Review

The transport systems of mammalian lysosomes

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I. Introduction

The present era of recognizing the transport systems of lysosomes began in 1982 with the demonstration by

Gahl et al. [1] and Jonas et al. [2] that the extraordinarily high lysosomal cystine accumulation observed in the genetic disorder, nephropathic cystinosis, was due to a defect in the lysosomal transport of cystine. This discovery served as a catalyst for the search and characterization of other lysosomal transport systems such that, 9 years later, 16 different lysosomal transport systems are now known with probably many others yet to be discovered (Fig. 1). These transport systems support the primary function of lysosomes as a major intracellular site for the degradation of a large variety of both endogenous and exogenous macromolecules. Many of the carrier proteins provide pathways for the release of degradation products from lysosomes while others transport substrates into lysosomes to aid processes of macromolecule breakdown. The lysosomal transport systems display some properties and substrate specificities that are similar to carrier-mediated routes in other cellular membranes, but significant differences are observed as well, permitting these transporters to function within the constraints of the acidic environment of the lysosome. None of the lysosomal transporters characterized thus far have been found to be dependent on Na^+ and in some cases transport activity is especially sensitive to the pH gradient and/or membrane potential existing across the lysosomal membrane. Nearly half of the known lysosomal transport systems have been described in just the

last 2 years, as the techniques for isolating lysosomes and kinetic analysis of lysosomal transport activities now allow for rapid detection and characterization of lysosomal transport activities. The major focus of this review will be to describe the characteristics of the different lysosomal transport systems with special emphasis on the new transport routes discovered since previous reviews on this subject [3-6].

II. Isolation of lysosomes and methods of analysis

I-A. Methyl ester loading technique

In addition to the techniques commonly used in kinetic analysis of transport, several specialized techniques have played important roles in the study of lysosomal transport systems. The first of these is the methyl ester loading technique which was initially described by Goldman and Kaplan and later modified by Reeves for loading lysosomes with radiolabelled amino acids [7-10]. In this technique, the carboxyl group(s) of an amino acid are converted to the methyl ester derivative by reaction with methanolic HCl. Methyl esters of neutral or acidic amino acids have only the α-NH_2 group (pKa approx. 7.5) that can be protonated. When intact cells or isolated organelles are incubated with amino acid methyl esters in the neutral pH range (pH 7-8), a significant proportion of the amino acid methyl
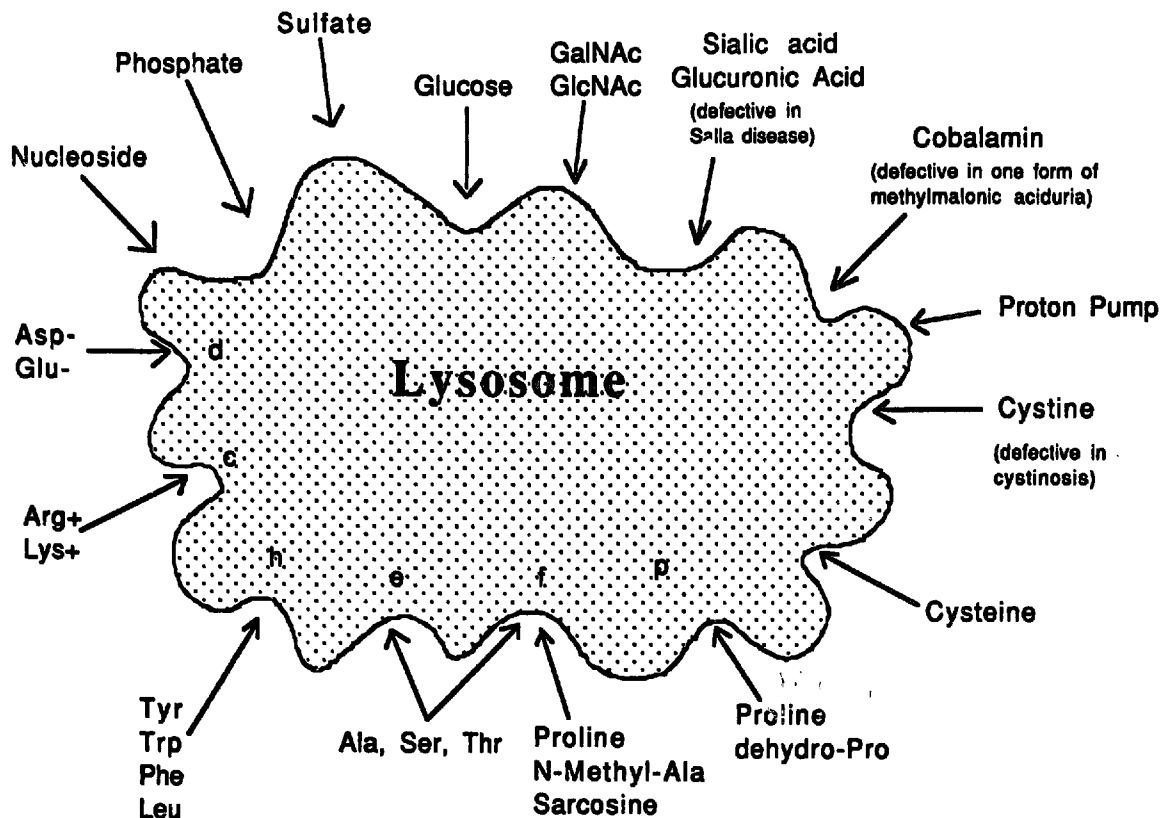

Fig. 1. Schematic diagram of the currently known lysosomal transport systems. The amoeboid form in which the lysosome depicted is not typical of lysosomal morphology but is used for ease of indicating the different lysosomal transport routes.

ester exists in an uncharged form, which rapidly crosses cellular membranes. Esterase activities within lysosomes convert the amino acid methyl esters back to the parent amino acids. Since the amino acids are charged at the intralysosomal pH, these polar molecules are now unable to passively diffuse from lysosomes and instead accumulate, requiring mediation by specific transport routes for their release. The high esterase activity in lysosomes relative to other cellular organelles allows amino acids to be specifically loaded into lysosomes even in the presence of other organelles. The methyl ester loading technique was applied by Gahl et al. [1] to load cystine into lysosomes of human leucocytes and show that lysosomal cystine efflux was greatly impaired in leucocytes from persons with nephropathic cystinosis.

II-B. Lysosomal uptake studies

Whereas the methyl ester loading technique allows one to study factors affecting exodus of amino acids from lysosomal preparations, often more quantitative information is gathered from lysosomal uptake studies which require that lysosomal preparations must be free of other organelles. Highly purified lysosomes are generally obtained by centrifuging granular fractions on a Percoll density gradient [11–14]. Typically, lysosomes are the only organelle detectable in the bottom one-third of these gradients ($d = 1.07-1.12$ g/cm^3) as determined by analysis of marker enzymes associated with specific cellular organelles [14]. This method has been used in isolating lysosomes from many different cell types. The integrity of lysosomal preparations is usually found to be 80–90% intact as judged by the latency of β-hexosaminidase activity, defined as the difference in activity of this lysosomal enzyme in the presence or absence of 0.1% triton [1,15]. Uptake of substrates by intact lysosomes is terminated rapidly by collecting lysosomes on glass fiber filters, washing the filters with ice-cold buffer and then counting the filters for radioactivity [14]. Alternatively, isolated lysosomes can be separated from unincorporated radioactivity by centrifugation through silicone oil [15]. Substrate uptake by intact lysosomes is often expressed per unit of latent β-hexosaminidase activity.

Membrane vesicles, prepared by lysing and resealing purified lysosomes, have become important for characterizing some lysosomal transport systems [16–19]. For instance, lysosomal sulfate transport has been demonstrated in uptake studies with vesicles prepared from rat liver lysosomes but apparently is not detectable using intact rat liver lysosomes [18]. Membrane vesicles may be especially useful for studying lysosomal transport systems which are highly dependent on proton gradients and/or membrane potential, because of the ability to experimentally impose these conditions in a

direction and magnitude favorable for analysis. In contrast, uptakes performed with isolated, intact lysosomes are subject to the pH gradient and membrane potential present after isolation of lysosomes, which may not be favorable for observing the desired transport activity. Assays using membrane vesicles prepared from rat liver lysosomes have used 0.22 μm filters [18] or gel filtration on small Sephadex columns [16] to separate vesicles from the uptake incubation medium. Uptakes are expressed per milligram of vesicle protein.

The application of these methods has now allowed for characterization of many different lysosomal transport systems. It must be stressed that many of the lysosomal transport systems to be described have been studied in only one cell type. The characteristics of lysosomal transport systems may vary markedly in lysosomes from different cell types, as already found for lysosomal system h [20] and to a lesser degree for the lysosomal cystine transporter [21].

Although it is apparent that many of the lysosomal transport systems must serve primarily for the net removal of their substrates from lysosomes, the net flux across the lysosomal membrane for most small molecular weight compounds at steady state is not known. This lack of knowledge with regard to fluxes is largely due to the difficulty of accurately measuring the intralysosomal concentration of metabolites because of the heterogeneous nature of isolated lysosomes, their small size and their small contribution to the total intracellular volume. Future improvements in analysis will allow a better understanding of the degree to which different transport systems are concentrative, their net direction of transport, their relationship to one another in determining the net flux of a given metabolite and how the lysosomal pH gradients and any relevant ion gradients affect the directionality of transport.

Carrier-mediated translocation of solutes across membrane barriers often involves significant structural movement and constant reorientation of components within the membrane as hydrogen bonds and hydrophobic interactions constantly break and reform. The ease with which these interactions can occur within the lipid environment of the membrane are highly temperature-dependent, with membrane fluidity and the nature of the various lipid phases changing appreciably over the temperature range from 5 to 37°C. The effect of these physical interactions is reflected by the difference in temperature coefficients between simple diffusion and carrier-mediated facilitated diffusion. Christensen has noted that simple diffusion has a low temperature coefficient with a Q_{10} nearer 1 than 2, whereas higher Q_{10} values are observed for carrier-mediated translocation of solutes across cellular membranes, displaying temperature coefficients as high as those characteristic of chemical reactions, i.e., two or more [22]. Temperature coefficients for the lysosomal

TABLE I

Temperature coefficients (Q_{10}) determined from Arrhenius plots for the lysosomal transport of different substrates

Lysosomal transport system	Cell type	Substrate assayed	Q_{10}	Reference
Phosphate	human fibroblast	phosphate uptake	1.8	121
Cobalamin	rat liver lysosomal vesicles	cobalamin uptake	1.8	130
System h	FRTL-5	tyrosine uptake	1.9	20
System h	FRTL-5	monoiodotyrosine uptake	1.95	53
Nucleoside	human fibroblast	adenosine uptake	2.0	15
Cystine	human leucocyte	cystine uptake	2.0	25
Sulfate	rat liver lysosomal vesicles	sulfate uptake	2.0	18
System c	human fibroblast	lysine efflux	2.2	31
GalNAc/GlcNAc	rat liver lysosomes	GalNAc, GlcNAc uptake	2.3	82
Neutral sugar	rat liver lysosomes	glycose and fucose efflux	2.3	86
Acidic sugar	human fibroblast	sialic acid efflux	2.4	80
Cysteine-specific	human fibroblast	cysteine uptake	3.2	58

passage of different substrates have been determined from Arrhenius plots in a variety of studies (Table I). These Q_{10} values range from 1.8 to 3.2 with a mean of 2.15 and a median of 2.0.

As an aid for comparing rates of lysosomal exodus of various substrates, we have tabulated the half-times for lysosomal exodus of various organic solutes which have been reported in the literature (Table II). Some of the lysosomal transport systems rapidly transport their substrates out of lysosomes, displaying half-times less than 10 min at 37 °C. It should be mentioned that half-times of exodus reflect the initial velocity only when lysosomes are loaded with a substrate below the saturation level of the transport route involved. Half-times of exodus can be influenced strongly by the level

of loading when lysosomes are loaded with substrate above the saturation level of the transport route.

