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Lysosomal sulfate efflux following glycosaminoglycan degradation: measurements in enzyme-supplemented Maroteaux–Lamy syndrome fibroblasts and isolated lysosomes

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Studies using lysosomal membrane vesicles have suggested that efflux of the sulfate that results from lysosomal glycosaminoglycan degradation is carrier-mediated. In this study, glycosaminoglycan degradation and sulfate efflux were examined using cultured skin fibroblasts and lysosomes deficient in the lysosomal enzyme *N*-acetylgalactosamine-4-sulfatase. Such fibroblasts store dermatan sulfate lysosomally, which could be labelled biosynthetically with $Na_2{}^{35}SO_4$. The addition of recombinant *N*-acetylgalactosamine-4-sulfatase to the media of ^{35}S labelled fibroblasts degraded up to 82% of the stored dermatan [^{35}S] sulfate over a subsequent 96 h chase and released inorganic [^{35}S] sulfate into the medium. In the presence of 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), sulfate was reused to a minor extent in newly synthesized proteoglycan. Isolated granules from recombinant enzyme supplemented fibroblasts degraded stored dermatan [^{35}S]sulfate to sulfate which was rapidly released into the medium at a rate that was reduced by the extra-lysosomal presence of the lysosomal sulfate transport inhibitors SITS, Na_2SO_4 and Na_2MoO_4. SITS also inhibited dermatan sulfate turnover, although it had no effect on the action of purified recombinant enzyme *in vitro*. These data imply that sulfate clearance occurred concomitantly with dermatan sulfate turnover in the lysosome even at high substrate loading, and that lysosome-derived sulfate, while available, is reutilized minimally in synthetic pathways.

Keywords: sulfate, lysosomes, lysosomal transport, glycosaminoglycans, fibroblasts, dermatan sulfate.

Abbreviations: SITS, 4-acetamido-4′-isothiocyanatostilbene-2,-2′-disulfonic acid; GAG, glycosaminoglycan; 4S, *N*-acetylgalactosamine-4-sulfatase; r4S, recombinant human *N*-acetylgalactosamine-4-sulfatase; PBS, phosphate buffered saline; BME, basal modified Eagle's medium; FBS, fetal bovine serum; GalNAc4S-GlcA-GalitolNAc4S, β-(*N*-acetyl-D-galactosamine-4-sulfate)-(1-4)-β-D-glucuronic acid)-(1-3)-*N*-acetyl-D-[1-^{3}H]galactosaminitol-4-sulfate; DS, dermatan sulfate; MPS, mucopolysaccharidosis.

Degradation of the glycosaminoglycans (GAG) dermatan sulfate (DS) and heparan sulfate occurs within the lysosome by the concerted action of groups of enzymes with different substrate specificities to produce monosaccharides and inorganic sulfate [1]. The selective permeability of the lysosomal membrane and the action of transporters that are present in the lysosomal membrane are fundamental to lysosomal function, in that they determine the influx of environmental ions and specific cofactors [2–4], as well as the efflux of degradation end-products. A family of facilitating transporters accounts for the efflux of these end-products [5–15]. Jonas and Jobe [14] have used isolated lysosomal membrane vesicles to demonstrate sulfate transport activity, and indicated several properties in common with the band 3 anion exchange protein of the erythrocyte plasma membrane. In particular, sulfate fluxes were significantly stimulated by proton fluxes, which may suggest a mechanism by which sulfate fluxes out of the lysosome are coupled. While much has been gained through investigation of transport in isolated lysosomal membrane vesicles, transport into and out of intact organelles will need to be studied to answer specific mechanistic questions about the functional associations between GAG degradation, product efflux and cofactor influx such as acetyl CoA.

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Several studies have dealt with efflux of exogenously introduced solutes from granules [5–7, 10, 16], but the associations between this transport phenomenon and the degradation of polymeric substrates have received little experimental attention.

Rome and co-workers [2, 17, 18] approached these associations by studying GAG degradation in fibroblasts and isolated granules. In intact fibroblasts, they found that GAG degradation and lysosomal efflux led to recycling of *N*-acetylhexosamines into newly synthesized GAG, but found no reutilization of sulfate. This may be explained by differences between the relative rates of cellular efflux of hexosamines and sulfate and/or the size of their respective nucleotide precursor pools, but such an explanation is difficult to test without methods for blocking cellular efflux and modulating nucleotide precursor pools.

Individuals with the mucopolysaccharidoses (MPS) lack specific lysosomal enzymes that degrade glycoconjugate substrates, and hence partially degraded substrates store within the lysosomes of their cells [19]. The enzyme deficiency can be corrected *in vitro* by exposure of fibroblasts to the deficient enzyme in the correctly processed form [19]. GAG turnover commences and continues to a point where the cells are essentially cleared of stored substrate. MPS fibroblasts therefore provide a unique experimental system for the study of the fate of lysosomal degradation products, since metabolism of substrates in cells with enzyme-deficient lysosomes can be compared directly to metabolism in the same cells with enzyme-supplemented lysosomes.

