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EVIDENCE FOR AN ATP-DRIVEN "PROTON PUMP" IN RAT LIVER LYSOSOMES BY BASIC DYES UPTAKE

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SUMMARY

When rat liver lysosomes are suspended in a medium containing acridine orange at neutral pH, accumulation of the dye may be observed within the vesicles. The uptake appears driven by a pH gradient between the external medium and the interior of the lysosomes since it is inhibited by NH_4^+, nigericin and other electroneutral proton-cation exchangers. FCCP is ineffective in inhibiting the uptake. In the presence of Mg^{++} and anions such as Cl^-, ATP promoted a further and more extensive but slower oligomycin and ouabain-insensitive dye uptake, which was also inhibited by FCCP. Very similar results were obtained with neutral red and atebrin. When the rate of the ATP-induced acridine uptake in preparations of different purification grade was compared, it was observed that the uptake rate increased in parallel with lysosomal enzymatic activity. These results suggest that an electrogenic ATP-driven-Mg^{++} dependent "proton pump" is operating in the lysosomal membrane, as previously proposed.

It has been reported that lysosomes in cells may accumulate weak bases, especially in the presence of an energy source (1-3). De Duve et al. suggest that uptake of basic dyes in lysosomes follows the classical pathway for uptake of weak bases: the dye permeates the lysosomes in its unprotonated form, and is trapped inside by protonation; a proton pump located in the lysosomal membrane provides the driving force for continuous uptake (4). In isolated lysosomes, uptake of weak bases (5,6), such as methyl--amine, has been observed and its distribution has been used to determine the internal pH. However, attempts to demonstrate uptake stimulation by an energy source have been thus far unsuccessful (7). In this work direct evidence is given for an ATP-Mg^{++}driven uptake associated with a proton pump (8-10) in isolated lysosomes

ABBREVIATIONS

FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone
HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
TCA, trichloro-acetic acid

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for the dyes acridine orange, neutral red and atebrin. Dye movement from the outer medium to the inner lysosomal compartment was followed by monitoring the absorption or fluorescence change (11-13) already reported to accompany their uptake in cellular organelles. As expected for an acidic lysosomal interior, uptake by lysosomes of the dyes mentioned was also observed in an ATP-free medium at neutral (or near) pH. In agreement with previous observations, no uptake was observed for 9 aminoacridine (6).

MATERIALS AND METHODS

Lysosomes from Triton untreated rats, were prepared according to P.L. Sawant et al. (14). Acid phosphatase activity was determined according to the method of Gianetto and De Duve (15), employing β-glycerolphosphate as substrate at pH 5, by means of 15 min assays at 37° in a medium containing 50 mM acetate buffer and Triton at a final concentration of 0.1-0.2%. The reaction was halted by addition of TCA (final concentration, 11%). Released P_i was measured by the method of Taussky and Shorr (16). Control experiments were also performed by adding TCA before β-glycerolphosphate. Reported data are the average of three measurements. Protein was determined according to Lowry et al. (17). Acridine orange and neutral red uptake by lysosomes was followed spectrophotometrically at 20°, with an Aminco DW-2a UV-VIS spectrophotometer by measuring the change in dye absorbance at 492 nm, with 540 as the reference lengthwave for acridine orange, and at 530 nm, with 620 nm as the reference lengthwave for neutral red. In order to detect any uptake of atebrin and 9-aminoacridine by lysosomes, dye fluorescence was measured employing a Hitachi MP-2A spectrofluorimeter at room temperature (20° ± 2°). The wavelength pairs for excitation and emission, 420 nm and 500 nm, and 400 nm and 440 nm were employed for atebrin and 9-amino acridine, respectively. The basic medium in experiments of dye uptake contained 0.1 M KCl, 5-10 mM $MgSO_4$, and 20 mM Hepes at pH 7. Acridine orange (Merck) was purified according to Pal and Schubert (18), while neutral red and 9-aminoacridine (Merck) and atebrin (Sigma) were used without further purification. All the other reagents employed were of the highest analytical grade commercially available. The lysosomes enriched fractions were used within a few hours following their preparation and were maintained before use at 0° in a preparation medium containing 0.25 M Sucrose and 1 mM EDTA at pH 7.