III. Amino acid transport systems

III-A. Lysosomal cystine transport and cystinosis

The autosomal recessive disease, nephropathic cystinosis, has been known since 1907 when Abderhalden described a family in which three infants had died of apparent malnutrition [23]. Autopsy of one of the three children demonstrated white crystalline material in the liver which was shown to be the amino acid, cystine. After initial confusion of this disorder with the unrelated inborn error of metabolism, 'cystinuria', cysti-

TABLE II

Half-time of exodus of various organic solutes from lysosomes

In each experiment referenced below lysosomes were loaded with the indicated solute and, subsequently, the time required for 50% of the solute to egress from lysosomes at the indicated pH and temp was determined. This time interval is referred to as the half-time of exodus.

| Solute	Half-time of exodus	Reference
Tyrosine	approx. 2 min at pH 7.0 and 37 °C in FRTL-5 cells	20
Glucuronic acid	2–3 min at pH 7.0 and 37 °C in human fibroblasts	78
D-Glucose	3 min at pH 7.0 and 25 °C in rat liver	86
L-Glucose	1–2 min at pH 7.0 and 25 °C in rat liver	86
Glutamate	5–7 min at pH 7.0 and 37 °C in human fibroblasts	51
Uridine	6 min at pH 7.0 and 37 °C in human fibroblasts	15
Inosine	7.5 min at pH 7.0 and 37 °C in human fibroblasts	15
Methionine	17 min at pH 7.0 and 37 °C in human leucocytes	54
Cysteine	18 min at pH 7.0 and 37 °C in human leucocytes	54
Lysine	24 min at pH 6.5 and 25 °C in human fibroblasts	31
Tryptophan	26 min at pH 7.0 and 37 °C in human leucocytes	54
Cystine	26 min at pH 7.0 and 37 °C in human leucocytes	54
Cystine	30 min at pH 7.0 and 37 °C in human fibroblasts	31
Cystine	approx. 45 min at pH 7.0 and 37 °C in Epstein Barr-transformed human lymphoblasts	28
Proline	39 min at pH 6.5 and 28 °C in human fibroblasts	a
Tyrosine	$\gg$ 45 min at pH 7.0 and 37 °C in human leucocytes	20

[a] Unpublished Data, R. Pisoni.
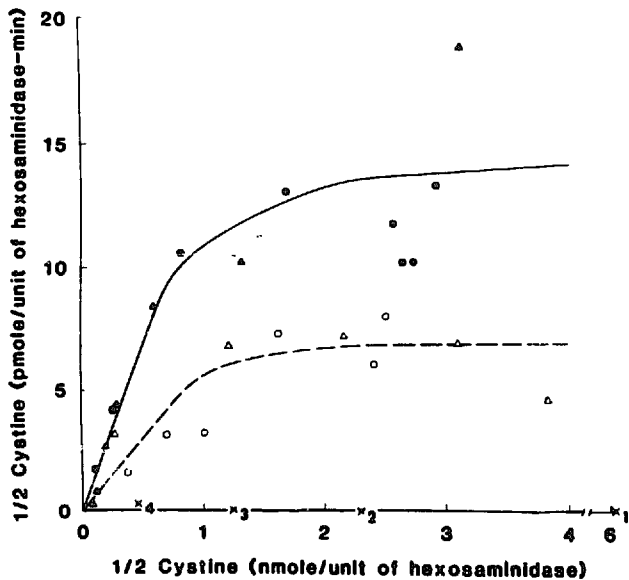
Fig. 2. Velocity of cystine egress from granular fractions isolated from leukocytes of normal subjects, cystinotic patients and subjects heterozygous for cystinosis. Leukocytes from two normal subjects (●, ▲) and two obligate heterozygotes for cystinosis (○, △) were loaded with 0.1 to 2.0 mM unlabelled cystine dimethyl ester. Granular fractions were prepared and velocities of cystine egress were determined at each level of loading. Leukocytes from four different cystinosis patients (X_1 to X_4) were loaded to a single cystine level and the velocity of cystine egress from isolated granular fractions was determined [1].

nosis became recognized as the most common underlying genetic cause of the renal Fanconi syndrome. In 1967–68, subcellular fractionation and electron microscopic studies definitively showed that the cystine accumulations which occur in this condition are contained within lysosomes [24].

Cystine, although poorly soluble, occurs as the free amino acid in various body fluids. Cystine is also a component of many proteins, stabilizing their structures through disulfide bridges. When endogenous and exogenous proteins which contain disulfides are degraded within lysosomes, the cystine released by this hydrolysis is normally transported from lysosomes back into the cytosol by a transport system with high specificity for cystine. Evidence for this transport route and its failure in nephropathic cystinosis, was first provided by Gahl et al. and Jonas et al. [1,2]. Gahl et al. applied the methyl ester loading technique to load leukocyte lysosomes with cystine and demonstrate egress of cystine from lysosomes in the granular fraction of normal cells, whereas such activity was lacking in lysosomes from persons with nephropathic cystinosis (Fig. 2). Obligate heterozygotes for nephropathic cystinosis displayed velocities of lysosomal cystine exodus which were close to 50% of that of normal individuals [1]. Jonas et al. loaded lysosomes with cystine by incubating fibroblasts with 30 mM cysteine-glutathione mixed disulfide. Intact normal and heterozygous fibroblasts

rapidly lost their lysosomal cystine ($t_{1/2}=20$ min), whereas cystinotic fibroblasts showed no loss of cystine during a 90 min incubation period at 37°C [2]. Although cystine loss from lysosomes could be observed when lysosomes were present within intact or heterozygous fibroblasts by this method, cystine egress could not be demonstrated using isolated lysosomes [2].

Since this discovery, many other characteristics of the lysosomal cystine transport system have been elucidated in a variety of cell types including human leucocytes, human lymphoblasts, human fibroblasts, mouse fibroblasts, rat liver cells and FRTL-5 cells [1,2,20,21,25–33]. This transport route recognizes the L-isomer of cystine and selenocystine with the highest affinity and weakly recognizes cystathionine, cysteamine-cysteine mixed disulfide and cystamine as indicated by their ability to moderately inhibit lysosomal cystine transport [21,25]. Lysosomal cystine uptake displays a pH optimum between pH 5.5 and 6.5 in human leucocyte lysosomes [26] and a K_m of 0.3–0.5 mM has been found for cystine uptake at pH 7.0 and 37°C by lysosomes of human leucocytes and mouse fibroblasts [21,25]. The lysosomal cystine transport system exhibits a *trans*-stimulation property in human leucocytes, FRTL-5 cells and mouse fibroblasts [20,21,25], showing much greater uptake of radiolabelled cystine when uptakes are performed with lysosomes pre-loaded with a high concentration of unlabelled cystine. In addition, Gahl et al. [25] have shown that [^{35}S]cystathionine uptake is trans-stimulated 7-fold using leucocyte lysosomes pre-loaded with unlabelled cystine. *trans*-Stimulation of lysosomal cystine transport has not been observed, however, in human fibroblasts (R. Pisoni, unpublished data).

Efflux of cystine from lysosomes occurs with a half-time of 25–45 min at 37°C and pH 7.0 in human leucocytes, fibroblasts and lymphoblasts and is stimulated 2–3-fold by MgATP [1,2,27–32]. Several studies have shown that this stimulatory effect of MgATP on lysosomal cystine exodus correlates with an increase in the transmembrane proton gradient and/or a decrease in the potential across the lysosomal membrane [28,29,32]. In addition, Jonas et al. [33] have found that the polyamines, spermidine, putrescine, cadaverine and spermine, at a concentration of 2 mM can stimulate lysosomal cystine efflux from rat liver lysosomes 1.3–2.1-fold.

Three different forms of cystinosis are known, all of which are inherited as autosomal recessive disorders [24]. Nephropathic cystinosis is the most severe and is observed more often than the other two forms, benign and intermediate cystinosis. The molecular basis for the differences between these conditions, all of which manifest intralysosomal cystine storage, are not known. One report suggests that significant residual lysosomal transport activity (9–29%) remains in the benign form,

but no significant lysosomal transport of cystine was seen in fibroblasts from an intermediate type [34]. Complementation analysis by somatic cell hybridization between the nephropathic and benign types, or between the nephropathic and intermediate types, showed no complementation with either cross [35]. These results suggest that all three forms of cystinosis are allelic mutations.

There is some indication that an alternative pathway may exist in lysosomes which acts as a minor route for the release of cystine from lysosomes. Lemons et al. [36] found that cystine was lost at a substantially accelerated rate from lysosomes of cystinotic cells when exposed to elevated temperatures of 40 or 43 °C. This temperature effect was observed in five different cystinotic cell lines from unrelated families including nephropathic, intermediate and benign forms of cystinosis. In contrast, exodus of cystathionine from lysosomes was the same at 37 °C or 43 °C indicating that this effect of temperature was not a general phenomenon affecting lysosomal permeability. It was speculated that lysosomal cystine loss at elevated temperatures was due to a transport route different from that which is defective in cystinosis, with the alternate pathway significantly contributing to lysosomal cystine exodus only at elevated temperatures. Recently, Greene et al. [21] have observed a portion of cystine uptake by mouse fibroblast lysosomes which appears to be insensitive to inhibition by various known competitive inhibitors of lysosomal cystine uptake. Forster et al. [37] have offered an alternate explanation for the enhanced loss of cystine from cystinotic fibroblasts at elevated temperatures. They suggest that the genetic defect in cystinosis decreases but does not totally eliminate lysosomal cystine transport activity, such that residual cystine transport activity is increased at elevated temperatures due to an increase in membrane fluidity and porter mobility. This hypothesis requires the unlikely possibility that the genetic defect in the three different forms of cystinosis be similar, in order for this hypothesis to be compatible with the observation of enhanced cystine loss from cystinotic fibroblasts at elevated temperatures by Lemons et al. [36] in five different unrelated cystinotic families representing the three different forms of cystinosis.

III-B. The cystine dimethyl ester selection technique

The methyl ester loading technique has been exploited to develop a selection method highly cytotoxic for cystinotic fibroblasts but not normal fibroblasts [38]. Cystine is specifically loaded into lysosomes of normal and cystinotic cells with cystine dimethyl ester (CDME). Since lysosomal cystine transport is impaired in cystinotic cells, exposure to CDME results in continuous loading of cystine into cystinotic lysosomes without

any means of cystine escape, eventually causing cell death. In contrast, lysosomes of normal cells are able to transport cystine out of their lysosomes, thereby protecting lysosomes from rupturing if the rate at which cystine forms within lysosomes does not greatly exceed the rate of lysosomal cystine exodus. Exposure to 2 mM CDME for 20 min was found to completely destroy some cystinotic fibroblast cell lines yet retain good cell viability in normal fibroblast cell lines [38]. The conditions for CDME cytotoxic selection vary somewhat for different cell lines. After transfecting a cystinotic cell line with normal human genomic DNA in an appropriate vector, exposure to cystine dimethyl ester should permit selecting only those cells which have incorporated and expressed the lysosomal cystine transport gene. Standard techniques of gene rescue could then be applied to identify the lysosomal cystine transport gene.

III-C. Cationic amino acid transport: lysosomal system c

Carrier-mediated transport of cationic amino acids across the lysosomal membrane has been demonstrated in human fibroblasts by analog inhibition analysis of the lysosomal uptake of L-[^{14}C]arginine [39] and characterization of a trans-stimulation property associated with L-[^{14}C]lysine exodus from human fibroblast lysosomes [31]. In the latter study, 2 mM L-lysine, added to the buffer in which lysosomes were suspended, trans-stimulated L-[^{14}C]lysine exodus from fibroblast lysosomes approx. 2-fold, decreasing the half-time of exodus from approx. 24 min to approx. 12 min at pH 6.5 and 25 °C (Fig. 3). trans-stimulation of L-[^{14}C]lysine exodus from fibroblast lysosomes was selective for the L-isomer of lysine and was dependent on the concentration of external lysine, obtaining maximal trans-stimulation using extralysosomal [Lys] $\geq$ 2 mM (Fig. 4). In addition to lysine, the cationic amino acids arginine, ornithine, 2-aminoethyl-L-cysteine and L-2,4-diaminobutyrate were all capable of trans-stimulating L-[^{14}C]lysine efflux from human fibroblast lysosomes. In contrast, neutral and anionic amino acids had no effect on L-[^{14}C]lysine efflux from fibroblast lysosomes demonstrating the specificity of this transport system for cationic amino acids. The rate of L-[^{14}C]lysine exodus and its trans-stimulation by cationic amino acids was very similar for lysosomes isolated from either normal or cystinotic human fibroblasts indicating that cystinotic fibroblasts have normal levels of lysosomal system c activity.

The same general substrate specificity for this carrier of cationic amino acids was observed in a subsequent investigation analyzing the ability of different amino acid analogs to inhibit the lysosomal uptake of a model cationic amino acid, L-[^{14}C]arginine [39]. Arginine uptake by human fibroblast lysosomes exhibited
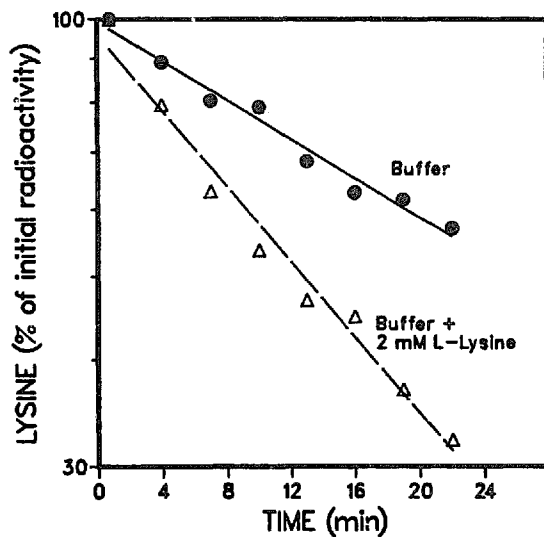
Fig. 3. *trans*-Stimulation of L-[^{14}C]lysine exodus from human fibroblast lysosomes by extralysosomal L-lysine. Lysosomes from normal human fibroblasts were loaded with L-[^{14}C]lysine by incubation with [^{14}C]lysine methyl ester. Lysine-loaded lysosomes were incubated at 25°C in the presence or absence of 2 mM L-lysine in 50 mM Mops-Tris buffer (pH 6.5) containing 0.25 M sucrose and 1 mM Na$_2$EDTA. At the indicated time points, lysosomes were collected on glass fiber filters, washed and counted for radioactivity [31].
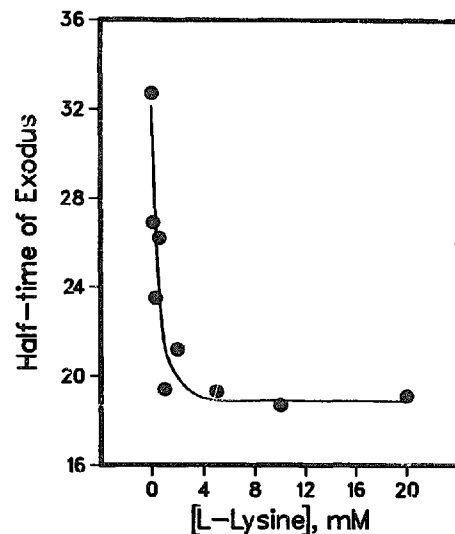
Fig. 4. Dependence of *trans*-stimulation of L-[^{14}C]lysine exodus from human fibroblast lysosomes on the concentration of external lysine. The half-time of L-[^{14}C]lysine exodus from human fibroblast lysosomes was determined for lysosomes incubated at 25°C, in pH 6.5 buffer containing unlabelled L-lysine at the concentration indicated [31].