In this paper, fibroblasts were chosen from clinically severe patients with the Maroteaux–Lamy syndrome (mucopolysaccharidosis type VI, MPS VI), which are deficient in lysosomal 4S. These patients store both DS and chondroitin sulfate but, since DS proteoglycans are a major sulfated product of the fibroblast [20], these cells store predominantly DS. DS turnover and lysosomal sulfate efflux were studied using both intact fibroblasts and isolated lysosomal organelles, following enzyme supplementation. This approach allowed pulse/chase experiments to be focused on lysosome-derived sulfate as opposed to sulfate derived from other sources, such as sulfur-containing amino acids or the culture medium.

Materials and methods

Materials

Phosphate buffered saline (PBS) was purchased from Commonwealth Serum Laboratories (Melbourne, Australia). Tissue culture flasks (75 cm^2) were obtained from Costar (Cambridge, USA). Basal modified Eagle's medium (BME), penicillin and glutamine were from Flow Laboratories (Melbourne, Australia). $Na_2{}^{35}SO_4$ (450–600 mCi $mmol^{-1}$) and other radiochemicals were purchased from DuPont-NEN Research Products (Sydney, Australia). Ham's F-12 nutrient mixture and fetal bovine serum (FBS) were from Gibco (Glen Waverley, Australia). Percoll and Sepharose CL-6B were purchased from Pharmacia LKB Biotechnology Inc. (Australia). SITS and sucrose were purchased from Sigma Chemical Co. (St. Louis, USA). CHO-DKI cells containing the full-length 4S construct (CHO4S2) were a gift from Dr D. Anson. Their construction has been described elsewhere [21].

Culture of fibroblasts

Human diploid fibroblasts were established from skin biopsies submitted to the hospital for diagnosis [22]. Cell lines were maintained at 37 °C in 5% CO_2 in BME, with antibiotics, non-essential amino acids and 10% (v/v) FBS (complete BME), as previously described [23]. The Maroteaux–Lamy syndrome (MPS VI) cultured skin fibroblast cell lines chosen for this study were all from clinically severe patients demonstrating dermatansulfaturia and were all found to be deficient in lysosomal 4S activity [24].

Fibroblast labelling

Fibroblasts from the MPS VI patients were grown to confluency (75 cm^2 flasks). Two hours prior to addition of radiolabel, the culture medium was exchanged with 10 ml of Ham's F12 medium supplemented with non-essential amino acids, 10% (v/v) FBS and 100 μM Na_2SO_4, but without antibiotics. GAG was then labelled biosynthetically by culturing for 24–48 h in the presence of 10 μCi of $Na_2{}^{35}SO_4$ per ml of medium. The cells were then washed with PBS (30 ml per culture), the medium exchanged for complete BME and the cells maintained in this medium for 16 h. The medium was then removed, the cells rinsed with PBS, harvested by trypsinization and washed with PBS by centrifugation. This procedure removed the extracellular labelled GAG [25]. The cells were pooled, replated in complete BME and incubated for 1 h to allow the cells to attach.

Expression and preparation of r4S

The CHO4S2 cells were maintained in Ham's F12 with antibiotics and 10% (v/v) FBS (complete F12) at 37 °C in a 5% CO_2 atmosphere. Cells were treated with medium containing 10 mM NH_4Cl for 3 days followed by medium without NH_4Cl for 2 days and then passaged. The medium from NH_4Cl treated cells was collected, dialysed against BME and concentrated sevenfold by ultrafiltration over a PM10 (Amicon, USA) filter. This stock solution was found to contain recombinant 4-sulfatase (r4S) with an activity of 610 nmol min^{-1} ml^{-1}. This corresponds to an enzyme concentration of 6.5 μg r4S per ml, calculated using a specific activity of 94 μmol min mg^{-1} [21]. This stock solution was stored at 4 °C until used in supplementation experiments. In the experiments shown in Table 2 and Fig. 4, aliquots of r4S were taken from stocks of

immunopurified enzyme, prepared and stored as previously described [21]. Cultures were supplemented to the same concentration of r4S protein.

Supplementation with r4S

The medium for fibroblasts prelabelled with $Na_2{}^{35}SO_4$ was changed to complete BME supplemented with 0.26 μg ml^{-1} of the r4S preparation. The cells were incubated for at least 3 h, such that enzyme was endocytosed and transported to the lysosomes [21].

