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Identification and characterization of a proton pump on lysosomes by fluorescein isothiocyanate-dextran fluorescence

(H$^+$-ATPase/sulfhydryl enzyme/electrogenic pump/vanadate insensitivity/cupric ion sensitivity)

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ABSTRACT **Fluorescein isothiocyanate-conjugated dextran was introduced preferentially into hepatic lysosomes by intraperitoneal injection into rats. The pH in isolated lysosomes, measured by fluorescein fluorescence, was ≈5 and gradually increased in KCl (to 7.0) at 25°C. In the presence of Mg^{2+}, ATP caused acidification of lysosomes that was reversed by the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Mn^{2+}, Co^{2+}, and Fe^{2+} could replace Mg^{2+} but Ca^{2+} could not. Cu^{2+}, Zn^{2+}, and Cd^{2+} were inhibitory. A membrane-permeant anion, in practice chloride, was required for this acidification. ATP analogues, including 5′-adenylyl imidodiphosphate, could not be substituted for ATP. ATP-driven acidification was sensitive to *N*-ethylmaleimide and quercetin but insensitive to oligomycin, ouabain, and vanadate. There were some differences between "normal" lysosomes and tritosomes; the acidification was resistant to azide and *N,N′*-dicyclohexylcarbodiimide in normal lysosomes but sensitive to these reagents in tritosomes. These results provide evidence for the presence of an electrogenic proton pump driven by MgATP (H$^+$-ATPase) on lysosomes.**

Lysosomes are acidic inside (1–3). The mechanism whereby this low pH is maintained is unknown, but two different mechanisms have been proposed. (*i*) A Donnan-type equilibrium or a modification of this equilibrium has been proposed from studies of the permeability properties of the lysosomal membrane (4–6). (*ii*) The existence of an ATP-driven proton pump on lysosomes has been proposed from the finding that proteolysis is stimulated by addition of ATP to intact lysosomes *in vitro* (7). The latter hypothesis is supported by observations and theoretical considerations of massive uptake of basic substances by lysosomes in living cells (8). Schneider found relatively high ATPase activity on lysosomal membranes (9) and observed that MgATP stimulates the uptake of methyl amine by tritosomes 1.5- to 2-fold (10). In the mitochondria/lysosomes fraction, similar enhancement has been reported for the uptake of weakly basic dyes (11) and amino acid methyl esters (12). Recently, Schneider found evidence for the presence of a MgATP-driven proton pump on lysosomes by using the methyl amine uptake method and suggested that this pump may be electroneutral (13).

We measured the intralysosomal pH of living macrophages by determining the fluorescence spectrum of fluorescein isothiocyanate-conjugated dextran (FITC-dex) that had been introduced preferentially into lysosomes and found a value of pH 4.7–4.8, which was 2 to 3 pH units lower than that of the medium and provided evidence for the presence of an active proton accumulation mechanism(s) in lysosomes (2).

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In this work, we obtained direct evidence for the presence of a MgATP-driven proton pump (H$^+$-ATPase) on lysosomes and also evidence that this pump is electrogenic in nature.

MATERIALS AND METHODS

Materials. Male Wistar rats (150–250 g) were purchased from Sankyo (Tokyo). FITC-dex (average M_r, 67,000; 0.007 mol of fluorescein per mol of glucose residue), creatine kinase, ATP, and other nucleotide derivatives were obtained from Sigma. Triton WR-1339 was obtained from Ruger (Irvington, NJ). Nigericin was a gift from Lilly. All other chemicals were of the highest grade available commercially. They were mainly obtained from Sigma and were used without further purification.

