Mechanisms of Loss of Latency of Lysosomal Enzymes

EFFECTS OF INCUBATION ON THE PROPERTIES OF LYSOSOMAL MEMBRANES

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1. The effects of sucrose and KCl on the loss of latency of lysosomal enzymes caused by incubation at 37°C, pH 7.4, were examined by using Triton-filled lysosomes from rat liver and two fractions from livers of rats not injected with Triton. 2. After incubation, the percentage free activity of lysosomal enzymes was measured before and after cooling to 0°C in order to determine the amount of latency lost at 37°C without cooling and the additional amount lost on cooling the incubated lysosomes to 0°C. 3. The latency that is lost without cooling is first decreased and then increased by increasing the osmotic strength of the incubation medium with KCl, or with sucrose in the presence of KCl. However, if the osmotic strength is increased with sucrose alone, loss of latency is decreased up to 0.25 M-sucrose, but is increased only slightly at higher sucrose concentrations. Apparently the lysosome is permeated by hyperosmolar KCl but not by sucrose during incubation. 4. If the osmotic strength of the assay medium is increased with KCl, the loss of latency caused by incubation for 60 min in hyperosmolar KCl is repressed. Thus it appears that a KCl-permeated lysosome can be obtained which is relatively stable until exposure to lower osmolarities. 5. The loss of latency caused by cooling incubated lysosomes to 0°C is largely eliminated if the osmotic strength of the medium in which the lysosomes are cooled is raised sufficiently with either sucrose or KCl. 6. Osmotic-fragility curves were obtained after incubation for 1 and 60 min at iso-osmoticity (0.2 M-KCl or 0.25 M-sucrose). Although little loss of latency occurs at iso-osmoticity, lysosomes incubated for 60 min display greatly increased fragility on exposure to hypo-osmolar KCl, hypo-osmolar sucrose or hyperosmolar KCl. 7. It is suggested that permeability to KCl at 37°C and the increase in fragility on exposure to hypo-osmolar conditions are both consequences of injury, probably from enzymic action, sustained by the lysosomal membrane during incubation at 37°C.

We are concerned with understanding the mechanisms by which the enzyme latency of lysosomes is lost during incubation *in vitro*. In studies on Triton-filled lysosomes from rat liver (Ruth & Weglicki, 1978), we found that incubation for up to 50 min at 37°C in 0.25 M-sucrose/0.04 M-Mops (4-morpholinepropanesulphonic acid), pH 7.4, did not cause loss of latency of the lysosomal enzymes unless the incubated lysosomes were first cooled to at least 15°C. This suggested that, on cooling, the lysosomal membrane undergoes a phase transition starting at approx. 15°C. We postulated that when lysosomes are incubated at 37°C enzymic action causes the production of potentially lytic substances, such

as lysophospholipids and non-esterified fatty acids. During the phase transition these substances may become more locally concentrated as the fluid areas in the membrane decrease; the consequence of this could be lysis of the lysosomal membrane, as manifested by loss of latency of the lysosomal enzymes. We reported that the enzyme latency of normal hepatic lysosomes (from rats not injected with Triton WR-1339) was also lost more rapidly on incubation at 37°C if the incubated lysosomes were cooled before assay, indicating that a phase transition was not unique to Triton-filled lysosomes. Subsequently we have found, however, that the extent to which incubation at 37°C results in loss of enzyme

latency, regardless of whether the incubated lysosomes are or are not cooled before assay, varies greatly depending on the type of lysosomal preparation being studied and on the composition of the incubation medium. Incubation at 37°C of normal hepatic lysosomes results in a more rapid loss of enzyme latency if heavy lysosomes (obtained between 1000g and 3300g on differential centrifugation) are studied than if light lysosomes (obtained between 3300g and 20000g) are studied. The effects of cooling are not so pronounced for normal as for Triton-filled lysosomes, and for incubations in 0.25 M-sucrose very little effect of cooling can be seen with light lysosomes. However, when incubations were carried out in 0.15 M-KCl instead of 0.25 M-sucrose, we observed a much more rapid loss of enzyme latency for both heavy and light lysosomes; in addition to a greater loss of latency at 37°C without cooling, a much greater effect of cooling was seen with both lysosomal preparations. The greater stability of light than of heavy lysosomes has been previously reported (Tanaka & Iizuka, 1968; Ignarro, 1971). These investigators also found that the lysosome-stabilizing effects of drugs at neutral pH was much greater for heavy than for light lysosomes.