Pulse/chase experiments

To investigate the fate of $^{35}SO_4^{2-}$ subsequent to its release from DS substrate in the granule, a series of pulse/chase protocols was employed. Fibroblast cultures were pulse-labelled, as has been described above, then supplemented with r4S for various times between 3 h and 30 h to achieve enzyme uptake and correction of the enzyme deficiency. The culture medium was completely changed for unsupplemented complete BME at each time point, to achieve the chase. The cells were washed with PBS and either solubilized with 4 M GuHCl: 50 mM sodium acetate: 2% (by vol) Triton X-100, pH 5.8, for experiments to determine the nature of the storage material, or harvested by trypsinization and disrupted by freeze–thawing in 0.02 M Tris-HCl: 0.5 M NaCl, pH 6.8, for measurement of the endocytosed enzyme activity. The ^{35}S radioactivity in all fractions was summed so that the kinetics of $^{35}SO_4^{2-}$ release could be reconstructed. The proportion of the ^{35}S radioactivity that was $^{35}SO_4^{2-}$ as opposed to [^{35}S]DS was determined by ethanol precipitation [18] and gel chromatography on Sepharose CL-6B. The latter separations were performed in 2 M GuHCl: 50 mM sodium acetate: 100 mM Na_2SO_4, pH 7.5, so that molecular weights of undegraded DS could be estimated by the method of Wasteson [26]. Samples were centrifuged in an Eppendorf microfuge prior to chromatography.

Variation of medium SO_4^{2-} was achieved by performing the supplementation experiments in (a) complete BME approximately 1100 μM SO_4^{2-}; (b) Ham's F12 with 1% (v/v) FBS and containing a total of 320 μM SO_4^{2-}; (c) reduced SO_4^{2-} medium (Ham's F12 with 1% (v/v) FBS, containing a total of 20 μM SO_4^{2-}.

The SITS experiments used the standard protocol except that SITS was first added 2 h after addition of the r4S to avoid any adverse effects that this compound may have had on binding of the enzyme to the cells. As judged by some cellular rounding and detachment, SITS at 0.5–1 mM was mildly toxic to fibroblasts, consistent with the findings of Keller and Keller [27]. Sulfate transport studies [28] have indicated that this concentration range of SITS was required to achieve maximal inhibition, hence, as a compromise, the cells were exposed to 0.5 or 1 mM SITS for 8 h, followed by the chase at 0.2 mM SITS. This treatment did not adversely affect cellular morphology.

[^{35}S]DS turnover in isolated granules

Fibroblasts that had been pulse radiolabelled, chased and exposed to r4S were washed with PBS and harvested by trypsinization. The cells were pelleted by centrifugation and washed in 0.25 M sucrose which had been titrated to pH 7 with solid Tris base. The cells were then resuspended in 1 ml of the same buffer and disrupted by 15–20 hypobaric shocks [29]. The homogenate was centrifuged at 200 × **g** for 10 min to pellet the nuclear debris and unbroken cells. The post-nuclear supernatant was brought to a final volume of 1.8 ml by adding aliquots of concentrated stock solutions such that the final composition was 20 mM Tris-HCl, 0.1 mM ATP, 5 mM $MgCl_2$, 0.5 M sucrose, pH 7. The increased sucrose concentration was present in an attempt to compensate for the increased fragility of lysosomes from MPS cells [18]. Data presented in Table 2, footnote 2, suggest that this change in sucrose concentration did not completely overcome the intrinsic fragility. Aliquots of the isolated granules were then incubated at 37 °C for the indicated time with extra ATP (1 μl of a 10 mM stock added to 300 μl) added at 20 min and 40 min. At the indicated incubation times, aliquots were centrifuged at approximately 12000 × **g** in an Eppendorf microfuge for 10 min at 4 °C. The supernatants were separated from the granular pellets and frozen until required for analysis. The granular pellets were homogenized in a final volume of 400 μl 2 M NaCl, 0.5% (by vol). Triton X-100. Supernatant and granular fractions were analysed by ethanol precipitation of [^{35}S]DS [18].

DS degradation assay

Degradation of endogenous DS by granules was followed by release of $^{35}SO_4^{2-}$. This was defined as ethanol soluble radioactivity, isolated after ethanol precipitation of [^{35}S]DS [18].

The distribution of ^{35}S radioactivity between two compartments (granules and supernatant) and two molecular weight pools ($^{35}SO_4^{2-}$ and [^{35}S]DS) was analysed by proportions. Radioactivity in a particular incubation mixture was normalized so that each mixture had equal total radioactivity. As incubation mixtures differed by less than 10% in radioactivity, introduced errors will be minimal.