Preparation of Lysosomes Containing FITC-dex. FITC-dex was introduced into rats intraperitoneally and the livers were fractionated by the procedure of de Duve *et al.* (14). FITC-dex was found to be localized predominantly in lysosomes. Two types of lysosomes were prepared. (*i*) As "normal" (unmodified) lysosomes, a lysosome-rich fraction was prepared by the method of de Duve *et al.* (14) with modifications to increase the speed of the procedure. Rats were injected intraperitoneally with FITC-dex (20 mg of FITC-dex/150 g of body weight), starved for 12 hr, and then decapitated. The liver homogenate was made in 3 vol of sucrose buffer (0.25 M sucrose/1 mM EDTA/0.1% ethanol) by a single up-and-down stroke of a Teflon pestle and centrifuged in the cold for 10,000 *g* min. The resultant supernatant was made up to 10 times the original volume with sucrose buffer and centrifuged for 33,000 *g* min and then for 250,000 *g* min. The precipitate was washed twice with a volume of sucrose buffer equivalent to half the volume of the supernatant. The final precipitate (the lysosome-rich fraction) was resuspended in sucrose buffer. (*ii*) Tritosomes were obtained by using the procedure of Trouet (15) as described by Leighton *et al.* (16). For introduction of FITC-dex into tritosomes, rats were injected first with Triton WR-1339 (85 mg/100 g of body weight) and then, 3 days later, with FITC-dex (20 mg/100 g of body weight), starved for 24 hr, and killed. The extent of purification of lysosomal enzymes was as reported by Leighton *et al.* (16).

Fluorescence pH Measurement. Unless otherwise indicated, the lysosome-rich fraction or the tritosome fraction was added to 2 ml of incubation mixture and the fluorescence was measured at 25°C in a Hitachi 650-10S fluorescence spectrophotometer with an ACC mode. The pH in lysosomes was determined either from the fluorescence spectrum [fluorescence measured with excitation at 495 nm/fluorescence measured

Abbreviations: FITC-dex, fluorescein isothiocyanate-conjugated dextran; DCCD, *N,N′*-dicyclohexylcarbodiimide: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta F/F$, relative fluorescence intensity.

with excitation at 450 nm (2)] or from the fluorescence intensity at 495 nm relative to that after addition of Triton X-100 to 0.02%, using the calibration curve shown in Fig. 1. In both cases, corrections were made for the fluorescence of FITC-dex released from lysosomes; extralysosomal FITC-dex was measured either by the filtration method (0.45-μm Millipore filter) or by determining the instantaneous decrease in fluorescence intensity after addition of acidic buffer to bring the pH of the incubation medium to 6. Emission was measured either at 519 nm (tritosome fraction) or at 550 nm (lysosome-rich fraction) to minimize the effect of light scattering. Additions were made in water or ethanol in a volume of <1% of that of the incubation medium. ATP solutions were adjusted to pH 7.0 before addition. No fluorescence spectrum skew was observed during the experiments. The lysosome fraction was used within 6 hr of preparation and was stored at 0°C in sucrose before use.

RESULTS

Internal pH and Permeability Properties of Isolated Lysosomes. Lysosomal pH measured by FITC-dex fluorescence was ≈5 in sucrose buffer. When the lysosome-rich fraction was suspended in 100 mM KCl/10 mM $MgCl_2$/20 mM Hepes/KOH, pH 7.0, at 25°C, the fluorescence intensity increased gradually with time (Fig. 2). The release of FITC-dex from disrupted lysosomes was too small to explain this increase, suggesting that it was due to gradual alkalinization of lysosomes. The calculated increase in pH was 0.1–0.2 pH unit in 4 min. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or valinomycin added alone increased the fluorescence intensity slightly, indicating very limited permeability of lysosomal membrane to protons and potassium (Fig. 2, curves a and b). When added together, FCCP and valinomycin caused almost instantaneous increase in fluorescence intensity, as did nigericin (Fig. 2, curve c). Release of FITC-dex from lysosomes treated with these reagents was also small, suggesting that rapid alkalinization of lysosomes was induced through exchange of internal protons for

