Lysosomal degradation of glycoproteins and glycosaminoglycans

Efflux and recycling of sulphate and *N*-acetylhexosamines

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Lysosomal degradation of the carbohydrate portion of glycoproteins and glycosaminoglycans produces monosaccharides and sulphate, which must efflux from the lysosomes before re-entering biosynthetic pathways. We examined the degradation of glycoproteins and glycosaminoglycans by lysosomes isolated from cultured human diploid fibroblasts. Cells were grown for 24 h in medium containing [³H]glucosamine and [³⁵S]sulphate. When lysosomes are isolated from these cells, they contain label primarily in macromolecules (glycoproteins and glycosaminoglycans). Glycoprotein degradation by isolated lysosomes was followed by measuring the release of tritiated sugars from macromolecules and efflux of these sugars from the organelles. Glycosaminoglycan degradation was monitored by the release of both tritiated sugars and [³⁵S]sulphate. During macromolecule degradation, the total amounts of free [³⁵S]sulphate, *N*-acetyl[³H]glucosamine and *N*-acetyl[³H]galactosamine found outside the lysosome parallels the amounts of these products released by degradation. The total degradation of glycoproteins and glycosaminoglycans by intact cultured cells was also examined. The lysosomal contribution to degradation was assessed by measuring inhibition by the lysosomotropic amine NH₄Cl. After 48 h incubation, inhibition by NH₄Cl exceeded 55% of glycoprotein and 72% of glycosaminoglycan degradation. Recycling of [³H]hexosamines and [³⁵S]sulphate by intact cells was estimated by measuring the appearance of 'newly synthesized' radioactively labelled macromolecules in the medium. Sulphate does not appear to be appreciably recycled. *N*-Acetylglucosamine and *N*-acetylgalactosamine, on the other hand, are reutilized to a significant extent.

INTRODUCTION

The lysosomal degradation of glycosaminoglycans and glycoproteins involves the concerted action of about 20 hydrolytic enzymes. The amino acids and carbohydrates that result from the degradative process are released from the lysosome and presumably recycled. The permeability properties of the lysosomal membrane to products of hydrolysis have been examined mainly by measuring entry of materials into the lysosome. These studies usually involve measurement of lysosome stability after suspension of the organelles in solutions of the compound whose permeability is under examination (for review see [1]). Only for amino acids have actual efflux rates been measured. The method used for these studies was modelled on experiments by Goldman and Kaplan [2,3], involving the incubation of lysosomes with amino acid methyl esters, which can freely cross the lysosomal membrane to be hydrolysed to free amino acids inside. Owing to their high polarity, the free amino acids do not readily diffuse back out of the lysosome and instead they accumulate inside. The release of these pre-accumulated amino acids is a direct measure of efflux [4–9].

We have previously examined the degradation of endogenous glycosaminoglycans by isolated lysosomes [10]. Intact lysosomes containing glycosaminoglycan chains metabolically labelled with [³⁵S]sulphate were shown to degrade over 30% of these chains *in vitro*. The degradative process was stimulated by ATP and acetyl-CoA. ATP presumably acts by stimulation of the

lysosomal proton pump. Acetyl-CoA is necessary, since it is a cofactor for one of the enzymes in the heparan sulphate degradative pathway [11,12]. In the present paper we have examined the efflux of sulphate and *N*-acetylhexosamines from isolated lysosomes after degradation of macromolecules and the relative extents of reutilization of these compounds by intact cultured fibroblasts.

EXPERIMENTAL

Reagents

H₂³⁵SO₄ (carrier free) and [³H]glucosamine (31 Ci/mmol) were purchased from New England Nuclear (Boston, MA). DMEM (powdered) and tissue-culture-grade trypsin were from Grand Island Biological Co. All other reagents were purchased from standard commercial suppliers.

Cell culture

Normal diploid human fibroblasts were obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ, U.S.A.). Cells were grown in 175 cm² flasks and maintained in DMEM containing 9% (v/v) fetal-calf serum; the NaHCO₃ concentration was lowered to 2.2 g/l and that of NaCl was increased to 7.44 g/l. Each litre of medium was supplemented with 10⁶ units of penicillin, 0.1 g of streptomycin sulphate and 0.25 mg of polymyxin B

Abbreviation used: DMEM, Dulbecco's modified Eagle's medium.

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sulphate. Cultures were maintained at 37 °C in an atmosphere of air/CO₂ (19:1).

