Biochimica et Biophysica Acta, 375 (1975) 274–285

BBA 76826

A THERMALLY INDUCED ALTERATION IN LYSOSOME MEMBRANES: SALT PERMEABILITY AT 0 AND 37 °C*

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(Received June 21st, 1974)

SUMMARY

Preparations of radioactive lysosomes were obtained from mouse kidney after injection of radioactive iodine-labeled bovine ribonuclease. Stability of these lysosomes in various media was estimated from measurements of proteolytic activity towards the ribonuclease, and of ribonuclease retention in particles. The lysosomes were stable at 37 °C in isotonic, sucrose-free solutions of KCl, NaCl, and potassium acetate, and in mixtures of these with $MgCl_2$, showing that these salts are relatively impermeant through the lysosomal membranes. The membranes were less permeable to Na^+ than to K^+. Both KCl and NaCl exerted their optimal protective effects over a broad concentration range above 0.125 M in 0.025 M acetate buffer. Mg^{2+} enhanced the protective effect of both K^+ and Na^+; the osmotic effect of 0.075 M NaCl–0.05 M $MgCl_2$ was indistinguishable during the entire course of ribonuclease digestion from that of isotonic sucrose. Osmotic protection by KCl–$MgCl_2$ was demonstrated over the pH range 5.5–7.0. A marked alteration in membrane properties occurs at lower temperatures in 0.11 M KCl–0.01 M $MgCl_2$ such that, at 0 °C, K^+ permeability is much higher than at 37 °C, as shown by a several-fold decrease in stability at the lower temperature.

INTRODUCTION

Lysosomal stability has usually been assayed by the criteria of enzyme latency or retention of enzymes within lysosomes. Because isotonic sucrose provides relatively good osmotic protection (Berthet et al. [1]), it has been used in most lysosome research. This is not a physiological medium. Salts can replace sucrose under appropriate circumstances. Rat liver lysosomes show fair stability in isotonic KCl over a

Abbreviation: MES, 2-(*N*-morpholino)ethane sulfonic acid.

* A preliminary report of some of this work was presented at the meeting of the Biophysical Society and the American Society of Biological Chemists, Minneapolis, June 1974.

range of temperatures (Berthet et al. [1], Allen and Lee [2], Ignarro [3], Berthet and de Duve [4]) and in sodium salts at 25 °C (Lloyd [5]). Bertini et al. [6] made the suggestive observation that, in 0.25 M $MgCl_2$ and at 0 °C, rat liver lysosomes are stable for prolonged intervals. Ignarro [7] recently demonstrated that such lysosomes are as stable at 37 °C, pH 7.4, in Hanks Balanced Salt Solution as in sucrose. Using this medium, Ignarro studied lysosome properties under the most nearly physiological conditions yet used.

Measurements of in vitro digestion of exogenous labeled proteins within intact lysosomes provide a sensitive and convenient tool for assessing lysosomal stability. The proteins are injected and absorbed via pinocytosis prior to sacrifice and fractionation. Damage is immediately reflected in decreased digestive activity. In this way, stability changes which occur over a range of a few seconds to 1 h or more can be studied (Davidson [8, 9], Mego and McQueen [10]). Lysosome stability can also be measured by release of lysosomal constituents or of pinocytosed exogenous protein (Mego and McQueen [10]). The stability of lysosomes in various solutes, in turn, is inversely related to the ease with which these solutes penetrate the lysosome membrane, because only non-penetrating solutes can provide osmotic protection (Lloyd [5, 11]). Mego et al. [12] observed that mouse liver lysosomes are stable and digestively active in 1 M NaCl and KCl at 37 °C. By the criterion of retention of pinocytosed, exogenous labeled albumin, these workers showed such lysosomes to be relatively stable in isotonic KCl.

One purpose of the present paper is to present data on the comparative permeabilities of the salts used for stabilization. We find that in isotonic NaCl, mouse kidney lysosomes are quite stable for 30 min at 37 °C, as shown by their digestive activity and retention of intact, labeled exogenous ribonuclease under these circumstances. In the presence of dilute Mg^{2+}, this stability is further enhanced. K^+ also offers osmotic protection, but is rather more permeant than Na^+. However, by both tests, stability in KCl solution is strongly temperature dependent, indicating that lysosomal membrane permeability is markedly altered with changes in temperature.