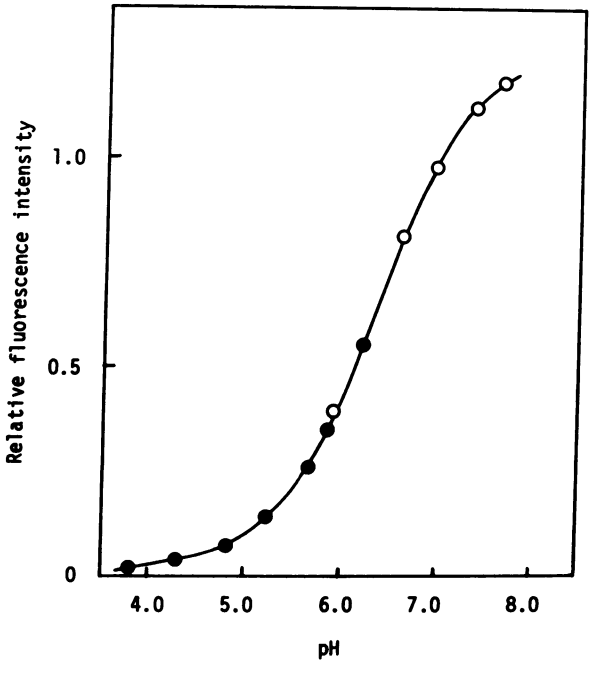

FIG. 1. Relative fluorescence intensity of FITC-dex at various pH values. Intensity at pH 7.0 is taken as unity. Excitation was at 495 nm and emission was measured at 550 nm with 5-nm slits on both monochromators. FITC-dex (1 μg/ml) was in 50 mM NaCl/10 mM buffer (●, NaOAc; ○, Na phosphate).

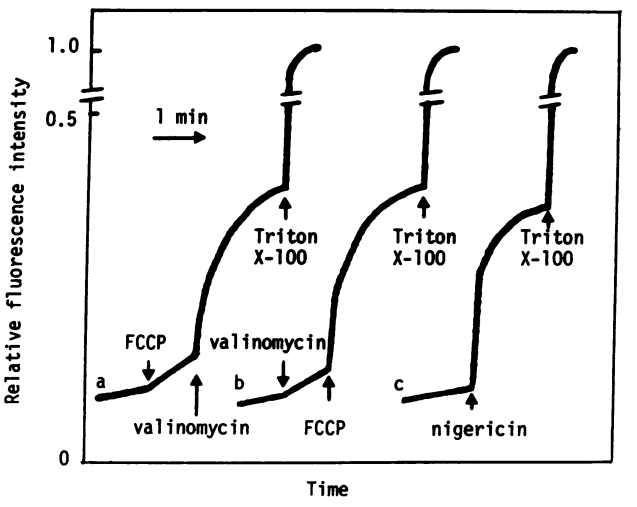

FIG. 2. Effects of ionophores on FITC-dex fluorescence in isolated lysosomes. Lysosome-rich fractions containing FITC-dex were added to 2 ml of incubation mixture (100 mM KCl/10 mM $MgCl_2$/20 mM Hepes/KOH, pH 7.0) at 25°C. Fluorescence intensity was normalized by taking that after addition of Triton X-100 as unity. Additions: FCCP, 2.5 μM; valinomycin, 2.5 μM; nigericin, 2.5 μM; Triton X-100, 0.02%. Addition of equal volumes of ethanol had no effect on the fluorescence. Lysosome samples contained 34 μg of protein.

external potassium ions. Addition of Triton X-100 disrupted the lysosomes and exposed FITC-dex to the incubation medium (pH 7.0), which gave us the total amount of FITC-dex in the system. Tritosome fractions gave essentially the same results.

