Electrogenicity of the lysosomal proton pump

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Using acridine orange as a pH gradient probe, the effects of valinomycin and FCCP on the pH gradient across lysosomal membranes in an ATP-free medium as well as on the rate of the inward ATP-driven proton translocation were investigated. Both lysosome-enriched and highly purified lysosomal preparations from rat liver were used with identical results. Ionophore effects were found to be different depending upon whether passive ion fluxes or ATP-driven H^+ translocation were involved and supported the existence of a membrane potential in the latter case. Anions stimulated the rate of ATP-driven proton translocation and stimulation increased with increasing anion lipophilicity. These results strongly support the electrogenic nature of the lysosomal proton pump.

Lysosome *Proton transport* *ATPase* *Acridine orange* *Electrogenic proton pump* *(Rat liver)*

1. INTRODUCTION

The experimental findings in recent years in support of the thesis of a proton 'pump' as the main mechanism for internal lysosomal acidification [1–4] have been based on various techniques: measurement of transmembrane pH gradient by means of methylamine distribution [5]; determination of energy-dependent amino acid accumulation in lysosomes [6]; use of the fluorescent pH indicator, fluorescein isothiocyanate-conjugated dextran, trapped within lysosomes by endocytosis [7], and spectrophotometric [8,9] or fluorimetric [10] monitoring of the dye acridine orange, which accumulates in lysosomes as a weak base. It was questioned in [5,7,8] whether the claimed H^+-ATPase was electrogenic or electroneutral. While the requirement for anion in order to observe ATP-driven basic dye uptake [8], as well as ATP-driven acidification of the lysosomal interior (monitored by the fluorescent pH indicator) [7] seems to support the electrogenicity of the pump, other findings obtained using the methylamine technique support its electroneutrality [5].

We used here the acridine technique to follow the development or dissipation of pH gradients across lysosomal membranes in lysosome-enriched or highly purified lysosomal preparations, also measuring a few times K^+ and H^+ with conventional electrodes. Following our previous observations [8], this report presents a deeper analysis of the question of the electrogenicity of ATP-driven H^+ translocation, based mostly on the effects of valinomycin and FCCP (well-known K^+ and H^+ carriers, respectively) on 'passive' and ATP-driven acridine orange uptake. From a comparison of the results obtained under two different sets of conditions, it is concluded that in the presence of ATP, the electrochemical potential of the inner K^+ is shifted to a higher value. This effect is attributed to an internally positive membrane potential created by an electrogenic proton-translocating ATPase.

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

2. MATERIALS AND METHODS

Lysosome-enriched preparations were obtained from rat liver as in [11], except that the procedure

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was arrested at fraction II [11]. A rather variable increase in the specific activity of acid phosphatase over that in homogenate was obtained; purification grade, defined as the ratio between acid phosphatase of the fraction and that of the homogenate was 5–18 (average ~12) in more than 40 preparations. In some trials, the technique in [10] was employed to obtain a lysosome-enriched preparation; average purification grade of these preparations was close to that reported above, but variability was smaller. Identical results were obtained when either preparation was employed. Highly purified lysosomes were prepared as in [13], by centrifugation of a lysosome-enriched fraction in a metrizamide gradient; in a few trials the metrizamide solutions were supplemented with 10–15 mM KCl for the purpose described below.

The activities of the following enzymes were determined as markers for various subcellular organelles: 5′-nucleotidase for plasma membranes [14], cytochrome *c* oxidase for mitochondria [15], glucose-6-phosphatase [14] for endoplasmic reticulum, and acid phosphatase [16] with minor modifications [9] for lysosomes. P_i was measured as in [17]. In some trials purified lysosome preparations were obtained by centrifugation of the lysosome-enriched fraction in a Percoll gradient. The heavier density fractions were collected as in [9,18] and subjected to acid phosphatase and 5′-nucleotidase assay. Enrichment in specific acid phosphatase activity over the homogenate of the subfraction L_2 defined in [13] was as high as 60–70-fold in the best preparations. The lysosomal preparations were stored at 0–4°C, the lysosome-enriched preparations were suspended in 0.25–0.3 M sucrose and 5 mM Hepes (pH 7.2) at 8–15 mg protein/ml, and the highly purified lysosomes, usually obtained at lower concentra-