The medium used for radioactive labelling of cells with [³⁵S]sulphate was identical with the normal medium, except that MgCl₂ was substituted for MgSO₄ and streptomycin sulphate and polymyxin B sulphate were omitted. If the cells were labelled with [³H]glucosamine, the medium used was made up with 100 mg of glucose/l, which is 10% of the normal glucose concentration. For double labelling (i.e. H₂³⁵SO₄ and [³H]glucosamine), both glucose and sulphate contents of the medium were decreased. Cells were labelled for 24 h with slow shaking (100 rev./min) on a Jr. Orbit Laboratory Shaker (Labline Instrument Co., Melrose Park, IL, U.S.A.) at 37 °C with 100–200 μCi of H₂³⁵SO₄ and 25–30 μCi of [³H]glucosamine in 15 ml of medium. The slow shaking ensured that the low volume of medium adequately covered the cell monolayer.

Preparation and incubation of lysosomes

In those experiments in which efflux of sulphate and/or sugars from isolated lysosomes was being measured, the cells were harvested and disrupted, and a mitochondrial + lysosomal (M + L) pellet was obtained as described previously [13]. This pellet was resuspended in 0.25 M-sucrose and incubated at 37 °C in 20 mM-Tris/HCl (pH 7.2), 5 mM-MgCl₂, 0.5 mM-ATP and 10 μM-acetyl-CoA. At various times, two 100 μl samples of this incubation mixture were removed for duplicate determinations of the extent of glycosaminoglycan degradation by using the ethanol precipitation method that we have previously described [10]. In addition, 300 μl was removed, placed in a 1.5 ml Brinkman polypropylene tube and centrifuged at 15000 rev./min for 15 min at 4 °C. The supernatant was carefully removed and the pellet was dissolved in 300 μl of 0.4% taurodeoxycholate. Duplicate 100 μl samples of both the supernatant and the solubilized pellet were assayed for glycosaminoglycan degradation. Lysosomal latency was measured throughout the 3 h incubation as described previously [13].

Sugar analysis by h.p.l.c.

To monitor the efflux of [³H]hexosamines from the lysosomes, it was necessary to first separate these sugars from other low-*M_r* tritiated compounds in the mixture (a crude lysosomal preparation from fibroblasts labelled with [³H]glucosamine contains label primarily in glucose, glucosamine, *N*-acetylglucosamine and *N*-acetylgalactosamine). Samples (50 μl) of either the crude incubation mixture or the taurodeoxycholate-solubilized M + L pellets were injected into a Hewlett–Packard liquid chromatograph (model 1084B) equipped with an Aminex HPX-87 column (300 mm × 7.8 mm). H.p.l.c.-grade water was used to make 0.005 M-H₂SO₄, which was pumped isocratically at 0.6 ml/min. Column effluent was monitored on line at 200 nm and collected directly into scintillation vials.

Cell treatment for recycling experiments

Cells were plated in 35 mm-diam. dishes and labelled for 24 h with 20 μCi of H₂³⁵SO₄ and 3 μCi of [³H]glucosamine in 1.5 ml of medium per dish. After labelling, the cells were washed twice with regular (DMEM) medium; the medium was removed and various unlabelled media were added to the cells. This was designated as zero time. At various time points up to 48 h

later, the medium was removed from dishes and frozen. The cells were washed with 1 ml of Hanks buffered saline (lacking Mg²⁺, Ca²⁺ and glucose, dissolved in 0.5 ml of guanidine extraction buffer {4 M-guanidine hydrochloride, 0.1 M-ε-aminohexanoic acid, 5 mM-benzamidine hydrochloride, 50 mM-EDTA, 50 mM-sodium acetate, 0.1 mM-phenylmethanesulphonyl fluoride, 10 mM-*N*-ethylmaleimide, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate ('CHAPS')}) and the dish was rinsed with an additional 200 μl of the latter. These samples were kept frozen before analysis.

Separation of glycoproteins and glycosaminoglycans

Media, trypsin digests and/or cells (dissolved in 0.5 ml of guanidine extraction buffer) were desalted on PD 10 columns (Pharmacia, Uppsala, Sweden). Glycoproteins were then separated from glycosaminoglycans on 2 ml DEAE-Sephacel columns [14]. The elution buffer for macromolecules (PD 10 column) and glycoproteins (DEAE-Sephacel column) was 8 M-urea containing 0.15 M-NaCl, 0.05 M-sodium acetate and 0.5% Triton X-100. The elution of glycosaminoglycans from the DEAE-Sephacel column was performed with the same buffer containing 1.4 M-NaCl. Samples (0.5 ml) were taken for scintillation counting.