RESULTS

Fig. 1 shows some results obtained using acridine orange as the "photometric" probe. Beside the "probe", the medium, which was buffered at pH 7, also contained 0.1 M KCl and 5 mM $MgSO_4$. Addition of the lysosomal suspension (fractions F I or F II of the Sawant et al. preparation (14)) caused a decrease in the absorbance of the dye, after an apparent rapid increase due to turbidity of the lysosomal

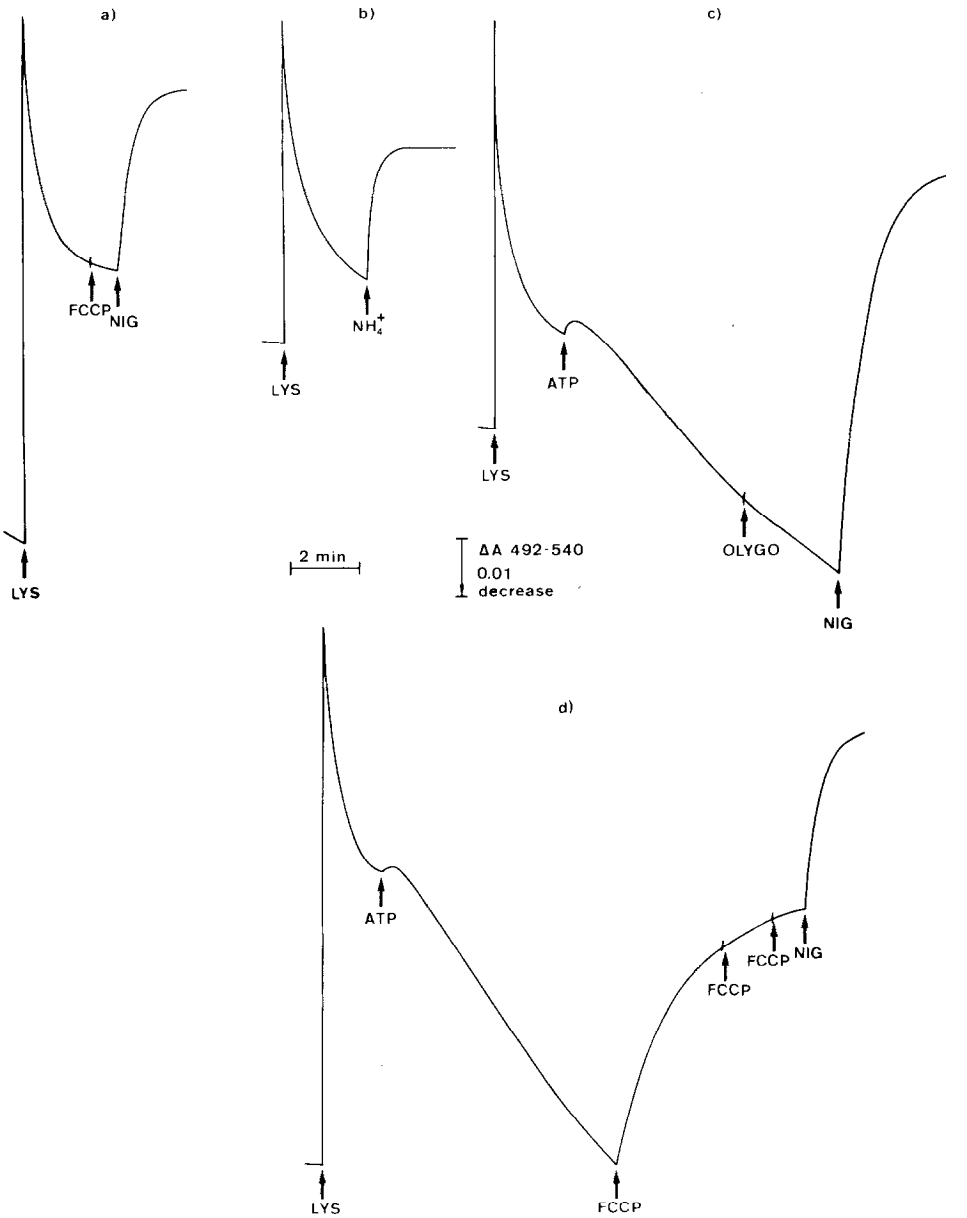

Fig. 1 - Acridine orange uptake by lysosomes and stimulation of the uptake by ATP. Inhibitory effects of NH_4^+, nigericin and FCCP. The medium (2.5 ml) contained: 8 μM acridine orange, 0.1 M KCl, 10 mM $MgSO_4$, 20 mM Hepes pH 7. ATP addition 1 mM. Other additions were: a) lysosomes 0.34 mg/ml of protein, 3 μM FCCP, 2 μg nigericin, b) lysosomes 0.23 mg/ml of protein 10 mM $(NH_4)_2SO_4$, c) lysosomes 0.23 mg/ml of protein, 5 μg oligomycin, 2 μg nigericin each addition, d) lysosomes 0.34 mg/ml of protein, 2 μM FCCP each addition, 2 μg nigericin. The acid phosphatase activity (pH 5) of the lysosomal suspensions was: a) d) 4.9γ P_i/mg x min, b) c) 2.6γ P_i/mg x min.

suspension. The decrease was reversed by NH_4^+, or nigericin (plus K^+) (fig. 1 a,b). Ca^{++} plus A23187 and Triton at very low concentrations ($\sim$ 0.01%) had a similar effect (not shown), while FCCP (fig. 1 a) was ineffective, unless valinomycin was also added (not shown). Subsequent addition of 1 mM ATP started a more extensive, though slower phase of absorbance change (fig. 1c), preceeded by a small enhancement phase. This latter effect, already observed in similar studies (12,19,20) will not be commented here(for discussion see however refs. 19,20). The absorbance decrease was reversed again by nigericin plus K^+, NH_4^+ and now also by FCCP. Oligomycin added after ATP, or preincubated ouabain (0.5-1 