complete saturability with a K_m of 0.32 mM at pH 7.0 and 37°C (Fig. 5) and was competitively inhibited by L-homoarginine, L-lysine, L-ornithine and 2-aminoethyl-L-cysteine with inhibition constants (K_i) ranging from 0.8–1.2 mM. This K_m of lysosomal arginine uptake is 8-times greater than the K_m for arginine transport

across the plasma membrane of human fibroblasts by system y$^+$ [40]. Several other differences were revealed for cationic amino acid transport by lysosomal system c and by system y$^+$ of the plasma membrane: (i) arginine transport across the plasma membrane by system y$^+$ is relatively insensitive to pH over the range from pH 5–8 [40], whereas arginine uptake by fibroblast lysosomes is
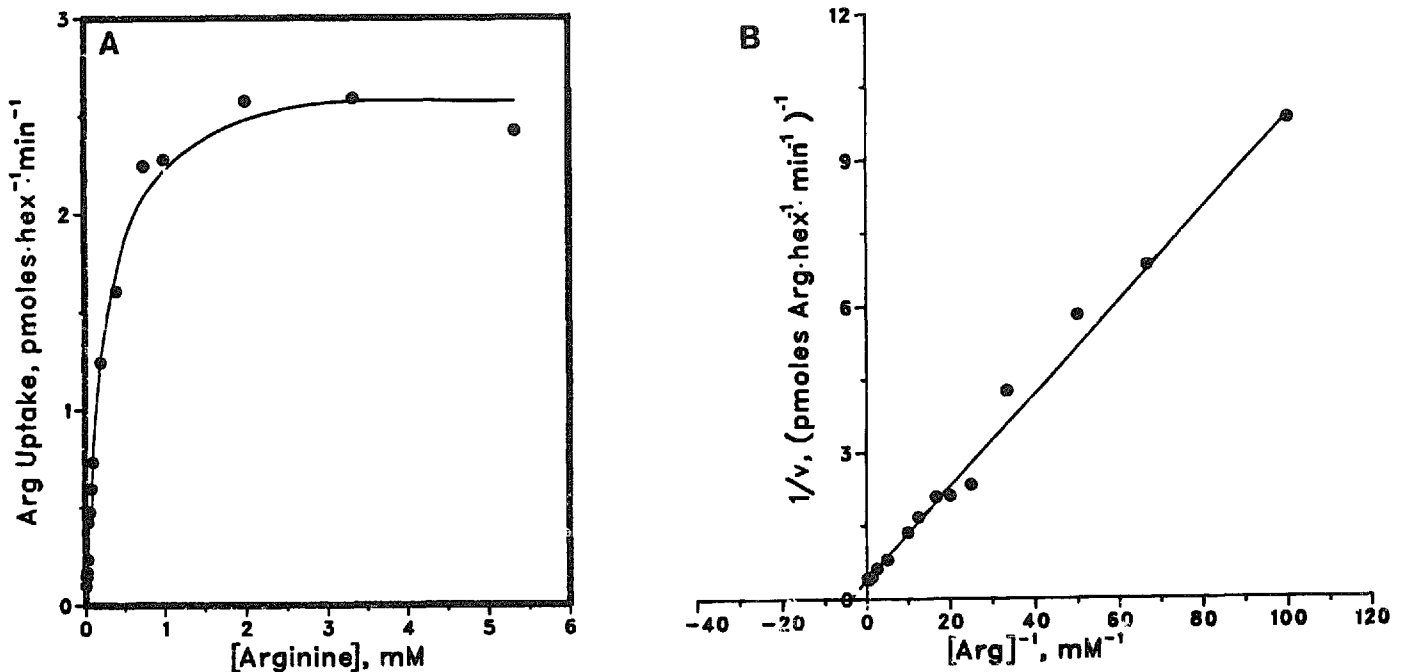
Fig. 5. Kinetics of the initial rate of arginine uptake into human fibroblast lysosomes as a function of L-arginine concentration. Fibroblast lysosomes were incubated for 1.5 min at 37°C in pH 7.0 buffer containing L-[^{14}C]arginine at the indicated concentration. At the completion of the incubation period, lysosomes were filtered, washed and counted for radioactivity. A, Michaelis-Menten plot of the initial rate of arginine uptake as a function of the arginine concentration; B, Lineweaver-Burk plot of the same data. A small non-saturable component of $K_d = 0.1$ pmol of arginine·hex^{-1} min^{-1} mM^{-1} has been subtracted from the data [39].

10-times faster at pH 7 than at pH 5 [39], (ii) in the presence of Na^+, certain neutral amino acids such as homoserine and ϵ-hydroxy-α-aminocaproate are accepted as surrogate substrates by system y^+ [41,42] but not by lysosomal system c [39], (iii) α-N-methyl-L-arginine ($K_i = 0.5$ mM), D-arginine ($K_i = 1.5$ mM), and ϵ-trimethyl-L-lysine ($K_i = 4.5$ mM) are recognized by lysosomal system c, whereas these analogs are poorly recognized by system y^+ [40]. This latter finding indicates that lysosomal system c has a slightly broader substrate specificity than system y^+, tolerating methylation of the α-amino group or side chain distal amino group and only moderately discriminates between L- and D-stereoisomers. Methylation of the α-carboxyl group is not accepted, however, as demonstrated by the failure of lysine methyl ester to inhibit lysosomal arginine uptake [39]. L-2,4-Diaminobutyrate is the shortest cationic amino acid recognized by lysosomal system c, as indicated by its ability to inhibit lysosomal arginine uptake and trans-stimulate lysosomal lysine exodus. In contrast, L-2,3-diaminopropionate has no effect on lysosomal arginine uptake.

The effects of the lysosomal transmembrane potential and proton gradient on lysosomal system c transport activity have not been studied. However, MgATP has been shown to exert opposite effects on efflux and influx. Lysine efflux is accelerated 2-fold upon exposing lysosomes to 2 mM MgATP at pH 6.5 and 25°C [31]. In contrast, the initial rate of lysosomal arginine uptake is retarded 3-fold when 2 mM MgATP is added to incubation mixtures at pH 7 and 37°C [39]. In addition, lysine efflux from fibroblast lysosomes increases approx. 1.4-fold as the extralysosomal buffer pH is raised from pH 5.5 to 7.6 [31], whereas arginine uptake increases 10-fold as the pH of the incubation medium is raised from pH 5 to 7 [39].

III-D. Cysteamine treatment of cystinosis and lysosomal transport system c

Addition of cysteamine to cultures of cystinotic fibroblasts produces rapid intralysosomal cystine depletion [43]. This effect is concentration-dependent and has been shown to be the result of mixed disulfide formation inside lysosomes between cysteamine and cystine [31,39,44]. The two products of this reaction, cysteine and the mixed disulfide of cysteamine and cysteine, are transported out of cystinotic lysosomes by other transport routes which are not defective (Fig. 6) [31,39,44]. The mixed disulfide of cysteamine and cysteine is an analog of lysine and is recognized by lysosomal system c with an affinity equal to that of lysine [39]. Cysteamine is relatively nontoxic and well tolerated. Treatment of a large population of cystinotic patients with cysteamine for periods of several years showed improved linear growth and stabilization of renal function incidental to this treatment. Cysteamine therapy of nephropathic cystinosis has become accepted as useful in averting the otherwise catastrophic renal failure which occurs in cystinosis [45].

Any compound that can enter lysosomes, form a mixed disulfide with cystine and whose product can be transported by existing carrier systems in the lysosomal membrane will be a potential cystine depleter and a potentially effective agent in the treatment of nephropathic cystinosis. Other agents known which perform in this way include thiocholine, which reacts to form a mixed disulfide recognized by lysosomal system c [39]

Cysteamine   Cystine   Mixed Disulfide   Cysteine
                                                             of cysteamine
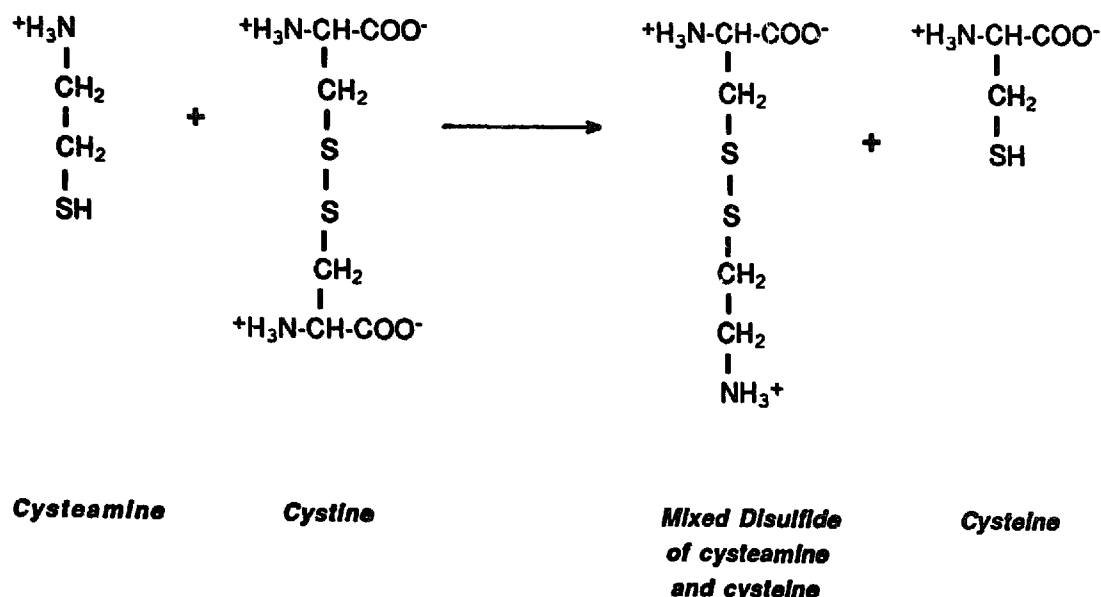   and cysteine

Fig. 6. Reaction of cysteamine and cystine [43].

and mercaptoethylgluconamide which may undergo hydrolysis inside the cell to liberate free cysteamine prior to mixed disulfide formation [46].

III-E. Dicarboxylic amino acid transport: lysosomal system d

Collarini et al. [47] have characterized a transport system, designated as lysosomal system d, that serves for the passage of anionic amino acids across the membrane of human fibroblast lysosomes. Glutamate and aspartate were taken up by fibroblast lysosomes by a single, high affinity transport system displaying a K_m of 4–12 μM for glutamate and aspartate. Lysosomal aspartate uptake linearly increases over the pH range from 5.5 to 8.0 displaying 2–3-fold greater uptake at pH 8 than at pH 5.5. A small trans-stimulation of [^{3}H]glutamate uptake is observed when uptakes are performed with lysosomes pre-loaded with unlabelled glutamate. Efflux of glutamate from human fibroblast lysosomes occurs rapidly with a $t_{1/2}$ of 5–7 min at pH 7.0 and 37°C. This transport system is similar in specificity to the plasma membrane anionic amino acid transport system, X_{AG}^-, with the exception that lysosomal system d is not dependent on Na^+ ions and has a broader substrate specificity in terms of substrate length, size and stereospecificity [48–51]. The maximum chain length permissible for recognition by lysosomal system d appears to be six carbons as indicated by the ability of L-α-aminoadipate to inhibit glutamate uptake strongly, whereas L-α-aminopimelate is ineffective. Only a small discrimination is made between the D- and L-stereoisomers of aspartate and glutamate, but as the chain length increases to 6 carbons, in the case of α-aminoadipate, strong preference is given to the L-stereoisomer. N-Methylation of either L-glutamate or L-aspartate greatly reduces their recognition by lysosomal system d. The cyclic glutamate analog, kainate, is poorly recognized as is the β-anionic amino acid, 3-aminoglutarate.