RESULTS

Efflux of sulphate from isolated lysosomes

We have previously shown that, after labelling of fibroblasts with [³⁵S]sulphate and isolation of intact lysosomes, over 90% of the label associated with these organelles is in [³⁵S]glycosaminoglycans. The isolated lysosomes can degrade up to 30% of these labelled macromolecules. [³⁵S]Glycosaminoglycans inside the lysosomes can be distinguished from free [³⁵S]sulphate released during degradation by precipitation with 80% (v/v) ethanol. The percentage of total radioactivity that remains soluble is a reflection of the extent of glycosaminoglycan degradation at any time during lysosome incubation [10]. In order to determine the rate of release of free sulphate from the lysosome after glycosaminoglycan degradation, we incubated lysosomes containing [³⁵S]glycosaminoglycans at 37 °C, and at various time points the lysosomes and the extralysosomal supernatant were separated and analysed for free [³⁵S]sulphate. Total glycosaminoglycan degradation was also measured by determining the percentage of free [³⁵S]sulphate released in the total lysosome incubation mixture. Thus the amount of [³⁵S]sulphate released from the glycosaminoglycan chains could be compared at each time point with the amount of [³⁵S]sulphate released from the lysosome. The results (Fig. 1) demonstrated that virtually all free [³⁵S]sulphate arising from glycosaminoglycan degradation could be simultaneously accounted for outside the lysosomes and that the rates of [³⁵S]sulphate production and [³⁵S]sulphate efflux were parallel.

Efflux of hexosamines from isolated lysosomes

When fibroblasts were incubated with [³H]glucosamine for 24 h, followed by a short (20 min) chase, an isolated lysosome-enriched fraction contained radioactivity in macromolecules (glycosaminoglycans and glycoproteins) and in low-*M_r* compounds. The amount of radioactivity

Fig. 1. Generation and efflux of [^{35}S]sulphate from lysosomes with time

Partially purified lysosomes (see the Experimental section) from cells labelled with [^{35}S]sulphate were incubated in 20 mM-Tris/HCl (pH 7.2)/5 mM- MgCl_2 /0.5 mM-ATP/10 μM -acetyl-CoA/0.25 M-sucrose at 37 °C. At the indicated times samples were removed for determination of free [^{35}S]sulphate inside (○) and outside (△) the lysosomes (see the Experimental section).

that was free glucosamine was less than 5% of the total. Although we expected most of the label to be incorporated into macromolecules as *N*-acetyl- ^3H glucosamine and *N*-acetyl ^3H galactosamine, labelling of other sugars was also possible, owing to entry of glucosamine into the glycolytic cycle. When lysosomes containing ^3H -labelled macromolecules were incubated at 37 °C, a time-dependent degradation of these compounds occurred similar to that which we previously observed with ^{35}S -labelled macromolecules.

Fig. 2(a) shows a chromatogram of a h.p.l.c. separation of various sugar standards. Fig. 2(b) is a histogram showing the separation of ^3H released from lysosomes into the incubation supernatant after 0 and 180 min incubation at 37 °C. The only radioactivity apparent at zero time (continuous line) is glucosamine, whereas after 180 min (broken line) most of the radioactivity has appeared in peaks corresponding to *N*-acetylglucosamine and *N*-acetylgalactosamine and some radioactivity in a peak that was overlapping but not coincident with the glucose peak. Free *N*-acetyl ^3H glucosamine and *N*-acetyl ^3H galactosamine would appear outside the lysosome after cleavage of these terminal sugars from glycosaminoglycans and glycoproteins within intact lysosomes and subsequent efflux. To determine the rate of lysosome degradation and release of ^3H hexosamines, h.p.l.c. was performed on the total lysosome incubation mixture and on the lysosome-free supernatants at various time points. Summations of the radioactivity in *N*-acetylglucosamine and *N*-acetylgalactosamine fractions (13–16 min) of the h.p.l.c. run are plotted in Fig. 3. The total *N*-acetyl ^3H glucosamine and *N*-acetyl ^3H galactosamine outside the lysosomes paralleled the amounts of these acetylated sugars produced by degradation.

