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pH GRADIENT ACROSS THE LYSOSOMAL MEMBRANE GENERATED BY SELECTIVE CATION PERMEABILITY AND DONNAN EQUILIBRIUM

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SUMMARY

The pH within isolated Triton WR 1339-filled rat liver lysosomes was determined by measuring the distribution of [^{14}C]methylamine between the intra- and extralysosomal space. The intralysosomal pH was found to be approximately one pH unit lower than that of the surrounding medium. Increasing the extralysosomal cation concentration lowered the pH gradient by a cation exchange indicating the presence of a Donnan equilibrium. The lysosomal membrane was found to be significantly more permeable to protons than to other cations. The relative mobility of cations through the lysosomal membrane is $H^+ \gg Cs^+ > Rb^+ > K^+ > Na^+ > Li^+ \gg Mg^{2+}, Ca^{2+}$. The presented data suggest that the acidity within isolated Triton WR 1339-filled lysosomes is maintained by: (1) a Donnan equilibrium resulting from the intralysosomal accumulation of nondiffusible anions and (2) a selective permeability of the lysosomal membrane to cations.

INTRODUCTION

The fact that most of the lysosomal enzymes in mammalian tissues exhibit an acidic pH optimum suggests that the intralysosomal milieu must be acid [1]. Indicator dyes have been applied to demonstrate the acidity within the lysosomes [2–4] but accurate measurements of the intralysosomal pH have proved difficult and little is known about the mechanisms that maintain the pH gradient across the lysosomal membrane. Recently, the intralysosomal pH has been determined by the measurement of the distribution of the weak base methylamine between the intra- and the extralysosomal space [5, 6].

As one of the conceivable mechanisms for the generation of an intralysosomal acid pH, Coffey and de Duve [7] postulated the presence of a Donnan equilibrium produced by an intralysosomal accumulation of nondiffusible anions. Several authors have observed the presence of acidic components in lysosomal fractions of

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kidney and liver [8, 9]. Recent data show that in isolated, native rat liver lysosomes the intralysosomal pH is influenced by the extralysosomal potassium concentration [6].

Results of this laboratory demonstrated that the membrane fraction of Triton WR 1339-filled rat liver lysosomes contains sialic acid at an even higher concentration than the plasma membrane of rat liver cells [10, 11]. Approximately 50 % of the membrane-bound sialic acid in lysosomes could be extracted with the total lipid fraction and was found in the ganglioside fraction which consisted mainly of hematoside and GM_1-ganglioside [12]. The membrane-bound sialic acid acts as a strong anion as shown by a strong reversible binding of acid phosphatase and β-glucuronidase to isolated lysosomal membranes at pH 4 [11]. Electron microscopy demonstrated that the sialic acid was localized on the inside of the lysosomal membrane [11]. These results provide the molecular and the structural basis for the occurrence of intralysosomal nondiffusible strong anions as a condition for a Donnan equilibrium.

In this communication I present evidence for a selective cation permeability and for a Donnan potential across the lysosomal membrane of Triton WR 1339-filled rat liver lysosomes. The data suggest that the mobility of protons across the lysosomal membrane is higher by orders of magnitude than those of metal cations. Their mobility depends on the diameter of the hydrated ion as well as on the charge.

EXPERIMENTAL PROCEDURE

Materials

Sucrose, salts and buffer substances were of analytical grade (Merck, Darmstadt, Germany). ^3H_2O (10^9 dpm/ml), [^{14}C]methylamine (22.4 mCi/ml), [1,2-^{14}C]-polyethyleneglycol (M_r 4000, 0.6 μCi/mg), Na^{36}Cl (aqueous solution 0.191 Ci/mol) were purchased from The Radiochemical Centre (Amersham, England). ^{3}H-labeled Triton WR 1339 (0.05 Ci/g) was kindly prepared by Professor G. Stöcklin, Kernforschungsanlage, Jülich, Germany) by the Wilzbach technique.