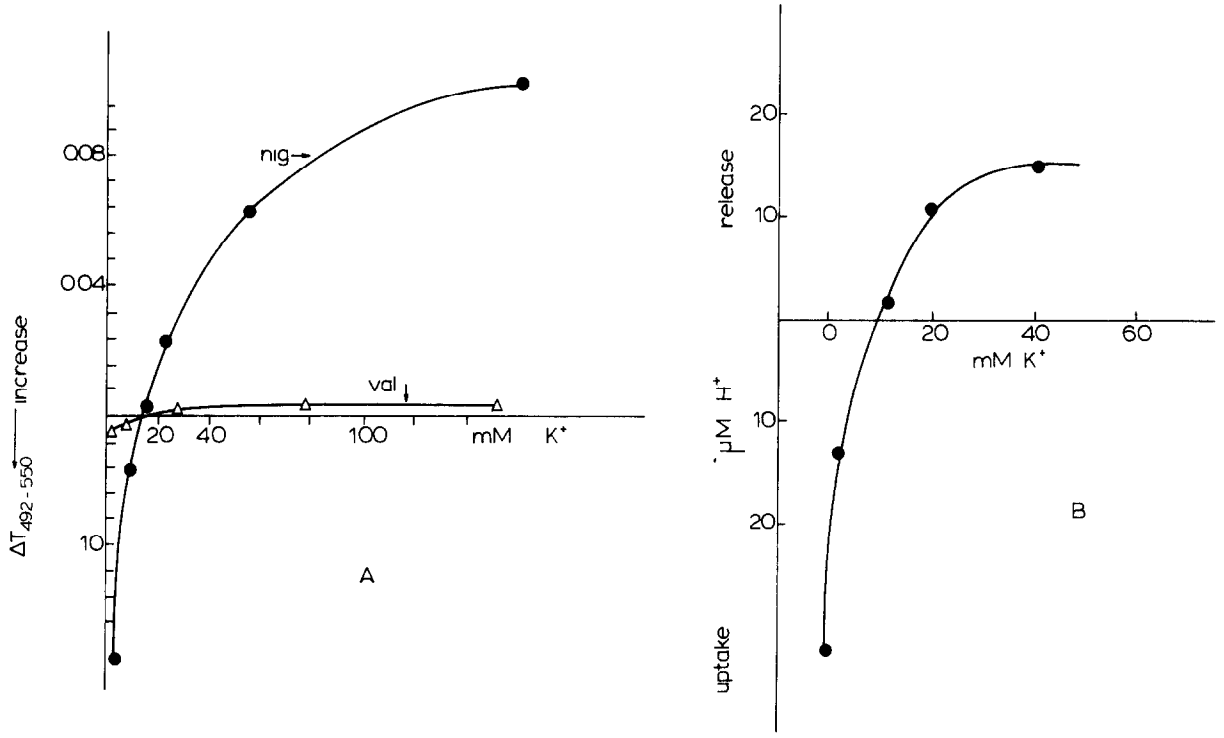

Fig.1. Comparison between H^+ electrode and dye response to ionophore addition to the lysosomal suspension. Effect of changing K^+ medium concentration. (A) Dye response (the extent of absorbance change is shown) to addition of nigericin (1 μg/ml) or valinomycin (1 μg/ml). The medium (2 ml) contained the indicated K^+ concentration (as the SO_4^{2-} salt) and sucrose to give a total (sucrose plus K_2SO_4) osmolarity of 0.24 osM, 3–5 mM $MgSO_4$, 15 mM Hepes plus Tris to bring the pH to 7.1, 12 μM acridine orange. Protein content was 0.35 mg/ml. (B) H^+ electrode response to nigericin addition (1 μg/ml) to the lysosomal suspension. Medium (3 ml) as in A, except Hepes was 3–4 mM and protein was 0.9 mg/ml. Lysosome-enriched preparations were used.

