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EFFECT OF IONOPHORES ON INTRALYSOSOMAL pH

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SUMMARY

1. The effect of ionophores on the intralysosomal pH (as estimated from the distribution of a weak acid or base), on the distribution of $^{42}K^+$ across the lysosomal membrane, and on the intralysosomal degradation of ^{125}I-labelled bovine serum albumin has been studied.

2. Nigericin and X537A equilibrate both $^{42}K^+$ and H^+ across the lysosomal membrane. Gramicidin equilibrates H^+ across the lysosomal membrane, this equilibration being more effective in a NaCl than in a KCl medium. Thus all three ionophores exhibit the same ion specificity as in other membranes.

3. The effect of the exchange-diffusion ionophores cannot be imitated by the combination of valinomycin with an uncoupler. Valinomycin by itself also has no effect.

4. X537A and nigericin inhibit the intralysosomal degradation of ^{125}I-labelled albumin only when potassium is present. In a sucrose-containing medium no effect is found. Similar results were obtained with gramicidin.

5. These data suggest that the lysosomal membrane is impermeable to monovalent cations at 25 or 37 °C, and that the transport of protons is organised in such a way that electroneutrality is maintained.

INTRODUCTION

The lysosomal system of the cell is the site of degradation of phagocytosed macromolecules by hydrolases with an acid pH optimum [1]. Thus the pH inside the lysosomes should be low enough to allow the hydrolases to function efficiently. In the accompanying paper [2] the methods that have been used to estimate the intralysosomal pH are discussed. Both in intact cells [3–6] and in isolated lysosomes [7–9] suspended in media of about pH 7 an intralysosomal pH 1–3 units lower than that of the ambient solution has been estimated. Using two independent methods,

Abbreviations: DMO, 5,5-dimethyloxazolidinedione-2,4; MES, 2-(*N*-morpholino)ethane sulphonic acid; MOPS, morpholinopropane sulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

we have shown that there is a pH difference across the lysosomal membrane that is a function of the pH of the medium [2].

Three mechanisms have been proposed by means of which a gradient of protons across the lysosomal membrane is set up and maintained. Firstly, Mego and coworkers [10, 11] have proposed that an ATP-dependent proton pump brings about an active transport of protons into the lysosomes. Secondly, Goldman and Rottenberg [8] and Henning [9] have proposed that the Δ pH is brought about by a Donnan equilibrium, caused by the presence of immobile, negatively charged groups within the lysosomes and free permeation of protons and monovalent cations across the lysosomal membrane. The third mechanism, proposed by Reijngoud and Tager [12], also invokes the presence of intralysosomal negatively charged groups. However, since the lysosomal membrane is impermeable to monovalent cations at 25 and 37 °C [13], Reijngoud and Tager [12] proposed that the dependence of the intralysosomal pH on that of the medium is brought about by a movement of protons accompanied by a charge-compensating movement of anions. Goldman and Rottenberg [8] and Henning [9] carried out their experiments at 0 °C, a temperature at which the lysosomal membrane is completely permeable to monovalent cations [12, 13], so that under those conditions the mechanism they propose does indeed operate (see ref. 12).

In this paper the results of a study on the effect of ionophores on the distribution of methylamine or 5,5-dimethyloxazolidinedione-2,4 (DMO) across the lysosomal membrane, and on the rate of hydrolysis of endocytosed albumin in isolated lysosomes, are presented. The results provide further evidence that the lysosomal membrane is impermeable to monovalent cations at 25 or 37 °C unless ionophores are added.

MATERIALS AND METHODS

Materials. ^3H_2O, [^{14}C]methylamine, 5,5-[^{14}C]dimethyloxazolidinedione-2,4, and [^{14}C]sucrose were obtained from Radiochemical Centre, Amersham, England, ^{125}I and ^{42}KCl from Philips Duphar, Petten, The Netherlands, bovine serum albumin (fraction V) from Sigma Chemical Co., St. Louis, USA., Triton WR 1339 from Rohm and Haas, Philadelphia, USA., nigericin from Eli Lilly and Co., Indianapolis, U.S.A., and X537A from Hoffman-LaRoche, Basle, Switzerland.

Isolation of rat liver lysosomes. Lysosomes were isolated by the flotation method of Trouet [14] as described by Kussendrager et al. [15] from the livers of Triton WR 1339-treated rats.

