ION DISTRIBUTION IN LYSOSOMAL SUSPENSIONS

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Received 3 April 1973

1. Introduction

Lysosomes are vesicular subcellular organelles containing a variety of hydrolytic enzymes with a characteristic acid pH optima. It has been inferred that the pH within the lysosomes is low although no direct evidence is available to support this claim [1]. Various basic dyes and other cationic molecules have been shown to be concentrated within lysosomes [2]. Amine distribution has been used to determine the internal pH in subcellular organelles in which the internal pH is lower than that of the medium [3]. We studied the distribution of amines and other cations in a lysosomal suspension in order to find whether there is a ΔpH and if there is what is its origin. Despite their apparent permeability to salts lysosomes demonstrate remarkable osmotic stability in salt solution [4]. Our results demonstrate the existence of various ion gradients, including a proton gradient, which are due to Donnan distribution, and explain the osmotic stability of lysosomes on the basis of a Donnan dependent salt exclusion.

2. Materials and methods

2.1. Materials

^3H_2O, [^{14}C]sucrose and [^{14}C]methyl amine were obtained from The Radiochemical Centre, Amersham. ^{42}K was obtained from the Nuclear Research Centre, Nahal Sorek, Israel. 9-Amino-acridine was a product of Fluka, and acridine orange (NA 0408) a product of Allied Chemical (Morristown, N.J.).

9-Acetamidoacridine and 9-ethyl acridine were gifts from Dr. M. Shinitzky of this Institute, The gramicidin was from Calbiochem and FCCP (carbonylcyanide-*p*-trifluromethoxyphenyl hydrazone) was a gift from Dr. P.G. Heytler (Du Pont).

2.2. Isolation of rat liver lysosomes

Liver lysosomes were prepared from male CR strain rats (150–300 g) that were starved for 16–24 hr. The livers were homogenized in 2.5 vol of 0.25 M sucrose, 0.5 mM in EDTA, with a teflon homogenizer and subjected to differential centrifugation according to Sawant et al. [5]. The final lysosomal pellet was resuspended in 0.25 M sucrose pH 7. The specific activity of acid phosphatase in the lysosomal fraction was 20–25-fold in comparison with the original homogenate.

2.3. Determination of the distribution of various compounds between the lysosomal pellet and the suspending medium

2.3.1. The water content of the pellet was calculated from ^3H_2O distribution. The extralysosomal water of the pellet was calculated from the ratio of [^{14}C]sucrose/^3H_2O in the pellet to that of the supernatant. 10 μCi ^3H_2O and 2 μCi [^{14}C]sucrose were added to 1.2 ml of lysosomal suspension which contained 2–3 mg of protein, The mixture was incubated for about 10 min in an ice bath and then transferred to 3 microcentrifuge tubes (0.4 ml each). The lysosomes were then sedimented in a Sorvall RC-2 centrifuge (head SS-34) at 12000 rpm for 30 min. After centrifugation,

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50 μl aliquots were removed from each supernatant and diluted into 1 ml of 1% SDS. A slice from the bottom of the pellet (containing about a third of the pellet) was cut and suspended in 1 ml of 1% SDS. After the pellet has completely dissolved, 0.2 ml samples of both diluted supernatant and dissolved pellet were mixed wiyh 10 ml of Aquasol (New England Nuclear) and counted in a Tricarb liquid scintillation spectrometer (Packard, USA).

2.3.2. The distribution of [^{14}C]methylamine and ^{42}K was determined by the same technique as that of the [^{14}C]sucrose; i.e., by comparing the ratio of [^{14}C]methylamine/$^{3}H_2O$ (or $^{42}K/^{3}H_2O$) in the pellet to that of the supernatant. The ratio obtained was then corrected for the extralysosomal water space (in the pellet) by use of the simultaneously determined sucrose distribution. ^{42}K was counted in a γ-counter and after its decay the samples were treated as before.

2.3.3. The uptake of acridine derivatives was determined as described above except that the concentration of the acridines was measured fluorimetrically. Fluorescence was measured with an Eppendorf-fluorimeter. The exciting light was filtered through a 405/436 filter and the emitted light through a 470-3000 filter.

3. Results

Table 1 shows the water content and the sucrose permeable space of lysosomal pellets and two sucrose concentrations. Although the amount of total water seems to increase slightly at high sucrose concentration, the fraction of osmotic water (i.e., the water content of the sucrose impermeable space) decreases, as expected for a higher osmolarity. The increase in the total water content is probably the result of a looser packing of the lysosomal pellet caused by the higher density of the medium and is reflected in an increased sucrose space. The data indicates that the [^{14}C]sucrose distribution is an adequate marker for the fraction of extralysosomal water in the pellet.

