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Infantile Sialic Acid Storage Disease: The Fate of Biosynthetically Labeled N-Acetyl-(^{3}H)-Neuraminic Acid in Cultured Human Fibroblasts

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ABSTRACT. N-acetyl-(^{3}H)-mannosamine [(^{3}H)-ManNAc] was used as a precursor for the metabolic labeling of N-acetyl-(^{3}H)-neuraminic acid [(^{3}H)-NANA] in cultured fibroblasts of a patient with infantile sialic acid storage disease (ISSD). The metabolic fate of free and bound (^{3}H)-NANA, isolated by high-performance liquid chromatography, was followed under pulse–chase labeling conditions. Nonsaturable accumulation of free (^{3}H)-NANA was observed in ISSD, while the metabolic flux from (^{3}H)-ManNAc to NANA-glycoconjugates was unaffected. Accumulated free (^{3}H)-NANA could not effectively be chased from ISSD cells although N-acetyl-(^{3}H)-hexosamines [(^{3}H)-HexNAc] were appearing in the chase medium. These metabolites could arise from (^{3}H)-NANA bound to glycoconjugates which were cleaved at normal rates in ISSD. The finding that free (^{3}H)-NANA was markedly increased relative to its major products (^{3}H)-HexNAc is suggestive for an impaired degradation and reutilization of (^{3}H)-NANA due to trapping in a metabolically unaccessible pool. In titration experiments with digitonin a clear-cut increase in the latency of labeled NANA relative to a cytoplasmic marker enzyme was evident in ISSD. The release of (^{3}H)-NANA, however, followed closely the digitonin–induced release of the lysosomal enzyme β-hexosaminidase. This is suggestive for a lysosomal location of the stored material. (*Pediatr Res* 20: 773–777, 1986)

Abbreviations

ISSD, infantile sialic acid storage disease
NANA, N-acetyl-neuraminic acid
ManNAc, N-acetyl-mannosamine
HexNAc, N-acetyl-hexosamines
TCA, trichloracetic acid
HPLC, high performance liquid chromatography
LDH, lactate dehydrogenase
β-HEX, β-N-acetylhexosaminidase
HVE, high voltage electrophoresis

ISSD has recently been identified as a very rare inherited disturbance in sialic acid metabolism (1, 2). This condition is characterized by hepatosplenomegaly and severe mental and motor retardation of early onset accompanied by a pronounced tissue storage and hyperexcretion of free sialic acid (NANA). Signs of lysosomal storage can be detected by morphological and ultrastructural criteria (2). The biochemical and ultrastructural features are similar to those occurring in another distinct disease entity which is, however, restricted to Finnish patients ("Salla disease") and exhibits a much more protracted clinical course (4).

So far, the underlying biochemical cause of both conditions is unknown. No abnormalities in the composition of cellular glycoconjugates and in the enzymes involved in the lysosomal and cytoplasmic metabolism of NANA were detected. Therefore, a defective transport of free sialic acid across the lysosomal membrane was suggested (5, 6).

Alternatively, high intracellular concentrations of free NANA may arise from increased biosynthesis caused by a defect of metabolic control as recently shown by Thomas *et al.* (7) in a patient with sialuria but without evidence of lysosomal storage.

Only limited information on the flux of NANA through the biosynthetic and catabolic pathways is available so far in Salla disease or ISSD.

In this study we used (^{3}H)-ManNAc to investigate the accumulation, distribution and metabolic fate of labeled (^{3}H)-NANA in cultured fibroblasts of a patient with ISSD.

MATERIALS AND METHODS

N-acetyl-(^{3}H)-mannosamine (26.6 Ci/mmol) was purchased from New England Nuclear, Boston, MA. N-acetyl-(4,5,6,7,8,9-^{14}C)–neuraminic acid (200 mCi/mmol) was from Amersham (Buckinghamshire, England). Unlabeled NANA and ManNAc were obtained from Sigma (Munich, FRG) and digitonin from Merck (Darmstadt, FRG). Culture medium, enzyme solutions, and additions for the culture media were from Serva (Heidelberg, FRG). All other reagents were of analytical grade and obtained from commercial sources.