III-F. Transport of the small neutral amino acids: systems e, f and p

The lysosome membrane, similar to the plasma membrane, contains multiple pathways for the transport of small neutral amino acids. Studies of lysosomal proline, alanine, serine and threonine uptakes have revealed the existence of at least three separate Na^+-independent routes mediating the passage of small neutral amino acids across the human fibroblast lysosomal membrane [14]. Although the broad outlines distinguishing these transport systems from one another have been established, considerably more investigation is required to understand the many specific characteristics of each route. The majority of information has

come from the investigation of lysosomal proline uptake. Lysosomal proline uptake displays a broad maximum in the neutral pH range centered near pH 6.4 and declines at pH values below 6.0 or above 7.0. Proline uptake is highly selective for the L-stereoisomer of proline and is strongly inhibited by small neutral amino acids with sidechains 1–2 carbons in length including the β-amino acids, 3-aminoisobutyrate and β-alanine. In contrast, anionic and cationic amino acids, or neutral amino acids having side chains ≥ three carbons in length are poor inhibitors of lysosomal proline uptake. The analogs, 2-(methylamino)-isobutyrate [MeAIB] and 2-aminoisobutyrate [2-AIB], which strongly inhibit proline transport across the plasma membrane of the human fibroblast, only weakly inhibit proline uptake by fibroblast lysosomes. The weak inhibition by MeAIB and 2-AIB suggests that excessive branching off of the α-carbon is not permissible for recognition by the transport protein. Finally, small neutral amino acid analogs in which the α-amino group is mono-N-methylated [e.g. sarcosine, α-N-methyl-L-alanine] are good inhibitors of lysosomal proline uptake but analogs in which the α-amino group is further methylated [N,N-dimethylglycine, betaine] are incapable of inhibiting proline uptake.

Proline uptake by human fibroblast lysosomes shows complete saturation at [Pro] ≥ 0.4 mM. Lineweaver-Burk and V/S vs. V plots are non-linear, however, indicating that at least two different pathways are involved in lysosomal proline uptake. K_m values of 0.01 mM and 0.07 mM were found for the two different routes of carrier mediation with the higher affinity route accounting for 60–70% of the total proline uptake. Additional support for the existence of at least two transport routes mediating lysosomal proline uptake came from studies showing that approx. 75% of total proline uptake is sensitive to inhibition by alanine, whereas the remaining 25% of proline uptake is not inhibited by alanine (Fig. 7). The route serving for the majority of lysosomal proline uptake, which also recognizes alanine, was designated lysosomal system f. Nearly complete inhibition of L-[^{14}C]proline uptake was obtained with L-proline and 3,4-dehydro-L-proline, suggesting that these two amino acids are recognized not only by the alanine-sensitive route (system f) but also by the second route of lower affinity which is not inhibited by alanine. This latter route was designated system p, for proline specific route (Fig. 8).

Studies of [^{14}C]alanine, [^{14}C]serine and [^{14}C]threonine uptake by human fibroblast lysosomes revealed that, in addition to systems f and p, at least one other transport system serves for carrier mediation of small neutral amino acids across the fibroblast lysosomal membrane [14]. This transport system, designated system e, mediates the majority of alanine, serine and threonine uptake by human fibroblast lysosomes but
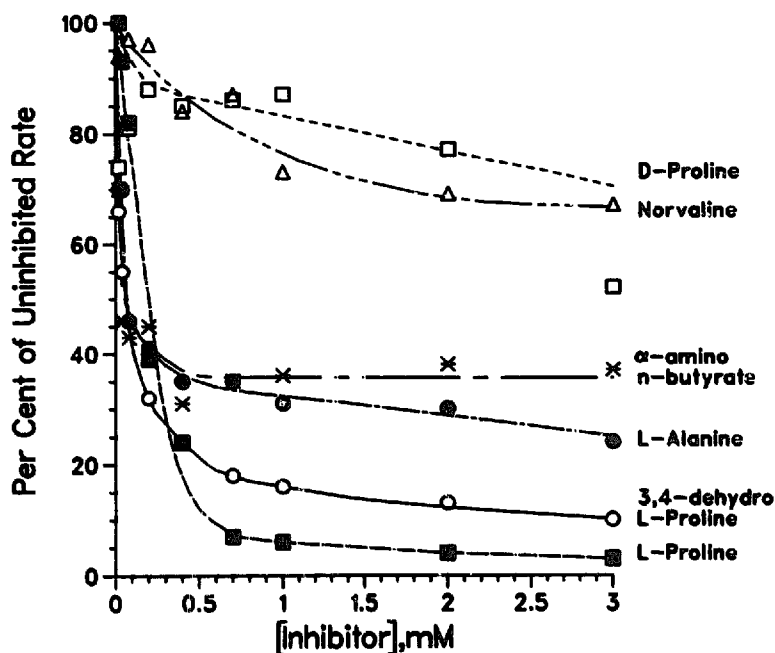
Fig. 7. Concentration dependence of inhibition of proline uptake into human fibroblast lysosomes by various amino acids. The uptake of 0.03 mM L-[^{14}C]proline into lysosomes was measured for 2.5 min at pH 7.0 and 37°C in the presence of various amino acids at concentrations ranging from 0 to 3.0 mM. The uninhibited control uptake was 0.23 pmol proline/unit of latent hexosaminidase activity [14].

does not recognize proline or other small neutral amino acids in which the α-amino group is a secondary amine (e.g. sarcosine, α-N-methyl-L-alanine). Whereas alanine inhibits L-[^{14}C]proline uptake by 70-80%, high concentrations of proline inhibit alanine, serine or threonine uptakes by only 10-20%. In addition, leucine, which has no effect on lysosomal proline uptake, inhibits lysosomal alanine and threonine uptakes by 60-70%. In one respect, the lysosomal transport systems shown in Fig. 8 bear a strong analogy to the Na$^+$-dependent transport systems, ASC and A, present on the plasma membrane of human fibroblasts. System ASC, similar to lysosomal system e, provides for the majority of alanine and serine transport across the fibroblast plasma membrane [50-52] but excludes amino acids in which the α-amino group is methylated. The minor route of transport, system A, similar to lysosomal sys-

tem f, recognizes small neutral amino acids including those in which the α-amino group is a secondary amine.

III-G. Transport of the large neutral, hydrophobic amino acids: lysosomal system h

A carrier-mediated transport system recognizing the large neutral amino acids was first demonstrated by Bernar et al. [20]. They observed *trans*stimulation of [^{3}H]tyrosine uptake by lysosomes of FRTL-5 cells after lysosomes were pre-loaded with tyrosine using the methyl ester loading technique. The specificity of this trans effect was shown by the inability of pre-loaded cystine to *trans*stimulate [^{3}H]tyrosine uptake. The initial rate of [^{3}H]tyrosine uptake by FRTL-5 lysosomes increased proportionately to the amount of unlabelled tyrosine loaded into the lysosomes until apparent saturation was achieved with intralysosomal tyrosine concentrations of 1-2 nmol of tyrosine loaded/unit of hexosaminidase activity. Analog inhibition analysis of [^{3}H]tyrosine uptake revealed that this transport system specifically recognizes the large neutral amino acids. Isoleucine, leucine, tryptophan, tyrosine, phenylalanine and histidine strongly inhibited [^{3}H]tyrosine uptake by FRTL-5 lysosomes. Valine and methionine moderately inhibited tyrosine uptake, whereas cystine, and anionic, cationic and small neutral amino acids did not inhibit lysosomal tyrosine uptake. The L-stereoisomer of the large neutral amino acids was strongly preferred compared to the D-stereoisomer [20] and tyrosine uptake obeyed Michaelis-Menten kinetics displaying a K_m of 20 μM at pH 7.0 and 37°C [53]. Exposure of FRTL-5
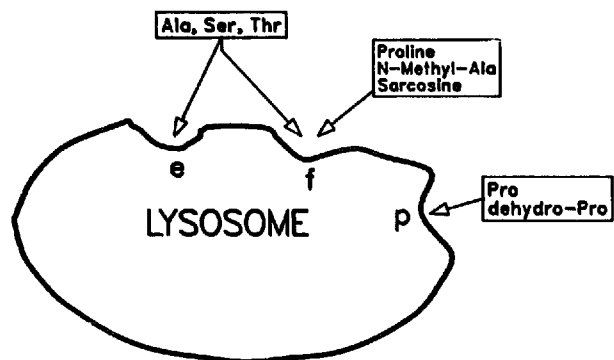

Fig. 8. Schematic diagram of the proposed transport systems for small neutral amino acids in the lysosome of the human fibroblast [14].

lysosomes to 2 mM MgATP or 5 mM N-ethylmaleimide had little effect on tyrosine uptake [20].

Exodus of tyrosine from lysosomes of FRTL-5 cells occurs very quickly at 37°C, with a $t_{1/2}$ of approx. 2 min. In contrast, tyrosine exodus from human leucocytes is very slow at 37°C showing only about a 10% loss after a 30 min incubation [20]. Other large neutral amino acids, however, were found to egress from leucocyte lysosomes at a moderate rate displaying halftimes of 17–26 min at 37°C for methionine, cysteine and tryptophan [54].

Lysosomal transport of large neutral amino acids has also been observed in human fibroblasts which exhibit some notable differences from lysosomal system h of FRTL-5 cells. Stewart et al. [55] found a low level of lysosomal uptake of large neutral amino acids by human fibroblast lysosomes [55]. Non-saturable uptake predominated when lysosomal uptakes were performed with millimolar concentrations of phenylalanine, leucine, or tryptophan. At much lower concentrations of these substrates, however, uptake was saturable, displaying K_m values of 5–30 μM which are similar to the K_m values found for lysosomal system h transport activity in FRTL-5 cells. Patterns of analog inhibition analysis, however, suggest that passage of large neutral amino acids across the fibroblast lysosomal membrane may be mediated by more than one route. The L-stereoisomer of inhibitory analogs was only slightly more effective than D-stereoisomers in striking contrast to lysosomal system h of FRTL-5 cells which is highly selective for the L-stereoisomer.

The Fischer rat thyroid cell line, FRTL-5, in which lysosomal system h has been characterized, retains many of the functional characteristics of thyroid tissue such as thyroglobulin synthesis, iodide uptake, cyclic nucleotide metabolism and response to thyrotropin. Harper et al. [56] found that lysosomal tyrosine countertransport increased an average of 7-fold when 0.1 nM TSH was added to the culture medium in which FRTL-5 cells were grown. An exposure time of 2–4 days was required in order to observe this level of stimulation in lysosomal tyrosine countertransport by TSH. In addition, lysosomal countertransport of two other lysosomal system h substrates, phenylalanine and leucine, was increased in FRTL-5 cells incubated with TSH. In contrast, lysosomal cystine countertransport which has been observed in FRTL-5 cells is not affected by culturing in media containing TSH. The enhancement of tyrosine countertransport activity by TSH could be mimicked by cAMP and cholera toxin suggesting that the TSH stimulatory activity was mediated by a cAMP signal. TSH stimulation of lysosomal tyrosine countertransport activity was completely inhibited by 100 μM cycloheximide or 25 nM actinomycin D indicating that de novo protein synthesis was required in order to observe TSH stimulation of tyrosine countertransport. Neither cycloheximide or actinomycin D affected the ability of TSH to increase cAMP levels in FRTL-5 cells. These results provide the first demonstration of a lysosomal transport system under hormonal regulation. Since lysosomes are a major site for thyroglobulin degradation, up-regulation of lysosomal system h by TSH in thyroid cells may be important to salvage tyrosine efficiently for maintaining a high overall rate of thyroglobulin synthesis thereby supporting thyroxine formation and release. As suggested by Harper et al., hormonal regulation of other lysosomal transport systems may exist when the carriers fulfill salvage roles or are inherently involved in production of regulatory signals relevant to the differentiated functions of a particular cell type.

The role of lysosomal system h in salvaging metabolites from thyroglobulin degradation in FRTL-5 cells was further shown by Tietze et al. [57] and Andersson et al. [53] who demonstrated that the metabolite, monoiodotyrosine, is also transported by lysosomal system h. Lysosomes pre-loaded with monoiodotyrosine *trans*stimulated the lysosomal uptake of tyrosine, leucine, phenylalanine and moniodotyrosine but not cystine. Monoiodotyrosine uptake by FRTL-5 lysosomes was competitively inhibited by other system h substrates, displayed maximal uptake near pH 7.5 and was stimulated 4–9-fold by culturing FRTL-5 cells in media containing TSH. Lysosomal system h recognizes monoiodotyrosine with greater affinity than tyrosine, displaying a K_m of 1.5–1.8 μM for monoiodotyrosine compared to the K_m of 20 μM for tyrosine uptake [53]. Similar to monoiodotyrosine, phenylalanine derivatives containing a halogen substituent on the phenyl ring were stronger competitive inhibitors than phenylalanine. In addition, leucine inhibited lysosomal monoiodotyrosine and tyrosine uptake with a K_i of 3.2 μM and 3.5 μM, respectively. Tyramine and 3-(p-hydroxyphenyl)-propionic acid do not inhibit lysosomal monoiodotyrosine uptake, indicating that both the α-carboxyl and α-amino groups of tyrosine are important for recognition by the system h transport protein.