Fig. 2. Efflux of hexosamines from lysosomes

(a) A mixture of approx. 10 μmol each of glucosamine (GlcN), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) and glucose was analysed by h.p.l.c. as described in the Experimental section (—). Superimposed (---) is the elution profile of approx. 100 μmol of glucose. (b) Samples (50 μl) of lysosome-free supernatant (see the Experimental section) at zero time (■) and after 180 min of incubation (□) of [^3H]glucosamine-labelled lysosomes were chromatographed, and 0.6 ml samples were collected into scintillation vials and counted for radioactivity.

Efflux of hexosamines and sulphate from lysosomes was compared by using organelles isolated from cells doubly labelled with [^{35}S]sulphate and [^3H]glucosamine. We could examine both of these degradation products by measuring the ethanol-soluble ^3H and [^{35}S]sulphate radioactivity in the lysosome-free supernatants. The results indicated a simultaneous release from lysosomes of [^3H]hexosamines and [^{35}S]sulphate (results not shown).

Degradation of glycosaminoglycans and glycoproteins by intact fibroblasts

In order to examine the degradation and recycling of glycosaminoglycans and glycoproteins, intact fibroblasts were labelled for 24 h with [^3H]glucosamine and [^{35}S]sulphate, chased for 20 min, replated in 35 mm dishes, chased for an additional 35 min and incubated in unlabelled media for up to 48 h. The turnover of glycosaminoglycans and glycoproteins by intact cells was examined after separation of these compounds on DEAE-Sephacel (see the Experimental section). The loss of radioactivity for both glycosaminoglycans (Fig. 4) and glycoproteins (Fig. 5) was unchanged if the cells were cultured in a low-glucose medium or in a low-glucose

Fig. 3. Production and efflux of *N*-acetylhexosamines from [^3H]glucosamine-labelled lysosomes

Samples (50 μl each) of both lysosome-free supernatant (Δ) and total lysosome incubation mixture ($\circ$) were chromatographed (see Fig. 2), and 0.6 ml fractions were collected and counted. The ^3H radioactivity corresponding to *N*-acetylglucosamine and *N*-acetylgalactosamine (13–16 min) of the h.l.p.c. run was totalled for each incubation time point.

medium with unlabelled glucosamine added (1 mM). Lowering the glucose concentration and adding glucosamine should greatly expand the pool of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine. The addition of 10 mM- NH_4Cl to the medium caused a marked inhibition of both glycosaminoglycan and glycoprotein degradation, to 18% and 33% of normal respectively. As expected, a similar rate of glycosaminoglycan degradation was observed when either ^3H or [^{35}S]sulphate was measured in the partially purified glycosaminoglycan fraction (cf. Figs 4a and 4b).

Recycling of [^3H]hexosamines

For measurement of recycling of [^3H]hexosamines and [^{35}S]sulphate, cells were cultured in 35 mm dishes and labelled overnight with [^3H]glucosamine and [^{35}S]sulphate (see the Experimental section). The cells were washed with media and then incubated (chased) for various times up to 48 h in media alone or media with added 10 mM- NH_4Cl or 1 mM-glucosamine. The protocol for these experiments was different from the previous protocol in that the cells were not treated with trypsin and replated after labelling. Therefore, during the subsequent incubation, labelled cell-surface glycoproteins and proteoglycans were free to enter lysosomes or the culture medium. Crude glycoprotein and glycosaminoglycan fractions were isolated and quantified in both the cells and the medium (see the Experimental section). At the start of the chase period, ^3H -labelled cell-associated macromolecules were 54% glycoproteins and 46% glycosaminoglycans. After 48 h in regular medium 42% of the glycoprotein radioactivity and 66% of the glycosaminoglycan radioactivity was either degraded or lost from the cells. Fig. 6(a) shows the loss of [^{35}S]sulphate-labelled macromolecules (glycosaminoglycans only) from the cells in the presence and

Fig. 4. Degradation of labelled glycosaminoglycans in intact cells

Fibroblasts labelled with [^3H]glucosamine and [^{35}S]sulphate were incubated at 37 $^{\circ}\text{C}$ in various media: $\circ$ normal DMEM; $\bullet$, low glucose (100 mg/l), $\square$, low glucose + 1 mM-glucosamine; Δ , normal DMEM + 10 mM- NH_4Cl . Radioactivity in glycosaminoglycans was measured at different times up to 48 h (see the Experimental section). (a) ^3H radioactivity in glycosaminoglycans per mg of cellular protein. (b) [^{35}S]Sulphate radioactivity per mg of cellular protein in glycosaminoglycans.