ATP-Driven Acidification of Lysosomes. When ATP was added to this incubation mixture after a few minutes of incubation, the fluorescence intensity decreased rapidly to nearly the original level, indicating acidification of lysosomes (Fig. 3*A*, curve a). The calculated decrease in pH was 0.3–0.5 unit. When the protonophore FCCP was added to the incubation mixture, the fluorescence intensity increased rapidly to almost the control value. Valinomycin had little effect. This ATP-driven acidification of lysosomes was insensitive to oligomycin (Fig. 3*A*, curve b). Atractyloside, a specific inhibitor of the mitochondrial ADP/ATP transport system, had no effect at 25 μM. The

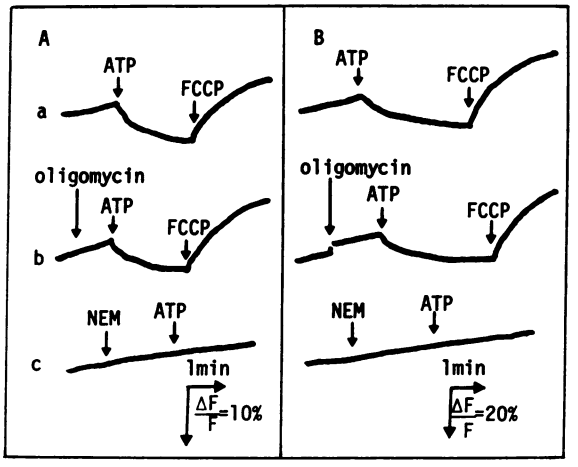

FIG. 3. ATP-dependent quenching of FITC-dex fluorescence. Procedures were as for Fig. 2, except that the concentration of KCl was 100 mM for the lysosome-rich fraction (*A*) and 150 mM for the tritosome fraction (*B*). Additions: ATP, 1 mM; FCCP, 1 μM; oligomycin, 5 μM; *N*-ethylmaleimide (NEM), 1 mM. Fluorescence intensities are shown as percentages of that just before addition of ATP/($\Delta F/F$). Lysosome samples contained 60 μg of protein (*A*) or 10 μg of protein (*B*).

sulfhydryl reagents *N*-ethylmaleimide (Fig. 3*A*, curve c) and *p*-chloromercuribenzenesulfonic acid (10 μM; data not shown) inhibited the ATP-driven acidification, indicating that this acidification was due to a sulfhydryl enzyme(s). Exactly the same results were obtained with FITC-dex-containing tritosomes (Fig. 3*B*). Furthermore, this ATP-driven acidification of lysosomes showed saturation kinetics with an apparent K_m value for ATP of 0.1–0.2 mM. These results indicate the presence of an ATP-driven proton pump on lysosomes.

Characterization of the ATP-Driven Acidification of Lysosomes. In the following section, only results obtained with the lysosome-rich fraction are presented to show the nature of the proton pump of normal lysosomes. But, unless otherwise indicated, essentially the same results were obtained using the tritosome fraction.

The effects of divalent cations other than Mg^{2+} on the ATP-driven acidification of lysosomes are shown in Fig. 4. Mg^{2+} could be replaced by Mn^{2+}, Co^{2+}, or Fe^{2+} but not by Ca^{2+}, Sr^{2+}, or Ba^{2+}. Mg^{2+} could also be partially replaced by Ni^{2+}, but Ni^{2+} was inhibitory to Mg^{2+}. On the other hand, Zn^{2+} and Cu^{2+} inhibited the Mg^{2+}/ATP-driven acidification of lysosomes. Cd^{2+} was also inhibitory.

The effects of various electrolytes on MgATP-driven acidification are shown in Fig. 5. When sodium or choline was added in place of potassium, the rate of acidification was almost the same, indicating that there was little requirement for a specific cation species (curves a and f). When potassium chloride was replaced by potassium sulfate, the acidification rate was very low (curve e). The effectiveness of anion species tested as potassium salts increased in the order sulfate = fluoride < phosphate < chloride = bromide (curves a–e). Addition of a low concentration (25 mM) of KSCN to K_2SO_4 medium resulted in a significant increase in the rate of acidification (curve h). These results indicate that a membrane-permeant anion is required for the MgATP-driven acidification of lysosomes. In sucrose or mannitol buffer, the rate of MgATP-driven acidification was almost the same as in K_2SO_4 or in Na_2SO_4 medium (curves g and i), though it took a long time to increase the intralysosomal pH before the addition of MgATP.

