor peaks of a size suggestive of oligosaccharides, with only a small peak in the sialic acid position; by 48 h, these fractions were only moderately decreased.

T.l.c. of the material eluting in the position of free sialic acid showed it to have the same R_F value (0.58) as the C_7-sialic acid used as a standard, both in normal control and SASD fibroblasts (Fig. 3).

Loss of cell-associated radioactivity during chase was quantitatively accounted for by its appearance in the culture medium. In normal cells, about 80% of the medium radioactivity after a 48 h chase period behaved as free sialic acid when analysed by gel chromatography, the remainder being found in the void volume and therefore consisting of large oligosaccharides or glycopeptides (results not shown). Material eluting in the position of free sialic acid was identified as C_7-sialic acid upon t.l.c. (Fig. 3). In the medium from SASD fibroblasts, only very little free C_7-sialic acid could be detected (results not shown).

Subcellular localization of ^{3}H-labelled sialic acid

To examine whether the site of storage in SASD cells is indeed the lysosomes, the subcellular distribution of the sialic acid-labelled degradation products obtained after a 48 h chase period was analysed by subjecting postnuclear supernatants to fractionation in Percoll

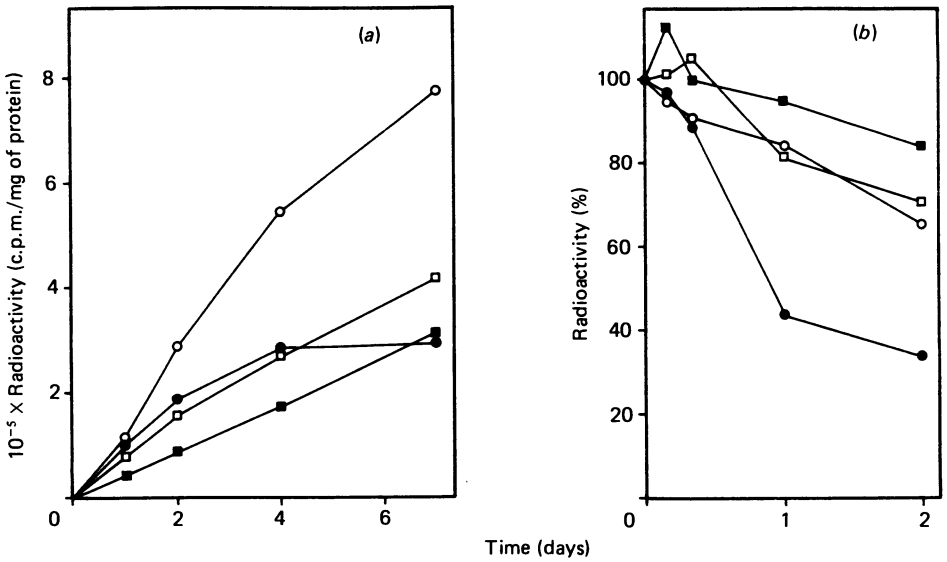

Fig. 1. Accumulation (*a*) and chase (*b*) of intracellular TCA-soluble radioactivity from sialic acid-labelled fetuin in fibroblasts of patients and controls

Confluent cultures were incubated in the presence of radioactive fetuin as described in the Materials and methods section. At intervals, the cells were homogenized and the TCA-soluble radioactivity determined. After 7 days, labelled medium was replaced by unlabelled and the disappearance of TCA-soluble radioactivity recorded. ○, SASD patient; □, sialidosis; ■, mucolipidosis II; ●, normal control.

