Lysosomes were purified approximately 40-fold from rat kidney cortex by differential and Percoll density gradient centrifugation. In a sucrose medium, the lysosomes quenched the fluorescence of the potential sensitive dye diS-C₃-(5) (3,3'-dipropylthiodicarbocyanine iodide) in a time-dependent manner, indicating that the dye accumulates within the lysosomal interior. After treatment of the lysosomes with valinomycin, the dye fluorescence displayed a logarithmic dependence upon the external K⁺ concentration; thus, the fluorescence signal provides a semiquantitative measure of the lysosomal membrane potential (Δψ). In the absence of valinomycin, lysosomal quenching of diS-C₃-(5) fluorescence was partially reversed by agents which collapse the lysosomal pH gradient (ammonium sulfate, chloroquine, and K nigericin), suggesting that the proton gradient across the lysosomal membrane contributes to Δψ. A rapid increase in diS-C₃-(5) fluorescence, indicative of an increase in Δψ, was observed upon the addition of Mg-ATP to the lysosomes. The ATP-dependent fluorescence change was inhibited by protonophores, K valinomycin, permeable anions, and N-ethylmaleimide, but was unaffected by ammonium sulfate, K nigericin, or sodium valinomycin. Oligomycin had no effect at concentrations below 2 μg/ml; at higher concentrations, oligomycin partially inhibited the fluorescence response to Mg-ATP, but it also inhibited the fluorescence response to K valinomycin, suggesting that it had modified the permeability of the lysosomal membrane. Dicyclohexylcarbodiimide behaved similarly to oligomycin. Mg-ATP also altered the lysosomal distribution of ⁶⁶Rh⁺ (in the presence of valinomycin) and SI⁴⁺[¹⁴C]CN⁻, consistent with an increase in the potential of the lysosomal interior of 40–50 mV. The results demonstrate that the lysosomal proton pump is electrogenic.

The acidic pH within the interior of lysosomes is generated and maintained by an ATP-dependent proton pump (Refs. 1–9; reviewed in Ref. 10). At present, it is uncertain whether this pump operates in an electroneutral or an electrogenic fashion, i.e., whether or not proton transport is directly coupled to the movement of other ions such that no net charge transfer occurs as a result of the operation of the pump. Schneider (5, 11) observed no change in the lysosomal membrane potential during proton transport and concluded that the pump was electroneutral. He has recently suggested (11) that external phosphate ions may enter the lysosome along with protons during the operation of the pump. On the other hand, experiments with plant vacuolysosomes (8, 9) suggest that activation of the proton pump by Mg-ATP causes an increase in the potential of the lysosomal interior of approximately 60 mV. Moreover, Okamura et al. (7) have stated that the lysosomal proton pump can generate a membrane potential under certain conditions, although the data supporting this conclusion are as yet unpublished.

In the present paper, we describe a new procedure for the isolation of lysosomes in high purity and good yield from rat kidney cortex. This procedure avoids the use of density altering agents such as Triton WR-1339 which could modify the permeability properties of the lysosomal membrane. Using the membrane potential sensitive fluorescent dye diS-C₃-(5)¹ as well as measurements of permeable ion distributions, we have obtained evidence that the activation of the lysosomal proton pump with Mg-ATP causes a rapid increase in the lysosomal membrane potential. This ATP-dependent change in membrane potential is blocked by protonophores and by N-ethylmaleimide and is reduced in magnitude by the presence of permeable anions. The results provide strong evidence that the lysosomal proton pump is electrogenic.

MATERIALS AND METHODS

Preparation of Lysosomes—Male albino rats (Charles River CD) weighing 350–450 g were used in these studies; in general, the best yields of lysosomes were obtained with older rats. Rats were killed by decapitation, and the kidneys were dissected and chilled in cold 0.3 M sucrose containing 1 mM EDTA adjusted to pH 7.0 with Tris (sucrose/EDTA buffer). Renal cortex was separated with a razor blade, weighed, minced, and homogenized in sucrose/EDTA buffer with four strokes in a Potter-Elvehjem glass homogenizer with a Teflon pestle rotating at approximately 1000 rpm. The volume of the homogenate was adjusted to 10 ml/g of wet tissue and centrifuged at 3000 g(maximum) for 5 min. The sediment was discarded, and the supernatant was spun at 3000 × g (maximum) for 10 min in a Sorvall SS-34 rotor. The sediment thus obtained consisted of a dark greenish pellet of lysosomes surrounded by red blood cells and overlaid with a brown zone which contained mitochondria, as well as some nuclear debris and unbroken cells. The supernatant was removed by aspiration and discarded along with the loosely packed upper portion of the pellet, which slid down the sides of the tube during aspiration. The pellet was then resuspended in a large volume (20-fold or more) of sucrose/EDTA buffer and subjected to two consecutive spins at 480 g(maximum) for 5 min so as to remove the erythrocytes which sedimented at this speed. The remaining supernatant was centrifuged at 3000 × g (maximum) for 10 min to yield the crude lysosomal preparation (heavy lysosomes). The pellet was resuspended in a minimum volume (300 μl/g of tissue) of sucrose/EDTA buffer and fractionated by density gradient centrifugation as described below.