Table 2 shows the distribution of [^{14}C]methylamine between the pellet and the medium at pH 7.0 (first column). Using the sucrose distribution a corrected ratio for the intralysosomal space is obtained

Table 1
Water compartments of lysosomal pellets.

Sucrose concn. in the medium (M)	Sucrose permeable space (Fraction of total pellet water)	Total pellet water (μl/mg Protein)	Osmotic water (μl/mg Protein)
0.25	0.64	3.07	1.10
0.61	0.69	3.14	0.98

The medium contained 1 mM Na-Hepes (pH 7.0) and the indicated sucrose concn. The method of determination and calculation is described in the experimental section.

Table 2
[^{14}C]Methylamine distribution in a lysosomal suspension.

Medium	[$^{14}C_a$]Pellet / [$^{14}C_a$]Supernatant	Osmotic space (Fraction of total pellet water)	[$^{14}C_a$]Lysosome / [$^{14}C_a$]Medium
Control	15.0	0.35	41
FCCP (25 μM)	17.4		48
Gramicidin (5 μM)	16.9		46

The medium contained 0.25 M sucrose. 1 mM Na-Hepes (pH 7.0) and in addition the indicated compounds. The procedure of determination and calculation is described in the Methods. [$^{14}C_a$] = [^{14}C]methylamine/$^{3}H_2O$.

Table 3
Effect of salts on [^{14}C]methylamine distribution in a lysosomal suspension.

Salt	Concn. (mM)	[$^{14}C_a$]Lysosome / [$^{14}C_a$]Medium (% Of control)
–	–	100
Na-Hepes	1	80
Na-phosphate	50	14
NaCl + gramicidin (5 μM)	50	10*
NaCl + gramicidin (5 μM)	100	7*
NaCl (–gramicidin)	100	7*
NaCl (–gramicidin)	200	7*
Methylamine chloride	0.08	100**
Methylamine chloride	0.18	85**
Methylamine chloride	1.1	70**
Methylamine chloride	10.1	24**

* Control contains 1 mM Na-Hepes (pH 7.0).
** Control contains 2 mM Na-Hepes (pH 7.0). Lysosomes were suspended in 0.25 M sucrose and the indicated compounds added. [^{14}C]methylamine distribution was determined as described.

(last column). Assuming an equilibration of the neutral amine a concentration ratio of 40 is equivalent to a ΔpH of 1.6. It is further observed (table 2) that compounds that are known to induce proton permeability [6] such as FCCP and gramicidin do not reduce the amine distribution ratio, indicating that the proton gradient is an equilibrium distribution and not an energy dependent gradient. The latter observation suggests that the distribution of methylamine and that of proton is governed by a Donnan potential. We therefore examined the effect of various salts on methylamine distribution. Table 3 shows that the sodium salts of either Hepes, phosphate or chloride decrease the methylamine distribution ratio. The fact that gramicidin which increases sodium ion permeability is not required for the sodium effect, implies that sodium is sufficiently permeable to equilibrate across the lysosomal membrane and to suppress the Donnan potential. Furthermore methylamine chloride is also effective in suppressing its own distribution ratio, this being compatible with its uptake by the lysosomes.

Fig. 1 shows the results of an experiment in which the distribution ratio of [^{14}C]methylamine and ^{42}K were determined simultaneously. These distribution

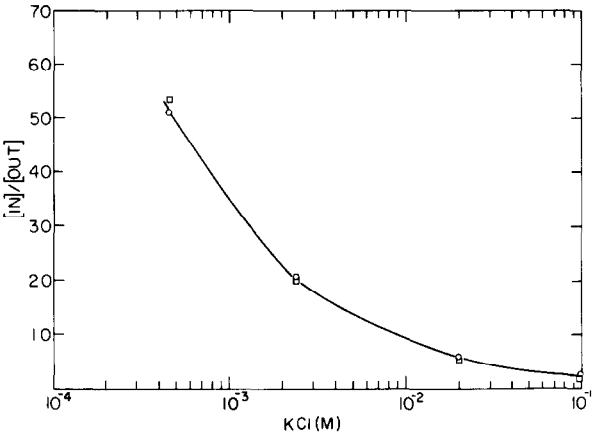

Fig. 1. Distribution ratio of [^{14}C]methylamine (□—□—□), and ^{42}K (○—○—○) as a function of the external KCl concentration. The medium consisted of 0.25 M sucrose, 1 mM Na-Hepes (pH 7) and the indicated KCl concentrations. Experimental conditions are given in the Methods.