Determination of the distribution of radioactive substances. The measurements of the distribution of radioactive methylamine, sucrose or DMO across the lysosomal membrane and the calculation of the inside pH on the basis of these measurements, were performed exactly as described previously [2, 7]. When ^{42}KCl was also present in incubations containing ^3H_2O and ^{14}C-labelled compounds, a minor modification was introduced for counting the samples. ^{42}K was counted by using the Chernkov method, and after the ^{42}K had decayed sufficiently (after 1 week) the ^{3}H and the ^{14}C radioactivity was measured by liquid scintillation. The sucrose-impermeable space, used in the calculations of intralysosomal accumulated material, was 0.45 ± 0.10 (10 determinations).

Degradation of denatured ^{125}I-labelled bovine serum albumin. The experiments

concerning the breakdown of denatured ^{125}I-labelled bovine serum albumin were performed exactly as described in the accompanying paper [2].

RESULTS

In Fig. 1 a schematic representation is given of the mechanism of action of the cation-proton exchange-diffusion ionophores nigericin and X537A [16] which were used in this study with lysosomes. These ionophores have the following specificity in other biological and artificial phospholipid membranes. Nigericin is highly specific for potassium and protons. It is an acid which is lipid soluble only in the form of the undissociated acid or undissociated potassium salt. X537A has a rather broad specificity and can transport a number of cations in exchange for protons (see ref. 17). In both cases there is a strictly stoichiometric exchange of one cation for one proton. On the other hand, gramicidin, the third ionophore used in this study, is a small lipid-soluble peptide that forms a pore in the membrane through which cations and protons can permeate freely [16]. It has a rather wide specificity, but it is generally considered that Na^+ permeates through the gramicidin pore more readily than K^+ (see ref. 16 for a discussion).

In Table I the effect is shown of the ionophore nigericin on the intralysosomal pH, calculated from the accumulation data of the weak acid DMO and the weak base methylamine. In a salt-poor, mannitol-containing medium to which no potassium was added, nigericin had no influence on the transmembrane pH difference. On the contrary, when potassium was present, a marked increase in the inside pH was observed. Taking into account the mechanism of action of the ionophore, it is clear that an exchange of potassium outside for protons inside was made possible, which resulted in a decrease of Δ pH. This decrease is dependent on the concentration of K^+ in the medium and becomes greater as the concentration of KCl is increased (see ref. 2).

In the accompanying paper [2] we have proposed a model for the interaction of methylamine and small cations with the lysosomes in which we recognize only one type of adsorption site, some of which are on the outside of the membrane and others towards the lysosomal matrix. On addition of nigericin all sites should become available for both methylamine and potassium with equal affinity. The effect of

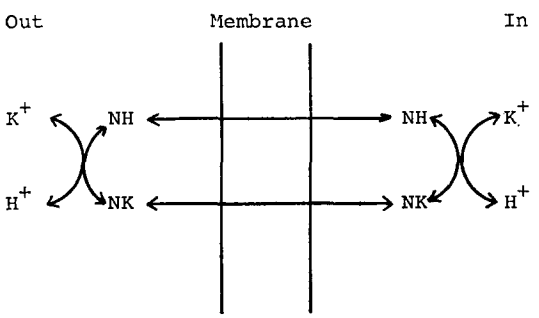

Fig. 1. Mechanism of action of a proton-cation exchange-diffusion carrier like nigericin. NH represents the protonated form of nigericin and NK its potassium salt.

TABLE I

INFLUENCE OF NIGERICIN ON THE INTRALYSOSOMAL pH CALCULATED FROM THE DISTRIBUTION OF DMO AND METHYLAMINE

Rat liver lysosomes were incubated in a medium (final volume, 1 ml) containing 10 mM MES, 10 mM MOPS and sufficient Tris to adjust the pH to 7.5, 250 mM mannitol or 130 mM KCl, 1 % ethanol and, where indicated, 2 μg nigericin. The medium also contained ^3H_2O, and [^{14}C]methylamine, [^{14}C]DMO or [^{14}C]sucrose. Incubation temperature, 25 °C. Time 1 min.

Medium	pH_{in} calculated from distribution of	
	DMO	Methylamine
Mannitol	6.71	6.22
Mannitol + nigericin	6.75	6.21
KCl	6.68	6.53
KCl + nigericin	7.50	7.21

nigericin and K^+ on the accumulation of methylamine is illustrated in Fig. 2 in the form of a Scatchard plot. The lines obtained at different K^+ concentrations converge to a single point on the abscissa, corresponding to a concentration of methylamine in the lysosomes of approx. 130 mM. In contrast, parallel lines were obtained in the absence of nigericin (see Fig. 3 of ref. 2).

