The function of isolated lysosomes was studied by measuring mucopolysaccharide degradation. Cultured human diploid skin fibroblasts were grown in medium containing H$_2$SO$_4$ to label endogenous mucopolysaccharide. Lysosome containing preparations at various stages of purity were isolated from disrupted cells. These preparations degraded mucopolysaccharide as indicated by the release of radioactive sulfate. Degradation was temperature-dependent, required intact lysosomes, and was optimal when incubation was carried out at neutral pH in a buffer of low ionic strength. Lysosomes from Harleb fibroblasts were unable to carry out the degradative process. ATP at 0.5 mM was found to stimulate both the rate and the extent of mucopolysaccharide degradation; GTP, UTP, and CTP had similar effects, whereas the noncleavable ATP analog adenosine 5'-(pyrimido)triphosphate gave no stimulation. The ATP stimulation was inhibited by nigericin. ATP also stimulated chloroquine accumulation in lysosomes, the magnitude of which was used to measure the change in intralysosomal pH. The presence of ATP was associated with acidification of lysosome pH by 0.23 units.

Acetyl coenzyme A was also found to stimulate lysosome function. This reagent, however, had no effect on chloroquine accumulation and thus appears to stimulate mucopolysaccharide degradation by a mechanism different from that caused by ATP.

Lysosomes are highly specialized organelles that have a low internal pH and contain hydrolytic enzymes which have acid pH optima. Much information is available on characterization of individual lysosomal enzymes. However, much less is known about how these enzymes function together in intact organelles and how the organelles themselves are influenced by the intracellular environment in which they exist. We decided to address this problem by examining the degradation of mucopolysaccharide in intact isolated lysosomes.

When cultured human fibroblasts are grown in medium containing radioactive sulfate (H$_2$SO$_4$), the label is incorporated exclusively into cellular mucopolysaccharide (1). After about 24 h a steady state is reached when intake of SO$_4^{2-}$ is balanced by secretion and degradation of labeled mucopolysaccharide (2). Degradation of the mucopolysaccharide (primarily dermatan sulfate and heparan sulfate) takes place in lysosomes by the consecutive action of at least nine different exoglycosidases (3). If any one of the enzymes needed for the degradation of the carbohydrate chains is absent, a block in the normal stepwise degradation will result and the mucopolysaccharide will accumulate in the lysosomes (4). The present paper demonstrates that lysosomes isolated from normal cells are able to degrade endogenous mucopolysaccharide and that this process can be stimulated by nucleotide triphosphates and acetyl coenzyme A.

**MATERIALS AND METHODS**

**Reagents**—4-Methylumbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyranoside was purchased from Research Products International. H$_2$SO$_4$ (carrier-free) and [ring-3,5-C]chloroquine were from New England Nuclear. Dulbecco’s modified Eagle’s medium (powdered) and tissue culture grade trypsin were purchased from Grand Island Biological Co. Nigericin was a gift of Lilly. All other reagents were purchased from standard commercial suppliers and were the best grade available.

**Cell Culture**—Diploid human fibroblasts were obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ) or from samples submitted for diagnosis. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 0.1 g of streptomycin sulfate, and 0.25 mg of polymyxin B sulfate. Cultures were maintained at 37 °C in an atmosphere of 5% CO$_2$ and 95% air.

For studies of mucopolysaccharide degradation, a low sulfate medium was prepared that was identical to the normal medium except that MgCl$_2$ was substituted for MgSO$_4$, and streptomycin sulfate and polymyxin B sulfate were omitted. Cells were labeled with H$_2$SO$_4$ (100 µCi in 35 ml of low sulfate medium) for 2 days before harvest. Preparation of Lysosomes—The cells were harvested and disrupted as described (5) except that cells were not incubated at 37 °C after trypsinization. In most experiments either the postnuclear supernatant or a lysosomal-mitochondrial (M + L) fraction was used as the source of lysosomes. The M + L fraction was obtained by centrifugation of the postnuclear supernatant at 12,000 rpm (17,750 × g) for 20 min (Serval SM-24 rotor) and resuspended in 0.25 M sucrose before use. Highly purified lysosomes were obtained by fractionation of the postnuclear supernatant on colloidal silica gradients (5).

