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Are lysosomes involved in hexose transport regulation? Turnover of hexose carriers and the activity of thiol cathepsins are arrested by cyanate and ammonia

(cycloheximide/acid proteases/carbamoyl phosphate/*N*-ethylmaleimide)

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ABSTRACT **The cycloheximide-related loss of transport activity (manifested as a decrease in V_{max} for transport) in cultured Nil hamster fibroblasts was blocked by the addition of carbamoyl phosphate, cyanate (a product of spontaneous phosphate elimination from carbamoyl phosphate), or ammonium salts to the culture medium. Acid proteases capable of hydrolyzing α-*N*-benzoyl-D,L-arginine-β-naphthylamine (cathepsins B_1, H, and L) were also inhibited *in situ* by ammonia and cyanate. The inactivation of these cathepsins by ammonia was irreversible and probably was related to the increase in the intralysosomal pH known to be caused by an accumulation of ammonia in the lysosomes. The inhibition of the cathepsin activity by cyanate *in situ* (and in cell-free extracts) was completely reversible and blocked irreversible inhibition of the cathepsin(s) by *N*-ethylmaleimide. The inactivation of the cathepsins caused by cyanate was deduced to be the result of reversible blocking of sulhydryl groups essential to the thiol cathepsin activity. The concomitant inhibition of thiol cathepsins and hexose carrier inactivation provided further evidence for the involvement of lysosomal proteases in at least part of the mechanism that regulates the rate of hexose transport in animal cells.**

Cultured animal cells are capable of modulating their rates of hexose and amino acid transport in response to the availability of nutrients in their culture medium (1–11). These changes in the rates of nutrient transport in response to nutritional shifts have been termed "adaptive" (6, 7) and have been found to be related to changes in the number of functional carriers (1, 3, 5, 9). Under normal culture conditions, the control of the number of carriers in Nil hamster cells appears to be a balance between carrier synthesis and carrier inactivation (5, 8). When new carrier synthesis is blocked by including cycloheximide (or puromycin) in otherwise unmodified culture medium, Nil cells lose as much as 95% of their hexose transport and about 50% of their A-system amino acid transport capabilities within 24 hr (4, 5, 9, 10). These losses of activity, irreversible without protein synthesis, suggest a degradative (perhaps proteolytic) mechanism of inactivation (5, 8–10).

Several indirect observations support the inference of the involvement of proteolytic degradation of carriers in the control: (*i*) decreases in carrier activity are accompanied by decreases in the V_{max} for transport with little, if any, changes in K_m (8); (*ii*) carrier inactivation is irreversible without protein synthesis (unpublished data); and (*iii*) excess levels of cycloheximide, previously shown to interfere with protein degradation in autophagic vacuoles (12), block the loss of carrier activity (4, 5). More direct evidence came from the observation that changes in the activity of the lysosomal cathepsins of the sulfhydryl-requiring group (cathepsins B_1, H, and L) were accompanied by exactly opposite changes in hexose transport activity (13). The inverse relationship between transport activity and thiol cathepsin activity (13) suggested that hexose and amino acid carriers are inactivated when lysosomal cathepsin B_1-like proteases are functional and are spared degradation when these proteases are not functional (8, 13).

In this report, we present evidence demonstrating that thiol cathepsin activity can be affected *in situ* by including the readily reversible sulfhydryl blocking reagent cyanate or the lysosomotropic ammonium ion in the culture medium for 24 hr. When present in the culture medium along with cycloheximide, these reagents also arrest cycloheximide-induced losses of hexose carrier activity. This evidence reinforces the idea that thiol cathepsins may be involved in the regulation of hexose transport activity in animal cells (13).

MATERIALS AND METHODS

Reagents. Uniformly labeled D-[^{14}C]galactose and general labeled L-[^{3}H]glucose were purchased from New England Nuclear. Culture medium and fetal calf serum were obtained from GIBCO. Reagents for enzyme assays and cycloheximide were from Sigma.