A preformed density gradient of Percoll was prepared by spinning 14 ml of a gradient mixture consisting of 75% Percoll, 15% 2 M sucrose, and 10% 1 M sucrose, respectively; in equilibrium potential for protons.

¹ The abbreviations used are: diS-C₃-(5), 3,3'-dipropylthiodicarbocyanine iodide; Mops, 3-(N-morpholino)propanesulfonic acid; FCCP, carboxyl cyanide p-trifluoromethoxyphenylhydrazone; DCCD, dicyclohexylcarbodiimide; NEM, N-ethylmaleimide; Δψ, membrane potential (inside with respect to outside); E_n, equilibrium potential for protons.
sucrose, and 10% 0.1 M Mops buffered to pH 7.4 with Tris at 48,000 \( \times g \) (maximum) for 60 min in a glass centrifuge tube (SS-34 rotor). The heavy lysosome fraction was layered on the gradient and centrifuged for 30 min at 48,000 \( \times g \) (maximum). After centrifugation, the densest zone of turbidity (Fig. 1, fraction 4) was collected by aspiration, suspended in a 10-fold excess of sucrose/EDTA buffer, and centrifuged for 30 min at 3,000 \( \times g \) (maximum) to separate mitochondria from lysosomes. The pellet was resuspended in sucrose/EDTA buffer and centrifuged once again at 3,000 \( \times g \) (maximum) for 10 min. Most of the Percoll was removed by these centrifugations because it did not sediment at such low speeds. The final pellet, which was dark green in color, was resuspended in sucrose/EDTA buffer at a final protein concentration of 5-10 mg/ml. All the solutions used in this procedure were chilled on ice, and all centrifugation steps were carried out at 4 °C.

**Ion Distribution and \( \Delta pK \) Measurements**—A novel procedure was developed for the ion distribution measurements using ion exchange resins to trap the labeled ion in the lysosomal extracts. The advantage of this approach is that the total water space, the external water space (measured with \([\text{L}^\text{4C}]\)sucrose), and the labeled ion content of the lysosomal pellets can all be determined with the same sample. Lysosomes (50 \( \mu l \)) were mixed with 50 \( \mu l \) of 0.3 M sucrose, 50 mM Mops adjusted to pH 7.0 with Tris which contained 100 \( \mu l \) of \([\mu H^3O]^+\)/ml of \([\mu H^3O]^+\)/ml of Mops buffer, 50 mM Mops adjusted to pH 7.0 with Tris which contained 100 \( \mu l \) of \([\mu H^3O]^+\)/ml of Mops buffer, 50 mM Mops adjusted to pH 7.0 with Tris which contained 100 \( \mu l \) of \([\mu H^3O]^+\)/ml of Mops buffer. Lysosomes were separated from contaminated mitochondria on a percoll gradient prepared from 75% Percoll in 0.3 M sucrose, pH 7.0, to remove Percoll and finally resuspended in the above buffer at 5-10 mg of protein/ml. Data on the purity of these lysosomal preparations are presented in Table I which shows the activities of marker enzymes for lysosomes (aryl sulfatase), mitochondria (succinate-p-iodonitrotetrazolium violet reductase), endoplasmic reticulum (glucose 6-phosphatase), and plasma membranes (5'-nucleotidase). As shown, the average (±S.D.) purification factor for arylsulfatase is 7-fold.
37.8 ± 6.8 \textit{SE} \textit{N} = 7, with an overall recovery of 5.7 ± 2.1% of the total activity in the crude homogenate. Similar values were obtained for N-acetyl-β-glucosaminidase, another lysosomal marker enzyme (data not shown). The activities of these enzymes were 80–90% latent, indicating that the lysosomes were isolated as intact organelles. In contrast, the specific activity of the other marker enzymes was considerably reduced in the lysosomal preparations compared to the crude homogenate.