mM) (not shown), were both ineffective (fig. 1c). Similar results were obtained with other fractions of the lysosomal preparation (14) but, as expected, with different rates and extents of absorbance changes (see below). The experiments carried out using acridine orange as the "probe" were repeated successfully by using neutral red and atebrin (by following the fluorescence change for the latter) and some findings (obtained using high purity preparations) are shown in fig. 2. No effect of the lysosomal preparation on the dye fluorescence was observed when 9-aminoacridine was employed as the "probe". When considering, in the above experiments, the overall absorption spectrum of the dyes acridine orange and neutral red (not shown), it may be concluded that the change in absorption corrisponds to a change in the dye spectrum from the "ortho" to the "meta" form (21). These changes are generally accepted as indicative of the dye uptake (11) and accumulation within the interior of the vesicular organelles (13,20) where, presumably the dye binds to anionic sites (20), much as for the fluorescence quenching of 9-aminoacridine or atebrin. The ATP-induced uptake shown above appeared dependent upon Mg^{++} and anions. In fig. 3a, the medium did not contain Mg^{++}. In fig. 3b, Cl^- was replaced by the less lipophilic anion SO_4^{--} (K^+ concentration was invaried), and in order to observe the usual ATP-driven uptake, Mg^{++} not Ca^{++} and KCl respectively, had to be added. In a KCl-free medium (fig. 3c), stimulation was also obtained by adding valinomycin. No anion dependence was, instead, observed for the ATP-independent uptake (compare fig.3a and 3b). The ATP-Mg^{++} induced dye uptake rate appeared to depend linearly on the protein content of the lysosomal addition, especially with the lower protein concentrations (fig.