The present paper reports a systematic study of the effects of buffer composition on the loss of enzyme latency caused by incubation at 37°C. These studies were limited to using, as the osmotically protecting solute, either sucrose or KCl; lysosomes have been regarded as being relatively impermeable both to sucrose at various temperatures (Appelmans & de Duve, 1955; Mego *et al.*, 1967; Lloyd, 1969) and to KCl at 37°C (Davidson & Song, 1975; see also Reijngoud & Tager, 1975). Heavy and light lysosomes from rats not injected with Triton, as well as Triton-filled lysosomes, have been studied, in order to look for possible differences among these preparations in mechanisms of latency loss. The experiments described below were designed principally to determine: (1) the effects of increasing concentration of sucrose or KCl on the amount of enzyme latency lost as a result of incubation at 37°C when assays for latency are performed without cooling; (2) the effects, after incubation at 37°C, of increasing concentration of sucrose or KCl on the additional amount of latency, over and above that lost at 37°C, which is lost as a result of cooling the incubated lysosomes to 0°C; (3) the effects of incubation at 37°C at iso-osmoticity (0.25 M-sucrose or 0.2 M-KCl) on the osmotic-fragility properties at 37°C of lysosomes. In the Discussion section we use the results of these studies (1) to distinguish various stages involved in the loss of enzyme latency that results from incubation at 37°C and (2) to postulate a basic mechanism by which this loss of latency is potentiated by incubation.

Experimental

Preparation of lysosomes

Sprague-Dawley rats obtained either from Charles River Breeding Laboratories, Wilmington, MA, U.S.A., or from Flow Laboratories, Dublin, VA, U.S.A., and weighing 200–300 g were used for these experiments. Triton-filled lysosomes were prepared as described by Ruth & Weglicki (1978). These lysosomes are purified 20–40-fold over the original homogenate (Weglicki *et al.*, 1974). Lysosomes were also prepared from rats not injected with Triton WR-1339 as follows. Livers were extracted in buffer containing 0.25 M-sucrose and 0.01 M-Mops, pH adjusted to 7.4 with NaOH, by homogenization for 2 s in a Sorvall Omni-Mixer, followed by three up-and-down strokes in a Thomas Teflon-pestle homogenizer. Extraction buffer was added to this homogenate to adjust the volume to 150 ml/liver. Centrifugation was then carried out at 1000g for 10 min. The pellet from this centrifugation step was discarded and the supernatant was centrifuged at 3300g for 10 min. The pellet from this centrifugation step was designated as the 'heavy lysosomal fraction'. The specific activity of *N*-acetyl- β -glucosaminidase in this fraction was approx. 2.5 times that of the original homogenate. The supernatant from the 3300g centrifugation step was centrifuged at 20000g for 20 min. The pellet from this centrifugation step was designated as the 'light lysosomal fraction'. The specific activity of *N*-acetyl- β -glucosaminidase in this fraction was approx. 3.5 times that of the original homogenate.

Enzyme assays and determination of percentage free activity

N-Acetyl- β -glucosaminidase and *p*-nitrophenyl phosphatase were assayed in assay medium containing either 0.25 M-sucrose or 0.1% Triton X-100 as described by Ruth & Weglicki (1978). Percentage free activity was calculated as 100 $\times$ activity in 0.25 M-sucrose/activity in 0.1% Triton X-100. Assays were for 3 or 5 min.

Incubation of lysosomes and determination of osmotic fragility

Incubations were performed in a Precision Scientific Shaker Bath (Fisher Scientific, Medford, MA, U.S.A.). All buffers used for incubations or osmotic-fragility determinations contained 0.01 M-Mops, adjusted at 22°C to pH 7.4 with NaOH. In addition, buffers contained various concentrations of sucrose and/or KCl (see the Results section).

For determinations of the effects of concentration of sucrose or KCl on loss of latency resulting from incubation at 37°C, lysosomal pellets were drained of buffer and homogenized without additional buffer with a Teflon-pestle homogenizer. The suspension

was then diluted with buffers containing the desired concentrations of sucrose or KCl. Pellets were usually diluted 10–15-fold and final sucrose or KCl concentrations reported are always those of the diluting buffers. (The pellets of Triton-filled lysosomes were so small that they were simply homogenized directly in buffers containing the desired concentrations of sucrose or KCl.) After incubation of the lysosomes at 37°C for given lengths of time, some samples were assayed immediately at 37°C without an intermittent cooling in order to determine percentage free activity without cooling. Other samples were placed on ice for 15 min, after which they were assayed at 37°C in order to determine percentage free activity after cooling.

For determinations of osmotic fragility, the lysosomal pellets were homogenized as described above and were then diluted 4-fold with either 0.25 M-sucrose or 0.2 M-KCl. For simplicity, the KCl samples are referred to as having a KCl concentration of 0.2 M. The samples were incubated at 37°C for stated times and were then diluted 4-fold with buffers containing appropriate concentrations of sucrose or KCl so that the desired final concentrations of sucrose or KCl were achieved; 3 min after dilution, 0.1 ml samples were added directly to 0.9 ml of assay medium.

Results

Fig. 1 shows results obtained when a preparation of heavy lysosomes was incubated under three different conditions. Figs. 1(a) and 1(b) show increases in percentage free activity when the incubation was in the presence of 0.25 M-sucrose or 0.15 M-KCl respectively. Loss of latency at 37°C without an intermittent cooling before assay occurred much more rapidly in 0.15 M-KCl, especially after a lag period of about 60 min. Also increased, when incubation was in 0.15 M-KCl, was the loss of latency caused by cooling the incubated lysosomes to 0°C before assay. In Fig. 1(c) the same lysosomal preparation was incubated in 0.15 M-KCl plus 0.25 M-sucrose. The rate of increase in percentage free activity without cooling was about the same as that obtained in 0.15 M-KCl alone; however, the effects of cooling before assay were completely eliminated. Fig. 2 compares results of incubating the light lysosomal fraction in 0.25 M-sucrose (Fig. 2a) or 0.15 M-KCl (Fig. 2b). This lysosomal fraction seems to be extremely stable in 0.25 M-sucrose: in this experiment the increase in percentage free activity at 37°C without cooling was very low even after prolonged incubation (2 h), and only a small additional increase occurred on cooling to 0°C.