Culture Conditions and Transport Assays. Nil hamster fibroblasts were maintained in a humidified Napco (model 7341) automatic CO_2 incubator (10% CO_2/90% air) in Falcon plastic culture dishes containing Dulbecco's modified Eagle's minimal essential medium (Dulbecco's medium) supplemented with 10% fetal calf serum. Cultures were then washed with sterile Dulbecco's phosphate-buffered saline (pH 7.4). Fresh medium (Dulbecco's containing 22 mM D-glucose, 10% dialyzed fetal calf serum, and ±7 μM cycloheximide) was then added and the cultures were incubated for 24 hr as described (4, 5). Prior to assays of uptake or transport and collection of cells for enzyme assays, the cultures were washed with the phosphate-buffered saline.

The washed cells were assayed for a 5-min uptake of D-[^{14}C]galactose as described (5). In transport kinetics experiments, the transport of D-galactose (0.1–1 mM) was for 15 sec at room temperature after exposure of the cells to 0.5 mM *N*-ethylmaleimide (MalNEt) for 15 min at 37°C in order to inactivate the intracellular galactokinase (8). Uptake and transport were corrected for simple diffusion/adsorption of the hexoses by subtracting the molar equivalents of the L-glucose associated with each assay from the molar equivalents of D-galactose (8). The carrier-mediated influx of galactose (by a 5-min uptake assay or a 15-sec transport assay) is referred to as "transport"

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Abbreviations: BANA, α-*N*-benzoyl-D,L-arginine-β-naphthylamine; MalNEt, *N*-ethylmaleimide; Cbm P_i, carbamoyl phosphate.

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because galactokinase does not appear to be rate limiting (5, 8) and all changes in transport rates caused by changes in culture conditions have been shown to be due to changes in the transport step (8).

After transport assays [and freeze/thaw lysis (see below)], proteins were dissolved in 0.1 M NaOH containing 0.1% sodium dodecyl sulfate and assayed by the method of Lowry *et al.* (14). Transport and enzyme activities were normalized for protein content and are referred to as activity per mg of cell protein per unit of time.

Enzyme Assays. Washed cultures were scraped with a Teflon policeman into 0.1 M phosphate buffer, pH 6/88 mM KH_2PO_4/12 mM Na_2HPO_4/1.3 mM Na_2 EDTA (referred to as pH 6 buffer) and the cells were lysed by five cycles of freezing (−15°C) and thawing (+25°C). Cathepsin B_1-like activity in the freeze/thaw lysates was measured as the release of β-naphthylamine from α-*N*-benzoyl-D,L-arginine-β-naphthylamine (BANA) essentially as described by Barrett and Heath (15). Briefly, 100-μl aliquots of the lysates (approximately 50 μg of protein) were preincubated for 10 min in 400 μl pH 6 buffer containing 2.5 mM L-cysteine in order to reduce the enzyme (15). Ten microliters of the substrate, BANA (dissolved in dimethyl sulfoxide), was then added (final concentration, 2 mM) and the mixture was allowed to incubate for 30 min at 40°C. The reaction was stopped by the addition of 500 μl of 2% (wt/vol) Brij 35 containing mersalyl acid and $NaNO_2$ (15). After they had been kept at room temperature (15), the samples were cleared by centrifugation in an Eppendorf 5412 centrifuge and the absorbance at 520 nm was measured. Blanks for standards and samples were obtained by adding the substrate after the stop reagent. Although this assay can be used to detect cathepsins B_1, H, and L [thiol cathepsins (15–17)], in this report the assay is referred to as the cathepsin B assay (15) and activity is expressed as nmol of naphthylamine released from BANA per mg of lysate protein per min.

β-Glucuronidase activity in lysates was measured as the release of 4-methylumbelliferone from 4-methylumbelliferone-β-D-glucuronide according to the method of Robins *et al.* (18). Aliquots (20 μl) of the lysate were incubated at 37°C for 1 hr in 80 mM acetate buffer at pH 4.6 containing 166 nM 4-methylumbelliferone glucuronide in a total volume of 120 μl. After the reaction was stopped by addition of 2 ml of 20 mM 2-amino-2-methyl-1-propanol·HCl (pH 10.4), the mixture was kept at room temperature for 15 min and then the fluorescence was determined by using excitation at 365 nm and emission at 450 nm. β-Glucuronidase activity is expressed as nmol of umbelliferone released per mg of protein per min.