III-H. The cysteine-specific lysosomal transport system

Whereas many of the transport systems associated with lysosomes are thought to function mainly for the export of metabolites out of lysosomes, a cysteine-specific lysosomal transport system has been described which appears to serve as a major route for the delivery of thiol into human fibroblast lysosomes [58]. The initial evidence for the existence of this transport system came from an investigation of the uptake of L-[35S]cystine by intact human fibroblasts. When human fibroblasts were incubated for 2–5 min with 20 μM L-[35S]cystine, 50–60% of the total radioactivity taken up by the cells was found to be associated with the

lysosomal compartment in the form of cysteine. This amount of cysteine sequestration in such a short time interval is amazing when one considers that lysosomes constitute only 4% of the intracellular volume of a normal human fibroblast [59]. Using Percoll-purified lysosomes it was found that a single transport route, highly specific for cysteine, served for the uptake of cysteine into human fibroblast lysosomes. The cysteine-specific transport system is present in both normal and cystinotic human fibroblasts, displays a K_m of 53 μM for cysteine uptake at pH 7.0 and 37°C and exhibits the highest activation energy of any known lysosomal transport system ($E_a = 21$ kCal/mol, $Q_{10} = 3.2$). The highly selective substrate specificity of this transport system is reflected by the lack of inhibitory effect of 5 mM concentrations of other neutral, cationic, or anionic amino acids on the lysosomal uptake of 35 μM L-[^{35}S]cysteine. Analogs very similar in size and structure to cysteine, such as serine, penicillamine, homocysteine and alanine are at best only weakly inhibitory. In addition, D-cysteine and the deaminated analog of cysteine, β-mercaptopropionate, have little effect on lysosomal cysteine uptake, whereas the decarboxylated analog of cysteine, cysteamine, is a good inhibitor of lysosomal cysteine uptake. These results indicate that the cysteine-specific transport route is highly selective for the L-stereoisomer and has a stringent requirement for a sidechain thiol and an α-amino group while not accomodating amino acids having sidechains longer than that found in cysteine. The α-carboxy group of cysteine, however, does not appear to be required for recognition in that cysteamine strongly inhibits lysosomal cysteine uptake.

The pH curve of the cysteine-specific lysosomal transport system is well suited for the delivery of cysteine into lysosomes. Very little uptake of cysteine by fibroblast lysosomes is observed over the acidic pH range from pH 4.8–6.0, but then the transport rate increases 7–10-fold between pH 6 and 7.5 (Fig. 9). If this pH dependence exists on both sides of the lysosomal membrane, then cysteine influx into fibroblast lysosomes originating at the cytosolic pH of 7.0 would be greatly favored over cysteine efflux from lysosomes originating at the intralysosomal pH of 5.3, thereby favoring net cysteine accumulation within lysosomes.

The large amounts of cysteine delivered into lysosomes by the cysteine-specific transport route may support lysosomal proteolysis in two different ways: by providing thiol necessary to maintain maximal activity of the lysosomal thiol-dependent proteinases and secondly by reducing protein disulfide bridges during proteolysis. Several studies have shown that thiols such as cysteine and GSH increase the rate at which disulfide-rich proteins are degraded by lysosomal proteinases, including degradation by proteinases which are not thiol-dependent [60–62]. Kooistra et al. [60] postulated
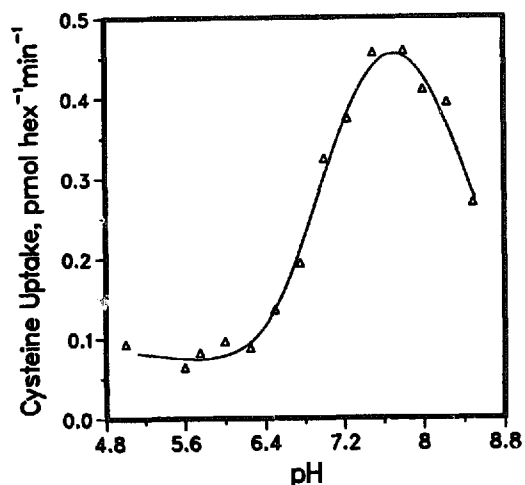

Fig. 9. pH profile of 0.034 mM L-[^{35}S]cysteine uptake by human fibroblast lysosomes [58].

that thiols reduce protein disulfide bridges during proteolysis within lysosomes, allowing proteins to unfold, thereby providing proteinases greater access to newly exposed, susceptible peptide linkages within the substrate [60]. A recent investigation by Feener et al. [63] has challenged whether protein disulfides can be reduced within lysosomes. In this latter study, Chinese hamster ovary (CHO) cells were allowed to endocytose the synthetic disulfide, [^{125}I]tyn-SS-PDL, which was prepared by conjugating poly-D-lysine (molecular mass approx. 60 kDa) to tyramine via a 3-(propionyldithio) propionic acid linkage and then iodinating the conjugated tyramine residues. Following endocytic uptake, [^{125}I]tyn-SS-PDL was found to be delivered to lysosomes but only 5% of its disulfide bonds were reduced within the lysosomes of intact cells during a 4 h incubation at 37°C. When 0.2 mM cystamine was added to the culture medium during the 4 h incubation, 29% of the disulfide bonds in [^{125}I]tyn-SS-PDL were reduced within the lysosomes of CHO cells. Feener et al. concluded that under normal culture conditions, reduction of [^{125}I]tyn-SS-PDL is not detectable within lysosomes, ruling out lysosomes as a meaningful site of reductive cleavage, but exposing lysosomes to cysteamine can transform lysosomes into a reducing compartment. Thoene et al. [43] have shown previously that 0.1 mM cysteamine can rapidly reduce large quantities of cystine within lysosomes of cystinotic fibroblasts, causing 50% reduction of the disulfide bonds within 15 min. In comparison, cleavage of only 29% of the disulfide bonds in [^{125}I]tyn-SS-PDL when cells are exposed to 0.2 mM cystamine for 4 h is an extremely slow rate of disulfide bond reduction which raises questions regarding the validity of using [^{125}I]tyn-SS-PDL for measuring lysosomal disulfide bond reduction. If such a slow rate of reducing the disulfide bonds in tyn-SS-PDL is observed when cells are cultured in media containing cystamine, it is not unexpected that even fewer of the

disulfide bonds in tyn-SS-PDL are reduced when cells are cultured in media lacking cystamine. Furthermore, in view of the recent finding of long chains of inorganic polyphosphate within human fibroblast lysosomes (see section VI-C), the tyramine-poly-D-lysine conjugate, being highly cationic, likely will become tightly associated with lysosomal inorganic polyphosphate which may restrict access of thiols to susceptible disulfide linkages. In conclusion, the slow rate of disulfide reduction of [125I]tyn-SS-PDL in lysosomes may be more a reflection of limited access to the disulfides of this highly cationic synthetic substrate than an indication of the overall ability of thiols to reduce protein disulfide bridges within lysosomes.

Two different cycles involving the interchange between cystine and cysteine are now apparent which serve the requirements for cellular metabolism within the cytosolic and lysosomal compartments of the human fibroblast (Fig. 10). Cystine is transported across the plasma membrane into the cytosol by the X_{CG}^- transport system [64]. Upon entering the cytosol, cystine is reduced by GSH to form cysteine and the mixed

disulfide of glutathione and cysteine, with much of this latter product being further metabolized. Cysteine formed in the cytosol can be transported back into the extracellular space by several transport routes, where it is reoxidized to form cystine [50,65-68]. Cytosolic cysteine can also be used for the synthesis of glutathione and proteins, metabolized for the production of other cellular constituents, or sequestered into lysosomes by the cysteine-specific lysosomal transport system to aid lysosomal proteolysis. Once within the lysosomal compartment, cysteine can react with protein disulfide bridges, as suggested by Lloyd [69], forming equimolar amounts of protein-linked cysteine and cystine at the site of each original disulfide bridge, which after proteolysis are released into the lysosome as free cystine and cysteine. Cystine is transported out of the lysosome into the cytosol by the lysosomal cystine transport system. Upon reaching the cytosol, cystine is reduced back to cysteine by GSH. The manner by which cysteine is transported from lysosomes remains to be determined. Lysosomal cysteine exodus could be facilitated in part by the cysteine-specific lysosomal trans-
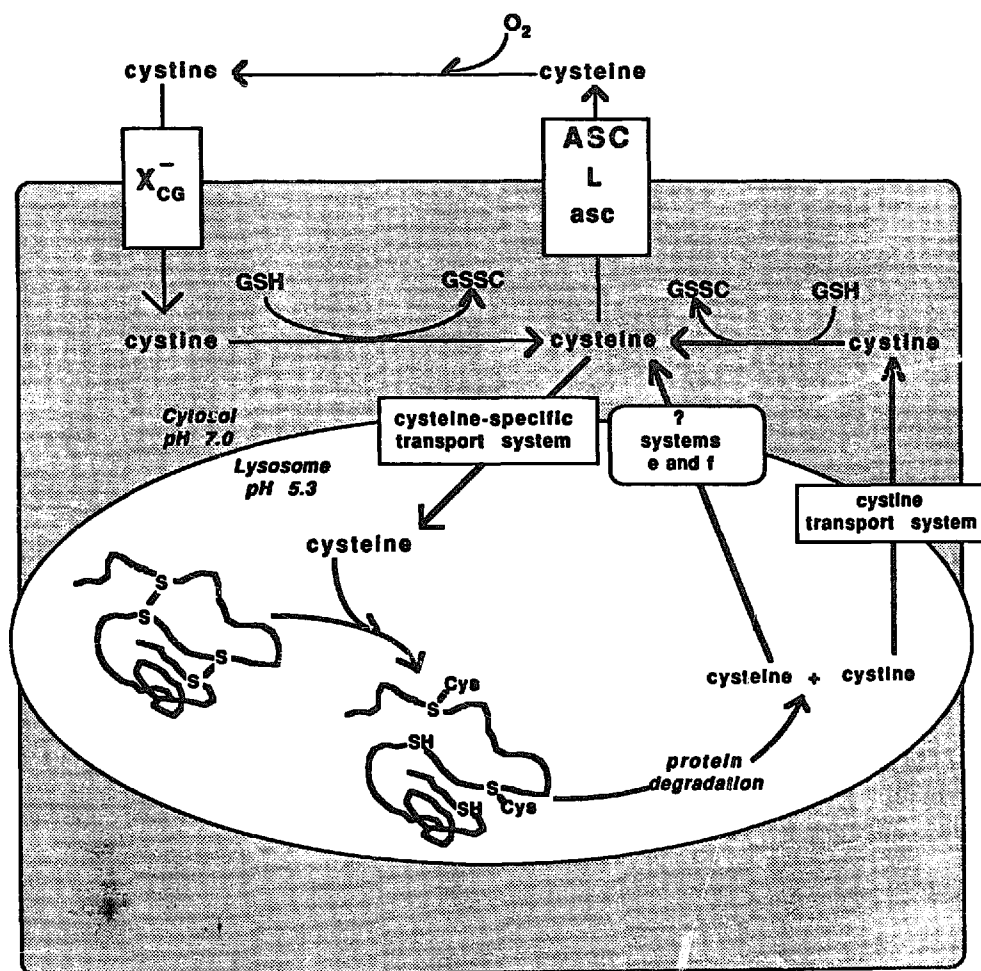

Fig. 10. Schematic diagram showing (a) the interconversion between cystine and cysteine in different cellular compartments, (b) the role of transport systems in maintaining this cycle and (c) the role of cysteine in supporting lysosomal proteolysis. The shaded area represents the cytosol, whereas the clear area represents the lysosomal compartment; ASC,L,asc and X_{CG}^- are plasma membrane amino acid transport systems [58].

port route and also by lysosomal systems e and f, which both recognize a broad range of small neutral amino acids. For the present time, we have shown these latter two transport routes as being likely candidates in playing a major role in mediating lysosomal cysteine egress.

Cysteamine and thiocholine are each capable of rapidly depleting cystinotic fibroblasts of intralysosomal cystine accumulations, yet the mechanisms which serve for the delivery of these two agents into lysosomes are not known. The ability of cysteamine to strongly inhibit cysteine uptake by the cysteine-specific lysosomal transport system indicates that cysteamine is recognized by this transporter. Analog inhibition studies of radiolabelled cysteamine uptake by lysosomes are necessary, however, to prove that this transport route actually serves for the delivery of cysteamine into lysosomes.

IV. Sugar transport systems

IV-A. Salla disease and the lysosomal transport of acidic monosaccharides

Similar to the discovery of the lysosomal cystine transport system, the detection of a lysosomal transport route mediating the passage of sialic acid and other acidic monosaccharides across the lysosomal membrane resulted from efforts by several investigators to understand the basis of two rare genetic disorders, Salla disease and infantile sialic acid storage disease (ISSD). In these two disorders, excessively high concentrations of free sialic acid are found in various tissues [24]. Biochemical studies did not reveal any defect in the major cellular enzymes involved in metabolism of sialic acid, and the concentration of glycoproteins, glycolipids and gangliosides within affected tissues were in the normal range. The clue to the role of lysosomes in this disorder came from electron microscopic and histochemical studies which indicated that the large accumulations of free sialic acid in Salla disease were contained within the lysosomal compartment [70–72]. Renlund et al. [73,74] then demonstrated that lysosomal degradation of sialic acid-containing glycoconjugates was normal but egress of free sialic acid from lysosomes was retarded greatly in Salla disease fibroblasts. In this latter study, lysosomes of normal and Salla disease fibroblasts were loaded with sialic acid to comparable levels by incubating cells with high concentrations of the sialic acid precursor, N-acetylmannosamine. Subsequent measurement of sialic acid loss from lysosomes demonstrated almost no exodus of sialic acid from Salla disease fibroblasts, whereas substantial efflux of sialic acid was observed from lysosomes of normal fibroblasts, with the rate of exodus being linearly proportional to the amount of sialic acid initially loaded into the normal lysosomes. Defective
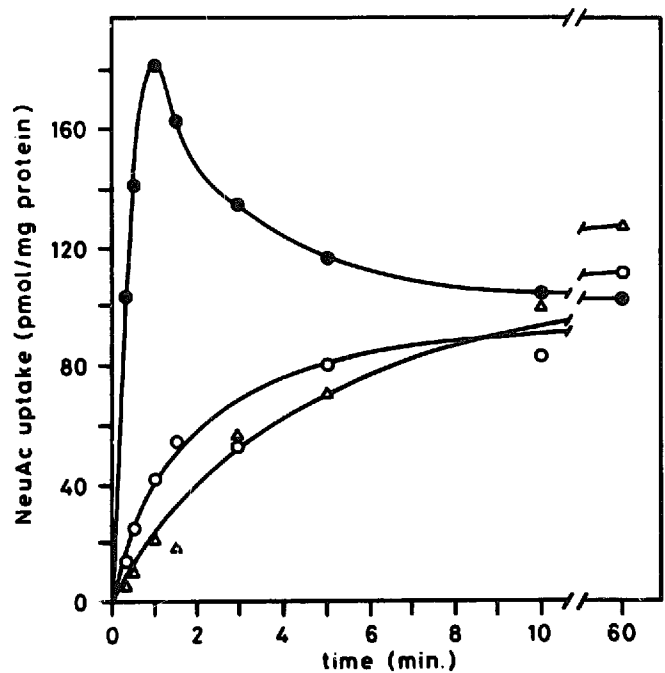