Cell culture. A fibroblast cell strain was established from a male infant with ISSD diagnosed in our laboratory. A detailed description of clinical, morphological, and biochemical findings will be published elsewhere. In brief, the patient presented with marked hepatosplenomegaly and severe progressive mental and motor retardation. Peripheral lymphocytes and cultured fibroblasts showed pronounced vacuolization. The activities of lysosomal enzymes including sialidase were found to be normal. Ten- to 30-fold levels of free NANA in urine, leucocytes, and cultured fibroblasts confirmed the diagnosis.

Stock cultures were grown in Eagle's basal medium supplemented with Earle's salts, 10% fetal calf serum and 2 mM glutamine (standard medium) as previously described (8). Subcultures were plated in 60-mm dishes and grown to confluency.

Metabolic labeling of NANA. For pulse-chase experiments growth medium was removed, cells washed with phosphate–buffered saline and incubated for up to 72 h at 37° C with 2 ml of a modified standard medium containing 13% fetal calf serum, reduced glucose concentration (0.48 mM), 25 mM Hepes, 10

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mM $NaHCO_3$, and 50 μCi/ml (^{3}H)-ManNAc (specific activity 26.6 Ci/mmol).

In chase experiments the radioactive medium was replaced by 2 ml of standard growth medium after extensive washing with phosphate-buffered saline. At the times indicated the cells were harvested by trypsinization, collected by centrifugation, sonicated in 0.5 ml of water, and further processed exactly as described (9). In brief, after addition of appropriate carrier compounds, TCA-soluble supernatants were analysed for free (^{3}H)-NANA. TCA pellets were subjected to hydrolysis with trifluoroacetic acid. Released (^{3}H)-NANA was taken as a measure for total NANA-sialoglycoconjugates [bound (^{3}H)-NANA]. In analogy, radioactive chase media were treated with TCA and processed according to the same protocol. Final fractions were lyophilized and reconstituted in 300 μl bidistilled water.

Analyses. Isolation of (^{3}H)-NANA was accomplished by HPLC (3). One hundred-μl aliquots of the radioactive material were chromatographed on a HPX-87 H column (300 × 7.8 mm ID, Biorad Laboratories, Vienna, Austria) equipped with a guard cartridge (40 × 4.6 mm ID). Isocratic elution was performed at 22°C for 15 min with 0.0045 N H_2SO_4 (pH 2.5) at a flow rate of 0.6 ml/min. Fractions of 0.4 ml were collected and assessed for radioactivity by liquid scintillation counting. (^{3}H)-NANA was eluted as a single peak at a retention time of 7 min coeluting with unlabeled NANA as detected by UV absorption at 200 nm (pool I). Another radioactive peak (pool II) eluted at a retention time identical to that of (^{3}H)-labeled or unlabeled ManNAc standard.

Radioactive fractions were subjected to ion exchange chromatography on Dowex 1 × 8 (9), thin-layer chromatography (10) and HVE at pH 5.5 (13) to confirm identity and purity. Authentic standards eluted from the HPLC column in the following order: NANA, 2,3-dehydro-NANA, ManNAc, glucosamine, mannose, galactose, and fucose.

Digitonin titration experiments. It has repeatedly been shown that digitonin makes membranes permeable by forming a complex with cholesterol (11, 12). The plasma membrane appears to be especially susceptible for leakage even at very low digitonin concentrations. Therefore, we attempted to define conditions for a maximal release of cytosolic components while leaving lysosomal structures largely intact: After metabolic labeling with (^{3}H)-ManNAc for 4 days cells were harvested by trypsinization, centrifuged, and carefully resuspended in Ca^{++} and Mg^{++}–free Hanks' solution supplemented with 10 mM Hepes (pH 7.0), 0.1% bovine serum albumin, 1 mM EDTA (12).

Aliquots containing approximately 400 μg of cell protein/ml and up to 50 μg/ml digitonin were prepared with the same buffer.

Suspensions were incubated under gentle shaking for 7.5 min at 25° C. Digitonin exposure was terminated by rapid centrifugation at 12,000 × *g* in an Eppendorf centrifuge for 30 s. Pellets and supernatants were separated immediately and analyzed for LDH and β-HEX activities by standard methods (13, 14). (^{3}H)-NANA was analyzed in the same fractions as described above. The sum of digitonin soluble and digitonin resistant fractions was taken as 100% both for enzyme activities and (^{3}H)-NANA content.