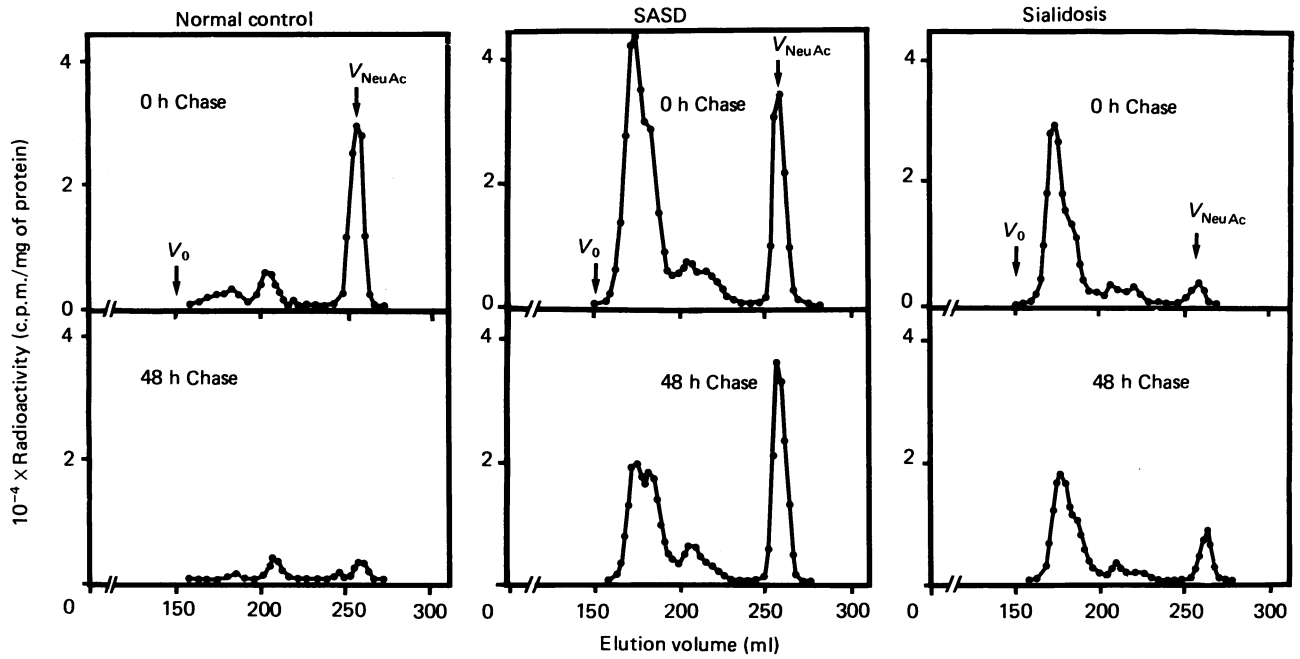

Fig. 2. Gel chromatography of intracellular TCA-soluble radioactivity in normal, SASD and sialidosis fibroblasts

TCA-soluble material, obtained after 7 days of incubation in the presence of labelled fetuin (0 h chase) and 2 days of subsequent chase (48 h chase), was fractionated on a column of Fractogel TSK HW-40S, as described in the Materials and methods section. Abbreviation: NeuAc, 5-acetamido-3,5-dideoxy-L-heptulosonic acid.

gradients (Fig. 4). In normal control cells, the bulk of the radioactivity was associated with the marker for the cytosol, lactate dehydrogenase, whereas the remainder co-distributed with β-hexosaminidase, the marker for the 'heavy' and 'light' lysosomal fractions. The amount of 'cytosolic' radioactivity was far in excess of the contribution from broken lysosomes, estimated at 15% from determinations of β-hexosaminidase latency. In SASD cells, most of the radioactivity fractionated with the lysosomal marker which, unexpectedly, was shifted to a position of lower density. The contribution to the 'cytosolic' radioactivity paralleled that of β-hexosaminidase (25%), indicating its exclusive lysosomal origin.

