ATP-dependent Acidification of Intact and Disrupted Lysosomes

EVIDENCE FOR AN ATP-DRIVEN PROTON PUMP

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Intralysosomal acidification, necessary for hydrolytic activities that permit reutilization of the component parts of proteins and other macromolecules, was studied by measuring the uptake of [14C]methylamine in purified and disrupted rat liver lysosomes. When MgATP was added to intact lysosomes, acidification was stimulated by 1 pH unit within 20 min at 25 °C. The acidification process involved nucleotide specificity for ATP; GTP stimulated less than ATP, whereas the non-hydrolyzable analog, adenosine 5'-(βγ-imido)triphosphate, did not stimulate. Acidification of lysosomes was inhibited by the proton ionophore, carbonyl cyanide m-chlorophenylhydrazone. ATP had little effect on lysosomal membrane potential, and the potassium ionophore, valinomycin, which has effects on membrane potential, had little effect on ATP-dependent acidification of lysosomes. In addition, an inhibitor of anion transport, disothiocyanostilbene disulfonic acid, inhibited ATP-dependent acidification. Therefore, it is concluded that ATP-dependent acidification of lysosomes occurs electroneutrally.

ATP-dependent acidification and the membrane ATPase of lysosomes have several common characteristics. Both activities are stimulated by preincubation of lysosomes in salts that elevate intralysosomal pH; both are inhibited by dicyclohexylcarbodiimide; and both have pH optima slightly above pH 7. These characteristics further suggest that acidification and ATPase activities may be coordinated by the same lysosomal membrane protein.

Lysosomal preparations disrupted by freezing were shown to be capable of ATP-dependent acidification despite the loss of soluble lysosomal enzymes that act as Donnan effectors (93–100% loss). Furthermore, acidification occurred only when proteinase inhibitors were present during freezing. These results provide evidence for an ATP-driven pump that operates electroneutrally and enzymatically and results in acidification of lysosomes.

Liver lysosomes are known to be necessary for degradation of extracellular proteins such as asialooruloplasmin (1–9), lipoproteins (4, 5) and altered proteins (6). In addition, lysosomes are probably necessary for degradation of intracellular protein (7–11). Degradation is required for reutilization of component parts of proteins and other macromolecules and, when impaired, results in serious disorders such as lysosomal storage diseases (12). The proper functioning of lysosomes depends on an acid pH inside; drugs such as chloroquine (13) and inhibitors of energy metabolism (14) elevate intralysosomal pH and inhibit protein degradation (4, 15–17).

Although intralysosomal pH is thought to be about pH 4.5 in cells (14, 18, 19), lysosomes purified from livers of rats treated with Triton WR-1339 have an internal pH of about 6 (20–22). The latter pH has been attributed to a Donnan equilibrium dependent on polyanions inside lysosomes and a membrane selectively permeable to protons (22). The polyanions acting as Donnan effectors are presumably soluble lysosomal enzymes, most of which are glycoproteins with acidic isoelectric points. However, it is questionable whether such a Donnan equilibrium can maintain the proper intralysosomal pH under in vivo conditions: these involve (a) a high concentration of cytoplasmic potassium ion with a probable leak rate into lysosomes of 152 = 20 min (23), and (b) a considerable flow into lysosomes of protein buffered at neutral pH, probably 10% of plasma protein daily (24).

Evidence in support of ATP-dependent acidification of lysosomes has been reported. First, Mege and co-workers (25) isolated intact lysosomes containing radiolabeled albumin and showed that proteinolysis of the albumin was stimulated by addition of ATP when the lysosomes were incubated in medium at neutral pH and not when incubated at acid pH. The effect of ATP was diminished by the proton ionophore dinitrophenol (26). Second, we and others have measured intralysosomal pH and observed acidification after addition of ATP (27–30). In a preliminary report, the acidification activity was correlated with membrane ATPase activity in terms of effects of different salts, gramicidin, and uncoupler (29). Here, these studies are extended to show the effects of valinomycin, of different nucleotides, and of the ATPase inhibitors oligomycin, diane, and dicyclohexylcarbodiimide. Also, the time course of acidification is examined, and the effects of ATP on the membrane potential and the internal volume of lysosomes are considered. Furthermore, it is shown that disrupted lysosomes, in which the soluble enzymes that have been postulated to be Donnan effectors are no longer latent, are active in ATP-dependent acidification.

