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A CRITICAL EXAMINATION OF THE EVIDENCE FOR AN MgATP-DEPENDENT PROTON PUMP IN RAT LIVER LYSOSOMES

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Summary

(1) When lysosomes isolated from the livers of Triton WR 1339-treated rats were incubated for 30 min in the presence of 100 mM KCl and $^{14}CH_3NH_2$, a stimulation by MgATP of the calculated accumulation of the base was observed, in agreement with previous results (Schneider, D.L. (1979) Biochem. Biophys. Res. Commun. 87, 559—565). A similar stimulation was seen with MgITP. Excess EDTA had very little effect on the stimulation by MgATP.

(2) There was little effect of MgATP or MgITP on the calculated accumulation of $^{14}CH_3NH_2$ if the base was added to the incubation medium 1, 3, 4 or 5 min before terminating the incubation instead of being present for the total incubation period of 30 min.

(3) The accumulation of the basic dye, acridine orange, by a crude lysosomal preparation isolated from the livers of untreated rats was found to be stimulated by MgATP, in agreement with earlier results (Dell'Antone, P. (1979) Biochem. Biophys. Res. Commun. 86, 180—189). Similar results were obtained with a crude lysosomal preparation isolated from the livers of Triton WR 1339-treated rats. In both cases, the stimulation was partly oligomycin-sensitive.

(4) There was very little or no effect of MgATP on the accumulation of acridine orange by preparations of pure lysosomes isolated from the livers of Triton WR 1339-treated rats.

(5) Our data do not require us to postulate the existence of an MgATP-dependent proton pump in lysosomes.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Introduction

It is now well-established that the intralysosomal pH is low in comparison to that of the surrounding medium, both in vitro, at extralysosomal pH values above about 5.5, and in vivo (see Refs. 1 and 2 for reviews). Two mechanisms for setting up and maintaining the pH difference across the lysosomal membranes have been postulated (see Ref. 2). According to one of these postulated mechanisms, a Donnan-type equilibrium maintains a ΔpH across the lysosomal membrane. The second mechanism invokes the presence of an ATP-dependent proton pump in the lysosomal membrane.

Indirect evidence for an ATP-dependent proton pump in lysosomes has been brought forward by Mego and coworkers [3—7] who studied the effect of added ATP on intralysosomal proteolysis (for a critical appraisal of this evidence see Refs. 2 and 8). More direct evidence for an ATP-driven proton pump has been obtained by Dell'Antone [9] and by Schneider [10]. Dell'Antone's conclusion that there is an ATP-dependent proton pump in lysosomes is based on the finding that ATP stimulates the accumulation of the base, acridine orange, in a lysosomal preparation isolated from rat liver according to the method of Sawant et al. [11].

The evidence originally obtained by Schneider and Cornell [12] in favour of an ATP-dependent proton pump has been shown by Hollemans et al. [8] to be due to artifacts (see also Ref. 10). More recently, however, Schneider [10] has presented new data showing that ATP enhances the accumulation of the weak base, CH_3NH_2, in lysosomes isolated from the livers of Triton WR 1339-treated rats. The enhancement occurs only if the lysosomes are preincubated in the presence of KCl, NaCl or LiCl. Furthermore, the preincubation brings about a concomitant stimulation of the ATPase activity of the lysosomes. These results are indeed indicative of an ATP-driven proton pump in lysosomes.

In contrast, Reijngoud and Tager [2], Hollemans et al. [8] and Henning [13] have been unable to observe any effect of ATP on the distribution of CH_3NH_2 across the lysosomal membrane. The results obtained by these groups [2,8,13] and analogous results obtained by Goldman and Rottenberg [1,14] indicate that the ΔpH across the lysosomal membrane is maintained by a Donnan-type equilibrium.

We have carried out further studies in order to elucidate the reasons for the apparent discrepancies between the results obtained by Dell'Antone [9] and Schneider [10] on the one hand, and those of Henning [13], Reijngoud and Tager [2] and Hollemans et al. [8] on the other. Although it has been possible to duplicate exactly the results obtained by Dell'Antone [9] and Schneider [10], additional control experiments have been carried out which show that the data are not in accordance with the presence of an MgATP-driven proton pump in rat-liver lysosomes.