tions (1–3 mg/ml), were kept in the same metrizamide or Percoll solution collected from the centrifugation tubes and used, in dye uptake experiments, as soon as prepared. Protein was determined as in [19], or with the Coomassie blue method [20] using bovine serum albumin as standard. This latter method was preferred with lysosomes obtained with the metrizamide or Percoll gradient, since metrizamide and Percoll do not interfere with color development, as occurs in the former [13,18]. Percoll and metrizamide instead did not interfere with any of the enzymatic assays.

Acridine orange (Merck, purified as in [9]) uptake (or release) was followed spectrophotometrically, by measuring the decrease (or increase) in dye absorbance at 492 nm, with 550 nm as the reference wavelength [21], using a double-wavelength apparatus, constructed in the workshop of the Institute of Physics of the University of Padova. pH changes were measured with a glass electrode and a Radiometer pHM 84 pH-meter connected to a Linseis recorder. K^+ uptake or release by lysosomes was measured with a Schott K^+ electrode connected to the above recording apparatus. All chemicals were analytical grade.

3. RESULTS

3.1. *Passive H^+/K^+ exchange*

It has been previously shown that nigericin, a known catalyzer of H^+/K^+ exchange, may induce lysosome uptake or release of acridine dye, depending on the medium's K^+ concentration [9]. When, in addition to the dye technique (fig.1A), an H^+ electrode was used to follow nigericin-induced H^+ translocation, it was observed that lysosomal uptake or release of H^+, or no H^+ movement also depended on the K^+ concentration of the medium (fig.1B). Using either technique, it was found that when nigericin-induced H^+ movement did not occur (null point), K^+ concentrations were similar (10–15 mM) when the medium pH was about 7.1 (fig.1). Thus, at these K^+ and H^+ medium concentrations, the two ions are in equilibrium across the lysosomal membrane, according to the classical equation,

$$H^+_{in}/H^+_{out} = K^+_{in}/K^+_{out}$$

Fig.1A also illustrates the results obtained in a set of identical experiments in which valinomycin was added, and the dye technique was used to follow proton translocation. The pattern seen was similar to that observed with nigericin, but a very low rate and extent (at similar ionophore concentration) of H^+/K^+ exchange, when detectable, were found. Valinomycin + FCCP in micromolar concentration, but not FCCP alone, mimicked the nigericin effect (not shown); FCCP (fig.2) did not affect the pH gradient, acid inside, set up by nigericin, in the way described above (fig.1).

3.2. *Valinomycin and anion stimulation of ATP-driven dye uptake*

It has also been reported that in a medium containing Mg^{2+} and anions like Cl^-, ATP induces