Correction of efflux data for latency

The $^{35}SO_4^{2-}$ appearing in the post-granular supernatant was assumed to result from two processes: lysosomal efflux and lysosomal rupture. Measurement of lysosomal transport has previously been corrected for rupture by reference to the latency of the lysosomal enzyme β-*N*-acetylglucosaminidase [16]. This analysis has been found to be inappropriate for correction of $^{35}SO_4^{2-}$ efflux, since binding of the enzyme to the lysosomal membrane [30] leads to a significant overestimation of the lysosomal latency. A more

appropriate estimate would take into account the membrane impermeability of macromolecular solutes. Therefore, we have analysed the amount of ethanol insoluble radioactivity ([^{35}S]DS) in the incubation supernatant as a measure of lysosomal rupture (lysosomal latency), calculated by dividing the difference between the supernatant [^{35}S]DS at zero time and the various incubation times, by the total [^{35}S]DS in the pellet at zero time. The latency was used to correct and then calculate the $^{35}SO_4^{2-}$ efflux rate. For example, the amount of sulfate outside the organelles due to changes in lysosomal latency are calculated as: sulfate in the pellet plus the change in sulfate in the supernatant times the change in percentage lysosomal latency.

Analytical procedures

Protein concentrations were determined using the bicinchoninic acid assay [31] with the modification that all samples and standards had a final Triton X-100 concentration of 0.4% (by vol) to ensure dissolution of the membrane proteins. Assays were performed at 37 °C for 1 h to reduce the non-specific reaction of lipid [32]. Total sulfatase activity was measured using the fluorogenic substrate, 4-methylumbelliferyl sulfate [33]. 4S activity was measured using the radiolabelled trisaccharide substrate GalNAc4S-GlcA-GalNAc-ol-4S [34]. Scintillation counting was performed with LKB (North Ryde, Australia) Optiphase Hisafe 3 scintillant, in which $^{35}SO_4^{2-}$ was counted with 60% efficiency. Uronic acid concentrations were determined by the method of Blumenkrantz and Asboe-Hansen [35]. Chondroitinase ABC (Miles, Japan) digestions were performed as described in Harper *et al.* [36]. The chemical identity of the low molecular weight ^{35}S radioactivity (ethanol soluble and totally included in Sepharose CL-6B) was confirmed as $^{35}SO_4^{2-}$ using high voltage electrophoresis following established procedures [37].

Results and discussion

Fibroblasts from MPS Type VI patients stored primarily DS glycosaminoglycans that were polydisperse, ranging in molecular weight between 12000 and 35000 with an average of 17000 (data not shown). Neither proteoglycans, consistent with previous data [38], nor sulfated *N*-acetylhexosamines previously observed in MPS VI patient urine [39] were stored. Our previous experiments suggested that enzyme correction, indicated by an onset of [^{35}S]DS turnover, occurred during a 92 h supplementation of MPS VI fibroblasts with r4S [21]. Other preliminary experiments defined experimental conditions for pulse labelling and enzyme supplementation which would allow description of the fate of lysosome-derived sulfate.

The experiments described here involved short-term (< 10 h) exposure to r4S, but resulted in at least twofold higher rates of clearance of stored [^{35}S]DS from the supplemented cultures as compared with the corresponding unsupplemented controls. The final intracellular ^{35}S radioactivity of the supplemented cultures averaged 7.9 ± 1.1-fold (n = 5) lower than the unsupplemented controls.

The fate of lysosome-derived $^{35}SO_4^{2-}$

Pulse/chase experiments were performed as described in the Materials and methods section. The experimental conditions were chosen such that [^{35}S]DS turnover proceeded in intact fibroblasts with different cytosolic SO_4^{2-} concentrations. Figure 1 shows that variation of the medium SO_4^{2-} concentration, and hence indirectly the cytosolic SO_4^{2-} concentrations, led to no significant change in the kinetics of $^{35}SO_4^{2-}$ release into the medium. Ethanol precipitation suggested that >90% of ^{35}S radioactivity appearing in the medium was $^{35}SO_4^{2-}$ independent of the medium sulfate concentration. This conclusion was supported by analysis of the radioactive components separated by high voltage electrophoresis (data not shown). Approximately 20% of the cell layer ^{35}S radioactivity was $^{35}SO_4^{2-}$, again with no apparent effect of medium sulfate concentration. A repeatable observation was the high proportion of polymeric [^{35}S]DS appearing in the medium of the unsupplemented cultures (42%), presumably due to cellular defecation of undegraded storage product [40]. The data show that in cultured fibroblasts, cellular efflux is the primary fate of sulfate produced from DS degradation in the lysosome. Manipulation of the intracellular sulfate pool size apparently did little to change this fate.

Effect of a sulfate-efflux inhibitor

The stilbene, SITS, is known to inhibit plasma membrane sulfate transport in cultured fibroblasts [41]. The effect of SITS was tested on the efflux of $^{35}SO_4^{2-}$ produced during r4S-supplementation of MPS VI fibroblasts. SITS did not block the enzymatic activity of r4S (< 5% inhibition between 0.01 mM and 1 mM SITS), nor the uptake of r4S enzyme in the MPS VI cells (Table 1). It did, however, inhibit the r4S-induced efflux of $^{35}SO_4^{2-}$ (Table 1). This observation is consistent with the major pathway of $^{35}SO_4^{2-}$ handling being lysosomal efflux, equilibration with the cytosolic SO_4^{2-} pool and efflux through the plasma membrane via a SITS-sensitive anion transporter. The unsupplemented rate was also inhibited, presumably via a similar mechanism, although the effect of SITS on cellular defecation is unknown.