**Mucopolysaccharide Degradation Assay**—Degradation of endogenous 35S-labeled mucopolysaccharide by lysosomes was followed by the release of free 35SO$_4^{2-}$. Aliquots of incubation mixtures (100 µl) were combined with 400 µl of unlabeled mucopolysaccharide (sodium chondroitin sulfate, 5 mg/ml in 2.0 M sodium chloride) in polycarbonate centrifuge tubes (Naigene, 12 ml; 16 × 100 mm). The undegraded mucopolysaccharide was precipitated by addition of 2.0 ml of 95% ethanol. Samples were mixed (Vertex Genie-mixer), heated for about 2 min in a 95°C water bath, and placed on ice for 5 min. The pellets were collected by centrifugation (12,000 × g, 10 min), and the supernatants were immediately decanted into scintillation vials. The pellets were dissolved in 0.5 ml of 2.0 M sodium chloride by gentle heating in the 95°C water bath and reprecipitated with 2.0 ml of ethanol. The tubes were again heated, cooled, and cenrifuged, and the supernatants were decanted as described above. This second extraction was necessary to release 35SO$_4^{2-}$ that had been trapped in the first precipitate. The final pellets were dissolved in 0.5 ml of 1.0 M NaCl and transferred to scintillation vials. The tubes were washed once with 0.5 ml of 1.0 M NaCl and once with 0.5 ml of water to optimize the transfer. Liquid scintillation mixture (10 ml of Aquasol, West Chem Products) was added and the samples were counted in a Packard Model 3320 liquid scintillation spectrometer. The per cent of labeled mucopolysaccharide degraded in each sample was represented.
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by the ethanol soluble radioactivity, expressed as a percentage of the total radioactivity (combined ethanol extracts plus pellet).

**Lysosome Latency Assay**—The extent of lysosome latency was determined by measuring the activity of β-hexosaminidase (EC 3.2.1.30) in each incubation mixture in the presence and absence of detergent (6, 7). The assay mixtures contained 25 μl of a diluted lysosome preparation plus 275 μl of 1.2 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 13 mM citric acid, 20 mM sodium phosphate, pH 4.4, containing 0.25 M sucrose. Assays with detergent were identical with that described above, but reaction mixtures contained 0.1% Triton X-100. After 2 min at 37 °C, the assays were quenched with 1 ml of glycine/carbonate buffer, pH 10 (6). The liberated 4-methylumbelliferyl was measured in a Farrand MK I spectrophotofluorometer (365 nm excitation and 450 nm emission). One unit represents 1 nmol of substrate hydrolyzed/min. The percentage of lysosomes that were broken was represented by the free β-hexosaminidase activity (measured in the absence of detergent), expressed as a percentage of the total enzyme activity (measured in the presence of detergent).

### RESULTS

**Mucopolysaccharide Assay**—Crude fibroblast mucopolysaccharide, labeled by addition of H$_3$SO$_4$ to the cell growth medium, could be rapidly separated from free $^{35}$S0$_4^{2-}$ by precipitation with 80% ethanol (1). When $^{35}$S0$_4^{2-}$ was mixed with unlabeled mucopolysaccharide and an ethanol precipitation was carried out, 98.6% of the added radioactivity remained soluble (Table I). [$^{35}$S]Mucopolysaccharide, subjected to the same procedure, was found almost entirely in the insoluble residue. Mixtures of mucopolysaccharide and sulfate at 1:1 and 1:10 ratios were separated by the precipitation procedure in the expected proportions. This assay appears valid for separation of free $^{35}$S0$_4^{2-}$ and [$^{35}$S]mucopolysaccharide.

### Lysosome Stability and Function—Fibroblasts were harvested and disrupted, and the suspension was freed of nuclei and unbroken cells (5). The postnuclear supernatant which contains lysosomes was incubated in 0.25 M sucrose and the time course of lysosomal stability (determined by measuring latency) and function (viz. mucopolysaccharide degradation) was examined at different temperatures (Fig. 1). The extent of lysosome latency varied among different lysosome preparations and among different cell types but generally indicated that 85-95% of the lysosomes were intact following cell disruption and removal of nuclei. Addition of detergent (0.1% Triton X-100) prior to assay completely abolished the observed latency. Lysosomes could also be broken, although not as consistently, by sonication or repeated freezing and thawing. Organelles kept at 0 °C remained intact over a 5-h period (Fig. 1A). Increased incubation temperatures resulted in an increased rate of lysosome disruption. Lysosomes incubated at 37 °C broke at a rate of approximately 8%/h.