MATERIALS AND METHODS

Lysosomes

These were purified from rat liver using Triton WR-1339 treatment. Rats were injected intraperitoneally with Triton WR-1339 and 3½ days later liver lysosomes were isolated by differential centrifugation and flotation through 34.5% sucrose, w/v, as described (31), except that, by not fasting the rats the night before killing, a higher enrichment was obtained (32). Isolation of lysosomes from rats fed ad libitum by this Triton technique provides highly purified preparations that have no detectable catalase nor cytochrome oxidase activity and are, thus, essentially free of peroxosomal and mitochondrial contamination. Glucose-6-phosphatase measurements indicate less than 10% contamination by endoplasmic reticulum. Purity was assayed by measuring N-acetylglucosaminidase and protein (32) and found to be

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at least 60-fold enriched over homogenate. As isolated at the 14-34R were prepared free of the soluble hydrolases by washing with 0.2 M NaCl at pH 8 as described previously (33).

ATPase Assay

Lysosomes were mixed with 200 mM sucrose, 75 mM salt as indicated, 0.5 mM MgCl₂, 1.5 mM ATP, 0.5 mM EGTA, and 0.5 mg of bovine serum albumin/mL. The assay at 37°C was initiated by addition of lysosomes and terminated after 10 min by addition of trichloroacetic acid to 3% final concentration. After centrifugation to remove denatured protein, released phosphate was determined colorimetrically with ammonium molybdate-ascorbate (34, 35).

Intralysosomal pH Measurements

Microforce Assay—These measurements were carried out as described by others (20-22). Duplicate, 1-ml samples of lysosomes containing either [³⁵S]methylamine and [³¹P]water or [³⁵S]succrose and [³¹P]water were incubated at 25°C for the times indicated, usually 20 min. Then lysosomes were collected by centrifugation at 15,000 × g for 2 min in a Brinkmann Model 3200 microfuge. Supernatants were removed by using a Pasteur pipette drawn out to a fine tip, and the pellets were analyzed for radioactivity and protein. The amounts of radioactivity used were such that [³¹P] was about 10,000 cpm/μl and [³⁵S] was about 500 cpm. Methylamine was 7 μM throughout, i.e., unlabeled methylamine was added to samples containing [³⁵S]succrose. The internal volumes of lysosomes were found to be about 4 μl/mg of protein in the absence and presence of ATP, respectively.

Microforce Assays with methylamine, a 150 μl aliquot of each mixture was generated by addition of ATP (Fig. 1b). In each case, addition of external MgATP promoted acidification to about pH 5.6 inside. After preincubation, lysosomes in ATP were 1.15 pH units more acidic inside than lysosomes incubated in EDTA and 1.35 pH units more acidic inside than out. ATP-dependent acidification occurred on a time scale of minutes and was complete by 20 min. In these acidification experiments, EDTA was added to lysosomes that were incubated without ATP as a control. The rationale is that both ATP and EDTA have chelating properties and similar negative charge. Although EDTA did not promote acidification, it was desirable to investigate further the requirement for ATP.

Nucleotide Specificity—Acidification showed nucleotide

Membrane Potential

Potassium [³¹C]thiocyanate uptake by lysosomes was used to measure membrane potential. The technique is based on the high permeability of membranes to thiocyanate anion; net uptake values permit calculation of membrane potentials from the Nerst equation (40). Measurements were made by mixing lysosomes in the same buffered salt media used for methylamine uptake except that potassium thiocyanate was 7 μM (60 mM/mmol).

Valinomycin and C-CCP were from Calbiochem; DCCD was from Eastman; [³¹C]succrose, [³¹C]thiocyanate, and [³¹P]water were from New England Nuclear, and other chemicals were purchased from Sigma.