Appearance of ^{35}S radioactivity in proteoglycans

To test for utilization of lysosome-derived $^{35}SO_4^{2-}$ in *de novo* proteolygcan synthesis, medium fractions from SITS-treated, supplemented cultures were analysed by Sepharose CL-6B chromatography relative to SITS-treated, unsupplemented cells (Fig. 2). The unsupplemented cells released [^{35}S]DS as previously described, as well as $^{35}SO_4^{2-}$. Little [^{35}S]DS was present in higher molecular weight species such as proteoglycans, known to appear at

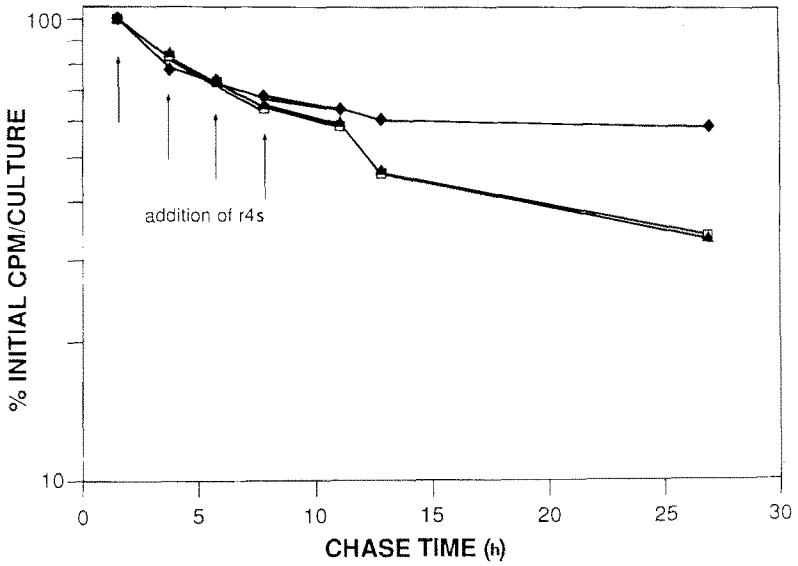

Figure 1. Kinetics of loss of ^{35}S radioactivity after supplementation as a function of medium sulfate concentration. Fibroblasts from MPS VI patients were labelled with $Na_2{}^{35}SO_4$ at 10 μCi ml^{-1} for 24 h in complete Ham's F12 medium as described in the Materials and methods section. One hour before addition of r4S, cultures were equilibrated with Ham's F12/1% FBS with (▲) or without (□) 300 μM Na_2SO_4. A control culture (◆) was supplemented with neither r4S nor Na_2SO_4 (hence 20 μM sulfate). The media were changed for fresh media at the time points shown: r4S (0.26 μg ml^{-1}) was present during the first four chase intervals of the corrected cultures (arrowed).

Table 1. Effect of SITS on the efflux of $^{35}SO_4^{2-}$ into the medium of r4S-supplemented MPS VI fibroblasts.[a]

Experiment	*$^{35}SO_4^{2-}$ efflux rate*	*Total sulfatase activity*[b]
	10^4 cpm per mg protein per 24 h	nmol min^{-1} mg^{-1}
Control	6.2	1.2
Control + 1 mM SITS[c]	3.4	0.8
Supplemented	30.3	11.2
Supplemented + 0.5 mM SITS[d]	19.0	13.7
Supplemented + 1 mM SITS[c]	13.5	9.2

[a] MPS VI fibroblasts were labelled with $Na_2{}^{35}SO_4$ at 10 μCi ml^{-1} for 24 h in complete Ham's F12 as described, chased for 16 h in complete BME, the cells trypsinized, distributed amongst 10 flasks and allowed to reattach over 1 h. The medium of all cultures was exchanged for fresh complete BME supplemented to 0.26 μg ml^{-1} r4S in supplemented cultures, or unsupplemented for the control cultures. SITS was included 2 h after addition of the r4S, and the chase continued for 19.7 h. Total sulfatase activity was measured using the fluorogenic assay described in the Materials and methods section.

[b] Measured in cell homogenates after the chase experiment was complete. Control values represent arylsulfatase A and C activities rather than residual 4S.

[c] Initial concentration of 1 mM SITS reduced after 8 h to 0.2 mM.