These preparations showed only low levels of contamination by mitochondria, a reflection of the excellent separation of mitochondrial and lysosomal markers on the Percoll gradients (Fig. 1). Thus, the specific activity of the mitochondrial enzyme succinate-p-iodonitrotetrazolium violet reductase was only 4% of its specific activity in the crude homogenate (Table I). Since mitochondria comprise only a fraction of the total protein of the crude homogenate, it follows that mitochondrial contamination of the lysosomal preparations must be considerably less than 4%. In some preparations, cytochrome oxidase activity was also measured as a mitochondrial marker, with results similar to those obtained for succinate-p-iodonitrotetrazolium violet reductase (data not shown). The relative absence of mitochondria in these preparations was confirmed by electron microscopic observations (data not shown). The vast majority of the particles consisted of spherical membrane-bound electron-dense bodies with diameters ranging from 0.5 to 1.4 μm; mitochondrial profiles were observed with a frequency of only 1/160 lysosomes. The results indicate that lysosomes can be obtained from rat kidney cortex in high purity and good yield following the procedure described under "Materials and Methods."

**Potential Sensitive Dye Fluorescence Measurements**—The carbocyanine dye diS-C₃(5) has been widely used to monitor the membrane potential in a variety of cellular and subcellular systems (18, 19). The positively charged dye accumulates within compartments that are electrically negative with respect to their surroundings, a process that leads to quenching of the dye fluorescence. As shown in Figs. 2 and 3, when lysosomes are added to a solution of 300 mM sucrose, 50 mM Mops/Tris, pH 7.0, containing 0.5 μM diS-C₃(5), 60–70% of the dye fluorescence is quenched in a time-dependent manner. Experiments at more rapid chart speeds have shown that 15–25% of the total quenching occurs rapidly, within the experimental mixing time (2–3 s), while the remainder occurs more gradually. Lysosomes which have been frozen and thawed or exposed to the nonionic detergent Nonidet P-40 (0.1%), procedures which destroy the integrity of the lysosomal membrane, show the rapid phase of fluorescence quenching but not the time-dependent phase. The rapid phase of quenching...
cannot be calibrated in terms of the magnitude of $\Delta \Psi$. Nevertheless, the signals clearly provide a semiquantitative indication of changes in the membrane potential.

**Relationship between $\Delta \Psi$ and $\Delta \rho$**—The average pH difference across the lysosomal membrane ($\Delta \rho$), as determined by the distribution of methylamine, is $1.2 \pm 0.3$ (n = 3) pH units (acid inside) at an external pH of 7.0. What is the contribution of this proton gradient to the apparent lysosomal membrane potential? This issue can be approached in a qualitative manner by assessing the effects on diS-C$_3$-(5) fluorescence of agents which collapse the pH gradient. As shown in Fig. 3A, when a weak base (5 mM ammonium sulfate) is added to the lysosomes in the presence of the dye, a rapid increase in fluorescence is observed. Similar results were obtained with 0.1 mM chloroquine and, as described later in connection with Fig. 4C, with the combination of nigericin and external K$^+$. The results suggest that the lysosomal membrane exhibits a significant permeability toward protons and that the pH gradient contributes to the apparent membrane potential (negative inside) observed under these conditions.

This conclusion is reinforced by the effects of protonophores on the fluorescence signal. As shown in Fig. 3B, when the lysosomes are treated with 1 $\mu$M FCCP, only a small degree of additional quenching of dye fluorescence is observed. Moreover, the magnitude of the effect of ammonium sulfate on dye fluorescence is similar to that seen in the absence of FCCP. The results suggest that protons are not far from equilibrium, even in the absence of FCCP, and that the membrane potential is approximately equal to the equilibrium potential for protons, i.e. $\Delta \Psi = E_H = -RT \ln (H_0/H_1)$. A different pattern of results is obtained, however, if the sucrose in the medium is replaced by K gluconate. In this case (Fig. 3C), the amount of fluorescence quenching is reduced in comparison to that observed in the sucrose medium (Fig. 3A and B), suggesting that the higher external K$^+$ concentration has caused the membrane potential to become more positive inside. Furthermore, when FCCP is added to the lysosomes in the K gluconate medium, a dramatic decrease in fluorescence is observed. The FCCP-induced fluorescence decrease is only partially reversed by agents which collapse the pH gradient such as 5 mM ammonium sulfate or nigericin plus external K$^+$ (data not shown); indeed, the magnitude of the fluorescence increase produced by such agents under these conditions is similar to that observed in the sucrose medium (cf. traces A and B in Fig. 3). The results suggest that in the K gluconate medium, protons are not in equilibrium with the lysosomal membrane potential and that increasing the membrane proton conductance with FCCP shifts $\Delta \Psi$ to a more negative (inside) value, presumably one that approximates $E_H$. The fact that the original level of fluorescence quenching is not restored by ammonium sulfate and K nigericin (agents that shift $E_H$ toward zero) suggests that $\Delta \Psi > 0$ in the K gluconate medium prior to the addition of the protonophores. The results are consistent with the idea, expressed previously (6, 7, 10), that the lysosomal membrane shows only a limited permeability toward both protons and potassium ions.