Fig. 5. Degradation of labelled glycoproteins in intact cells

Cells, under conditions described in Fig. 4, were also measured for their radioactive glycoprotein content (^3H radioactivity per mg of cellular protein).

Fig. 6. Degradation and recycling of glycosaminoglycans and total macromolecules by intact fibroblasts.

Cells were double-labelled as described in Fig. 4. (a) Loss of ^{35}S -labelled glycosaminoglycans from the cells incubated in normal DMEM (●) or DMEM containing 10 mM- NH_4Cl (○). The appearance of ^{35}S -labelled macromolecules in the medium at the same time is shown by the broken lines: ▲, normal DMEM; △, DMEM containing 10 mM- NH_4Cl . (b) Loss of ^3H -labelled macromolecules from the cells and appearance of ^3H -labelled macromolecules in the medium. Symbols are as in (a).

absence of NH_4Cl . The inhibition of ^{35}S -glycosaminoglycan degradation by NH_4Cl was approx. 68%. Fig. 6(b) shows the time course for loss of total ^3H -labelled macromolecules (^{35}S -glycosaminoglycans plus ^{35}S -glycoproteins) from the cells in the presence and absence of NH_4Cl . Loss of tritiated macromolecules from the cells was inhibited by approx. 61% by NH_4Cl .

The appearance of ^{35}S -labelled macromolecules (proteoglycans) in the medium was not inhibited by NH_4Cl (Fig. 6a) and was not competed for by the addition of unlabelled sulphate to the medium (results not shown) or unlabelled glucosamine (Fig. 7b). Furthermore, considerably less of this material was observed in the medium if cells were treated with trypsin between the pulse and the chase. From these results, it appears likely that these ^{35}S -labelled macromolecules appearing in the medium represent loss from the cells of initially labelled material and not synthesis of new glycosaminoglycans.

In contrast, the appearance of ^3H -labelled macromolecules (proteoglycans and glycoproteins) in the medium (Fig. 6b, broken lines) is inhibited by NH_4Cl

Fig. 7. Effect of glucosamine in recycling of ^3H -hexosamines and ^{35}S -sulphate.

Cells were double-labelled as described in Fig. 4. (a) The appearance of ^3H -labelled macromolecules in the medium of cells incubated in normal DMEM (▲) and in the DMEM containing 1 mM-glucosamine (□). (b) Appearance of ^{35}S -glycosaminoglycans in the media of cells incubated in normal DMEM (▲) and in DMEM containing 1 mM-glucosamine (□).

(Fig. 6b) and competed for by the addition of unlabelled glucosamine in the chase (Fig. 7a). Furthermore, there was only a slight decrease in the appearance of this material if the cells were treated with trypsin between the pulse and the chase. We concluded that a substantial portion of the ^3H -labelled macromolecules represented newly synthesized material containing sugars derived from lysosomal degradation. As shown in Figs. 6 and 7, the extent of ^3H -hexosamine reutilization is substantial; by 48 h over 50% of the total ^3H -labelled macromolecules lost from the cells (Fig. 6b, ●) can be accounted for in this 'newly synthesized' pool (Fig. 6b, ▲).

DISCUSSION

The experiments described here indicate that, after lysosomal degradation of proteoglycans and glycoproteins, there is a rapid efflux of sulphate and hexosamines (*N*-acetylglucosamine and *N*-acetylgalactosamine) across the lysosomal membrane which appears only limited by the rate of production of these metabolites in the lysosome. The rapid release of sulphate from the lysosome appears to be inconsistent with a detailed study by Casey *et al.* [15] of the permeability (penetration) properties of the lysosomal membrane to anions and cations. Lysosome penetration as measured by osmotic lysis was found to agree largely with the lyotropic series for both types of inorganic ions, suggesting that penetration was dependent on the ionic field strength of the permeant substance and not dependent on a specific transport system in the lysosomal membrane. Sulphate

was the least permeant of all the anions tested; however, as pointed out by Reijngoud & Tager [1], the measurement of ion penetration by using osmotic protection as a criterion of impermeability may not reflect the ability of these compounds to efflux from the lysosomes, since influx studies measure the movement of compounds from the neutral cytosol to the acidic lysosome, while effluxing substances move in the opposite direction.