Fig. 11. Sialic acid uptake by rat liver lysosomal vesicles in the presence or absence of a proton gradient. Rat liver lysosomal membrane vesicles, prepared in 20 mM NaHepes buffer (pH 7.4), were pre-incubated for 10 min at 20°C in 20 mM NaHepes buffer (pH 7.4) (●———●; △———△), or 20 mM NaHepes/40 mM MES buffer (pH 5.5) containing 0.01 mM monensin (○———○). Subsequently, transport of 0.1 mM [^{14}C]NeuAc was measured at 20°C in 20 mM NaHepes buffer (pH 7.4) (△———△; $pH_{in} = pH_{out} = 7.4$), or in 20 mM NaHepes/40 mM MES buffer (pH 5.5) containing 0.01 mM monensin (○———○; $pH_{in} = pH_{out} = 5.5$), or in 20 mM NaHepes/40 mM MES (pH 5.5) (●———●; $pH_{in} = 7.4 > pH_{out} = 5.5$) [16].

sialic acid transport in lysosomes of Salla disease fibroblasts was confirmed subsequently by several other investigators [75–77].

Further investigation by Mancini et al. [16] using rat liver lysosomal membrane vesicles provided an understanding of the kinetic properties and overall substrate specificity of this carrier serving for lysosomal transport of sialic acid. The initial rate of sialic acid uptake by rat liver lysosomal vesicles is increased 10–25-fold by the presence of an inwardly directed proton gradient ($pH_{out} = 5.5 > pH_{in} = 7.4$) (Fig. 11). Under these conditions, sialic acid uptake by rat liver lysosomal vesicles is saturable with a K_m of 0.24 mM at 20°C. The K_m of sialic acid uptake at pH 5.5 in the absence of a proton gradient, however, is 3-fold greater than the K_m at pH 5.5 in the presence of a proton gradient. A Hill plot of the effect of [H$^+$] on proton gradient-dependent sialic acid uptake did not detect any significant degree of cooperativity of protons in accelerating the rate of lysosomal sialic acid uptake. In the absence of a proton gradient, the carrier fails to exhibit an acidic or alkaline pH optimum giving similar rates of sialic acid uptake at pH 5.5 (in = out) and at pH 7.4 (in = out). Furthermore, sialic acid uptake is unaffected by expos-

ing vesicles to valinomycin under conditions expected to change the membrane potential across the lysosomal vesicular membrane.

Analog inhibition and transstimulation experiments revealed that the carrier which recognizes sialic acid is actually a group-specific transporter for acidic monosaccharides [16]. The uronic and aldonic sugars, glucuronate, galacturonate, gluconate and galactonate were strong competitive inhibitors of [^{14}C]sialic acid uptake. In contrast, neutral sugars, anionic amino acids and the lactones-gulonolactone and mannuronolactone- had little effect on sialic acid uptake. Analogs modified at the carboxyl group of carbon 1 or at carbon 2 were only moderately inhibitory suggesting that both of these sites are important in recognition in addition to the strict requirement for a negatively charged terminus. Competitive inhibition constants of 0.11, 0.22, 0.26 and 1.6 mM were found for glucuronate, N-glycolylneuraminic acid, gluconate and 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, respectively. When lysosomal vesicles were pre-loaded with unlabelled sialic acid, [^{14}C]sialic acid uptake was transstimulated approx. 10-fold. A similar transstimulation of [^{14}C]sialic acid uptake occurred when vesicles were pre-loaded with glucuronate proving actual transport of glucuronate by this carrier.

Similar to the findings with rat liver lysosomal vesicles, the sialic acid carrier of human fibroblast lysosomes also was shown to transport glucuronic acid, displaying a half-time of 2–3 min for exodus of glucuronic acid at 37°C [78] and a K_m of 0.28 mM [79]. Furthermore, not only is free sialic acid stored within lysosomes of Salla disease fibroblasts but also glucuronic acid at about 5% of the amount of sialic acid. Tietze et al. [80] have demonstrated that the defect in infantile sialic acid storage diease (ISSD), like that in Salla disease, is due to an impairment in the transport system mediating lysosomal passage of sialic acid and glucuronate. Salla disease fibroblasts were shown to contain approx. 10-times the normal level of free sialic acid, whereas ISSD fibroblasts have approx. 100-times the normal level of sialic acid. The cause for the significantly more severe clinical manifestations and greater lysosomal storage of sialic acid in ISSD versus Salla disease patients is not yet known. However, one speculation is that lysosomes from Salla disease patients may have a small amount of residual sialic acid carrying capacity that is not apparent by current methods of analysis [80].

IV-B. The GalNAc / GlcNAc transport system

Lysosomal degradation of glycosaminoglycans and glycoproteins has been shown to produce large quantities of N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) within the lysosomal compart-

ment followed by rapid lysosomal efflux of these metabolites and their efficient re-incorporation into new complex carbohydrates [81]. Recently, Jonas et al. [82] described a transport system that displays high specificity for GlcNAc and GalNAc in rat liver lysosomes. Lysosomal uptake of these acetylated, amino sugars exhibits saturation kinetics with a K_m of 4.4 mM at pH 7.0 and 25°C. Uptake of 1 mM [^{3}H]GlcNAc or [^{3}H]GalNAc is transstimulated 3–4-fold when uptakes are performed with rat liver lysosomes pre-loaded with approx. 10 nmol unlabelled GlcNAc/unit β-hexosaminidase activity. The high specificity of this transport system is indicated by GalNAc and GlcNAc strongly inhibiting the uptake of one another, whereas ManNAc (25 mM) inhibited 1 mM [^{3}H]GlcNAc uptake by only 30%. Furthermore, 25 mM glucosamine, glucose, glucuronic acid, N-acetylneuraminic acid, mannose, fucose, or various amino acids have little effect on the lysosomal uptake of 1 mM [^{3}H]GlcNAc. Lysosomal GlcNAc uptake is insensitive to pH in the range from pH 6–8 and is not affected by the addition of 5 mM MgATP or dithiothreitol to incubation mixtures. Similar to the lysosomal glucose transporter, cytochalasin B strongly inhibits this lysosomal GalNAc/GlcNAc carrier with a 50 μM concentration producing 90% inhibition of GlcNAc uptake.

Recently, Jonas and Jobe showed that many of the properties of the rat liver lysosomal GlcNAc/GalNAc transporter can also be observed using lysosomal membrane vesicles [19]. Lysosomal membrane vesicles were prepared from Percoll-purified rat liver lysosomes by osmotic lysis, facilitated by exposure to 5 mM methionine methyl ester at pH 7 and 37°C in the presence of protease inhibitors to preserve transport activity [19,83]. Membrane vesicles formed in this manner have a random membrane orientation and are able to acidify the intravesicular space in the presence of MgATP indicating preservation of lysosomal proton pump activity. Lysosomal membrane vesicles pre-loaded with 50 mM GlcNAc displayed saturable uptake of [^{3}H]GlcNAc, whereas very little uptake of [^{3}H]GlcNAc is observed with vesicles not pre-loaded with unlabelled GlcNAc. A K_m of 1.3 mM was found for GlcNAc countertransport at 25°C and pH 7.0 by these lysosomal vesicles; this is slightly lower than the K_m of 4.4 mM found for GlcNAc uptake by intact rat liver lysosomes. GlcNAc countertransport by rat liver lysosomal membrane vesicles also displays a pattern of analog inhibition which is similar to that seen with intact rat liver lysosomes. Using this system additional characteristics of the GalNAc/GlcNAc carrier were revealed: (i) recognition by the transport protein does not tolerate phosphorylation or sulfation of GlcNAc at the 1 or 6 position (ii) 2-N-acetylallosamine which differs from GlcNAc in the orientation of the hydroxyl groups at positions 1 and 3 is recognized nearly as well as GlcNAc and GalNAc;

however, moving the acetyl group from position 2 to position 6 on GlcNAc or to position 3 of AllNAc results in poor recognition by the transporter. Thus, orientation of the acetyl group and net charge of the sugar are critical structural elements for carrier recognition, whereas various orientations of the hydroxyl groups are permissible.

Finally, a phenylisothiocyanate derivative of GlcNAc (PITC-GlcNAc) was shown to inhibit GlcNAc countertransport by 70% at pH 7.0. This inhibition by PITC-GlcNAc is believed to be due to competition at the binding site and not due to inactivation since covalent modification by this agent is not favorable at pH 7. PITC-GlcNAc may serve as a useful probe in future studies to specifically label the receptor site of the lysosomal GlcNAc/GalNAc transport protein.

IV-C. Lysosomal transport of glucose and other neutral hexoses

Docherty et al. [84] provided the first experimental evidence supporting the existence of a lysosomal transport system for neutral sugars. They demonstrated that loss of latency by rat liver lysosomes in osmotic protection experiments varied with different neutral hexoses and pentoses, occurred more rapidly with D-stereoisomers than L-stereoisomers and could be retarded by exposure to 1 mM phlorrhizin or 50 μM cytochalasin B which have been shown to inhibit other sugar transport systems. Subsequently, Maguire et al. [85] demonstrated [14C]glucose uptake by rat liver tritosomes obtaining an apparent K_m of 48 ± 18 mM at pH 7.4 and 25°C, although the highest concentration used in this determination was 20 mM, well below that necessary to demonstrate complete saturability of the transport route. D-[14C]Glucose (10 mM) uptake was inhibited 40–60% by 50 mM concentrations of D-mannose, D-glucose or D-2-deoxyglucose and cytochalasin B inhibited glucose uptake in a dose-dependent manner with 200 μM cytochalasin B causing 75% inhibition. The strong inhibitory effect of cytochalasin B upon lysosomal glucose transport has also been confirmed by Mancini et al. and Jonas et al. [17,86].

Recently, Mancini et al. and Jonas et al. have provided a further detailed characterization of this lysosomal neutral sugar transport system. Mancini et al. [17] demonstrated saturable glucose uptake by membrane vesicles prepared from purified rat liver lysosomes. Glucose uptake was approx. 2-fold greater when the extravesicular pH was in the acidic range from pH 5–6 than in the neutral pH range from pH 7–8. Imposing proton gradients across the vesicular membrane had no effect on vesicular glucose uptake indicating that, in sharp contrast to lysosomal sialic acid transport, glucose transport is not coupled to proton gradients. At

pH 7.4 and 20°C, a K_m of 75 mM was obtained for glucose uptake by lysosomal vesicles, whereas at pH 5.5 a K_m of 90 mM was found with a V_{max} 2-fold greater at pH 5.5 than at pH 7.4. Similarly, uptake of the sugars, D-galactose, D-mannose and L-fucose displayed K_m values ranging from 50–75 mM at pH 5.5. For all these analogs, Lineweaver-Burk and V/S vs. V plots were linear suggesting that only one transport route is involved in their uptake by rat liver lysosomal vesicles. Most importantly, a transstimulation property, observed as a 3-fold greater D-[14C]glucose uptake when vesicles were pre-loaded with 100 mM D-glucose, was used to determine the nature of the substrates transported by this carrier and what structural features are important for recognition. In addition to glucose, 100 mM concentrations of the neutral hexoses, D-galactose, D-mannose, D-fucose, L-fucose and D-fructose transstimulated D-[14C]glucose uptake 1.7–3-fold. A small transstimulation of D-[14C]glucose uptake was also observed with the pentose, D-ribose. In contrast, L-stereoisomers of glucose, galactose and rhamnose or the acidic sugars, D-gluconic acid, D-glucuronic acid and sialic acid had no transstimulatory effect on glucose uptake. Furthermore, N-acetyl-D-glucosamine, 6-amino-D-deoxyglucose, the pentose, D-arabinose and the lactones, D-galactonolactone, D-mannuronolactone and L-ascorbic acid were also ineffective in transstimulating glucose uptake by rat liver lysosomal vesicles. In conclusion, the lysosomal glucose transport system recognizes neutral hexoses with preference for the D-stereoisomer except in the case, thus far, for fucose in which both isomers are recognized. Sugars which are N-acetylated, or which contain a lactone, or a charged substituent are poorly recognized. The poor ability of pentoses to transstimulate glucose uptake suggests that pentoses generally are not transported by this carrier. Bird et al. [87] and Maguire et al. [85] have shown that D-ribose, at concentrations of 125–250 mM, enters rat liver lysosomes at a higher rate than glucose. Since D-ribose is poorly recognized by the glucose transporter, the majority of D-ribose passage across the lysosomal membrane most likely occurs by a different pathway. Whether this pathway is simple diffusion as suggested by Bird et al. [87] or by a carrier-mediated route remains to be determined.