[d] Initial concentration of 0.5 mM SITS reduced after 8 h to 0.2 mM.

the void volume of this column. Supplementation of the cells with r4S and addition of SITS during the chase led to a significant increase in the incorporation of radioactivity into this proteolygcan material, as well as a decrease in the radioactivity eluting between 17 ml and 30 ml. The proportions of total ^{35}S radioactivity in the medium fractions as $^{35}SO_4^{2-}$ were 93% and 69% for supplemented and unsupplemented cultures, respectively, consistent with ethanol precipitation data presented above. The total amount of ^{35}S radioactivity in the proteoglycan peak (12–15 ml) was fourfold higher in r4S supplemented, SITS-treated cultures (2760 disintegrations per min per mg protein compared with 660 per mg in unsupplemented cultures). These data suggest some recycling of $^{35}SO_4^{2-}$ released from lysosomes during enzyme supplementation. SITS apparently increased the proportion of the lysosome-derived sulfate that was recycled into sulfated macromolecules. Since the profile for supplemented cells which had not been SITS-treated also exhibited low amounts of proteoglycans (data not shown, but not significantly different from Fig. 2, unsupplemented), this

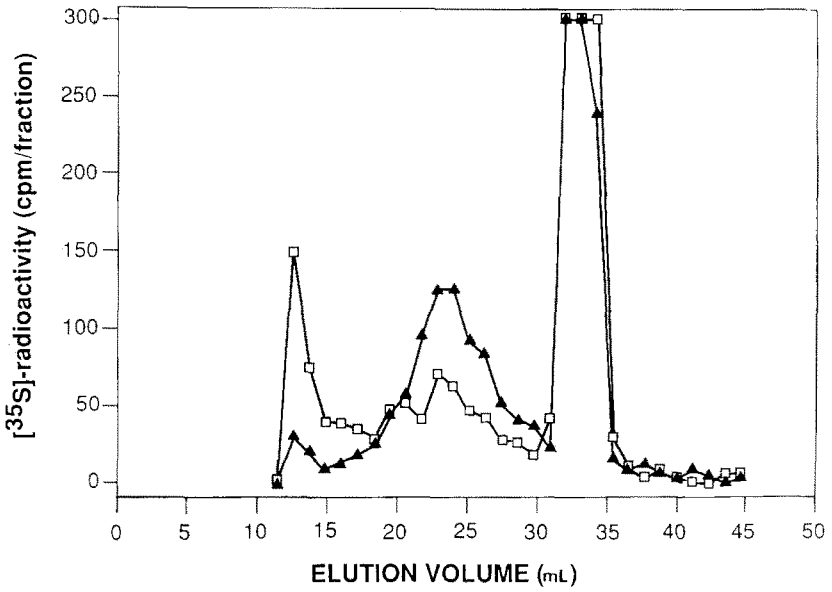

Figure 2. Sepharose CL-6B separation of ^{35}S radioactivity in the medium of SITS-treated MPS VI fibroblasts, with or without r4S supplementation. MPS VI fibroblasts were treated as described in Table 2. The medium fractions were separated on Sepharose CL-6B, equilibrated in 2 M GuHCl: 50 mM sodium acetate: 100 mM Na_2SO_4, pH 7.5. The elution profiles refer to r4S-supplemented (□) and unsupplemented (▲) cultures.

presumably resulted from inhibition of the normal processes of radiolabelled sulfate efflux and unlabelled sulfate influx. Interestingly, there was no evidence for recycling in the reduced sulfate experiments described above (Fig. 1).

[^{35}S]*DS turnover in isolated organelles*

To examine inhibitors of sulfate transport at the level of the lysosome, [^{35}S]DS loaded granules were isolated from enzyme-supplemented MPS VI cells and incubated *in vitro*. The supernatant radioactivity increased during the course of the 60 min incubation, over and above the zero time value (Table 2) as did the proportion of $^{35}SO_4^{2-}$ in the supernatant (Fig. 3(a)). The rate of $^{35}SO_4^{2-}$ appearance in the supernatant due to rupture of the organelles was estimated from the lysosomal latency (described in the Materials and methods section), i.e., the total $^{35}SO_4^{2-}$ was calculated as the sum of pellet and supernatant $^{35}SO_4^{2-}$ and this was multiplied by the lysosomal latency. This derived value also increased during the incubation, but did not account for all the $^{35}SO_4^{2-}$ appearing in the supernatant. The remainder probably came from transmembrane transport. Supporting this conclusion are the data obtained with granules from cells which had been supplemented with r4S. In this case (Fig. 3(b)), the rate of appearance of $^{35}SO_4^{2-}$ in the supernatant could be totally accounted for by rupture of the organelles. This type of experiment was repeated three times with measurable differences between supplemented and unsupplemented organelles in each.