RESULTS

Highly specific metabolic labeling of NANA can be achieved by the use of (^{3}H)-ManNAc as radioactive precursor for phosphorylation and aldol condensation with phosphoenolpyruvate (13).

Incubation of fibroblasts with (^{3}H)-ManNAc for up to 3 days and subsequent HPLC isolation of radioactive products in cells and media by HPLC revealed two major peaks that eluted within 15 min (Fig. 1): The first peak (pool I) could be identified as pure (^{3}H)-NANA by elution with 1 M formic acid from Dowex

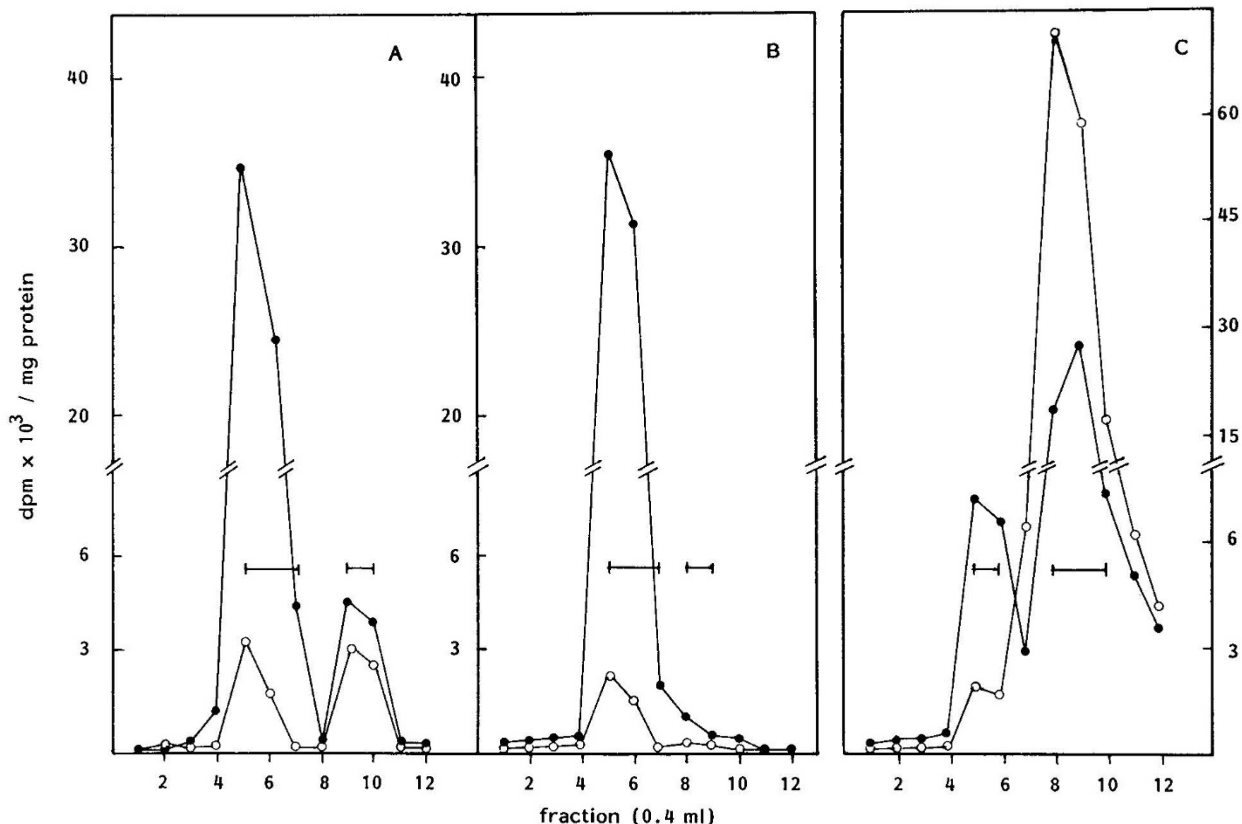

Fig. 1. HPLC profiles of intra- and extracellular TCA-soluble radioactivity after metabolic labeling of ISSD (●——●) and control (○——○) fibroblasts with (^{3}H)-ManNAc. Examples are shown for intracellular material obtained after 48 h pulse labeling (*A*) and a 24 h chase (*B*). Analysis of the culture medium after 72 h a chase is depicted in *C*. Processing of cells and medium and HPLC conditions are described in "Materials and methods." Combined radioactive fractions coeluting with standard NANA (pool I) and ManNAc (pool II) were used for calculations.