RESULTS

Arrest of Hexose Carrier Inactivation. In the course of testing ATP and other high-energy compounds for stimulation of proteolysis in extracts of cultured Nil hamster fibroblasts, we adventitiously discovered that carbamoyl phosphate (Cbm P_i) inhibited acid protease activity. Because proteases have been suspected of being involved in the inactivation of hexose carriers (5, 8, 13), it was of interest to determine if Cbm P_i could arrest the inactivation of the carriers. It has been shown (1, 3, 5) that carrier inactivation could be distinguished from other potential control mechanisms (e.g., carrier synthesis resulting from derepression) by including cycloheximide (1–10 μl/ml in Dulbecco's medium) for 18–24 hr. At these concentrations, cycloheximide arrested protein synthesis by more than 90% and promoted the loss of 80–90% of the hexose carrier activity (4). Table 1 shows examples of major decreases in the rate of D-galactose transport resulting from the inclusion of cycloheximide in the culture medium of the confluent cells. When Cbm P_i was included in the culture medium along with cycloheximide, the loss of galactose transport activity related to the presence of cycloheximide was blocked. Cyanate, a reagent that Jones and Lipmann (19) showed was a product of spontaneous phosphate elimination from Cbm P_i and Stark (20) showed was a potent, reversible sulfhydryl reagent, was also effective in blocking the cycloheximide-associated loss of transport activity. Whereas Cbm P_i (or cyanate) always blocked the loss of hexose transport, stimulations (or inhibitions) of transport by Cbm P_i or cyanate alone were not consistent findings.

Table 1. Effects of cycloheximide, Cbm P_i, and potassium cyanate on D-galactose transport

Addition to culture medium	D-Galactose transport,* pmol/mg/5 min	Relative transport, %
None	260 ± 10	100
Cycloheximide	20 ± 5	8
Cbm P_i	590 ± 140	227
Cycloheximide + Cbm P_i	240 ± 5	92
None	751 ± 132	100
Cycloheximide	45 ± 16	6
Cyanate	516 ± 46	69
Cycloheximide + cyanate	606 ± 88	81

Confluent cultures of Nil cells were washed and maintained for 24 hr in Dulbecco's minimal essential medium containing 7 μM cycloheximide, (2 μg per ml), 10 mM dilithium carbamoyl phosphate (Cbm P_i), 10 mM potassium cyanate, 7 μM cycloheximide and 10 mM Cbm P_i, or 7 μM cycloheximide and 10 mM cyanate.

* Cultures were washed and assayed for D-galactose transport (0.1 mM D-[^{14}C]galactose). Values are shown as mean (± range) amount of galactose taken up per mg of cell protein per 5 min in duplicate cultures.

Because cycloheximide-mediated decreases in hexose carrier activity have been shown to be due to decreases in the V_{max} (with little or no change in K_m) for transport (13), it was of interest to determine the effects of Cbm P_i in transport kinetics experiments. The results of a typical galactose transport kinetics experiment are shown in Table 2. The presence of cycloheximide in the medium for 24 hr resulted in an approximately 90% decrease in the V_{max} for galactose transport. The presence of Cbm P_i in the culture medium along with cycloheximide effectively prevented this decrease in V_{max}. Although there also appeared to be changes in the K_m, they are small compared with the changes in V_{max}. The significance of the apparent changes

Table 2. Effects of cycloheximide and Cbm P_i on galactose transport kinetics

Culture condition	Galactose transport K_m*	Galactose transport V_{max}†
Control (no additions)	2.4	1.31
Cycloheximide	0.9	0.10
Cycloheximide + Cbm P_i	2.0	1.14

Confluent cultures were washed with buffered saline and maintained for 24 hr in Dulbecco's medium (control), Dulbecco's medium containing 7 μM cycloheximide, or Dulbecco's medium containing 7 μM cycloheximide and 20 mM Cbm P_i. Triplicate cultures were washed with buffered saline and then preincubated for 15 min at 37°C in buffered saline containing 0.5 mM MalNEt. The preincubation medium was removed and the transport of D-galactose (0.1, 0.2, 0.4, and 1.0 mM) was determined in the 15-sec assay. The reciprocal of the mean transport rates was plotted vs. the reciprocal of D-galactose concentration and the lines through the data points were obtained from linear regression analysis.

* K_m values are in mM (D-galactose).