Methods

Preparation of lysosomes. 200–250 g Sprague-Dawley rats were injected with Triton WR 1339 (170 mg/100 g rat). After 4 days, Triton WR 1339-filled lysosomes were prepared by slight modifications [10] of the original method of Wattiaux et al. [13]. This lysosome fraction is free of other subcellular material (plasma membranes, mitochondria, microsomes, peroxisomes) as ascertained by the determination of the correspondent marker enzymes [10], by the absence of cytochromes aa_3 and c [15] and by electron microscopy [16].

pH determinations in lysosomes. The intralysosomal pH was determined by measuring the distribution of the weak base methylamine between the intralysosomal and the extralysosomal space as worked out by Rottenberg et al. for the determination of the pH in chloroplasts [17, 18]. The total volumes of lysosomal pellets and the methylamine concentrations in lysosomes were calculated after filtering centrifugation of lysosomes through a layer of silicone oil according to Klingenberg and Pfaff [19] in a Beckman Microfuge. The total volume of the pellet was determined by the use of ^3H_2O and the extralysosomal volume was measured by the addition of poly[1,2-^{14}C]ethyleneglycol (M_r 4000) which does not permeate the lysosomal mem-

brane. The intralysosomal volume was calculated by subtraction of the extralysosomal volume from the total pellet volume. The intralysosomal pH was calculated according to the following equation. Its derivation is discussed by Rottenberg et al. [17, 18].

$$\frac{[[^{14}C]\text{methylamine}]_{in}}{[[^{14}C]\text{methylamine}]_{out}} = \frac{[H^+]_{in}}{[H^+]_{out}}$$

after transformation:

$$pH_{in} = pH_{out} - \log \frac{[[^{14}C]\text{methylamine}]_{in}}{[[^{14}C]\text{methylamine}]_{out}}$$

Experimental procedure. The suspension of lysosomes (0.35 M sucrose, Tris-acetate buffer, 0.01 M, pH 7.0, final protein concentration 5–10 mg/ml) contained either of the following pairs of tracers: ^3H_2O (10^6 dpm/ml) and poly[1,2-^{14}C]ethyleneglycol (10^6 dpm/ml) for the determination of the total pellet volume and the extralysosomal volume, or ^3H_2O (10^6 dpm/ml) and [^{14}C]methylamine (10^6 dpm/ml) for the calculation of the distribution of this base between the intralysosomal and the extralysosomal space. Filtering centrifugation was performed in Beckman Microfuge tubes which contained from bottom to top: 0.02 ml 0.45 M sucrose, 0.04 ml silicone oil (AR 200/AR 20 7 : 1, Wacker Chemie, München, Germany) and 0.1 ml incubation mixture containing 0.5–1.0 mg lysosomal protein, 0.35 M sucrose, 0.01 M Tris-acetate buffer, pH 7.0 and the appropriate radioactive materials described above. Pellet volume (^3H_2O, poly[^{14}C]ethyleneglycol) and intralysosomal pH determinations (^3H_2O, [^{14}C]methylamine) were performed in parallel assays each containing the corresponding concentration of cold polyethyleneglycol or methylamine. All operations were performed at 2 °C in a cold room. Lysosomes were sedimented through the silicone oil layer usually in a centrifugation time of 30 min at approximately 11 000 rev./min. For kinetic studies the centrifugation time was shortened to 1 or 2 min, which resulted in a sediment containing a sufficient amount of lysosomes for accurate measurements. After centrifugation the tubes were frozen in acetone/solid CO_2 and the bottom tip of the tube containing the lysosomal pellet was cut off with a razor blade and transferred into a counting vial containing Bray's solution [20] for scintillation counting. The supernatant in the upper part of the tube representing the extralysosomal medium after centrifugation was used for both radioactivity measurements and enzyme determinations.

Ions were added as 2.0 M or 0.1 M stock solutions after suspension of lysosomes in 0.35 M sucrose containing the radioactive tracers. The distribution of $^{36}Cl^-$ in the lysosomal pellet obtained by filtering centrifugation was examined after adding Na^{36}Cl (10^6 dpm/ml, 1.31 μmol/ml and KCl 30 μmol/ml) and poly[^{14}C]-ethyleneglycol (10^6 dpm/ml) prior to centrifugation. In parallel assays the intra- and extralysosomal spaces were determined using poly[^{14}C]ethyleneglycol and ^3H_2O as described for the pH determination.