Fig. 1. Heavy lysosomes: increase in percentage free activity caused by incubation at 37°C in buffers containing sucrose and KCl

Heavy lysosomes were incubated at 37°C in 0.01 M-Mops, pH 7.4, plus 0.25 M-sucrose (a), 0.15 M-KCl (b) or 0.25 M-sucrose and 0.15 M-KCl (c). Shown are percentage free activities of *N*-acetyl- β -glucosaminidase when samples were removed at the indicated times and assayed at 37°C either immediately (O) or after being placed on ice for 15 min (Δ).

However, when the same preparation was incubated in 0.15M-KCl there was a much more rapid increase in free activity without cooling and a significant additional increase after cooling.

Fig. 3 demonstrates that, as suggested by the results shown in Fig. 1(c), the increase in free activity caused by cooling can be eliminated by increased osmolarity. In Fig. 3(a), a preparation of heavy lysosomes was incubated for 60 min in buffer containing 0.15M-KCl plus increasing concentrations of sucrose. The increase in free activity caused

by cooling decreased progressively as the sucrose concentration was increased from 0 to 0.25M. However, the increase in free activity without cooling was found first to decrease, up to about 0.1M-sucrose, and then to increase as the sucrose concentration was increased further. In Fig. 3(b), the sucrose was not present throughout the incubation, but instead was added after 57 min of incubation; 3 min later the lysosomes were either assayed immediately without cooling or were cooled to 0°C for 15 min and then assayed. The effects of the added sucrose seemed to

Fig. 2. Light lysosomes: increase in percentage free activity caused by incubation at 37°C in buffers containing sucrose and KCl

Light lysosomes were incubated at 37°C in 0.01M-Mops, pH 7.4, plus 0.25M-sucrose (a) or 0.15M-KCl (b). Shown are percentage free activities of *N*-acetyl- β -glucosaminidase when samples were removed at the indicated times and assayed at 37°C either immediately (O) or after being placed on ice for 15 min (Δ).

Table 1. Heavy lysosomes: percentage free activity after cooling minus percentage free activity before cooling, after incubation for 60 min at 37°C in buffer containing 0.15M-KCl plus increasing concentrations of sucrose

As described in the legend for Fig. 3, lysosomes were incubated for 60 min at 37°C in buffers containing 0.15M-KCl plus increasing concentrations of sucrose added either at the beginning of the incubation or at 57 min. At 60 min samples were removed and assayed for percentage free activity of *N*-acetyl- β -glucosaminidase either immediately without cooling or after being placed on ice for 15 min. The numbers shown in this Table were calculated by subtracting percentage free activity before cooling from percentage free activity after cooling. Values given are means $\pm$ ranges from two experiments.

Concn. of sucrose added to 0.15M-KCl (M)	Percentage free activity after cooling minus percentage free activity before cooling	
	Sucrose added at beginning of incubation	Sucrose added at end of incubation
0.00	42.9 $\pm$ 2.3	44.5 $\pm$ 2.8
0.05	32.9 $\pm$ 6.9	29.2 $\pm$ 2.4
0.10	22.5 $\pm$ 6.7	23.2 $\pm$ 3.9
0.15	13.9 $\pm$ 3.5	15.2 $\pm$ 4.0
0.20	6.0 $\pm$ 0.8	7.8 $\pm$ 1.9
0.25	6.5 $\pm$ 1.7	—
0.30	—	-3.5 $\pm$ 1.2

Fig. 3. Heavy lysosomes: percentage free activity after incubation for 60 min at 37°C in buffer containing 0.15 M-KCl plus increasing concentrations of sucrose present throughout incubation (a) or added at end of incubation (b)

In (a), heavy lysosomes were incubated for 60 min at 37°C in 0.01 M-Mops, pH 7.4, plus 0.15 M-KCl and sucrose at the concentrations indicated. At 60 min samples were removed and assayed for percentage free activity of *N*-acetyl- β -glucosaminidase either immediately (O) or after being placed on ice for 15 min (Δ). In (b), heavy lysosomes were incubated for 57 min at 37°C in 0.01 M-Mops, pH 7.4, plus 0.15 M-KCl. At 57 min samples were diluted 4-fold with buffers containing 0.01 M-Mops, pH 7.4, plus 0.15 M-KCl and appropriate concentrations of sucrose so that the indicated final concentrations of sucrose were obtained. After 3 min samples were removed and assayed for percentage free activity of *N*-acetyl- β -glucosaminidase either immediately (O) or after being placed on ice for 15 min (Δ).

be manifested fully even when the addition was made only 3 min before assay or cooling. As shown in Table 1, the increase in percentage free activity caused by cooling (percentage free activity after cooling minus that before cooling) was basically the same whether sucrose was added at the beginning or at the end of the incubation. Two conclusions are suggested from these results: (1) the increase in free activity obtained when incubated lysosomes are cooled can be largely eliminated if the osmolarity of the medium in which the lysosomes are cooled is sufficiently high, and (2) incubated lysosomes display a sensitivity to hyperosmolarity (in this case 0.15 M-KCl plus greater than 0.1 M-sucrose), as

manifested by a loss of enzyme latency in the absence of cooling.