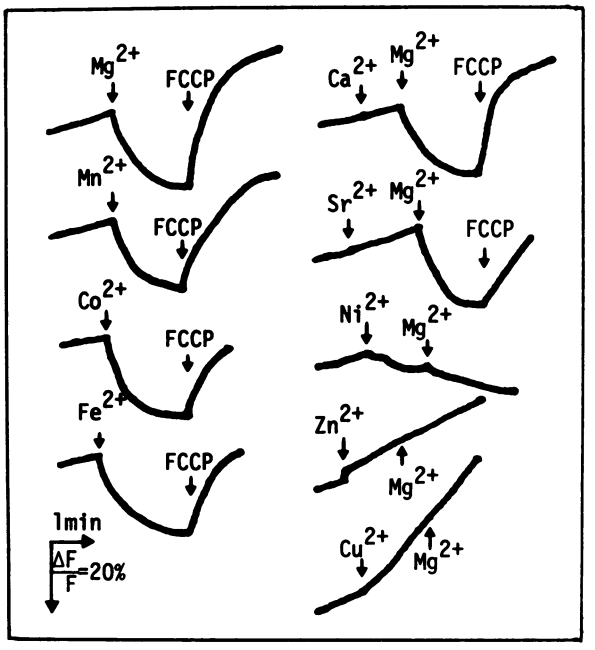

FIG. 4. Effects of divalent cations on the ATP-driven acidification of lysosomes. Procedures were as for Fig. 2. Incubation was in 100 mM KCl/1 mM ATP/20 mM Hepes/KOH, pH 7.0. Metal ions were added as 1 mM chlorides. Lysosome samples contained 66 μg of protein.

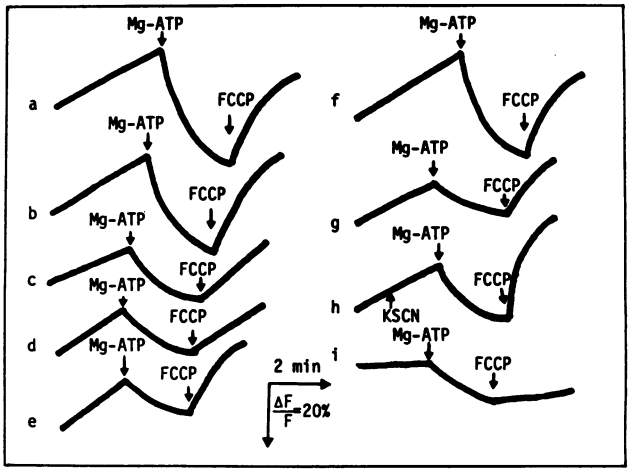

FIG. 5. Electrolyte requirement of the MgATP-driven acidification of lysosomes. Incubation mixtures contained 20 mM Hepes/tetramethylammonium hydroxide, pH 7.0; 100 mM KCl (curve a), KBr (curve b), K phosphate (curve c), KF (curve d), K_2SO_4 (curves e and h), NaCl (curve f), or Na_2SO_4 (curve g) or 300 mM sucrose (curve i); and (except i) 200 mM sucrose to increase the stability of lysosomes. ATP was added at the same intralysosomal pH after appropriate incubation periods. Additions: MgATP, 1 mM; FCCP, 2.5 μM; KSCN, 25 mM. Results are corrected for quenching of extralysosomal FITC-dex fluorescence by KSCN ($\Delta F/F$ = 6%). The lysosome sample contained 74 μg of protein.