Determination of protein concentrations, enzymes and radioactivity. Protein was determined by the Lowry method [21]. Acid phosphatase was assayed according to Linhardt and Walter [22] using *p*-nitrophenylphosphate (Merck, Darmstadt) as substrate. Radioactivities were measured in a Tri-Carb scintillation counter (Packard

Model 3380) with automated quench correction using Bray's solution [20]. Lysosomal pellets were shaken vigorously to achieve perfect suspension in the scintillation solution.

RESULTS

After lysis of lysosomes by repeated freezing and thawing (five times), by brief sonication or by osmotic shock, no pellet was observed after filtering centrifugation. Total lysis was monitored by the release of acid phosphatase. Lysosomal ghosts produced by this method apparently do not pass the silicone oil layer. The determination of the intralysosomal pH by the distribution of [^{14}C]methylamine between the intra- and extralysosomal space requires precise measurements of the intralysosomal volume. As shown in Fig. 1, the lysosomal volume was found to be proportional to the lysosome concentrations present before the filtering centrifugation. Therefore, this method allows pH determinations without previous adjustment of the lysosomal concentration within the range described in Fig. 1. The pH of the medium, previously adjusted to pH 7.0 with Tris-acetic acid buffer, remained constant at the different lysosomal concentrations used in the assay (0.3–1.0 mg/0.1 ml). Furthermore it was found that variations of the lysosomal concentrations within this range had no influence on the results of the determination of the intralysosomal pH ($\pm$ 0.05 pH units).

To establish that methylamine does not significantly change the intralysosomal pH, the concentration of [^{14}C]methylamine (25 μM) was increased by the addition of unlabelled methylamine up to 225 μM. As shown in Fig. 2, an increase of the methylamine concentration from 25 μM (used in all subsequent experiments) to 45 μM had no demonstrable effect. A slight increase was observed only upon further increase to 225 μM. In addition, 3-fold dilution with unlabeled polyethyleneglycol did not alter the results of the pH calculations. These experiments as well as inability of lysosomal ghosts to pass the silicone layer exclude a significant adsorption of either compound to lysosomal membranes. It should be mentioned that Triton WR 1339 might alter the permeability properties (i.e. for methylamine) since the pH

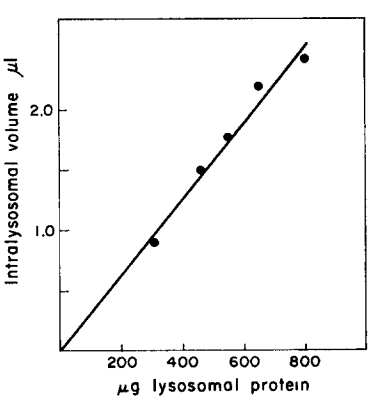

Fig. 1. Determination of the intralysosomal volume of Triton WR 1339-filled rat liver lysosomes. Incubation conditions and volume calculations are described under Methods.

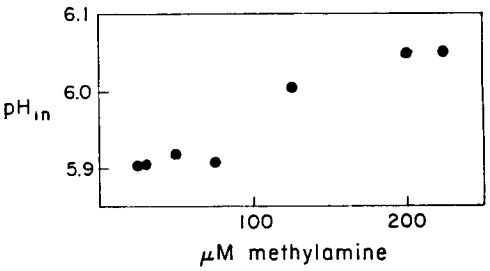

Fig. 2. Influence of increasing concentrations of methylamine on the intralysosomal pH. The intralysosomal pH was determined by measuring the distribution of [^{14}C]methylamine between the intra- and the extralysosomal space as described under Methods. Lysosomes were incubated under same conditions as in Fig. 1 with $^{3}H_2O$ (10^6 dpm/ml) and [^{14}C]methylamine (10^6 dpm/ml, 25 μM). The methylamine concentration was increased from 25 μM up to 225 μM by adding unlabelled methylamine hydrochloride. The volumes of the intra- and extralysosomal spaces were determined in parallel assays using identical conditions as described in Fig. 1, after addition of the same concentrations of unlabelled methylamine as used for the pH calculation.

values observed in normal rat liver lysosomes are considerably lower [6].

The pH gradient across the lysosomal membrane is strictly governed by the extralysosomal pH. This is demonstrated in Fig. 3. The pH gradient increases from 0.7 to 1.3 pH units when the extralysosomal pH is changed from 7.1 to 8.5. These data agree with the results obtained by Reijngoud and Tager [5] using the same type of lysosomes.