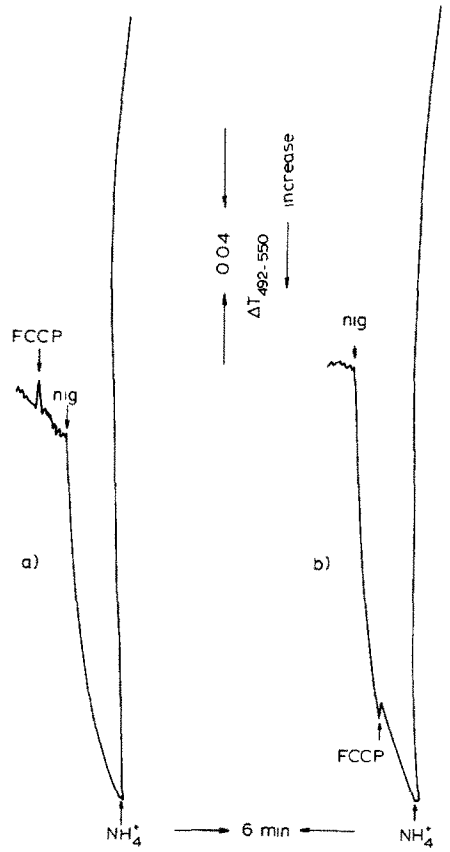

Fig.2. FCCP insensitivity of the pH gradient driven by nigericin in lysosomes, at low medium K^+. Medium: 12 μM acridine orange, 0.25 M sucrose, 40 mM choline chloride, 3 mM KCl, 15 mM Hepes (plus Tris) (pH 7.1). Protein (from a lysosome-enriched preparation) was about 0.3 mg/ml. FCCP and nigericin addition, 8 μM and 0.5 μg/ml, respectively.

further dye uptake by lysosomes in addition to that observed in an ATP-free medium, thus indicating an ATP-driven acidification of the lysosomal interior. This finding was obtained using a lysosome-enriched preparation from normal rats [8,9], as well as lysosomes from Triton-treated rats [10]. Fig.3A shows that valinomycin addition after ATP, in an experiment with a lysosome-enriched