MATERIALS AND METHODS

Bovine ribonuclease labeled with ^{125}I was prepared by labeling with *N*-bromosuccinimide as previously described (Davidson [9] and Davidson et al. [13]). Efficiency of labeling was improved to slightly over 80 % by using more dilute (0.01 M) phosphate buffer, pH 7. Some of these experiments were performed with ribonuclease B rather than A; uptake characteristics were similar for each form. 0.1 mg of enzyme in 0.05 ml of isotonic saline was injected into the tail veins of 6–8 weeks-old female mice. Mice were sacrificed after 20 min and kidneys were homogenized by means of a teflon pestle, in 0.25 M sucrose. Homogenates were centrifuged at $250 \times g$ for 10 min to remove debris, then at $2250 \times g$ for 20 min to sediment digestively active particles containing [^{125}I]-labeled ribonuclease. For details, see Davidson [9]. These precipitates were resuspended in 0.025 M sodium acetate, 0.25 M sucrose, 0.05 M mercaptoethanol, pH 5.5, 0.19 ml per kidney. Incubations were performed as previously described [9]. Briefly, aliquots of particle suspensions were diluted 100-fold with incubation media, in a bath regulated at 37 ± 0.15 °C unless otherwise stated. Incubations at 0 °C were performed by packing the tubes deeply in wet ice. In addition

to buffers and sucrose or salt as described in captions, media included 0.05 M mercaptoethanol and EDTA, except that EDTA was omitted when digestion of protein was only monitored for 10 min. In most experiments, EDTA was $3 \cdot 10^{-5}$ M but was $1 \cdot 10^{-3}$ M in several. Experimental results were not affected by these variations. The progress of ribonuclease digestion was followed by the release of ^{125}I in acid-soluble form, as determined by centrifugation of samples after addition of phosphotungstic acid in HCl [13]. When samples were removed only after 30 min of incubation, the mixture was stirred at intervals before this. Zero-time values were obtained for each incubation from similar particle dilutions which were not warmed: the difference between zero-time values and those obtained during incubation represent ribonuclease digestion. Acidified samples from KCl media showed a precipitate, presumably a phosphotungstate salt of K^+. Zero-time values were usually somewhat lower in KCl media (about 6–8 %) than without salt (10–13 %) and intermediate with NaCl. In experiments comparing incubations in KCl, in sucrose, and in hypotonic solution, when the acidified samples were adjusted to the same salt and sucrose concentrations before centrifugation, results were similar to experiments in which this was not done. Almost all incubations were either performed in duplicate, or 2–4 samples were removed in rapid succession from the incubation mixture, with duplicates agreeing within 3 % of the total radioactivity. Lysosome breakage was determined by centrifugation at 8900 $\cdot$ g to sediment intact lysosomes, followed by phosphotungstic acid treatment of the supernatant to bring down intact ribonuclease (Davidson [9]). This precipitate represents undigested exogenous protein released by lysosome breakage during incubation (Mego and McQueen [10]). In practice, supernatant radioactivities were measured, and those of precipitates determined by difference.

RESULTS

In our examination of protein degradation by lysosomes in salt medium, initial experiments were performed at neutral pH with MES buffer 2-(*N*-morpholino)-ethane sulfonic acid (Good et al. [14]) on the premise that, in the less artificial conditions of an aqueous salt medium, stability might be favored near physiological pH. With sucrose or salt for osmotic stability, the rate of ribonuclease digestion was not much less than that at the optimal pH of 5.5 (Table I), confirming that the pH–activity curve for proteolysis by intact lysosomes (Mego and McQueen [10]) is much flatter than that observed with lysosome extracts (Coffey and de Duve [15], Goettlich-Riemann et al. [16]).