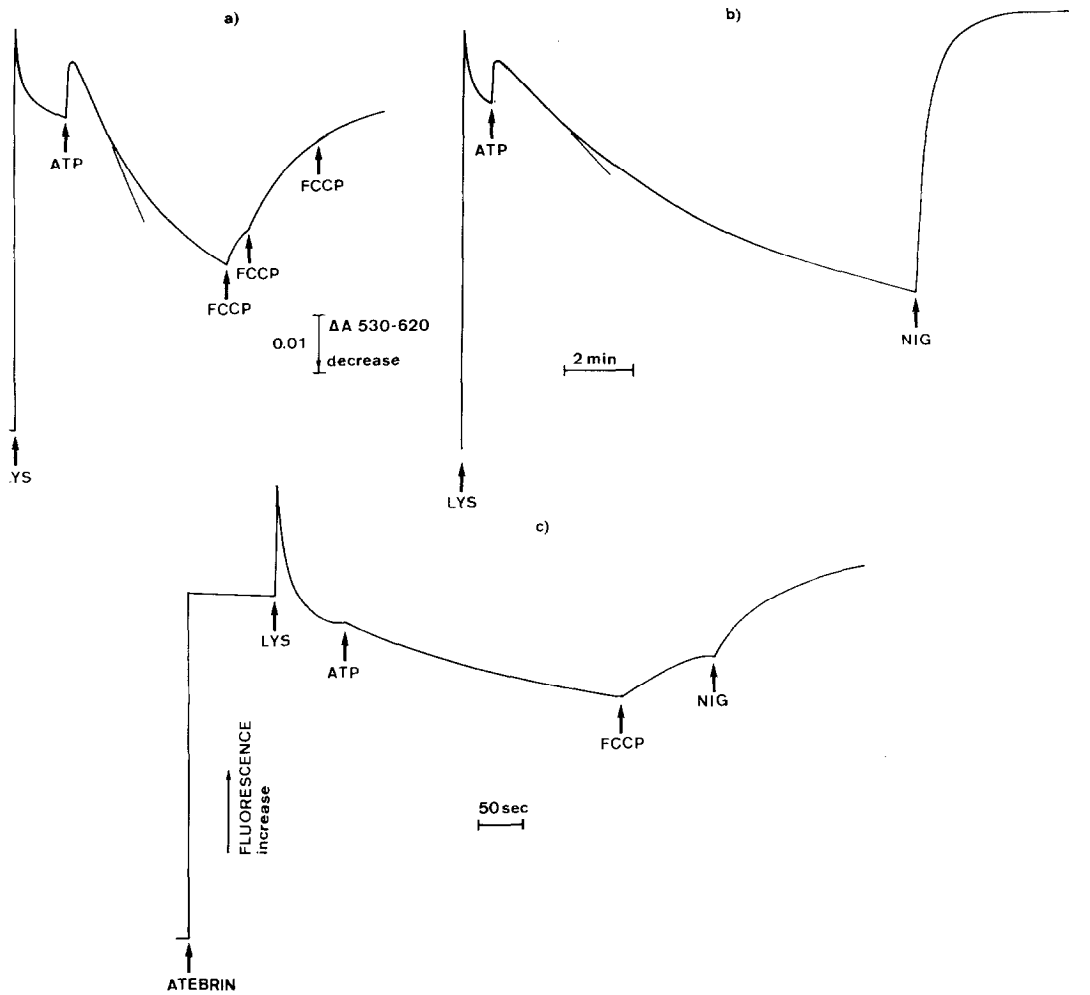

Fig. 2 - Neutral red and atebrin uptake by lysosomes. The medium (2.5 ml) contained 0.1 M KCl, 5 mM $MgSO_4$ and a) b) 10 µM neutral red, 10 mM Hepes pH 6.5, c) 10 µM atebrin, 10 mM Hepes pH 7.5. Additions: 1 mM ATP, 2 µg nigericin, 3 µM FCCP. Lysosomes additions were: a) 0.88 mg/ml, b) 0.22 mg/ml c) 0.35 mg/ml of protein. Acid phosphatase activity of the lysosomal suspensions (pH 5) was: a) 7.5, b) c) 18.4 γ P_i/mg x min.

