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EVIDENCE AGAINST A MgATP-DEPENDENT PROTON PUMP IN RAT-LIVER LYSOSOMES

MARJA HOLLEMANS, DIRK-JAN REIJNGOUD and JOSEPH M. TAGER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

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Summary

1. The effect of MgATP has been studied on the accumulation of the lipid-soluble anion thiocyanate, the accumulation of the lipid-soluble base methylamine, and the fluorescence of bound anilinonaphthalene sulphonate in rat-liver lysosomes. The lysosomes used were isolated from the livers of rats pretreated with Triton WR 1339.

2. The accumulation of thiocyanate is stimulated by the addition of valinomycin in the presence of K^+ but not by the addition of MgATP.

3. The fluorescence of anilinonaphthalene sulphonate bound to lysosomes is enhanced by valinomycin in the presence of K^+, the extent of the enhancement being dependent on the concentration of K^+. In contrast, MgATP has no effect on the fluorescence.

4. The intralysosomal pH, as estimated from the distribution of methylamine, is not affected by the addition of MgATP in media with or without K^+, Na^+ or HCO_3^-.

5. These data strongly suggest that there is no MgATP-dependent proton pump in rat-liver lysosomes.

Introduction

The function of lysosomes is the degradation of phagocytosed material by hydrolases with an acid pH optimum [1]. It has been proposed by de Duve et

Abbreviations: MES, 2-(*N*-morpholino)ethane sulphonic acid; MOPS, morpholinopropane sulphonic acid; ANS, 1-anilinonaphthalene-8-sulphonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid; S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide; EGTA, ethyleneglycol-bis(β-aminoethyl-ether)-*N*,*N*-tetraacetic acid.

al. [1,2] that the intralysosomal pH must be low compared to that of the surrounding medium. Studies on the distribution of the weak base methylamine have demonstrated a pH difference across the lysosomal membrane of approx. 1 unit at physiological pH values [3—6]. Therefore, a mechanism should be present to maintain the pH difference across the lysosomal membrane (for recent reviews see refs. 7 and 8).

Two general mechanisms by means of which a ΔpH across the lysosomal membrane is maintained have been proposed, one being energy independent [3—6] and the other energy dependent [9—16]. In the energy-independent mechanism it is proposed that the ΔpH is due to a Donnan-type of distribution of protons brought about by the presence within the lysosomes of indiffusible negatively charged groups [3—6]. According to the energy-dependent mechanism, an ATPase delivers the energy to bring about an inward directed H^+ flux [9—16]. Although the observations (see Tables I and II of ref. 8) that the intralysosomal pH in isolated lysosomes is comparable to values measured in situ obviate the necessity of postulating the existence of an ATP-driven proton pump in the lysosomal membrane, it cannot be excluded that an energy-dependent pump functions as an auxiliary mechanism.

If an ATP-dependent proton pump exists in a membrane, there are two possibilities with regard to its mode of action. The first is that transport of the proton across the membrane is stoichiometrically coupled to the flux of another (charge compensating) ion. In this case the proton flux across the membrane must be described by the chemical potentials of the ions transported by the ATPase [17]. The second possibility is that protons are pumped into the lysosomes without being stoichiometrically coupled to an accompanying flux of another ion. In this case a transmembrane electrical potential is generated in addition to the chemical potential, and the proton flux has to be described by the electrochemical proton potential [18].

In membrane-bound organelles like mitochondria [19], chloroplasts [20] and chromaffin granules [21], addition of ATP induces an electrogenic proton transport. In the absence of a charge-compensating flux the electrochemical proton gradient is measured mainly as $\Delta\psi$. In the presence of a permeant anion the electrochemical proton gradient will be monitored as a pH difference.

We have investigated the effect of ATP on the intralysosomal pH under a variety of conditions, including those used by investigators [9,14—16] who have brought forward evidence for the operation of a proton pump in lysosomes. In our studies the transmembrane proton gradient (ΔpH) was calculated from the distribution of the lipid-soluble base methylamine. In addition, the transmembrane electrical potential ($\Delta\psi$) was monitored as the response of the fluorescent probe anilinonaphthalene sulphonate (ANS) or calculated from the distribution of the lipid-soluble anion thiocyanate. The effect of ATP was compared with that of ionophores expected to influence ΔpH and/or $\Delta\psi$. The results of the investigation are described in this paper.