As the same table shows, digestion was much reduced in media of low osmolarity due to lysis of the lysosomes. Because the incubations were performed in dilute suspensions, lysosome breakdown results in dilution of the released proteases to such an extent that the digestion rate due to released proteases is barely detectable in this system. In these experiments, the particle suspension, in buffered, isotonic sucrose, was diluted 100-fold in 0.025 M buffer, 0.05 M mercaptoethanol at 37 °C. Lysosome breakdown in hypotonic solution is not instantaneous, and the figure of 8.2 % ribonuclease breakdown in 10 min reflects some action of intact lysosomes. When osmotic lysis was produced prior to digestion by dilution of preparation in cold hypotonic buffer before warming the mixture to 37 °C, digestion was cut by half

TABLE I

OSMOTIC PROTECTION OF LYSOSOMES BY SUCROSE OR SALT MEDIUM, AS SHOWN BY RIBONUCLEASE DIGESTION AT pH 5.5–7.0

Buffers were 0.025 M. At 0 time, portions of kidney particle suspension were added to 100 times their volume of incubation medium at 37 °C, except that in the indicated trials, the dilution was made at 0 prior to warming to 37 °C. Similar mixtures were kept on ice to provide 0-time values. Samples were removed at the indicated times and treated with phosphotungstic acid. The acid-soluble label was determined in each case as a percentage of the total label in the sample. Ribonuclease digestion at 10 and 30 min was found by subtracting the percentage of label which was acid soluble at 0-time from that found after 10 or 30 min of incubation. If several experiments were performed with the same medium, standard errors are given.

Buffer and additions	No. of experiments	Ribonuclease digestion (percentage of total [^{125}I]-labeled ribonuclease)	
		10 min	30 min
MES buffer, pH 7.0			
0.25 M sucrose	4	19.3±1.3	
0.11 M KCl–0.01 M $MgCl_2$	3	19.4±1.6	
0.11 M KCl–0.01 M $MgCl_2$			
0 °C for 5 min, prior to warming	1	13.3	
0 °C for 13 min, prior to warming	1	8.8	
Dialyzed against above salts at 0 °C	1	7.8	
None	3	8.2±1.5	
Sodium citrate, pH 6.5	1		
0.25 M sucrose		17.8	28.6
0.11 M KCl–0.01 M $MgCl_2$		18.9	27.0
None		8.0	12.1
Sodium citrate, pH 5.5	1		
0.25 M sucrose		19.3	44.3
0.11 M KCl–0.01 M $MgCl_2$		24.2	45.3
None		11.5	19.7
Sodium acetate, pH 5.5			
0.25 M sucrose	4	27.9±0.8	49.4±1.6
0.11 M KCl–0.01M $MgCl_2$	4	27.2±1.1	42.1±1.6
0 °C for 5 min, prior to warming	2	14.6±0.8	24.1±0.3
None	4	9.4±1.0	17.2±1.9
None	2	4.4±0.1	8.3±0.2
0 °C for 13 min, prior to warming			

(Table I, last line).

In 0.11 M KCl and 0.01 M $MgCl_2$, corresponding to the principal intracellular cations, with MES buffer and 0.05 M mercaptoethanol to enhance lysosomal proteolysis (Mego and McQueen [10]), digestion was nearly the same as with 0.25 M sucrose (Table I). Since a readily permeant solute cannot provide osmotic protection, this stabilization by salt demonstrates the relative impermeability of the lysosomes to KCl. Potassium acetate gave similar results to those seen with the chloride.

When the lysosome preparation was added to the KCl–$MgCl_2$–MES buffer at 0 °C; a few minutes before warming to 37 °C, no digestion occurred after warming. The same was true in acetate buffer with these salts at pH 5.5 (Table I). Thus, the lysosomes were not stable in salt at 0 °C, in contrast to their stability in sucrose at

this temperature. Their membranes underwent a change between 0 and 37 °C lowering permeability to ions at the higher temperature. Independent confirmation of this permeability change will be described below.

Table I shows that KCl offers osmotic protection with other buffers besides MES, and at lower pH. Optimal digestion occurs in the pH range 5–5.5, and at this unphysiological pH, also, membrane permeability at 37 °C is low to KCl. After incubation for 30 min instead of 10, the extent of digestion in the salt medium was almost equal to that in sucrose, showing that the salt offers protection for at least that long. We have previously reported that, to obtain maximal digestion in this system, it is necessary to avoid the inhibitory effects of very small concentrations of contaminant metal ions, either by incubation of very concentrated particle suspensions or by addition of very dilute EDTA [8] (and manuscript in preparation). In the experiments reported here, EDTA was added to the medium when digestion was monitored for more than 10 min. At pH 6.5, citrate buffer possessed sufficient chelating power to render negligible the effect of added EDTA.