In addition to maintaining structural integrity, the lysosomes present in the postnuclear supernatant retained the ability to degrade endogenous labeled mucopolysaccharide (Fig. 1B). Degradation of [$^{35}$S]mucopolysaccharide was dependent on temperature; the suspension kept at 0 °C was unable to degrade its [$^{35}$S]mucopolysaccharide, whereas those maintained at 25 °C and 37 °C for 5 h degraded 5% and 12%, respectively. Degradation required intact lysosomes since disruption of the organelles by sonication, freeze-thawing, or detergent completely prevented sulfate release.

The stability of lysosomes in the postnuclear supernatant was determined in a number of buffers at different pH values. Lysosomes broke rapidly in acidic (pH 4-5) medium, as determined by loss of latency (Fig. 2A). Lysosomes in the postnuclear supernatant buffered between pH 6 and pH 7.5 showed the greatest stability. Lysosomal breakage was much increased above pH 8. Degradation of [$^{35}$S]mucopolysaccharide was also measured for each of the incubations described in Fig. 2 after a 24-h incubation. Some ethanol-soluble radioactivity was released at pH 4 (9.5%) and even more at pH 5.

### Table I

**Separation of mucopolysaccharide from free sulfate by ethanol precipitation**

| Assay       | Ethanol-soluble | Ethanol-insoluble | Soluble | Insoluble |
|-------------|----------------|------------------|--------|----------|
|             | Extract        | Extract          | %      | cm³      | %        | cm³     |
| 1. [{$^3$}S]MPS | 443            | 109              | 68,808 | 0.8      | 99.2     |
| 2. H$_3$SO$_4$ | 62,628         | 7,641            | 1,016  | 98.6     | 1.4      |
| 3. [{$^3$}S]MPS + H$_3$SO$_4$ | 7,142          | 791              | 67,255 | 10.5     | 89.5     |
| (1:10)      |                |                  |        |          |          |
| 4. [{$^3$}S]MPS + H$_3$SO$_4$ | 61,446         | 7,547            | 7,798  | 89.9     | 10.1     |
| (1:10)      |                |                  |        |          |          |

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![Fig. 1. The effects of temperature on lysosome latency and function. Normal fibroblasts, cultured to confluency in 150-cm$^2$ flasks, were labeled with H$_3$SO$_4$ as described under "Materials and Methods." A postnuclear supernatant was prepared (5), and aliquots were incubated under the following conditions: A—-A, 37 °C; □—□, 25 °C; O—O, 0 °C; ▽—▽, 37 °C with 0.1% Triton X-100 added. After the indicated times, samples of each incubation were withdrawn for determination of lysosome latency (A) and mucopolysaccharide degradation (B).](image-url)
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Fig. 2. The effect of pH on latency of isolated normal and Hurler lysosomes. A, the postnuclear supernatant was incubated at 37 °C in 0.25 M sucrose and the following buffers: ○—○, 25 mM sodium acetate, pH 4.0; △—△, 25 mM sodium acetate, pH 5.0; ●—●, 25 mM sodium phosphate, pH 6.0; ▲—▲, 25 mM sodium phosphate, pH 7.0; ■—■, 25 mM sodium phosphate, pH 8.0. B, normal (●—●) and Hurler (○—○) postnuclear supernatant incubated at 37 °C in 20 mM Tris-HCl (pH 7.0), 0.25 M sucrose.

Lysozyme stability was the same at pH 7 in phosphate (Fig. 2A) or Tris-HCl buffer (Fig. 2B). However, lysosomes incubated in phosphate buffer (Fig. 2A, pH 6–8) were unable to degrade [35S]mucopolysaccharide, probably because of inhibition of lysosomal sulfatases by inorganic phosphate (8) since pH 7 Tris-HCl was not inhibitory. [35S]Mucopolysaccharide degradation in 0.02 M Tris-HCl, pH 7, was 29.4%.