Materials and Methods

Materials. ^3H_2O, [^{14}C]methylamine, KS^{14}CN and [^{14}C]sucrose were obtained from Radiochemical Centre (Amersham, England), Triton WR 1339

from Rohm and Haas (Philadelphia, U.S.A.), valinomycin from Sigma (St. Louis, MO, U.S.A.) and ATP (disodium salt) from Boehringer (Mannheim, Germany). In our experiments, an equimolar mixture of disodium ATP and $MgSO_4$ was used. 5-Chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S-13) was a gift from Dr. P. Hamm, Monsanto (St. Louis, U.S.A.) and ANS was a gift from Dr. G.K. Radda (Oxford, England).

Isolation of rat-liver lysosomes. Lysosomes were isolated by the flotation method of Trouet [22] as described by Kussendrager et al. [23] from the livers of Triton WR 1339-treated rats.

Fluorescence measurements. The fluorescence intensity of ANS was measured with a Hitachi Perkin-Elmer MPF-2A fluorimeter at 22°C, using an excitation wavelength of 380 nm and a wavelength of 480 nm to measure the emission.

Measurement of the distribution of radioactive methylamine, thiocyanate and sucrose across the lysosomal membrane. Rat-liver lysosomes were incubated in the media indicated in the legends to the tables and figures in the presence of ^3H_2O and either [^{14}C]methylamine or KS^{14}CN. After incubation for the times indicated the lysosomes were separated from the medium by rapid centrifugation (2 min in an Eppendorf centrifuge, Model 3200, run at full speed) and the ^{14}C and ^3H radioactivity in the pellet and supernatant determined as described in ref. 4. In parallel incubations containing exactly the same components except methylamine or thiocyanate, the lysosomes were incubated with [^{14}C]sucrose. The distribution of methylamine between the lysosomes and the medium was corrected for adherent water as follows:

$$f_{MA} = \frac{r_{MA} - r_{suc}}{1 - r_{suc}} \tag{1}$$

where f_{MA} = accumulation factor for methylamine

$$r_{MA} = \frac{([^{14}C]\text{methylamine}/^3H_2O)_{pellet}}{([^{14}C]\text{methylamine}/^3H_2O)_{supernatant}} \tag{2}$$

and

$$r_{suc} = \frac{([^{14}C]\text{sucrose}/^3H_2O)_{pellet}}{([^{14}C]\text{sucrose}/^3H_2O)_{supernatant}} \tag{3}$$

The accumulation factor for thiocyanate (f_{SCN^-}) was determined in exactly the same way.

Calculation of the intralysosomal pH from f_{MA} and of $\Delta\psi$ from f_{SCN^-}. The intralysosomal pH was calculated from the accumulation of the lipid-soluble base methylamine using the following formula (see ref. 4):

$$pH_{in} = pH_{out} - \log f_{MA} \tag{4}$$

For the calculation of $\Delta\psi$, f_{SCN^-} was used (see e.g. refs. 24 and 25). The rationale is as follows. An ion (i) which is in electrochemical equilibrium across a membrane is distributed according to the Nernst equation. Since at equilibrium

$$\Delta\mu_i = RT \ln \frac{[i]_{in}}{[i]_{out}} + ZF\Delta\psi = 0 \tag{5}$$

it follows that

$$\Delta\psi = -\frac{RT}{ZF} \ln \frac{[i]_{in}}{[i]_{out}} \quad (6)$$

At room temperature (22° C) this expression is

$$\Delta\psi = 60 \log \frac{[i]_{in}}{[i]_{out}} \text{ (in mV)} \quad (7)$$

In order to use Eqn. 7 for the determination of $\Delta\psi$ it is necessary to have a freely permeant ion present, like SCN^-.

Results

Mego and coworkers [9—12] were the first to bring forward evidence that ATP can bring about a decrease in intralysosomal pH in isolated lysosomes. Since this evidence was indirect, we studied the effect of ATP on the intralysosomal pH more directly by measuring the distribution of the lipid-soluble weak base methylamine, which will be distributed according to the pH difference across the lysosomal membrane (see refs. 4—8).