A decrease of the pH gradient across the lysosomal membrane induced by the addition of metal cations is well compatible with the presence of a Donnan potential. The influence of different cations on the Donnan equilibrium suggests mechanisms which restrict the mobilities of different cations. Fig. 4 demonstrates the influence of increasing concentrations of a variety of cations on the intralysosomal pH. The observed metal cation-proton exchange decreases with the atomic weight and with

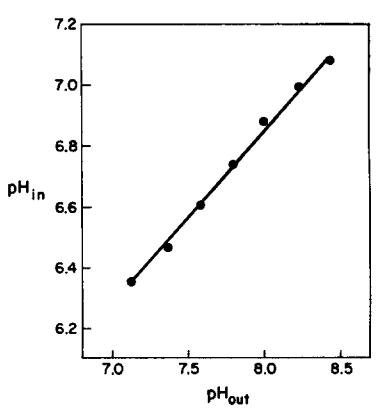

Fig. 3. Intralysosomal pH measured in dependence of the extralysosomal pH. Lysosomes were incubated under conditions described in Figs 1 and 2. The pH of the medium was adjusted with 0.01 M Tris-acetate buffer of the appropriate pH. The pH of the medium was checked before and after filtering centrifugation. In no case was a change of pH of more than 0.05 pH units observed.

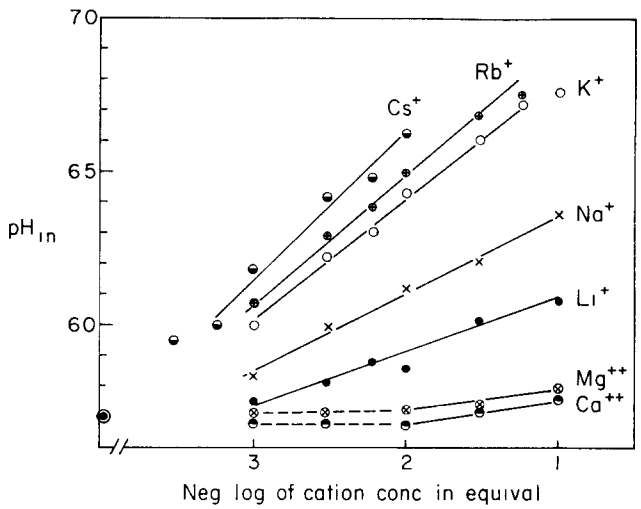

Fig. 4. Influence of different concentration of various cations added to the incubation medium. Various cations were added to lysosomal incubation mixtures as used in the preceding figures using 2.0 and 0.1 M stock solutions. All the salts contained Cl^- as the anion. The data shown are taken from one experiment using an incubation time of 45 min. The pH point at the origin of the abscissa (O) was measured without any addition of salts.

increasing charge of the cation. The rate of the cation-proton exchange across the lysosomal membrane follows the order: $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+ \gg Mg^{2+}$, Ca^{2+}. All the data shown in Fig. 4 were taken from one experiment which is representative of four experiments. In three other experiments using different times of incubation (between 30–90 min) the ions followed the same order of exchange. The measurements were not made under equilibrium conditions, however, in each experiment the incubation times were the same for all the samples.

To establish further that the data shown in Fig. 4 in fact result from different cation mobilities, the exchange of K^+ and Na^+ with intralysosomal protons was followed by measuring the intralysosomal pH at various times after the addition of 30 mM KCl or NaCl. To achieve this, the time of filtering centrifugation was shortened to 1 min. This results in lower yield of lysosomes, however, the pellet size is sufficient for accurate measurements. The results, shown in Fig. 5, demonstrate that intralysosomal protons are exchanged significantly faster with K^+ than with Na^+. It should be mentioned, however, that the results of pH determination vary significantly when different preparations are used. This can be seen comparing the results of Figs 4 at 45 min and 5 at 10 min after addition of 30 mM KCl.