RESULTS

Intralysosomal Acidification

Measurement of Intralysosomal pH—The pH of lysosomes isolated in unbuffered sucrose media was measured using the technique of radioactive methylamine uptake and was found to be acid (Fig. 1a). Since the pH outside of the lysosomes was 7.0, the ΔpH across the lysosomal membrane was about 0.6. This ΔpH was diminished to about 0.1 pH unit when lysosomes were preincubated at 0°C for 1 h in 75 mM KCl (Fig. 1b). In each case, addition of external MgATP promoted acidification to about pH 5.6 inside. After preincubation, lysosomes in ATP were 1.15 pH units more acidic inside than lysosomes incubated in EDTA and 1.35 pH units more acidic inside than out. ATP-dependent acidification occurred on a time scale of minutes and was complete by 20 min. In these acidification experiments, EDTA was added to lysosomes that were incubated without ATP as a control. The rationale is that both ATP and EDTA have chelating properties and similar negative charge. Although EDTA did not promote acidification, it was desirable to investigate further the requirement for ATP.
specificity in that ATP was more effective than GTP (Table I). Under these conditions intralysosomal pH in the absence of nucleotide was 6.35; thus adenosine 5′-(β,γ-imido)triphosphate, a nonhydrolyzable analog of ATP, resulted in no acidification. On the other hand, GTP did support significant acidification inasmuch as a ΔpH of 0.25 corresponds to a 1.8 × proton gradient. In view of the nucleotide specificity, it appeared that ATP may promote acidification by driving ion movements across the lysosomal membrane. Accordingly, the effects of various salts on intralysosomal pH were studied.

**Salt Effects**—Preincubation of lysosomes in various salts and subsequent measurement of internal pH showed that all were nearly equally effective at raising the intralysosomal pH and subsequent measurement of internal pH showed that all I. Under these conditions intralysosomal pH in the absence was inhibited nearly completely by ATP (Table I). Since 1 μM diisothiocyanostilbene disulfonic acid is known to inhibit anion transport in erythrocytes (41), ATP-dependent movement of protons and anions appears possible. Inasmuch as paired movement of protons and anions would be electroneutral, whereas proton movement alone would be electrogenic, the effect of ATP on lysosomal membrane potential was investigated.

**Table I**

**Nucleotide specificity for acidification of lysosomes and for phosphate release by lysosomal membranes**

| Nucleotide | Intralysosomal pH | Nucleotide-dependent ΔpH | Phosphate release (μmol/min/mg protein) |
|------------|-------------------|--------------------------|----------------------------------------|
| None       | 6.35              | 0.00                     | 0.03                                   |
| AMP-PNPa   | 6.35              | 0.00                     | 0.03                                   |
| ADP        | 6.30              | 0.05                     | 0.79                                   |
| GTP        | 6.10              | 0.25                     | 1.36                                   |
| ATP        | 5.65              | 0.70                     | 1.27                                   |

a AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate.

**Table II**

**Effects of salts and ATP on intralysosomal pH**

Freshly prepared lysosomes were incubated for 1 h at 4°C either as isolated in about 0.75 M sucrose or diluted 20% to give 30 mM Tris- HCl, pH 8, 7.5 mM salt as indicated, and about 0.6 M sucrose. "None" denotes lysosomes that were not preincubated in buffered salt and were incubated, as described in the legend to Fig. 1a, without KCl. ATPase activity was measured colorimetrically with ammonium molybdate-ascorbate as described under "Materials and Methods." ATP-dependent ΔpH is defined as the intralysosomal pH value obtained for lysosomes in EDTA minus that value obtained for the same lysosomes in ATP.

| Salt     | Intralysosomal pH | ATP-dependent ΔpH | ATPase activity (μmol/min/mg) |
|----------|-------------------|-------------------|-------------------------------|
|          | - ATP  | + ATP  | - ATP  | + ATP  | - ATP  | + ATP  |
| None     | 5.85   | 5.65   | 0.20   | 0.15   |        |        |
| KCl      | 6.75   | 6.55   | 1.10   | 0.30   |        |        |
| NaCl     | 6.65   | 5.90   | 0.75   | 0.29   |        |        |
| K2SO4    | 6.85   | 5.85   | 0.90   | 0.29   |        |        |
| LiCl     | 6.85   | 6.05   | 0.80   | 0.31   |        |        |

**Table III**

**Effect of diisothiocyanostilbene disulfonic acid on ATP-dependent acidification and ATPase activity of lysosomes**

Diisothiocyanostilbene disulfonic acid (DIDS) was prepared in dimethyl sulfoxide at a concentration of 50 μM and was added to reaction mixtures containing lysosomes in sucrose and potassium chloride as in Fig. 1a. After incubation at 25°C for 20 min, the lysosomes were isolated and ATP-dependent ΔpH was determined as in Table II. ATPase activity was measured colorimetrically with ammonium molybdate-ascorbate as described under "Materials and Methods."