In the experiments of Table I, rat-liver lysosomes were incubated in the presence and absence of different concentrations of MgATP. It is clear that ATP has hardly any effect on the internal pH. In the absence of a salt at pH 7.5 the internal pH increased slightly from 5.57 to 5.79 when ATP was present. In a salt-containing medium a slight decrease was observed. At a medium pH of 6.5 no effect of MgATP could be detected.

An explanation for the inability of MgATP to bring about a decrease in the internal pH in the experiment of Table I might be the relatively short incubation time used (5 min); in the experiments of Mego and coworkers [9—12] incubation periods of about 1 h were generally used. Therefore, the intralysosomal pH was calculated in the absence and presence of MgATP at different time intervals up to 40 min in a medium which was essentially the same as

TABLE I

EFFECT OF ATP ON THE INTRALYSOSOMAL pH

Rat-liver lysosomes were incubated for 5 min at room temperature in 1 ml of a medium containing 250 mM mannitol or 130 mM KCl, ^3H_2O, and either [^{14}C]methylamine (1.80 μM; 0.1 μCi) or [^{14}C]sucrose (0.16 μM; 0.1 μCi). In addition the medium contained 25 mM MES and 25 mM MOPS together with sufficient Tris to adjust the pH to 6.5, or 25 mM Tris and sufficient MES and MOPS, in equimolar ratio, to adjust the pH to 7.5. Where indicated MgATP was added at a concentration of 2 or 5 mM.

pH of medium	MgATP (mM)	pH_{in}	
		Mannitol medium	KCl medium
6.5	0	—	5.72
6.5	2	5.25	5.65
6.5	5	5.37	5.72
7.5	0	5.57	6.09
7.5	2	5.47	6.06
7.5	5	5.79	5.94

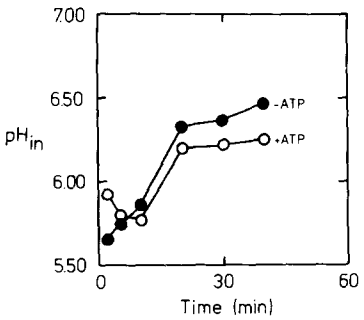

Fig. 1. The influence of ATP on the intralysosomal pH during prolonged incubation times. Rat-liver lysosomes were incubated at 25°C in a medium (final volume: 7 ml) containing 250 mM sucrose, 50 mM mercaptoethanol, 25 mM Tris, sufficient acetic acid to bring the pH to 7.40, ^3H_2O, and either [^{14}C]-methylamine (1.80 μM; 0.7 μCi) or [^{14}C]sucrose (0.16 μM; 0.7 μCi). Where indicated MgATP (3.5 mM; pH 7.4) was also present. At the times indicated 1-ml samples were removed from the medium and centrifuged for 4 min at 20 000 × g_{max}.

that used by Mego et al. [9]. Fig. 1 shows that upon prolonged incubation times the intralysosomal pH was about 0.2 unit lower in the presence of ATP than in its absence. However, the pH of the medium also decreased in the presence of ATP, from 7.5 to 7.1 at the end of the incubation. A decrease in external pH of this magnitude has been shown previously to correspond to a decrease in the internal pH of 0.2 unit (see Fig. 1 of ref. 4). Thus the observed decrease in the internal pH of the lysosomes after prolonged incubation in the presence of ATP is due to a decrease in the pH of the medium, probably due to ATP hydrolysis by phosphatases released during the incubation.

Iritani and Wells [14] and Hegner [15] have postulated that there is a membrane-bound ATPase in lysosomes. According to the former authors [14] this ATPase is a bicarbonate-stimulated Mg^{2+}-ATPase, whereas Hegner [15] suggests that it is a cation-dependent Mg^{2+}-ATPase. We have investigated the effect of MgATP on the intralysosomal pH under conditions which, according to these authors, would favour the action of such a Mg^{2+}-ATPase.