Materials and Methods

Rat liver lysosomes. These were isolated by using the flotation method of Trouet [15] as described by Kussendrager et al. [16] from the livers of Triton

TABLE I

EFFECT OF MgATP AND MgITP ON ΔpH AND Δψ IN RAT-LIVER LYSOSOMES

Lysosomes isolated from the livers of Triton WR 1339-treated rats were preincubated for 20 min at 20°C in the presence of 100 mM KCl, ^3H_2O and 0.4 mM EDTA. After 20 min, 1 mM ATP + 2 mM $MgSO_4$, or 1 mM ITP + 2 mM $MgSO_4$ were added as indicated. In some incubations, excess EDTA (final concentration 8 mM) was present from the start of this preincubation period. $^{14}CH_3NH_2$ (0.1 μCi; 1.80 μM), or [^{14}C]sucrose (0.1 μCi; 0.16 μM), or $KS^{14}CN$ (0.1 μCi; 1.67 μM) were added in parallel incubations at the start of the preincubation or 3 or 4 min before separating the lysosomes from the incubation medium by centrifugation. After measuring the ^{14}C and ^3H radioactivity in the pellet and in the supernatant the accumulation factors (f) for CH_3NH_2 and KSCN, corrected for the [^{14}C]sucrose space ($r_{sucrose}$), were calculated as follows:

$f_i = \frac{r_i - r_{sucrose}}{1 - r_{sucrose}}$, where i is the indicator used.

ΔpH and $\Delta\tilde{\mu}_{H^+}$ were calculated as described in Ref. 8.

Expt.	Additons	Time of incubation with ^{14}C-labelled compounds (min)	$r_{sucrose}$	$f_{CH_3NH_2}$	ΔpH	f_{KSCN}	Δψ (mV)	$\Delta\tilde{\mu}_H$ (mV)
1	None	30	0.8	4.4	0.6	6.5	49	85
	MgATP	30	0.8	11.1	1.0	7.3	52	112
	MgITP	30	0.8	8.9	0.9	7.4	52	106
	MgATP + EDTA	30	0.8	7.7	0.9	7.0	51	105
2	None	3	0.7	3.5	0.6	7.9	54	90
	MgATP	3	0.6	4.6	0.7	5.3	44	86
	MgITP	3	0.7	3.2	0.5	5.1	42	72
	MgATP + EDTA	3	0.7	3.9	0.6	7.1	51	87
3	None	30	0.5	4.8	0.7			
	MgATP	30	0.5	8.4	0.9			
	None	4	0.5	3.6	0.6			
	MgATP	4	0.5	5.2	0.7			

WR 1339-treated rats. In some of the experiments in which the uptake of acridine orange was measured, the lysosomes were isolated as described by Sawant et al. [11].

Distribution of $^{14}CH_3NH_2$, [^{14}C]sucrose and $KS^{14}CN$ across the lysosomal membrane. The distribution was measured and calculated as described in Ref. 8 after incubation of the lysosomes in the media and under the conditions indicated in the legend to Table I. The lysosomes were separated from the medium as described in Ref. 8. It is important to note that the sucrose space was measured in parallel incubations for each condition tested (cf. Ref. 8; see also Ref. 10).

Uptake of acridine orange. The uptake of acridine orange, a basic dye, was measured exactly as described by Dell'Antone [9], using an Aminco DW2 spectrophotometer.

Materials. ^3H_2O, $^{14}CH_3NH_2$, $KS^{14}CN$ and [^{14}C]sucrose were obtained from the Radiochemical Centre (Amersham, U.K.), Triton WR 1339 from Rohm and Haas (Philadelphia, U.S.A.), acridine orange from Eastman Kodak (Rochester,

NY, U.S.A.), and ATP and ITP from Boehringer (Mannheim, F.R.G.). Nigericin was a gift from Eli Lilly Benelux (Brussels, Belgium).

Results and Discussion

Examination of the evidence brought forward by Schneider [10] in favour of an MgATP-driven proton pump

In Expt. 1 of Table I, lysosomes isolated from the livers of Triton WR 1339-treated rats were incubated in the presence of KCl exactly as described by Schneider [10]. In agreement with his results, we found that MgATP enhanced the accumulation factor for CH_3NH_2, so that the calculated ΔpH was increased from 0.6 to 1.0. However, MgITP had a similar effect. Furthermore, addition of an excess of EDTA in order to bind all Mg^{2+} had relatively little effect on the enhancement by MgATP of the accumulation factor for CH_3NH_2.

Schneider [10] has stressed that the lysosomes must be incubated for 20 min in the presence of KCl or LiCl in order to elicit a response to MgATP. The results of Expts. 2 and 3 of Table I indicate that $^{14}CH_3NH_2$ and/or [^{14}C]-sucrose must also be present during this long preincubation in the presence of MgATP; when $^{14}CH_3NH_2$ and [^{14}C]sucrose were added 3 or 4 min before terminating the incubation, the enhancement by MgATP of the accumulation factor for CH_3NH_2 largely disappeared. It should be stressed that MgATP was present throughout the 10 min incubation. Schneider [10] suggests that the postulated proton pump is electrogenic. If this were the case, one would expect to find an increase in the membrane potential on addition of ATP; this was not observed (Table I; see also Ref. 8).