Lysosomes that have been incubated at 37°C in 0.25 M-sucrose also exhibit a sensitivity to hyperosmolarity brought about by addition of increasing amounts of KCl. In Table 2, heavy lysosomes were incubated for 60 min in 0.25 M-sucrose; percentage free activities were determined both when increasing concentrations of KCl had been present throughout the incubation and when incubation was in the presence of 0.25 M-sucrose alone, with increasing concentrations of KCl added 3 min before assay without cooling. The lysosomes that were incubated in 0.25 M-sucrose alone displayed a sensitivity to

Table 2. *Heavy lysosomes: percentage free activity after incubation for 60 min at 37°C in buffer containing 0.25 M-sucrose plus increasing concentrations of KCl*

Lysosomes were incubated for 60 min at 37°C in buffers containing 0.01 M-Mops, pH 7.4, plus 0.25 M-sucrose and increasing concentrations of KCl added either at the beginning of the incubation or at 57 min. At 60 min samples were assayed for percentage free activity of *N*-acetyl- β -glucosaminidase immediately without cooling. Values given are the means $\pm$ ranges from two experiments.

Concn. of KCl added to 0.25 M-sucrose (M)	Percentage free activity at 60 min	
	KCl added at beginning of incubation	KCl added at end of incubation
0.00	6.9 $\pm$ 0.6	7.6 $\pm$ 0.9
0.09	11.5 $\pm$ 2.0	12.0 $\pm$ 1.2
0.19	37.7 $\pm$ 1.5	18.3 $\pm$ 2.1
0.28	55.2 $\pm$ 1.5	26.6 $\pm$ 2.2
0.38	65.8 $\pm$ 1.6	31.8 $\pm$ 1.4

Table 3. *Heavy lysosomes: effect of adding KCl to assay medium on percentage free activity observed after incubation for 60 min at 37°C in buffers containing hyperosmolar KCl*

Lysosomes were incubated for 60 min at 37°C in buffers containing various concentrations of KCl. At 60 min samples were removed and assayed without cooling either in the usual assay medium (containing 0.25 M-sucrose) or in assay medium containing 0.25 M-sucrose plus KCl at the same concentration used for the incubation. Values given are means $\pm$ ranges from two experiments.

Concn. of KCl in incubation or assay (M)	Percentage free activity at 60 min	
	Assayed in 0.25 M-sucrose	Assayed in 0.25 M-sucrose + KCl to indicated concentration*
0.38†	65.8 $\pm$ 1.6	20.1 $\pm$ 3.7
0.28†	52.2 $\pm$ 1.5	18.2 $\pm$ 2.6
0.33‡	59.9 $\pm$ 1.9	18.6 $\pm$ 0.3
0.00†	10.5 $\pm$ 1.3	—

* KCl was included also in the assay medium containing 0.1% Triton X-100; there was no change in total activity compared with that obtained in assay medium with 0.1% Triton X-100 alone.

† Incubation medium also contained 0.25 M-sucrose.

‡ Incubation medium did not contain sucrose.

hyperosmolar KCl added only 3 min before assay; however, the percentage free activities after exposure to KCl for only 3 min were not as great as when KCl was present throughout the incubation.

This increase in percentage free activity caused by incubation at 37°C in the presence of 0.25 M-sucrose plus hyperosmolar KCl was largely eliminated if the lysosomes were assayed in medium that had been adjusted to the same KCl concentration as that present in the incubation (Table 3). Table 3 also demonstrates that, when lysosomes were incubated at 37°C in 0.33 M-KCl without sucrose, the increase in free activity caused by incubation was largely eliminated by addition of KCl to the assay medium. Thus it appears that, when lysosomes are exposed to hyperosmolar KCl at 37°C, loss of latency is minimal until the lysosomes are subsequently exposed to a lower osmolarity.

In Fig. 4 lysosomes were suspended at 0°C in buffers containing increasing concentrations of KCl (Fig. 4a) or sucrose (Fig. 4b) and, after being maintained for approx. 15–30 min at 0°C, were

assayed for percentage free activity. The osmotic strength of the suspension medium had relatively little effect on free activities obtained for these unincubated lysosomes when compared with the effect of osmotic strength on free activities obtained after incubating lysosomes for 60 min at 37°C (Fig. 5). In Fig. 5 the percentage free activities after incubating heavy lysosomes for 60 min in buffers containing increasing concentrations of KCl (Fig. 5a) or of sucrose (Fig. 5b) are shown. Fig. 6 shows similar data obtained with light lysosomes (incubated for 60 min at 37°C) and with Triton-filled lysosomes (incubated for 40 min at 37°C). For these three lysosomal preparations, there is a large 'hyperosmolar effect' of KCl after incubation at 37°C; increasing the concentration of KCl above 0.2 M causes increasing losses of latency to occur when the lysosomes are subsequently assayed in 0.25 M-sucrose. However, hyperosmolar sucrose alone (up to 0.6 M) causes relatively little loss of latency over that obtained by incubation in 0.25 M-sucrose.