The substrate requirement for the acidification of lysosomes is shown in Fig. 6. Neither AMP nor ADP served as a substrate, and ADP inhibited the effect of ATP added afterward. This inhibition was overcome by addition of a high concentration of ATP. Among the various ATP analogues, none of which serve as substrates for known ATPases, neither adenosine 5′-[α,β-methylene]- nor adenosine 5′-[β,γ-methylene]triphosphate could support lysosomal acidification. Even adenosine 5′-[β,γ-imido]triphosphate could not replace ATP and, like ADP, it

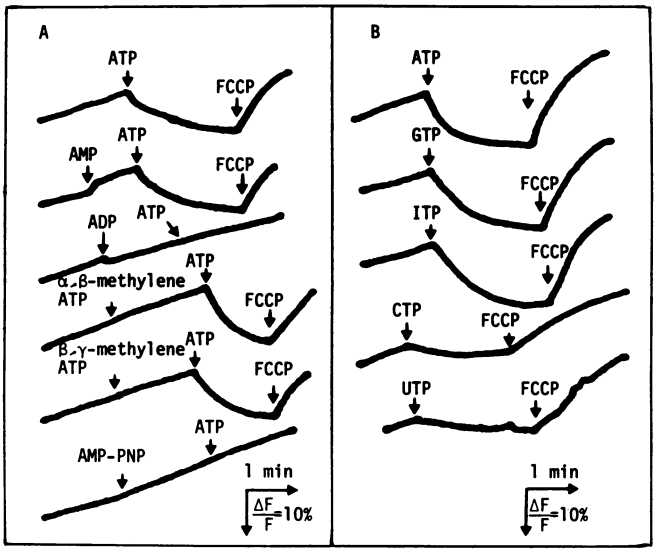

FIG. 6. Substrate requirement of the lysosomal proton pump. Procedures were as for Fig. 2. Additions were made at 1 mM. Lysosome samples contained 54 μg of protein. α,β-Methylene ATP, adenosine 5′-[α,β-methylene]triphosphate; β,γ-methylene ATP, adenosine 5′-[β,γ-methylene]triphosphate; AMP-PNP, adenosine 5′-[β,γ-imido]triphosphate.

inhibited the effect of ATP added afterward, indicating its interaction with the active site of the proton pump. Nor did any other substrate having a high energy phosphate bond—carbamoyl phosphate, acetyl phosphate, creatine phosphate, phospho*enol*pyruvate, NADH, or NADPH (1 mM each)—or *p*-nitrophenyl phosphate (0.25 mM), or even succinate (5 mM) support lysosomal acidification. These results indicate that the energy conserved in the β,γ-pyrophosphate bond of ATP is used for the acidification of lysosomes. Supporting evidence for this conclusion was obtained by using an ATP-regenerating system—creatine phosphate, creatine kinase, and a limited amount of ATP. A limited amount of ATP alone produced only transient acidification of lysosomes whereas, in combination with creatine phosphate and creatine kinase, it produced a continued acidification of lysosomes, resulting in significant decrease in the intralysosomal pH (Fig. 7). Among the other nucleoside triphosphates tested, GTP and ITP showed comparable activity with ATP, but UTP and CTP were only slightly effective (Fig. 6*B*). However, the nucleoside triphosphate specificity of tritosomal proton pump was in the order ATP > GTP = ITP > UTP = CTP, with the rate of proton translocation by ATP being ≈3 times that by GTP (data not shown).

The effects of various ATPase inhibitors other than oligomycin and sulfhydryl reagents (Fig. 3) are summarized in Fig. 8. The lysosomal proton pump activity was not affected by ouabain or vanadate (Na_3VO_4 or $NaVO_3$) but was strongly inhibited by quercetin. There were some differences in the effect of *N,N'*-dicyclohexylcarbodiimide (DCCD) and azide between normal lysosomes and tritosomes. The activity was resistant to these reagents in normal lysosomes (Fig. 8, curves e and f) but was highly sensitive in tritosomes (data not shown).