In Table II an experiment is shown in which the intralysosomal pH was

TABLE II

INFLUENCE OF MgATP ON THE INTRALYSOSOMAL pH AT DIFFERENT BICARBONATE CONCENTRATIONS

Rat-liver lysosomes were incubated for 4 min at room temperature in 1 ml of a medium containing 130 mM KCl, 25 mM Tris, sufficient MES and MOPS to bring the pH to 7.4, ^3H_2O and either [^{14}C]methylamine (1.80 μM; 0.1 μCi) or [^{14}C]sucrose (0.16 μM, 0.1 μCi). In addition where indicated MgATP (5 or 10 mM) and $NaHCO_3$ (5, 10 or 20 mM) were present. $NaHCO_3$ was added immediately before the rat-liver lysosomes. During incubation the Eppendorf incubation tubes were kept closed to prevent the escape of CO_2.

$NaHCO_3$ (mM)	pH_{in} at ATP concentration of		
	0 mM	5 mM	10 mM
0	6.34	6.26	6.27
5	6.35	6.28	6.29
10	6.29	6.24	6.23
20	6.28	6.22	6.23

TABLE III

INFLUENCE OF MgATP ON THE INTRALYSOSOMAL pH IN MEDIA OF DIFFERENT COMPOSITION

Rat-liver lysosomes were incubated for 2 min at room temperature in 1 ml of a medium containing the indicated salt composition, 25 mM Tris, sufficient MES and MOPS in equimolar ratio, to adjust the pH to 7.50, ^3H_2O, and either [^{14}C]methylamine (1.80 μM; 0.1 μCi) or [^{14}C]sucrose (0.16 μM; 0.1 μCi). Where indicated MgATP was added at a concentration of 2 or 5 mM.

MgATP (mM)	pH_{in} in media containing	
	KCl (130 mM), NaCl (10 mM)	NaCl (130 mM) KCl (10 mM)
0	6.09	6.00
2	6.06	5.98
5	5.94	5.95

calculated in the presence of increasing concentrations of bicarbonate. It is clear that MgATP in combination with bicarbonate has no influence on the intralysosomal pH. Under the conditions tested the intralysosomal pH remained about 6.2—6.3. Furthermore, the ion composition of the medium was ineffective in unmasking an ATPase-driven proton pump. In the experiment of Table III the ion composition of the incubation medium was either 130 mM KCl and 10 mM NaCl, comparable to the cytosolic ion composition, or 130 mM NaCl and 10 mM KCl, comparable to the extracellular fluid. Addition of MgATP had no influence on the intralysosomal pH in either case.

The most direct evidence for a MgATP-dependent proton pump in lysosomes has been presented by Schneider and Cornell [16], who measured methylamine distribution. In the experiment of Table IV the effect of MgATP on the accumulation of methylamine in rat-liver lysosomes was compared with that of MgADP and that of MgATP in the presence of uncoupler, under exactly the same experimental conditions as those described by Schneider and Cornell [16]. It is clear that in our hands MgATP has no effect on the accumulation of methylamine under these circumstances. It is important to note that Schneider and Cornell [16] report the results of two experiments in which the effect of MgATP on methylamine uptake was tested. The results differ considerably, the stimulation being about 100% in one experiment and only about 40% in the other. We have also observed that in some preparations MgATP brings about

TABLE IV

INFLUENCE OF MgATP OR MgADP ON METHYLAMINE UPTAKE BY LYSOSOMES

Rat-liver lysosomes were suspended in 200 μl 20 mM potassium MOPS, pH 7.0, 250 mM sucrose, 0.5 mM EGTA, ^3H_2O and either [^{14}C]methylamine (6 μM; 0.1 μCi) or [^{14}C]sucrose (0.54 μM; 0.1 μCi). After incubation for 5 min at room temperature, 1 mM MgATP, 1 mM MgADP, or 1 mM MgATP and 1.5 μM S-13 were added. After an additional incubation for 3 min centrifugation was performed.

Addition	f_{MA}	pH_{in}
MgATP	17.37	5.76
MgADP	19.44	5.71
MgATP + S-13	18.50	5.73

TABLE V

INFLUENCE OF MgATP ON THE INTRALYSOSOMAL pH IN A KCl MEDIUM

Rat-liver lysosomes were incubated for 1 min at room temperature in 1 ml of a medium containing 130 mM KCl, 35 mM Tris, sufficient MES and MOPS, in equimolar ratio, to bring the pH to 7.5, 10 µl ethanol, ^3H_2O, and either [^{14}C]methylamine (1.80 µM; 0.1 µCi) or [^{14}C]sucrose (0.16 µM; 0.1 µCi). Where indicated MgATP (5 mM, pH 7.5) and 10 µg valinomycin were also present. Abbreviations: R_{MA}, ratio [^{14}C]-methylamine/^3H_2O) in pellet/([^{14}C]methylamine/^3H_2O) in supernatant; R_{SU}, ratio ([^{14}C]sucrose/^3H_2O) in pellet/([^{14}C]sucrose/^3H_2O) in supernatant; f_{MA}, accumulation ratio of methylamine in lysosomes after correction of R_{MA} for adherent water. A, correction for adherent water made using mean RSU. B, correction for adherent water made using R_{SU} deteremined in a parallel incubation.