In additional experiments (not shown) it was found that low ionic strength (<50 mM) gave best lysosome stability. Unless otherwise stated the remaining incubations were carried out in 0.02 M Tris-HCl (pH 7.2), 0.25 M sucrose.

In the postnuclear supernatant of fibroblasts isolated from Hurler patients (and therefore lacking the enzyme α-L-iduronidase), the rate of lysosome breakage at 37 °C was somewhat higher than that seen with normal cells (Fig. 2B). This increased fragility was reproducible and was observed with four different Hurler cell cultures (GM0415, GM1391, and two cultures obtained from samples submitted for diagnosis). The Hurler lysosomes were completely unable to degrade their endogenous [35S]mucopolysaccharide.

ATP Stimulation of Lysosomal Function—Of the numerous chemicals evaluated for a stimulatory effect, ATP plus Mg2+ consistently activated [35S]mucopolysaccharide degradation in the cell-free system. Lysosomes incubated in the presence of 100 μM ATP and 5 mM MgCl2 degraded 50% more [35S]mucopolysaccharide than controls without ATP (Fig. 3). Addition of ATP did not affect lysosome stability.

The effect of ATP concentrations was examined with a partially purified preparation of lysosomes (M + L fraction) as well as the postnuclear supernatant. [35S]Mucopolysaccharide degradation in both preparations was stimulated by ATP (Fig. 4). The stimulation almost reached a plateau at approximately 0.5 mM ATP. In the experiment shown in Fig. 4 the maximum stimulation of [35S]mucopolysaccharide degradation by ATP (5 mM) was similar in both the postnuclear supernatant (50%) and the L + M fraction (65%). However, in other experiments stimulation of the M + L fraction by ATP was as high as 120% (Fig. 6, Table II).

ATP was not the only nucleotide that could stimulate [35S]mucopolysaccharide degradation; GTP, CTP, and UTP were equally effective. However, there was little or no stimulation by ADP, AMP-PNP (1) (a nonhydrolyzable ATP analog), and cAMP (not shown) (Table II). Lysosome stability was unaltered by these reagents.

1 The abbreviation used is: AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate.
fraction, described above). \[^{35}S\]Mucopolysaccharide degradation in isolated lysosomes was stimulated by ATP and acetyl-CoA together was additive. CoA gave only approximately 30% by 5 \times 10^{-6} M CoA, but ATP doubled this stimulation (Table II). None other nucleotides tested showed a slight enhancement over the controls (no additions or with ATP) (Table III). This was found to be the case. Lysosomes (M + L fraction) were incubated for 60 min with \[^{35}S\]chloroquine in the absence of ATP, nigericin was only slightly inhibitory. The second line of evidence indicating that ATP might act by stimulating a lysosomal proton pump came from studies of the distribution of \[^{35}S\]chloroquine in lysosomes. An increased proton concentration in lysosomes should be accompanied by an increased uptake of \[^{35}S\]chloroquine into these organelles due to trapping of the protonated form of the weak base (10^-12). The second line of evidence indicating that ATP might act by stimulating a proton pump came from studies of the distribution of \[^{35}S\]chloroquine in lysosomes. An increased proton concentration in lysosomes should be accompanied by an increased uptake of \[^{35}S\]chloroquine into these organelles due to trapping of the protonated form of the weak base (10^-12). The second line of evidence indicating that ATP might act by stimulating a proton pump came from studies of the distribution of \[^{35}S\]chloroquine in lysosomes. An increased proton concentration in lysosomes should be accompanied by an increased uptake of \[^{35}S\]chloroquine into these organelles due to trapping of the protonated form of the weak base (10^-12). The second line of evidence indicating that ATP might act by stimulating a proton pump came from studies of the distribution of \[^{35}S\]chloroquine in lysosomes. An increased proton concentration in lysosomes should be accompanied by an increased uptake of \[^{35}S\]chloroquine into these organelles due to trapping of the protonated form of the weak base (10^-12).