† V_{max} values are pmol of D-galactose transported per mg of protein per 15 sec.

in K_m should be considered cautiously, especially because a decrease in K_m should contributed to an increase (not decrease) in the transport rate if the affinity of the substrate for the carrier site is the property involved.

Differential Effects of Cycloheximide, Cyanate, and Ammonia on Lysosomal Enzymes. Cbm P_i inhibited acid protease activity in extracts of cultured Nil cells. An earlier study had shown that ammonium ions increased transport and prevented cycloheximide-related losses of carrier activity (13). Moreover, with lysates of cultured cells with large differences in transport activity, an inverse relationship between cathepsin B activity and hexose transport was found (13). It was of interest, therefore, to determine what effects cycloheximide, ammonia, and cyanate had on cathepsin B-like activity *in situ*. Cultures were exposed to cycloheximide, NH_4Cl, or potassium cyanate for 24 hr and assayed for cathepsin B and β-glucuronidase activities by using frozen-thawed lysates of washed cells. Cycloheximide had not affected the recovery of either enzyme (Table 3). After cells were exposed to NH_4Cl, only about 10% of the cathepsin B activity could be recovered and β-glucuronidase was comparatively unaffected. A decrease in recoverable β-glucuronidase was seen in extracts of cultures exposed to cyanate. However, in striking contrast with the effects of ammonia, extracts of cultures treated with cyanate revealed an interesting increase in the activity of the recovered cathepsin B. This increase appeared to contradict the idea that the enzyme(s) should have been inhibited by cyanate *in situ*. This apparent contradiction could be rationalized by proposing that the enzyme within the lysosomes had been inactivated by cyanate during the culture period and that the inhibition was reversed as a result of lysis and dilution of the cyanate. In support of this hypothesis, it is known that cyanate is a readily reversible sulfhydryl reagent (20) and purified cathepsin B_1 is known to be protected from what has been described as autolytic degradation by reversible sulfhydryl reagents (21). Hence, the increase in cathepsin B activity in lysates of cells treated with cyanate indirectly supported the argument that cathepsin B was actually inhibited by cyanate *in situ*.

Cyanate Reversibly Inhibits Cathepsin B in Lysates. More direct support for the above hypothesis came by taking advantage of the observations that cathepsins B_1, H, and L are the only known acid proteases that specifically hydrolyze BANA and are also the only known sulfhydryl-requiring ("thiol") cathepsins (15–17, 21). It was reasoned that, if the cathepsin(s) from Nil cells was a thiol cathepsin, it should be inhibited by cyanate, a readily reversible sulfhydryl reagent (20). Table 4 shows the results of a typical competition experiment in which cyanate and the irreversible sulfhydryl reagent MalNEt were added to the cell-free lysate and then the lysate was assayed for cathepsin activity. Cyanate was at least as effective as MalNEt in ability to inactivate the cathepsin. In addition, the cyanate (but not the MalNEt) inhibition was fully reversed during a 4-hr dialysis.† Cyanate competed with MalNEt for the inhibition of the cathepsin(s), further indicating that the cathepsin activity is of the sulfhydryl-requiring (thiol cathepsin) type. Another consistent finding was that exposure of the lysate to cyanate protected the thiol cathepsin against losses of activity during extended dialysis. In the experiment shown, hydrolysis of BANA decreased about 33% during the 4-hr dialysis of the control lysate but did not decrease at all during dialysis of the cyanate-treated lysate.

Table 3. Effects of exposing cells to cycloheximide, ammonium ions, and cyanate on lysosomal enzyme activities

Addition to medium*	BANA hydrolysis, nmol naphthylamine/ mg/min	β-Glucuronidase activity, nmol umbelliferone/ mg/min
None	3.29 ± 0.15 (1.00)	1.90 ± 0.25 (1.00)
Cycloheximide	3.28 ± 0.05 (1.00)	2.63 ± 0.67 (1.38)
None	5.44 ± 0.09 (1.00)	2.68 ± 0.34 (1.00)
Cyanate	8.9 ± 0.3 (1.64)	2.05 ± 0.15 (0.76)
None	2.55 ± 0.08 (1.00)	5.9 ± 1.0 (1.00)
NH_4Cl	0.29 ± 0.02 (0.11)	4.74 ± 0.28 (0.80)

Confluent cultures were maintained for 24 hr in the presence of the reagents listed. The cultures were washed twice with buffered saline and once with pH 6 buffer and then scraped into pH 6 buffer. Freeze/thaw lysates of the suspended cells were assayed for cathepsin and β-glucuronidase activities. Values for cathepsin activity are means ± SD for quadruplicate assays; values for β-glucuronidase are means ± SD for eight assays. Relative values are in parentheses.