Expt.	Additions	R_{MA}	R_{SU}	f_{MA}		pH_{in} calculated from	
				A	B	A	B
1	None	7.05	0.64	16.44	17.80	6.28	6.25
	MgATP	8.51	0.55	20.25	17.69	6.19	6.25
	Valinomycin	4.53	0.74	10.08	14.58	6.50	6.34
	Valinomycin + MgATP	6.85	0.57	15.91	14.60	6.30	6.34
	Mean		0.61				
2	None	5.51	0.48	9.84	9.67	6.52	6.51
	MgATP	5.71	0.50	10.12	10.52	6.50	6.48
	Mean		0.49				

an apparent increase in the accumulation of methylamine. This is illustrated by the data given in Table V. In Expt. 1 of Table V the addition of MgATP brought about an increase in the distribution factor for methylamine (r_{MA}; column 3). If the mean value for the sucrose space (see column 4) is applied in the correction for adhering water (see Eqn. 1), an apparent increase in the accumulation factor for methylamine is observed (column 5). In the presence of valinomycin this increase is more pronounced than in the absence of the ionophore. However, if the distribution factor for methylamine is corrected for adhering water by applying the appropriate values for the sucrose-permeable space, obtained from parallel incubations in which [^{14}C]sucrose was substituted for [^{14}C]methylamine, the apparent increase in the accumulation factor disappeared completely, both in the presence and absence of valinomycin (column 6). Thus the MgATP-induced increase in the distribution factor of methylamine appears to be due to a change in the volume of lysosomes brought about by the different incubation conditions. In preparations where the volume remains constant, as indicated by a constant value for the sucrose-permeable space, there is also no MgATP-induced shift in the distribution factor of methylamine (Expt. 2 of Table V). The data presented in Table V also indicate that it is unlikely that ATP induces an electrogenic proton movement in lysosomes, for the following reason. If an electrogenic proton influx occurs across the lysosomal membrane in the absence of a permeant anion, the electrochemical proton gradient is mainly determined by $\Delta\psi$. On the other hand, in the presence of a permeant anion, an electrogenic proton movement can induce a shift in the intralysosomal pH. Since Cl^- has been shown to be a slightly permeant anion for the lysosomal membrane [26], a small decrease in pH inside lysosomes would be expected if such an electrogenic proton influx is induced by MgATP. However, Table V shows that no such decrease can be ob-

served if the appropriate correction for adhering water is applied (see also ref. 27).

The data presented in Tables I—V refer to the chemical gradient of protons across the lysosomal membrane only. The possibility that MgATP might induce an electrogenic movement of protons, which would lead to an electrochemical proton potential, was investigated further by testing the effect of addition of ATP on the transmembrane potential. Indeed, it might be expected that in lysosomes the transmembrane electrical potential would be more suitable than the chemical potential of protons for the detection of electrogenic proton influx. Due to the high intralysosomal buffering capacity [28] a considerable influx of protons would have to take place to be able to induce a measurable change in intralysosomal pH.

One method, albeit controversial [29—32], of measuring $\Delta\psi$ is to make use of the fluorescence enhancement of ANS. In order to test whether the fluorescence enhancement can be correlated with a transmembrane potential across the lysosomal membrane, the fluorescence response of ANS was studied in lysosomes suspended in a KCl-containing medium after inducing a transmembrane potential, positive inside, by addition of valinomycin. Due to the rapid influx of K^+ a diffusion potential is generated. In Fig. 2A a typical experiment is shown. If $\Delta F/F$ is plotted against log $[K^+]_{medium}$ (Fig. 2B), a straight line is obtained. Such a linear relationship indicates that the ANS fluorescence is correlated with the diffusion potential.