1 × 8 (9) and subsequent thin layer chromatography (10). The second peak (pool II) had the same retention time as ManNAc on HPLC. On HVE two radioactive peaks with a mobility identical to (^{14}C)-NANA and (^{3}H)-ManNAc were detected. Their quantitative relationship was very close to HPLC results. Pool II showed no electrophoretic mobility, as is described for N-acetylhexosamines (6, 13) (data not shown).

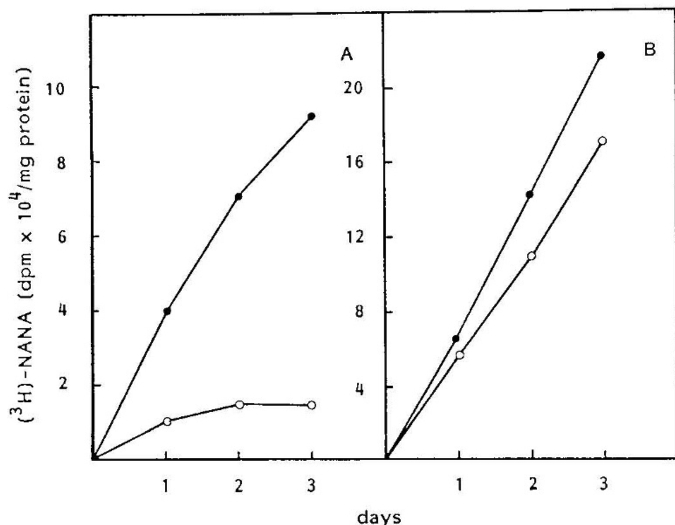

Fig. 2. Time course of radioactivity in NANA after pulse labeling of ISSD (●——●) and control (○——○) fibroblasts with (^{3}H)-ManNAc; *A*: TCA-soluble (free NANA) *B*: TCA-insoluble (protein bound NANA). The mean of duplicate determinations is shown.

Representative HPLC elution profiles of TCA soluble intracellular radioactivity after 48 h of pulse labeling and after a 24 h chase incubation are depicted in Figure 1*A* and *B*. Under both conditions a marked accumulation of (^{3}H)-NANA (pool I) was evident in ISSD cells while pool II showed a marked decay during the chase incubation. Analysis of the medium after a 72-h chase (Fig. 1*C*) revealed large amounts of pool II but also a small (^{3}H)-NANA peak, at least in ISSD cells.

Following the time course of pulse labeling in normal fibroblasts the accumulation of (^{3}H)-NANA approached a steady state within 48 h (Fig. 2*A*).

In ISSD cells (^{3}H)-NANA increased 5- to 10-fold above control levels without reaching an equilibrium after 3 days. No significant difference in the amount of bound (^{3}H)-NANA, liberated by trifluoroacetolysis from the TCA pellets, was observed (Fig. 2*B*). So far, these results confirm the previous data of Thomas *et al.* (9).

In chase experiments, free intracellular (^{3}H)-NANA decreased by 80% within 2 days in controls while no loss at all was observed in ISSD (Fig. 3*A*). A slight increase in the amount of stored (^{3}H)-NANA was observed within the initial chase period. In contrast, intracellular bound (^{3}H)-NANA showed a normal disappearance rate in ISSD (Fig. 3*B*). This is in agreement to the finding of normal sialidase activities in our patient (not shown).

Small amounts of (^{3}H)-NANA were detected in the chase media being markedly higher in ISSD (Fig. 3*D*). Bound (^{3}H)-NANA of culture media increased at a somewhat lower rate than in controls (Fig. 3*E*). As shown above (Fig. 1*C*), the radioactivity in the medium after 72 h of chase incubation consisted almost exclusively (>98%) of (^{3}H)-N-acetylhexosamines (pool II) in control cells. This is in marked contrast to the data in ISSD, where approximately 20% of total (^{3}H) counts are associated with the NANA peak (pool I). The relative amount of radioac-