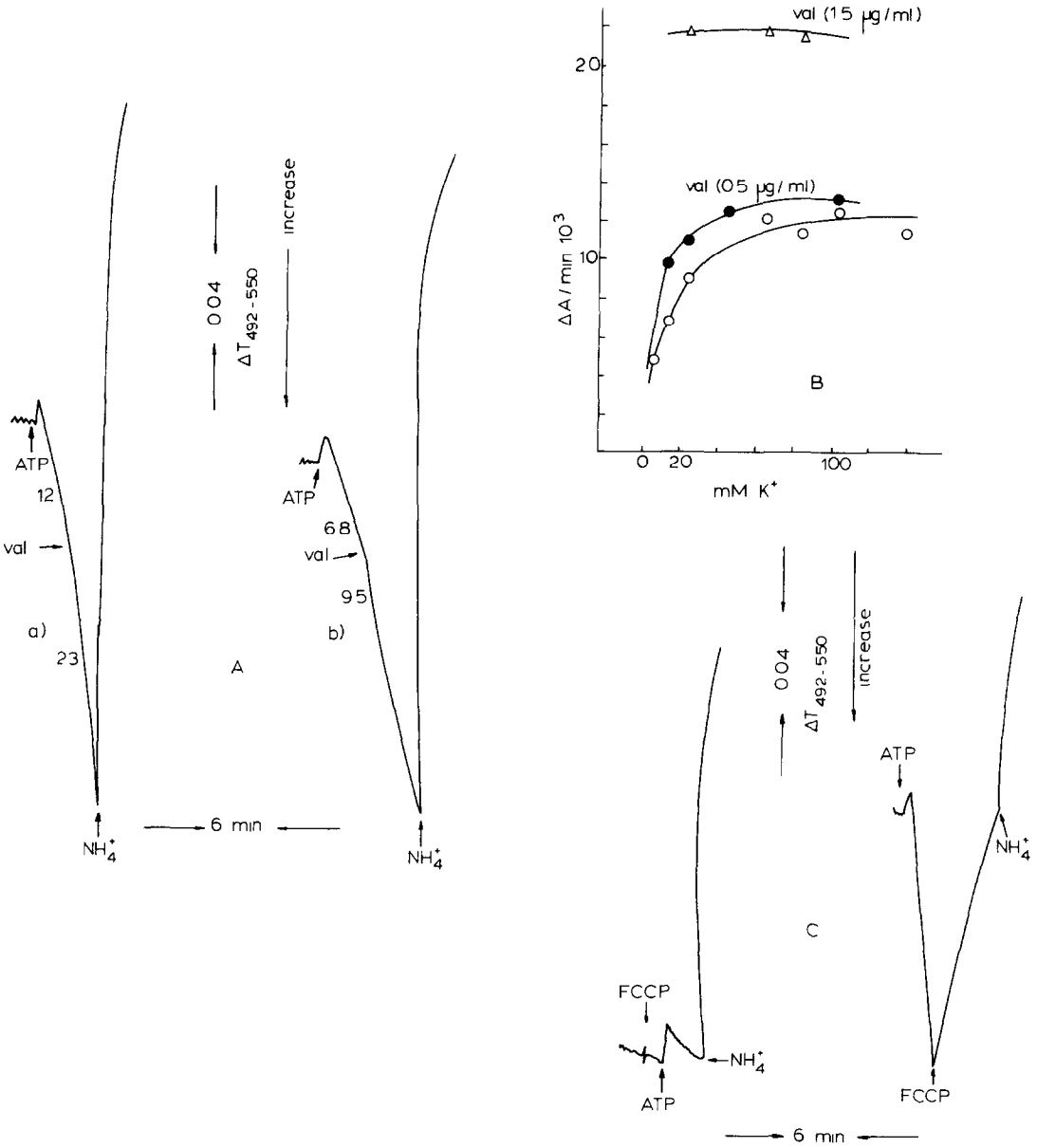

Fig.3. Valinomycin or FCCP effect on the rate of ATP-driven dye uptake by lysosomes. (A,B) Traces a and b, 60 mM K^+ (as SO_4^{2-} salt) and 5 mM K^+ (as SO_4^{2-} salt), respectively, was present in the medium (2 ml), which also contained sucrose to give an (sucrose plus K_2SO_4) osmolarity of 0.24 osM, 3–5 mM $MgSO_4$, 15 mM Hepes (plus Tris) (pH 7.1), 12 μM acridine orange. ATP was added (1 mM) from a 0.4 M solution neutralized (pH ~7.1) by Tris. Valinomycin additions: (a) 1.5 μg/ml, (b) 0.5 μg/ml. NH_4^+ (as SO_4^{2-} salt) addition. 10 μl from a saturated solution. Protein (a lysosome-enriched preparation was used) about 0.35 mg/ml In A, numbers near traces indicate initial slopes in $\Delta A \cdot min^{-1} \times 10^3$. (C) FCCP addition, 8 μM; medium, protein content and other additions as in fig.2.

preparation at both low (5 mM) and high (60 mM) external K^+, added as the SO_4^{2-} salt, stimulated the dye uptake rate; results obtained at these and other concentrations are summarized in fig.3B. At high valinomycin concentrations (1.5 μg/ml), a 2–2.5-fold stimulation was observed. The valinomycin effect was also observed in a KCl medium, but the effect was less significant due to a higher dye uptake rate at high KCl concentration. It is noteworthy that valinomycin stimulation was observed at K^+ concentrations (>20 mM) where nigericin, and to a much lower extent, valinomycin in the absence of ATP induced instead H^+ release (and thus K^+ uptake) as shown in fig.1. Moreover, FCCP prevented ATP-driven dye uptake; when FCCP was added after ATP, it blocked and partially reversed the effect (fig.3C). Attempts to measure K^+ extrusion by valinomycin, following ATP addition, with a K^+ electrode at low medium K^+ were unsuccessful. Even though K^+ release can be inferred in exchange with H^+, its occurrence was most likely below the sensitivity of the apparatus.