If there is a Mg^{2+}-ATPase-driven electrogenic proton pump in lysosomes, the expection is that a similar enhancement of ANS fluorescence would be seen on ATP addition. However, as shown in Fig. 3a, on addition of MgATP only an instantaneous slight increase in fluorescence intensity (cf. Fig. 1 of ref. 16) is observed, which can also be reproduced by addition of MgADP (Fig. 3b) or $MgSO_4$ (not shown). Subsequent addition of the uncoupler S-13 leads to a

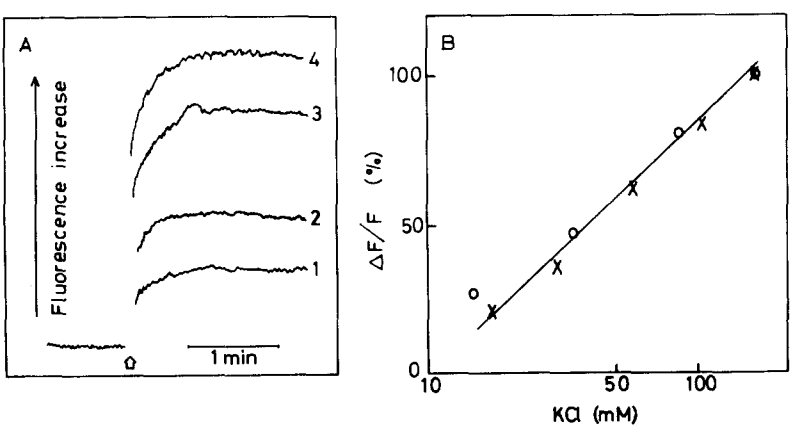

Fig. 2. Enhancement by KCl (plus valinomycin) of fluorescence of ANS bound to lysosomes. (A) KCl at a concentration of 15 mM (curve 1), 36 mM (curve 2), 85 mM (curve 3), or 165 mM (curve 4) were added at the white arrow to a cuvette containing, in a final volume of 2 ml, 300 mM sucrose, 10 mM HEPES-KOH (pH 7.5), 10 μg valinomycin, 5 μM ANS and lysosomes (about 200 μg protein). Incubations were at room temperature. (B) Relative fluorescence enhancement of ANS as a function of KCl concentration. o———o, KCl was added after the addition of 10 μg valinomycin (as in A); X———X, valinomycin (10 μg) was added after the addition of KCl. Experimental conditions were as in A.

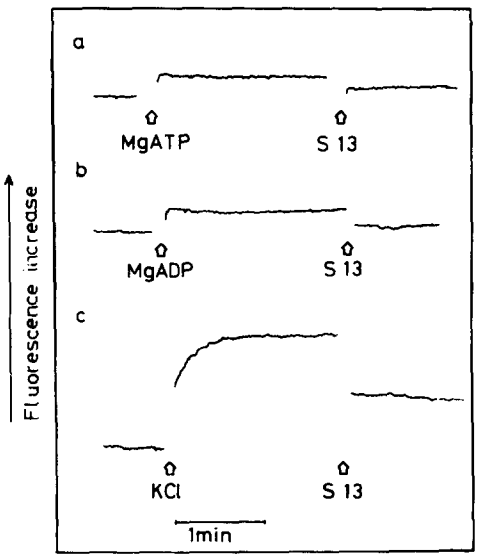

Fig. 3. Effect of MgATP, MgADP and valinomycin plus KCl, followed by S-13 on fluorescence of ANS bound to lysosomes. Lysosomes (about 200 μg protein) were incubated at room temperature in 2 ml of a medium containing 300 mM sucrose, 10 mM HEPES-KOH (pH 7.5) and 5 μM ANS. At the first arrow the following additions were made: a, 12.5 mM MgATP; b, 10 mM MgADP and c, 10 μg valinomycin plus 30 mM KCl. At the second arrow 2 μM S-13 was added.

slight instantaneous decrease in fluorescence in the presence of either MgATP (Fig. 3a, cf. Fig. 1 of ref. 16) or MgADP (Fig. 3b). For comparison, the effect of valinomycin is shown in Fig. 3c. Subsequent addition of S-13 in the presence of valinomycin leads to a substantial decrease in fluorescence.