Although K^+ is the dominant cation within cells, Na^+ offers greater osmotic protection under the conditions of these experiments. Representative curves showing the complete course of digestion are presented in Fig. 1. Digestion was more extensive after 30 min in Na^+ medium than with K^+, whether both salts were compared in the presence or in the absence of added Mg^{2+} (Table II). It follows that the membrane permeability to Na^+ is significantly lower than to K^+. Lyso some stability is near maximal in 0.125 M salt, both for NaCl (Fig. 2) and KCl (Fig. 3). Extent of digestion falls rapidly with lower concentrations, as may be seen from the figures. There is a broad plateau of stability in hypertonic salt, with some diminution of activity when

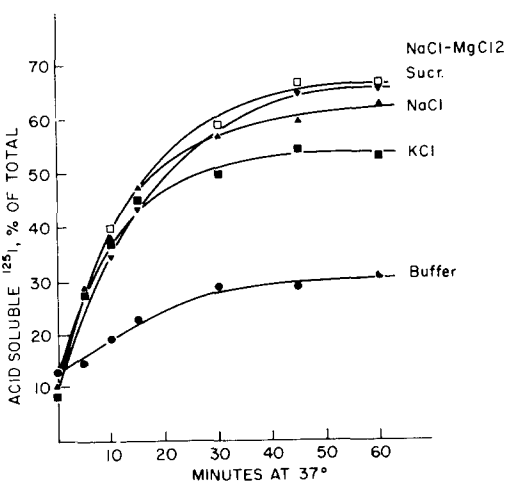

Fig. 1. Ribonuclease digestion as an index of osmotic stabilization by salts or sucrose. Digestion is low in the absence of an isotonic impermeant solute. Duplicate incubations were performed in each medium as described in Materials and Methods and Table I, and samples were removed at the indicated times and treated with phosphotungstic acid. The graph points shown averages from the duplicate incubations. The medium was 0.025 M sodium acetate buffer, pH 5.5, 0.05 M mercaptoethanol, $3 \cdot 10^{-5}$ M EDTA, and the following additions: ▼, 0.25 M sucrose; ⎵, 0.075 M NaCl 0.05 M $MgCl_2$; ▲, 0.15 M NaCl; ■, 0.15 M KCl; ●, no additions.

TABLE II

OSMOTIC STABILITY OF LYSOSOMES WITH VARYING SALT COMPOSITION

Incubations and calculations were performed as described for Table I, and the extent of ribonuclease digestion was determined after 30 min at 37 °C. Buffer was 0.025 M sodium acetate, pH 5.5. The average values for acid-soluble label are given, with standard errors. Probabilities, P, were computed by t-test upon paired values from parallel incubations. There were 3–5 such comparisons for each probability determined.

Addition	No. of incubations	Ribonuclease digestion (percentage of total ±S.E.)
0.025 M sucrose	7	46.3±0.8
0.075 M NaCl, 0.05 M $MgCl_2$	5	49.2±2.0*
0.15 M NaCl	5	44.9±1.9*,**,***
0.075 M KCl, 0.05 M $MgCl_2$	5	42.4±2.0*
0.15 M KCl	6	37.4±1.8**,***
None	5	16.4±0.6

* Difference from 0.25 M sucrose not significant at $P = 0.1$,

** Difference between NaCl and KCl significant at $P = 0.05$. and difference between KCl with Mg^{2+} and NaCl with Mg^{2+} significant at $P = 0.02$.

*** Difference from same salt with Mg^{2+} significant at $P = 0.01$.

NaCl concentration reaches 1 M (Fig. 2). K^+ was not tested above 0.4 M.

When some of K^+ or Na^+ was replaced by Mg^{2+}, digestive activity was further enhanced. In 0.05 M $MgCl_2$ and either KCl or NaCl, 0.075 M, ribonuclease breakdown in 30 min was not significantly different from that in isotonic sucrose. As noted, here too activity was greater with Na^+ than with K^+. In fact, NaCl plus $MgCl_2$ allow digestion at 37 °C to proceed to completion at a rate at least as great as with sucrose,