4B). Thus, the initial rate of uptake per mg of protein, which progressed increasingly in order for the fractions of increasing purification grade could be estimated directly. A similar dependence was found when the extent of the ATP-independent photometric change (lysosomes induced) was plotted against the protein concentration (Fig. 4A). A plot of the dye uptake rate per mg of protein against the acid phosphatase activity (fig. 4C), which summa-

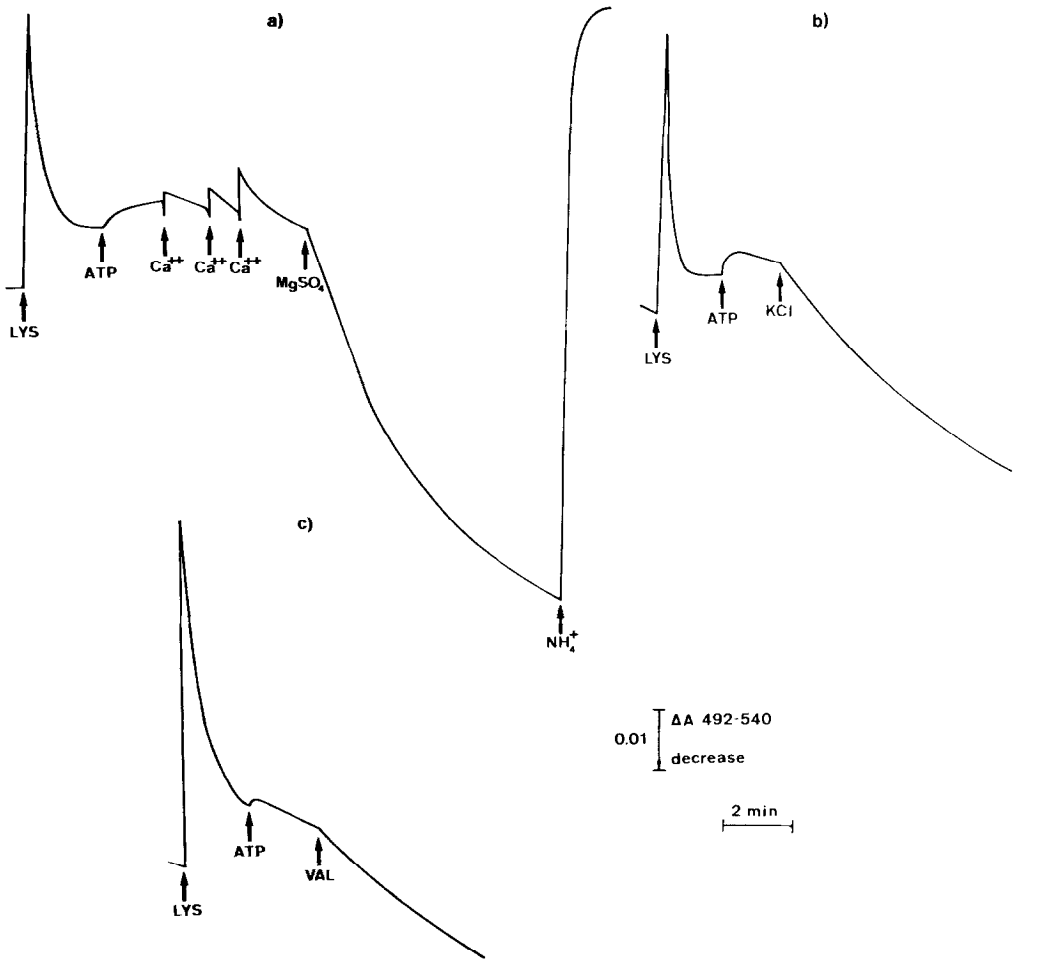

Fig. 3 - Mg^{++} and anion effect on the ATP-driven acridine orange uptake by lysosomes.
The medium (2.5 ml) contained: 8 μM acridine orange, 10 mM Hepes pH 7 and a) 0.1 M KCl, b) 40 mM K_2SO_4, 5 mM $MgSO_4$, c) 0.2 M sucrose, 10 mM $MgSO_4$. Lysosomes additions were: a) b) 0.48 mg/ml, c) 0.26 mg/ml of protein. Acid phosphatase activity of the lysosomal suspensions (pH 5) was a) b) 7.0 γ P_i/mg x min, c) 10.1 γ P_i/mg x min. In experiment a) Ca^{++} and Mg^{++} additions as indicated in the Figure. Other additions: 1 mM ATP, 1.5 μg valinomycin, 8 mM $(NH_4)_2SO_4$, 30 mM KCl.

rizes the results obtained with preparations of different purification grade, revealed thus a direct correlation between the photometric and enzymatic parameter.