As with sulphate, the efflux of *N*-acetylhexosamines from isolated lysosomes was found to occur in parallel with release from macromolecular precursors. This finding agrees with influx studies by Lloyd [16], which indicated that uncharged monosaccharides could freely penetrate rat liver lysosomes. Although Lloyd's data argue against a specific carbohydrate carrier mechanism, studies by Docherty *et al.* [17, 18] and McGuire *et al.* [19] indicate that glucose transport into rat liver lysosomes occurs by a substrate-specific facilitated diffusion. A recently recognized disorder where sialic acid accumulates in lysosomes lends additional support to the notion that sugar carriers may exist in the lysosomal membrane [20, 21]. Our results do not argue for or against a specific carrier for hexosamines or sulphate, since, under the conditions that we have examined, the efflux of these compounds parallels their production.

Specific lysosomal membrane transport systems have also been shown to exist for amino acids, H^+ ions and acetyl moieties. Transport of the amino acid cysteine out of the lysosome is mediated by a specific lysosomal membrane carrier that has been shown to be defective in cystinosis [7-9]. In addition, a system that transports cationic amino acids across the lysosomal membrane of human fibroblasts has been characterized [22]. Lysosomal acidification (uptake of H^+) has received considerable attention. There is now widespread agreement that a lysosomal-membrane proton-translocating ATPase is responsible for generating a transmembrane pH gradient (for review see [23]).

The lysosomal membrane also has the ability to transport acetyl moieties from the cytoplasm to the interior of the lysosome during degradation of heparan sulphate. We have previously presented evidence that net transport of acetyl groups occurs as the result of a transmembrane acetylation reaction [12, 24], which results in the formation of terminal α -linked *N*-acetylglucosamine moieties of heparan sulphate. These groups are then cleaved by α -*N*-acetylglucosaminidase and, according to the data presented here, they rapidly efflux out of the lysosome.

Although both sulphate and *N*-acetylhexosamines are rapidly released from lysosomes, their subsequent metabolic fate is quite different. Sulphate released by lysosomal degradation rapidly exchanges with sulphate in the culture medium and, consequently, there is little or no recycling detected. On the other hand, hexosamines produced by lysosomal degradation do not appear to be lost from the cells, and over 50% of these sugars are reincorporated into newly synthesized secreted macromolecules (Fig. 7).

The degradation of glycosaminoglycans and glycoproteins by intact fibroblasts was significantly inhibited by the lysosomotropic amine NH_4Cl (82% inhibition for glycosaminoglycans and 67% inhibition for glycoproteins). Although this would indicate that some degradation may occur by a non-lysosomal mechanism, studies

by Ahlberg *et al.* [25] suggest that certain lysosomotropic agents are not able completely to suppress lysosomal proteolysis. Furthermore, owing to the lysosomal localization of the enzymes required to degrade proteoglycans, it is reasonable to assume that all of the proteoglycan degradation that we have observed with intact cells has occurred in lysosomes.

$[^3H]$ Hexosamines that were released by lysosomal degradation were recycled into secreted macromolecules with a high efficiency. Since we have only measured secreted macromolecules (glycoproteins and proteoglycans in the medium), the amount of recycling that actually occurs may be greater, since our measurements do not take into account newly synthesized macromolecules which may have been rapidly degraded. *N*-Acetylglucosamine and *N*-acetylgalactosamine can be rapidly converted into phosphate derivatives by kinase activities present in mammalian tissues [26] and subsequently activated to UDP derivatives. There have been deacetylation activities reported; however, free hexosamines formed can be rapidly phosphorylated and reacylated.

The usual precursor of *N*-acetylglucosamine and *N*-acetylgalactosamine in glycoproteins and proteoglycans is glucosamine 6-phosphate, which can be produced either from fructose 6-phosphate and glutamine or from glucosamine (in tissue-culture cells this latter precursor is obtained from the culture medium). Glucosamine 6-phosphate is acetylated and converted into *N*-acetylglucosamine 1-phosphate before activation to UDP-*N*-acetylglucosamine (which can rapidly epimerize to UDP-*N*-acetylgalactosamine). A mutant of mouse fibroblasts, AD6, isolated by Pouyssegur & Pastan [27] was found to be defective in acetylation of glucosamine 6-phosphate [28, 29]. Although no acetyltransferase activity could be detected in these cells, they did synthesize glycoproteins to about 50% of wild-type values. The high extent of *N*-acetylhexosamine recycling that we have observed may indicate that, in AD6 cells, overall glycoprotein biosynthesis is almost entirely supported by recycling of *N*-acetylhexosamines produced during lysosomal degradation.

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