The demonstrated permeability of the lysosomal membrane for cations does not exclude an independent flow of salts, anions as well as cations, due to an osmotically damaged membrane. A high osmotic pressure is produced within lysosomes during sucrose density gradient centrifugation which strongly decreases the osmotic stability of Triton WR 1339-filled lysosomes (Henning, R., unpublished observations). To exclude this possibility, the influx of the anion present in the previous experiments was determined by filtering centrifugation using $^{36}Cl^-$ and poly[^{14}C]-ethyleneglycol for the determination of the extralysosomal volume in the pellet. The

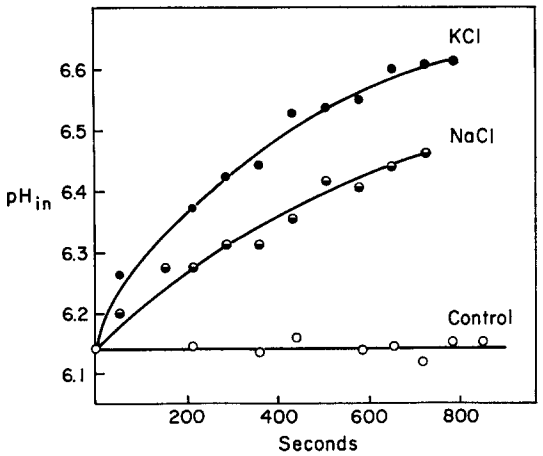

Fig. 5. Time dependence of the exchange of K^+ and Na^+ with intralysosomal protons. Lysosomes were incubated under conditions as described in Fig. 4. At time zero an aliquot was taken for the pH determination and then KCl or NaCl was added in a final concentration of 30 mM. The incubation medium was rapidly mixed. The filtering centrifugation time was 1 min. Controls were run without the addition of salt. ●–●, after addition of 30 mM KCl; ◒–◒, after addition of 30 mM NaCl; ○–○, controls.

$^{36}Cl^-$ distribution between the intra- and extralysosomal space was measured following the same time schedule used in Fig. 5 after addition of $^{36}Cl^-$ and 30 mM KCl. The results showed neither significant increase nor any change of the $^{36}Cl^-$ concentration in the intralysosomal space over a period of 15 min. The intralysosomal $^{36}Cl^-$ concentration remained constantly less than 5 % of the total $^{36}Cl^-$ concentration in the pellet while the intralysosomal volume contributed 60 % to the total pellet volume. (Total dpm $^{36}Cl^-$ in the pellets: 1000–1500 dpm.) These results exclude a significant influx of anions within 15 min after external addition of salt.

Several experiments were performed to detect an influence of ATP on the pH gradient because Mego and associates [23, 24] postulated an ATP-dependent proton transport mechanism or a proton-potassium exchange pump in the lysosomal membrane. Preincubation of lysosomal mixtures (10–30 min at room temperature or at 37 °C, addition of 1–10 mM ATP, 0.1–1.0 mM $MgCl_2$, 1–5 mM KCl) indicated that ATP had no influence on the pH gradient under these conditions.

DISCUSSION

Two mechanisms for the generation of a pH gradient across the lysosomal membrane have been discussed; an energy dependent proton transport mechanism or an energy-independent Donnan equilibrium produced by particular compositional and structural properties of the lysosomal membrane. As outlined in the introduction, the structure of the lysosomal membrane is compatible with the scheme of a Donnan equilibrium. The measurement of the distribution of the weak base methylamine appeared to be the most suitable method for the accurate determination of the pH within subcellular organelles with an internal acid pH [5, 6, 17, 18]. In the present study this procedure was modified to allow the processing of a large number of sam-

ples in one experiment by the use of the silicone oil filtering centrifugation procedure developed by Klingenberg and Pfaff [19]. The application of this method improved significantly the accuracy of the determination of the intra- and extralysosomal volumes as shown in Fig. 1 for a wide range of lysosome concentrations. It should be mentioned that a space permeable to sucrose has been found in normal lysosomes [1] which might influence the pH determination when sucrose is used as a marker for the extralysosomal volume. For this reason sucrose was replaced by polyethyleneglycol with a molecular weight being tenfold higher than that of sucrose.

Intralysosomal pH determinations after varying the pH of the medium from 7.1 to 8.5 show a practically linear dependence of the intralysosomal pH on the extralysosomal pH (Fig. 3). These data have been found to be independent of the times of incubation after changing the pH of the incubation buffer (30–120 min). This confirms the results of Reijngoud and Tager [5] who observed that a pH equilibrium is reached 2 min after changing the pH in the medium. This means that the proton moves freely through the lysosomal membrane under the applied experimental conditions.