* Concentrations used: cycloheximide, 7 μM; potassium cyanate, 10 mM; NH_4Cl, 100 mM.

Cyanate Reversibly Inhibits Thiol Cathepsin(s) *in Situ*. Because the cyanate inhibition was easily reversed, a direct demonstration of cyanate inhibition of the thiol cathepsin(s) believed to occur *in situ* was not readily available. However, the competition between cyanate and MalNEt for sulfhydryl groups [plus the expectation that the cyanate inhibition of the thiol cathepsin(s) *in situ* would be reversed after washing and lysis of the cells] provided a means to determine whether or not cyanate could penetrate through the cell to the lysosome and, in fact, inactivate the thiol cathepsins. Cells that had been treated for 2 hr with MalNEt, cyanate, or a combination of the two were exposed to dithiothreitol to inactivate any unreacted sulfhydryl reagents, washed, and lysed. MalNEt irreversibly inactivated the cathepsin activity *in situ* and cyanate blocked that inactivation (Table 5). β-Glucuronidase was unaffected by exposure of the cells to the sulfhydryl reagents. The cathepsin(s) from the cyanate-treated cells showed an increase in activity compared with the activity from the control cells. Even though the increase in activity (25%) was within experimental error, the apparent protective effect of the reversible sulfhydryl reagent on the thiol cathepsin(s) was consistent and similar to the effect seen in Table 3.

DISCUSSION

It has been suggested that control of nutrient transport in cultured animal cells involves a catabolically controlled balance between carrier synthesis and carrier degradation (4, 5, 8, 13, 22). This model of regulation is based primarily on two findings: (*i*) animal cells totally deprived of D-glucose for 18–24 hr require protein synthesis in order to develop severalfold enchanced (derepressed) rates of hexose and A-system amino acid transport (1, 3–5, 8–10, 22), and (*ii*) cells maintained in the presence of cycloheximide (to inhibit new carrier synthesis) and D-glucose progressively lose carrier activity over a culture period of 18–24 hr (4, 5, 8, 9). Although some form of cellular energy is required for carrier inactivation (11), glycolysis does not appear to be solely involved in this aspect of the carrier regulation (4, 5, 11). There is evidence to indicate that a macromolecular com-

† The resistance to reversal of the carbamoylation by addition of excess dithiothreitol (or L-cysteine) was a consistent finding. In the presence of 2.5 mM L-cysteine (in the BANA hydrolysis assay), 40–50 μM cyanate caused 50% inhibition of the cathepsin activity (unpublished data). Similarly, only 0.2 mM mercuric chloride was required to inactivate 50% of the cathepsin activity, and 1 mM iodoacetate (or iodoacetamide) caused >95% inhibition of the cathepsin activity under the same conditions. Phenylmethylsulfonyl fluoride (1 mM) had no effect on the activity of the cathepsins in the BANA hydrolase assay (unpublished data).

Table 4. Effects of MalNEt and cyanate on cathepsin B activity in cell-free lysates

Addition	BANA hydrolysis, nmol naphthylamine/mg/min		
		At t = 4 hr	
	At t = 0	No dialysis	With dialysis
None	1.45 ± 0.04 (1.00)	1.14 ± 0.23 (1.00)	0.97 ± 0.02 (1.00)
Cyanate	0.09 ± 0.02 (0.06)	0.09 ± 0.01 (0.08)	1.72 ± 0.1 (1.77)
MalNEt	0.18 ± 0.03 (0.12)	0.14 ± 0.02 (0.12)	0.05 ± 0.01 (0.05)
Cyanate and then MalNEt	0.02 ± 0.01 (0.02)	0.08 ± 0.02 (0.07)	0.78 ± 0.02 (0.80)