The effect of the point in time at which $^{14}CH_3NH_2$ and [^{14}C]sucrose were added was examined in more detail in the experiment of Table II. Similar values for the distribution of CH_3NH_2 and sucrose in the absence and presence of ATP were obtained when the labelled compounds were added 1, 3 or 5 min before terminating the incubations. However, when the labelled compounds were present for 10 or 30 min, there was a change in the distribution of the labelled compounds, resulting in an apparent increase in ΔpH which was somewhat greater in the presence of ATP than in its absence (cf. Table I). We have no explanation at present for the anomalous distribution of CH_3NH_2 and sucrose after prolonged incubation.

Examination of the evidence brought forward by Dell'Antone [9] suggesting that there is an ATP-dependent proton pump in lysosomes

When a lysosomal preparation isolated from rat liver by using the method of Sawant et al . [11] was incubated with acridine orange, there was a decrease in the absorbance at 492—540 nm (Fig. 1a) indicative of the disappearance of the dye from the medium and presumably due to uptake (or binding) of the dye by components in the preparation. In agreement with the results of Dell'Antone [9], the uptake of acridine orange was enhanced by the addition of ATP (Fig. 1a). Subsequent addition of oligomycin led to an inhibition of uptake of the dye of about 30%; a similar inhibition was observed, but not commented on, by Dell'Antone (see Fig. 1 of Ref. 9). On addition of nigericin,

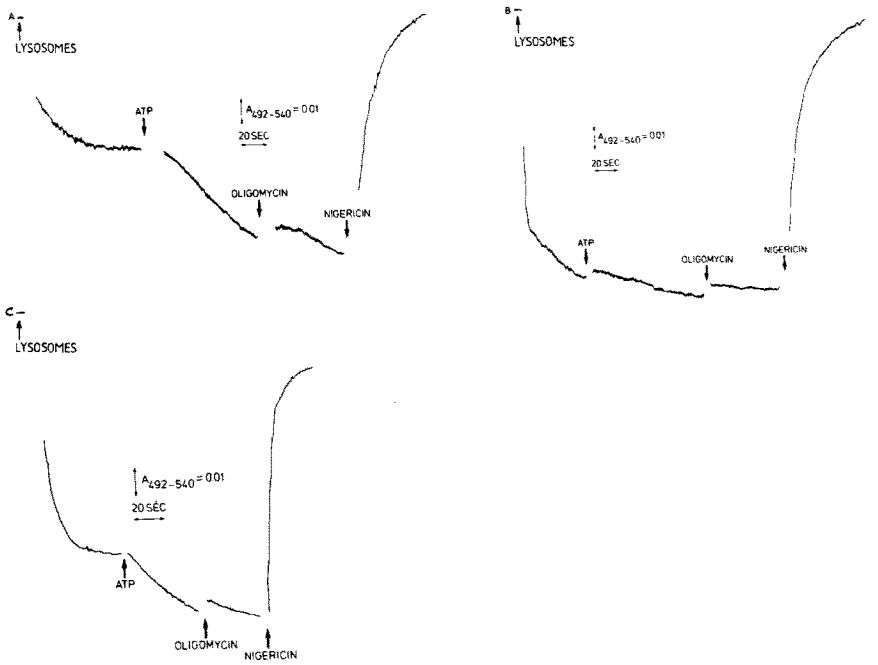

Fig. 1. Acridine orange uptake by lysosomes and the effect of addition of ATP. The medium (final volume 2.5 ml) contained 8 μM acridine orange, 100 mM KCl, 10 mM $MgSO_4$ and 20 mM Hepes (pH 7.0). The consecutive additions were as follows. (A) Lysosomes prepared according to the method of Sawant et al. [11] (0.26 mg protein); ATP (final concentration 1 mM); 5 μg oligomycin; and 5 μg nigericin. (B) Lysosomes prepared according to Ref. 16 from Triton WR 1339-treated rats (0.07 mg protein); ATP; oligomycin; and nigericin. (C) Lysosomes prepared according to the method of Sawant et al. [11] from livers of rats pretreated with Triton WR 1339 (0.30 mg protein); ATP; oligomycin; and nigericin.

the $A_{492-540}$ value increased to the initial value with all three preparations (cf. Ref. 9), indicating that acridine orange had been accumulated as a consequence of a pH difference between the medium and the interior of the vesicles present in the preparation.