Not only nigericin but also X537A makes the lysosomal membrane permeable to K^+. As shown in Table II, in the absence of the ionophores, the ΔpH is not equal to the logarithm of the accumulation factor for potassium. However, these two values do become approximately equal in the presence of nigericin or X537A, indicating that the membrane becomes permeable to K^+ only when a K^+-specific ionophore is present.

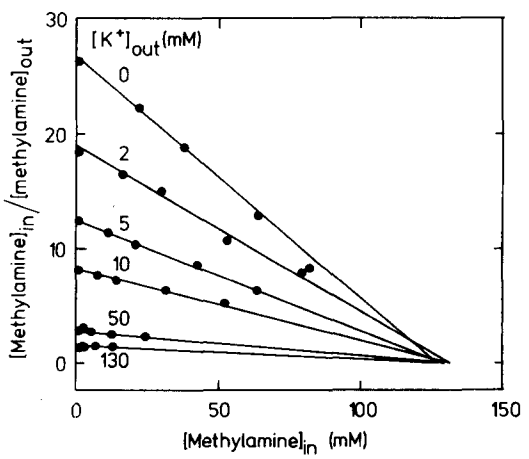

Fig. 2. Effect of concentration of methylamine on accumulation of [^{14}C]methylamine in rat liver lysosomes in the presence of nigericin and different concentrations of K^+. The incubation conditions were as described in Table I, except that unlabelled methylamine was added at concentrations of 0, 1, 2, 5 and 10 mM. Nigericin (2 μg) was present. To maintain tonicity mannitol was added up to 250 mosM. Separation of supernatant and the lysosomal pellet was effected by using the silicone technique as described by Harris and Van Dam [18].

TABLE II

INFLUENCE OF NIGERICIN AND X537A ON THE ΔpH AND THE DISTRIBUTION OF $^{42}K^+$ ACROSS THE LYSOSOMAL MEMBRANE

Rat liver lysosomes were incubated under the conditions described in Table I, except that $^{42}K^+$ was also present. 20 μM X537A was used where indicated. The ΔpH was calculated from the distribution of [^{14}C]methylamine.

Medium	Addition	ΔpH	K^+ accumulation	
			Factor	Log factor
Mannitol	None	1.45	4.0	0.6
	Nigericin	1.10	14.2	1.15
	X537A	1.05	11.6	1.06
KCl	None	1.26	0.7	−0.15
	Nigericin	0.27	1.3	0.12
	X537A	0.26	1.7	0.23

In Fig. 3 the effect of nigericin on the ΔpH at different outside pH values is shown, in a mannitol medium (A) or a KCl medium (B). In a mannitol-containing medium hardly any influence of nigericin on the ΔpH was found. In the presence of potassium, however, the ΔpH was strongly diminished by nigericin at an outside pH of 6.5–8.5.

Fig. 4 shows the effect of nigericin on the accumulation of K^+ in a mannitol medium. In this case nigericin increased the accumulation factor of K^+ at all pH values tested.

All these data give support to the idea that nigericin and X537A act as K^+-H^+ exchange-diffusion carriers in lysosomal membranes exactly as they do in other membranes. The data in Table III show that the same ion specificity can be demon-

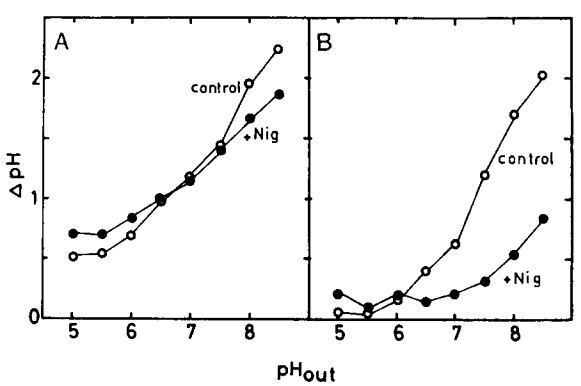

Fig. 3. Effect of nigericin on ΔpH in rat liver lysosomes at different outside pH values. Rat liver lysosomes were incubated in a medium (final volume, 1 ml) containing 10 mM MES, 10 mM MOPS and sufficient Tris to bring the pH to 5–7.5, as indicated. For the pH values 8 and 8.5, 10 mM Tris was adjusted to the pH indicated with an equimolar solution of MES and MOPS. Furthermore, the medium contained 250 mM mannitol (A) or 130 mM KCl (B), ^3H_2O, either [^{14}C]methylamine or [^{14}C]sucrose, 1 % ethanol and, where indicated, 2 μg nigericin. The incubation temperature was 25 °C, and the incubation time 1 min.