The influence of various cations on the pH gradient as shown in Fig. 4 is compatible with the presence of a Donnan equilibrium as well as with the capacity of the lysosomal membrane to discriminate different types of cations. According to the data shown in Fig. 4, the concentration of most of the externally added cations must exceed 10^{-3} M in order to decrease the intralysosomal proton concentration by a metal cation-proton exchange. It agrees with observations [6] showing the influence of external K^+ on the pH in normal rat liver lysosomes. However, the external cation concentration necessary for a demonstrable change of the internal H^+ concentration must be some orders of magnitudes higher ($\approx 10^{-3}$ M) suggesting that the mobility of metal cations across the lysosomal membrane is considerably lower than that of protons. Therefore, an equilibrium of positive and negative charges inside and outside the lysosomal membrane favors the proton as a cation for the intralysosomal space.

The influence of the various cations on the pH gradient as monitored by lowering the proton concentration inside the lysosome shows remarkable differences. The order of the metal cation-proton exchange is: $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+ \gg Mg^{2+}, Ca^{2+}$. This corresponds to the mobilities of these cations in an aqueous environment which is determined by the degree of hydration being high around cations with a low atomic weight ($Li^+ : 4.01 \cdot 10^{-4}$ (cm/s)/(V/cm), $K^+ : 7.6 \cdot 10^{-4}$ (cm/s)/(V/cm)) [25, 26]. With respect to this rule, the proton reaching the highest mobility is an exception because it exists in the form of a hydronium ion and its charge is conducted via hydrogen bonds and not by the dislocation of the proton ($H^+ : 36.3 \cdot 10^{-4}$ (cm/s)/(V/cm)) [25, 26]. The comparative measurement of the exchange of K^+ or Na^+ with intralysosomal protons as a function of time as shown in Fig. 5 strongly supports these considerations. These data are compatible with a cation movement across the lysosomal membrane through hydrophilic regions which operationally might behave like pores. The observed differences of the mobilities seem to originate from their own physical properties rather than from chemical or structural features of the lysosomal membrane. It should be emphasized, however, that not all of the data shown in Fig. 4 are consistent with this hypothesis. The divalent cations exchange only very poorly with intralysosomal protons while the ionic mobil-

ity of divalent cations in an aqueous environment such as Ca^{2+} is even higher (Ca^{2+}: $6.16 \cdot 10^{-4}$ (cm/s)/(V/cm)) than that of Na^{+} ($5.19 \cdot 10^{-4}$ (cm/s)/(V/cm)) [25, 26]. One possibility is that the size of hydrated divalent cations is too large for such hydrophilic regions within the lysosomal membrane to allow an unrestricted mobility across this membrane.

All attempts to influence the pH gradient by incubations or preincubations with ATP and mono- or divalent cations had no effect. This might be in part due to the low temperature applied for the pH determinations. The presence of an energy-dependent proton pump in the lysosomal membrane has been reported recently by Mego and associates [23], who studied the effects of ATP on the function of isolated heterolysosomes as examined by the degradation of ^{121}I-labeled albumin at various pH. These authors also showed that the lysosomal protein degradation in mouse liver slices can be inhibited by blocking the energy metabolism [24]. The degradation of ^{125}I-labeled DNA, however, was not affected by these inhibitors. Because rat liver lysosomal DNAase also has an acid pH optimum [27] these data do not show conclusively the presence of a proton pump in the lysosomal membrane. Very recently, de Duve et al. [28] presented indirect evidence for a proton pump in the lysosomal membrane. It should be mentioned, however, that no direct evidence for an energy-dependent proton pump is available to date.

Additional mechanisms might be present to support the pH gradient generated by a Donnan equilibrium in vivo. The permanent cleavage of ester bonds within the functioning lysosome could serve as a means to produce protons. This intralysosomal proton-producing machinery could decrease the concentration of other cations and generate a much stronger pH gradient than that observed in vitro. Although no influence of ATP on the pH gradient could be observed in this study, this does not rule out that there exists a proton pump in the lysosomal membrane since all of the described experiments have been done in isolated lysosomes in the cold.

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