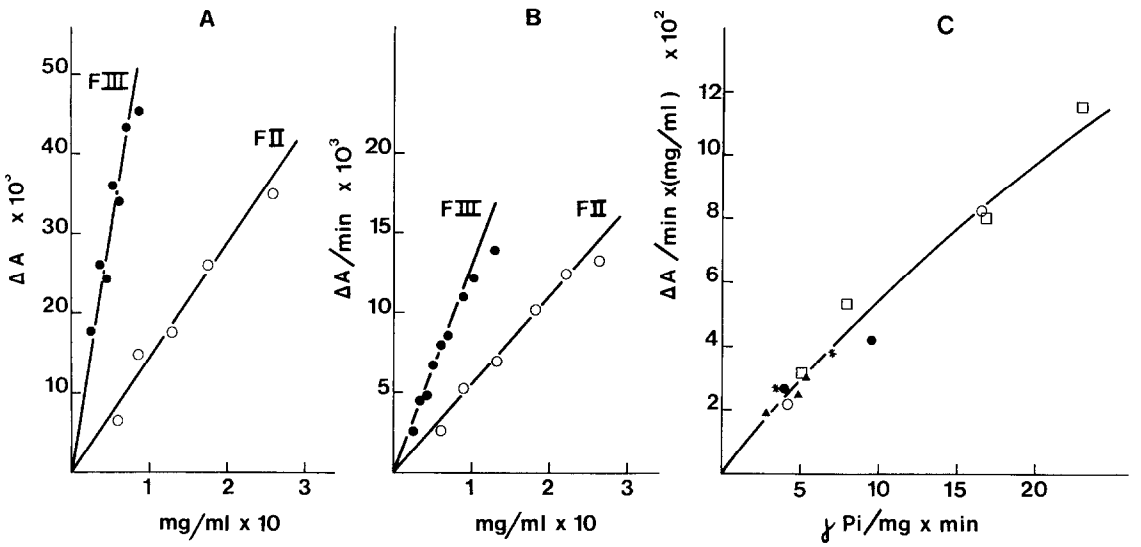

Fig. 4 - Comparison between photometric response and lysosomal enzymatic activity.
In A) the extent of the acridine orange photometric change induced by the addition of lysosomes, in the absence of ATP, is plotted versus protein concentration, for two lysosomal preparations, indicated as F II and F III, of different acid phosphatase activity (7.2 and 23.4γ P_i/mg x min, respectively). In B) as in experiment A, except in ordinate the rate of the ATP-induced acridine orange change is plotted. The rates were evaluated at the beginning of the decrease phase, as shown in Fig. 2. In C) the rate of the ATP-induced acridine orange uptake per mg/ml is plotted against the acid phosphatase activity. The rates were measured in the same basic medium as A and B (see Methods). Identical symbols corrispond to different fractions obtained in a single preparation. Pellets, especially those corresponding to fraction F I (14), were not well defined and, depending on the criterion adopted for pellet removal, some variability in the lysosomal enzymatic activity in the different preparations, was recorded. Acid phosphatase activity was determinate at pH 5. B) C) ATP addition 1 mM.