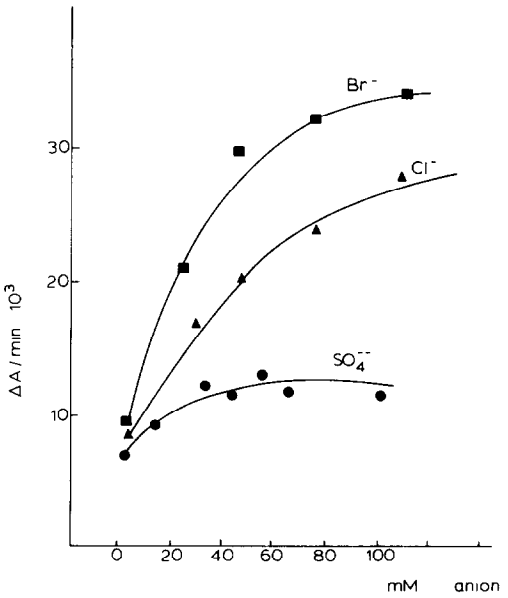

Fig.4. Effect of anion on the rate of the ATP-driven dye uptake. Medium (2 ml) contained 3–5 mM $MgSO_4$, 15 mM Hepes plus Tris to bring the pH to 7.1, anion (as K^+ salt) at the indicated concentration and sucrose to bring the salt plus sucrose osmolarity to 0.24 osM, and 12 μM acridine orange. The rate was estimated at the beginning of the ATP-driven dye uptake which was linear, in the transmittance scale, for several minutes. ATP addition and protein content (a lysosome-enriched preparation was used) as in fig.3A,B.

Fig.4 illustrates the effect of some anions on the rate of ATP-driven dye uptake, which increased with increasing anion concentration until a maximum was reached. The maximum value depended on the anion tested: it increased in the order $SO_4^{2-} < Cl^- < Br^-$, which corresponds to the increase in anion lipophilicity.

3.3. *Valinomycin effect on ATP-driven proton uptake in highly purified lysosomal preparations*

Experiments similar to those illustrated in fig.3 with lysosome-enriched preparations were repeated using highly purified lysosomal preparations obtained by a metrizamide gradient as in [13] and in a few trials lysosome preparations obtained by a Percoll gradient [9,18]. The former preparation, highly purified in lysosomes (>60-fold), contained a low level of the endoplasmic reticulum marker glucose-6-phosphatase and of the mitochondrial marker cytochrome *c* oxidase (relative specific activity over the homogenate, 0.2–0.3 and 0.3–0.5, respectively) but was enriched in the plasma membrane marker 5′-nucleotidase (4–6-fold). Thus, in agreement with [13] the main contaminant in this preparation appears to be plasma membrane. In the latter preparation the level of purification in lysosomes was lower, not more than 20-fold, but as also reported [18] a low level of 5′-nucleotidase was found (relative specific activity over the homogenate 1–2.5 in our preparations). Moreover, no enrichment in the Golgi enzyme, galactosyltransferase, in the heavier density fractions of a Percoll gradient has been reported [18]. Thus this preparation is also suitable for control experiments.

We have previously reported, in agreement with [10], but in contrast with [22], that ATP-driven dye uptake could also be observed in highly purified lysosome preparations [9], obtained with a metrizamide gradient as in [13]. However, considering the increased purification grade, the dye uptake rate was not as high as expected, and this was attributed in part to some inhibitory action by metrizamide. In initial experiments with highly purified lysosomes (not shown), similar to those illustrated in fig.3 with lysosome-enriched prepara-