**Uptake of Chloroquine**—The enhancement of \[^{35}S\]mucopolysaccharide degradation by ATP may be due, at least in part, to stimulation of a lysosomal neutral pump. Two lines of evidence lead us to this conclusion. First, the proton ionophore nigericin (5 \mu g/ml) in the presence of 10 mM KCl completely prevented the stimulation of \[^{35}S\]mucopolysaccharide degradation by ATP, even at high ATP concentrations (Fig. 4). In the absence of ATP, nigericin was only slightly inhibitory. The second line of evidence indicating that ATP might act by stimulating a proton pump came from studies of the distribution of \[^{35}S\]chloroquine in lysosomes. An increased proton concentration in lysosomes should be accompanied by an increased uptake of \[^{35}S\]chloroquine into these organelles due to trapping of the protonated form of the weak base (10^-12). This was found to be the case. Lysosomes (M + L fraction) incubated for 60 min with \[^{35}S\]chloroquine in the absence of ATP accumulated 12.8% of the added radioactivity (Table IV). Addition of 0.5 mM ATP doubled this accumulation. There was only a very slight accumulation of \[^{35}S\]chloroquine.
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TABLE IV
Accumulation of [14C]chloroquine in isolated lysosomes

Lysosomes (M + L fraction, derived from 3 confluent 155-cm² flasks) were incubated at 37°C in 0.02 mM Tris-HCl (pH 7.2), 5 mM MgCl₂, 0.25 M sucrose, 1 mM [14C]chloroquine (3 x 10⁻⁶ Ci/μmol), and additions listed. The concentrations of ATP and AMP-PNP were 0.5 mM, acetyl-CoA was 5 μM, and nigericin was used at a level of 25 μg/ml with 10 mM KCl. After a 1-h incubation, samples were assayed for lysosome latency (see "Materials and Methods") and a 100-μl aliquot of each mixture was transferred to a 1.5-ml Brinkmann test tube. Lysosomes were collected by centrifugation for 10 min in an Eppendorf model 5412 microcentrifuge at 15,000 × g. Supernatants were transferred to scintillation vials for measurement of radioactivity. The pellets were dissolved in 0.25 M sucrose, 0.1% Triton X-100 and assayed for β-hexosaminidase activity and radioactivity.

| Additions | [14C]chloroquine radioactivity |Fraction of lysosomes broken | Corrected [%] chloroquine in pellet | Change in pH |
|-----------|--------------------------|-----------------------------|---------------------------------|-------------|
| none      | 12.8                     | 0.28                        | 17.8                            |             |
| Acetyl-CoA | 11.4                     | 0.22                        | 14.6                            | 0.05        |
| ATP       | 26.6                     | 0.30                        | 38.0                            | -0.23       |
| ATP + acetyl-CoA | 26.6                | 0.29                        | 37.5                            | -0.22       |
| AMP-PNP   | 15.0                     | 0.28                        | 20.7                            | -0.04       |
| ATP + nigericin | 2.5                 | 0.75                        | 10.2                            | 0.15        |

* [%]Chloroquine radioactivity was corrected by dividing the percent [%]chloroquine in pellet by the fraction of lysosomes that were intact.

The change in the intralysosomal pH that resulted when the various reagents were added to the control (no additions) was calculated from the corrected data by the following formula: change in pH = \[ \frac{1}{\text{pellet}} \times \log(\% \text{[14C]chloroquine in the control pellet}/\% \text{[14C]chloroquine in the supernatant}) - (\%\log(\% \text{[14C]chloroquine in pellet}/\% \text{[14C]chloroquine in the supernatant}). \]

The calculations assume that the lysosome volume is the same in each incubation and that each molecule of chloroquine binds two protons in the lysosome. The theoretical basis for similar pH calculations has been discussed (18, 21, 23).