DISCUSSION

The involvement of a pH gradient in the acridine response has been discussed elsewhere (12,20,22),however, it seems further supported by the present findings that the acridine response may be inhibited by high NH_4^+concentrations, proton ionophores, or proton-cation exchangers. A major problem arises when the above result is evaluated in regard to the question of the heterogeneity of the lysosomal preparation (see below).

The finding of a lysosomal ATP-driven proton pump, which is suggested by the acridine uptake observed, is in agreement with a

recent report of a lysosomal ATP-ase (23). However, in contrast with ref. 23, in the present data, the ATP-driven proton translocation appeared activated only by Mg^{++} and not also by Ca^{++} (fig. 3a). It must be underlined that only the ATP-Mg^{++} induced uptake is FCCP sensitive, and therefore it should be associated with an electrogenic proton pump. The first uptake phase seems due to a residual pH gradient without a charge "unbalance" (24). However,in the absence of ATP, which occurs during the preparation, it is possible that the membrane potential, if it existed "in vivo", could perhaps relax, in consideration also of the cation permeability of lysosomes at low (0°)temperature (25). Obviously this point would deserve greater comment. Another point supporting the "in vitro" electrogenic nature of the observed proton translocation is the stimulation of the dye uptake by lipophilic anions, or by valinomycin in a K^+-free medium (fig.3c) which would cause K^+ efflux. Both ion translocations would transiently collapse the membrane potential and permit new proton translocation. The ionophore requirement fulfilled in order to observe ΔpH collapse (FCCP plus valinomycin or nigericin for the ATP-independent, FCCP or nigericin for the ATP-dependent dye uptake) confirms the low, if any, permeability of H^+ and K^+ at 20° of the lysosomal membrane (24). In this regard, it may be observed that the rate of the ATP-dependent dye uptake is much lower than the energy independent uptake. Therefore, not acridine, but the anion or primary H^+ translocation may be considered as the rate limiting step, for the ATP-driven uptake. As reported above, no fluorescence quenching was observed when 9-aminoacridine was used. This acridine derivative has been shown to have a lower affinity towards the organic phase, as compared to the other dyes here tested, and this parameter appeared critical in observing uptake in submitochondrial particles (20) (for a thorough theoretical analysis of the problem see ref. 4). In fact in experiments on acridine uptake by lysosomes (6), in a ATP-free medium 9-aminoacridine was reported much less bound,than acridine orange, and thus it may be presumed that 9-aminoacridine is too low in the interior of lysosomes for the mechanism of dye fluorescence quenching to operate (20). However, most probably, the dye is only bound to external sites of the membranes, considering that the binding seems affected by cations, such as K^+ or Na^+ (see also ref. 26). Similar arguments or perhaps lipophilic anion deficiency could explain the lack of stimulation by ATP on amine uptake (7).

In regard to the problem concerning the heterogeneity of the lysosomal preparation, it may be observed that 1) acridine dyes are taken up by cells and concentrate within the lysosomes (1-3); 2) the uptake observed in the present study, parallels the lysosomal enzymatic activity; 3) contamination by submitochondrial particles that in energized conditions are active in taking up protons (20,27) may be excluded because the dye uptake is not inhibited by oligomycin; 4) Ca^{++} or ouabain do not affect the uptake, suggesting that sarcoplasmic reticulum or plasmatic membranes are not involved.

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