**Effect of ATP on $\Delta \Psi$**—As shown in Fig. 4A, when Mg-ATP is added to the lysosomes in the presence of diS-C$_3$-(5), a rapid increase in fluorescence is observed. This fluorescence increase is reversed by the subsequent addition of carbonyl cyanide $m$-chlorophenylhydrazone (Fig. 4A) or FCCP (Fig. 4B) and is completely prevented by adding the protonophores prior to the addition of Mg-ATP. Valinomycin (0.5 $\mu$M), in the presence of 5 mM K$_2$SO$_4$, also blocks the effect of Mg-ATP on diS-C$_3$-(5) fluorescence (data not shown). The effect of ATP is not observed if Mg$^{2+}$ is omitted from the medium; Ca$^{2+}$ will partially substitute for Mg$^{2+}$ although the fluorescence change develops more slowly and achieves only 40-50% of the magnitude of the response shown by Mg$^{2+}$ (data not shown). Neither Mg$^{2+}$ nor Ca$^{2+}$ have an effect on diS-C$_3$-(5) fluorescence in the absence of ATP.

The fluorescence increase induced by Mg-ATP cannot be attributed to the displacement of the dye from internal binding sites by protons as a secondary result of ATP-dependent acidification of the lysosomal interior. This conclusion is based on the results shown in Fig. 4, which indicate that Mg-ATP still produces the fluorescence increase in the presence of ammonium sulfate (B) or the combination of nigericin plus external K$^+$ (C), agents which prevent the internal acidification of lysosomes (2, 7, 10). These results and the inhibitory

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**Fig. 3.** Effect of (NH$_4$)$_2$SO$_4$ and protonophores on the quenching of diS-C$_3$-(5) fluorescence by lysosomes. Lysosomes were added at the upper arrow to cuvettes containing 2 ml of 0.5 $\mu$M diS-C$_3$-(5) in either 300 mM sucrose, 50 mM Mops/Tris, pH 7.0 (A and B), or 150 mM K gluconate, 50 mM Mops/Tris, pH 7.0 (C). At the points indicated, 10 $\mu$L of 1 M (NH$_4$)$_2$SO$_4$ or 0.2 $\mu$L of 10 mM FCCP in ethanol) were added to the cuvettes.

**Fig. 4.** Effect of Mg-ATP on the fluorescence of diS-C$_3$-(5) in the presence of lysosomes. The cuvettes contained 0.5 $\mu$M diS-C$_3$-(5) in the sucrose medium described in the legend to Fig. 2; lysosomes were added at the point shown by the drop in fluorescence. At the point indicated by Mg-ATP, 20 $\mu$L of 0.1 M MgSO$_4$ and 20 $\mu$L of 0.1 M ATP (disodium salt) were added to the cuvette; the Tris salt of ATP gave identical responses. Other additions were 10 $\mu$L of 1 M (NH$_4$)$_2$SO$_4$, 20 $\mu$L of 0.5 M K$_2$SO$_4$, 0.2 $\mu$L of 10 mM FCCP or carbonyl cyanide $m$-chlorophenylhydrazone (CI-CCP) in dimethyl sulfoxide, 1 $\mu$L of 2 mg/mL of nigericin in ethanol, and 1 $\mu$L of 2 mM valinomycin (Val) in dimethyl sulfoxide, as indicated.
effects of the ionophores described above indicate that Mg-
ATP induces a shift (positive inside) in the lysosomal mem-
brane potential, presumably by activating the lysosomal pro-
ton pump.