Previous studies of [35S]mucopolysaccharide metabolism in foreskin fibroblasts revealed a biphasic rate of degradation; 70% of intracellular [35S]mucopolysaccharide was degraded in 12 h, whereas the remaining radioactivity was released from cells at a rate of approximately 5%/day (1). In the present study the degradation of [35S]mucopolysaccharide by isolated lysosomes under optimal conditions appears to plateau after 3-5 h incubation with about 30% of the total [35S]mucopolysaccharide degraded. Although more than half of the lysosomes were intact when degradation slowed, [35S]Mucopolysaccharide degradation could have stopped due to the production of an inhibitor or the depletion of a cofactor. However, addition of unlabeled intact or disrupted lysosomes (freshly prepared or preincubated for 3 h at 37°C) to lysosome suspensions did not inhibit or activate the degradation (not shown). Acetyl-CoA and ATP did not appear to be depleted during [35S]mucopolysaccharide degradation; hourly additions of these reagents to actively degrading lysosomes did not extend the period of [35S]mucopolysaccharide breakdown. Although some yet unidentified cofactor or inhibitor could be involved, it is possible that some [35S]mucopolysaccharide may be inaccessible to the hydrolytic enzymes in our system, perhaps as part of the mucopolysaccharide pool that is only slowly degraded in intact cells (1).

The marked increase in [35S]mucopolysaccharide degradation upon addition of ATP is accompanied by a lowering of intralysosomal pH. Various methods to measure intralysosomal pH, including the distribution of weak bases or acids (for review, see Ref. 18) and the degradation of endocytosed proteins (19, 20), published values for intralysosomal pH vary from below 4 to as high as 6.5, depending on the system studied and the method of pH measurement. Two models have been proposed to account for a low intralysosomal pH. One model is based on a Donnan-type equilibrium where fixed negatively charged groups within the lysosome can induce an asymmetric distribution of acid-base equivalents across the lysosomal membrane (18). The second model invokes an energy-dependent proton pump in the lysosomal membrane, presumably driven by the hydrolysis of ATP (21).

The effects of ATP on lysosome function that we have observed lend support to the existence of an ATP-driven pump. ATP stimulated both the degradation of endogenous mucopolysaccharide and the uptake of [14C]chloroquine in isolated lysosomes. The lack of similar effects by the ATP analog AMP-PNP indicates that ATP utilization may be a necessary step in the action of ATP. In addition, both degradation of mucopolysaccharide and uptake of [14C]chloroquine were inhibited by the proton ionophore nigericin, which would dissipate a proton gradient. Nigericin, however, did not completely block either lysosome function or base accumulation, a result that may indicate that other factors, such as fixed negative charges, may contribute to maintenance of the lysosomal

DISCUSSION

Lysosome function in a cell-free system was first studied by Mego and McQueen (13) who injected 125I-labeled albumin into mice and followed the release of acid-soluble radioactivity in isolated kidney and liver phagolysosomes. Studies of labeled-albumin degradation have also been conducted in rat liver phagolysosomes (14), and the digestion of 125I-labeled ribonuclease has been examined in mouse kidney lysosomes (15-17). The rate of digestion of these labeled proteins both in vivo and in vitro is a much more rapid process than the degradation of [35S]mucopolysaccharide that we have studied.

The t₁/₂ for 125I-labeled ribonuclease breakdown in vivo is approximately 14 min, while the rate in isolated phagolysosomes has been estimated at 11 min (15, 17). This contrasts with the initial rate of [35S]mucopolysaccharide degradation that we have observed with isolated fibroblast lysosomes (10-15%/h). However, this rate is at least as rapid as that seen in intact cultured cells (1, 5).
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The stimulation of $[^{35}S]$mucopolysaccharide degradation by acetyl-CoA appears to be by a mechanism that is different from that caused by ATP since the effects of the two compounds were additive and since acetyl-CoA was unable to stimulate chloroquine accumulation. The stimulation of degradation by acetyl-CoA probably occurs because this compound takes part directly in the degradation of heparan sulfate as a cofactor for acetylation of terminal glucosamine residues exposed by the action of heparan N-sulfatase (3, 9). These glucosamine residues are presumably resistant to further cleavage unless they are first $N$-acyetylated via the enzyme $N$-acetylglucosamine $N$-acetyltransferase (9). The source of acetyl-CoA for the transferase reaction is not known. The stimulation of $[^{35}S]$mucopolysaccharide degradation by acetyl-CoA in isolated lysosomes suggests that in intact cells lysosomes may be supplied with this substrate from the cytosol. The development of a system to study lysosome degradation of mucopolysaccharide in isolated intact organelles should allow us to determine if lysosomes have a specific transport system for acetyl-CoA.

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