Fig. 7 shows the difference curves (percentage

Fig. 4. Heavy lysosomes: percentage free activity after suspension at 0°C in buffers containing increasing concentrations of KCl (a) or sucrose (b)

Heavy lysosomes were suspended at 0°C in buffers containing 0.01 M-Mops, pH 7.4, plus either KCl (a) or sucrose (b) at the concentrations indicated. After the suspension had remained for approx. 15–30 min at 0°C, samples were assayed for percentage free activity of *N*-acetyl- β -glucosaminidase. Results are the means $\pm$ S.E.M. from four experiments.

Fig. 5. Heavy lysosomes: percentage free activity after incubation for 60 min at 37°C in buffers containing increasing concentrations of KCl (a) or sucrose (b)

Heavy lysosomes were suspended at 0°C in buffers containing 0.01 M-Mops, pH 7.4, plus either KCl (a) or sucrose (b) at the concentrations indicated. After incubation at 37°C for 60 min samples were assayed for percentage free activity of *N*-acetyl- β -glucosaminidase either immediately (O) or after being placed on ice for 15 min (Δ). Results are the means $\pm$ S.E.M. from four experiments.

Fig. 6. Light lysosomes (a, b) and Triton-filled lysosomes (c, d): percentage free activity after incubation at 37°C in buffers containing increasing concentrations of KCl (a, c) or sucrose (b, d)

In (a) and (b), light lysosomes were suspended in buffers containing 0.01 M-Mops, pH 7.4, plus either KCl (a) or sucrose (b) at the concentrations indicated. After incubation for 60 min at 37°C, samples were assayed for percentage free activity of *N*-acetyl- β -glucosaminidase either immediately (O) or after being placed on ice for 15 min (Δ). In parts (c) and (d), Triton-filled lysosomes were suspended in buffers containing 0.01 M-Mops, pH 7.4, plus either KCl (c) or sucrose (d) at the concentrations indicated. After incubation for 40 min at 37°C samples were assayed for percentage free activity of *p*-nitrophenyl phosphatase either immediately (O) or after being placed on ice for 15 min (Δ).

free activity after cooling minus percentage free activity before cooling) that were calculated from the data shown in Figs. 5 and 6 for lysosomes incubated at 37°C. These curves further illustrate the inhibition, by sufficiently high osmotic strength, of the loss of enzyme latency caused by cooling. The

shape of these difference curves is also influenced by the fact that increased amounts of free activity are obtained even without cooling when the incubation medium contains hypo-osmolar concentrations of KCl or sucrose or hyperosmolar concentrations of KCl (and assays are performed in 0.25 M-sucrose).

Fig. 7. Light lysosomes (a), heavy lysosomes (b) and Triton-filled lysosomes (c): percentage free activity after cooling minus percentage free activity before cooling, after incubation at 37°C in buffers containing sucrose or KCl

These curves were constructed from the values shown in Fig. 5 (heavy lysosomes) and Fig. 6 (light lysosomes and Triton-filled lysosomes) for percentage free activity after incubation for 60 min (40 min for Triton-filled lysosomes) at 37°C in the presence of increasing concentrations of sucrose (O) or KCl (Δ). At each indicated concentration of sucrose or KCl, percentage free activity before cooling was subtracted from percentage free activity after cooling.

The curves in Figs. 5 and 6 have the form of osmotic-fragility curves. However, the lysosomes in these experiments had been exposed to the various osmotic conditions for 60 min at 37°C. At 37°C enzymic reactions may cause the properties of the lysosomal membrane to undergo continual change. In order to determine the osmotic fragility at 37°C at a given instant, it is necessary to minimize the time of exposure to the various osmotic conditions, as was done in the experiments illustrated in Fig. 3(b) and in the right-hand column of Table 2. Fig. 8(a) shows osmotic-fragility curves for heavy lysosomes that had been incubated in 0.2M-KCl for either 1 or 60 min at 37°C. After the incubation, samples were diluted at 37°C with buffers containing various amounts of KCl so that the indicated final concentrations of KCl were obtained; 3 min later the diluted samples were assayed for percentage free activity. Fig. 8(b) shows results from a duplicate experiment. Fig. 8(c) shows osmotic-fragility curves for heavy lysosomes that had been incubated in 0.25M-sucrose for either 1 or 60 min at 37°C. After the incubation, samples were diluted at 37°C with buffers containing various amounts of sucrose so that the indicated final concentrations of sucrose were obtained; 3 min later the diluted samples were assayed for percentage free activity.