DISCUSSION

It is difficult to prepare normal (unmodified) lysosomes in a pure state without previous treatment of animals with reagents such as Triton WR-1339 that change the density of the lysosomes. Triton WR-1339 has been reported to change the lipid composition of lysosomes (17), thus probably changing that of the lysosomal membrane, and this may affect the proton pump activity of lysosomes. Our method permitted us to estimate the pH within lysosomes even in the presence of other cellular organelles and, thus, within normal lysosomes. By using this

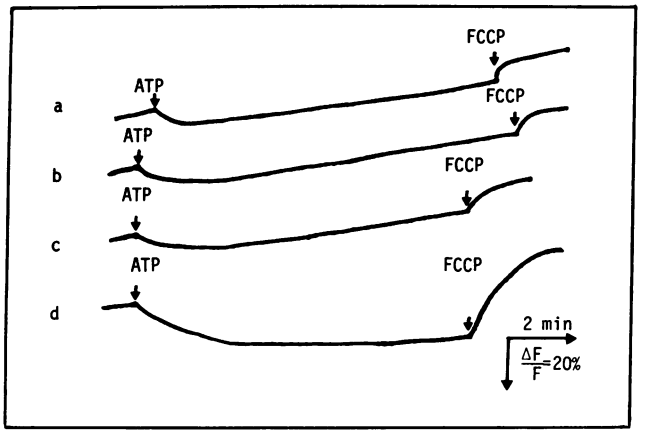

FIG. 7. Requirement of ATP hydrolysis for ATP-driven acidification of lysosomes. Procedures were as for Fig. 2, except that the incubation mixture (1 ml) was supplemented with 0.1% bovine serum albumin and 0.4 mM atractyloside. Curves: a, no additions; b, 2 mM creatine phosphate; c, 20 μg of creatine kinase; d, 2 mM creatine phosphate/20 μg of creatine kinase. Other additions were ATP at 10 μM and FCCP at 1 μM. Lysosome samples contained 800 μg of protein.

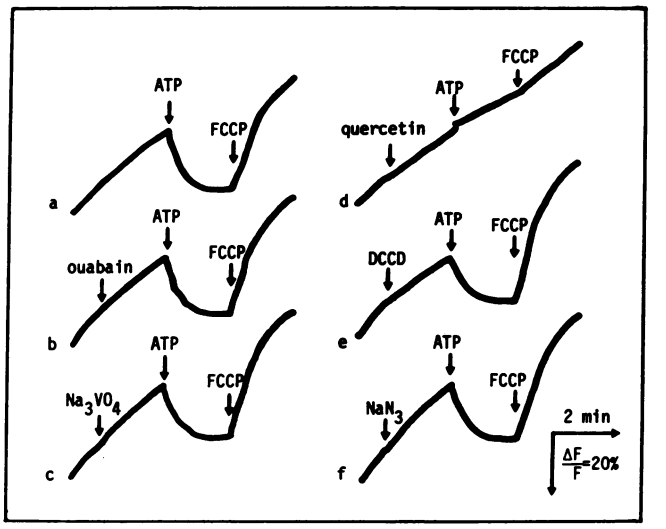

FIG. 8. Effects of various ATPase inhibitors on ATP-driven acidification of lysosomes. Various additions were made before adding ATP (1 mM). Curves: a, no additions (control); b, 500 μM ouabain; c, 100 μM Na_3VO_4; d, 100 μM quercetin; e, 25 μM DCCD; f, 1 mM NaN_3. Results are corrected for quenching of extralysosomal fluorescence by quercetin ($\Delta F/F$ = 10%). Lysosome samples contained 68 μg of protein.

method, we obtained evidence for the presence of a proton pump on normal lysosomes, as well as on tritosomes, and confirmed most of the data of Schneider (13).

This unique MgATP-driven proton pump was found to be a kind of sulfhydryl enzyme(s). It was inhibited by quercetin, like various other ion translocating ATPases. But it differs from the ATPase of mitochondria in being insensitive to oligomycin. Also, it differs from plasma membrane Na^+/K^+-ATPase by its insensitivity to ouabain and from microsomal Ca^{2+}-ATPase in that it does not require Ca^{2+}. It is unique in its sensitivity to Cu^{2+} and Zn^{2+}. Nevertheless, its insensitivity to vanadate, which inhibits various ATPases forming phosphorylated intermediates (18, 19), suggests that its fundamental mechanism of proton translocation may resemble that of mitochondrial H^+-ATPase, which is also insensitive to vanadate (19) and has never been shown to form a phosphorylated intermediate.