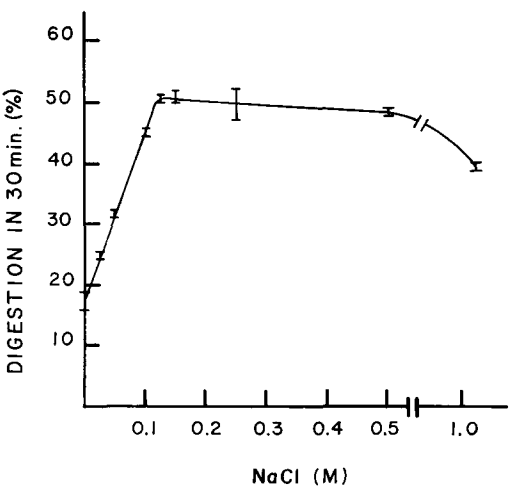

Fig. 2. Stability of digestively active lysosomes as a function of NaCl concentration; extent of ribonuclease digestion after 30 min in media of increasing NaCl concentration. Experiment was performed as described in Fig. 1, with particle suspension diluted into incubation media at 37 °C. Three samples were removed from each incubation mixture for treatment with phosphotungstic acid; the points represent averages and the bars, standard errors.

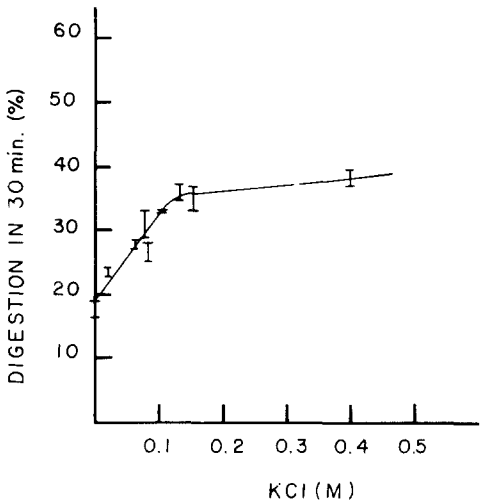

Fig. 3. Stability of digestively active lysosomes as a function of KCl concentration; extent of ribonuclease digestion after 30 min in media of increasing salt concentration. Procedure was as for Fig. 2. The points represent averages for two samples, and the range is given by the bars.

over a span of almost 1 h (Fig. 1). It follows that Mg^{2+} is impermeant, but the effect of Mg^{2+} in this case may be more than merely osmotic (see Discussion).

The demonstrations of lysosomal stability described so far have been indirect, depending upon a property, digestive activity, which is known to require intact lysosomes (Davidson [9], Mego et al. [12]). It is also possible to estimate lysosome stability independently and more directly, by measuring release of undigested ribonuclease due to lysosome breakage. This is done by centrifuging, after incubation, to bring down intact lysosomes, and then adding acid to the supernatant, to precipitate undigested, labeled protein (Mego and McQueen [10]). In this type of experiment, the phosphotungstic acid precipitate includes only that intact protein which has become dissociated from lysosomes, because the first spin removes intact particles. Using this approach we showed that in KCl–$MgCl_2$ mixtures lysosomal breakage at 37 °C is greater than in sucrose (Table III), probably accounting for the lesser hydrolysis in the former medium. Even in sucrose thermal breakdown occurred, in agreement with the early observations of Berthet and de Duve [4]. At 0 °C, in contrast to 37 °C, the salt mixture offered little more protection than a hypotonic medium. After 30 min in cold salt the extent of breakage was not significantly different from that in hypotonic solution at 37 °C. Even after only 10 min at 0 °C, breakdown in salt was about 80 % complete, if ribonuclease release in hypotonic medium is considered maximal. This is a reasonable assumption considering the adsorption of lysosomal components in tissue preparations (Baccino et al. [17]) and the retention of bovine ribonuclease by damaged lysosomes (Davidson et al. [13]). This shows clearly the markedly greater permeability of lysosome membranes to K^+ at 0 than at 37 °C. An alternative hypothesis, that breakdown at 0 °C was due directly to ion binding by the membranes, was tested and excluded by suspending lysosome preparations at 0 °C in sucrose with KCl and $MgCl_2$ (Table III). In this medium, despite the resulting hypertonicity, the particles were much more stable than in isotonic KCl–$MgCl_2$ alone at this tem-