The total $^{35}SO_4^{2-}$ present after the 60 min incubation divided by the initial pellet [^{35}S]DS was used to calculate the percentage degradation of total DS. This was 2.7% in the unsupplemented case and 14.2% in the supplemented case. Hence, it appears that the reaction was incomplete. Others have also noted the limitations on complete degradation in isolated granules [18]. Further, the relatively high rate of degradation in the unsupplemented granules suggests some degradation of sulfated polymers other than DS.

This correctable system provided an opportunity to study DS degradation and $^{35}SO_4^{2-}$ efflux from intact lysosomes under conditions of high substrate load. Jonas and Jobe [14] have found that sulfate transport across the lysosomal membrane is mediated by a system that is inhibited by the stilbene DIDS. We reasoned that the closely related compound SITS should inhibit sulfate efflux from the intact granule. SITS at 0.5 mM, and to a lesser extent at 0.1 mM, inhibited $^{35}SO_4^{2-}$ efflux (Table 3). Since the sulfate flux was generated by glycosaminoglycan turnover, the rate of degradation was also measured and also found to be reduced by SITS. In standard enzyme assays, SITS did not inhibit r4S activity in the range 0.01–1 mM, and so its action is likely to result from interactions of SITS with ion transport activities of the lysosomal membrane, which then influence glycosaminoglycan turnover by a mechanism not yet defined. Consistent with this conclusion, intralysosomal $^{35}SO_4^{2-}$ was not increased by SITS treatment (Table 4). Further, 5 mM SO_4^{2-} and MoO_4^{2-} inhibited $^{35}SO_4^{2-}$ efflux as well as DS degradation (Table 3). Both ions have been found to inhibit sulfate transport across the plasma membrane [43] and the lysosomal membrane [14], but, again, neither resulted in an accumulation of $^{35}SO_4^{2-}$ within the lysosomes (Table 4). It is possible that inhibition of

Table 2. Distribution of ^{35}S radioactivity into four fractions during incubation of post-nuclear supernatants from r4S supplemented and unsupplemented MPS VI fibroblasts. Experiment procedure as described in the legend to Fig. 3.

Experiment	*Time* (min)	*Pellet fraction* (dpm)		*Supernatant fraction* (dpm)			% *Latency*
		$^{35}SO_4^{2-}$ [a]	[^{35}S]DS [b]	$^{35}SO_4^{2-}$ [a]	Δ	[^{35}S]DS [b]	
Control	0	202 [c,d]	5294 [d,e]	308 [d]	–	3802	–
	10	128	4170	329	21	5024	23
	30	263	3500	294	−12	5006	23
	60	305 [d]	3928	348 [d]	40	5113	25
Supplemented	0	67	5173	286	–	3867	–
	10	154	4329	395	109	4578	14
	30	181	3972	686	300	5001	22
	60	197	3345	891	605	5270	27

[a] Ethanol soluble ^{35}S radioactivity; >90% $^{35}SO_4^{2-}$.
[b] Ethanol insoluble ^{35}S radioactivity.
[c] All data corrected for minor (<10%) variation between experimental aliquots.
[d] Percentage DS degradation can be calculated from these data as ((305 − 202) + (348 − 308))/5294, i.e., 2.7%.
[e] Zero time, [^{35}S]DS used in calculation of lysosomal latency. Initial non-latency during preparation 42%.

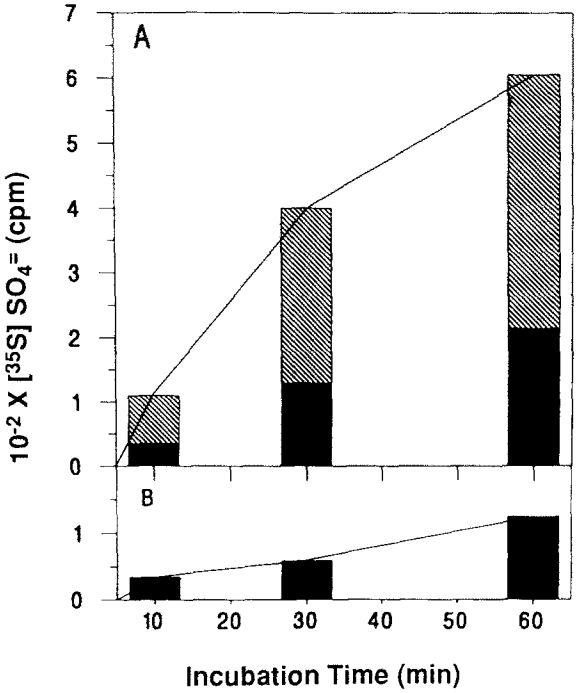

Figure 3. Loss of $^{35}SO_4^{2-}$ from intact organelles of r4S-supplemented MPS VI fibroblasts. Fibroblasts from MPS VI patients were labelled, chased for 16 h as described in Table 1; r4S (0.26 μg ml^{-1}) was added for 3 h after replating. Cells were harvested, disrupted and fractioned as described in the Materials and methods section. Cell post-nuclear fractions were suspended in buffer with a final concentration of 0.5 M sucrose, 20 mM Tris-HCl, 0.1 mM ATP, 5 mM $MgCl_2$, pH 7, and were then incubated at 37 °C for 10, 30 or 60 min. The zero time point sample was centrifuged immediately after suspension of the nuclear fraction. At the completion of the incubation, aliquots were centrifuged at approximately 12 000 × **g** in an Eppendorf microfuge for 10 min. The supernatants and pellets were separated. The