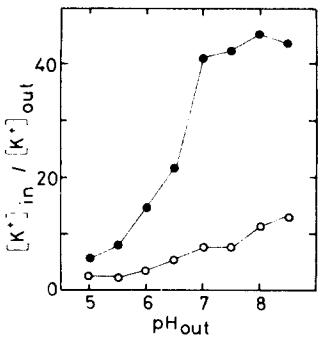

Fig. 4. Effect of nigericin on the distribution of K^+ in rat liver lysosomes at different outside pH values. The experiment was that described in Fig. 3. ^{42}KCl was present in the incubation medium containing mannitol and the ^{14}C-labelled compounds. ●—●, nigericin; ○—○, control.

strated in lysosomes as in other membrane systems. The decrease of the ΔpH in the presence of nigericin was more pronounced when K^+ was present than when Na^+ was in the incubation medium. Gramicidin, like nigericin, also caused a decrease in ΔpH. However, the effect was more pronounced in NaCl than in KCl.

These effects of ionophores on the inside pH can also be demonstrated with an intralysosomal degradation process (cf. ref. 2). In Fig. 5 the effect of ionophores in a sucrose- and in a KCl-containing medium on the breakdown of phagocytosed ^{125}I-labelled bovine serum albumin is shown. When no KCl was present the ionophores had only a small effect on the rate of protein degradation. Nigericin and gramicidin consistently caused a small stimulation, and X537A a small inhibition. When the same experiment was performed in a KCl-containing medium a strong inhibition by nigericin and X537A and a smaller inhibition by gramicidin were observed.

It is known that X537A acts as an exchange-diffusion carrier at a wide range of pH values [16]. The effect of X537A on the intralysosomal degradation of albumin at different outside pH values is shown in Fig. 6. In this experiment, the effect of the ionophore on protein breakdown by lysed lysosomes was also tested. Under each condition, the activities were expressed as a percentage of that obtained at

TABLE III

EFFECT OF NIGERICIN AND GRAMICIDIN ON ΔpH ACROSS THE LYSOSOMAL MEMBRANE

For conditions, see Table I. The final osmolarity of 250 mosM was obtained by adding the appropriate amount of KCl or NaCl. The ΔpH was calculated from the distribution of [^{14}C]methylamine. Where indicated, 2 μg gramicidin was added.

Addition	ΔpH in a medium containing	
	KCl	NaCl
None	1.01	1.10
Nigericin	0.30	0.80
Gramicidin	0.56	0.38

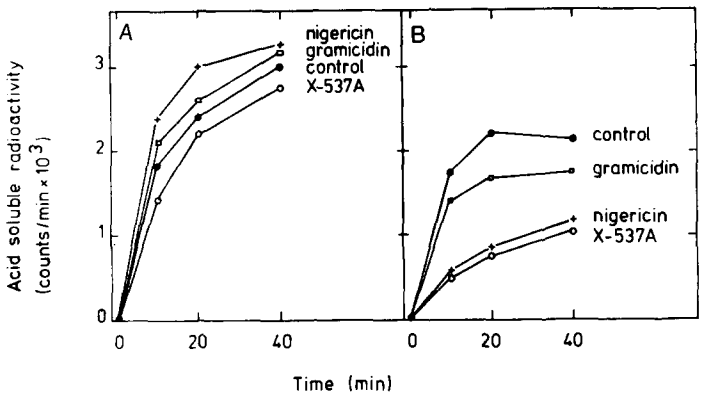

Fig. 5. Effect of ionophores on the degradation of endocytosed ^{125}I-labelled albumin by intact isolated rat liver lysosomes. A crude mitochondrial-lysosomal fraction, obtained from rats injected with ^{125}I-labelled albumin 30 min prior to decapitation, was incubated as described in ref. 2 in a medium containing either 250 mM sucrose (A) or 130 mM KCl (B). The pH of the medium was 7.5. Nigericin (2 μg), gramicidin (2 μg) and X537A (20 μM) were present when indicated. 1 % ethanol was also present.

the optimum pH. In lysed lysosomes there was a sharp optimum at pH 5.5 (cf. ref. 2). In intact lysosomes there was also an optimum at 5.5. However, the activities at higher pH were greater than in lysed lysosomes. In the accompanying paper [2] this effect is interpreted as being due to the pH difference maintained across the intact lysosomal membrane. In the presence of X537A the optimum in intact lysosomes remained the same, but the shape of the curve changed; there was a sharp drop in activity as the pH was increased, which was not observed in the absence of the ionophore. This effect is interpreted as being due to an increase of intralysosomal pH, brought about by the ionophore (see Discussion).