Concerning the sensitivity to DCCD, which is a well-known inhibitor of F_1-like H^+-ATPase (20), the proton pump activity was highly sensitive in the tritosome fraction but was resistant in the lysosome-rich fraction. Addition of a large excess of the lysosome-rich fraction to FITC-dex-containing tritosomes did not affect the DCCD sensitivity of the tritosomal proton pump. This suggests that the observed resistance in the lysosome-rich fraction is probably not due to consumption of DCCD by the F_1-ATPase of mitochondria present in this fraction. A high concentration of DCCD caused breakage of lysosomes. The difference in sensitivity to DCCD (and also to azide), as well as in nucleotide specificity, might be related to the change in lysosomal membrane after Triton WR-1339 treatment.

The acidification of lysosomes induced by MgATP required a permeant anion and was reversed by FCCP, in accordance with the results of Dell'Antone (11). The order of anion requirement was almost the lyotropic order and was similar to that of anion permeation through the lysosomal membrane (21). These results suggest that the lysosomal proton pump is electrogenic. Such a pump would not be expected to generate a detectable membrane potential in the presence of sufficient amounts of a permeant anion, as shown by Johnson *et al.* (22) for the electrogenic proton pump of chromaffin granules. Therefore, the finding by Schneider (13) that ATP does not sig-

nificantly affect the lysosomal membrane potential in the presence of 100 mM KCl does not, as he claims, argue against the electrogenic nature of the lysosomal proton pump. As will be shown in another paper, evidence that ATP can generate a lysosomal membrane potential under certain conditions has recently been obtained in our laboratory by using a fluorescent membrane potential indicator and the distribution of labeled permeant ions.

Contrary to Schneider's claim (13), sensitivity to inhibition by diisothiocyanostilbene disulfonic acid, an inhibitor of anion transport in erythrocytes, does not argue against the electrogenic nature of the lysosomal proton pump, since there is no reason why anion permeation into the lysosomes should involve the participation of a carrier protein sensitive to this disulfonic acid. In our experience, the disulfonic acid also inhibits the electrogenic mitochondrial proton pump.

The lack of inhibition by valinomycin is also put forward by Schneider (13) as evidence against the lysosomal proton pump being electrogenic. Before this conclusion can be accepted, more information is needed on the effect of valinomycin on lysosomes. According to Schneider (13), exposure to valinomycin in the presence of 150 mM KCl induces a lysosomal membrane potential of +40 mV, which is maintained for at least 30 min at 25°C. We have found the lysosomes remain intact for quite a while in the presence of valinomycin but eventually become lysed. These results suggest that penetration of the chloride ion is slow under these conditions and limits the rate of entry of the potassium ion. Our results with different anions indicate that the rate of ATP-driven proton transport may also be affected by the rate of anion permeation. As shown here, the lysosomal proton pump seems to be similar to the electrogenic proton pump of chromaffin granules (22) but different from the electroneutral proton pump (K^+/H^+-ATPase) of gastric mucosa (23). The characteristics of the lysosomal proton pump observed so far are similar to those of the H^+-ATPase recently found in yeast vacuoles (24).

Results with ATP analogues and an ATP-regenerating system suggest that cleavage of the β,γ-pyrophosphate bond of ATP is required for the acidification of lysosomes; that is, the lysosomal proton pump is in fact a kind of ATPase (proton translocating ATPase, H^+-ATPase). ATPase activity was reported to be present on lysosomal membranes by Schneider (9), and his finding was recently confirmed by Mego and his colleagues (25). Furthermore, Schneider presented evidence that this ATPase(s) acts as a proton pump (10, 13). However, this ATPase(s) has been shown to be activated by Ca^{2+} as well as by Mg^{2+} (10), which is different from the characteristics of the proton pump activity.

Identification of H^+-ATPase awaits further studies, especially purification of the ATPase and reconstitution of the proton pump with purified enzyme.

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