Fig. 8(d) shows results from a duplicate experiment. The results show that incubation at 37°C at iso-osmoticity (0.2M-KCl or 0.25M-sucrose) causes lysosomes to undergo a large change in osmotic fragility, even though very little increase in free activity may occur at iso-osmoticity. Lysosomes that have been incubated at 37°C for 60 min are much more unstable on exposure to hypo-osmotic conditions, as well as on exposure to hyperosmolar concentrations of KCl, than are lysosomes which have been incubated for only 1 min. Fig. 9 shows similar results from experiments on light lysosomes (Figs. 9a, 9b) and on Triton-filled lysosomes (Fig. 9c).

Figs. 10(a) and 10(b) show the rate of increase in percentage free activity when heavy lysosomes are incubated in 0.15M- and 0.3M-KCl, respectively, at constant temperature, either 0°C or 37°C. For both concentrations of KCl, the rate of loss of latency is considerably greater, especially after an initial lag period, at 37°C than at 0°C.

Discussion

The stabilizing or labilizing effects *in vitro* of various agents on the lysosomal membrane have often been tested by determining whether they de-

Fig. 8. Heavy lysosomes: osmotic fragility after 1 and 60 min of incubation at 37°C in buffers containing 0.2 M-KCl (a, b) or 0.25 M-sucrose (c, d)

Heavy lysosomes were suspended with either 0.2 M-KCl (a, b) or 0.25 M-sucrose (c, d). After incubation for 1 min (○) or 60 min (△) at 37°C, samples of the lysosomes in 0.2 M-KCl were diluted 4-fold with buffers (warmed to 37°C) containing appropriate amounts of KCl so that the indicated final concentrations of KCl were obtained. Samples of the lysosomes in 0.25 M-sucrose were diluted with buffers containing appropriate amounts of sucrose. After 3 min the diluted samples were assayed immediately at 37°C for percentage free activity of *N*-acetyl- β -glucosaminidase. Part (b) is a duplicate experiment of part (a) and part (d) is a duplicate experiment of part (c).

crease or increase the rate of appearance of free activity of lysosomal enzymes during incubation at 37°C (see Wattiaux, 1977). However, the mechanisms by which the latency of the lysosomal enzymes is lost during such incubations have been unclear, since it is difficult to distinguish between: (a) direct osmotic disruption, resulting from an inherent permeability of the lysosomal membrane to a given solute, and (b) disruption resulting from alterations in the membrane during the incubation, sometimes called 'non-osmotic lysosomal breakdown' (see Casey *et al.*, 1978). Consequently, the mechanisms by which stabilizing or labilizing agents exert their effects have also been unclear. As discussed below, it is possible to distinguish various mechanistic routes

that can result in loss of enzyme latency after incubation of lysosomes at 37°C. Moreover, the data in the present paper indicate that the mechanisms of loss of latency caused by incubation at 37°C are fundamentally similar for the three different lysosomal preparations studied, in spite of the fact that their membranes may have different lipid compositions and may be largely derived from different subcellular sources (Henning & Heidrich, 1974). Quantitative differences in stability among the preparations do seem to exist, as illustrated by comparing Figs. 1(a) and 2(a).

The effect of salts on lysosomes has been a subject of investigation for many years. Berthet *et al.* (1951) and Appelmans & de Duve (1955) found

Fig. 9. Light lysosomes (a, b) and Triton-filled lysosomes (c): osmotic fragility after 1 and 60 min of incubation at 37°C in buffers containing 0.2 M-KCl (a) or 0.25 M-sucrose (b, c)

Light lysosomes were suspended in either 0.2 M-KCl (a) or 0.25 M-sucrose (b); Triton-filled lysosomes were suspended in 0.25 M-sucrose (c). After incubation for 1 min (O) or 60 min (Δ) at 37°C, samples of lysosomes in 0.2 M-KCl were diluted 4-fold with buffers (warmed to 37°C) containing appropriate amounts of KCl so that the final concentrations of KCl as indicated were obtained. Lysosomes in 0.25 M-sucrose were diluted with buffers containing appropriate amounts of sucrose. After 3 min the diluted samples were assayed immediately at 37°C for percentage free activity of *N*-acetyl- β -glucosaminidase.

that KCl and NaCl were unable at 0°C to prevent a progressive solubilization of acid phosphatase in the absence of sucrose. Allen & Lee (1972) found a gradual loss of lysosomal enzyme latency in 0.15 M-KCl at 0°C and suggested that the disruptive effect of KCl on liver lysosomes at 0°C was concentration-dependent and could be due to transient osmotic-pressure gradients produced when the concentration of KCl was subsequently lowered, but direct effects on the lysosomal membrane could not be excluded. Studies on liver lysosomes that had taken up injected ^{131}I -labelled protein found that, at 0°C, 0.25 M-KCl did not protect well against release of radioactive protein (Bertini *et al.*, 1967), but, at 37°C, 1 M-KCl or 1 M-NaCl were almost as effective as 0.2 M-sucrose in maintaining intact lysosomes (Mego *et al.*, 1967). Davidson & Song (1975) found that 0.11 M-KCl/0.01 M-MgCl₂ was able to protect lysosomes osmotically at 37°C. At 0°C, however, exposure to this same concentration of KCl/MgCl₂ for only 13 min resulted in a subsequent decrease of more than 50% in intralysosomal proteolysis at 37°C, indicating that osmotic lysis had occurred at 0°C. It was suggested that a phase transition occurred in the lysosomal membrane such that permeability to K⁺ was much greater at 0°C than at 37°C. Reijngoud & Tager (1975) also obtained evidence that the lysosomal membrane is more permeable to K⁺ at 0°C than at 37°C and con-