A completely different way of achieving a proton-cation exchange is schemat-

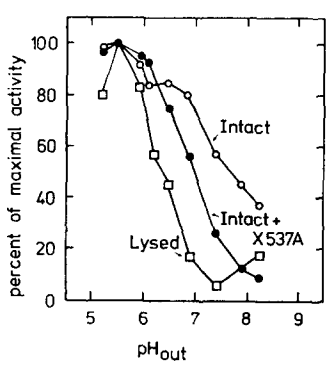

Fig. 6. Effect of X537A on the intralysosomal degradation of ^{125}I-labelled albumin and on the degradation by lysed lysosomes. The medium contained 130 mM KCl, 1 % ethanol and either 10 mM MES+10 mM MOPS adjusted to pH 5–7.5 with Tris as indicated, or 10 mM Tris adjusted to pH 8 or 8.5 with MES+MOPS. Where indicated, 20 μM X537A was added. For the experimental procedure with intact lysosomes, see legend to Fig. 5 and ref. 2. For the experimental procedure with lysed lysosomes, see ref. 2.

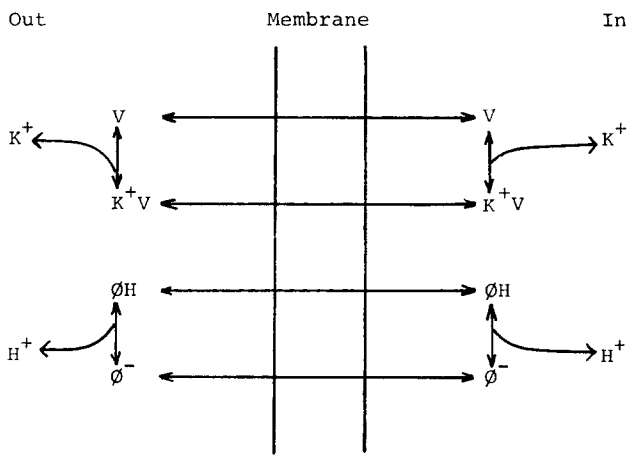

Fig. 7. Mechanism of action of valinomycin and uncoupler. V represents the ionophore valinomycin and ϕ^- and ϕH the dissociated and undissociated form of the uncoupler, respectively.

ically presented in Fig. 7. In this case two different types of ionophores are added to a membrane-bound system, namely valinomycin and an uncoupler. Valinomycin makes the membrane permeable to potassium in an electrogenic way [16].The positively charged complex of potassium · valinomycin is lipid soluble, as is valinomycin itself. Uncouplers of oxidative phosphorylation bring about a charge-uncompensated transport of protons across the membrane [19, 20]. Most of them are weak acids. Both the dissociated and the undissociated form of the uncoupler are lipid soluble. On addition of both ionophores an electroneutral exchange of potassium and protons is achieved. In Table IV an experiment of this type with lysosomes is shown. It is clear from these data that the combination of valinomycin and FCCP at the concentrations used has no influence on the potassium distribution and the ΔpH. Furthermore no influence was found when 2,4-dinitrophenol, another acidic uncoupler, or a basic uncoupler (either chlorpromazin or atebrin) was added

TABLE IV

EFFECT OF IONOPHORES ON ΔpH AND K^+ DISTRIBUTION ACROSS THE LYSOSOMAL MEMBRANE

For conditions, see Table II. The osmolarity was obtained by either 250 mM mannitol or 130 mM KCl. Where indicated, 2 μg nigericin, 1 μg valinomycin, or 20 μM FCCP was added. The incubation time was 2 min.

Addition	ΔpH in a medium containing		K^+ accumulation in a medium containing	
	KCl	Mannitol	KCl	Mannitol
None	1.18	1.55	1.1	9.7
Nigericin	0.25	1.43	1.3	69.7
FCCP	1.13	1.54	0.7	11.1
Valinomycin	1.20	1.55	0.7	12.8
Valinomycin + FCCP	1.30	1.45	0.9	12.1

in combination with valinomycin (not shown). Thus the combination of valinomycin and uncoupler is ineffective in suppressing the ΔpH across the lysosomal membrane even when the incubation medium contains the appropriate cation.