As the intracellular radioactive degradation products

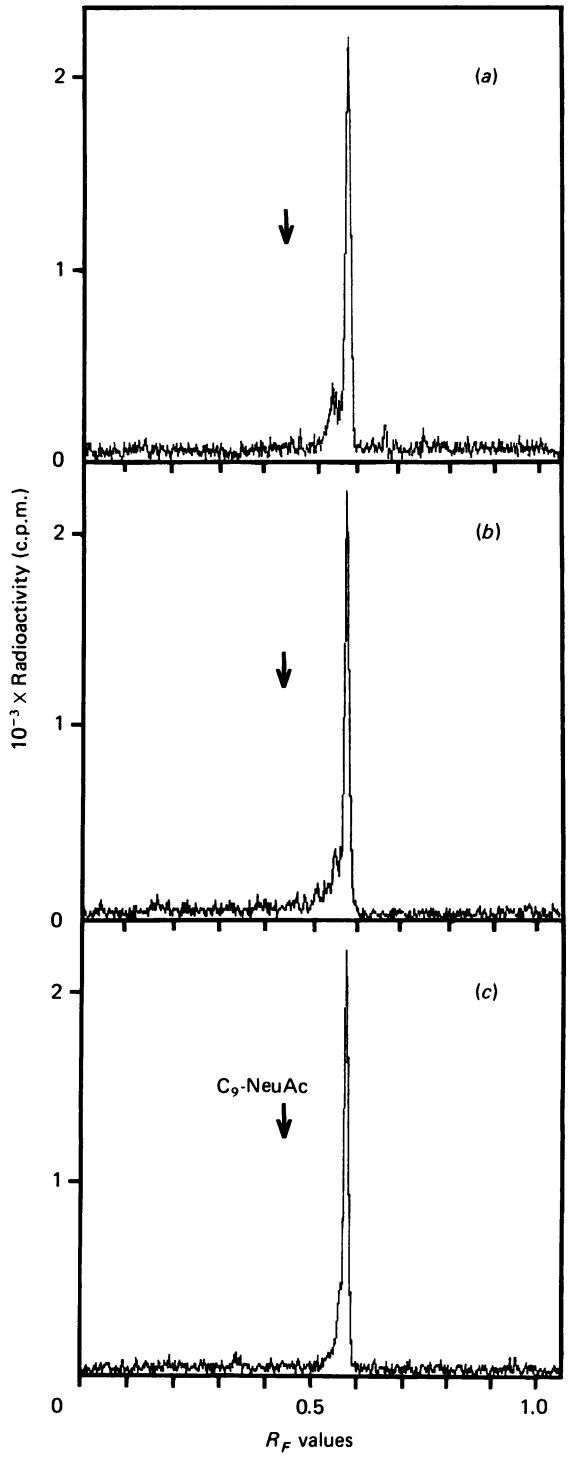

Fig. 3. T.l.c. of labelled sialic acids

(*a*) Normal cells: [^{3}H]sialic acid released into the medium after 48 h chase. (*b*) Normal cells: cell-associated [^{3}H]sialic acid after 48 h chase. (*c*) SASD cells: cell-associated [^{3}H]sialic acid after 48 h chase. The arrow indicates the position of standard C_9-Neu5Ac (5-acetamido-3,5-dideoxy-D-nonulosonic acid) (R_F 0.43); the labelled C_7-sialic acid standard had an R_F of 0.58. Other details are in the Materials and methods section.

consisted of a mixture of free and oligosaccharide-bound sialic acid, the fractions corresponding to the lysosomal and cytosolic compartments, respectively, were pooled and fractionated by ion-exchange chromatography as described in the Materials and methods section. In normal cells, the 'heavy' lysosomal pool contained about 80% of species characteristic of oligosaccharides, the remainder being free C_7-sialic acid, whereas the 'light' lysosomal and 'cytosolic' pools contained nearly exclusively (approx. 90%) material characteristic of free C_7-sialic acid. In SASD fibroblasts, free C_7-sialic acid was the predominant species (about 60%) in the 'lysosomal' as well as the 'cytosolic' pools.

DISCUSSION

Our studies show that the modified glycoprotein fetuin with labelled C_7-sialic acid residues is readily endocytosed by the fibroblasts and subsequently degraded. In normal control cells, the degradation proceeds to the formation of free C_7-sialic acid which is then excreted into the culture medium. In fibroblasts from patients with the lysosomal storage diseases sialidosis and mucolipidosis II, the degradation is impaired because of the deficiency of sialidase and other glycosidases, leading to an accumulation of labelled sialyloligosaccharides. This indicates that the site of the fetuin degradation is indeed the lysosomes. SASD fibroblasts, on the other hand, despite their ability to degrade the glycoprotein, exhibit an abnormal accumulation of free C_7-sialic acid and, in addition, sialyloligosaccharides. Such oligosaccharide accumulation may seen surprising, but an increase of bound beside free sialic acid had previously been observed upon chemical quantification of the storage material (Baumkötter *et al.*, 1985), and may be explained by a secondary inhibition of the sialidase by the accumulated sialic acid, a known competitive inhibitor of this enzyme (Corfield & Schauer, 1982; Mendla & Cantz, 1984). In normal fibroblasts, the C_7-sialic acid that was produced upon the degradation of fetuin was released into the culture medium without further modification. Therefore, the accumulation of sialic acid in SASD cells must be due to a defective cellular release mechanism rather than a block in its metabolism.

Further insight into the pathogenetic mechanism was obtained by an investigation of the subcellular distribution of the labelled C_7-sialic acid. Fractionation of postnuclear supernatants in Percoll gradients from fibroblasts that had been 'chased' for 48 h showed that, in normal cells, most of the sialic acid was localized in the cytosol, the remainder being associated with lysosomal fractions. In SASD cells, on the other hand, the bulk of the sialic acid was found in the lysosomes. Unexpectedly, these lysosomes exhibited a marked decrease in their density which may be due to an influx of water following the osmotic gradient produced by the accumulated sialic acid. Taken together, the findings show that the sialic acid that is generated in the lysosomes upon the degradation of the glycoprotein is normally released into the cytosol. There, it would be utilized further were it not the C_7-analogue that is not amenable to such reactions and is therefore excreted into the culture medium. In SASD fibroblasts, the intralysosomal accumulation of free C_7-sialic acid must be due to a defect in its translocation across the lysosomal membrane, presumably because of a mutated transporter protein specific for sialic acid. By inference, this transporter would recognize both the native C_9- as well as the C_7-sialic acid.