| DIDS  | ATP-dependent ΔpH | ATPase activity (μmol/min/mg) |
|-------|-------------------|-------------------------------|
| 0 μM  | 0.70              | 0.35                          |
| 1 μM  | 0.65              | 0.31                          |
| 50 μM | 0.10              | 0.30                          |

**Effect of ATP on Membrane Potential**—Using [3H]thiocyanate distribution in lysosomes as a measure of membrane potential, ATP-dependent changes in membrane potential were found to be small (Fig. 2a) and can presumably be attributed to magnesium ion, because addition of MgEDTA gave similar changes. It is worth noting that the lack of a membrane potential is not due to membrane leakiness. When lysosomes at 25°C were suspended in medium containing 150 mM KCl and 1 μg/ml of valinomycin, a potassium ion-specific ionophore, the distribution of [3H]thiocyanate indicated a membrane potential of +40 mV. A value of +40 mV is predicted by the diffusion potential if one assumes that the intralysosomal potassium concentration is 33 mM. In the absence or presence of ATP, the membrane potential of +40 mV was not diminished after 30 min of incubation at 25°C. Thus, the lysosomal membrane is not freely permeable to ions, and the absence of a membrane potential during ATP-dependent acidification of lysosomes is, then, indicative of an electroneutral mechanism. If ATP-dependent acidification of lysosomes is electroneutral, then it should not be inhibited by the membrane potential imposed by a potassium chloride gradient and valinomycin. On the other hand, if the addition of ATP to lysosomes were to cause a membrane permeability change that would allow a Donnan equilibrium to establish itself, then a potassium chloride gradient and valinomycin should be inhibitory. These possibilities were considered next.

**Effects of Ionophores and Inhibitors on ATP-dependent Acidification**—Table IV shows that ATP-dependent acidification was not inhibited by valinomycin plus potassium ion. In addition, the proton ionophore, C-CCP did inhibit the ATP effect which shows that ATP leads to formation of a net proton gradient. If ATP promotes acidification of lysosomes by driving ion pump(s), then acidification might be sensitive to inhibition by specific compounds. ATP-dependent acidification of lysosomes was inhibited by 10 μM dicyclohexylcarbodiimide and 50 μM diamide; oligomycin (0.5 μg/ml) did not inhibit acidification. The relatively small amounts of the inhibitors employed, 10 nmol of dicyclohexylcarbodiimide and 50 nmol of diamide, would not be expected to react with all of the soluble lysosomal enzymes that are possible Donnan effectors but rather with specific proteins. Therefore, the presence of a lysosomal protein that requires ATP to drive catalytically proton translocation across the lysosomal membrane seems more probable. An ATP-driven proton pump would be expected to have a pH optimum, a point at which the ΔpH across the lysosomal membrane is maximal; in contrast, a Donnan equilibrium should maintain a constant ΔpH as the outside pH is varied.

**Effect of External pH on ATP-dependent Acidification**—Intact lysosomes were incubated in the absence and presence of external MgATP at various pH values, and intralysosomal...
Lysosomal membranes are known to have ATPase activity (35). Table I compares the phosphohydrolase activity of membranes acting on various nucleotides with the ability of these nucleotides to promote acidification of intact lysosomes. ADP is hydrolyzed even though it does not promote acidification; this activity corresponds to the previously reported nucleotide diphosphatase activity (49) and has been recently reported to co-sediment with ATPase activity when Triton X-100-solubilized lysosomal membranes are centrifuged into sucrose gradients (50). Although GTP was a better substrate than ATP at 1.5 mM, previous results reveal that ATP is the preferred substrate at 0.2 mM and below (35). ATP, above 2 mM, has an inhibitory effect on ATPase activity which is probably related to the fact that 1.5 mM GTP is a superior substrate. The observations that neither ADP and GTP promote acidification as well as ATP may indicate that ATP is the normal substrate for the ATPase, but further comment is required. It is possible that both ADP and GTP uncouple phosphohydrolase activity from proton pump activity. Alternatively, it is possible that there are multiple nucleoside phosphatases in the lysosomal membrane and that ADP and GTP phosphohydrolases are different activities than the ATPase.