Acridine orange was also taken up by lysosomes prepared from Triton WR 1339-treated rats [16], but in this case there was no effect of ATP (Fig. 1b). Since the difference in behaviour between lysosomes from untreated and treated rats may have been due to the presence of Triton WR 1339 in the latter, a lysosomal preparation was isolated according to the procedure of Sawant et al. [11] from the livers of rats previously treated with Triton WR 1339. This preparation responded to the addition of MgATP in exactly the same way as those isolated from the livers of untreated rats (cf. Fig. 1a and c). Thus, the lack of effect of ATP on the uptake of acridine orange by the lysosomes prepared from treated rats is not due to inactivation by Triton WR 1339 of an ATP-linked proton pump in the lysosomes.

TABLE II

EFFECT OF TIME OF ADDITION OF $^{14}CH_3NH_2$ AND [^{14}C]SUCROSE ON THE ΔpH

For incubation conditions and calculations see Table I.

Time of incubation with $^{14}CH_3NH_2$ or [^{14}C]sucrose (min)	$r_{CH_3NH_2}$		$r_{sucrose}$		$f_{CH_3NH_2}$		ΔpH	
	—ATP	+ATP	—ATP	+ATP	—ATP	+ATP	—ATP	+ATP
1	1.9	2.1	0.7	0.7	4.5	4.3	0.6	0.6
3	2.1	2.4	0.7	0.7	4.4	5.2	0.6	0.7
5	2.4	2.8	0.6	0.6	4.4	5.6	0.6	0.7
10	2.5	3.4	0.9	0.8	10.8	13.0	1.0	1.1
30	2.5	4.1	0.8	0.8	9.8	17.4	1.0	1.2

Conclusions

Firstly, the results presented in this paper indicate that the enhancement by MgATP of the accumulation factor for $^{14}CH_3NH_2$ in lysosomes incubated under the conditions described by Schneider [10] is not due to a specific MgATP-dependent proton pump. It is a non-specific effect of ATP requiring the preincubation of the lysosomes not only with salt, as shown by Schneider [10], but also with $^{14}CH_3NH_2$ and/or [^{14}C]sucrose. Furthermore, ATP can be replaced by ITP.

Secondly, the results show that the stimulation by MgATP of the uptake of acridine orange by a rat liver lysosomal preparation isolated according to the method of Sawant et al. [11] is due not to an ATP-dependent proton pump in the lysosomes as suggested by Dell'Antone [9], but to an ATP-dependent process in impurities present in the preparation. Since the effect of ATP is partly oligomycin-sensitive (Fig. 1a and c; see also Fig. 1 of Ref. 9), mitochondrial contamination must be responsible for at least part of the effect. Indeed, in submitochondrial particles, ATP addition will lead to acidification of the interior of the particles. Furthermore, the mitochondrial component of the effect may consist, in part, of an energy-dependent binding of acridine orange to mitochondrial membrane fragments (cf. Ref. 17). Other contaminants such as microsomes (see Ref. 18) may also contribute to the effect. There is little or no enhancement by ATP of the uptake of acridine orange by a pure lysosomal preparation from Triton WR 1339-treated rats.

Thus, the available evidence does not require us to postulate the existence of an ATP-dependent proton pump in isolated rat liver lysosomes (contrast Refs. [3—7,9,10 and 12]). Ohkuma and Poole [19] have concluded that there is an energy-dependent proton pump in lysosomes in vivo. Although their elegant technique of following spectral changes of an endocytosed dye has enabled them to measure the intralysosomal pH in living cells, the results of their experiments with metabolic inhibitors cannot be interpreted unequivocally; they merely show that the overall functioning of the lysosomal apparatus is energy-dependent.

Our earlier results [8,20,21] and those of others [1,13,14] have shown that

the ΔpH across the lysosomal membrane in isolated rat liver lysosomes is due to a Donnan-type equilibrium brought about by the presence within the lysosomes of non-diffusible negatively charged groups like the sialic acid in haematoside [22] and glycoproteins [23]. It should be stressed that these results do not exclude the possibility that there is an ATP-dependent proton pump in lysosomes. However, the Donnan-type equilibrium across the lysosomal membrane and the buffering capacity within the lysosomes are sufficient to account for such phenomena as the massive accumulation of chloroquine in lysosomes both in vitro [24] and in vivo in cultured human skin fibroblasts (Oude Elferink, R., Hollemans, M. and de Groot, P.G., unpublished observations).

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