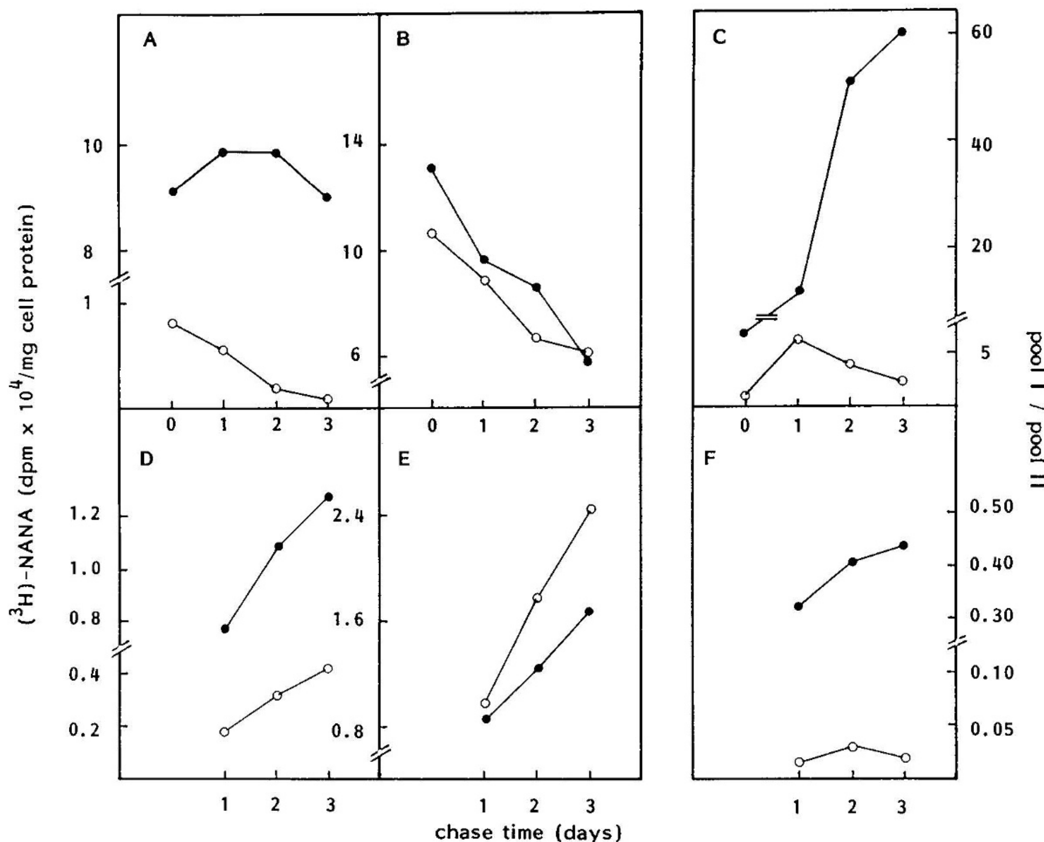

Fig. 3. Decay of (^{3}H) radioactivity associated with NANA. ISSD (●——●) and normal (○——○) fibroblasts were pulse-labeled with (^{3}H)-ManNAc for 48 h. A subsequent chase incubation with standard medium was performed for the times indicated. TCA-soluble (*A*, *D*) and TCA-insoluble (*B*, *E*) (^{3}H)-NANA of cells (*upper panels*) and medium (*lower panels*) was analysed as described in "Materials and methods." *C*, *F*: relative amounts of free (^{3}H)-NANA (pool I) related to (^{3}H)-N-acetylhexosamines (pool II) found in TCA-supernatants of cells and medium.

tivity expressed as the ratio of pool I over pool II varied with increasing chase time (Fig. 3*C* and *F*). At any time point this value was elevated and showed a marked increment with increasing chase time in ISSD cells.

Thus our results demonstrate an intracellular accumulation and an impaired metabolic utilization of biosynthetically labeled free (^{3}H)-NANA. These findings might be explained by an intralysosomal trapping of free (^{3}H)-NANA as previously suggested (2, 6). In order to establish a difference in the subcellular distribution, the latency of free intracellular (^{3}H)-NANA after exposure to digitonin was investigated (Fig. 4).

When fibroblast suspensions were exposed to digitonin concentrations above 7.5 μg/mg cell protein, LDH was solubilized almost quantitatively (more than 90%). Under these conditions only 20 to 35% of β-HEX were extracted. Both cell lines showed identical enzyme release in response to the detergent. In contrast, the shape of the latency curve of (^{3}H)-NANA was clearly different. In ISSD (*panel B*) the (^{3}H)-NANA release closely followed the lysosomal marker enzyme.