However, the results of fluorescence probe studies are difficult to interpret [31—33]. For instance, Haynes and Simkowitz [33] have shown that ANS forms a ternary complex with valinomycin and K^+ and that this complex permeates across phospholipid vesicle membranes. We have therefore used an alternative approach, in which the transmembrane potential was calculated from the distribution of a radioactively labelled lipid-soluble anion according to Eqn. 7. In Table VI an experiment is shown in which the transmembrane electrical potential, ΔpH, and the resulting electrochemical proton gradient were measured under different conditions. In the absence of any addition the slow permeation of K^+ [26] generates a membrane potential of 18 mV, which is clearly enhanced after addition of valinomycin. If S-13 is added under these conditions the membrane potential decreases from 18 to 7 mV in the absence of valinomycin and from 40 to 27 mV in its presence. The effects of valinomycin and S-13 on ΔpH shown in Table VI are in agreement with those observed by Reijngoud and Tager [8]. Valinomycin or S-13 added separately induces only a small decrease of the pH difference across the lysosomal membrane, wheras the combination of both ionophores nearly abolishes the pH difference. The resulting electrochemical proton gradients under these conditions are shown in the last column of Table VI. If no ionophores are present the electrochemical proton gradient is 71 mV. This clearly indicates that no equilibrium has been reached between the potassium gradient and proton

TABLE VI

THE INFLUENCE OF VALINOMYCIN, S-13 AND MgATP ON THE DISTRIBUTION OF METHYLAMINE OR THIOCYANATE ACROSS THE LYSOSOMAL MEMBRANE

Rat-liver lysosomes were incubated for 1 min at room temperature in 1 ml of a medium containing 90 mM K_2SO_4, 35 mM Tris, sufficient MES and MOPS, in equimolar ratio, to bring the pH to 7.6, 5 mM $MgSO_4$, 20 µl ethanol, ^3H_2O and either [^{14}C]methylamine (1.80 µM; 0.1 µCi), [^{14}C]thiocyanate (1.67 µM; 0.1 µCi) or [^{14}C]sucrose (0.16 µM; 0.1 µCi). Where indicated 10 µg valinomycin and 4 µM S-13 were also present. When 5 mM MgATP was added $MgSO_4$ was omitted. Abbreviations: f_{CNS^-} and f_{MA}, accumulation ratio in lysosomes of thiocyanate and of methylamine respectively, after correction for adherent water using R_{SU} (see Table V) as determined in a parallel incubation. $\Delta\tilde{\mu}_{H^+} = \Delta\Psi + 60\ \Delta pH$.

Additions	f_{CNS^-}	$\Delta\Psi$ (mV)	f_{MA}	ΔpH	$\Delta\tilde{\mu}_{H^+}$ (mV)
None	1.98	18.0	7.68	0.89	71
Valinomycin	4.53	39.6	4.75	0.68	80
S-13	1.31	7.2	3.21	0.51	38
Valinomycin + S-13	3.02	28.8	1.13	0.05	32
MgATP	2.26	21.6	9.20	0.96	79
MgATP + valinomycin	3.72	34.2	6.49	0.81	83
MgATP + S-13	1.72	14.4	3.68	0.57	49
MgATP + valinomycin + S-13	2.62	25.2	1.17	0.07	29

gradient. If S-13 is added the $\Delta\tilde{\mu}_{H^+}$ clearly decreases, but does not vanish completely. In the presence of valinomycin a small increase in the electrochemical proton gradient is observed, which decreases after addition of S-13.

Finally, the data shown in Table VI clearly show that addition of MgATP has no effect either on the $\Delta\tilde{\mu}_{H^+}$ or on its constituent parts.

When lysosomes were incubated in a chloride medium (Cl^- is slightly permeant in contrast to SO_4^{2-} [26]), analogous results were obtained. Whereas addition of valinomycin and S-13 affects $\Delta\tilde{\mu}_{H^+}$ and its constituent parts, albeit to a smaller extent than in K_2SO_4, addition of MgATP has no effect on ΔpH, $\Delta\psi$ or $\Delta\tilde{\mu}_{H^+}$ (results not shown). Thus it is very unlikely that in lysosomes a Mg^{2+}-ATPase exists which induces an electrogenic proton transport.