The effect of Mg-ATP on \( \Delta \Psi \) is also evident from measure-
ments of the distribution of permeable ions (Table II). Three
different ion probes were used for these determinations: the lipophilic cation \([\text{H}]^+\) [20, 21], \( ^{86}\text{Rb}^+ \) in the presence of
valinomycin, and the permeable anion \( ^{14}\text{C}]^\text{CN}^- \). As shown
in Table II, each probe gave different values for \( \Delta \Psi \) in the
absence of ATP, ranging from \(-120 \text{ mV} \) for \([\text{H}]^+\) to \(-9 \text{ mV} \)
for \( ^{86}\text{Rb}^+ \). In the presence of Mg-ATP, the value for \( \Delta \Psi \) measured with either \( ^{86}\text{Rb}^+ \) or \( ^{14}\text{C}]^\text{CN}^- \) shifted in a positive
direction by approximately \( 40 \text{ mV} \) (36.1 ± 4.5 mV \( n = 3 \)) for
\( ^{86}\text{Rb}^+ \) and \( 46.2 ± 8.7 \text{ mV} \) \( n = 4 \) for \( ^{14}\text{C}]^\text{CN}^- \). In other
words, Mg-ATP produced a loss of \( ^{86}\text{Rb}^+ \) from the lysosomes
and a gain in \( ^{14}\text{C}]^\text{CN}^- \). In the absence of valinomycin, the loss
of \( ^{86}\text{Rb}^+ \) from the preloaded lysosomes was smaller in
magnitude and occurred more slowly (data not shown). In
contrast, Mg-ATP produced no change in the distribution of
\([\text{H}]^+\) [20, 21]. The reasons for the lack of response by \([\text{H}]^+\) are
unclear and have not been further investigated. It seems
likely, however, that \([\text{H}]^+\) may be tightly bound to sites
within the lysosome and that this binding might account for
the highly negative \( \Delta \Psi \) obtained and its lack of response to
Mg-ATP. (When 20 mM KCl was added to the lysosomes in
the presence of valinomycin, \( \Delta \Psi \), as measured by \( ^{86}\text{Rb}^+ \),
increased only slightly, from \(-129 \text{ to } -104 \text{ mV} \).) Despite the
anomalous behavior of \([\text{H}]^+\), the data in Table II provide
strong confirmation of the conclusions reached in the fluo-
cescence experiments, i.e. Mg-ATP causes the lysosomal
membrane potential to become more positive inside.

Inhibitors—As shown in Fig. 5A, oligomycin (6.2 \( \mu \)g/ml)
produced a 50% decrease in the magnitude of the fluorescence
change induced by Mg-ATP. Other lysosome preparations
frequently showed lesser degrees of inhibition, but in these
instances, higher concentrations of oligomycin invariably
brought on the inhibitory response. Oligomycin concentra-
tions less than 2 \( \mu \)g/ml had no effect on the ATP-dependent
fluorescence response. These concentrations of oligomycin
are approximately 50-fold higher than those required to sup-
press completely the effects of Mg-ATP on \( \text{di}-\text{C}_{3}(5) \) fluo-
rescence in kidney mitochondria (data not shown). Thus, it
seems unlikely that the graded effects of oligomycin within
such a high range of concentrations could be attributed to
contamination of the lysosome preparations by submitochon-
drial particles. This conclusion is supported by the data
shown in Fig. 5B, which indicate that oligomycin also inhib-
ited by 50% the increase in fluorescence produced by estab-
lishing an inwardly directed K gradient in the presence of
valinomycin. Therefore, oligomycin either interferes with
the response of the dye to a change in membrane potential or
alters the permeability properties of the lysosomal membrane
such that potentials generated by either Mg-ATP or K vali-
nomycin are reduced in magnitude.

As shown in Fig. 5, C and D, DCCD (50 \( \mu \)M) markedly
inhibited the effects of Mg-ATP on \( \text{di}-\text{C}_{3}(5) \) fluorescence,
and like oligomycin, it altered the response to K valinomycin.
In this case, the fluorescence change caused by the addition of
10 mM KCl (prior to valinomycin) is dramatically increased

\[ \text{Mg-ATP} \]

\[ \Delta \Psi \]

\[ \text{K}^+ \text{-valinomycin} \]

\[ \text{Inhibitor} \]

\[ - \]

\[ + \]

\[ \text{Oligomycin (6.2 \( \mu \)g/ml)} \]

\[ \text{DCCD (50 \( \mu \)M)} \]

\[ \text{NEM (100 \( \mu \)M)} \]

\[ \text{Na}_3\text{VO}_4 \]

\[ (1 \text{mM)} \]