A rough estimation of the quantitative distribution of free (^{3}H)-NANA between the LDH-associated ("cytosolic") and β-HEX-associated ("lysosomal") compartments was made on the basis of the following assumptions: 1) The recovery of digitonin-soluble (^{3}H)-NANA is comparable to the recovery of digitonin-soluble marker enzymes. 2) The activity of β-HEX can be taken as an indicator for a minor leakage of intralysosomal material occurring at low digitonin concentrations ("carry over").

The amount of free (^{3}H)-NANA found in the supernatants or in the digitonin-resistent cell pellets was related to the percentage of the marker enzymes in the same fractions. At any given digitonin concentration, which resulted in a clear-cut difference in the distribution between LDH and β-HEX, two equations with two unknowns allowed us to quantitate the LDH-associated and β-HEX associated (^{3}H)-NANA content (Table 1). Based on these calculations the main portion (73%) of stored (^{3}H)-NANA resides in the "lysosomal compartment" in ISSD. This is in marked contrast to control cells which showed a reverse relative distribution of (^{3}H)-NANA (68% in the "cytoplasmic compartment"). Moreover, in controls, the absolute amount of (^{3}H)-NANA associated with β-HEX was only 5% of that of ISSD cells.

DISCUSSION

This work is concerned with the transport and utilization of NANA in cultured fibroblasts of a patient with ISSD. Since NANA itself is poorly incorporated into intact cells (13) we made use of the precursor (^{3}H)-ManNAc for the relatively specific metabolic labeling of NANA (10). In normal cells (^{3}H)-NANA accumulation reached a plateau within 3 days indicating an equilibrium between biosynthesis and degradation (Fig. 2*A*). In ISSD, however, the cellular accumulation of (^{3}H)-NANA was not saturable within 3 days, which is consistent with the hypothesis that a concentration gradient for (^{3}H)-NANA is continuously maintained in favor of the intracellular compartment.

Following the fate of bound (^{3}H)-NANA (Fig. 2*B*) we observed a nearly linear increase with time in both phenotypes showing that the biosynthetic flux from (^{3}H)-ManNAc via cytidine monophosphate-NANA to NANA-glycoconjugates is apparently unaffected in ISSD. This is in agreement with the finding of normal cytidine monophosphate-NANA levels in ISSD fibroblasts (9). In controls, incorporated (^{3}H)-NANA was effectively chased

Table 1. *Estimation of (^{3}H)-NANA associated with the activities of LDH and β-HEX**

	LDH-associated NANA ("cytoplasmic")		β-HEX associated NANA ("lysosomal")	
	dpm/mg protein	%	dpm/mg protein	%
Control fibroblasts	5284 ± 319	68	2517 ± 490	32
ISSD fibroblasts	18871 ± 4635	27	52377 ± 6503	73

* Calculations are based on two equations with two unknowns: Equation 1 (supernatant):

$$(\%\ \text{LDH})\cdot x + (\%\ \beta\text{-HEX})\cdot y = \text{dpm}\ (^3\text{H})\text{-NANA}$$

Equation 2 (cell pellet):

$$(\%\ \text{LDH})\cdot x + (\%\ \beta\text{-HEX})\cdot y = \text{dpm}\ (^3\text{H})\text{-NANA};$$

x is the total LDH, and y is the total β-HEX activity associated (^{3}H)-NANA. Values (n = 4) are expressed as mean ± 1 S.D., obtained at the digitonin concentrations withe near maximal LDH release.