Discussion

The studies of Reijngoud and Tager [4], Goldman and Rottenberg [3] and Henning [6] have shown clearly that the main mechanism to set up and maintain a pH difference across the lysosomal membrane is a Donnan-type of equilibrium [8]. The necessity for an energy-dependent proton pump has been questioned since the intralysosomal pH values measured in isolated lysosomes are comparable to those observed in lysosomes in situ [8]. However, it does not rule out the possibility of an auxiliary mechanism in which ATP is involved and which regulates the intralysosomal pH.

In general two types of proton pumps can be distinguished, one which brings about an electroneutral exchange of protons for cations and one which accomplishes an electrogenic proton transport. The ATP-driven K^+-H^+ exchange in vesicles from gastric mucosa is illustrative of the first type [17]. In their review, Reijngoud and Tager [8] suggested that on the basis of the published results on ATP-driven proton transport in lysosomes, an electroneutral exchange could

not be excluded. However, the data presented here suggest the opposite. The K^+ concentration in isolated lysosomes is about 10 mM [27]; the ANS fluorescence response indicates a similar concentration (see Fig. 2B). In view of the lysosomal buffering capacity, complete replacement of the internal K^+ pool by an influx of protons would result in a decrease of the internal pH of about 0.2 unit. Such a decrease was not observed in any of our experiments.

The second type of energy-driven proton transport is electrogenic. The combination of a Donnan equilibrium and an electrogenic ATP-driven proton transport has been documented in chromaffin granules [34]. In the absence of ATP the pH inside the granules is 5.8 at an external pH of 7.5. If ATP is added to a suspension of granules in the presence of chloride, which is a permeant ion in these granules, the internal pH decreases an additional 0.5 unit.

The possibility of electrogenic proton transport in lysosomes has been studied by using the distribution of methylamine and of thiocyanate to monitor the effects of ATP addition on the chemical (ΔpH) and the electrical ($\Delta\psi$) part of the electrochemical proton gradient across the membrane. The $\Delta\psi$ has also been studied by measuring the ANS fluorescence intensity. The results presented clearly demonstrate that no electrogenic ATP-driven proton transport occurs across the lysosomal membrane. In agreement with our results, Henning [6] stated that he, too, has been unable to detect any influence of ATP on the intralysosomal pH at room temperature or 37° C, and using varying Mg^{2+} and K^+ concentrations. In contrast Schneider and Cornell [16] concluded from their study that an ATP-driven proton pump is present in the lysosomal membrane. They reported an ATP-dependent increase in methylamine accumulation and an enhancement in ANS fluorescence intensity. However, in our hands this apparent ATP-dependent increase in methylamine accumulation disappears if the appropriate correction for adherent water is applied.

With respect to the very small apparent enhancement of the ANS fluorescence intensity, our results indicate that this enhancement is due to an increase in the binding of ANS to the lysosomal membrane brought about by the presence of Mg^{2+} salts in the medium. In addition, the specificity in the effects of ATP on the methylamine distribution and ANS fluorescence observed by Schneider and Cornell [16] are clearly different from the properties of a lysosomal membrane-bound ATPase presented by Schneider in a previous paper [35]. For instance, Schneider and Cornell [16] show that CaATP is ineffective in bringing about an increase in methylamine accumulation, whereas in the previous paper [35] it was reported that Ca^{2+} is as effective as Mg^{2+} in eliciting an ATPase in the membrane fraction from lysed lysosomes. Furthermore it is noteworthy that the increase in methylamine accumulation induced by ATP was measured by Schneider and Cornell [16] in the presence of EGTA, although this compound is highly effective in inhibiting the ATPase observed by Schneider [35] in the membrane fraction of lysed Triton-filled lysosomes.

Thus our conclusion is that no ATP-dependent proton pump, whether electroneutral or electrogenic, is present in the lysosomal membrane.

It will be obvious that in lysosomes in situ, the energetic state of the cell may influence the intralysosomal pH indirectly (see ref. 27 for a discussion). Thus the observations of De Duve et al. [36] on the effect of respiratory inhibitors on the accumulation of basic compounds and on the intralysosomal

pH (see also ref. 37) must be due to an inhibition of intracellular processes like membrane fusion, which are dependent on the energy level of the cell.

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