\[ \text{FIG. 5. Effect of inhibitors on the increase in fluorescence}
induced by either 1 mM Mg-ATP (A, C, E, and G) or 10 mM
KCl plus 1 \( \mu \)M valinomycin (B, D, and F). In each panel,
two traces are shown. The first arrow in each trace indicates the
time of addition of either the inhibitors (right trace) or solvent alone (left
trace). (For oligomycin and DCCD (B and D), the last arrow shown
on the left traces indicates the addition of the inhibitor following the
completion of the fluorescence response to K valinomycin.) For the
sake of clarity and space, the initial decline in fluorescence due to
quenching by lysosomes is not shown. All experiments were conducted
in sucrose media with Mg-ATP additions as described in the legends
to Figs. 2 and 4. Other additions are as follows: \( K \), 20 \( \mu \)l of 1 M KCl,
\( C \), 1 \( \mu \)l of 2 mM valinomycin in dimethyl sulfoxide, 0.25 \( \mu \)l of 50 mg/
ml of oligomycin in ethanol, 2 \( \mu \)l of 50 mM DCCD in ethanol, 2 \( \mu \)l of
0.1 M NEM, and 20 \( \mu \)l of 0.1 M Na_3VO_4.\]
**Discussion**

Lysosomes from rat kidney were first isolated by Straus (23-26) as large (0.1-5.0 μm) "droplets" which sedimented at relatively low centrifugal forces and were further characterized by Shibiko and Tappel (27) and by Maunsbach (28, 29). The procedure described here can be carried out rapidly without specialized equipment and yields lysosomes that are approximately 40-fold purified over the crude homogenate (Table I). Its success depends upon the fact that, unlike liver lysosomes, kidney lysosomes are quite dense and can readily be separated from mitochondria and other organelles by density gradient centrifugation. This feature of kidney lysosomes has been previously described by Maunsbach (28). The present procedure differs from that of Maunsbach in that a relatively low centrifugation speed was used to obtain the initial crude lysosomal preparation from the homogenate and that subsequent purification of the lysosomes was accomplished using isosmotic gradients of Percoll rather than sucrose gradients. The latter factor is particularly important because it avoids the osmotic stress that sucrose gradients of the required density would place upon the lysosomes. An added advantage of the present procedure is that density-altering agents such as Triton WR-1339 have not been employed; thereby minimizing the risk of inducing nonphysiological changes in the permeability properties of the lysosomal membrane.

In a sucrose medium buffered at pH 7.0, the pH gradient across the membrane of the isolated kidney lysosomes is approximately 1.2 pH units, as determined from the distribution of [14C]methylamine. The relatively minor influence of protonophores on the fluorescence of diS-C₂-(5) (Figs. 3 and 4) suggest that protons are nearly in equilibrium under these conditions. This means that ΔΨ is approximately equal to the equilibrium potential for protons, given by $E_H = -60 \Delta pH = -72 \, \text{mV}$. This value is considerably more positive than that obtained from the distribution of [14C]methylamine and yet more positive than that obtained from [3H]TPP⁺ distribution (Table II). These discrepancies probably reflect the binding of SCN⁻ and TPP⁺ to sites within the lysosomes. On the other hand, $E_H$ is only slightly more positive than the value of ΔΨ obtained from the distribution of Rhb in the presence of valinomycin. Indeed, the difference between these two values may reflect the fact that under the conditions of these measurements, valinomycin causes a negative shift in ΔΨ because of the presence of K⁺ within the lysosomes (Fig. 2A). As discussed in connection with Fig. 2B, valinomycin produces no changes in fluorescence at $K_0 = 0.3 \, \text{mM}$; assuming that this external K⁺ concentration represents the point at which $K/K_0 = \exp(-\Delta \psi_r/RT)$ (see "Results") and that $\Delta \psi_r = E_H = -72 \, \text{mV}$, one can calculate that $K_0 \approx 4.8 \, \text{mM}$.

Although protons may be in equilibrium in a buffered sucrose medium, this does not appear to be the case in potassium gluconate. Under these conditions, ΔΨ reflects the high external K⁺ concentration and may well attain a positive value (see "Results"). In any event, ΔΨ is considerably more positive than $E_H$, as shown by the large decrease in diS-C₂-(5) fluorescence when the lysosomal membrane is made permeable to protons by treatment with FCCP (Fig. 3C). This suggests that in a high K⁺ medium such as the cell cytoplasm, the intralysosomal acidity represents a distinctly nonequilibrium distribution of protons. If this is the case, then a Donnan equilibrium can play little or no role in the development or maintenance of the lysosomal pH gradient (cf. Ref. 10).