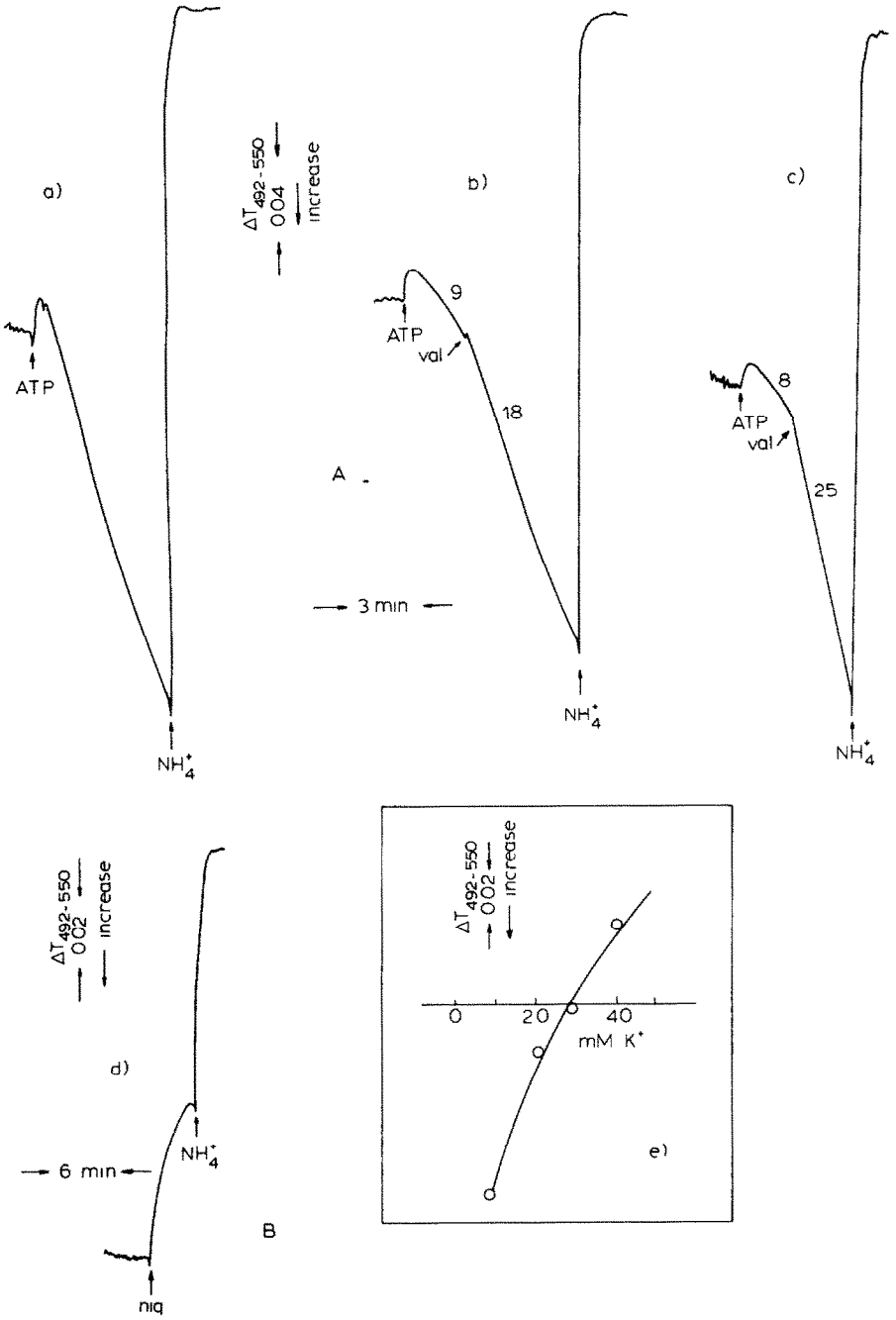

Fig.5. (A) ATP-driven dye uptake from highly purified lysosomes. Effect of valinomycin. Highly purified lysosomes were prepared as in [13] with minor modifications (see section 2). Protein content in experiments was 0.05–0.06 mg/ml. Medium contained 3–5 mM $MgSO_4$, 15 mM Hepes (plus Tris) (pH 7.1), 12 μM acridine orange and (a) 125 mM KCl, (b) 25 mM K_2SO_4 plus sucrose to bring the salt plus sucrose osmolarity to 0.24 osM and about 1.6 mM KCl (deriving from the metrizamide solutions, see section 2), (c) about 1.6 mM KCl (deriving as in b) plus sucrose to bring the salt plus sucrose osmolarity to 0.24 osM. ATP, valinomycin and NH_4^+ addition as in fig.3. Numbers near traces indicate initial slopes in $\Delta A \cdot min^{-1} \times 10^3$ (approximated to whole numbers). (B) H^+/K^+ exchange driven by nigericin in highly purified lysosomes. Effect of changing K^+ medium concentration. Medium as in A, experiment c, except: in (d) K^+ was 40 mM and in the inset (e) K^+ concentration as indicated. Protein content was 0.02–0.03 mg/ml.