Evidence for defective lysosomal sialic acid transport

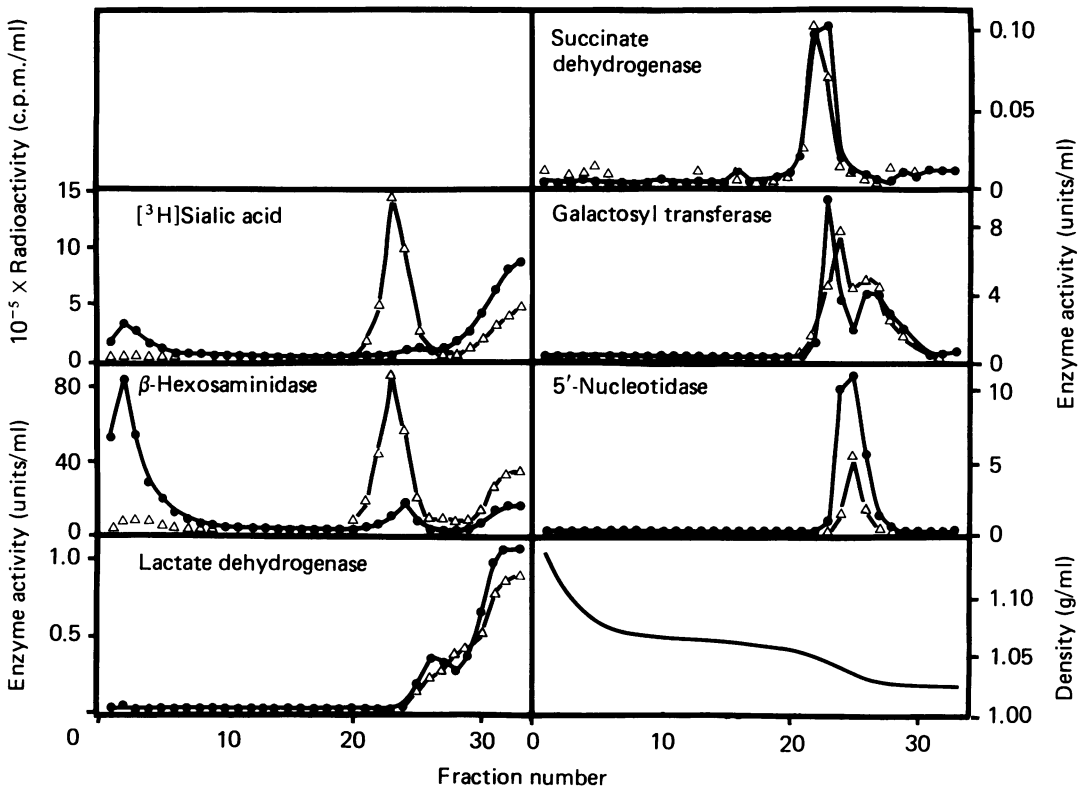

Fig. 4. Subcellular distribution of [^{3}H]sialic acid radioactivity

Fibroblast cultures were incubated for 4 days in the presence of [^{3}H]sialic acid-labelled fetuin and subsequently 'chased' for 48 h. Normal control cells (●; 4.2 mg of cell protein) and SASD cells (△; 1.6 mg of protein) were fractionated on Percoll gradients, as described in the Materials and methods section.

in fibroblasts of patients with various forms of SASD has recently been presented by other investigators (Hildreth *et al.*, 1986; Renlund *et al.*, 1986*a*; Mancini *et al.*, 1986; Paschke *et al.*, 1986). Working with isolated lysosomal fractions from Salla fibroblasts, a defective sialic acid egress (Renlund *et al.*, 1986*b*), or abnormal retention of sialic acid (Jonas, 1986), have been reported. Our present work confirms and extends these studies and shows that fetuin containing labelled C_7-sialic acid residues can conveniently be used to follow the fate of the liberated sialic acid in SASD cells. Indeed, this method has recently been applied to the prenatal diagnosis and confirmation of a foetus with infantile SASD (Vamos *et al.*, 1986).

SASD is a new example of an inherited lysosomal transport disorder, and the first one involving a carbohydrate molecule. Evidence for a transport system of facilitated diffusion of simple sugars has been reported in rat liver lysosomes (Hales *et al.*, 1984). A genetic defect in the lysosomal transport of the amino acid cystine has been shown to cause the lysosomal storage disease cystinosis (Gahl *et al.*, 1982; Schneider & Schulman, 1983), and a disorder of vitamin B_{12} storage appears to be due to an impaired transport of free vitamin B_{12} out of lysosomes (Rosenblatt *et al.*, 1985). In none of these diseases has it so far been possible, however, to characterize the defective carrier at the protein level. Study of lysosomal transport processes may be of increasing importance in defining a whole new group of lysosomal storage diseases.

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