(*continued in next column*)

Table 3. Effect of various additives on the rates of DS degradation and sulfate efflux from post-nuclear supernatants of r4S supplemented MPS VI fibroblasts. The experiment was performed as described in the Materials and methods section and the legend to Fig. 3. Incubations were for 60 min at 37 °C.

Additive	$^{35}SO_4^{2-}$ *efflux rate* [a] *disintegrations* (min^{-1} h^{-1})	% *Inhibition of efflux*	% DS *degradation* [b]	% *Inhibition of degradation*
None (n = 5)	227 ± 12	–	6.5	–
SITS 0.1 mM	165	27	4.6	29
0.5 mM	0.5	99.5	0.8	87
SO_4^{2-} 5 mM	48	79	2.1	68
MoO_4^{2-} 5 mM	139	39	3.8	41

[a] Supernatant $^{35}SO_4^{2-}$ corrected for efflux due to lysosomal rupture.
[b] Calculated as described in Table 2, footnote d. The unsupplemented residual rate has been subtracted. n = number of full experimental repeats.

r4S within the organelle is achieved by very low amounts of SO_4^{2-} associated in stoichiometric amounts with the enzyme as opposed to macroscopic accumulation of the anion to levels equivalent to those used in an *in vitro* assay.

GAG turnover in the lysosome is known to be a rapid process [25] and studied with isolated lysosomal membrane vesicles suggest that product transport may also be rapid [8, 11, 14]. In normal fibroblasts, where the steady state

proportion of counts present as DS and SO_4^{2-} was determined by ethanol precipitation. The raw counts were corrected for the zero time supernatant SO_4^{2-}, the change in lysosomal latency and minor variations in the size of aliquots between experiments, to give estimates of the total supernatant $^{35}SO_4^{2-}$ (bars) and that appearing in the supernatant due to organellar rupture (filled section of each bar). (a) r4S-supplemented granules; (b) unsupplemented granules.

Table 4. Distribution of ^{35}S radioactivity into four fractions during incubation of post-nuclear supernatants from r4s supplemented MPS VI fibroblasts in the presence of transport inhibitors. The experiment was performed as described in the Materials and methods section and the legend to Fig. 3. Incubations were for 60 min at 37 °C.

Experiment	*Time* (min)	*Pellet fraction* (dpm)		*Supernatant fraction* (dpm)			% *Latency*
		$^{35}SO_4^{2-}$ [a]	[^{35}S]DS [b]	$^{35}SO_4^{2-}$ [a]	Δ	[^{35}S]DS [b]	
Supplemented	0	293 [c]	4970 [d]	424	–	3936	–
	60	298	4322	814	391	5028	22
	60	247	4068	724	301	4714	16
+SITS (100 lm)	60	236	3183	707	284	5076	23
+SITS (500 lm)	60	260	3836	497	74	5023	22
+$MgSO_4$ (5 mM)	60	201	3140	621	197	5799	38
+Na_2MoO_4 (5 mM)	60	256	4012	650	226	4834	18

[a] Ethanol soluble ^{35}S radioactivity; >90% $^{35}SO_4^{2-}$.
[b] Ethanol insoluble ^{35}S radioactivity.
[c] All data corrected for minor (<10%) variation between experimental aliquots.
[d] Zero time, [^{35}S]DS used in calculation of lysosomal latency. Initial latency during preparation 42%.

levels of undegraded GAG and end-products should be low, Rome and Hill [17] found that sulfate efflux paralleled GAG degradation. Efflux and degradation again occurred concomitantly in the enzyme-supplemented MPS VI lysosome, where the steady state level of undegraded GAG and the rate of production of sulfate are higher than normal by design. Accumulation of sulfate within the lysosome was not measurable, even in the presence of transport inhibitors. This result may reflect a non-saturable component of sulfate transport, which is often observable in reconstituted membrane vesicle transport experiments (G. S. Harper, unpublished observations).

The enzyme corrected lysosome system described in this paper will enable more detailed studies of the potentially coupled processes of GAG degradation and the transport of monosaccharide and inorganic sulfate products from the lysosome [1]. However, the possibility that genetic deficiency of *N*-acetylgalactosamine-4-sulfatase indirectly affects other normal properties of lysosomes, such as membrane integrity or sulfate transporter activity, has yet to be investigated.

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