When Mg-ATP is added to the lysosomes in the presence of diS-C₂-(5), an increase in fluorescence is observed. This
represents a positive shift in the membrane potential generated by the activity of the lysosomal proton pump. This conclusion is based on the following lines of evidence. (a) The ATP-dependent fluorescence shift is blocked by protonophores and by K valinomycin, agents which increase the conductivity of the lysosomal membrane and thereby short circuit the current generated by the proton pump (Fig. 4). A similar inhibitory effect is produced by the presence of permeable anions in the external medium, the degree of inhibition increasing with the permeability of the lysosomal membrane to the anion in question (Fig. 7). (b) Since the effect of ATP is not blocked by agents that collapse the lysosomal pH gradient (NH₄Cl and K nigericin) (Fig. 4), it cannot be attributed to a secondary effect of lysosomal acidification and must therefore represent the electrogenic operation of the pump itself. (c) The characteristics of the ATP-dependent fluorescence response are similar to the known characteristics of the lysosomal proton pump. Thus, the activity is inhibited by NEM, it is insensitive to vanadate, and it displays a nucleotide specificity similar to that reported for lysosomal acidification (1, 6, 7). (d) Finally, the activity cannot be attributed to other organelles, most notably mitochondria or submitochondrial particles that might contaminate the lysosomal preparation. This is evident from the fact that the ATP-dependent fluorescence response represents a reversal of quenching and that the recovery itself is clearly of lysosomal origin. Thus, mitochondrial membranes, which represent only a minute fraction of the total protein present in the lysosomal preparations (Table 1), could not possibly account for the magnitude of the fluorescence response to Mg-ATP. Furthermore, the fluorescence response shows little sensitivity to the potent mitochondrial ATPase inhibitor oligomycin; some inhibition is observed at high concentrations of oligomycin, but this probably reflects an effect of the agent on the lysosomal membrane permeability rather than a direct effect on the ATPase (Fig. 5B). Finally, NEM (0.1 mM) completely blocks the effect of Mg-ATP on diS-C₃(5) fluorescence of lysosomes (Fig. 5E) although it has no effect on the mitochondrial fluorescence response to ATP (data not shown).

The present findings are in agreement with those of Marin et al. (8) and Cretin (9) who observed that Mg-ATP produced a positive shift in membrane potential in plant vacuoliysosomes (lutos) obtained from the rubber tree. Moreover, electrogenicity is also a feature of proton pumps in other intracellular organelles, including chromaffin granules, platelet granules, neurophysiological granules, insulin secretory granules, sperm acrosomes, and yeast vacuoles (reviewed in Refs. 30 and 31). Schneider (4, 11) has claimed that the proton pump of rat liver lysosomes is not electrogenic and has suggested that this is because phosphate ions are cotransported with protons during ATP-dependent acidification. However, as shown in Fig. 7A, the presence of phosphate in the external medium does not eliminate the ATP-dependent shift in ΔΨ; therefore, if phosphate accumulates within lysosomes during ATP-dependent acidification as indicated by Schneider's data (11), it does so by some mechanism other than electroneutral proton-phosphate co-transport. Other evidence cited by Schneider (4, 11) for an electroneutral proton pump is that Mg-ATP failed to alter significantly the distribution of S¹³⁴Cl⁻CN⁻ in rat liver lysosomes. However, the data in Table I demonstrate that in rat kidney lysosomes, Mg-ATP produces a marked shift in S¹³⁴Cl⁻CN⁻ distribution, consistent with a change in ΔΨ of more than 40 mV. As pointed out by Okumura et al. (7), Schneider's results may have been affected by the presence of high concentrations of KCl (100 mM) in his experiments; this concentration of a relatively permeable anion such as Cl⁻ would greatly reduce the magnitude of any membrane potential generated by the proton pump. This is evident from Fig. 7, which shows that permeable anions (including Cl⁻ at 20 mM) do indeed reduce the magnitude of the shift in ΔΨ produced by Mg-ATP.