The ATPase activity of intact lysosomes was stimulated by preincubation in buffered salt solution in parallel with ATP-dependent acidification (Table II). The increase in activity may be due to (a) an increase in intralysosomal pH, (b) a dependency on ions, or (c) lysis of the lysosomal membrane. An increase in intralysosomal pH is known to occur (Fig. 1). Preincubation with salt led to an increase in intralysosomal pH when the lysosomes were buffered at pH 8 (Table II) or at pH 7 (Fig. 2). There was an apparent lack of ion specificity (Table II), and the latency of N-acetylglucosaminidase was only slightly affected by preincubation (27). By the process of elimination, the major factor determining the increase in ATPase activity may be the increase in intralysosomal pH.

The inhibitors of ATP-dependent acidification, diisothiocyanostilbene disulfonic acid, dicyclohexylcarbodiimide, and diamide were also found to inhibit lysosomal membrane ATPase activity and thus suggest a link between ATPase and acidification activities. Dicyclohexylcarbodiimide completely inhibited ATP-dependent acidification, while it inhibited ATPase activity 60%. However, diisothiocyanostilbene disulfonic acid inhibited ATP-dependent acidification with little effect on ATPase activity (Table III). One possibility is that proton pump activity is more sensitive to inhibitors and inactivation than ATPase activity. An illustration of the apparent lability of the pump activity is the observation that it usually cannot be measured in disrupted lysosome preparations even though the ATPase activity is unaffected.

**ATP-dependent Acidification of Disrupted Lysosomal Preparations**

Lysosomal preparations were disrupted by freezing and storing overnight at −20 °C. Disruption was complete as evidenced by a lack of latent N-acetylglucosaminidase activity. Freshly prepared lysosomes were 80-90% latent; frozen-stored ones were, 0 to at most 7%, latent. The membranes of disrupted lysosomes apparently resell spontaneously because an amount of [14C]sucrose that corresponds to less than 1 μl/mg of protein was included in the membranes after gel filtration.
tion. The lack of latency indicates release of most of the soluble enzymes from the lysosomes. Since the soluble enzymes are postulated to be the polyanions that create a Donnan effect, the disrupted preparations should not be capable of acidification if the only mechanism of acidification is Donnan equilibrium.

To study acidification of disrupted lysosome preparations, it was necessary to use rapid gel filtration for separation of lysosomal vesicles from their external medium because centrifugation at 15,000 × g for 2 min did not result in complete pelleting. Table VI shows results obtained with intact lysosomes analyzed by the two methods. ATP-dependent acidification was observed in both cases; the apparent ΔpH across the lysosomal membrane was 0.65 and 0.70 pH units by the centrifugation and rapid gel filtration method, respectively. This good agreement between the two methods supports the validity of the rapid filtration technique.

When disrupted lysosome preparations were incubated with [14C]methylamine in the absence of ATP, considerably less methylamine uptake was seen than with intact lysosomes (Table VII). Less uptake is to be expected because the internal volumes of membrane vesicles are nearly 10 times smaller than those of intact lysosomes. However, ATP-dependent acidification of the vesicles occurred provided that the preparations were stored with proteinase inhibitor (Table VII). Furthermore, the magnitudes of the proton gradients in the disrupted lysosomal preparations were equal to those of intact preparations. Acidification occurred in disrupted preparations despite the possibility of increased leakage to protons and the possibility of outside-in, as well as outside-out, orientation of the presumed proton pump. An absolute requirement for proteinase inhibitor was observed. Aprotinin (shown), soybean trypsin inhibitor, and β-aminobenzamide were all effective; phenylmethylsulfonyl fluoride and N-ethylmaleimide were not (not shown). It is interesting to note that disrupted lysosomes in the L-fraction preparation displayed a greater capacity for methylamine uptake than those in the Triton WR-1339 preparation. First, this greater uptake may indicate that Triton can cause membrane damage and leakiness in protons. Second, and perhaps more significant, the contaminants in the L-fraction preparation, mitochondria, peroxisomes, and endoplasmic reticulum, may act as substrates for the proteinases in the preparation and thereby spare the lysosomal membrane proteins. These data are the first demonstration of acidification of disrupted lysosomes and may have significant implications for the relative roles of membrane pumps and Donnan effectors, as will be discussed below.