tions, valinomycin-induced stimulation of the ATP-driven dye uptake rate was not significant, while ATP-independent dye uptake monitoring of the pre-existing pH gradient [9] was strongly enhanced, and the null point for H^+/K^+ exchange was drastically lowered. These findings both suggest that an H^+/K^+ exchange takes place during the long metrizamide centrifugation step (see section 2). In fact (fig.5B), when the metrizamide solutions were supplemented with 10–15 mM KCl, a higher null point value was observed, indeed consisting of 10–15 mM more than expected (see section 4). Moreover, a higher rate of ATP-driven dye uptake per unit of protein concentration was found (note the difference in protein content and time scale in comparison to fig.3 referring to lysosome-enriched preparations); furthermore valinomycin stimulation increased 2–3-fold, nearly the same as before (fig.5A). Similar experiments (not shown) using lysosome preparations obtained by a Percoll gradient or performed in the presence of oligomycin at high concentration (>2.5 μg/ml) with either preparations gave identical results.

4. DISCUSSION

4.1. *Use of the dye as a probe for ΔpH*

As shown in [8,9], the decrease or increase of acridine orange absorbance is associated with a respective increase or decrease in the pH gradient across the lysosomal membrane. In poorly buffered medium, an H^+ electrode indicated nigericin-driven pH changes which were in good agreement with the dye response, in that a similar value for the null point was obtained with both techniques (fig.1). This should make calibration of the dye response possible, in order to determine the H^+ that are translocated (see [20]). For the purposes of this study, however, a qualitative use of the probe was sufficient, although it is possible to measure the internal pH of isolated lysosomes before [9] and after the proton 'pump' is allowed to operate (in preparation).

4.2. *Electrogenic nature of the lysosomal proton pump*

Nigericin, and to a very much lesser extent valinomycin, induced release of H^+ via leak in the case of valinomycin (and thus K^+ uptake) from lysosomes at K^+ concentrations greater than about 15 mM, the opposite being found for K^+ concentrations below this value (fig.1), when the pH of the medium was about 7.1. Nevertheless, also at K^+ medium concentrations over 20 mM, valinomycin induced an enhancement in ATP-driven dye uptake rate, most likely by extrusion of K^+. These findings are easily interpreted if we assume that the ATP-driven H^+ translocation creates an internally positive membrane potential. Thus, the electrochemical potential of internal K^+ is increased with respect to external K^+, and this causes extrusion of K^+ from the lysosomes even at external K^+ concentrations where a K^+ influx would be expected for an electroneutral H^+/K^+ exchange. The K^+ efflux in turn transiently reduces the membrane's potential, allowing further H^+ and dye uptake. Accordingly, in ATP-supplemented medium, FCCP is able to equilibrate H^+, without a permeant counterion, such as valinomycin–K^+, or to prevent the pH gradient established by ATP (fig.3C); this situation is reversed when the gradient is established by nigericin, at low medium K^+ (fig.2).

Essentially the same results were obtained with the highly purified preparations (prepared as in [13]) except that the null point for the H^+/K^+ exchange in preliminary trials was found at higher K^+ values when the metrizamide solutions were supplemented with 10–15 mM KCl, on the basis of the null point found with lysosome-enriched preparation, in order to minimize the H^+/K^+ exchange. This point was not further explored. One possibility is that an H^+ efflux may occur via an H^+-anion co-transport, at a rate higher than that of the H^+/K^+ exchange (the opposite is likely to occur in the absence of K^+ in the medium due to the high K^+ gradient). Thus H^+ and K^+ are no longer in equilibrium. K^+ efflux and H^+ re-uptake are required for attainment of the 'new' equilibrium and this would become apparent, when testing H^+/K^+ exchange driven by nigericin, as a shift of the null point to a higher value.

The electrogenic nature of the pump is also supported by the stimulatory effect of anions on the rate of ATP-driven dye uptake. In fact, the uptake rate increased in the same order of increasing lipophilicity of the anion ($SO_4^{2-} < Cl^- < Br^-$) and this was also observed when ATP-driven acidification was monitored by the pH indicator, fluorescein isothiocyanate [7].

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