Recently, Jonas et al. [86] using intact rat liver lysosomes applied analog inhibition analysis to determine the characteristics of the lysosomal glucose transporter in intact rat liver lysosomes. The same general substrate specificity was found as that described above for lysosomal membrane vesicles. trans-Stimulation, however, was not observed when D-glucose or L-fucose uptakes were performed with intact lysosomes preloaded by equilibration with 100 mM D-glucose. Lysosomal glucose uptake was insensitive to pH over the range from pH 6–8 and was not affected by the pres-

ence of 2 mM MgATP, 5 mM dithiothreitol, 25 mM NaCl, or 25 mM KCl in incubation mixtures.

The ability of this transport system to remove neutral sugars quickly from the lysosomal compartment is suggested by the rapid efflux of D-glucose from rat liver lysosomes which occurs with a half-time of 3 min at 25°C and pH 7.0. trans-Stimulation of D-[^{14}C]glucose exodus was not observed when 50 mM D-glucose was present in the extralysosomal buffer.

V. Lysosomal nucleoside transport

For many years, lysosomes have been known to contain enzymatic activities capable of degrading nucleic acids completely to nucleosides and inorganic phosphate, although further degradation of nucleosides to their free base and sugar has not been observed [88–93]. Several studies indicate that one of the major applications of these lysosomal enzymatic activities is in degradation of cytoplasmic RNA. In 1981, Sameshima et al. [94] found that in WI-38 fibroblasts, a pathway of RNA turnover is activated during serum deprivation which is inhibited by the lysosomotropic amine, NH_4Cl. Subsequently, Lardeux and Mortimore demonstrated that in perfused rat liver, under conditions of nutritional deprivation, 65% of total cytoplasmic RNA is degraded per day, with the autophagolysosomal pathway accounting for 70–85% of this catabolism [95]. Similar to endogenous protein breakdown, cytoplasmic RNA degradation is subject to regulation by insulin and the intracellular concentration of various amino acids, showing a close correspondence between the fractional turnover of protein and RNA over the range of amino acid deprivation. Furthermore, the lysosomotropic amine, chloroquine, inhibits RNA turnover in perfused rat liver by 70%. Thus, lysosomes appear to play a major role in cytoplasmic RNA turnover, leading to the production of large quantities of nucleosides within this compartment. Recently, a lysosomal nucleoside transport system has been characterized that provides a major route for the lysosomal passage of nucleosides across the lysosomal membrane [15]. In this investigation, adenosine uptake by human fibroblast lysosomes was found to be a saturable process, displaying a K_m of 9 mM at pH 7.0 and 37°C. This K_m of 9 mM for lysosomal adenosine uptake is 25–100-times larger than that reported for adenosine transport across the plasma membrane of many types of eukaryotic cells [96–99]. The V_{max} of lysosomal adenosine uptake, 21 pmol min^{-1} hex^{-1}, is several-fold greater than that for any of the fibroblast lysosomal amino acid transport systems, making it one of the major carriers in the lysosomal membrane in terms of net transport capacity. The large V_{max} of the lysosomal nucleoside transport system coupled with its large K_m enables this transport system to efficiently deal with

high intralysosomal concentrations of nucleosides which easily could be generated during degradation of nucleic acids within the relatively small volume of the lysosomal compartment.

Lysosomal adenosine uptake is insensitive to pH over the range from pH 5–8, is not affected by the addition of 2 mM MgATP to incubation mixtures and displays an activation energy of 12.9 kCal/mol (Q_{10} = 2.0). Analog inhibition analysis revealed that this transport system recognizes both purine and pyrimidine nucleosides. The purine nucleosides, 2'-deoxyadenosine, inosine, 6-dimethylaminopurine riboside and purine riboside competitively inhibit lysosomal adenosine uptake, displaying K_i's ranging from 4–26 mM, whereas the pyrimidine nucleosides, cytidine, uridine, thymidine and cytosine-β-D-arabinoside are recognized with lower affinity with K_i's of 23–42 mM. Nucleotides are not accepted by the nucleoside carrier as demonstrated by the lack of competitive inhibition by 5'-ADP, 5'-AMP and 3'-AMP. Two features of the pattern of analog inhibition suggest that recognition is directed primarily towards the base portion of the nucleoside: (i) changing the nature of the nucleoside sugar from ribose to deoxyribose or arabinose has little effect on recognition of nucleosides by the transporter (ii) the sugar, D-ribose, is a very poor competitive inhibitor of lysosomal adenosine uptake, in contrast to the nucleobase, 6-dimethylaminopurine (K_i = 11 mM) which is recognized nearly as well as adenosine and approx. half as well as its nucleoside, 6-dimethylaminopurine riboside (K_i = 4.4 mM).

Addition of hydrophobic substituents at the 6 position of the purine ring enhances recognition by the nucleoside transporter. This is especially apparent with the nucleoside analog, nitrobenzylthioinosine. Dipyridamole and nitrobenzylthioinosine are extremely potent inhibitors (K_i approx. 1 nM) of nucleoside transport across the plasma membrane of many types of eukaryotic cells [96–99]. Lysosomal adenosine uptake is inhibited 50% by 25 μM dipyridamole or 27 μM nitrobenzylthioinosine. This level of inhibition suggests that the lysosomal nucleoside carrier recognizes dipyridamole and nitrobenzylthioinosine with 10^3–10^4-fold greater affinity than adenosine.

Efflux experiments demonstrated that nucleosides are transported relatively quickly out of human fibroblast lysosomes. The half-time of uridine and inosine exodus from fibroblast lysosomes was 6 min ± 0.9 and 7.5 min ± 1.0, respectively, at 37°C and pH 7.0 (Fig. 12). trans-Stimulation of [^{3}H]uridine or [^{3}H]inosine exodus was not observed when saturating levels of inosine or uridine were added to the buffer in which lysosomes were suspended. Experiments involved in loading [^{3}H]adenosine into human fibroblast lysosomes revealed that adenosine is quickly deaminated to inosine within the lysosomal compartment. After a 2.5 min
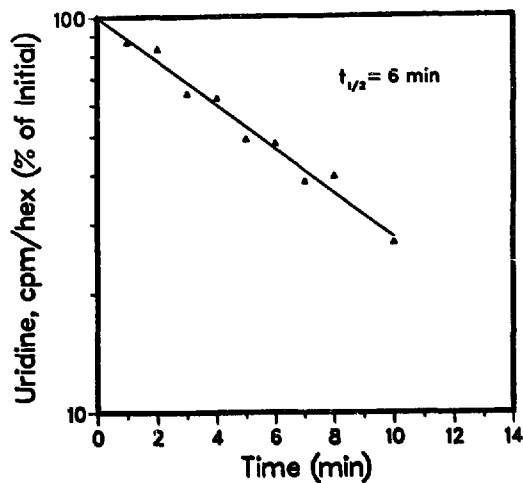
Fig. 12. Exodus of uridine from human fibroblast lysosomes. Fibroblast lysosomes were pre-loaded by incubation with 180 mM [³H]uridine for 20 min at pH 7.0 and 37°C. Lysosomes were then washed of unaccumulated radioactivity by centrifugation at 4°C, resuspended in pH 7.0 buffer and incubated at 37°C. At the indicated time points, lysosomes were collected on glass fiber filters, washed and counted for radioactivity [15].

adenosine uptake, 85% of the [³H]adenosine taken up by fibroblast lysosomes was recovered as adenosine within lysosomes but after a 15–20 min incubation nearly all of the radioactivity contained within the lysosomes was in the form of inosine. Recently, the enzyme responsible for this deamination of adenosine within lysosomes has been characterized and was found to be very similar to the well-characterized adenosine deaminase from human fibroblasts and human lymphocytes which is deficient in adenosine deaminase deficiency [100]. Questions still remain as to how adenosine deaminase activity is delivered to fibroblast lysosomes, what its half-life is in this compartment and what specific role deamination of lysosomal adenosine has in overall cellular metabolism.

Lysosomal passage of nucleobases has been investigated only briefly [15]. [³H]Adenine was found to enter human fibroblast lysosomes by a route not saturable either by high concentrations of various nucleosides or by adenine itself. This non-saturable passage of adenine across the lysosomal membrane is distinct from the carrier-mediated, saturable route serving for lysosomal nucleoside transport.

VI. Inorganic ion transport systems

VI-A. The lysosomal proton pump

The most prominent of the lysosomal ion transporters is the lysosomal proton pump or proton translocating ATPase which utilizes the energy from ATP hydrolysis to pump protons into the lysosomal interior and generate an acidic intralysosomal environ-

ment [101–106]. The lysosomal H⁺-ATPase is electrogenic and displays maximal ATPase activity at pH 6.8 and [MgATP] > 1 mM [107,108]. The lysosomal proton pump is similar to other vacuolar type H⁺-ATPases by its characteristic response to several inhibitors. Lysosomal acidification is insensitive to oligomycin, azide, ouabain, or vanadate but is strongly inhibited by N-ethylmaleimide and nitrate [102,107]. In contrast, the mitochondrial F_0F_1 ATPases are strongly inhibited by oligomycin or azide and E_1E_2-phosphoenzyme ATPases of the plasma membrane are inhibited by vanadate. Vacuolar ATPases have been found in membranes of various intracellular acidic compartments in eukaryotic cells, chromaffin granules, coated vesicles, the Golgi apparatus, plant and yeast vacuoles and proton-transporting microsomal vesicles [109–114]. Purification of several of the vacuolar proton pumps indicates that they are organized as a large complex often composed of at least nine different subunits [112,115,116]. Although structural studies of the lysosomal H⁺-ATPase have not yet disclosed its quaternary structure, several reports suggest that its subunit composition is very similar to vacuolar proton pumps purified from other membranes. Vacuolar H⁺-ATPases undergo an irreversible inhibition when exposed to MgATP and KNO_3 at 0–4°C [117]. These conditions result in the release of a water-soluble moiety of the H⁺-ATPase from the membrane. Five polypeptides having apparent molecular masses of 72, 57, 41, 34 and 33 kDa on SDS-PAGE are released from chromaffin granules under these conditions. Moriyama and Nelson found that lysosomal membranes from rat liver or rat kidney cortex release the same pattern of polypeptides when incubated with MgATP and KNO3 at 0°C [117]. Antibodies against the vacuolar H⁺-ATPase of chromaffin granules or human gastric mucosa also cross react with the lysosomal H⁺-ATPase indicating shared epitopes [117–119] and have allowed for the immunoprecipitation of lysosomal polypeptides of 70, 56 and 31 kDa [120]. Hopefully, future studies will be able to elucidate the role of the different subunits in the function and regulation of the lysosomal H⁺-ATPase and determine how its activity is related to other pathways of lysosomal ion conductance.

VI-B. Lysosomal transport of sulfate and molybdate

The investigation of sulfate transport by rat liver lysosomes provides a good example of the importance of incorporating several different approaches of analysis in studying a transport process. Jonas and Jobe were unable to observe any appreciable uptake of 100 μM [³⁵S]Na₂SO₄ by intact rat liver lysosomes during a 20 min incubation period at 25°C and pH 7.0. In contrast, saturable uptake of 100 μM [³⁵S]Na₂SO₄ was readily demonstrated when uptakes were performed

with rat liver lysosomal membrane vesicles having an internal pH of 7.0 [18]. Sulfate uptake was 2-fold faster in the presence of a proton gradient (pH 5.0_{out} > pH 7.0_{in}) than in the absence of a gradient (pH 7.0_{in} = pH 7.0_{out}) and displayed a K_m of 160 μM.

Uptake of [^{35}S]sulfate by lysosomal vesicles is *trans*stimulated 5–20-fold when vesicles are pre-loaded with 10 mM Na_2SO_4 at pH 7.0 in the absence of a proton gradient. NaMoO4 also strongly *trans*stimulates [^{35}S]sulfate uptake under these conditions indicating that molybdate is transported by this carrier. The specificity of this transport system for sulfate and molybdate is demonstrated by the failure of 10 mM sodium phosphate, sodium chloride, sodium bicarbonate and sodium metabisulfite to *trans*stimulate [^{35}S]sulfate uptake to any significant degree. However, sulfate uptake by rat liver lysosomal membrane vesicles is strongly inhibited by 0.1 mM DIDS and 1 mM NAP-taurine which also strongly inhibit the band 3 anion transporter. Although 10 mM chloride ion does not *trans*stimulate sulfate uptake, it causes approx. 50% *cis* inhibition of sulfate uptake. At the present time it is not established whether this inhibitory effect of Cl⁻ is due to Cl⁻ directly binding to the transport protein or is an indirect effect, such as causing a change in membrane potential via a chloride conduc-tance channel which then affects sulfate transport activity.

Sulfate uptake by vesicles is not observed at pH 5.0 in the absence of a proton gradient (pH 5.0_{out} = pH 5.0_{in}). Furthermore, agents that alter the membrane potential across the lysosomal vesicular membrane produce substantial changes in sulfate uptake. Thus, the lysosomal sulfate/molybdate carrier appears to be regulated, in part, by the membrane potential across the lysosomal membrane and possibly by different states of protonation. Rome and Hill have shown that sulfate is rapidly released from human fibroblast lysosomes following lysosomal degradation of [^{35}S]glycosaminogly-cans [81]. In contrast to N-acetylhexosamines, however, sulfate released from lysosomes rapidly exchanges with sulfate in the culture medium resulting in little or no detectable re-incorporation of this released sulfate into newly synthesized macromolecules. Although the majority of sulfate released from lysosomes does not appear to be immediately salvaged for biosynthetic processes, it will be interesting to determine the fate of molybdate released within lysosomes following degradation of molybdate-containing metalloenzymes.