Lysate protein (approximately 3 mg/ml in pH 6 buffer) was incubated in the presence of 0.5 mM L-cysteine at 40°C for 10 min in order to reduce the thiol cathepsin enzymes. The sample was then divided into two equal volumes; one was made 10 mM in cyanate, and the other received the same volume of pH 6 buffer. The two samples were then incubated at 40°C for 30 min. Half of the portion that had been preincubated in pH 6 buffer was made 10 mM in MalNEt, and the other half received the same volume of pH 6 buffer (control). The sample that had been preincubated in cyanate was also divided into two equal volumes; one was made 10 mM in MalNEt and the other received pH 6 buffer. At this point, all volumes were equal and the four samples were incubated at 40°C for 15 min. At the end of this preincubation time, all samples were made 20 mM in dithiothreitol and aliquots from each sample were immediately assayed for cathepsin B activity (t = 0). Additional aliquots of the preincubation mixes were removed, placed in stoppered tubes, and allowed to stand at room temperature for 4 hr (t = 0, no dialysis). The remainders of the four preincubation mixtures were placed in dialysis bags and dialyzed for 4 hr at room temperature against four changes of 100 vol of pH 6 buffer containing 2.5 mM L-cysteine (t = 4, with dialysis). Aliquots were assayed in the standard 10-min preincubation/30-min assay. Values shown are the means ± SD of quadruplicate assays. Relative values are in parentheses.

ponent (presumably a protein that is irreversibly inactivated during glucose starvation) is also required (4, 5, 8, 10, 13). The possibility that a labile protease could be such a macromolecular component of the carrier control mechanism has been suggested (5, 8, 10). Subsequently, it was found that cathepsin B was irreversibly inactivated during glucose starvation and that the reappearance of the enzyme activity in cell-free lysates (after administration of glucose to the starved cells) required protein synthesis (13). However, more direct evidence that cathepsins (or intracellular proteases) might be involved in transport control was required.

By taking advantage of the observation that >90% of hexose carrier activity can be lost within 24 hr when Nil cells are cultured in the presence of D-glucose and cycloheximide (4, 5, 8), a means of studying catabolically controlled degradation of carriers, uncomplicated by carrier synthesis, was available. Under these conditions, Cbm P_i (i.e., cyanate) preserved carrier activity against the cycloheximide-induced decreases (Table 1). In addition to Cbm P_i and cyanate, ammonium salts [$(NH_4)_2SO_4$, NH_4Cl, and $(NH_4)_2CO_3$] and sodium fluoride arrest the carrier inactivation when they are added to the culture medium along with cycloheximide (ref. 13; unpublished data). The transport kinetics experiments indicated that the preservation of carrier activity by Cbm P_i was due to a protection against the cycloheximide-related decrease in V_{max} (Table 2). Protection against the decrease in V_{max} for galactose transport also is characteristic of the block in the cycloheximide-related loss of transport activity in cultures treated with ammonia (unpublished data). In these cases, the protection against the cycloheximide-induced decreases in V_{max} most likely represented protection against decreases in the number of existing, functional carriers. Hence, in the absence of the synthesis of new carriers, ammonia and cyanate must have entered the lysosomes, inactivated the thiol cathepsins (by distinctly different mechanisms), and, apparently as one consequence, protected the existing carriers against inactivation.

Table 5. Cathepsin B and β-glucuronidase activities in extracts of cells treated with MalNEt and cyanate

Addition	BANA hydrolysis, nmol naphthylamine/ mg/min	β-Glucuronidase activity, nmol umbelliferone/ mg/min
None	3.04 ± 0.14 (1.00)	0.95 ± 0.05 (1.00)
Cyanate	3.83 ± 0.46 (1.26)	1.01 ± 0.06 (1.06)
MalNEt	0.19 ± 0.11 (0.06)	0.8 ± 0.04 (0.84)
Cyanate + MalNEt	2.03 ± 0.39 (0.67)	0.92 ± 0 03 (0.97)