ratios were found to strongly depend on KCl concentration in the suspending medium and were practically identical at various KCl concentrations. Valinomycin which is known to increase potassium permeability [6] did not affect these ratios at all (not shown). The results give additional support to the assumption that the methylamine, proton and potassium distributions are all governed by a Donnan potential. The distribution of [^{14}C]methylamine and the water content of freeze–thawed (several cycles) lysosomal suspensions was also determined. The freeze–thawing released about 30% of the lysosomal protein. The fraction of sucrose impermeable space was reduced from 0.36 to 0.18 while the water content of the osmotic space was reduced from 1.1 μl/mg protein to 0.85 μl/mg protein. In parallel, the distribution ratio of [^{14}C]methylamine was reduced from 56 to 13. These results indicate that the Donnan distribution is dependent on the proteins inside the lysosomal vesicles.

The quenching of fluorescent amines has been previously used to determine proton gradients in various vesicular suspensions [7,8]. Using 9-aminoacridine we could not detect fluorescence quenching in lysosomal suspensions. We have therefore studied the uptake of 9-aminoacridine by following its depletion from the supernatant (after centrifugation). In order to estimate the contribution of binding to the apparent up-

Table 4
Distribution of various acridine derivatives in a lysosomal suspension.

Compound	pK	NaCl concn. in the medium (mM)	Acridine uptake in pellet (%)	Lysosomal content of acridine derivatives (moles/mg Protein) × 10^{-10}
9-Aminoacridine	10.0	–	68	6.55
		100	38	3.68
9-Ethylacridine	6.2	–	81	7.75
		100	81	7.75
9-Acetamidoacridine	4.2	–	40	3.78
		100	67	6.42
Acridine orange	Tertiary amine	–	79	110
		100	88	122

The suspending medium was initially 4.17×10^{-6} M in the acridine derivatives. Acridine orange initial concentration was 1.04×10^{-4} M. The medium (1.2 ml) was 0.25 M sucrose, 1 mM in Na-Hepes (pH 7.0), 100 mM in NaCl where indicated and contained 5.22 mg of lysosomal protein. Determination of acridine uptake is described in the Methods. Percent uptake indicates the fraction of the acridine derivative taken up by the lysosomes from the medium.

take that has been measured, the uptake of other acridine derivatives, including acridine orange which is known to bind to lysosomes, were studied as well. Table 4 shows the uptake of 9-aminoacridine, 9-ethylacridine, 9-acetamidoacridine and acridine orange at low and high salt concentrations. All acridine derivatives including 9-acetamidoacridine (which is unionized at pH > 5) bind to lysosomes. A calculation of ΔpH based on the assumption that all of the amine taken up by the lysosomes is free is therefore not valid in this case. At high salt concentrations one expects that the internal concentration will be close to the external concentration. All of the acridines studied showed, however, quite high uptake values even at high salt concentrations, though they differed in the extent of uptake. Thus one cannot differentiate quantitatively on the basis of these experiments between the bound and the free species. It is observed, however, that those acridines which on the basis of their pK values, are not expected to be concentrated at low salt inside lysosomes (e.g., 9-acetamidoacridine and acridine orange) were taken up to a greater extent at high salt than at low salt. 9-Aminoacridine which is expected to be concentrated in its free form within lysosomes at low salt was indeed taken up to a greater extent at low salt. 9-Ethylacridine which has a lower pK and thus is expected to be concentrated less than 9-aminoacridine was taken up to the same extent at low and high salt. The uptake pattern of the various acridines is compatible with a ΔpH dependent distribution of 9-aminoacridine but a quantitative treatment is precluded because of the extensive binding of acridine derivatives.

4. Discussion

The data are best explained by assuming that the unequal distribution of permeable ions (such as protons, potassium, sodium and small amines) in lysosomes is determined by a Donnan potential. This conclusion is based on the finding that [^{14}C]methylamine and ^{42}K give the same distribution ratio and that this ratio decreased as the salt concentration in the suspending medium is increased. Since we have shown that protons are sufficiently permeable, their distribution must be the same as that of other permeable cations. The [^{14}C]methylamine distribution ratio allows the calculation of ΔpH even for cases in which the protons are not free to equilibrate throughout the system, if it is assumed that the neutral species permeates fast enough to equilibrate across the membrane. The fact that this ΔpH is unaffected by proton conductors indicates that proton distribution is also an equilibrium distribution, i.e., Donnan distribution.