The electrogenicity of the lysosomal proton pump may be important in promoting the osmotic stability of lysosomes (cf. Ref. 10). In vitro, the presence of ATP exerts a stabilizing effect on lysosomes in the presence of electrolytes; this effect may be due in part to the positive shift in membrane potential induced by Mg-ATP, which would retard the diffusion of salt across the lysosomal membrane by reducing the rate of influx of the cationic species (cf. Ref. 32). In situ, the osmotic stability of lysosomes may be critically dependent upon maintaining a relatively low internal K⁺ concentration, and the positive potential generated by the proton pump may well provide the mechanism by which this is accomplished. Of course, such a potential would also result in the accumulation of permeable anions within the lysosome, but this probably would not create a major osmotic stress because of the low concentration of such ions (e.g. Cl⁻) within the cytoplasm of most mammalian cells. A decline in the magnitude of the lysosomal membrane potential may be involved in the lysosomal alterations which occur during the development of pathological states, such as ischemia, that are accompanied by a fall in cellular ATP levels. For example, swelling of intracellular lysosomes is observed within 15 min of the onset of ischemia in cardiac tissue; after 30 min, there is a progressive loss of lysosomal integrity resulting in the release of lysosomal enzymes into the cell cytoplasm (reviewed in Refs. 33 and 34). Although many factors are undoubtedly involved in this complex process, it seems likely that the gradual influx of cytoplasmic K⁺ into the lysosomes, in response to a decline in the lysosomal membrane potential, may play a role in the swelling and eventual disruption of these organelles.

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REFERENCES
1. Mego, J. L., Farb, R. M., and Barnes, J. (1972) Biochem. J. 128, 763-769.
2. Okumura, S., and Poole, B. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3327-3331.
3. Puma, A., and Antoni, J. M. (1981) Biochem. Biophys. Res. Commun. 86, 180-189.
4. Schneider, D. L. (1979) Biochem. Biophys. Res. Commun. 87, 559-565.
5. Schneider, D. L. (1961) J. Biol. Chem. 236, 3856-3864.
6. Reeves, J. P., and Reames, T. (1981) J. Biol. Chem. 256, 6047-6053.
7. Okumura, S., Moriyama, Y., and Takaso, T. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2758-2762.
8. Marin, B., Marin-Lanza, M., and Kemer, E. (1981) Biochem. J. 199, 365-372.
9. Cretin, H. (1982) J. Memb. Biol. 65, 175-184.
10. Reeves, J. P. (1983) in Lysosomes in Biology and Pathology (Dingle, J. T., Dean, R. T., and Sly, P., eds) Elsevier Biomedical Press, Amsterdam, in press.
11. Schneider, D. L. (1983) J. Biol. Chem. 258, 1833-1838.
12. Hollemans, M., Elferink, R., O., de Groot, P. G., Strijland, A., and Tager, J. M. (1981) Biochim. Biophys. Acta 643, 149-151.
13. Morre, D. J. (1971) Methods Enzymol. 22, 130-148.
14. Wharton, D. C., and Strijland, A., (1967) Methods Enzymol. 10, 245-250.
15. Barrett, A. J., and Heath, M. F. (1977) in Lysosomes: A Laboratory Handbook (Dingle, J. T., ed) pp. 19-127, North-Holland Publishing Co., Amsterdam.
16. Reeves, J. P. (1979) J. Biol. Chem. 254, 8914-8921.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
18. Waggoner, A. S. (1979) Methods Enzymol. 55, 689-695.
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19. Bashford, C. L., and Smith, J. C. (1979) Meth. Enzymol. 55, 569–586
20. Skulachev, V. P. (1971) Curr. Top. Bioenerg. 4, 127–163
21. Felle, H., Porter, J. S., Slayman, C. L., and Kaback, H. R. (1980) Biochemistry 19, 3585–3590
22. Casey, R. P., Hollemans, M., and Tager, J. M. (1978) Biochim. Biophys. Acta 508, 15–26
23. Straus, W. (1954) J. Biol. Chem. 207, 745–755
24. Straus, W. (1956) J. Biophys. Biochem. Cytol. 2, 513–521
25. Straus, W. (1957) J. Biophys. Biochem. Cytol. 3, 933–947
26. Straus, W. (1962) J. Cell Biol. 12, 231–246
27. Shibko, S., and Tappel, A. L. (1966) Biochem. J. 95, 731–741
28. Maunsbach, A. B. (1969) in Lysosomes in Biology and Pathology (Dingle, J. T., and Fell, H. B., eds) Vol. 1, pp. 115–153, North-Holland Publishing Co., Amsterdam
29. Njus, D., Knoth, J., and Zallakian, M. (1981) Curr. Top. Bioenerg. 11, 107–147
30. Rudnick, G. (1983) in Physiology of Membrane Disorders (Andreoli, T. E., Fanestil, D. D., Hoffman, J. F., and Schultz, S. G., eds) Second Ed., Plenum Publishing Co., New York, in press
31. Ruth, R. C., and Weglicki, W. B. (1982) Am. J. Physiol. 242, C192–C199
32. Wildenthal, K. (1978) J. Mol. Cell. Cardiol. 10, 595–603
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