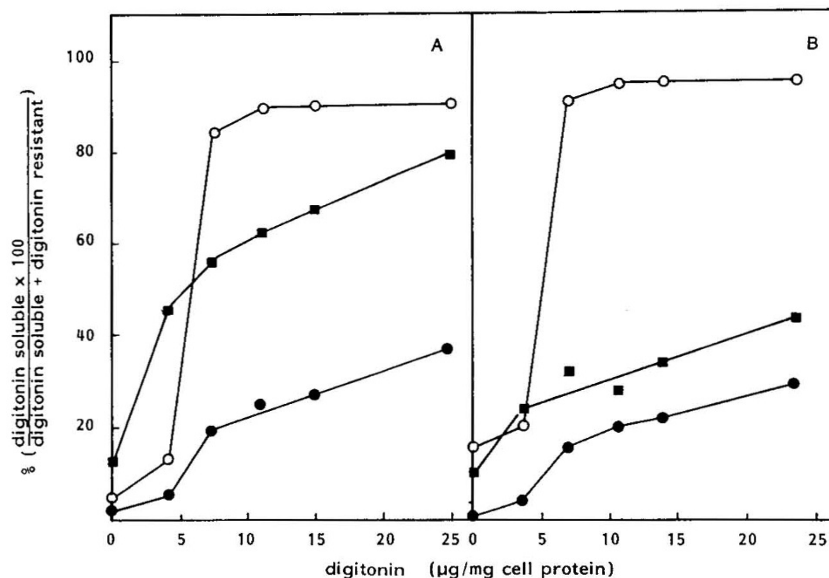

Fig. 4. Digitonin titration curves for (^{3}H)-NANA (■——■), LDH (○——○) and β-HEX (●——●). Normal (*A*) and ISSD fibroblasts (*B*) were exposed to increasing concentrations of digitonin under conditions as described in "Materials and methods." Values are expressed as the percentage of soluble material relative to the total content (sum of soluble + insoluble = 100%).

from the cells simply by the addition of nonradioactive culture medium. This might reflect cleavage of (^{3}H)-NANA to (^{3}H)-ManNAc and/or exchange of (^{3}H)-NANA with the extracellular compartment. In contrast, no substantial decrease of intracellular-free (^{3}H)-NANA was observed in ISSD cells during chase incubations. (^{3}H)-NANA bound to glycoconjugates, however, was apparently cleaved at normal rates in ISSD (Fig. 3*B*). It is conceivable that during a chase incubation with unlabeled medium (^{3}H)-NANA is not only diluted in its specific activity but also further metabolized. The use of a sensitive HPLC method allowed us to analyze the content of (^{3}H)-NANA and of its major catabolic products (^{3}H)-HexNAc in the chase media. As evidenced in Figure 1*C* relatively large amounts of (^{3}H)-HexNAc were found in the chase media of both phenotypes. Since the absolute amounts were exceeding those observed after the pulse-labeling period (Fig. 1*A*) one could speculate that (^{3}H)-HexNAc is arising due to metabolic conversion of (^{3}H)-NANA by a NANA-pyruvate lyase. So far, however, we and others (5, 6) have not been able to detect this enzyme activity in fibroblast homogenates. (^{3}H)-HexNAc in the chase medium of ISSD fibroblasts might stem from the cleavage of bound (^{3}H)-NANA by normal acting lysosomal sialidases and subsequent conversion via the pathway mentioned. Theoretically, extracellular enzyme activities could also contribute to metabolite levels of the media.

In contrast to Hancock *et al.* (6), we were able to detect small amounts of (^{3}H)-NANA in the media of ISSD cells. This finding is open to a number of explanations, *e.g.* excretion to the culture medium, exchange from the cytoplasmic pool, release by media neuraminidase from surface sialilated glycoconjugates, or cell death after prolonged culture at confluency. Regardless the exact mode of (^{3}H)-NANA catabolism, the observation of markedly increased (^{3}H)-NANA/(^{3}H)-HexNAc ratios in the intra- and extracellular compartments (Fig. 3*C* and *F*) of ISSD cells provides evidence that (^{3}H)-NANA is less accessible to the processes of degradation and reutilization. The clear-cut difference in the distribution of (^{3}H)-NANA relative to the marker enzymes LDH and β-HEX observed in the digitonin titration experiments (Fig. 4) strongly indicates that trapping of (^{3}H)-NANA in ISSD occurs within a digitonin-resistant compartment, presumably representing lysosomes. The quantitative data calculated by use of digitonin titration curves are in good agreement with data derived from subcellular fractionation studies (6).

In our experiments we observed nonsaturable accumulation of metabolically labeled free (^{3}H)-NANA in ISSD fibroblasts. Although the exact molecular mechanism of this phenomenon remains to be elucidated our data are consistent with the hypothesis that this accumulation is determined by concentrating free (^{3}H)-NANA within the lysosomal compartment. Our experiments also support the view that intralysosomal trapping of free (^{3}H)-NANA leads to impaired NANA catabolism and reutilization.

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