**DISCUSSION**

The results clearly show an ATP-dependent lowering of the intralysosomal pH by 1 pH unit (Fig. 1b) and thus substantiate preliminary communications (27–30). Furthermore, the kinetics of this *in vitro* acidification are in good agreement with those observed in leukocyte lysosomes after phagocytosis of dye-impregnated yeasts (18, 19). It is important to point out that methylamine uptake by lysosomes is a measure of their overall proton gradient and reflects both the rate of proton pumping and leaking. The pH lowering to about 5.5 is not as great as one would expect on the basis of measurements in living cells which indicate an intralysosomal pH of 4.5 (14, 18). This probably can be attributed to leakiness because Triton WR-1339 treatment, which was used in these experiments, is known to result in an increased permeability of lysosomal membranes to protons (42). ATP-dependent acidification of L-fraction lysosomes was greater than that of Triton lysosomes (Table VII); in fact, since L-fraction preparations are only about 20% pure, the actual lysosomal ΔpH might be significantly greater and correspond to an intralysosomal pH of 4.5.

ATP-dependent acidification suggests the presence of a proton pump in the lysosomal membrane which may be related to its known ATPase activity (35). Consistent with this suggestion are the similar and coordinate effects of salt on both ATPase and acidification activities (cf. Table II). The possibility that these activities are due to the same protein is further suggested from experiments in which DCCD completely inhibited ATP-dependent acidification while it inhibited ATPase activity 60%. In mitochondria, proton pump activity is also more sensitive to inhibition by DCCD than is ATPase activity: 1 nmol of DCCD/mg of submitochondrial particle protein completely inhibited energy-linked (pump) activity (51) but inhibited ATPase activity about 25% (52). The purported ATP-driven proton pump in lysosomes appears to operate electroneutrally, perhaps in conjunction with anion transport, because acidification is inhibited by the inhibitor of anion transport, disothiocyanostilbene disulfonic acid (Table III). Also, measurement of [14C]thiocyanate uptake (Fig. 2) shows that ATP does not significantly affect the membrane potential of lysosomes during the course of acidification. An electroneutral proton pump could be either due to a proton-cation exchange process or due to a proton-anion cotransport process. Although it is not yet possible to say with certainty which mechanism is operating, proton-anion cotransport would agree well with inhibition by disothiocyanostilbene disulfonic acid. It is uncertain whether the lysosomal membrane contains only one nucleoside triphosphatase (50) or multiple nucleoside triphosphatases, only one of which is a

**TABLE VI**

A comparison of methylamine uptake by intact lysosomes as measured by centrifugation and rapid gel filtration

Fresh lysosomes were prepared and incubated as under "Materials and Methods." After 30 min, the lysosomes were isolated by pelleting. Table VI shows results obtained with intact lysosomes analyzed by the two methods. ATP-dependent acidification was observed in both cases; the apparent ΔpH across the lysosomal membrane was 0.65 and 0.70 pH units by the centrifugation and rapid gel filtration method, respectively. This good agreement between the two methods supports the validity of the rapid filtration technique.

| Method            | Microfuge | Rapid gel filtration |
|-------------------|-----------|----------------------|
| Methylamine uptake| ~ ATP     | + ATP                | ATP-dependent ΔpH |
| 3,340             | 14,590    | 0.65                 |
| 16,300            | 85,900    | 0.70                 |

**TABLE VII**

ATP-dependent acidification of lysosome preparations measured by rapid gel filtration

L-fraction was prepared from normal rat liver without use of Triton WR-1339 by differential centrifugation as described (48) and was 15-fold enriched in N-acetyl-β-D-glucosaminidase relative to homogenate. Lysosomes containing Triton WR-1339 were isolated as described under "Materials and Methods." Preparations were frozen and stored either in sucrose as isolated or with Tris, free base, and aprotinin added to give 2 mM and 1.7 μM, respectively.

| Preparation        | Methylamine Uptake | ATP-dependent ΔpH |
|-------------------|-------------------|-------------------|
|                   | ~ ATP             | + ATP             |                   |
| 1. L-fraction      |                   |                   |
| a. Fresh           | 4,090             | 13,740            | 0.53              |
| b. Disrupted       | 430               | 1,810             | 0.62              |
| c. Disrupted without proteinase inhibitor | 260 | 306 | 0.07 |
| 2. Lysosomes (Triton WR-1339) |                   |                   |
| a. Fresh           | 12,200            | 31,000            | 0.40              |
| b. Disrupted       | 1,400             | 3,770             | 0.43              |
| c. Disrupted without proteinase inhibitor | 1,290 | 1,060 | 0.43 |
proton pump. Multiple phosphatases are certainly present in the lysosomal membrane because 5'-nucleotidase activity (43) and ATPase activity (30) are both known to be present. Purification of the ATPase(s) would provide useful information in this regard and is being pursued.