Table 5 shows several parameters of the lysosomal suspensions calculated from the experiment given in fig. 1, assuming that the potassium distribution and

Table 5
Intralysosomal parameters as affected by the Donnan distribution at various KCl concentrations in the suspending medium.

KCl concn. in the medium (mM)	$^{42}K_{in}/^{42}K_{out}$	$\frac{[^{14}C]_{in}\ methylamine}{[^{14}C]_{out}\ methylamine}$	ψ (mV)	pH_{in}	Internal salt concn. (mM)	Fixed charge concn. (mM)
0.42	51.0	53.0	99	5.3	0.008	20
2.0	20.4	19.8	76	5.7	0.1	40
20	5.7	5.7	44	6.25	3.5	110
100	2.3	2.0	21	6.64	43	230

Lysosomes were suspended in 0.25 M sucrose, 1 mM Na-Hepes, pH 7.0. The results are those of the experiments given in fig. 1. The concentration ratio of ^{42}K and [^{14}C]methylamine was determined as described in the Methods. The Donnan potential was calculated according to $\psi = 58 \log([K^+]_{in}/[K^+]_{out})$, pH_{in} in accordance with $\Delta pH = \log[^{14}Ca]_{in}/[^{14}Ca]_{out}$ at $pH_{out} = 7.0$. Chloride ion internal concn. was calculated from $[Cl^-]_{in} = ([K^+]_{out}/[K^+]_{in}) \times [Cl^-]_{out}$ and the concn. of the fixed charged ($n[R^{n-}]$) from $n[R^{n-}] = ([Cl^-_{out}]^2 - ([Cl^-]^2_{in}/[Cl^-]_{in}$.

methylamine distribution obey a Donnan distribution. Donnan potential, ΔpH, internal anion concentration [Cl^-] and fixed charge concentration are given. It is observed that the calculated fixed charge concentration increases 10-fold on increasing the salt concentration 200-fold. This increase might be partially due to osmotic shrinkage and changes in solute and water activity coefficients. However, it is probably mostly the result of an increase in the degree of ionization of charged groups which is brought about by the change in the internal pH from 5.3 to 6.64. It was previously found [9] that about 50% of the lysosomal protein is an acidic protein, and this protein together with phospholipids could account for this excess of fixed negative charge.

Fig. 2 shows a plot of the internal anion concentration, [Cl^-], as a function of the concentration of salt in the medium. Because of electroneutrality considerations the internal concentration of the anion represents maximal salt (KCl) penetration into lysosomes. The curve has a very distinct positive curvature, which is an important criterion for distinguishing Donnan phenomena from other types of binding in which case the curvature is negative [10]. The figure also shows that most of the salt is excluded from the lysosomes even when suspended in salt solution. This might explain their osmotic stability despite the permeability to small ions. Thus, in fact, the salt is excluded from the lysosome by the Donnan potential leading to protection from osmotic lysis.

Although the internal pH calculated for high salt concentration is smaller than that implicated for *in vivo* conditions, it is possible that *in vivo* the charges are more concentrated (i.e., lysosomes may be more contracted under physiological conditions) and thus less affected by isotonic salt concentration. Furthermore, intralysosomal hydrolytic reactions could generate local excess of protons.

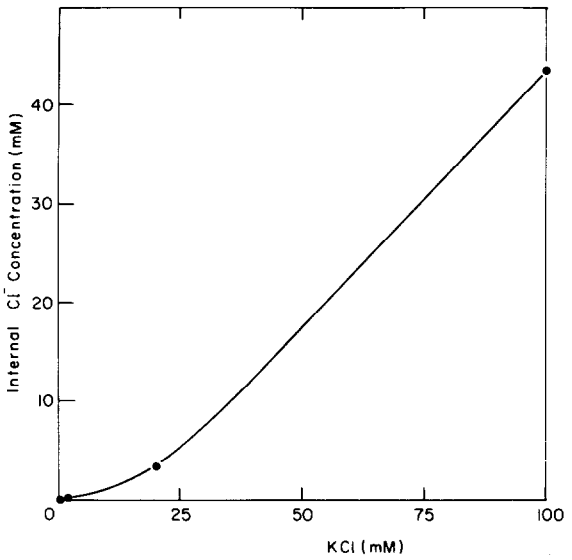

Fig. 2. Internal chloride concentration as a function of external KCl concentration. The values are taken from table 5.

After completion of this work a report on low intralysosomal pH measured by use of [^{14}C]methylamine distribution in Triton WR-1339 filled lysosomes has appeared [11].

Acknowledgements

We wish to thank Professor M. Avron and Dr. L. Goldstein for helpful discussions.

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