TABLE III

BREAKDOWN OF MOUSE KIDNEY LYSOSOMES AT 37 AND 0 °C IN ISOTONIC SALTS AND IN ISOTONIC SUCROSE

Portions of mouse kidney particle preparations were diluted 100-fold in the media and at the temperatures shown. Besides the additions described, each medium contained 0.025 M sodium acetate buffer, 0.05 M mercaptoethanol, and $3 \cdot 10^{-5}$ M EDTA at pH 5.5. After 30 min (except as shown), duplicate 1-ml samples of each incubation suspension were made up on ice to 2-ml volumes of identical composition: 0.25 M sucrose, 0.055 M KCl, 0.005 M $MgCl_2$. These mixtures were centrifuged for 15 min at 8940 $\times$ g in a Spinco No. 40.3 rotor to sediment intact lysosomes. The supernatants were treated with phosphotungstic acid reagent as described (Davidson [9]) to sediment undegraded, labeled protein, released upon breakdown of damaged lysosomes. A correction was made for quenching by phosphotungstic acid. Ribonuclease release is given as acid-insoluble ^{125}I in the 8900 $\times$ g supernatant, as a percentage of the total spun at 8900 $\times$ g. The averages for 2–4 experiments are given, where the value for each experiment was the average from duplicate samples, agreeing in almost every instance within 1·5 % of the total spun. Net lysosome breakdown at 0 °C was computed by subtracting the control value of soluble ribonuclease in sucrose at 0 °C, and dividing the corrected ribonuclease release in each test by that seen with maximal breakage. Maximal breakage was assumed to correspond to the average ribonuclease release after 30 min in sucrose at 37 °C.

Additions	Temperature (°C)	No. of experiments	Ribonuclease release (percentage ±S.E.)	Lysosome breakdown (percentage net)
0.25 M sucrose	37	4	23.7(±1.5)	
	0	4	9.2(±0.2)	0
0.11 M KCl, 0.01 M $MgCl_2$	37	3	36.2(±3.2)	
10 min	0	3	55.7(±5.8)	79.8
30 min	0	3	63.5(±5.1)	93.2
0.25 M sucrose, 0.11 M KCl, 0.01 M $MgCl_2$	0	2	26.7(±2.7)	21.4
None	37	4	67.5(±1.18)	100

perature. The data in Table III also show that lysosome breakdown in salt is not a consequence of warming from 0 °C, since it is seen when the particles are kept cold throughout the experiment.

DISCUSSION

When intralysosomal protein digestion is measured in dilute lysosome suspensions at moderately acid pH, rapid proteolysis indicates intactness of the lysosomes. Lysosome breakage causes a drop in proteolysis rate to levels barely detectable in short incubations, due to dilution of both protease and substrate by the incubation medium. The membranes of mouse kidney lysosomes are relatively impermeable to KCl and NaCl in vitro at 37 °C, as shown by the active lysosomal digestion in such media for well over 10 min (Tables I and II). Similar results have been obtained with potassium acetate. The persistence of digestive activity means that the lysosomes are intact (Mego and McQueen [10], Davidson [8, 9]), and their resistance to osmotic lysis in these media shows that the salts do not penetrate the membranes (Lloyd [5, 11]). Between 37 and 0 °C, the membranes undergo a transformation which

greatly increases their permeability to K^+, such that, at the lower temperature, KCl no longer provides osmotic protection. This follows from the lack of digestive activity in lysosomal preparations which have been chilled in KCl medium (Table I), and from the direct demonstration of rapid release of exogenous ribonuclease from lysosomes in such medium in the cold (Table III). The disruption in salt at 0 °C is not an effect of charge binding at this temperature – in 0.25 M sucrose, 0.11 M KCl, 0.01 M $MgCl_2$ at 0 °C, the lysosomes were not quite as stable as in 0.25 M sucrose alone, but much more so than in the same composition of salt without sucrose (Table III). This is a hypertonic solution which may explain the drop in stability.

Comparison of extents of digestion in various media leads to conclusions about the relative permeabilities of the lysosomal membrane in various species (Table II and Fig. 1). The differences in digestion shown in Table II do not indicate differences in initial reaction rates, since these are similar for sucrose and K^+ (Table I and Fig. 1), and not less for NaCl than for sucrose (Fig. 1). It follows that, at 37 °C, permeability of the lysosome membranes to Na^+ is less than that to K^+. This may reflect the greater hydrated radius of Na^+ than of K^+, or their greater hydration energy (Cotton and Wilkinson [18]) if penetration requires dehydration (Stein [19]).