VI-C. Lysosomal phosphate transport

Recently, a phosphate transport system has been described in lysosomes from human fibroblasts [121]. Phosphate uptake by fibroblast lysosomes exhibits saturable, Michaelis-Menten kinetics with a K_m of 5 μM

at pH 7.0 and 37°C. Lineweaver-Burk and V/S vs. V plots are linear suggesting that phosphate uptake is mediated by one transport route. High specificity for phosphate is indicated since high concentrations of Na_2SO_4, $NaHCO_3$, NaCl, KCl, 3'-AMP, 5'-AMP and the band 3 anion transport inhibitor, 4,4'-diiso-thiocyanatostilbene-2,2'-disulfonate (DIDS) have no effect on lysosomal phosphate uptake. In contrast, the phosphate analog, arsenate, is a strong competitive inhibitor of lysosomal phosphate uptake displaying a K_i of 7 μM. In addition, several compounds were found to inhibit lysosomal phosphate uptake in a non-competitive manner. These were glucose-6-phosphate (K_i = 210 μM), pyridoxal phosphate (K_i = 250 μM), CTP (K_i = 140 μM) and the non-hydrolyzable analog of ATP, AMP-PNP (K_i = 80 μM). This non-competitive inhibition suggests the existence of a site(s) distinct from the transport protein receptor site to which nu-cleotides, glucose-6-phosphate and pyridoxal phosphate can bind resulting in inhibition of lysosomal phosphate uptake. This site may reside on the transport protein or on a neighboring subunit if the phosphate transport activity exists as a complex with other proteins.

Lysosomal phosphate uptake is highly pH-dependent showing maximal uptake in the acidic pH range between pH 4.5 and 5.5, with half-maximal uptake occurring near pH 7.1 and no phosphate uptake observed at pH values > 8.5 (Fig. 13). This pH dependence of lysosomal phosphate uptake closely resembles the titration curve for the conversion of the monobasic form of phosphate to its dibasic form which has a pKa ≃ 7.1, suggesting that the lysosomal phosphate transport route specifically recognizes the monobasic form of phosphate.

The majority of [^{32}P]phosphate taken up by human fibroblast lysosomes is rapidly converted to TCA-solu-ble and TCA-insoluble products which appear to be

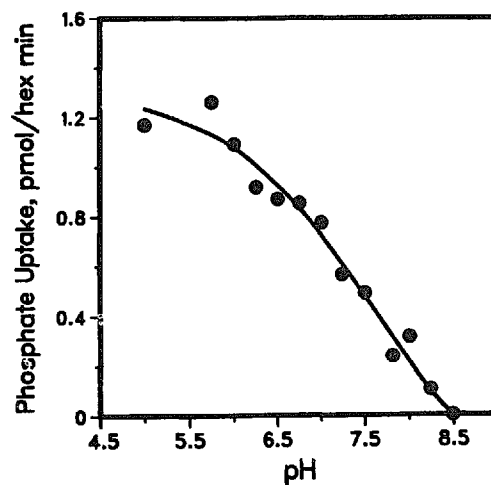Fig. 13. pH dependence of 0.04 mM [^{32}P]phosphate uptake by human fibroblast lysosomes [121].

unable to egress from fibroblast lysosomes. After a 1 min [^{32}P]phosphate uptake approx. 50% of the radioactivity recovered from lysosomes is inorganic phosphate and after incubations of 2.5–17 min only 22–27% of the radioactivity exists as inorganic phosphate. The nature of the TCA-soluble products formed within fibroblast lysosomes is not known at the present time. However, we have found the TCA-insoluble material to consist of long chains of inorganic polyphosphate ranging in size from 100–600 phosphate residues in length (E.R. Lindley and R.L. Pisoni, J. Biol. Chem., in press). It is noteworthy that large amounts of polyphosphate are present in yeast vacuoles which are functionally analogous to lysosomes of mammalian cells [122,123].

The K_m and pH dependence of the lysosomal phosphate transport system differs significantly from phosphate transport systems of mitochondria which display a K_m near 2 mM [124,125]. In addition, phosphate uptake by human fibroblast lysosomes also may differ substantially from that in rat liver lysosomes. Schneider has demonstrated [^{32}P]phosphate uptake by rat liver lysosomal vesicles prepared from tritosomes [126]. Both characteristics reported for [^{32}P]phosphate uptake by rat liver lysosomal vesicles are contrary to those found with phosphate uptake by human fibroblast lysosomes: (a) 0.03 mM DIDS causes approx. 80% inhibition of phosphate uptake by rat liver lysosomal vesicles, whereas phosphate uptake by human fibroblast lysosomes is not affected by [DIDS] ≤ 0.3 mM, (b) 4.8 mM MgATP stimulates [^{32}P]phosphate uptake by rat liver lysosomal vesicles; in contrast, 2 mM MgATP strongly inhibits [^{32}P]phosphate uptake by human fibroblast lysosomes. Some of these differences may reflect inherent differences between isolated intact lysosomes and tritosomes. Ohkuma et al. [102] have noted that the H$^+$-ATPase activity of isolated, intact rat liver lysosomes is not inhibited by DCCD or azide, whereas H$^+$-ATPase activity from rat liver tritosomes is highly sensitive to these two inhibitors. Hopefully, future investigations will help to elaborate the similarities and differences in lysosomal phosphate transport in different mammalian cell types.

Lysosomes appear to play a major role in cytoplasmic RNA turnover which would generate large quantities of inorganic phosphate within the lysosomal compartment. The phosphate uptake studies using intact fibroblast lysosomes clearly demonstrate the presence of a phosphate transport system in human fibroblast lysosomes but the role of this transport system in mediating the release of large quantities of inorganic phosphate from lysosomes is difficult to ascertain because of the large degree of intralysosomal phosphate metabolism. The possibility remains that a different route may serve for phosphate release from fibroblast lysosomes. Similar to lysosomal sulfate transport, a lysosomal phosphate transport system serving mainly for exodus may exist in human fibroblast lysosomes which is not detectable in studies of phosphate uptake using intact lysosomes but may become apparent using lysosomal membrane vesicles.

Lysosomes contain a membrane-bound ATPase which provides energy from the hydrolysis of ATP for operation of the lysosomal proton pump. When fibroblast lysosomes are incubated with radiolabelled ATP, radioactivity from [γ-^{32}P]ATP is taken up by fibroblast lysosomes, whereas radioactivity is not accumulated when lysosomes are exposed to [α-^{32}P]ATP. These results indicate that fibroblast lysosomes use the lysosomal phosphate transport system to take up the γ-phosphate released by hydrolysis of ATP, but ATP itself is not taken up. Thus, the activity of the lysosomal membrane ATPase is related to the operation of two different lysosomal ion transport routes: the proton pump and the phosphate transport system.

VII. Vitamin B$_{12}$ transport and methylmalonic aciduria

Vitamin B$_{12}$ (cobalamin) is a required co-factor for a number of mammalian enzymes. It is known to circulate in plasma bound to the protein transcobalamin II. Following endocytosis and lysosomal degradation, cobalamin is released from lysosomes into the cytosol where the holo-enzymes are synthesized [127]. In 1985, Rosenblatt et al. described a defect in the release of cobalamin from lysosomes of an individual exhibiting a new form of methymalonic aciduria [128]. They demonstrated that fibroblasts from this patient showed increased stores of ^{57}Co in lysosomes after exposure to [^{57}Co]cobalamin for 24 h. The fraction of cobalamin released into the cytosol was only one-tenth that found in control lysosomes isolated from normal fibroblasts, which led Rosenblatt et al to suggest that the lysosomal accumulation of cobalamin was due to a defect in lysosomal transport of cobalamin. Complementation analysis using fibroblasts from the patient described above and fibroblasts from patients with methylmalonic aciduria due to other defects showed that complementation occurred between all previously known methylmalonic aciduria mutations and cells with lysosomal cobalamin storage. The authors named this new defect involving lysosomal storage of free cobalamin, cblF [129].

Recently, it has been reported that rat liver lysosomal vesicles display a specific transport system for cobalamin. This system exhibits saturation kinetics with a K_m of 3.5 μM which was demonstrable after vesicles had been previously loaded with 100 μM B$_{12}$. Uptake specificity studies demonstrated that adenosylcobalamin, methylcobalamin and cobinamide dicyanide all competed for uptake of [^{57}Co]B$_{12}$ [130].

VIII. Lysosomal dipeptide transport

Two reports suggest the existence of dipeptide transport systems in lysosomal membranes. Mego has demonstrated that when exogenous cysteinyl-glycine is added to intact mouse kidney lysosomes pre-loaded with bovine serum albumin, the bovine serum albumin is degraded at an accelerated rate within the lysosomes presumably due to the ability of thiol to dissociate protein disulfide bridges thereby accelerating proteolytic degradation [131]. These results imply that the dipeptide, cysteinyl-glycine, is able to pass across the lysosomal membrane. Recently, Bird and Lloyd employed osmotic protection studies with rat liver lysosomes and found for seven pairs of dipeptide stereoisomers, the osmotic protection afforded by the D-stereoisomers was much better than protection provided by the L-stereoisomers [132]. These results suggest the existence of a transport mechanism in rat liver lysosomes which preferentially mediates lysosomal passage of the L-stereoisomer of some dipeptides. Of the seven pairs of dipeptides which were tested, loss of latency was rapid when lysosomes were exposed to 0.25 M L-Ala-L-Ala, L-Ala-Gly, or Gly-Gly, intermediate for Gly-L-Ala, Gly-L-Thr,and Gly-L-Ser and slow when exposed to Gly-L-Asn, Gly-L-Val or the D-stereoisomer of any of the above dipeptides.

IX. Acetyl coenzyme A: α-glucosaminide N-acetyltransferase

Rome and colleagues have described a lysosomal membrane enzyme, acetyl CoA:α-glucosaminide N-acetyltransferase, which catalyzes the transmembrane transfer of acetyl groups from cytosolic acetyl CoA to terminal α-linked glucosamine residues of heparan sulfate located within the lysosomal compartment [133,134]. This transferase has a broad pH optimum from pH 6–8 and displays K_m values of 0.55 mM and 0.3 mM for acetyl CoA and glucosamine, respectively, at pH 5.8 and 37°C. The kinetic analysis suggests that this enzyme works by a Di Iso Ping Pong Bi Bi mechanism. The proposed reaction cycle begins with acetyl-CoA binding to the enzyme on the cytoplasmic side of the lysosomal membrane; in the second step the enzyme is acetylated, followed by release of CoA; the acetylated enzyme then undergoes a conformational change resulting in transmembrane relocation of the bound acetyl moiety towards the lysosomal interior; the second substrate, a glucosamine residue on heparan sulfate, then binds to a separate site on the enzyme, becomes acetylated and the product released. The sulfhydryl reactive agent, p-chloromercuribenzoate at a concentration of 0.5 mM completely inhibits enzymatic activity, whereas 1 mM N-ethylmaleimide or 10 mM iodoacetamide have little effect. Heriditary deficiency

of acetyl CoA:α-glucosaminide N-acetyltransferase, known as Sanfilippo C syndrome, results in lysosomal accumulation of heparan sulfate because of the inability to degrade this mucopolysaccharide completely when glucosamine residues are not acetylated.

X. Concluding remarks

The lysosomal transport systems described in this review provide a firm base of knowledge for future investigations. Many new lysosomal transport systems are likely to be discovered in the future, yet there remain many interesting areas of inquiry involving the currently known lysosomal carriers. During these investigations, much has been learned beyond understanding the characteristics of individual transport systems. The basis of four different genetic disorders in lysosomal transport is now known and the techniques that have been developed greatly enhance the ability to characterize other lysosomal transport disorders. An understanding of the mechanism of cysteamine therapy of cystinosis has been obtained, allowing for discovery of other agents capable of depleting cystinotic cells of their accumulated cystine. Unexpected intralysosomal metabolism of adenosine and phosphate have pointed to the occurrence of lysosomal enzymatic activities previously not known. Hormonal regulation of lysosomal system h has demonstrated modulation of a specific lysosomal transport system to meet the metabolic needs of a specialized tissue. The properties of the lysosomal nucleoside transport system demonstrates the ability of lysosomes to deal effectively with the degradation of nucleic acids and to recycle their key metabolic components. In addition, the cysteine-specific lysosomal transport route delivers large quantities of cysteine into lysosomes, suggesting a role for this thiol in lysosomal metabolic activities.

Several photoaffinity probes and chemical modifying agents now have been identified which may be useful for specifically labelling some lysosomal transport proteins. Furthermore, the cystine dimethyl ester selection technique may aid identification of the lysosomal cystine transport gene. Efforts in these areas will ultimately lead to an understanding of the structure of the lysosomal transport proteins, the factors that affect their function within the lysosomal membrane and the way these carriers are targeted to lysosomes. Knowledge of the lysosomal transport systems also will be crucial for developing drug delivery strategies in which agents are specifically delivered to lysosomes by receptor-mediated endocytosis. Having developed the tools for investigation and a strong base of knowledge, one eagerly anticipates further advances in understanding the role of transport systems in lysosomal function and their contribution to health and disease.

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