cluded that this was due to a phase transition in the lysosomal membrane. These investigators found that exposure of Triton-filled lysosomes to 50 mM-KCl (in the presence of 0.25 M-sucrose) for only 3 min at 0°C was sufficient to eliminate almost completely the pH gradient across the membrane as measured by distribution of [^{14}C]methylamine between the lysosomes and the medium. At 25°C, however, the pH gradient was lost gradually over a period of about 40 min.

Like others, we find a transient osmotic protection of lysosomes by KCl, both at 0°C and 37°C. Reijngoud & Tager (1975) concluded that the lysosomal membrane is completely permeable to K⁺ at 0°C. However, exposure of unincubated lysosomes for only 3 min to a wide range of KCl concentrations at either 0°C or 37°C does not result in penetration by salt to an extent sufficient to cause appreciable osmotic rupture of the lysosomes. The only case in which we see evidence of a very rapid entry of KCl into the lysosome is when lysosomes that have been previously incubated for 60 min at 37°C at iso-osmoticity are exposed to hyperosmolar KCl. For such incubated lysosomes only 3 min exposure, even at 37°C, to 0.3 M-KCl is sufficient to cause a large amount of osmotic rupture when these lysosomes are subsequently placed in assay medium containing only 0.25 M-sucrose. We have shown (Table 3) that loss of enzyme latency of lysosomes

Fig. 10. Heavy lysosomes: increase in percentage free activity caused by incubation at 0°C and 37°C in buffer containing 0.15 M-KCl (a) or 0.3 M-KCl (b)

Heavy lysosomes were suspended in 0.01 M-Mops, pH 7.4, plus either 0.15 M-KCl (a) or 0.3 M-KCl (b) and were then incubated at either 0°C (O) or 37°C (Δ). At the indicated times samples were assayed for percentage free activity of *N*-acetyl- β -glucosaminidase. Samples incubated at 37°C were assayed without cooling. For incubation in 0.3 M-KCl, the rate of increase in percentage free activity at 37°C was $39.1 \pm 1.5\%/h$ and that at 0°C was $13.1 \pm 0.8\%/h$ (both means $\pm$ S.E.M. from four experiments).

incubated in hyperosmolar KCl does not occur for the most part until the KCl-permeated lysosomes are added to assay medium containing only 0.25 M-sucrose. Thus, at least at 37°C, lysosomes apparently can withstand some permeation by KCl without undergoing osmotic rupture as long as the osmotic strength of the medium is maintained sufficiently high. Consequently, hyperosmolar KCl solutions do provide protection against osmotic rupture, even though permeation by salt does occur. It is an important point that the loss of enzyme latency in the presence of KCl actually occurs more rapidly at

37°C than at 0°C, as illustrated in Fig. 10. Thus, if the inherent permeability of the lysosomal membrane to KCl is greater at 0°C than at 37°C, mechanisms other than a simple permeation of KCl into the lysosome must be involved in producing the loss of enzyme latency at 37°C. The probability that enzymic action is involved in the loss of enzyme latency caused by incubation at 37°C has been recognized for some time (Beaufay & de Duve, 1959).

As mentioned in the introduction, we have postulated (Ruth & Weglicki, 1978) that during incubation of Triton-filled lysosomes at 37°C potentially lytic products such as lysophospholipids and non-esterified fatty acids are formed which cause loss of latency of the lysosomal enzymes when the lysosomes are subsequently cooled through the phase transition; what occurs during the incubation at 37°C is an increase in potential for loss of latency on cooling. (As illustrated in the present paper, however, the expression of the effect of cooling can be repressed by increasing the osmotic strength of the medium in which the incubated lysosomes are cooled.) The data shown in Figs. 8 and 9 indicate that incubation at 37°C at iso-osmoticity results as well in an increased potential for loss of latency on subjection of the lysosomes to either hypo-osmotic or hyperosmotic stress.

The increased susceptibility of incubated lysosomes to hypo-osmotic stress might at first seem to be attributable to permeation, during the incubation, of the lysosomal membrane by solute. The resulting increased internal concentration of solute would then cause an increase in the external concentration of solute, which is necessary in order to prevent osmotic rupture. However, the increase in susceptibility to hypo-osmotic stress appears to be largely the same whether the lysosomes are incubated in the presence of KCl or in the presence of sucrose. Our data show that incubation at 37°C for as long as 60 min in up to 0.6 M-sucrose causes a minimal amount of osmotic rupture when the incubated lysosomes are subsequently assayed for 3 min in 0.25 M-sucrose. Thus it seems unlikely that, during incubation in 0.25 M-sucrose, enough sucrose could have entered the lysosome to cause the relatively large increase in osmotic fragility that is observed on exposure for 3 min to concentrations of sucrose below 0.25 M. Another possible explanation of the increased susceptibility to hypo-osmotic stress is that during the incubation at 37°C the lysosomal membrane is injured, probably by enzymic action. This injured membrane could be more fragile on exposure to hypo-osmotic conditions than was the original, uninjured membrane. This explanation could account for an increased susceptibility to hypo-osmotic stress even when incubation was carried out in the presence of a non-permeating solute such