The increased lysosomal activity seen with added Mg^{2+} (Table II and Fig. 1) is certainly partly due to an osmotic effect since otherwise lysis would occur with the diminished concentration of Na^+ or K^+. Mg^{2+} clearly does not penetrate these membranes. However, a direct effect upon lysosome membranes must be considered as well. Marked alterations upon binding of Mg^{2+} or other divalent cations are characteristic of biological membranes (Reinert and Davis [20], Steck et al. [21], Burger et al. [22]) and membrane lipids (Rand and Sengupta [23]). The superior osmotic protection with Na^+, and its further enhancement by Mg^{2+}, may explain Ignarro's [7] finding that in Hanks Balanced Salt Solution rat liver lysosomes are more stable than in KCl. However, the relative permeabilities may not be the same with rat liver lysosomes as in mouse kidney.

The stability changes with temperature can be approximated quantitatively as follows. Intralysosomal digestion in sucrose medium, as measured by release of acid-soluble iodine label, is a first-order reaction characterized by a rate constant, k_D, of 0.056 min^{-1} at 37 °C, pH 5.5, corresponding to a half-time of 12.4 min, and breakdown of ribonuclease-bearing lysosomes during the course of digestion is less than about 10 % of the total (Davidson, manuscript in preparation). Digestion is thus describable by the equation:

$$I_s^{\text{sucrose}} = R_0(1 - e^{-k_D t}) \tag{1}$$

where I_s is acid-soluble radioactivity released during incubation at 37 °C, R_0 is the quantity of ^{125}I-labeled ribonuclease in digestively active lysosomes at time 0 and t is time at 37 °C. We make the reasonable assumption that lysosome breakdown in salt is also first order with respect to concentration of intact lysosomes, having a breakdown constant k_B. We also note that digestion proceeds at very nearly the same rate within intact lysosomes in salt as in sucrose (Fig. 1), and so take the same rate constant for both media. Then, for acid-soluble iodine release

$$I_s^{\text{salt}} = \frac{k_D R_0}{k_D + k_B^{37\,°C}} \left[1 - e^{-(k_D + k_B^{37\,°C})t}\right] \tag{2}$$

Here, k_B describes the rate of breakdown in excess of any which may occur in sucrose, although breakdown in sucrose, as stated above, is very slow.

The ratio, $r = I_s^{salt}/I_s^{sucrose}$, which is readily measured experimentally, works out as

$$r = \frac{k_D}{k_D + k_B^{37\,^\circ C}} \cdot \frac{1 - e^{-(k_D + k_B^{37\,^\circ C})t}}{1 - e^{-k_D t}} \tag{3}$$

At sufficiently long time intervals, the ratio involving exponentials goes to unity, allowing determination of k_B from the known value of k_D and experimental measurement of the limiting value of r, designated r_e. r_e was determined for two kinetic experiments, each involving duplicate incubations in both sucrose and 0.11 M KCl, 0.01 M $MgCl_2$ at 37 °C, pH 5.5, for 60 min. Maximal digestion in each medium was determined from the visually determined asymptotes of the digestion curves, such as those in Fig. 1. The error in this visual determination is estimated as less than 5 % of the total release of acid-soluble ^{125}I. Values of 0.798 and 0.867 were obtained for r in these experiments, giving an average of 0.83(2). Taken together with the aforementioned value for k_D, this gives 0.011(3) for $k_B^{37\,^\circ C}$ (half-time, 63 min). The validity of this result can be checked by substituting $k_B^{37\,^\circ C}$ and k_D back into Eqn 3, calculating the predicted value of r_{30} (for 30 min of incubation) and comparing with the experimental results for pH 5.5, from the experiments summarized in Table I. The experimental value for r_{30} was obtained by calculating the ratio for each experiment, averaging, and computing the standard error; it is 0.84(7)±0.055. This is to be compared with the predicted value of 0.88(7). A predicted value of 0.790 for r_{30} is obtained if one takes 0.023, twice the calculated experimental value for $k_B^{37\,^\circ C,\,salt}$, and this provides an upper limit.