Inhibition of intralysosomal acidification by ATP in the presence of valinomycin plus potassium chloride would be expected if membrane potential were of critical importance in regulating intralysosomal pH; the slight stimulation observed in these experiments with resealed lysosomal membranes indicates that at least in this system, transmembrane pH is important in regulating ATPase activity (30) and is maximal when the external pH is slightly alkaline (Table V). The inhibition of acidification by C-CCP (Table IV) shows that ATP generates a proton gradient and is notable: the effect of C-CCP is not rather than inhibit, a Donnan equilibrium. Also, it is interesting to note that C-CCP stimulates ATPase activity (29).

It is necessary to point out that the determination of intralysosomal pH is dependent on measuring both internal volume and methylamine uptake. Although this represents a potential source of error, the effects of ATP on the internal volumes of lysosomes are interestingly slight, lysosomes suspended in sucrose alone being an exception. It seems quite possible that one difference between the results presented here and those reported by Hollemans and co-workers (44) is the determination of internal volume. Although [%]sucrose and [%]H2O were used in both cases, the absolute sucrose concentrations were very different, as these results were obtained with at least 200 mM sucrose throughout and Hollemans and co-workers (44) used submicromolar concentrations. Their results are possibly compromised by absorptive binding of sucrose. We have observed that lysosomes in 0.16 µm sucrose do bind sucrose and that the binding is diminished by addition of ATP. Another reason for the discrepancy between the results may be the quality of the lysosome preparation.

At present, one suspects that the major difference between the ATP-dependent acidification reported here and the lack of acidification observed by others (44) is due to proteolytic damage that occurred when their preparations were centrifuged to form a pellet, as a means of concentrating the lysosomes after flotation gradient centrifugation. ATP stimulation of methylamine uptake by lysosomes does decrease as the isolated lysosomes are stored on ice. Our measurements of acidification by intact lysosomes were made within 4 h after isolation. In addition, the lysosomes were used as isolated in hyperosmotic sucrose (14.3 to 34.5% sucrose interface, about 0.75 m) because centrifugation to form a pellet of concentrated lysosomes and resuspension in isosmotic medium significantly affects membrane integrity as indicated by changes in the latency of N-acetylglucosaminidase. Lysosomes were disrupted by freezing and storing overnight at -20 °C were active in ATP-dependent acidification, but only if proteinase inhibitors were employed (Table VII). Inasmuch as acidification of disrupted preparations was as great as that of intact preparations, disruption by freeze-thawing apparently causes neither greatly increased leakiness to protons nor a significant number of outside-in vesicles. ATP-dependent acidification of disrupted lysosomes is significant; if the Donnann effectors were released, then acidification must be due to ATP-driven ion movement across the lysosomal membrane. Presumably the Donnan effectors are the soluble lysosomal enzymes, and enzyme latency should be an accurate measure of their release. Disrupted preparations showed 100–93% loss of latency, which is indicative of nearly complete release. Experiments on acidification of lysosome ghosts separated from the soluble enzymes are in progress and will provide additional information on the role of ATP-driven ion movements that result in intralysosomal acidification.

The evidence reported here for the possibility of an ATP-driven proton pump in lysosomes agrees with a number of observations. Lysosomal proteases are known to require an acid pH for activity (45), and external ATP stimulates the degradation of intralysosomal albumin in vitro (25). Protein degradation is energy-dependent (15–17), and inhibitors of energy metabolism raise the intralysosomal pH of living cells (14). Permeant weak bases like the antimalarial drug chloroquine are accumulated in lysosomes of cells to such an extent that active processes seem likely to be involved (13, 36). Also, the acidification properties of lysosomes are similar to those of chromaffin granules in which a proton pump is generally accepted (37, 39, 40, 46). Acidification of the chromaffin granules requires about 20 min, which is in good agreement with the data reported here for lysosomes. However, the systems differ in that the chromaffin granule proton pump appears to be electronegenic (47), whereas the lysosomal proton pump appears to be electroneutral.

Although future experiments are required to evaluate more quantitatively the relative roles of ATP-driven proton pump and Donnann effectors in acidification of lysosomes, at present it seems very reasonable to conclude from these experiments on intact and disrupted lysosomes that a proton pump mechanism may be operable.

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