as sucrose. It is known that lysophosphatidylcholine causes an increased fragility of the erythrocyte membrane on exposure to hypo-osmotic conditions (Seeman, 1966). Thus it seems a reasonable hypothesis that increased susceptibility of the lysosomal membrane to hypo-osmotic stress could be a result of the presence of metabolic products resulting from enzymic action.

When lysosomes are incubated in 0.2M-KCl at 37°C for 60 min, loss of latency is minimal. Thus it appears that, within this time-span and at this concentration of KCl, net permeation of salt into the lysosome does not take place to an extent great enough to cause osmotic rupture either in the presence of 0.2M-KCl or when the lysosomes are subsequently assayed in 0.25M-sucrose. Nonetheless, if lysosomes that have been incubated for 60 min in 0.2M-KCl are exposed to 0.33M-KCl for 3 min, the amount of latency that is lost during the subsequent assay is roughly equivalent to the amount lost when lysosomes have been incubated continuously in 0.3M-KCl for 60 min (see Figs. 8 and 10). This, as well as comparison of Figs. 8 and 9 with Figs. 5 and 6, leads to the conclusion that the final amount of osmotic rupture caused by exposure of incubated lysosomes to hyperosmolar KCl is largely independent of the concentration of KCl that was present during the incubation. As illustrated in Table 2, even lysosomes that have been incubated for 60 min in 0.25M-sucrose alone display a fragility on exposure for only 3 min to hyperosmolar KCl,

whereas lysosomes incubated at 37°C in 0.2M-KCl for only 1 min can be exposed for 3 min to as high as 0.6M-KCl with very little resulting osmotic rupture. Thus it appears that incubation at 37°C in 0.2M-KCl or in 0.25M-sucrose potentiates a rapid permeation of the lysosome by hyperosmolar KCl. We suggest that at 37°C permeation of the lysosome by KCl occurs in a concentration-dependent manner only after an alteration (probably by enzymic action) of the lysosomal membrane, and the rate at which this alteration takes place is largely independent of the salt concentration of the incubation medium.

Scheme 1 illustrates three different mechanistic routes by which incubation at 37°C at iso-osmoticity can result in loss of latency of the lysosomal enzymes. The fundamental damaging process occurs during the incubation at 37°C, and we propose that this consists of enzymic action which injures the lysosomal membrane. For incubations as long as 60 min, damage is not sufficient to admit enough water or solute into the lysosome to cause appreciable osmotic rupture. However, the incubated lysosomes display an increased tendency to undergo physical damage on exposure to one of the forms of stress illustrated. With regard to stabilizing or labilizing agents, for each of the steps indicated in Scheme 1, it is conceivable that agents might be found which would act at that particular step so as to increase or decrease the loss of enzyme latency resulting from incubation. A conclusion that can be drawn from

Scheme 1. *Pathways by which incubation at 37°C results in loss of latency of lysosomal enzymes*

As described in the text, lysosomes that have been incubated at 37°C have become more susceptible to the three kinds of stress depicted in this Scheme. Loss of latency resulting from incubation does not for the most part occur until the incubated lysosomes are subjected to these stresses.

these studies is that loss of enzyme latency is not always a good criterion for judging whether or not alterations of lysosomal integrity have taken place. As shown in Scheme 1, lysosomes that have been incubated at iso-osmoticity may have undergone significant damage without this damage being expressed as a loss of enzyme latency. In addition, lysosomes exposed at 37°C to hyperosmolar KCl may be permeated by KCl, but this is not expressed as a loss of enzyme latency unless the KCl-permeated lysosomes are subsequently exposed to lower osmolarities. Even the loss of enzyme latency caused by cooling, which is itself a method of unmasking damage to the lysosome, will not be expressed if cooling is carried out at high osmotic strength.

Various questions remain unanswered. The data shown in Fig. 7 make it appear that the loss of latency caused by cooling incubated lysosomes to 0°C may be inherently greater in KCl solutions than in sucrose solutions. This may be due to more permeation of the lysosome by KCl than by sucrose during incubation, but effects exerted by KCl on the lysosomal membrane or on the enzymes responsible for the damage to the membrane might also be involved. Binding of K⁺ to the lysosomal membrane has been demonstrated (Reijngoud *et al.*, 1976) and a stimulation of a membrane-bound lysosomal ATPase by KCl has been claimed (Schneider, 1979). Also, although we postulate the existence of a KCl-permeated lysosome, that is relatively stable until exposed to lower osmolarities, we have no information at present on the actual quantities of either K⁺ or Cl⁻ that permeate the lysosomal membrane during incubation at iso-osmoticity or at hyper-osmoticity.

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