The rate of breakdown at 0 °C may be calculated from the extents of ribonuclease release from lysosomes in each medium, divided by the release after 30 min in the absence of osmotically protective additions (Table III). Thus, release without additions is assumed to represent total lysosome breakdown. If breakdown in salt is then assumed to be first order with respect to intact lysosomes, a rate constant of breakdown, $k_B^{0\,^\circ C}$, can be computed. Taking the data for the first 10 min at 0 °C in KCl–$MgCl_2$, this is 0.16 (half-time 4.3 min), about 14 times $k_B^{37\,^\circ C}$, and about 7 times the upper limit for $k_B^{37\,^\circ C}$ of 0.023. Calculation thus confirms the qualitative conclusion that the lysosomes break down much more rapidly at 0 than at 37 °C in KCl–$MgCl_2$. It follows that some marked change occurs in the membranes of these renal lysosomes between the two temperatures, causing the change in permeability. Goldman and Rottenberg [24] have shown that salt penetration into rat liver lysosomes in the cold is controlled by a Donnan equilibrium. In mouse kidney at 37° C, our results show that the membrane itself must be limiting.

It will be important to study the temperature dependence of permeability in detail, to learn the structural correlates of the permeability change. Possible causes include a thermal transition in membrane lipids (Blazyk and Steim [25], Oldfield and Chapman [26]), or a change in conformation or aggregation of membrane proteins. It seems unlikely that an ion-pump could be involved, removing ions at the higher temperature, because in our highly dilute incubation media (at least 100 times dilute relative to the intact kidney) concentrations of endogenous ATP should be quite low. Moreover, at pH 5.5, addition of ATP (10^{-3} M) to the medium did not increase

the extent of digestion seen in 30 min with 0.075 M KCl–0.05 M $MgCl_2$.

In previous studies, cited in the Introduction, isolated rat liver lysosomes were stable in KCl at low temperature as well as 37 °C. Possibly the difference in properties reflects the different origins of the lysosomes. Differences in pH may also be significant. Also, there are different populations of lysosomes within a given tissue (Malbica [27, 28], Baggiolini et al. [29], Beaufay et al. [30]). The extent of the differences in properties between these has not yet been thoroughly explored, and preparative methods selecting for one type or another may provide markedly different products.

These results confirm Ignarro's finding [7] that salts can be used to provide osmotic stabilization during studies upon lysosomes, allowing comparison of properties observed in sucrose with those in an ionic medium. Lysosomal proteolysis in Na^+–Mg^{2+} medium, the best salt medium tested, proceeds at a rate similar to that in sucrose, suggesting that digestion results obtained in sucrose are reliable. The most nearly physiological ion composition studied, KCl plus $MgCl_2$, did not give stabilities as great as those seen with Na^+, or in sucrose. In no medium has true lysosome stability been found, since even in 0.25 M sucrose, thermal degradation occurs (Berthet et al. [1], and see Table III of this paper). Moreover, although mouse kidney lysosomes are unstable in cold KCl plus $MgCl_2$ in vitro, we isolated active lysosomes from kidneys which had been kept on ice for 30 min or more during which time thelysosomes remained intact in the K^+-rich cytoplasm. Some of the other components of the intact cell must be required for optimal stability; specific anion effects may be involved. There could also be an effect of protein binding to the membrane surface, specifically or otherwise. Stoner and Sirak [31] present evidence that macromolecules are required to provide osmotic protection to mitochondria equivalent to that in vivo, a condition which may apply to lysosomes in view of the suggestion of Beaufay et al. [32] of a sucrose-permeable protein-impermeable "sucrose space" in these particles. ATP has been found to affect lysosome stability (Malbica [28]). Although we found no effect in our system at pH 5.5, ATP may energize an ion pump at physiological pH (Mego et al. [33], Hegner [34]). Moreover, the stabilizing effect of sucrose may be more than osmotic; perhaps polyhydroxylic solutes, such as the triose and hexose phosphates within cells, are determinants of lysosome membrane properties, in a manner analogous to the effect of polyhydroxylic alcohols in other membranes (Zimmer et al. [35]) or with proteins (Myers and Jacoby [36]). The data of Lee [37] also suggest a possible specific effect of some phosphorylated polyalcohols. Polyalcohols clearly cannot be considered inert, but it is not yet clear in what range their effects should be considered "physiological", and when otherwise.

ACKNOWLEDGEMENTS

Thanks are due Dr D. F. H. Wallach for helpful discussions. This work was partly supported by Research Grant BC-41 from the American Cancer Society.

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