DISCUSSION

Intrinsically related to the question of the pH of the environment in which the lysosomal hydrolases catalyse degradative processes is that of the permeability properties of the lysosomal membrane. During degradation within the lysosomes of polyelectrolytes like nucleic acids and proteins, low molecular weight compounds, many of which are acids and bases, are released. If the degradation products were to remain within the lysosomes, the resultant increase in osmolarity would lead to swelling and possibly disruption of the organelles. The products must thus be transported from the lysosomes and, if they are acids or bases, this transport must occur in such a way that electroneutrality is maintained. The only way in which both conditions can be met is if the acids or bases are transported in the undissociated form*. Other types of exchange-diffusion reactions would lead to an increase in osmolarity during degradation. In addition, transport of the basic or acidic degradation products in their undissociated form would ensure that the intralysosomal pH is stabilized.

In the accompanying paper [2] it was adduced that the lysosomal membrane is impermeable to monovalent cations at 25 or 37 °C. More direct evidence for this conclusion has been provided in this paper by studying the effect of ionophores on the distribution of methylamine and of K^+, and on the rate of an intralysosomal process, the degradation of endocytosed albumin. Both methods show that a K^+-H^+ exchange-diffusion ionophore brings about equilibration of the K^+ gradient and the ΔpH across the lysosomal membrane. In Fig. 8 a comparison is made of two methods of estimating the change in pH_{in} induced by the addition of X537A in the presence of K^+, as a function of the pH of the medium. Both methods give very similar results.

From the observed K^+ accumulation in the presence of nigericin or X537A,

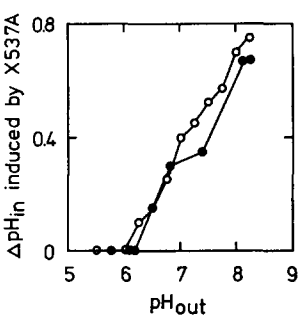

Fig. 8. Comparison between the change in intralysosomal pH brought about by addition of X537A calculated from the methylamine distribution (●—●), or from the rate of hydrolysis of intralysosomal pH (○—○).

* It should be pointed out that the transport of an acid (or base) in the undissociated form is formally equivalent to an exchange of the acid anion for hydroxyl (or of the protonated base for protons).

and the amount of protons that must be exchanged for this K^+, it is clear that the lysosomes must have a high buffering capacity. In the experiment of Table II, for instance, the K^+ accumulation factor in the presence of 130 mM KCl in the medium increased from 0.7 to 1.3. Thus there must have been an exchange of $0.6 \times 130 = 78$ mM K^+ for H^+. Since the lysosomal matrix space in this experiment contained about 3 μl water/mg lysosomal protein, and since the increase in pH was 0.99, it can be calculated that the buffering capacity of the lysosomes was about 0.25 μg equiv. H^+/pH unit per mg lysosomal protein at an inside pH of about 6.7. This value is of the same order of magnitude as that obtained by direct measurement (0.1 μg equiv./pH unit per mg lysosomal protein; see ref. 12).

It is noteworthy that a combination of two electrogenic ionophores, one bringing about a transport of K^+ (valinomycin) and the other a transport of H^+ (uncoupler), has no effect on the ΔpH across the lysosomal membrane. We have at present no explanation for this phenomenon.

The effect of nigericin and of uncoupler on the intralysosomal hydrolysis of ^{125}I-labelled albumin has also been studied by Mego[22]. Although his results are difficult to interpret, his observation that nigericin inhibits the hydrolysis at pH 8 in the absence of ATP is in agreement with our results.

Thus our results lead us to conclude that the lysosomal membrane is impermeable to monovalent cations unless an exchange-diffusion ionophore is present or the temperature is lowered [12]. At a temperature of 25 or 37 °C, in the absence of ionophores, there is a transport of protons together with a permeant anion (or there is an exchange of hydroxyl for anion). Methylamine is transported across the lysosomal membrane in the unprotonated form, becomes protonated in the lysosomes and is "bound" by electrostatic interaction to the negatively charged groups in the lysosomes (probably including sialic acid residues; see ref. 23). These negatively charged groups are in equilibrium with uncharged groups. If the temperature is lowered, or if an exchange-diffusion carrier is added, the "binding sites" become available to monovalent cations as well as to methylamine.

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