Triplicate cultures in 100-mm dishes were exposed to 1 mM MalNEt, 10 mM cyanate, or 1 mM MalNEt plus 10 mM cyanate by addition of 300 μl of concentrated reagent solutions to 15 ml of the culture medium. Control cultures received 300 μl of phosphate-buffered saline. After the cultures were incubated at 37°C for 2 hr, 1 ml of 300 mM dithiothreitol was added to each dish. After mixing, 300 μl of MalNEt was added to the medium of the control and cyanate-treated cultures (final MalNEt concentration, 1 mM) and 300 μl of cyanate was added to the control and MalNEt-treated cultures (final cyanate concentration, 10 mM). The medium was removed and the cultures were washed three times with 5 ml of phosphate-buffered saline and once with 5 ml pH 6 buffer. The cells were scraped into 3 ml of pH 6 buffer and lysed by freezing and thawing. BANA hydrolase assays for each dish were done in quadruplicate; assays for β-glucuronidase for each dish were done in triplicate. Values are the means ± SD of 12 samples for cathepsin B or 9 samples for β-glucuronidase. Numbers in parentheses are relative values.

The irreversible inactivation of the thiol cathepsins in the NH_4Cl-treated cells probably relates to ammonia-induced increases in intralysosomal pH. During the long (24 hr) exposure of cells to ammonium salts, accumulation of ammonia in the lysosomes was evident by microscopic examination of the cultures. Cultures exposed to NH_4^+ contained cells with numerous clear and distended vacuoles, indicating that water had been taken up by the lysosomes in response to a change in the intralysosomal ion balance (23). It is known that even during short (2 hr) exposures of cultured cells to ammonia there are significant increases in the intralysosomal pH concomitant with the accumulation of ammonia in the lysosomes (24). The increase in pH over the long culture period might have been sufficient to cause inhibition of the acid hydrolases cathepsin B and β-glucuronidase *in situ*. Cathepsins B_1 and L are known to be irreversibly inactivated at near neutral pH (15, 16). In contrast to the mechanism of ammonia-induced inactivation, the inhibition of thiol cathepsins by cyanate appears to have been the result of a reversible carbamoylation of essential sulfhydryl groups on the thiol cathepsins. The strongest support for the

involvement of sulfhydryl groups in the inactivation process came from the cyanate competition with MalNEt for the SH-groups that was revealed in the dialysis part of the experiment shown in Table 4 and in the experiment shown in Table 5.

With these results taken together, it seems reasonably certain that the thiol cathepsins are involved in at least part of the hexose carrier regulation mechanism in animal cells. Whether one or all of the thiol cathepsins (B_1, H, and L) is (or are) involved in the inactivation of carriers is not known. Moreover, because cells have a wide variety of proteases with pH optima at acid and neutral pH, it is important to keep in mind that other intracellular proteases may also be involved in the carrier inactivation mechanism.

Are lysosomes involved in hexose transport regulation? Although the evidence does suggest the involvement of cathepsins in the carrier regulation, two prominent (perhaps related) problems accompany the model of lysosomal enzyme interactions with plasma membrane carriers. The first is the role of energy in the overall process. It has been reported that inactivation of oxidative phosphorylation by 2,4-dinitrophenol or oligomycin promotes the enhancement of hexose transport and, similar to the effects of ammonia and cyanate, these inhibitors block the cycloheximide-associated loss of carrier activity (11). Sodium fluoride also blocks the cycloheximide-related inactivation of carriers, further implying an energy requirement in the inactivation process. The exact nature of the energy requirement for carrier inactivation is not known.

The second problem in the model involving lysosomes in the carrier inactivation process relates to the mechanism by which the carriers and the proteases come into physical contact. The model implies an obligatory movement of the carrier and lysosome relative to each other. Endocytosis of carrier-associated plasma membranes, in the form of vesicles, could satisfy this requirement as well as account for the energy requirement. The resulting speculative model would require the involution of carriers, perhaps as part of primary endocytotic vesicles, and eventual recycling of carriers to the functional position in the plasma membrane if not damaged en route by proteolysis. This model also leaves open areas of control that appear to come from some as yet unidentified aspect of glucose catabolism (1–5, 8–13).

It is interesting that transformed 3T3 cells and Nil cells appear to have low intracellular cathepsin activities compared with their nontransformed counterparts (25, 26). It is well known that these transformed cells have higher rates of hexose transport than do the nontransformed cells. Hence, at least in these two cell lines, the inverse relationship between lysosomal protease activity and carrier activity is consistent with the present model of transport control. It will be interesting to see if other lines of transformed cells have similar protease–carrier relationships.

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